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Inactivation of cytochrome P450 isozymes by spironolactone

Author
Decker, Caroline J.

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Inactivation of Cytochrome P450 Isozymes by Spironolactone

by

Caroline J. Decker

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Inactivation of Cytochrome P-450 Isozymes (P450) by Spironolactone (SPL)

Abstract

The diuretic SPL suicidally inactivates adrenal and testicular P450 17α-hydroxylases. SPL produces modest loss of constitutive hepatic P450 content acutely, and induces the P450IIIA isozymes chronically without increasing spectrally detectable P450. These studies were initiated to determine whether acutely, SPL inactivates hepatic P450IIIA and to examine the mechanism of this process.

SPL selectively inactivated rat hepatic P450IIIA and P450IIC11 through (1) inactivation of isozyme-selective marker activities; (2) enhancement of P450 destruction in rats administered dexamethasone (DEX), a P450IIIA inducer; (3) attenuation by P450IIIA inhibitors; and (4) inactivation of purified P450IIC11. P450 destruction was reproducible in vitro, and satisfied most criteria for mechanism-based inactivation (i.e. cofactor and temperature dependence). SPL deacetylation to SPL-SH was required for destruction; desulfurated analogs were inactive. Prosthetic heme was lost stoichiometrically with P450 content both in vivo and vitro, a fraction of which was found covalently bound to liver microsomal proteins. NADPH-dependent covalent binding of ^{14}C-SPL to microsomal proteins also occurred.

Examination of SPL metabolism in vitro led to the isolation of two polar metabolites, whose formation was NADPH- and O_2-dependent, P450IIIA-selective and inhibitable by carbon monoxide and n-

octylamine. SPL-mediated P450 destruction and polar metabolite formation was also observed in human liver and adrenal microsomal systems. HPLC and mass spectral analysis of the metabolites indicated their structures as the SPL sulfinic and sulfonic acid derivatives, a finding confirmed with chemically synthesized standards.

SPL administration significantly decreased hepatic glutathione (GSH) levels in DEX-pretreated rats, and GSH addition in vitro attenuated both SPL-mediated P450 destruction and polar metabolite formation significantly. A metabolite, detected in incubations containing both GSH and SPL was considerably enhanced by NADPH inclusion. After purification and mass spectral analysis, its structure was established as a disulfide adduct (SPL-SSG) of SPL-SH and GSH. SPL-SSG formation was greater in incubations containing purified hog liver flavin-containing monoxygenase (FMO), but without concomitant polar metabolite formation, thus distinguishing SPL metabolism by P450 and FMO.

Polar SPL metabolite formation provided evidence for formation of the reactive thiyl radical and/or sulfenic acid species - likely perpetrators of enzyme inactivation. Isolation of SPL-SSG provided evidence for reaction of either SPL-derived electrophile with GSH and for GSH-mediated attenuation of polar metabolite formation and P450 destruction.

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Abbreviations used

ßNF: β-naphthoflavone
BNPP: bis-*p*-nitrophenylphosphate
BZP: benzphetamine
CAN: canrenone
CAT: catalase
CO: carbon monoxide
CuOOH: cumene hydroperoxide
DEX: dexamethasone
DTT: dithiothreitol
EM: ethylmorphine
ER: ethoxyresorufin
ERY: erythromycin
GSH: glutathione
GSSG: oxidized glutathione
3-MC: 3-methylcholanthrene
NADPH: nicotine adenine dinucleotide phosphate, reduced form
NOA: n-octylamine
ND: N-demethylase
NT: non-pretreated
O_2: molecular oxygen
OHase: hydroxylase
P450: cytochrome P450 isozymes
PCN: pregnenolone 16α-carbonitrile
pCNMA: *p*-chloro-N-methylaniline
PB: phenobarbital
PTR: pentoxyresorufin
SOD: superoxide dismutase
SOLD: soldactone
SPL: spironolactone
SPL-SH: deacetylated spironolactone
T-OHase: testosterone hydroxylase

I. Background

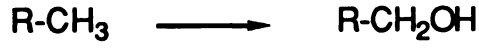
A. Cytochrome P450 monoxygenase system

1. Description

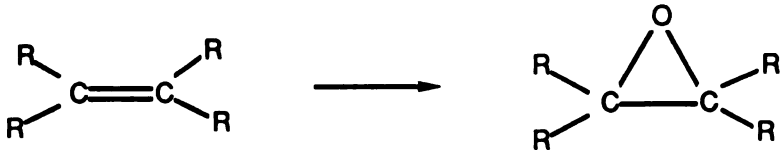
Cytochrome P450 (P450) consists of an isozyme family of microsomal or mitochondrial membrane bound hemoproteins (found in many organisms and tissues therein) which catalyze the oxidation of various endogenous and exogenous compounds (Estabrook et al., 1963; Cooper et al., 1965). Cytochrome P450 isozymes catalyze the hydroxylation, epoxidation and heteroatom oxidation of many compounds (Figure 1). Often dealkylation is a result of heteroatom oxidation. Under anaerobic conditions, P450 may catalyze the reductive metabolism of halogenated compounds such as carbon tetrachloride (Roland et al., 1977). P450 is classified as a "phase I" drug metabolizing enzyme [along with the flavin-containing monoxygenase (FMO)] because it inserts a polar group (such as a hydroxyl function) into a substrate. If the hydroxylated product is not sufficiently hydrophilic to be excreted, a phase II reaction enzyme such as glucuronyl transferase may act on it, thus further increasing the hydrophilic character of the product for ultimate excretion. P450 was first detected because of its characteristic reduced, carbon monoxide (CO)-complexed 450 vs. 490 nm difference spectrum (Omura and Sato, 1964). P450 has a molecular weight of ~50,000 D and contains one prosthetic heme (Fe^{+3}-protoporphyrin IX moiety)/mole of enzyme, which is involved in both electron and oxygen transfer. Cytochrome P450-reductase (P450-reductase) is required for microsomal P450-mediated reactions in that it ferries electrons (one at a time) from NADPH to the iron of P450 heme (Raftell and Orrenius, 1970).

Figure 1. Reactions catalyzed by P450

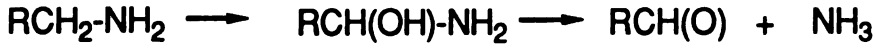
Carbon Hydroxylation



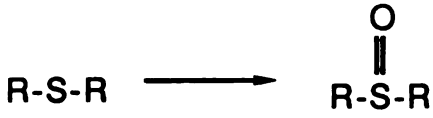
Olefin Epoxidation



Dealkylation



Heteroatom Oxidation



P450-reductase has a molecular weight of ~78,000 D and contains one mole each of flavin mononucleotide and flavin dinucleotide (Iyanagi and Mason, 1973). Reconstitution of purified P450 and P450-reductase requires the addition of lipids such as dilauroylphosphatidylcholine (Lu et al., 1969).

2. Catalytic Cycle

P450 is a monoxygenase (in that it catalyzes the introduction of one atom of oxygen or its redox equivalent into a substrate) which requires molecular O_2, NADPH, and P450-reductase for catalytic activity (Figure 2). P450 catalysis proceeds in a sequential fashion (from Ortiz de Montellano, 1986): 1. Substrate binding, which lowers the reduction potential of P450 heme; 2. NADPH-mediated reduction (by 2 electrons) of cytochrome P450-reductase; 3. Reduction of P450 Fe^{+3}-heme by one electron from P450-reductase; 4. Binding of O_2 to form the Fe^{+3}-heme dioxygen complex; 5. Reduction of the heme-oxygen complex with another electron from P450 reductase; 6. Cleavage of the oxygen-oxygen bond with release of one molecule of H_2O (the resultant "activated oxygen complex" may be represented as the ferryl Fe^{+5}=O[RH], or perferryl Fe^{+4}-O^-[RH] species); 7. Transfer of the "activated oxygen" into the substrate; 8. Finally, dissociation with liberation of the hydroxylated substrate and ferric iron-heme P450 species (Figure 2).

Step 7 is complex; it is generally believed that hydrogen atom abstraction from the subtrate to form Fe^{+3}OH·[·RH] followed by radical recombination to Fe^{+3}[ROH] occurs. This is supported by studies of deuterium isotope effects on hydroxylation rates (Groves et al., 1978;

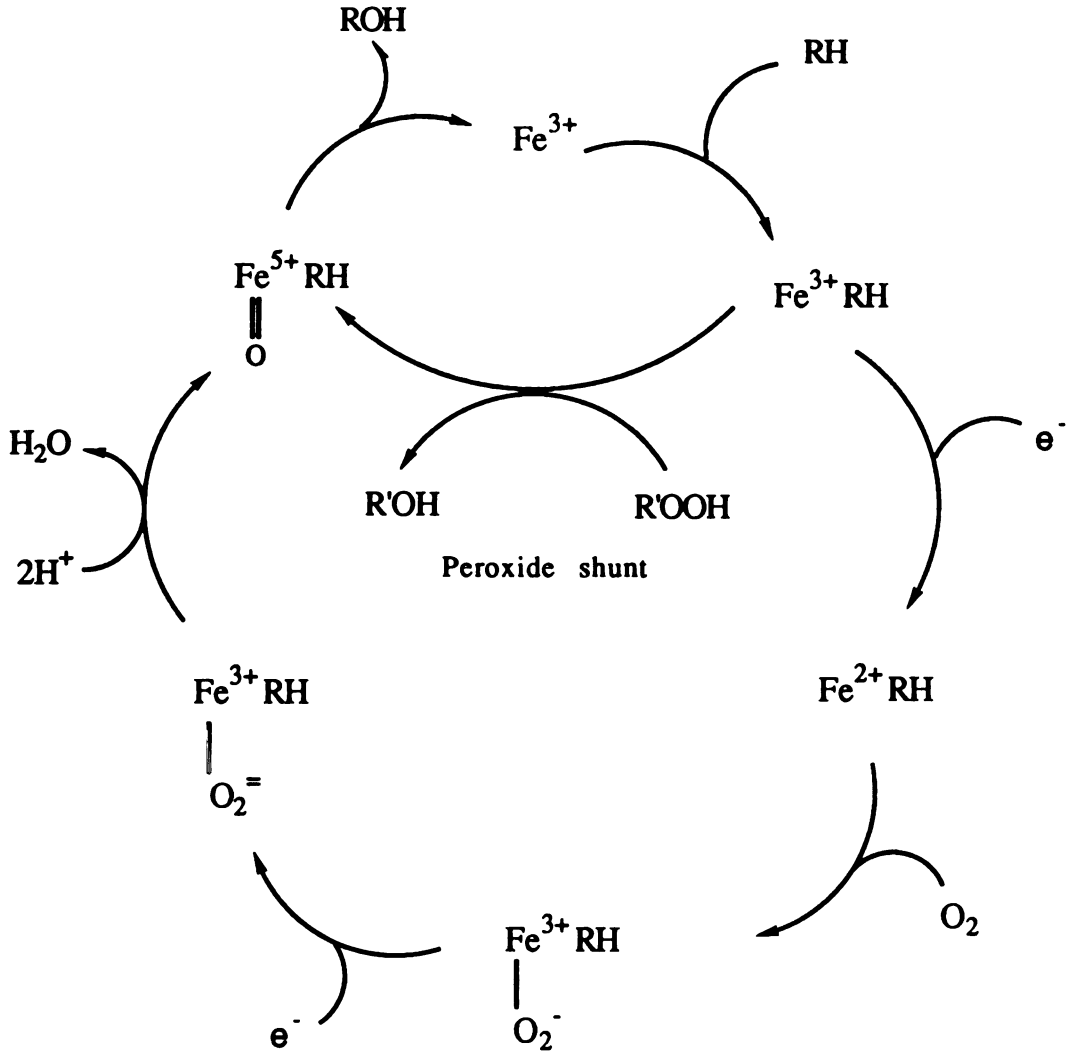
Lu et al., 1984) and rearrangement reactions of various cyclic compounds as a consequence of P450 catalysis (Stearns and Ortiz de Montellano, 1985). One electron oxidation of a heteroatom by P450 is supported by spin trapping studies of released 4-alkyl substituents of 4-alkyl-1,4-dihydropyridines, as such a phenomenon is predicted following a one electron oxidation of the pyridine nitrogen (Augusto et al., 1982). Evidence for one electron oxidation of heteroatoms however, has been recently challenged (Kennedy and Mason, 1990). For instance, N- and S-dealkylations are believed to involve an initial one electron oxidation of nitrogen or sulfur, followed by a hydrogen atom transfer from the adjacent carbon to the iron-bound oxygen, with resultant carbon radical formation and subsequent collapse to the hydroxylated carbon (Guengerich and MacDonald, 1984; Ortiz de Montellano, 1986).

Hydrogen peroxide or organic peroxides can act as oxygen donors for P450 (Figure 2). These compounds interact with the substrate-complexed P450 in its resting [Fe^{+3}] state to form the activated oxygen complex (i.e. Fe^{+5}=O[RH] or Fe^{+4}-O^{-}[RH]) with release of H_2O, in the case of hydrogen peroxide, or an alcohol in the case of organic hydroperoxides (peroxide shunt, Figure 2); such activated complexes can directly oxidize substrates without the usual requirement for O_2 and electrons from P450-reductase (Coon et al., 1977).

3. P450 Isozymes

In the liver, the P450 isozyme system plays an important detoxification role in that it permits an organism to cope with the plethora of xenobiotic substances which it comes in contact with,

Figure 2. Catalytic cycle of P450



Fe^{+3} represents the P450 heme iron; R: alkyl/aryl group; ROOH: alkylhydroperoxide; ROH: alcohol; e^-: electron; O_2: molecular oxygen. Refer to text for details.

through biotransformation. Such metabolism usually results in xenobiotic elimination. With some notable exceptions (e.g. cholesterol 7α-hydroxylase), P450 isozymes in the liver are primarily involved in the oxidation of xenobiotics, have broad substrate specificities, and may be induced by various chemicals. P450 isozymes in the endocrine glands (such as the adrenals, testis, ovary and placenta) differ in that they are involved in the synthesis of endogenous steroid hormones, have relatively restricted substrate specificities and are not inducible. P450 isozymes may be distinguished from one another by their amino acid sequence, molecular weight, substrate specificities, absorption spectra, immunological properties, as well as induction characteristics.

a. Hepatic forms

Substrate specificities for the hepatic P450 isozymes are broad and much overlap occurs. Table 1 lists several of the known rat hepatic forms characterized to date. Quite often regio- and stereoselective hydroxylation of testosterone is used as a functional probe for the identification of different P450 isozymes (Waxman, 1988; Figure 3), as hydroxylation of this substrate is highly isozyme-selective. This discussion will focus on the major forms in the rat, using the "Nebert" (gene-based) and "Levin" nomenclatures for the isozymes (Nebert et al., 1987; 1989; Ryan et al., 1982; 1984).

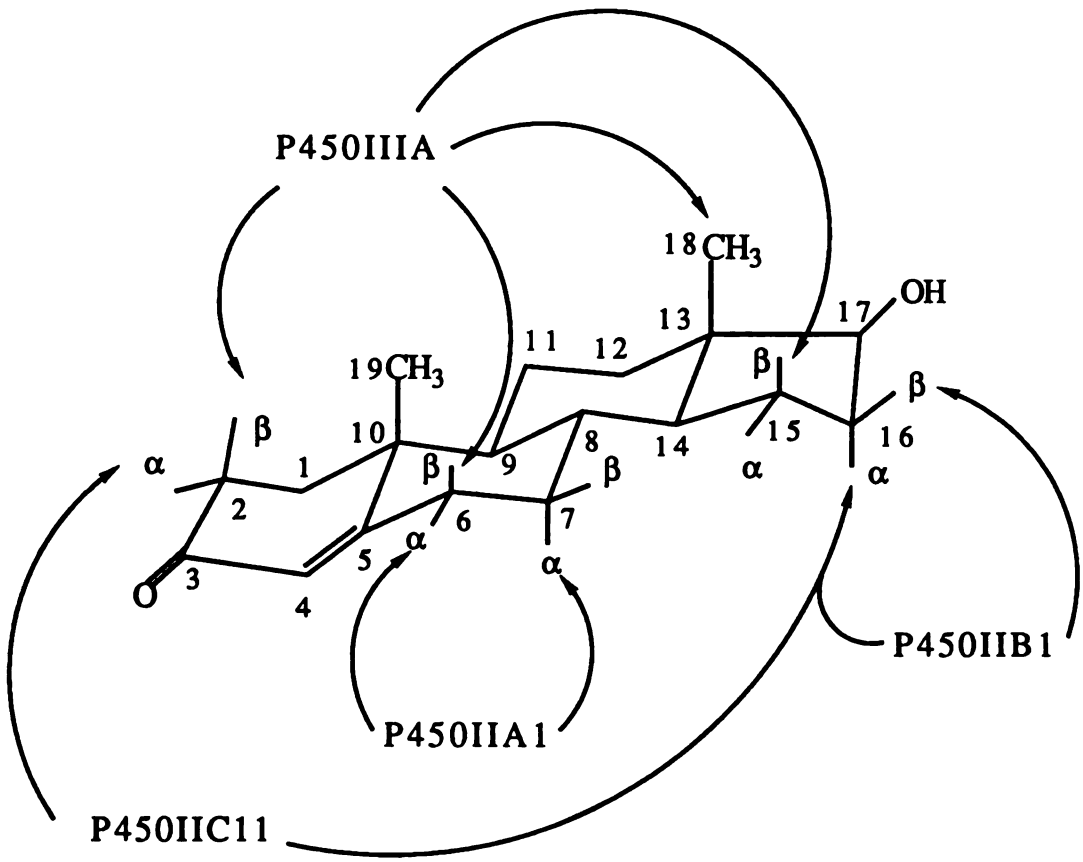
P450IIC11 or P450h (Guengerich et al., 1982) is a male specific form which increases at puberty and is regulated by both testosterone

Table 1. Major rat hepatic P450 isozymes

Nomenclature				
Nebert	Levin	Inducers	marker activities	sex specificity
P450IA1	c	ßNF, 3-MC	PCNMAND, EROD	
P450IA2	d	ßNF, 3-MC, SF	PCNMAND, EROD	
P450IIA1	a		T 7α-OHase	
P450IIB1	b	PB, chlordane	T 16ß-OHase, PROD, BZPND	
P450IIB2	e	PB		
P450IIC6	k	PB	W 7R-OHase	
P450IIC11	h		T 2α-OHase	male
P450IIC12	i			female
P450IIE1	j	EtOH, isoniazid	NDMAND, EtOH oxidation	
P450IIIA1	pcn1(p)	DEX, PCN, PB	T 6ß, 15ß, 2ß-OHases, ERYND, EMND	
P450IIIA2	pcn2	PB	T 6ß, 15ß, 2ß-OHases, ERYND, EMND	male
P450IVA1		clofibrate	Laω-OHase	

ßNF: ß-naphthoflavone; PB: phenobarbital; DEX: dexamethasone; 3-MC: 3-methylcholanthrene; SF: safrole; EtOH: ethanol; NDMAND: N-nitrosodimethylamine N-demethylation; Laω-OHase: Lauric acid ω-hydroxylase; WROHase: warfarin hydroxylase; PROD: pentoxyresorufin O-deethylase; EROD: ethoxyresorufin O-deethylase; PCNMAND: *p*-chloro-N-methylaniline N-demethylase; ERYND: erythromycin N-demethylase; EMND: ethylmorphine N-demethylase, BZPND: benzphetamine N-demethylase; W 7R-OHase: warfarin 7R-hydroxylase; T: testosterone; OHase: hydroxylase. See text or Guengerich, 1987 for references.

Figure 3. Regio- and steroselective hydroxylation of testosterone



Arrows refer to sites of hydroxylation of the testosterone molecule by various rat hepatic P450 isozymes. α: positions below the plane of the ring system. ß: positions above the plane of the ring system. See text for detailed description of P450 isozymes.

(Dannan et al., 1986) and growth hormone (Gustafsson et al., 1983). P450IIC11 is associated with high testosterone 2α and 16α-hydroxylase activities (T 2α-OHase and T 16α-OHase, respectively) as well as androstenedione formation from testosterone (Waxman, 1988). P450IIC11 is constitutive and is often actually decreased after the administration of various P450 inducers.

The P450 forms inducible by phenobarbital (PB)-type compounds include P450IIC6, IIB1, IIB2 and IIIA2, or k, b, e and pcn2, respectively, in Levin's nomenclature (Guengerich et al., 1982). P450IIB1 is considered to be the major PB-inducible form and catalyzes the 16β-hydroxylation of testosterone (T 16ß-OHase) (Waxman et al., 1983), but even more selectively the O-deethylation of pentoxyresorufin (Lubet et al., 1985). P450IIC6 selectively hydroxylates warfarin at the 7R or 7S position (Guengerich et al., 1982).

Isozymes inducible by polycyclic aromatic hydrocarbons [such as 3-methylcholanthrene (3MC) and ß-naphthoflavone (ßNF)] belong to the P450IA subfamily (i.e. P450IA1 and P450IA2), and are also known as c and d respectively, using Levin's nomenclature. These isozymes are typified by their ability to selectively O-deethylate ethoxyresorufin (Burke and Mayer, 1974). *p*-Chloro-N-methylaniline (PCNMA) N-demethylation is a less selective marker for P450IA isozymes than ethoxyresorufin O-deethylation, but is still indicative of these forms (Correia and Mannering, 1973).

The most recently characterized inducible P450s are those of the steroid inducible class P450IIIA, which will be referred to collectively as P450IIIA, because no functional activity has yet been found which is able to distinguish between the individual isozymes characterized. P450IIIA (also known as P450p) was first identified as a unique form in that pregnenolone 16α-carbonitrile (PCN) was found to stimulate ethylmorphine (EM) N-demethylase (EMND) activity relatively selectively in rats, as compared to the prototypical inducers PB and 3-MC (Lu et al., 1972). P450IIIA was first purified from female rats pretreated with PCN, and was characterized by its high EMND activity (Elshourbagy and Guzelian, 1985). Metabolic intermediate complex formation of P450 with the macrolide antibiotics troleandomycin (TAO) and erythromycin (ERY) was demonstrated to occur (Pessayre et al., 1981; Dannan et al., 1981) and was later shown to be somewhat selective for P450IIIA (Wrighton et al., 1985). Formation of the TAO intermediate complex (as that of norbenzphetamine) involves oxidation of the amine function of these compounds by P450 to a nitroso group which binds to ferrous heme, producing a tight, catalytically inactive, complex, which may be dissociated in vitro by the addition of the oxidizing agent $K_3Fe(CN)_6$ (Franklin, 1977). The nitroso TAO-Fe^{+2}(P450) complex has a characteristic absorbance at 456 nm (Pessayre et al., 1981). Formation of such complexes has been used to quantitate P450IIIA levels in vivo (Larrey et al., 1983) and in vitro (Wrighton et al., 1985). TAO treatment was subsequently shown to induce P450IIIA, by stabilizing the hemoprotein in its TAO-complexed state (Watkins et al., 1986).

P450IIIA was also found to be induced by dexamethasone (DEX) (Schuetz et al., 1984); as well as PB, methyltestosterone, and spironolactone (SPL) (Heuman et al., 1982). Many investigators have experienced difficulty in obtaining an active purified preparation of P450IIIA; higher activity has been reported when phosphatidylserine (Imaoka et al., 1988), or when a chloroform/methanol lipid extract from rat hepatic microsomes (Halvorson et al., 1990), is included as the lipid components. Recently, several catalytically active P450IIIA forms were purified (Nagata et al., 1990). Liver microsomes prepared from rats pretreated with PCN, DEX or PB exhibit high testosterone 6ß-hydroxylase (T 6ß-OHase) activity (Waxman et al., 1985). More selective marker activities for P450IIIA are testosterone 2ß-, 15ß- and 18-hydroxylation (Underwood et al., 1986; Sonderfan et al., 1987), and 4-6-testosterone diene formation (Nagata et al., 1986). Steroid-inducible P450IIIA has been shown to O-debenzylate benzyloxyphenoxazone, and to hydroxylate benzo(a)pyrene and 2-acetylaminofluorene (Namkung et al., 1989). Rat P450IIIA has recently been implicated in the oxidative cleavage of digitoxin (Eberhart et al., 1990).

To date, four rat P450IIIA isozymes have been purified (Nagata et al., 1990). Two of such P450IIIA forms, P450IIIA1 and P450IIIA2, have been well characterized. P450IIIA1 corresponds to P450 PCN1 and is inducible by steroids such as DEX and PCN as well as PB; whereas IIIA2 corresponds to the form PCN2 that is constitutive, male specific, and inducible by PB (Gonzalez et al., 1986). Neither P450IIIA form is present in uninduced mature female rats (Waxman et al., 1985).

Three forms of P450IIIA have been identified on the basis of differential responses to chloramphenicol (Graves et al., 1987; Halpert, 1988). Cannabidiol-mediated inactivation of mouse constitutive or PB-inducible P450IIIA isozymes, but not those that are DEX-inducible, also appears to distinguish between P450IIIA forms (Bornheim and Correia, 1990).

P450IIIA has been found to exist in human liver and is inducible by DEX, TAO and PB (Watkins et al., 1985). Four human P450IIIA forms have been identified to date, HLp (P450IIIA3), HLp2, HLp3 (P450IIIA5), and P450NF (P450IIIA4) (Wrighton et al., 1989; Aoyama et al., 1989; Guengerich et al., 1986). Unlike the rat, a P450IIIA form is the most prominent isozyme in the human fetal liver (Hulla and Juchau, 1989). Human liver P450IIIA hydroxylates testosterone primarily at the 6ß-position, in a similar manner as does its rat liver counterpart (Waxman et al.., 1988). The therapeutic agents diltiazem (Pichard et al., 1990b), nifedipine (Guengerich et al., 1986), gestodene (Guengerich, 1990), ethynylestradiol (Guengerich, 1988) and cyclosporine (Kronbach et al., 1988) are oxidized by human P450IIIA isozymes as are the endogenous hormones cortisol (Ged et al., 1989) and progesterone (Waxman et al., 1988). Human P450IIIA isozymes also participate in the metabolism of the hepatotoxin, aflatoxin (Shimada and Guengerich, 1989) and the environmental pollutant, 1-nitropyrene (Howard et al., 1990).

b. Steroidogenic P450 Isozymes

The steroidogenic P450 enzymes differ from the liver microsomal enzymes in that they are under very tight physiological control to

ensure that the proper hormones are produced at the proper location. Orientation of these enzymes in the membrane is also very important and shuttling of steroid hormone intermediates occurs (Hall, 1984). Steroidogenic P450 isozymes also differ from the majority of their hepatic counterparts in that several complex sequential oxidation steps are involved in the actions of several steroidogenic P450s such as cholesterol side chain cleavage (SCC) and aromatase (3 sequential cycles), and the 17,20-lyase (2 sequential cycles) (Jefcoate, 1988). Again, unlike the hepatic microsomal P450 isozyme system, several of the steroidogenic P450 enzymes are located in the outer membrane of the mitochondria (such as 11ß-hydroxylase) and require both adrenodoxin (an iron-sulfur protein) and adrenodoxin reductase (a flavoprotein) to shuttle electrons from NADPH to P450 instead of P450 reductase. The existence of different isozymic compositions confers species and/or organ selective production of particular hormones. For instance, lack of steroid 17α-hydroxylase (17α-OHase) in the adrenal cortex of the rat results in the production of corticosterone instead of cortisol. In contrast, guinea pigs and man possess adrenal 17α-OHase and produce cortisol.

B. Flavin-containing monoxygenase (FMO)

FMO is a microsomal flavin-containing enzyme of ~60,000 D molecular weight, found in the liver, lung and other tissues that is involved in the oxidation of many nitrogen- and sulfur-containing xenobiotics (Ziegler, 1980; 1988). FMO oxidizes various sulfides to the corresponding sulfoxide(s) or sulfone. FMO has recently been shown to oxidize the methylated SPL analog (SPL-SCH_3) to its corresponding sulfoxides (Cashman and Pena, 1989b). FMO was early on shown to be

involved in the oxidation of thiocarbamides (Ziegler, 1980). The oxidation of thiocarbamides to their sulfenic acid derivatives by FMO has been proposed on the basis of isolation of N-methylimidazole and sulfite metabolites of methimazole (Poulsen et al., 1974), and of the sulfinic acid derivative of phenylthiourea (Poulsen et al., 1979) from incubations containing purified hog liver FMO. The formation of such metabolites is postulated to be the result of either a disproportionation reaction (between two sulfenic acid molecules) or a second oxidation of the sulfenic acid species by FMO. Increased biliary excretion of oxidized glutathione (GSSG) following administration of the thiocarbamides thiourea, phenylthiourea and methimazole (Krieter et al., 1984), has also supported the belief that these sulfur compounds are oxidized by FMO to their corresponding formamidine sulfenic acids. Generation of GSSG and the parent thiocarbamide could arise from disulfide exchange reactions of excess glutathione (GSH) with a GSH-thiocarbamide mixed disulfide (formed via reduction of a formamidine sulfenic acid by GSH).

Many substrates oxidized by P450 are also oxidized by FMO. FMO differs from P450 in that it is not induced by any xenobiotic compound (Sum and Kasper, 1982), and is not selectively inactivated by any mechanism-based inhibitors. n-Octylamine (NOA, an inhibitor of many P450 isozymes) is a stimulator of FMO activity (Ziegler, 1980). The pH optimum for hepatic FMO activity is ~8.5 regardless of the substrate oxidized (Poulsen and Ziegler, 1979), another characteristic feature which distinguishes it from P450 (which has a pH optimum of 7.4). Thus, induction status, NOA addition, and variation of pH are

often employed to functionally distinguish between the two monooxygenases in liver microsomal systems.

The catalytic mechanism of FMO is believed to involve interaction of the substrate with the 4α-hydroperoxyflavin form of the enzyme (which may be generated in the absence of the substrate; Poulsen and Ziegler, 1979). If a substrate can be oxidized by an organic peroxide, then it also is apparently oxidizable by FMO. Oxidation is believed to occur via nucleophilic attack of the substrate on the 4α-hydroxyflavin (a two electron oxidation). While a nucleophilic mechanism of substrate oxidation is probably in operation, radical reaction mechanisms cannot be excluded.

C. Mechanism-based inactivators of P450

1. Description

Mechanism-based inactivators, otherwise known as suicide substrates are compounds which require metabolic activation by an enzyme in order to result in its inactivation. Mechanism-based inactivation of P450 is NADPH- and O_2- dependent, and may be attenuated by the addition of CO (Ortiz de Montellano and Correia, 1983; Ortiz de Montellano, 1988). A specific criterion for mechanism-based inactivation is that the reactive species which is responsible for enzyme inactivation should destroy the enzyme at its active site, before diffusing out of the enzyme-substrate cleft. Mechanism-based inactivation is time dependent and substrate-dependent (i.e. saturable by increasing inactivator concentration) and obeys pseudo first-order kinetics (Rando, 1984). Mechanism-based inactivators are often characterized by a partition ratio, representing the number of catalytic

cycles per inactivation event (Ortiz de Montellano, 1988). This value is a measure of the efficiency of a suicide substrate and represents the number of times a substrate is processed per molecule of enzyme inactivated. Thus the partition ratio for P450 suicide substrates such as 1-aminobenzotriazole and allylisopropylacetamide are <12 and ~200, respectively (Ortiz de Montellano and Reich, 1984; Walsh, 1984). Kinetic parameters for suicidal inactivation include K_I, the apparent dissociation constant for the inhibitor from the enzyme; $T_{1/2}$, the time taken to reach half maximal inactivation of the enzyme at saturating inactivator concentrations; and k_{inact}, the inactivation rate constant, which represents the maximal rate of inactivation, also determined at saturating inactivator concentrations (Rando, 1984; Waley, 1985).

2. Mechanism-based inactivators of P450: Classification

Mechanism-based P450 inactivators may be classified into three general categories: 1. Those which inactivate P450 by binding covalently to its apoprotein; 2. those which destroy the prosthetic heme moiety by N-alkylating it; or 3. those that cause degradation of the heme moiety with subsequent binding of heme or its derivatives to the apoprotein, presumably at its active site.

a. Compounds which bind to the apoprotein

Many compounds are activated by P450 to a species which binds covalently to the P450 apoprotein. Several unsaturated compounds such as 1-ethynylpyrene or secobarbital are activated by P450 to reactive species which bind to the P450 apoprotein, resulting in its catalytic inactivation (Lunetta et al., 1989; Gan et al., 1984). Two of the major classes of compounds which covalently bind to P450

apoprotein will be discussed in depth: those containing either a sulfur or a halogen atom in their structure.

i. Sulfur compounds

Many sulfur compounds inactivate P450 in a manner which requires oxidation by P450, although such inactivation is not genuine "suicidal" inhibition. GSH, an important cellular nucleophile, is often used to trap reactive electrophiles released from the active site of an enzyme. Because it apparently cannot gain access to the P450 active site, GSH serves as a probe of whether the P450 inactivation observed is truly suicidal. Compounds such as methimazole inactivate P450 in such a non-suicidal manner, wherein GSH is able to completely attenuate such inactivation (Kedderis and Rickert, 1985).

The mechanism of parathion activation by and subsequent binding to P450 has been extensively studied. When ^{35}S-labelled parathion was incubated with liver microsomes in the presence of NADPH, covalent binding to the microsomal protein was observed, whereas no detectable binding occurs when the compound was ^{14}C-radiolabelled (Neal et al., 1977; Halpert and Neal, 1981). ^{35}S-Parathion incubation with purified P450IIB1 (supplemented with NADPH) resulted in the covalent binding of approximately 4 nmol of sulfur per nmol of enzyme, 50-75% of which was removed by the addition of cyanide or dithiothreitol, suggesting the presence of hydrodisulfides in such covalent linkage. The prosthetic heme moiety of P450 as revealed by its chromophore was lost following parathion administration, although the fate of the heme was unclear.

CS_2-mediated inactivation of P450 is similar to that of parathion; after its P450-dependent metabolic activation, atomic sulfur appears to be the species which covalently binds to the P450 apoprotein (De Matteis, 1974). However, P450 heme is not destroyed concomitantly and has been speculated to be released intact (Jarivisalo et al., 1978) because of the observed increase in heme catabolism to bile pigments following carbon disulfide administration.

Diethylthiocarbamide destroys P450 in vivo in a manner which results in release of intact heme, which in turn induces heme oxygenase activity (Miller et al., 1983). δ-Aminolevulinic acid synthetase activity is induced as a consequence of the increased rate of heme degradation and depletion of the hepatic heme pool. Addition of GSH in vitro abolishes such P450 destruction (Miller et al., 1983).

Many sulfhydryl compounds such as n-butanethiol (Flowers et al., 1987), and various thiosteroids (Menard et al., 1979b, Bednarski and Nelson, 1989) have been shown to suicidally inactivate several forms of P450. It has been proposed that this inactivation occurs as a consequence of the binding of atomic sulfur to proteins. Covalent binding of 6-mercaptopurine to microsomal proteins is believed to be the result of a P450-catalyzed oxidation of the compound to a sulfenic acid species which binds to protein sulfhydryls (Hyslop and Jardine, 1981a; 1981b; Abraham et al., 1983). The relationship of this covalent binding to P450 inactivation has been speculated upon.

ii. Halogen-containing compounds

Chloramphenicol is an extensively studied halogenated P450 inactivator. P450-mediated hydroxylation of this substrate is followed by hydrochloric acid loss and the subsequent formation of an acyl chloride which is believed to efficiently acylate at least one lysine residue of the P450 apoprotein (Halpert, 1981). Such apoprotein alkylation appears to occur without disruption of the normal CO-induced Soret spectrum and therefore without heme loss (Halpert and Betner, 1983). Acylation of this lysine residue interferes with electron transport from P450-reductase to P450IIB1 (Halpert et al., 1985). Analogs of chloramphenicol have been assessed in different P450 systems, some of which possess differential isozyme specificity and are able to cause heme loss in addition to that of P450 (Miller et al., 1986).

b. Compounds which destroy P450 heme through formation of N-alkylporphyrins

The second category of P450 suicide substrates (covalent binding to the prosthetic heme moiety of P450) includes those which form N-alkylporphyrins or green pigments (so called because livers of animals treated with such compounds appear greenish). These suicide substrates can be further classified into 2 major structural classes; 1). Compounds possessing a terminal unsaturation (i.e. olefins or alkynes); and 2). Dihydropyridines or quinidines, and N-N containing compounds.

i. Terminal olefins and acetylenes

Metabolic activation of terminal olefins by P450 isozymes partitions between epoxide and N-alkylporphyrin formation, where the epoxide

intermediate does not participate in green pigment formation (Ortiz de Montellano et al., 1979). Much of the initial N-alkylporphyrin structure determination was done with novonal and allylisopropylamide (AIA) by Ortiz de Montellano and coworkers (Ortiz de Montellano et al., 1982; 1983a; 1983b). The ultimate N-alkylporphyrin or green pigment, was shown to result from the addition of activated oxygen to the internal carbon site of unsaturation, and the pyrrolic nitrogen of P450 heme to the terminal carbon (Ortiz de Montellano et al., 1983a). A metallaoxetane (or ene for alkyne) intermediate (a four membered ring structure between the heme iron, oxygen and the two carbons of the olefin or acetylene, with the internal carbon of the desaturation binding the oxygen) has been postulated as an intermediate in N-alkylporphyrin formation (Ortiz de Montellano et al., 1983b; Komives and Ortiz de Montellano, 1987).

ii. Dihydropyridines and N-N containing compounds

The second and third class of suicide inactivation include the 4-alkyl-substituted dihydropyridines and N-N function-containing compounds (such as hydrazines) which are believed to involve a one electron oxidation of a nitrogen atom followed by rearrangement and elimination of a radical species. Subsequent reaction of the resultant radical with one of the pyrrolic nitrogen(s) of the prosthetic heme yields the N-alkylporphyrin. In some cases (such as with some of the phenylhydrazines) binding of the radical to the heme iron of myoglobin (a model for P450) occurs before migration to the porphyrin nitrogen (Ringe et al., 1984). A complex between phenyldiazine and the heme iron of bacterial $P450_{cam}$ has recently been characterized (Raag et al., 1990). 1-Aminobenzotriazole

activation by P450 results in a species which apparently bridges the P450 porphyrin moiety through two of its pyrrole nitrogens (Ortiz de Montellano and Matthews, 1981).

The mechanism for 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine (DDEP) has been extensively studied and is believed to involve a one electron oxidation of the pyridine nitrogen by P450 followed by 4-ethyl radical extrusion and aromatization to the pyridine. The N-ethylated heme adduct has been structurally characterized (Ortiz de Montellano et al., 1981). The ethyl radical has been spin trapped with α-(4-pyridyl-1-oxide)-N-t-butyl-nitrone (POBN), and the resultant adduct detected by electron spin resonance (Augusto et al., 1982). The 4-isopropyl analog of DDEP has also been spin trapped by POBN and detected by electron spin resonance (Lee et al., 1988). Recently, the possibility of contaminating trace metal catalysis in the formation of such radicals has been raised (Kennedy and Mason, 1990), thereby challenging the notion that the spin adduct constitutes evidence of one electron oxidations by P450.

c. Compounds which inactivate P450 by producing covalent binding of heme derivatives to apoproteins

In the third mechanism of suicidal inactivation covalent binding of the heme (or fragments thereof) to the P450 apoprotein occurs. Peroxide-mediated destruction of P450 heme and accompanying microsomal bleaching has long been recognized (Blake and Coon, 1980). Guengerich observed such heme loss in a reconstituted P450IIB1 system, with covalent binding of ^{3}H-heme to P450IIB1 (Guengerich,

1978). Hydrogen peroxide was believed to be responsible for heme destruction, since inclusion of sodium azide, a catalase inhibitor, was required for such an observation. A small portion of the P450 heme destroyed in liver microsomal systems could be accounted by polar metabolites, identified as mono- and dipyrrolic heme fragments (Schaefer et al., 1985). Such binding of heme to the P450 apoprotein was shown to occur in the carbon tetrachloride-mediated destruction of P450 (Davies et al., 1985). Covalent binding of heme derivatives to microsomal proteins appears to account for a large fraction of the destroyed heme during cumene hydroperoxide-mediated heme destruction (Guengerich, 1986; Decker et al., 1986; Correia et al., 1987).

Covalent binding of heme derivatives to microsomal proteins is also associated with the destruction of heme by several other P450 suicide substrates, many of which, such as norethindrone (NOR) and AIA also result in N-alkyl porphyrin formation as well as irreversible substrate binding to the apoprotein (Davies et al., 1986; Bornheim et al., 1987). The mechanism for irreversible heme-protein binding remains to be established. At present however, it appears that all of the substrates which exhibit such irreversible binding of heme (or its derivatives) to microsomal proteins are also those which are potentially metabolized by P450 to free radical species. Conceivably, such radicals can react quite easily with the electron rich heme (porphyrin) moiety (Griesbaum, 1970).

Recently an intact heme moiety was found bound to myoglobin following $BrCCl_3$ treatment (Osawa and Pohl, 1989). A similar intact

heme-myoglobin adduct (where the heme moiety is linked to a tyrosine residue) was characterized from a reaction between horse myoglobin and H_2O_2 (Catalano et al., 1989). Irreversible P450 heme binding to protein also occurs after DDEP-mediated P450 inactivation in vivo, and is associated with enhanced loss of P450 apoproteins, conceivably through increased proteolytic degradation (Correia et al., 1987). Whether the heme-alkylation is a signal for enhanced degradation of proteins in cells, remains to be determined.

D. Spironolactone

1. Historical perspectives

Spironolactone (SPL), 3-(3-oxo-7α-acetylthio-17ß-hydroxy-4-androsten-17α-yl)propionic acid γ–lactone is an antimineralocorticoid potassium-sparing diuretic which functions as a competitive inhibitor of aldosterone at the receptor level in the collecting duct of the kidney cortex (Corvol, 1981). SPL (Aldactone$^{R)}$ is used clinically in the treatment of congestive heart failure, essential hypertension, cirrhosis of the liver, primary hyperaldosterism and other conditions causing edema (Overdiek and Merkus, 1987). Canrenone (CAN) and potassium canrenoate (SPL analogs lacking the sulfur moiety) are used clinically in Europe, though SPL is the most commonly used form throughout the world and differs from its analogs in that it possesses no agonist activity (Feldman, 1978). As SPL use was associated with side effects such as gynecomastia and decreased libido (Spark and Melby, 1968), and also with decreases in the levels of both testosterone (Dymling, 1978) and cortisol (Greiner et al., 1976), its effects on the steroidogenic P450 enzymes catalyzing the synthesis of these hormones were assessed by various groups in the 1970s. The effects

of SPL on hepatic P450 forms (Gerard and Feller, 1970) were also examined, as alteration of these P450 isozyme levels affect the metabolism of endogenous compounds including steroid hormones (Waxman, 1986).

2. Effect of SPL on hepatic drug metabolism

Pretreatment of female rats with SPL resulted in a significant attenuation of the pharmacological effects of administered pentobarbital (Selye et al., 1969). The observed attenuation of pentobarbital sleep time was subsequently found to be due to enhanced hepatic metabolism of pentobarbital following chronic SPL administration to female rats (Solymoss et al., 1969). Stripp et al. (1971), later found that chronic SPL administration (100 mg/kg, twice daily for 4 days) to female rats resulted in a 2-4 fold enhancement of EMND, hexobarbital (HB) oxidation, and 3,4-benzpyrene (BP) hydroxylation. In male rats treated similarly, only EMND activity was increased while HB and BP hydroxylation activities were significantly decreased. Hepatic P450 content however, was decreased in both sexes. The effect of SPL on BP- hydroxylase activity in male rats could be reversed if 17α-methyltestosterone (an androgen) were given in concert with SPL (Menard et al., 1974a). This finding together with the observation that SPL inhibited the effects of exogenous methyltestosterone on BP and HB hydroxylases in female rats (Stripp et al., 1971), indicated that reduced testosterone synthesis in males might be partially responsible for such SPL-mediated effects. These findings in male rats are consistent with the testosterone-dependent expression of several male-specific P450 forms such as P450s IIIA and IIC11 (Einarsson et al., 1973; Dannan et al., 1986). These findings

also provided explanations for earlier reports documenting the antiandrogenic effects of SPL, such as gynecomastia in humans (Spark and Melby, 1968). Interestingly, SPL antagonized androgen-induced weight increases of rat seminal vesicles (Steelman et al., 1969), indicating a possible direct competition between SPL and testosterone for the testosterone receptor. Thus, at that time, the effects of SPL on some hepatic P450 activities were believed to be indirectly mediated through its reduction of testosterone levels.

The paradoxical increases in EMND activity in male rats, and EMND, and HB- and BP-hydroxylases in female rats, were later shown to be due to the induction of the steroid-inducible P450IIIA isozymes (Lu and Conney, 1972; Elshourbagy and Guzelian, 1985; Heuman et al., 1982; Section IA3a). These isozymes exhibit efficient metabolism of EM and are inducible to a greater extent in female rather than male rats by SPL administration (Elshourbagy and Guzelian, 1985; Waxman et al., 1985). Thus, the increases in these functional activities could be attributed to P450IIIA induction by SPL.

SPL was also found to directly destroy ~20% of hepatic P450 content in vitro, when non-pretreated (NT) male rat hepatic microsomes were assessed for NADPH-dependent SPL-induced P450 loss (Menard et al., 1979a).

3. Effects of SPL on adrenal and testicular P450

When acute effects of SPL were assessed in vivo, an 80% decrease in testicular P450 content, with a concomitant heme loss was observed in rats (Menard and Gillette, 1974a). Steroid 17α–OHase activity

(attributable to a key P450 enzyme in testosterone synthesis from pregnenolone) was also found to be significantly inhibited, whereas steroid 17β–hydroxylase and cytochrome b_5 were unaffected (Menard and Gillette, 1974a) following SPL administration. Thus, SPL appeared to inhibit testosterone-dependent hormonal action not only by inhibiting an enzyme required for its formation, but also by competing with it for its receptor at its site of action. Interestingly, incubation of testicular microsomes with SPL in vitro, resulted in no P450 loss (Stripp et al., 1973); thus a metabolite of SPL was believed to produce the observed testicular P450 inactivation. When adrenal microsomes (preparations containing high P450 dependent 17α-OHase activity in species producing cortisol rather than corticosterone) were assessed for SPL-mediated P450 destruction in vivo, dramatic losses of both P450 content and 17α-OHase activity were observed in cortisol-producing species such as the guinea pig and dog (Menard et al., 1974b), confirming the susceptibility of this particular adrenal P450 to SPL-mediated inactivation. Greiner et al. (1976) observed decreased cortisol production in those species with functional 17α-OHase, but no loss of corticosterone in the rat, supporting the selective inactivation of P450 dependent 17α-OHase by SPL in vivo. In addition to 17α-OHase, progesterone 21-hydroxylase, another enzyme instrumental in cortisol synthesis was found to be decreased in parallel after SPL administration to cortisol-producing animals (Menard et al., 1976).

4. Previous studies employing SPL analogs

The structural requirements of several SPL analogs for P450 destruction have been assessed. Menard et al. (1978) found that administration (100 mg/kg, for 4 hr) to dogs of either SPL or 7α-thio-

SPL (SPL-SH, the deacetylated form of SPL) resulted in 50-65% decreases in testicular venous plasma testosterone levels, whereas the desulfurated SPL analogs CAN or soldactone [the γ-hydroxyacid of CAN; (SOLD)] failed to do so. SPL-SH was proposed to be a SPL metabolite based on the isolation of known urinary S-methyl SPL derivatives in human urine (Karim et al., 1975). Adrenal venous plasma cortisol concentrations were also dramatically decreased in this study after either SPL or SPL-SH administration.

Menard and coworkers extended these studies with SPL analogs in vitro (Menard et al., 1979a), using microsomes prepared from the adrenal or testicular glands of rats, guinea pigs and dogs, and found that NADPH was required, and that SPL-SH was more potent than SPL in its ability to induce P450 and associated heme loss. The desulfurated analogs (CAN and SOLD), as well as the S-methyl SPL metabolite (SPL-SCH_3) and its corresponding sulfoxide (SPL-$S(O)CH_3$) and sulfone (SPL-$S(O_2)CH_3$) derivatives produced virtually no P450 loss in vitro. These studies revealed an important role for the sulfur moiety (as either a thioester or thiol, but not S-methyl) of SPL in its destruction of P450.

5. Mechanistic studies

To examine the role of the thiol moiety of SPL in P450 destruction, thiol analogs of testosterone (a substrate metabolized more selectively by P450 isozymes in the liver than those in the adrenals and testes) were assessed relative to SPL-SH for their P450 destructive capacity in microsomal preparations from the three aforementioned guinea pig organs (Menard et al., 1979b). In vitro, SPL produced marginal (i.e.

10-20%) losses of rat hepatic P450, whereas 7α-thiotestosterone was found to produce larger (25-30%) losses of hepatic P450 with negligible effects on adrenal and testicular microsomal P450 content, thereby indicating the relative specificity of hepatic microsomal P450s in testosterone hydroxylation. The 7α-hydroxytestosterone derivative caused no P450 loss, indicating a critical role of the thiol moiety in such inactivation.

To provide insight into the mechanism of P450 destruction by SPL, NADPH-dependent metabolism of radiolabelled SPL (either ^{35}S- or C21-^{14}C-) by guinea pig adrenal and testicular microsomal proteins was assessed. These studies (Menard et al., 1979b) demonstrated that the sulfur atom of SPL appears to be eliminated during its metabolic activation, and covalently binds to the P450 apoprotein, resulting in spectrophotometrically detectable heme loss and P450 inactivation similar to those observed during parathion-mediated P450 inactivation.

6. Metabolism of SPL

SPL is extensively metabolized by many species to two major categories of products: those which retain the sulfur moiety and those which do not (Figure 4).

a. Non-sulfur containing metabolites

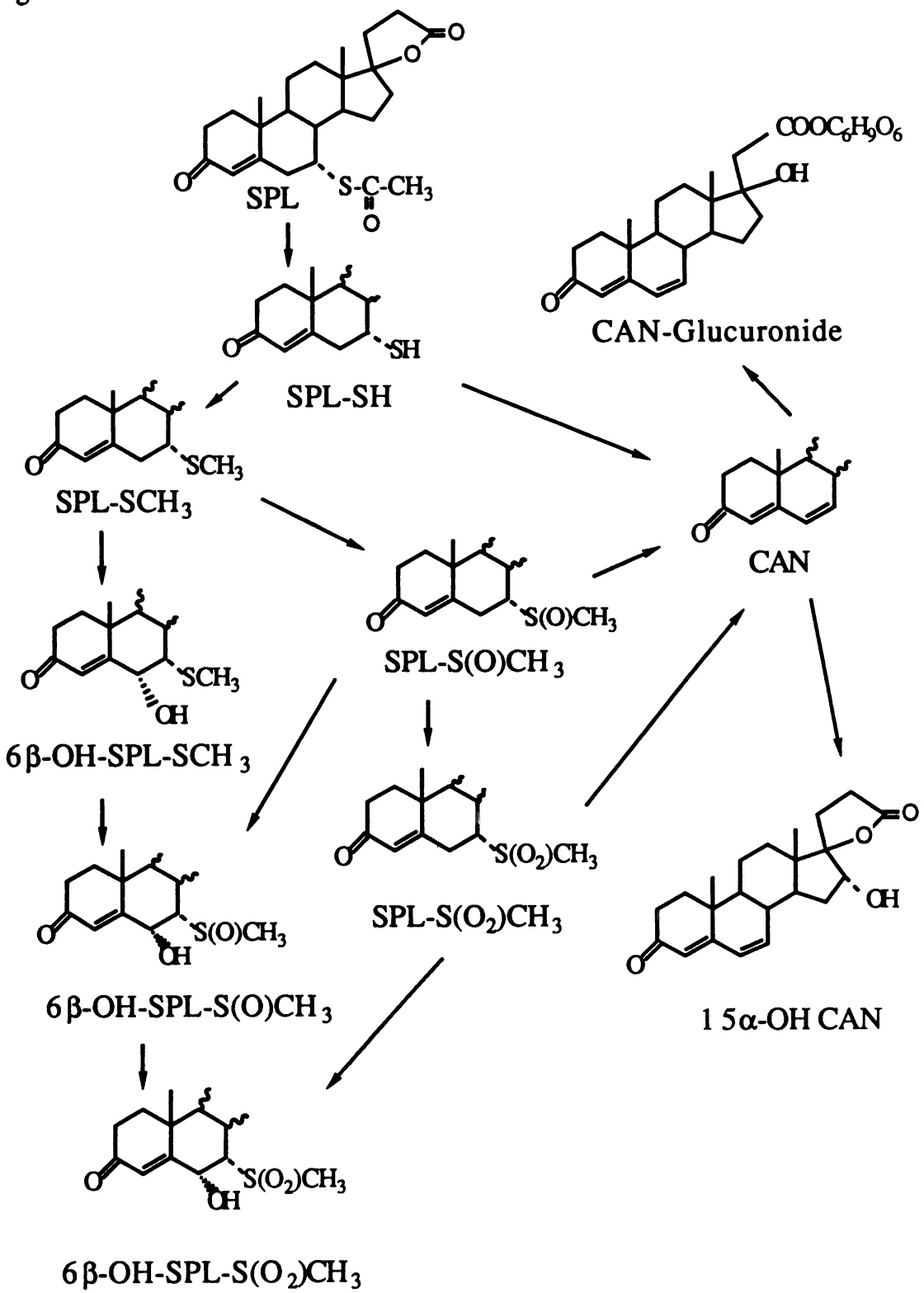
The major non-sulfur-containing metabolite of SPL in humans was CAN (Sadee et al., 1974), which was the primary SPL metabolite in vivo (Sadee et al., 1974; Karim, 1978) until different procedures (i.e. HPLC with products monitored at different absorbances) were employed to assay for the α,β-unsaturated ketone "240 nm"-

chromophore of SPL (Overdiek et al., 1985). Thioester deacetylation of SPL to SPL-SH followed by H_2S elimination was postulated to yield CAN. CAN undergoes hydrolysis of its lactone ring to canrenoate which was excreted in the urine primarily as the glucuronide ester conjugate, CAN-glucuronide (Karim et al., 1975). 15α-hydroxy CAN (15α–OH CAN) has also been identified as a minor urinary metabolite in man (Karim et al., 1975). Cook et al. (1988) subsequently found that CAN is metabolized to the 6α-7α or 6β-7β epoxide derivatives and that the 6β–7β epoxide may be further metabolized to its glutathione conjugate by rat hepatic cytosol. Both the 3α– and 3ß-hydroxy-derivatives of the 6ß-7ß epoxide have been found to act as direct mutagens in the mouse lymphoma assay (Cook et al., 1988).

b. Sulfur-containing metabolites

The earliest identified sulfur-containing metabolites were the epimeric sulfoxides and sulfone of SPL-SCH_3 as minor forms in human urine, which were believed to rapidly eliminate the sulfur entities to yield CAN (Karim and Brown, 1972). The sulfoxides 6ß-OH-SPL-$S(O)CH_3$ (Karim and Brown, 1972) and sulfone 6ß-OH-SPL-$S(O_2)CH_3$ (Abshagen et al., 1976) were identified as prominent metabolites in human urine, though the introduction of the 6β-hydroxyl group in these compounds did not permit sulfur elimination to CAN. The 6ß-hydroxy derivative of SPL-SCH_3, 6ß-OH-SPLSCH_3 had been isolated from human plasma and urine (Karim et al., 1975) and is the logical precursor of the above mentioned major urinary sulfoxide(s) and sulfone. The thiomethyl compounds were believed to arise from SPL deacetylation followed by its S-methylation, though neither the sulfhydryl nor S-methyl compound was isolated before 1980.

Figure 4. SPL Metabolism



Refer to text for description of structures.

Colby's group later isolated the thiol derivative of SPL in guinea pig microsomal preparations from different tissues with the capacity to deacetylate. The deacetylation order was found to be liver>kidney>adrenal>testes (Sherry et al., 1981). These studies were extended to show that SPL-SH formation could be blocked by the addition of the organophosphate esterase inhibitor bis-*p*-nitrophenylphosphate (BNPP), and that SPL-mediated guinea pig adrenal P450 loss required formation of SPL-SH (Sherry et al., 1986), consistent with Menard's earlier findings (Menard et al., 1979a). In vitro studies thus substantiated the previously proposed deacetylation of SPL as a required step in SPL-mediated P450 loss. In 1985, SPL-SCH_3 was indeed found to be the primary in vivo metabolite of SPL (Overdiek et al., 1985). Requirements for S-adenosylmethionine addition in the in vitro S-methylation of SPL to SPL-SCH_3 by microsomal S-methyltransferase activity of guinea pig hepatic and renal microsomal preparations were also established by Colby's group (Lacagnin et al., 1987). Methylation of SPL-SH by red blood cells had been previously shown to occur (Keith et al., 1984).

More recently, Cashman and Pena more elegantly showed that S-oxygenation of S-methyl SPL (to form the epimeric sulfoxides) is primarily catalyzed by the hog liver FMO (Cashman and Pena, 1989b), and that these S-oxide compounds rapidly eliminate to form CAN through a general base-catalyzed mechanism (Cashman and Pena, 1989a), thereby providing direct evidence for the pronounced CAN formation generally observed. These studies

experimentally confirmed the previous speculation that CAN is derived from oxidized SPL species.

II. Objectives

SPL is a commonly used diuretic. Its use has been contraindicated for porphyric patients, although the mechanistic basis for this has not been revealed. SPL has been shown to cause decreased libido and gynecomastia in patients (Spark and Melby, 1968; Section ID1). A partial explanation for the observed clinical manifestations comes from animal model systems where SPL suicidally inactivates adrenal and testicular P450 17α-OHase (a key enzyme in androgen biosynthesis) both in vivo and in vitro (Menard et al., 1974a; 1974b; Section ID3). Acutely administered, SPL produces modest decreases in rat hepatic P450 levels (Stripp et al., 1971; Menard et al., 1979a; Section ID2), whereas repeated SPL administration induces immunodectable levels of P450IIIA (Heuman et al., 1982). These findings are reminiscent of the effects of some compounds (such as SB and AIA) which are able to induce the same P450 isozyme which they initially inactivate (De Matteis, 1973; Levin et al., 1973).

Therefore, as SPL was observed to inactivate adrenal and testicular 17α-OHases and hepatic P450 acutely, and to induce hepatic P450IIIA when administered chronically, the initial objectives of this study were to assess its acute effects on rat hepatic P450IIIA to determine whether it is an inactivator/inducer of this hepatic isozyme. Initial studies proved that SPL does selectively inactivate both P450IIIA and P450IIC11 (Sections IVA; IVB).

The suicidal inactivation of P450 by sulfur-containing compounds is well known, and a variety of reactive species have been postulated to account for this inactivation (Neal et al., 1977; Hyslop and Jardine 1981a; Section IC2ai), although the precise mechanisms by which P450 oxidizes sulfur-containing compounds are at present poorly understood. The role of the thiol moiety of SPL in adrenal and testicular P450 inactivation had been established by (1) studies which showed the requirement for deacetylation of SPL to SPL-SH (Sherry et al., 1986; Section ID6b) for P450 destruction to occur and (2) the observed failure to destroy P450 when SPL is either desulfurated or when its thiol function is blocked (Menard et al., 1978; 1979a; Section ID4). At the time that these studies were embarked on, the mechanism of P450-mediated oxidation of thiol-containing compounds such as SPL-SH had not been elucidated, although its involvement in suicidal inactivation of P450 had been extensively documented.

Therefore, the second objective of this proposal was to examine the criteria for suicidal inactivation of P450IIIA by SPL-SH as well as to scrutinize the course of P450IIIA-dependent metabolism of SPL-SH, in order to investigate and establish the relationship between these two processes, to provide insight into the mechanism by which SPL-SH specifically inactivates P450, as well as to elucidate how sulfur-containing compounds, in general, are oxidized by P450.

III. Materials and Methods

Materials

DEX, GSH, isocitrate, isocitric dehydrogenase, catalase (CAT), superoxide dismutase (SOD), Protease type VII, Ellman's reagent, dimyristoyl phosphatidylcholine, bovine serum albumin (BSA), ERY, Tween 80 and hemin were purchased from Sigma Chemical Co, St Louis, MO. SPL was purchased from Calbiochem Inc., San Diego, CA or Sigma. The SPL analogs, SPL-SH, SPL-SCH_3, SOLD, CAN and the radiolabelled compounds, ^{14}C-SPL, ^{14}C-CAN and ^{14}C-SOLD (specific activity, 57.7, 70.5 and 60.7 μCi/mg, respectively) were generously donated by Searle Chemical Co., Skokie, Ill. 4-^{14}C-δ-Aminolevulinic acid (53.2 mCi/mmol), 1,4-^{14}C-testosterone (51 mCi/mmol), and (glycine 2-H)-^{3}H-glutathione (1 Ci/mmol) were purchased from New England Nuclear, Boston MA. TAO was a gift from Dr J. J. Korst, Pfizer Inc., CN. NOA, BNPP, PCNMA, cumene hydroperoxide and ßNF were obtained from Aldrich Chemical company. Scintiverse L.C. was purchased from Fisher Scientific, Pittsburgh, PA. Sodium phenobarbital came from Mallinckrodt Inc., Paris, KY. NADPH was obtained from Boerhinger Mannheim, West Germany. All other chemicals were of reagent grade and obtained from commercial sources.

Synthesis of SPL-SH

SPL (50 mg) was deacetylated using an equimolar amount of sodium methoxide. The reaction was performed in 20 ml of methanol and incubated at 33°C for 4 h. The resultant mixture was filtered and subjected to high pressure liquid chromatography (HPLC) using a C18 Rainin Dynamax column (8μ particle size, 21.4 mm X 25 cm; with guard

column 21.4 mm X 5 cm), connected to a Hewlett-Packard 1040A diode array (UV) detector set at 240 nm. The mobile phase consisted of an isocratic system of 80% methanol in water run at 10 ml/min. SPL-SH eluted at 13 min, as confirmed with an authentic sample run in parallel. Samples were collected, the methanol was evaporated under a stream of nitrogen and the resultant aqueous mixture (~5 ml) extracted three times with ethyl acetate (EtOAc; 5 ml, each). The EtOAc extracts were combined and evaporated under nitrogen to obtain SPL-SH.

Treatment of Rats

Male or female Sprague Dawley rats, from Bantin Kingman Labs, Gilroy, CA (200-250g) were injected intraperitoneally (i.p.) with PCN (100 mg/kg, in corn oil, daily for 3 days), DEX (100 mg/kg, in corn oil, daily for 3-4 days), PB (80 mg/kg, in water, daily for 4-5 days), or ßNF (80 mg/kg, in corn oil, daily for 4 days). When TAO pretreatment was included in the protocol, DEX- or PB-pretreated rats were given a single i.p. injection of TAO (500 mg/kg, in corn oil) 3 h before sacrifice. SPL (100-750 mg/kg, i.p., suspended in water with a few drops of Tween 80) was administered for 1-15.5 h. Maximal hepatic P450 destruction was observed at SPL doses of 150 mg/kg administered for 3 h.

Preparation of rat hepatic microsomes

Rats were decapitated, livers excised and perfused in ice cold 1.15% potassium chloride (KCl). A 50% (w/v) homogenate was prepared in 0.1M potassium phosphate buffer, pH 7.4 with a Blessig homogenizer

(50 ml). Homogenates were centrifuged at 9,000 xg at 4°C for 15 min to remove unbroken cells, nuclei, lysosomes and mitochondria. The resultant supernatant was centrifuged at 105,000 xg at 4°C for 60 min. The supernatant of this centrifugation step was discarded and the pellet resuspended in 1.15% KCl and recentrifuged for 30 min at 105,000 xg at 4°C to obtain 'washed microsomes'. The pellet of this centrifugation step was suspended in 8-15 ml of 0.1M potassium phosphate buffer, pH 7.4.

Preparation of human adrenal microsomes

Human adrenals were obtained from Dr. Allan Rettie, University of Seattle, WA. The tissue was minced with scissors and homogenized (25%, w/v homogenate) in a 0.02 M Tris-HCl buffer pH 7.4 at 4°C using a polytron homogenizer. All subsequent steps for microsomal preparation were identical to those for hepatic microsomal preparation, except that Tris buffer was used instead of phosphate buffer.

Purification of P450IIC11 and P450 reductase

P450IIC11 and P450-reductase was kindly supplied by Dr. K. Yao. The purification procedures are detailed in Sugiyama et al. (1989) and Shepard et al. (1983), respectively.

Purification of hog FMO

Hog liver FMO was generously supplied by Dr. J. R. Cashman, UCSF. The purification procedure is described in Sabourin et al. (1984).

Protein Determination

100- or 50-fold dilutions of liver or adrenal microsomes in double distilled water were made and protein concentration determined by the method of Lowry et al. (1951) using BSA standards.

Heme determination

Heme concentrations were determined by the method of Omura and Sato (1964). Microsomal preparations or in vitro microsomal incubation mixtures were dissolved in 2 volumes of a solution of 20% pyridine in 0.1N NaOH and mixed well by vortexing. A few grains of sodium dithionite were used to reduce the heme iron from its ferric to ferrous state. The reduced vs. oxidized pyridine-hemochromogen difference spectra were recorded between 500 and 620 nm on an Aminco DW-2(a) scanning UV-vis spectrophotometer. The difference in absorbance between 557 and 600 nm was measured and an extinction coefficient (ε) of 34.2 $mM^{-1}cm^{-1}$ used to determine microsomal heme concentrations.

P450 determination

P450 concentration was determined by the method of Estabrook et al. (1972) where the difference spectrum of the CO-bound reduced vs. CO-bound oxidized cytochrome was measured. Determinations were made using an Aminco DW-2(a) scanning uv/vis spectrophotometer. Microsomes (or aliquots of incubation mixtures) were diluted with 0.1M potassium phosphate buffer, pH 7.4 and gassed with CO (which had been passed through a deoxygenating solution consisting of 0.1g sodium anthraquinone sulfonic acid, and 1.0 g sodium dithionite

dissolved in 100 ml of 0.1N NaOH), and baselines between 400 and 500 nm established. Reduction of such samples were achieved by adding a few grains of sodium dithionite to the sample cuvette, and the difference in absorbance between 400 and 500 nm recorded. The difference in absorbance between 450 and 490 (ε=100 $mM^{-1}cm^{-1}$) was measured to determine P450 concentrations.

Determination of N-demethylase activities

N-demethylation of EM, ERY, benzphetamine (BZP), and PCNMA was assayed by determining formaldehyde produced by the method of Nash (1953). Reaction mixtures (5 ml or 1ml, final volume) contained 2 mM EM, 1 mM ERY, 1 mM BZP, or 1 mM PCNMA. A NADPH-regenerating system was utilized, consisting of NADPH (1 mM), isocitrate (5 mM), isocitrate dehydrogenase [0.1 unit (where 1 unit converts 1.0μmol of isocitrate to α-ketoglutarate per min at pH 7.4 at 37°C) per ml incubation], and magnesium chloride (2 mM). The buffer utilized consisted of: 7.5 mM semicarbazide-HCl in sodium potassium phosphate buffer, pH 7.4. Microsomes (1mg/ml, final concentration) were added to start the reaction after a 3 min preincubation period at 37°C. Reactions were allowed to proceed 15 min and were terminated by the addition of 40% of the incubation volume of a 5.5% w/v solution of zinc sulfate heptahydrate, shaken another 2 min, then 40% of the original incubation volume of a 4.5% w/v solution of barium hydroxide octahydrate added. The resultant mixtures were centrifuged in an International Centrifuge for 15 min (~5,000 rpm). For 1 ml-incubations, 1 ml of the supernatant was added to 0.4 ml Nash reagent [15% ammonium acetate (w/v), 0.2% acetylacetone (v/v), 0.3% glacial

acetic acid (v/v) in distilled water], thoroughly mixed and heated at 60°C for 20 min. The samples were allowed to cool to room temperature and were monitored at 412 nm (ε=7.7 $mM^{-1}cm^{-1}$) in a Beckman B or DU-20 spectrophotometer.

Testosterone hydroxylase activities

Regio- and steroselective testosterone hydroxylases were assayed by the method of Wood et al. (1983), with modifications by Bornheim et al. (1987). Incubations contained ^{14}C-testosterone (0.14 µCi, 0.25 mM), a NADPH-regenerating system (as previously described), 0.1 mg/ml microsomal protein, in 0.1 M potassium phosphate buffer pH 7.4. Reactions were initiated by the addition of microsomes and incubated for 10 min at 37°C. The reactions were terminated by the addition of EtOAc or methylene chloride (3 ml), and fixed amounts of cold, hydroxylated testosterone metabolites were added as carriers before extractions with the organic solvent originally used (2 additional times). The organic layers were combined, dried under nitrogen, and dissolved in 0.4 ml of Solvent A [43% methanol (MeOH), 1.1% acetonitrile, in water]. 0.2 ml of such extracts were injected on a Rainin C-18 Microsorb column (5µm particle size, 4.6 mm X 15 cm) equipped with a guard column (4.6 mm X 5 cm). The column was equilibrated with 80% solvent A and 20% solvent B (75% methanol, 1.9% acetonitrile, in water) and a solvent gradient developed at 3 min by a progressive increase in solvent B to 37.5, 60 and 100% for 15, 8, and 1.5 min, respectively. The metabolites were detected by absorbance at 254 nm, individually collected, added to 15.0 ml

Scintiverse LC and quantitated using a Beckman LS 7000 scintillation counter.

In vitro incubation systems with hepatic microsomes

A typical incubation mixture (final volume 1.5, 3, or 6 ml) contained hepatic microsomes (2-3 μM P450); NADPH (1 or 2 mM); ethylenetriaminetetraacetic acid (EDTA) or diethylenetriaminepenta-acetic acid (DTPC) (1.5 mM); and SPL, CAN, SOLD, SPL-SH or SPL-SCH_3 (0.5 or 1.0 mM final concentration); in 0.1 M phosphate buffer pH 7.4. The 6.0 mM steroid stock solutions were made up in PC buffer (25 mg phosphatidylcholine sonicated in 100 ml of 0.1M potassium phosphate buffer, pH 7.4) which was again sonicated for 3 min after addition of the appropriate steroid, and again immediately before addition to the incubation mixture. Reaction mixtures were preincubated for 15 min at 37°C in a shaking water bath to allow deacetylation of SPL to SPL-SH, then initiated by the addition of NADPH. Reactions were terminated at either 15 or 30 min with an atmosphere of CO and chilling in ice. In various experiments, SOD (0.1 mg/ml), CAT (0.5 mg/ml), BNPP (0.4 mM), NOA (3 or 5 mM), or GSH (1 or 5 mM) were included.

In vitro incubations with human liver microsomes

Incubations contained 3 μM P450, 0.5 mM SPL-SH (from a 6 mM stock solution in PC buffer), 1 mM NADPH, and 1.5 mM DTPC. Incubations were initiated with NADPH and allowed to proceed 15 min at 37°C before termination on ice. For determination of testosterone activities, 3.0 ml aliquots of the incubations were centrifuged at

105,000 xg for 30 min, the microsomes obtained, resuspended in 1.0 ml phosphate buffer and protein concentration determined. Microsomal protein (5 mg) was rehomogenized in 5 ml (final volume) 0.1 M phosphate buffer pH 7.4 containing 5 mg BSA. This step was performed to remove any non-specifically bound SPL-SH from the microsomes which could have interfered with the testosterone assay. The suspension was then sedimented at 105,000 xg, the supernatent decanted and the microsomal pellet resuspended in 0.1M phosphate buffer pH 7.4, and protein concentration determined. Such resuspended microsomes were then assayed for testosterone hydroxylase activities as previously described.

In vitro incubations with human adrenal microsomes

Incubations contained adrenal microsomes (0.5 μM P450); 1.5 mM EDTA; 1.15% KCl; SPL-SH (0.5 mM final concentration, from a 3 mM stock solution in PC buffer, identical to previously described except that Tris buffer was again utilized instead of phosphate); and NADPH (1 mM) in 0.05 M Tris-HCl buffer pH 7.4. Control incubations which excluded the steroid or NADPH were performed. NADPH was added to initiate the reactions, which were allowed to proceed for 15 min and were stopped by chilling on ice and with an atmosphere of CO.

In vitro incubations with purified P450IIC11 (P450h)

P450IIC11 was reconstituted by a 10 min preincubation of P450IIC11 (0.6 nmol/ml), NADPH-P450 reductase (~600 units/0.1 nmol of P450), and 20 μg of dilauroylphosphatidylcholine (sonicated in 0.1 M phosphate buffer, pH 7.4) at 37°C. The complete reaction mixture

(final volume, 2 ml), contained SPL-SH (0.5 mM in PC buffer), EDTA (1 mM), and catalase (0.5 mg/ml). The reaction was initiated with NADPH and allowed to proceed at 37°C for 30 min before termination as described for microsomal incubations. Control incubations that excluded either NADPH or SPL-SH were also run in parallel.

In vitro incubations with purified FMO

In vitro incubations (0.5 ml final volume) contained SPL-SH (0.5 mM, 25 µl of 10 mM stock solution in ethanol), 2.5 nmol of purified FMO, NADPH (1 mM), GSH (5 mM), and were initiated by the addition of SPL-SH, after a preincubation period of 2 min at 33°C. The reaction mixtures were incubated at 33°C for 15 min and stopped by the addition of perchloric acid (5%, final concentration). Incubations conducted at pH 7.4, consisted of solutions which were made in 0.1 M potassium phosphate, pH 7.4; incubations at pH 9.0 consisted of reagents made with 0.25 M sodium borate buffer, pH 9.0.

Deacetylation of SPL to SPL-SH

In vitro incubations (3.0 ml) contained SPL (0.01, 0.25, 0.50, or 1.0 mM), 3 µM P450 and DTPC (1.5 mM) in 0.1M potassium phosphate buffer pH 7.4. The mixtures were incubated for 15 min at 37°C and terminated by the addition of an equal volume of EtOAc (3 ml). The incubations were extracted 2 more times with equal volumes of EtOAc, the EtOAc layers collected, evaporated under nitrogen and the extract redissolved in 0.5 ml EtOAc. Aliquots (10µL) were injected on a Rainin Dynamax C-18 column [(5µ particle size, 4.6 mm X 20 cm), with a guard column (4.6 mm X 5 cm)] connected to a Hewlett-Packard

1040A diode array (UV) detector set at 240 nm. An isocratic system of 60% methanol/40% 0.02 M sodium acetate, pH 4.0 was run at 1.0 ml/min, with SPL and SPL-SH eluting at 16.4 and 17.6 min, respectively. Authentic standards were used to confirm retention times and quantification achieved using the integration function on the diode array detector, with standard curves of known quantities of authentic SPL or SPL-SH.

Substrate exhaustion technique for determination of the partition ratio for SPL-mediated P450 inactivation

Exceedingly low concentrations of substrate are employed in this method, to ensure that the substrate is depleted before all enzyme which is susceptible to inactivation is destroyed. The amount of P450 inactivated can be attributed to metabolism of all substrate present at the beginning of the reaction. Incubations were as those described except that P450 concentrations were 1.5 and 4.0 μM, and SPL concentrations (5.0 or 2.5 μM) were employed. Reactions were started by the addition of NADPH (1.0 mM, final concentration) after a 15 min preincubation period and allowed to proceed for 1 h with supplementation of NADPH after 30 min (3 additional μmol were added to the 3 ml incubation). P450 concentrations were determined before and at 0.25, 0.05 and 1 h after initiation of the reaction. The quantity of substrate added at the start of the reaction divided by the quantity of P450 destroyed thus represents the partition ratio or number of moles of substrate metabolized per mole of enzyme inactivated.

In vitro heme reconstitution

Livers from NT or DEX-pretreated rats (receiving or not receiving SPL) were prepared as previously mentioned but homogenized (50%, w/v) in 0.1 M potassium phosphate buffer, pH 7.0, containing 10 mM GSH and 0.25 M sucrose. Such homogenates were incubated in the presence or absence of 50 μM hemin at 37°C for 15 min. The reaction was stopped by the addition of 3 volumes of ice-cold 1.15% KCl and microsomes were prepared as previously described.

Tryptophan pyrrolase activity

Tryptophan pyrrolase activity was measured in fresh homogenates, as described by Badawy and Evans (1975). Reaction mixtures contained 2.5 ml of L-tryptophan (0.03 M), 11.0 ml sodium phosphate buffer (0.2 M, pH 7.0), 12.5 ml distilled water, and either hemin (0 or 2.0 μM, to measure apoenzyme and holoenzyme activity, respectively), and were initiated by the addition of 4 ml of a 25% liver homogenate (that was made in 0.1M potassium phosphate buffer, pH 7.4). Reactions were conducted at 37°C in a shaking water bath. 3 ml-aliquots were taken at 0 and 60 min, mixed with 2 ml cold 0.9 M trichloroacetic acid (TCA), vortexed, and centrifuged at 1500 rpm in an International Centrifuge. 2.5 ml of the resultant supernatant were added to 1.5 ml NaOH (0.6 N) and the amount of formylkynurenine formed determined by measuring the absorbance at 365 nm (ε= 4540 $mM^{-1}cm^{-1}$) in a Zeiss spectrophotometer. Percent saturation of TPO was calculated as activity of holoenzyme (-hemin value) divided by total enzyme activity (+hemin value) x 100.

Heme oxygenase activity

Heme oxygenase was determined by the method of Tenhunen et al. (1969). 1.5 ml incubations contained 0.5 ml of microsomes (10 mg/ml), 0.9 ml of heme solution (containing 0.5 mM heme, 2 mg/ml BSA, 51 mM sodium chloride, in 0.1 M potassium phosphate buffer, pH 7.4), and 0.1 ml of rat hepatic cytosol (which contains biliverdin reductase to convert the bilverdin formed by heme oxygenase to bilirubin). 50 µl of a 20 mM solution of NADPH was added to start the reaction, which was monitored with an Aminco UV-vis spectrophotometer at 468 nm, and bilirubin measured using an extinction coefficient of 60 $mM^{-1}cm^{-1}$.

N-alkylporphyrins

The presence of N-alkylporphyrins were assayed in 50% homogenates as described by Correia et al. (1981).

Determination of heme-protein adducts

To pulse-label P450 heme, DEX-pretreated or NT rats were injected with the heme precursor ^{14}C-δaminolevulinic acid (ALA, 25 µCi, i.p.) and sacrificed 3 h later. Hepatic microsomes were prepared and the total microsomal ^{14}C-heme and the ^{14}C-heme specifically incorporated into P450 determined by: 1) suspending 10 mg microsomal protein in 0.1 M phosphate buffer pH 7.0 (containing 10 mM nicotinamide, 2mM GSH, 20% glycerol); 2) digestion for 18 h at 4°C with subtilisin (Sigma protease type VII, 10 µg/mg microsomal protein) to remove all cytochrome b_5; 3) centrifugation at 105,000 xg to remove the digested cytochrome b_5; and 4) subsequent heme crystallization and P450

assay of microsomal aliquots to monitor radiolabelled P450 heme as described by Correia and Burk (1978).

Irreversible ^{14}C-heme binding to the microsomal protein was monitored after incubation of ^{14}C-heme labelled microsomes (2 mg/ml) with SPL (1 mM), NADPH (1 mM), NADPH regenerating system (as previously described), and EDTA (1.5 mM) for 30 min at 37°C. Controls consisted of incubations which excluded SPL or NADPH, or included cumene hydroperoxide (0.5 mM) as the sole oxygen donor and sodium azide NaN_3 (1 mM). Microsomes were resedimented from such incubation mixtures and the supernatants were used to assay for polar ^{14}C-heme derivatives as described by Schaefer et al. (1985). The ^{14}C-heme-derived material covalently bound to microsomal proteins was monitored as described by Rollins and Buckpitt (1979). Precipitation of the protein in 6 ml-aliquots of the incubation mixtures was achieved by adding an equal volume of 25% aqueous trichloroacetic acid (TCA), followed by centrifugation (using an International Centrifuge for 10 min at ~5,000 rpm) of the solution, discarding the supernatant (as was done in all subsequent steps), followed by 2 washes of 12.5% TCA, then 3 washes of ethanol/ether (3:1, v/v), followed by a final wash of 80% methanol in water. The vacuum-dried pellets containing radiolabeled protein were then weighed, dissolved in Protosol and quantitated in a scintillation counter.

Methanol/Sulfuric acid protocol to remove P450 heme

The method detailed by Davies et al. (1985) was employed. A 5% sulfuric acid in methanol solution was used for the initial precipitation step (30 ml added to a 3 ml of an incubation) and all subsequent washing steps (6 total) of incubations identical to heme-labelled microsomal incubations previously described. All supernatants were discarded and the pellet obtained dissolved in 1 ml of 1N sodium hydroxide (NaOH), heated at 60°C for 1 h. The protein concentration was determined by the Lowry method, and the radioactivity of each fraction determined by liquid scintillation counting.

Irreversible binding of SPL or analogs to rat liver microsomal protein

Incubation mixtures (3 or 5 ml) were as previously described above, but contained ^{14}C-SPL (0.015-0.5 μCi), ^{14}C-CAN (0.5 μCi), or ^{14}C-SOLD (0.5 μCi) in addition to either 0.5 or 1 mM of the corresponding unlabelled steroid dissolved in PC buffer. Incubations were conducted at 37°C for 30 min and terminated by addition of an equal volume of 25% TCA in H_2O or 5% H_2SO_4 in methanol (v/v). Precipitates were exhaustively and sequentially washed with organic solvents (acetone, ethanol, ether, ethyl acetate) until no radioactivity remained in such washes. The precipitates were dried and dissolved in 1 ml of 1N NaOH, heated to 60°C for 1 h to fully dissolve all protein. The protein concentration was determined by the method of Lowry, and 0.4 ml aliquots counted by liquid scintillation.

In vitro generation and isolation of SPL metabolites

Reaction mixtures were similar with the prototype described previously, except that in some cases they also included ^{14}C-SPL (~0.15 μCi). Incubations were carried out at 37°C for 30 min, in an atmosphere of O_2, and centrifuged at 105,000 xg for 30 min. The resultant supernatants were filtered through a 0.45 μm Rainin nylon 66 filter, then subjected to HPLC immediately or 3.0 ml-aliquots of such supernatants were passed through a Waters C18 SepPak and the polar metabolites of SPL eluted with 3.0 ml H_2O/MeOH (50/50, v/v) after a 3 ml-wash with double distilled water to elute the most polar compounds. The HPLC system used for separation of metabolites was equipped with a Rainin C-18 Dynamax column [(5μm particle size, 4.6 mm X 20 cm); guard column (4.6 mm, X 5 cm)] connected to a Hewlett-Packard diode array (UV) detector set at 240 nm. In some experiments, when radiolabeled SPL was omitted, soldactone (42.5 μM) was included as an internal standard. The mobile phase used was NaOAc (0.02M, pH 4.0)/MeOH (50/50, v/v), at a flow rate of 1.0 ml/min, and the metabolite peaks (a and b) eluting at 15 and 17 min respectively, were collected individually and subjected to scintillation counting or mass spectrometric analysis. Metabolites could also be quantitated by integration of the area under the 240 nm absorption peaks. Such values, calculated by using the extinction coefficient of SPL at 240 nm (determined by injecting 2-20 nmol authentic SPL), agreed well (within 5%) with those quantitated by inclusion of ^{14}C-SPL.

Mass spectrometric (MS) analyses of SPL polar metabolites: Liquid chromatography (LC)/MS

Thermospray (TSP) LC/MS spectra were generated using an LKB Model 2150 HPLC pump with LKB Model 2152 LC controller (LKB, Bromma, Sweden) coupled to a Vestec thermospray LC-MS Model 201 (Vestec Corporation, Houston, Texas) dedicated LC/MS system. The column used was an Ultrasphere C_{18} (5μm particle size, 4.6 mm X 15 cm) column protected with a 5μm Ultrasphere precolumn. The mobile phase consisted of 0.2 M ammonium acetate/methanol/glacial acetic acid (50:50:1, by volume) pumped isocratically at a flow rate of 1.0 ml/min. Operating temperatures for the TSP interface were T_1 (vaporizer) =135°C; T_2 (tip) = 174°C; Jet (vapor) = 258°C; source block=290°C. The system was operated in the positive ion mode with a repeller voltage of 40 V and a filament current of 300 μA. These analyses were performed by Drs. M. R. Rashed and T. A. Baillie of Univ of Washington, Seattle, WA.

Fast atom bombardment (FAB)/MS and FAB/MS/MS analyses of the SPL polar metabolites

FAB/MS analyses were performed on a VG 70 SEQ hybrid tandem instrument of EBQQ geometry (VG Analytical Ltd., Manchester, UK), equipped with an Ion Tech fast atom gun and VG 11/250 data system. The system was operated in the negative ion mode and a solution of sodium iodide in water (0.1M) was used for calibration. Ionization was achieved following bombardment with xenon (7 keV) as the primary beam, and conventional FAB spectra were recorded via the data system at an accelerating potential of 8 kV. Samples of

metabolites (1-2 μg), purified by HPLC were redissolved in deionized water (3 μl) and added to glycerol (3 μl) on a FAB target. Collisionally-activated dissociation (CAD) was performed in the first (rf-only) quadrupole region, using argon as the collision gas. The pressure in the quadrupole analyzer housing was maintained at 1 X 10^{-6} Torr, and daughter ion spectra were obtained by selection of the appropriate $(M-H)^-$ parent by adjusting the magnetic field, and scanning the quadrupole mass analyzer from m/z 1000-50 over a period of 5 sec. Daughter ion spectra were obtained with collision energies ranging from 20-235 eV in order to identify conditions yielding the maximum fragmentation of selected parent ions. These analyses were also performed by Drs. M. R. Rashed and T. A. Baillie of Univ of Washington, Seattle, WA.

Structural characterization of the P450-dependent metabolites of SPL

To determine the structural identity of these metabolites, each metabolite peak was collected from the original HPLC system used to separate the polar metabolites. For some analyses, the fractions were rechromatographed on the same HPLC column with an isocratic mobile phase of 30% MeOH in water. The compounds were monitored as previously at 240 nm and eluted at 12 and 15 min, respectively, in this second HPLC system. Each peak material was collected, the methanol evaporated under a stream of nitrogen, the samples lyophilized, and subjected to thermospray LC/MS, as well as FAB/MS and FAB/MS/MS analyses as previously described above.

Synthesis of authentic SPL-sulfinic and sulfonic acid derivatives

These compounds were chemically synthesized by the procedure described by Filby et al. (1973). SPL (0.05 mol) dissolved in 10 ml of methylene chloride (CH_2Cl_2) was cooled to -30°C, before addition of 10-ml aliquots of *m*-chloroperbenzoic acid (mCPBA; 0.1 mol, dissolved in 200 ml of CH_2Cl_2,), at half-hour intervals. The mixture was left standing overnight at -30°C, before further cooling at -70°C, rapid filtration through a Whatman filter to remove mCPBA, and subsequent evaporation of the filtrate under nitrogen. The residue was then dissolved in MeOH/H_2O (50:50, v/v) before HPLC to separate the sulfinic and sulfonic acid derivatives as described above. Each synthesized compound was subjected to the mass spectral procedures detailed previously.

Determination of GSH in liver

Liver homogenates (33%, w/v) from NT or DEX-pretreated rats either treated without or with SPL (200 mg/kg, suspended in water with Tween 80, i.p. 2 h) were made in 0.1M phosphate buffer pH 7.0 containing 0.25M sucrose and centrifuged at 6,500 rpm in a Sorvall centrifuge for 15 min. Two ml of the resultant supernatant were added to 0.5 ml of 25% TCA and vortexed. Such samples were left at 4°C for 30 min, then resedimented at 6,500 rpm for 15 min. One-tenth of the resultant supernatant was added to 0.03 ml of (4 mg/ml) Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid) and 2.0 ml of 0.5 M sodium phosphate, pH 8.0. The absorbance of such samples were

determined at 412 nm using a Beckman DU-20 spectrophotometer, and quantified using a standard GSH (10-100 nmol) curve.

Determination of GSSG in in vitro incubations

Supernatents from incubations identical to those described for polar metabolite isolation were assayed for GSSG as described by Hill and Burk (1982), using a standard curve of 5-50 µmol GSSG.

In vitro generation and isolation of a SPL-GSH adduct (SPL-SSG)

Reaction mixtures (3-6 ml) were identical to those previously described, except that GSH (5 mM) was included for adduct isolation. Parallel incubations were performed that excluded NADPH. Fifteen or 30 min incubations conducted at 37°C, were either terminated by (1) the addition of perchloric acid (5%, final concentration) and centrifuged for 15 min in an International Centrifuge (setting=40) for 15 min, or (2) centrifuged at 105,000 xg for 30 min. The resultant supernatants (0.5 to 1.0 ml) from either procedure were injected on a Rainin Dynamax C-18 column [(5µm particle size, 4.6 mm X 20 cm), with a guard column (4.6 mm X 5 cm)] connected to a Hewlett-Packard 1040A diode array (UV) detector set at 240 nm (to monitor the absorbance of the α-ß unsaturated ketone function of SPL). The column was equilibrated with 38% MeOH/62% 0.02 M NaOAc, pH 4.0, and a linear gradient of 38% to 100% MeOH in 25 min was applied immediately at the time of injection. The SPL-SSG adduct had a retention time of 16.9 min in the HPLC system described, and was quantified by either ^{3}H-GSH or integration of the area of the 240 nm

absorbance peak (using the diode array detector integration function, as was previously described for SPL metabolite quantification), with S-*p*-nitrobenzylglutathione as an internal standard. The SPL-SSG adduct was found to be recovered quantitatively (>98%) from acid precipitated incubations. In other incubations, ^{3}H-GSH (7μCi per 6 ml incubation) was included and peaks were collected and subjected to scintillation counting for quantitation. Either method of adduct quantification yielded comparable values (within 12%).

Adduct Derivatization for mass spectral analysis

The adduct isolated from incubations that were centrifuged at 105,000 xg was collected after the HPLC procedure detailed above, then injected on the same column with 50%, v/v of methanol in water as the mobile phase. The material with an absorbance at 240 nm and eluting at 8 min using this system was collected, and the methanol in the collected fractions evaporated under a stream of nitrogen. Such samples were then lyophilized on a Virtis Lyophilizer (model 12 Freezemobile). The lyophilized material was then esterified with acetyl chloride (0.2 N; 5μl) in dry hexanol at 45°C for 1 h (Falick and Maltby, 1989). Aliquots (1 μL) were then subjected to mass spectrometric analysis.

Mass spectral analysis of the SPL-SSG Adduct

Liquid secondary ion mass spectral analyses in the positive mode (+LSIMS) were performed on the derivitized samples as described by Falick and Maltby (1989). A MS-50S (Kratos Analytical Instruments, Manchester, U.K.) equipped with a 23-kG magnet and 10-kV

postacceleration detector was used for analysis. The LSIMS ion source, primary Cs+ ion gun, and coolable sample probe were developed at the UCSF MS Facility. Trifluoroacetic acid (1%) in thioglycerol was used as the matrix and probe temperatures were maintained at 5-15°C. These analyses were performed by David Maltby, Mass Spectrometry Facility, UCSF.

Synthesis of the SPL-SSG adduct

SPL-SH (~2.5 mg) was added to a 10 ml-solution of 50% sodium borate buffer pH 9.0/50% acetonitrile containing a 2-fold molar excess of oxidized GSH (GSSG). The mixture was stirred at room temperature for two h, then subjected to the HPLC procedure previously detailed for SPL-SSG adduct isolation. A compound eluted with both the absorbance maximum (i.e. 240 nm) and retention time (~16.5 min) of the putative adduct isolated from metabolic incubations. The yield of the synthetic matter was approximately 0.25 mg (6%).

IV. Results and Discussion

A. Preliminary in vivo findings

1. Effects of SPL administration on P450 content and N-demethylase activities in PCN-pretreated rats

Acute administration of SPL to animals had previously been found to decrease guinea pig adrenal and testicular P450 content dramatically and rat hepatic P450 content slightly (Menard et al., 1974a; 1974b; 1979a; Section ID3). When administered chronically, SPL was also found to stimulate rat liver microsomal activities such as EMND [a marker activity of P450IIIA, (Elshourbagy and Guzelian, 1985; Section IA3a)] in female and male rats (Stripp et al., 1971; Section ID2), and to induce immunodetectable levels of P450IIIA in rats (Heuman et al., 1982; Section II). These observations suggested a dual inactivation/induction effect of SPL upon different P450 forms, as is observed with compounds such as AIA and SB, known cytochrome P450 suicide substrates (Levin et al., 1973; DeMatteis, 1973; Section II). Therefore, we set out to determine whether SPL could also inactivate P450IIIA in rats pretreated with PCN [a proven inducer of P450IIIA, (Elsbourbagy and Guzelian, 1985)]. When administered intraperitoneally at 250, 500 or 750 mg/kg for two h, SPL markedly decreased P450 levels in PCN-pretreated rats (Table 2). At the concentrations and time periods administered, a range of 45-66% destruction of spectrally detectable P450 was observed (Table 2). Of the N-demethylase activities monitored, EM [which largely reflects P450IIIA function, but also P450s IIB1, IIC11 and IIC6 activity (Guengerich et al., 1982)] was significantly decreased; benzphetamine

Table 2. Effect of SPL[a] administration on hepatic P450 content and EM, BZP and PCNMA N-demethylase activities in PCN[b]-pretreated rats

SPL (mg/kg)/Time (h)	P450 content[c]	N-demethylase Activities[d] EM	BZP	PCNMA
0/0	1.19	521	261	151
250/15.5	0.40	133	77.4	104
500/2	0.66	172	124	85.7
500/15.5	0.46	177	112	90.6
750/2	0.56	100	190	122

[a]SPL administration and [b]PCN-pretreatment of rats as described in Section III. [c]P450 content (nmol/mg microsomal protein). [d]N-demethylase activities (nmol HCHO formed/mg protein/15 min). All values represent the mean of two determinations from one animal (i.e. n=1), except those for DEX where n=2.

[(BZP), an index of P450IIB1, IIC11 and IIC6-mediated function (Guengerich et al., 1982)] was modestly decreased; whereas PCNMA [a functional indicator of P450IA1 and IA2 activities (Correia and Mannering, 1973; Section IA3a)], was only slightly affected. Thus, PCN-inducible P450IIIA appeared to be relatively more susceptible to inactivation by SPL than the other isozymes monitored.

2. Effect of SPL dose in DEX-pretreated rats

SPL administration for 2 h (at different doses, Table 3) to rats that were pretreated with DEX [a compound reported to induce higher levels of P450IIIA than PCN (Schuetz et al., 1984; Section IA3a)] also resulted in significant losses (69-74%) of P450 content, with quantitatively greater loss of P450 content (nmol/mg) in the DEX-pretreated rats. Near maximal P450 destruction was observed at 100 mg/kg, the lowest dose employed (Table 3). EM N-demethylation, a P450IIIA functional marker, was most dramatically inactivated as observed in PCN-pretreated rats (Table 2). SPL doses of 150 or 200 mg/kg were subsequently employed to attain maximal P450 inactivation in DEX-pretreated rats.

3. Time course of SPL-mediated inactivation in DEX-pretreated rats

Following administration of SPL (150 mg/kg, i.p.) to DEX-pretreated rats, inactivation was maximal at ~3 h (Table 4). Thereafter, a 3 h-period of SPL administration was selected in vivo to attain maximal P450 loss.

Table 3. Effect of SPL[a] dose on hepatic P450 content and EM, BZP and PCNMA N-demethylase activities in DEX[b]-pretreated rats

SPL dose (mg/kg)	P450 content[c]	N-demethylase Activities[d] EM	BZP	PCNMA
0	1.39	836	255	108
100	0.43	74.0	48.0	39.2
200	0.35	50.8	38.3	31.5
300	0.42	28.8	23.7	60.0
400	0.36	59.7	69.4	38.7

[a]SPL (as in Table 2, for 2h), [b]DEX pretreatment (daily for 3 days, as described in Section III). [c]P450 content (nmol/mg microsomal protein), [d]N-demethylase activities (nmol HCHO formed/mg protein/15 min). All values represent the mean of two determinations for one animal, except at 0 mg/kg SPL, where values represent the mean of n=2.

Table 4. Time course of SPL[a]-mediated inactivation of P450 in DEX[b]-pretreated rats

SPL Treatment (h)	P450 content[c]	N-demethylase Activities[d]		
		EM	BZP	PCNMA
0	1.49	657	202	99.7
2	0.86	293	94	23.6
2.5	0.50	225	113	58.2
3	0.59	168	85.4	108
4	0.69	237	114	137

[a]SPL (150 mg/kg, 2h), [b]DEX (as in Table 3), [c]P450 content (nmol/mg microsomal protein), [d]N-demethylase activities (nmol HCHO formed/mg protein/15 min). Values at 0 and 3 h represent a mean of n=3, at 2 and 4 h represent the mean of n=2, and those at 2.5 h represent n=1.

4. Hepatic P450 and heme content, and N-demethylase activities in DEX-pretreated and NT rats following SPL administration

The effects of in vivo SPL administration were compared in DEX-pretreated and NT rats [which exhibit different isozymic compositions of liver microsomal P450 (Guengerich et al., 1982; Section IA3a)] to assess the effects of SPL on other hepatic isozymes [particularly P450IIC11, which is the prominent P450 form in NT male rats (Waxman et al., 1985)]. When SPL-mediated loss of P450 content in DEX-pretreated and NT rats was compared, a pronounced P450 loss (55%) was observed in DEX-pretreated rats, compared to that observed in the NT rats (21%; Table 5). Erythromycin N-demethylase (ERYND), a more selective marker activity for P450IIIA than EMND (Wrighton et al., 1985), was also dramatically diminished following SPL treatment. Decreased enzyme marker activities attested to the selective P450IIIA inactivation by SPL in both the DEX-pretreated and NT rats (Table 5).

5. Testosterone hydroxylase activities in DEX-pretreated rats following SPL administration

Testosterone hydroxylase activities [which are much more selective markers for the various hepatic P450 isozymes, (Waxman, 1986)] were monitored in vitro in liver microsomes from DEX-pretreated rats to ascertain more accurately the particular isozymes inactivated by SPL administration in vivo. The testosterone 2ß-, 15ß- and 18-hydroxylase activities [most selective markers for P450IIIA (Underwood et al., 1986; Sonderfan et al., 1987; Section IA3a) were

Table 5. Effect of SPL[a] administration on hepatic microsomal P450 and heme content, and N-demethylase activities in NT and DEX[b]-pretreated rats

Treatment	Content[c]		N-demethylase Activities[d]			
	P450	Heme	EM	ERY	BZP	PCNMA
NT	0.94 ± 0.05	1.59 ± 0.16	152 ± 48	ND	88 ± 28	136 ± 35
NT + SPL	0.74 ± 0.08	1.14 ± 0.15	38 ± 1.0	ND	37 ± 1	124 ± 27
% Loss	21	28	75		58	6
DEX	1.43 ± 0.26	2.22 ± 0.18	639 ± 122	141 ± 36	207 ± 19	115 ± 51
DEX + SPL	0.64 ± 0.18	1.12 ± 0.40	150 ± 117	15 ± 3	75 ± 59	106 ± 21
% Loss	55	50	77	89	64	8

[a]SPL (150 mg/kg, 3h), [b]DEX (as in Table 3), [c]P450 and heme content (nmol/mg microsomal protein), [d]EM, ERY, BZP, and PCNMA N-demethylase activities (nmol HCHO formed/mg protein/15 min). All values represent mean ± S.D. of n=3, except P450, EM, BZP and PCNMA N-demethylase values in DEX-pretreated rats where values represent the mean ± S.D. of n=6. ND: not determined.

those most affected by SPL administration (Figure 5). Testosterone 2α-hydroxylase [a functional marker for P450IIC11 (Waxman, 1988; Section IA3a)] was also inactivated to a substantial extent (to 16.5% of original). Testosterone 7α–hydroxylase (a P450IIA1 marker; Waxman et al., 1988) was diminished negligibly following SPL administration (Figure 5). The refractory nature of P450IIB1 to SPL-mediated inactivation has been established in vitro (Underwood and Correia, unpublished observations). Therefore, P450IIIA and P450IIC11 appeared to be selectively inactivated, while P450IIA1 was comparatively spared by SPL administration in vivo.

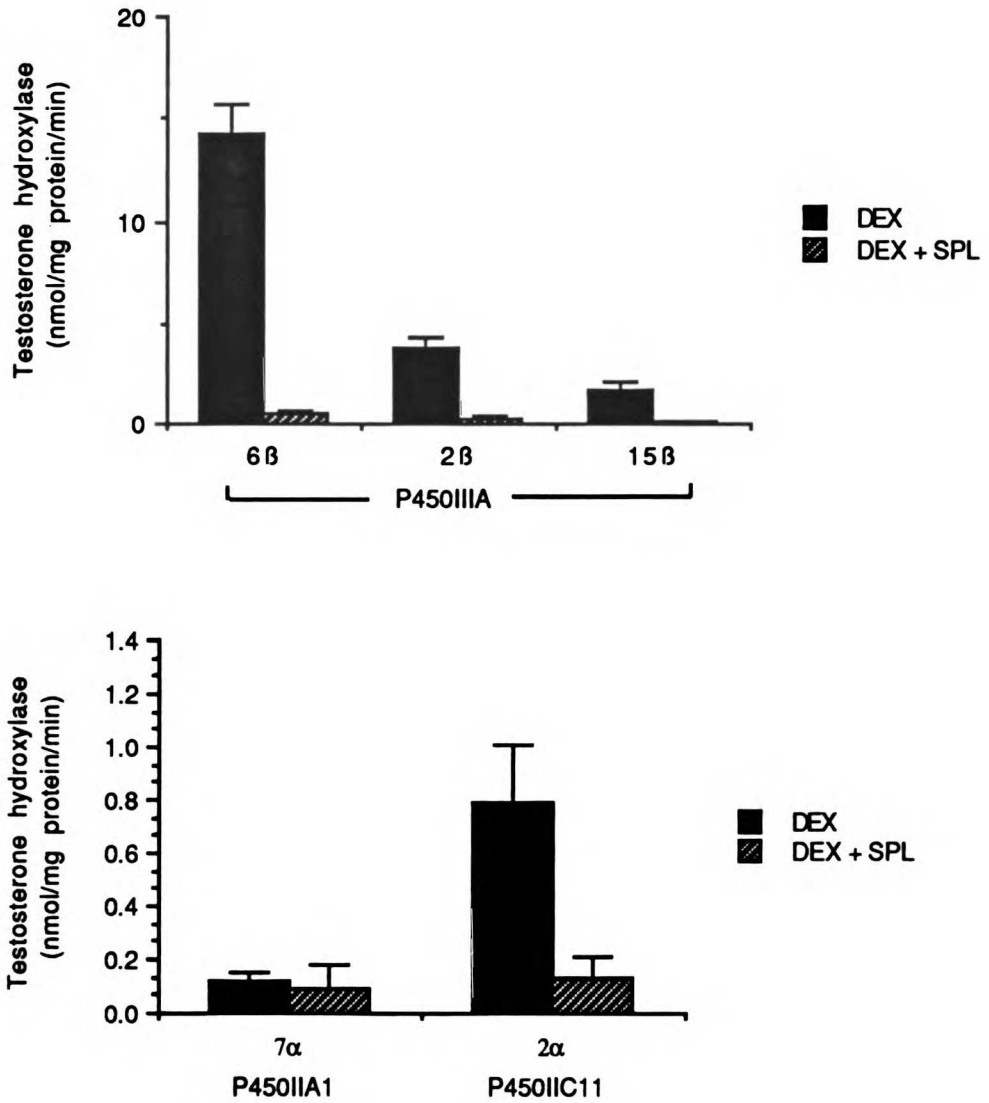
6. Effect of TAO complexation on SPL-mediated P450 inactivation in DEX-pretreated rats

Complexation of P450IIIA with the "P450IIIA-specific" inhibitor TAO in vivo (Wrighton et al., 1985; Section IA3a) before SPL administration, resulted in a significant attenuation of SPL-induced loss [69% in the absence of TAO complexation vs. 18% in its presence, (Table 6)]. Again, P450IIIA appeared to be a major target of SPL-mediated inactivation, as its inhibition through metabolic intermediate complex formation with TAO was found to significantly decrease the extent of P450 destruction by SPL.

7. P450 content and N-demethylase activities following SPL administration to DEX-pretreated and NT female rats

The effects of SPL on hepatic P450 content and isozyme activities in DEX-pretreated and NT female rats were assessed because P450IIIA is virtually absent in adult female rats, but can be immunochemically

Figure 5. Testosterone hydroxylase activities[a] in DEX[b]-pretreated rats following SPL[c] administration.



[a]Testosterone hydroxylase activities as determined in Section III, [b]DEX pretreatment (as in Table 3), [c]SPL (150 mg/kg, 3h). All values represent the mean ± S.D. (n=3).

Table 6. Effect of in vivo TAO[a] complexation on SPL[b]-mediated losses of hepatic P450 content and EM and ERY N-demethylase activities in DEX[c]-pretreated rats

Treatment	P450 content[d]	Heme content[d]	N-demethylase Activities[e] EM	ERY
None	1.18	1.72	368	91
+SPL	0.37	1.05	74	13
% loss	68.6	39.0	79.9	85.7
+TAO	1.21	2.18	325	109
+TAO/+ SPL	0.99	1.75	172	51.0
% loss	18.2	19.7	47.1	53.2

[a]TAO (administered as described in Section III, 400 mg/kg, 16 h before SPL administration), [b]SPL (200 mg/kg, 3 h), [c]DEX pretreatment (as in Table 3). [d]P450 and heme content (nmol/mg microsomal protein), [e]EM, and ERY N-demethylase activities (nmol HCHO formed/mg protein/15 min). All values represent the mean of 2 determinations (n=1).

induced ~70 fold by DEX-pretreatment (Waxman et al., 1985). Furthermore, P450IIC11 is a male specific P450 isozyme (Guengerich et al., 1982; Section IA3a), thus the two forms of P450 (IIIA and IIC11) believed to be susceptible to SPL-mediated inactivation (Sections IVA4, IVA5) would not be present in adult female rats, thus representing a rat model containing a hepatic microsomal P450 isozymic mixture theoretically resistant to SPL-mediated inactivation. Only 5% of the total hepatic P450 content was lost following SPL administration to non-pretreated female rats, whereas 29% of the P450 content was lost in their DEX-pretreated counterparts (Table 7). EMND activity was once again decreased to the greatest extent in the DEX-pretreated rats (by 89%) compared to other P450 activities. Though P450 content was not increased with DEX pretreatment, the large enhancement of EMND activity (454 vs. 58.5; Table 7) attested to induction of P450IIIA1 in these animals, as DEX selectively induces only P450IIIA1 in female rats (Waxman et al., 1985). The marginal P450 loss (5%) in the NT female rats, (Table 7) when compared to their male counterparts (21%; Table 5), directly reflected the low hepatic P450IIIA and P450IIC11 content present in the NT female rats. P450IIIA1 induction in female rats by DEX pretreatment thus increased the susceptibility of animals to SPL-mediated inactivation (Table 7).

8. Effect of CAN on rat hepatic P450 content and ERYND activity

To determine whether the sulfur moiety of SPL was required to produce P450IIIA inactivation, as previously observed in adrenal and testicular systems (Menard et al., 1978; 1979a; Section ID4), the

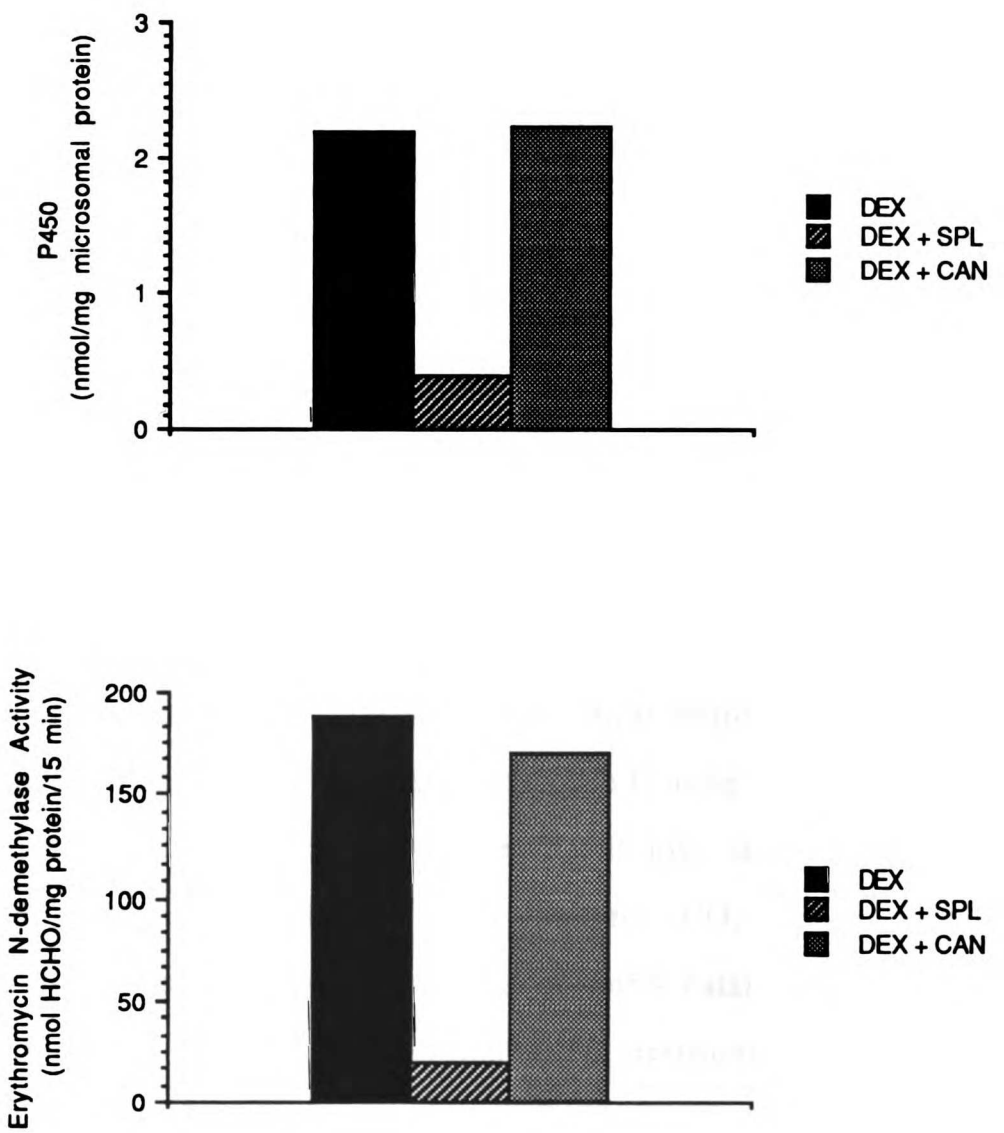
dethioacetylated SPL analog CAN [the 6,7-diene metabolite of SPL in vivo, (Sadee et al., 1974; Section ID6a)] was assessed for P450 inactivation. The administration of CAN to DEX-pretreated rats resulted in neither loss of P450 chromophore nor of ERYND activity in vivo (Figure 6), indicating a crucial role for the thioester moiety of SPL in P450IIIA inactivation, as previously reported for the SPL-mediated loss of dog testicular P450 (Menard et al., 1978).

Table 7. Effect of in vivo SPL[a] administration on hepatic microsomal P450 content and EM, BZP, and PCNMA N-demethylase activities in NT and DEX[b]-pretreated female rats

Treatment	P450 content[c]	N-demethylase Activities[d]		
		EM	BZP	PCNMA
NT	0.92	58.5	60.5	100
NT + SPL	0.87	36.5	29.0	72.5
% loss	5.4	37.6	52.1	27.5
DEX	0.80	454	166	104
DEX + SPL	0.57	66.0	76.5	89.5
% loss	28.8	85.5	53.9	13.9

[a]SPL (250 mg/kg, 2.5 h), [b]DEX pretreatment (as in Table 3), [c]P450 content (nmol/mg microsomal protein), [d]EM, BZP, and PCNMA demethylase activities (nmol HCHO formed/mg protein/15 min). All values represent the mean of two individual determinations.

Figure 6. Effect of CAN[a] or SPL[a] on P450 content and ERY N-demethylase activity in DEX-pretreated[b] rats



[a]CAN or SPL (administered as in Section III, 200 mg/kg, 3 h), [b]DEX pretreatment (as in Table 3). Values for DEX and DEX + SPL represent the mean of 2 determinations (n=1). Values for DEX + CAN represent the mean of values obtained from two separate experiments.

B. In vitro effects of SPL on P450

1. Requirements for SPL-mediated P450 destruction in vitro

The effects of O_2 and NADPH (cofactors of P450), reaction temperature and carbon monoxide (CO) on P450 destruction were assessed in an in vitro system employing liver microsomes prepared from DEX-pretreated rats. The experiments were performed to determine whether: (1) P450IIIA destruction could be reproduced in an isolated system, and (2) SPL was functioning as a mechanism-based inactivator of P450. Indeed, SPL-mediated destruction of P450IIIA could be reproduced in vitro (Table 8), with losses of P450 comparable to those observed in vivo with DEX-pretreated rats (i.e. 55%; Section IA4). Both NADPH and O_2 were required for SPL-mediated destruction of P450 in vitro (Table 8), indicating that metabolism of SPL by P450 was necessary for P450 destruction. Incubation of SPL, O_2 and NADPH with liver microsomes prepared from DEX-pretreated rats at 0°C, resulted in minimal P450 destruction, indicating the necessity for P450 enzymatic activity for SPL-mediated loss of P450 to occur. Gassing incubations with the P450 inhibitor, CO, resulted in no P450 loss, further substantiating the role of P450 catalysis in its inactivation. Thus, SPL-mediated P450 destruction appeared to satisfy most criteria for mechanism-based P450 inactivation (Rando, 1984; Section IC2), indicating that P450IIIA catalyzes its own destruction via its metabolic activation of SPL.

2. Kinetics of SPL-mediated P450 destruction

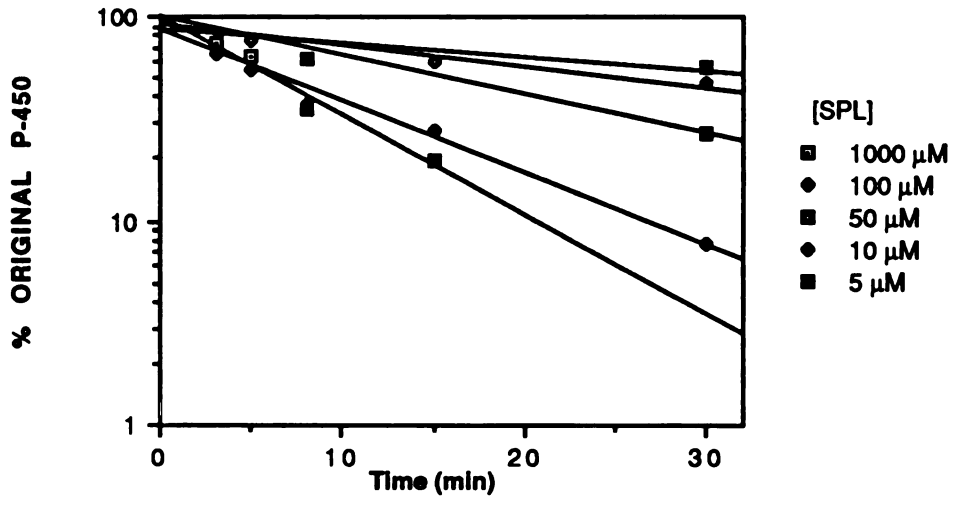
The effects of SPL concentration and incubation time on P450 destruction were assessed utilizing liver microsomes prepared from

Table 8. Requirements for in vitro SPL-mediated destruction utilizing microsomes from DEX-pretreated rats[a]

System	P450 Loss (% original)
[b]Complete	52
-NADPH	0
$+N_2$	3
+CO	1
0°C	2
-SPL	5

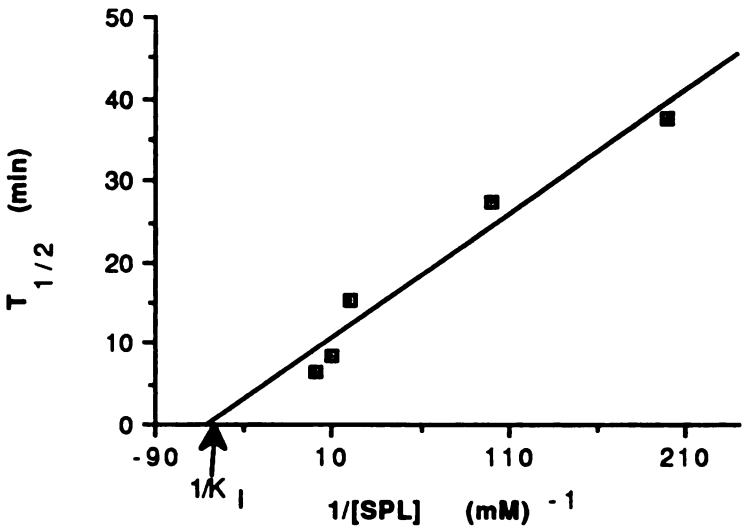
[a]DEX pretreatment (daily for 4 days) and hepatic microsome preparation as described in Section III. [b]Complete incubation system contained SPL (1.0 mM), NADPH (1.0 mM), P450 (3 μM), in 0.1 M phosphate buffer, pH 7.4 for a 30 min incubation at 37°C. Values represent the means of determinations obtained from two individual microsomal preparations.

Figure 7. Kinetics of SPL-mediated P450 destruction in vitro[a]



[a]In vitro incubations using microsomes prepared from DEX-pretreated rats (daily for 3 days) as described in Section III. The maximum possible spectrally detectable P450 loss observed after incubation with SPL (1 mM), in the presence of NADPH at 37°C for 30 min is depicted as 100% "original P450".

Figure 8. $T_{1/2}$ of SPL-mediated P450 destruction vs. SPL concentration



The $T_{1/2}$ values for inactivation from Figure 7 are plotted against the reciprocal of the SPL concentration to obtain K_I ~17μM.

DEX-pretreated rats in order to examine the kinetic features of this process. SPL-mediated destruction of P450IIIA was found to be maximal at SPL (1.0 mM) and at 15 min of incubation (Figure 7). When % P450 loss [wherein 100% corresponds to the maximal value of P450 content susceptible to SPL-mediated inactivation (i.e. 55%)] was plotted against time on a semi-log plot, the half-times ($t_{1/2}$) of P450 destruction at the various SPL concentrations employed were obtained (Figure 7). When the $t_{1/2}$'s were plotted against SPL concentration, the X-intercept represented the reciprocal of K_I, the dissociation constant of the inhibitor for the enzyme (Waley, 1984; Figure 8). K_I was found to equal 17 μM. K_{inact}, the rate constant for inactivation, equals $t_{1/2}/\ln 2$, when inactivation and $t_{1/2}$ is maximal (i.e. at saturating inhibitor concentrations), and was calculated to be 0.08 min^{-1}.

3. Partition ratios for destruction of P450 by SPL

Using the substrate disappearance method (Section III), a partition ratio of 22.4 ± 3.9 nmol of SPL metabolized per P450 nmol inactivated was obtained (Table 9). This finding demonstrated that approximately 25 moles of SPL were metabolized by each mole of P450 per inactivation event.

4. Effects of SPL on P450 destruction in microsomes prepared from various inducers

To determine whether SPL was as selective for P450IIIA in vitro as in vivo (Section IVA), SPL-mediated destruction was assessed utilizing liver microsomes prepared from DEX or PB-pretreated, and NT rats.

Table 9. Partition ratios using the substrate exhaustion technique[a] for destruction of P450 isozymes from DEX[b]-pretreated rats in vitro[c]

P450 [μM]	Partition Ratio (nmol SPL metabolized/nmol P450 destroyed) SPL [5.0 μM]	SPL [2.5 μM]
1.5	22.7	25.0
4.0	25.0	16.7

[a]Substrate exhaustion technique for determining partition ratios is described in Section III. [b]DEX pretreatment as in Table 8. [c]In vitro incubations as described in Section III. Each value represents the mean of two determinations for one microsomal preparation.

As in the in vivo experiments, SPL-mediated destruction was maximal in systems containing high P450IIIA content (i.e. using liver microsomes from DEX-pretreated rats). P450 losses of 55, 24 and 21% in systems containing liver microsomes prepared from DEX-pretreated, PB-pretreated and NT rats, respectively, were observed (Table 10), which was consistent with the individual P450IIIA content of each liver microsomal preparation.

5. Effect of TAO complexation on SPL-mediated P450 loss in vitro

To determine whether TAO could attenuate P450 destruction in vitro as in vivo (Section IVA6), liver microsomes prepared from either DEX or PB-pretreated rats administered TAO (before sacrifice), were employed. Indeed, in the in vitro system with liver microsomes prepared from DEX/TAO-pretreated rats, a 23% loss of hepatic P450 was observed, whereas a 55% loss was observed with liver microsomes obtained from DEX-pretreated rats (Table 10). Similarly, there was a 10% P450 loss in the PB/TAO system, in contrast to the 24% P450 loss observed in the PB system (Table 10). Thus, as in the in vivo system (Table 4), complexation of P450IIIA with TAO was found to significantly attenuate SPL-mediated P450 destruction, implicating a critical role for this isozyme as catalyst in SPL-mediated P450 destruction.

Table 10. Effect of rat pretreatment[a] and TAO[b] administration on SPL-induced loss of rat hepatic cytochrome P450 in vitro[c]

Rat pretreatment	Cytochrome P450	
	Initial content (100%) (nmol/mg protein)	SPL-Induced loss (%)
None	0.94 ± 0.05	21 ± 1
DEX	1.46 ± 0.26	55 ± 4
DEX/TAO	1.80 ± 0.19	23 ± 2
PB	2.07 ± 0.39	24 ± 6
PB/TAO	1.90 ± 0.17	10 ± 6

[a]DEX pretreatment (daily for 3 days), and PB pretreatment (daily for 4 days) as detailed in Section III. [b]TAO administration as in Table 6. [c]Incubations as described in III, but containing 2 mg/ml microsomal protein and 1.0 mM SPL. All values represent the mean ± S.D. (n=3).

6. Effects of various SPL analogs and esterase inhibitors on P450 destruction

The sulfur moiety of SPL was crucial for in vitro guinea pig adrenal and testicular P450 destruction (Menard et al., 1979a; Section ID4). Indeed, deacetylation of SPL was later shown to be required to produce P450 destruction in such preparations (Sherry et al., 1986; Section ID6b). To examine whether similar restrictions were operative for SPL-mediated destruction of rat hepatic P450IIIA, several analogs of SPL were assayed for destructive capacity in our in vitro system. SPL-SH was found to be equipotent to SPL in its NADPH-dependent ability to mediate P450 loss (51% vs. 56%; Table 11), consistent with earlier reports (Menard et al., 1978; 1979a; Section ID4). Inhibition of SPL deacetylation to SPL-SH by the inclusion of BNPP (a microsomal esterase inhibitor), resulted in a significant attenuation (87%; Table 11) of P450 destruction, implicating SPL-SH as the destructive species, as previously reported by Sherry et al., (1986). CAN and SOLD (the 6-7 diene analogs which lack the sulfur moiety of SPL) both produced minimal P450 loss (1 and 2% respectively; Table 11), consistent with earlier in vivo observations with CAN (Figure 6; Section IVA8) by this laboratory, as well as earlier findings of Menard and Gillette (1979a). Incubation with the S-methylated metabolite of SPL i.e. SPL-SCH_3 (Karim and Brown, 1972; Section IA6b) also resulted in minimal P450 loss (6%; Table 11), again indicating that SPL-SH was the species responsible for producing P450 destruction.

Table 11. In vitro effects of SPL and analogs on DEX[a]-pretreated rat hepatic cytochrome P450 isozymes in vitro[b]

Steroid/addition	P-450 Loss (%)
SPL	56
SPL-SH	51
NONE	4
SPL/BNPP (0.4mM)	7
CAN	1
SOLD	2
SPL-SCH_3	6

[a]DEX (as in Table 8). [b]In vitro incubations as described in Section III, containing 1.0 mM of the steroid analog. Each value represents the mean of results obtained from two individual microsomal preparations. The basal (100%) value for microsomal P450 content (nmol/mg of protein) was 1.62.

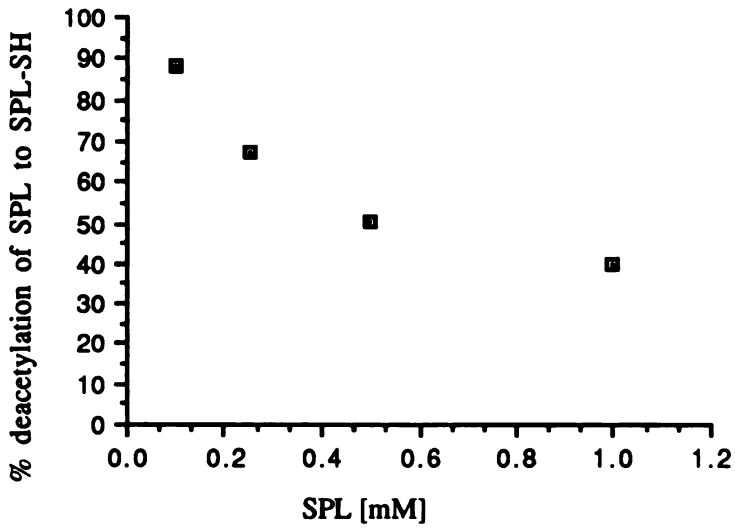
7. Extent of conversion of SPL to SPL-SH by rat liver microsomes prepared from DEX-pretreated rats

The conversion of SPL to SPL-SH was assessed in order to determine whether SPL-SH was being formed in adequate quantities to account for our observations utilizing SPL (Table 11). Indeed, incubation of SPL (1.0 mM) with liver microsomes prepared from DEX-pretreated rats for 15 min, resulted in 40% deacetylation of SPL to SPL-SH, equivalent to 0.4 mM SPL-SH available in standard in vitro incubation mixtures (Figure 9). SPL-SH generated in the in vitro system apparently accounts for the associated P450 loss observed [because deacetylation of SPL appears to be a prerequisite for P450 destruction (Table 11)]. Because generation of SPL-SH (which is essential for SPL-mediated P450 loss) is incomplete, the values listed for actual concentration of inactivating species required to destroy P450 are probably overestimated (Figures 7 and 8). It must be noted however, that in the kinetic studies discussed (Section IVB2), k_{inact}, K_I and $T_{1/2}$ values are determined primarily from data obtained at very low SPL concentrations [i.e. 4 of the five data points are at 0.10, 0.05, 0.001, and 0.005 mM SPL (Figures 7 and 8)] wherein SPL deacetylation is almost complete [70% deacetylation occurs at 0.25 mM SPL within 15 min (Figure 9)]. Thus at these particular concentrations, SPL concentrations closely approximate actual SPL-SH concentrations.

8. Effect of SPL-SH on purified P450IIC11

As SPL appeared to inactivate rat hepatic microsomal P450IIC11 in vivo (Section IVA4), SPL-SH was assayed for its ability to inactivate purified P450IIC11 in a reconstituted system (Section III). In this system, ~50% of the P450IIC11 present was destroyed by SPL, directly

Figure 9. In vitro[a] deacetylation of SPL to SPL-SH



[a]In vitro incubations for 15 min with liver microsomes from DEX-pretreated rats (as in Table 8) as described in Section III. SPL and SPL-SH quantified on HPLC as described in Section III. Each data point represents the mean of two individual experiments.

confirming its susceptibility to SPL-mediated inactivation, and accounting for the SPL-mediated inactivation of hepatic microsomal testosterone 2α-OHase activity in DEX-pretreated rats (Figure 2; Section IVA5). Incomplete in vitro P450IIC11 inactivation by SPL-SH may be due to its production of a SPL-SH metabolite which competes with SPL-SH for the active site of the enzyme. Dialysis of the enzyme followed by incubation with a fresh supply of SPL-SH may resolve whether this indeed is the case. This descrepancy may also be due to the presence of FMO in the intact NT rat which also contributes to the loss of P450IIC11 activity observed in vivo.

C. Effect of SPL on P450 prosthetic heme

1. Loss of P450 heme in vivo

It was previously observed that the P450 prosthetic heme moiety was lost after SPL-mediated inactivation in vivo, when hepatic microsomal heme content was monitored in parallel with P450 content following SPL administration to male rats (Table 5). Such heme was found to be lost concomitantly with and stoichiometrically to P450, similarly to that reported earlier with SPL-inactivated testicular and adrenal microsomal P450 (Menard et al., 1974a; Section ID3). These findings led us to investigate the fate of prosthetic heme in SPL-mediated P450IIIA destruction, because knowledge of this process should yield insight into the mechanism of P450 inactivation by SPL.

2. Assessment of intact P450 heme release

Loss of intact P450 heme occurs after the administration of diethyldithiocarbamide with the subsequent induction of heme oxygenase (Miller et al., 1983; Section IC2ai). To determine whether P450 heme was released intact following SPL administration in vivo, tryptophan pyrrolase (TPO) and heme oxygenase were monitored as indicators of increased intrahepatic heme levels. Tryptophan pyrrolase heme saturation has been used as an indicator of the "free" heme pool status and should reflect changes in hepatic "free" heme if and when they occur (Bissell and Hammaker, 1977). SPL administration to DEX-pretreated rats resulted in a slight decrease in TPO saturation, indicating that available "free" heme did not increase in these rat livers (Table 12). Interestingly, holoenzyme activities (i.e. TPO+heme values) decreased following administration of SPL, indicating that the levels of this enzyme are somehow affected by SPL.

Table 12. Effect of SPL[a] on hepatic microsomal P450 and heme content, tryptophan pyrrolase (TPO) and microsomal heme oxygenase (HO) activities in DEX-pretreated[b] rats

SPL Treatment (h)	Content[c] P450	Heme	TPO[d]-heme/TPO[d]+heme (% saturation)	Heme oxygenase[e]
0	1.71	2.68	7.52/17.0 (44.2)	747
1	0.79	1.29	4.72/14.3 (33.0)	492
2	0.51	1.07	2.40/9.21 (26.1)	120
3	0.49	0.93	3.68/10.4 (35.4)	254

[a]SPL administration (200 mg/kg), and [b]DEX pretreatment (daily for 3 days) as in Section III. [c]P450 and heme content (nmol/mg microsomal protein). [d]TPO activity (μmol kynurenine formed/g liver/h), and [e]Heme oxygenase activity (nmol/mg protein/10 min) determined as described in Section III. All values represent the mean of results obtained from two individual animals.

In SPL-treated rats, hepatic microsomal heme oxygenase was monitored because this heme degrading enzyme would be induced by an increase in the "free" heme pool, if it were to occur (Tenhunen et al., 1969). Heme oxygenase levels were found to decrease following SPL administration, thereby indicating lack of increased hepatic free heme (Table 13). Indeed, the observed time-dependent decrease in hepatic heme oxygenase levels may actually reflect depletion of the free heme pool following SPL administration, a point to be considered in the assessment of the porphyrinogenic potenial of this drug.

3. Release of P450 heme as N-alkylporphyrins (green pigments)

Because many P450 inactivators (such as AIA and norethindrone) which cause hepatic heme depletion result in green pigment formation (Ortiz de Montellano and Correia, 1983; Section IB2b) these products of heme breakdown were assayed in rats, in order to determine whether SPL inactivation of P450 also resulted in N-alkylporphyrin formation. Formation of these heme derivatives was not detected following SPL administration to DEX-pretreated rats, implying a different mechanism for prosthetic heme loss in this system, than that observed for AIA-mediated inactivation of P450 (Ortiz de Montellano et al., 1979).

4. In vitro reconstitution of SPL-inactivated P450 with heme

In some cases where the prosthetic heme moiety of P450 is lost during P450 inactivation, the enzyme may be reconstituted, (even with restoration of full functional activity) by incubation of the heme-stripped apo-P450 with fresh heme in vitro [e.g. heme restoration of P450IIB1 (Bornheim et al., 1987)]. To determine whether hepatic P450IIIA could be restored in a similar fashion after SPL-mediated

P450 heme loss, in vitro incubations containing liver homogenates prepared from DEX-pretreated and non-pretreated rats administered SPL, were incubated with exogenous hemin (Section III). Neither the P450 chromophore nor functional EM N-demethylase activity could be restored following such treatment (Table 13), indicating that SPL-mediated loss of P450 heme did not occur in a manner similar to that of AIA-mediated destruction of P450IIB1 heme, where the enzyme is structurally and functionally restored by in vitro incubation with heme (Bornheim et al., 1987). In fact, these effects were similar to those of AIA on P450IIIA, or SB on P450IIB1, where neither chromophore, nor activity could be restored by exogenous hemin after drug-mediated prosthetic heme loss (Bornheim et al., 1987; Lunetta et al., 1989). Thus, its appears that P450IIIA heme is inactivated by several compounds in a manner which is not amenable to in vitro reconstitution with heme as observed with other isozymes, yielding further supportive evidence for the fragile character of this particular isozyme [i.e. functional lability on purification (Elshourbagy and Guzelian, 1985; Section IA3a)].

5. Formation of heme-protein adducts after SPL-mediated P450 destruction

After administration of acute SPL to DEX-pretreated rats, liver microsomes appeared bleached rather than green, as observed after administration of N-alkylporphyrin-producing compounds (Ortiz de Montellano and Correia, 1983; Section IB2b). Because peroxidative heme damage caused by agents such as cumene hydroperoxide also results in such bleaching, and is associated with irreversible heme binding to the microsomal protein (Guengerich, 1978; Section IC2c)],

Table 13. Effect of in vitro heme reconstitution on hepatic P450 and EM N-demethylase activity after SPL[a] administration to NT and DEX[b]-pretreated rats

System	P450 content[c]	EM N-demethylase Activity[d]
NT	0.80	108
NT + heme	0.69	102
NT + SPL	0.54	34
NT + SPL + heme	0.65	35
DEX	1.56	741
DEX + SPL	1.04	324
DEX + SPL + heme	1.12	371

[a]SPL (200 mg/kg, for 3 h) and [b]DEX pretreatment (daily for 3 days) as in Section III. Heme reconstitution was carried out as described in Section III. [c]P450 content (nmol/mg protein). [d]EM N-demethylase Activity (nmol HCHO formed/mg protein/15 min). All values represent the mean of two determinations (n=1).

the possibility of a similar phenomenon in SPL-elicited microsomal bleaching was considered. Indeed, Menard et al. (1979a) previously noted that adrenal microsomes appeared bleached following SPL administration. For these reasons, P450 heme pools were pulse labelled with ^{14}C-ALA in DEX-pretreated rats (Section III), followed by in vitro SPL-mediated inactivation of liver microsomal P450 and assessment of covalent binding of ^{14}C-heme to microsomal protein. A significant enhancement of NADPH-dependent ^{14}C-heme binding to microsomal protein (31% of the total radioactivity in the presence of NADPH vs. 10% in its absence) was observed (Table 14). NADPH-dependent ^{14}C-heme binding, although lower than that observed in the corresponding system with cumene hydroperoxide (CuOOH; wherein ~70% of the total ^{14}C-heme radioactivity was found covalently bound to microsomal protein), still appeared to account for a significant fraction (~54%) of P450 ^{14}C-heme lost after incubation with SPL and NADPH (Table 14).

Methylvinylmaleimide (MVM) and hematinic acid (HA), two polar monopyrrolic heme degradative products were also monitored in these experiments because their formation was shown to be enhanced during peroxidative heme damage (Schaefer et al., 1985). Their formation however, was only slightly enhanced by SPL in the NADPH-supplemented liver microsomal incubations (Table 14).

Covalent binding experiments using an alternative method reported to remove heme more adequately [i.e. replacing H_2SO_4 washes for those with trichloroacetic acid; (Davies et al., 1986)] revealed that ~27% of

the P450 heme destroyed could be accounted for as covalently bound to microsomal protein (Table 15). Binding of heme derivatives to microsomal protein was also assessed using liver microsomes prepared from NT rats, wherein a fraction of the heme destroyed was also found irreversibly bound to microsomal proteins. Irreversible binding of ^{14}C-heme to microsomal protein and identification of polar ^{14}C-heme degradative products offered a partial explanation for SPL-mediated P450 heme loss, although the mechanistic basis for such an event remains unknown.

Table 14. SPL-induced degradation of microsomal ^{14}C-heme to irreversibly bound heme protein adducts and polar metabolites

System[a]	Radioactivity[b] (cpm/incubation)	P450 loss[c] (% of initial)	Irreversible ^{14}C-binding[d] cpm	Polar ^{14}C-metabolites[e] cpm
- NADPH - SPL	250,000	0	13,000 (5.12%)	2,750 (1.1%)
+ NADPH - SPL	250,000	4	25,100 (10%)	11,320 (4.5%)
+ NADPH + SPL	250,000	57	77,500 (31%)	15,600 (6.2%)
+ CuOOH	250,000	89	175,000 (70%)	18,500 (7.4%)

[a]Incubation mixtures containing hepatic microsomes from DEX-pretreated rats (as in Table 3) that received 4-^{14}C-ALA. The complete system is described in Section III where 1.0 mM SPL was used.

[b]Isolation of the subtilisin resistant ^{14}C-heme content of microsomes (which exclusively contain cytochrome P450 heme) allowed the estimate that ~84% of the initial radioactivity in the incubation specifically pertains to cytochrome P450 heme (Section III).

[c]P450 loss as described in Section III. Corresponding heme losses are 62% and 87% for NADPH + SPL and CuOOH systems, respectively.

[d]Irreversibly bound ^{14}C-heme-derivatives monitored in the microsomal fraction after incubation as described in Section III.

[e]Determined in aliquots of the incubation medium after removal of microsomal fraction and possibly containing hematinic acid, MVM and propentdyopents. Values in brackets represent % of initial radioactivity.

Table 15. Binding of ^{14}C-heme derivatives to microsomal proteins of DEX and NT rats in vitro.

Rat Pretreatment	Heme bound[a]	Heme destroyed[b]	Heme bound/destroyed
DEX	0.18 ± 0.04	0.68 ± 0.22	27.3%
NT	0.08 ± 0.03	0.19 ± 0.06	42.1%

DEX-pretreatment of rats as in Section III (daily for 4 days), ^{14}C-ALA administration, and microsomal preparation as described in Section III. Incubation systems contained 3μM P450, 0.5 mM SPL, 1.5 mM NADPH, in 0.1M potassium phosphate buffer pH 7.4.

[a]Heme bound (nmol/mg microsomal protein), after subtracting the binding of the parallel incubation lacking NADPH, using the H_2SO_4/McOH precipitation method for covalent binding determination (Section III). [b]Heme destroyed (nmol/mg protein) measured by monitoring the heme chromophore as in Section III. Each value represents the mean ± S.D. (n=3).

D. Covalent binding of radiolabelled SPL and various analogs to liver microsomal protein

Many suicidal inactivators of P450 have been shown to bind covalently to P450 apoprotein (Ortiz de Montellano, 1988; Section IC2a). Menard et al. (1979b) previously demonstrated that during incubation of adrenal microsomes with SPL, the sulfur atom of SPL was covalently bound to adrenal P450 apoprotein (Section ID5), and that this was accompanied by heme loss. They proposed that such covalent sulfur binding [similar to that associated with parathion-mediated P450 inactivation (Neal et al., 1977; Section IC2ai)], accounted for inactivation of P450 by SPL. To determine whether similar apoprotein alkylation occurs during SPL-mediated inactivation of P450IIIA, the NADPH-dependent covalent binding of ^{14}C-SPL, ^{14}C-CAN and ^{14}C-SOLD to rat hepatic microsomal protein from NT and DEX-pretreated rats was assessed in vitro.

1. Metabolic requirements and effect of rat pretreatment on covalent binding of ^{14}C-SPL to protein in vitro

NADPH was found to significantly enhance the covalent binding of ^{14}C-SPL to microsomal protein from NT rats [Δ = (+NADPH) - (-NADPH) = 2.2 nmol/mg protein]. When microsomes from DEX-pretreated rats were employed, this binding was significantly enhanced (Δ = 6.3 nmol/mg protein; Table 16). This increase in covalent binding of ^{14}C-SPL to microsomal proteins is consistent with the greater SPL-mediated losses of P450 observed in microsomal systems enriched with P450IIIA after DEX-mediated induction (Table 9; Section IB5).

Thus, covalent binding of SPL to the P450 apoprotein may also contribute to P450 inactivation.

2. Effects of DTT, GSH, and BNPP on NADPH-independent and dependent ^{14}C-SPL binding to rat liver microsomal protein

The covalent binding of ^{14}C-SPL (nmol bound steroid/mg microsomal protein) was quite high (~12/1) compared to the corresponding P450 loss (nmol/mg microsomal protein); and also occurred in the incubations lacking NADPH, indicating the involvement of processes not dependent on P450 activity in such binding (Table 16). Similar findings were reported with guinea pig adrenal microsomes (Menard et al., 1979b), where the addition of GSH or dithiothreitol (DTT) was found to significantly decrease the NADPH-independent ^{14}C-SPL binding. In the rat hepatic system described above, the high NADPH-independent binding could also be largely attenuated by the addition of GSH and/or DTT. That is, inclusion of the two nucleophiles in the incubation mixture resulted in 50-90% attenuation of the covalent binding observed in their absence (Table 16). Thus, it seemed plausible that such high background binding could be due to interactions between protein sulfhydryls and the thiol moiety of SPL-SH to produce SPL-SH/protein mixed disulfides (via air oxidation) which could be reduced by the addition of the thiol nucleophiles, DTT or GSH. DTT was also found to decrease Δ, the difference in binding between the NADPH-supplemented and NADPH-devoid incubations, whereas GSH produced a smaller effect (Table 16). This result may be explained either by competition between SPL and DTT for the active

site of P450 (Dawson et al., 1983), or to the more efficient trapping of reactive alkylating SPL electrophilic species by DTT than GSH. Because microsomal esterases have been shown to deacetylate SPL to SPL-SH independently of NADPH inclusion (Figure 8; Section IB7), the effect of the esterase inhibitor BNPP on ^{14}C-SPL in the NADPH-devoid incubations was assessed to determine the importance of SPL-SH formation in the observed non-specific binding. Indeed, inclusion of BNPP diminished ^{14}C-SPL binding in incubations devoid of NADPH, consistent with the theory that formation of SPL-SH was necessary for the formation of mixed disulfides between SPL-SH and protein thiols and that their formation was responsible for the previously observed high covalent binding in the NADPH-devoid incubations (Table 16).

3. Binding of ^{14}C-SOLD and CAN to liver microsomal proteins prepared from NT and DEX-pretreated rats

NADPH-dependent covalent binding of ^{14}C-SOLD (the 6-7 diene, 21-hydroxyacid SPL analog) to liver microsomes prepared from NT rats was quite low (Δ= 0.4 nmol/mg), but was enhanced by DEX-pretreatment (Δ= 2.5 nmol/mg protein), implying a role of P450IIIA in such NADPH-dependent binding. This relatively low binding (as compared to ^{14}C-SPL) is consistent with the low levels of P450 loss produced by SOLD in vitro (Table 11; Section IVB6). NADPH-independent binding of SOLD (NT 5.57 nmol/mg; DEX 4.47 nmol/mg) was also lower than that observed with SPL (Figure 16). This further supports the possibility that ^{14}C-SPL-SH/protein-thiol mixed disulfide formation must indeed occur, because SOLD lacks a sulfur moiety.

Table 16. Irreversible binding of SPL and analogs to liver microsomal protein from DEX-pretreated and NT rats

Rat Pretreatment	Analog	Addition (mM)	Irreversible binding (nmol/mg protein) -NADPH	+NADPH	Δ
NONE	^{14}C-SPL*	-	11.5 ± 3.10	13.7 ± 0.21	2.2 ± 3.11
	^{14}C-SOLD*	-	5.57 ± 0.04	5.99 ± 0.13	0.4 ± 0.14
	^{14}C-CAN*	-	2.75 ± 0.07	4.51 ± 0.20	1.8 ± 0.21
DEX	^{14}C-SPL*	-	12.3 ± 1.53	18.7 ± 1.62[a]	6.3 ± 2.2
	^{14}C-SOLD*	-	4.47 ± 0.16	6.93 ± 0.37	2.5 ± 0.38[b]
	^{14}C-CAN*	-	3.27 ± 0.47	14.8 ± 2.47[b]	11.5 ± 2.5[c]
DEX	^{14}C-SPL	-	12.6 ± 4.12	20.0 ± 5.57	7.5 ± 4.2
	^{14}C-SPL	+BNPP(0.4)	5.75		
	^{14}C-SPL	+GSH(1)	6.51	10.9	4.4
	^{14}C-SPL	+GSH(1) +DTT(1)	3.61	5.39	1.8
	^{14}C-SPL	+GSH(5.0)	6.72 ± 2.17[d]	12.3 ± 4.04[e]	5.5 ± 2.7
	^{14}C-SPL	+GSH(5.0) +DTT(5.0)	1.68	2.14	0.5
	^{14}C-CAN	-	4.01 ± 2.94	14.2 ± 7.04	10.2 ± 4.2
	^{14}C-CAN	+GSH(5)	3.23 ± 1.98	5.64 ± 3.66	2.4 ± 1.8

DEX-pretreatment of rats as in Section III (daily for 3 days). Microsomal preparation and in vitro incubations performed as described in Section III. The concentration of SPL or analog was 0.5 mM, except where indicated (*), when the concentration was 1 mM. Values are the mean ± SD of at least 3 separate experiments, except where the mean of two individual experiments is given as a single value. [a,b,c]Statistically significant at $^{a}p < .01$, $^{b}p < .002$, $^{c}p < .005$ relative to the corresponding system using non-pretreated rats. [d,e]Statistically significant at $^{d}p < 0.01$ or $^{e}p < 0.05$ when compared with corresponding ^{14}C-SPL controls, incubated in the absence of GSH.

^{14}C-CAN [the 6,7-diene in vivo metabolite, (Sadee et al., 1972; Section ID6a)] produced even lower NADPH-independent binding (NT 2.75 nmol/mg; DEX 3.27 nmol/mg), than SOLD. This again supports the possibility that "mixed disulfide formation" theory might be responsible for the previously observed high NADPH-independent binding with ^{14}C-SPL (Menard et al., 1979b; Section IVD2), because CAN is identical to SOLD in the 6,7-region of the steroid (i.e. possesses no sulfur atom).

Interestingly, Δ ^{14}C-CAN binding was over 2-fold higher than that of SPL (i.e. 11.5 vs. 6.3 nmol/mg) in liver microsomes from DEX-pretreated rats, but comparable to that of ^{14}C-SPL in liver microsomes from NT rats (Table 16). These results indicated that P450IIIA induction increased the extent of ^{14}C-CAN binding and were initially puzzling as CAN produced no P450 inactivation either in vivo (Figure 5; Section IVA8) or in vitro (Table 11; Section IVB6). Inclusion of GSH in incubations of DEX-pretreated rat liver microsomes resulted in lowering the NADPH-induced binding (Δ) of CAN to values comparable to those of SOLD [i.e. to 2.4 nmol/mg; (Table 16)], indicating that the putative reactive (binding) metabolite of CAN could be trapped by the nucleophile GSH, and therefore was not confined to the active site of its generating enzyme.

Recently, the formation of both 6α,7α- and 6β,7β-epoxides of potassium canrenoate by rat hepatic S9 fractions has been reported

(Cook et al., 1988; Section ID6a). Furthermore, in vitro metabolism of potassium canrenoate in rat hepatic microsomes was inhibited by SPL, indicating either competition for the same enzyme or inactivation of an enzyme important for potassium canrenoate metabolism by SPL (Cook et al., 1988). These findings are consistent with our report of P450IIIA inactivation by SPL (Sections IVA, IVB) and P450IIIA-dependent activation of CAN to a species which covalently binds to microsomal proteins (Table 16). Oxidation of α,β-unsaturated ketones by P450 may result in epoxides which once formed are quite likely to bind to microsomal proteins. Thus, the observation of DEX-enhanced NADPH-dependent binding of CAN may be explained by the selective oxidation of CAN to either the 6α,7α- or 6β,7β- epoxide (either of which may avidly bind to microsomal proteins) by P450IIIA. Reaction of such a CAN epoxide with GSH may result in attenuation of ^{14}C-CAN binding. Indeed, a GSH-adduct of potassium canrenoate epoxides was recently identified (Cook et al., 1988).

Recently, P450IIIA has been shown to catalyze the desaturation of the 6,7-region of testosterone to the corresponding 4,6-diene metabolite (Nagata et al., 1986). P450IIIA hydroxylates testosterone at the 6ß-position (Waxman, 1988), and is suicidally inactivated by 6ß- or 7α-thiol derivatives of testosterone (Menard et al., 1979b; Underwood et al., submitted for publication), and SPL (Sections IVA; IVB). These findings together with the observation that P450IIIA appeared to oxidize CAN to (a) species which bind(s) to microsomal proteins

suggests considerable regioselectivity of P450IIIA for this 6,7-region of steroids. Furthermore, the reported finding of 6ß-OH-SPL-SCH_3 as a known metabolite of SPL (Karim et al., 1975; Section ID6b) is entirely consistent with P450IIIA-mediated oxidation.

Although both SOLD and CAN are desaturated at the 6-7 position, CAN bound to a greater extent than SOLD. The differential binding characteristics of these two molecules may be explained by the greater lipophilicity of CAN which would more easily gain access to the active site of P450IIIA and be oxidized at the 6-7 region. SOLD, a charged species (which possesses a hydroxyacid function at the D ring) may not gain access as readily to microsomal P450IIIA, thus accounting for its poor activation and consequent covalent binding to microsomal proteins.

Although P450IIIA-mediated CAN binding to microsomal protein was apparent, its involvement in the SPL-mediated inactivation of P450IIIA appears secondary as CAN failed to destroy the enzyme. It is conceivable that the observed binding of ^{14}C-SPL to microsomal proteins (Section IVD1) also may be due to the binding of its putative metabolite CAN in such incubations, and thus may not contribute to enzyme inactivation. CAN formation through elimination of the 7α-function of SPL-SCH_3, SPL-$S(O)CH_3$, or SPL-$S(O_2)CH_3$ is not expected in vitro, since in the absence of S-adenosyl methionine no SPL-SCH_3 can be generated (Lacagnin et al., 1987; Section ID6b). The formation of

CAN through elimination of H_2S from SPL-SH is however possible, although unlikely. Elucidation of the covalent binding characteristics of CAN revealed different pathways of metabolic activation in the NT and DEX-pretreated rat microsomal systems, and thus may be used to predict the possible effects that this clinically used drug and isolated metabolite of SPL may have.

E. Metabolism of SPL by P450IIIA

1. Rationale

Studies with radiolabelled SPL analogs yielded inconclusive mechanistic explanations for SPL-mediated inactivation of P450. To gain insight into the mechanism of SPL-mediated inactivation of P450IIIA, the metabolism of SPL was examined to determine whether P450IIIA-selective metabolic pathway(s) exist for this compound that could explain its selective inactivation of P450IIIA. Plausible mediators of SPL-mediated inactivation of P450IIIA include the highly reactive electrophilic sulfenic acid or thiyl radical, which would result from a P450-catalyzed 2- or 1- electron oxidation, respectively of SPL-SH.

Isolation of either the thiyl radical or sulfenic acid species is unlikely due to their high reactivity. Spontaneous oxidation to more stable derivatives (such as the sulfonic acid species) has been postulated (Ross et al., 1985; Ross and Moldeus, 1986; Tamba et al., 1986). Therefore, the formation of oxidized (and thus more polar) metabolites of SPL-SH by P450IIIA was investigated to determine whether there was selective involvement of this isozyme in the oxidation of SPL-SH.

2. Detection of polar SPL metabolites, and criteria for their formation

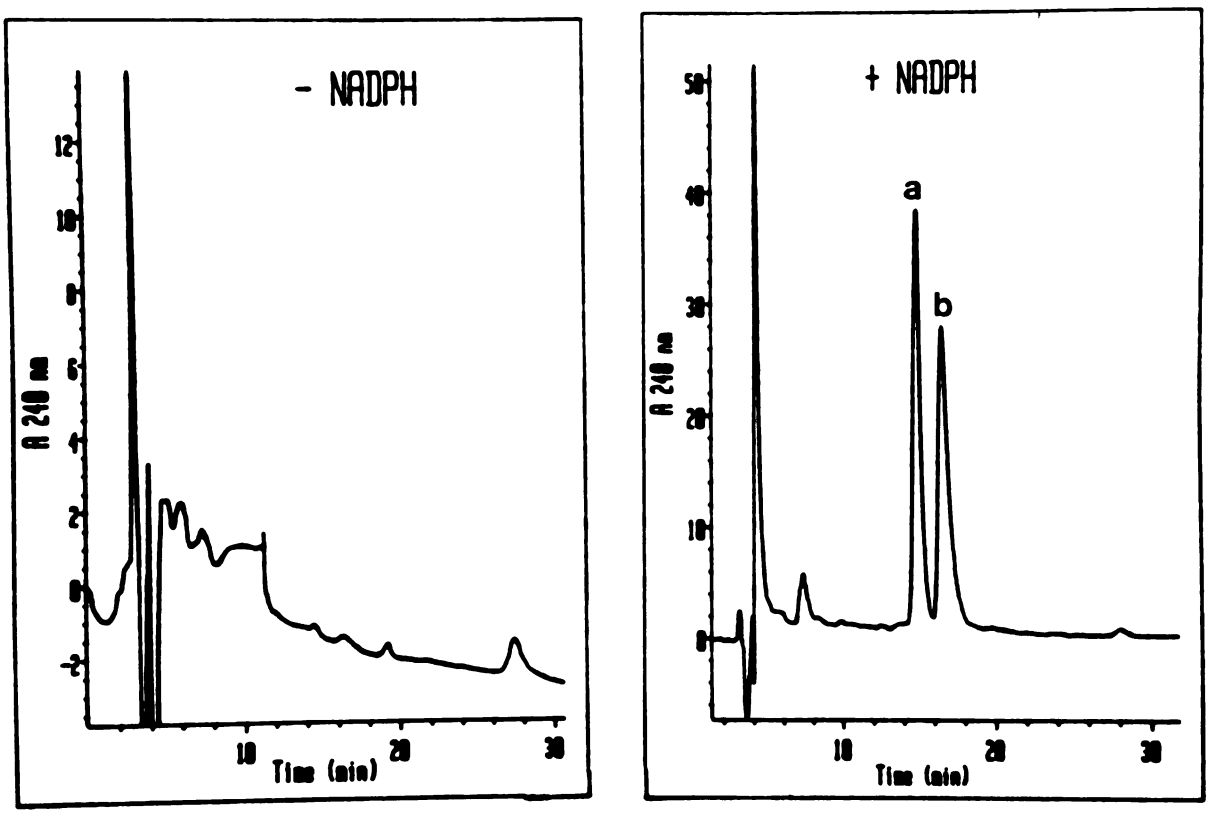
HPLC analysis (Section III) of supernatants of incubations containing liver microsomes prepared from DEX-pretreated rats (Section III) yielded the NADPH-specific formation of two very polar (relative to SPL) metabolites (a and b; Figure 10). These metabolites possessed

absorption maxima at 240 nm (as does SPL) and were quantified using the integration function on the diode array detector with SOLD as an internal standard, or by radioactivity after ^{14}C-SPL was included in microsomal incubations (Section III). As can be seen in Table 17, the formation of these metabolites was found to be dependent on SPL and P450 concentration, and was significantly impeded at 0°C, indicating the involvement of an enzymatic process. The formation of the polar metabolites was dependent on NADPH and O_2 inclusion, and addition of CO (a P450 inhibitor) significantly attenuated the yield of these metabolites, implying a crucial role of P450 in their generation (Table 17). The role of FMO in such polar metabolite production was excluded as inclusion of NOA [a P450 inhibitor, but stimulator of FMO (Ziegler, 1980; Section IB)] attenuated rather than increased polar metabolite formation.

3. Isozyme selectivity for SPL polar metabolite formation

Comparable incubations containing hepatic microsomes prepared from PB- or ßNF-pretreated and NT rats yielded significantly lower quantities of polar metabolites than those obtained with hepatic microsomes from DEX-pretreated rats, implicating a major role of P450IIIA in metabolite formation (Table 17). Polar metabolite formation paralleled P450 loss in all of the systems employed (despite their differing P450 isozymic compositions), suggesting a direct association between the two processes. Partition ratios (nmol metabolite formed/nmol of P450 inactivated) were approximately 20 for all microsomal systems used, indicating that P450 inactivation and

Figure 10. HPLC chromatography of SPL polar metabolites



Refer to Section III for HPLC conditions and experimental details.

Table 17. In vitro conversion of SPL to polar metabolites by rat liver microsomes

Rat Pretreatment	Incubation System[a]	P450 loss[b]	polar metabolites[c]
DEX	complete	48.5 ± 3.50	88.8 ± 21.9
	-NADPH	0	5.01 ± 10.0
	+CO	0	0
	0°C	1.6	19.0
	P450 [1μM]	50.0	36.3
	SPL [0.1mM]	39.6	48.0
	SPL [0.01mM]	19.1	6.5
	n-octylamine [3 mM]	26.5	25.6
DEX	Complete	48.5 ± 3.50	88.6 ± 19.2
	+Catalase (0.5mg/ml)	43.0 ± 3.68	77.4 ± 39.7
	+SOD[a] (0.1mg/ml)	52.3 ± 3.97	57.8 ± 19.1
	Complete	43.9 ± 7.54	78.7 ± 17.8
	+Catalase + SOD	43.1 ± 13.5	53.7 ± 19.8
DEX	Complete	42.6 ± 6.15	76.3 ± 15.7
NONE	Complete	21.4 ± 10.2[d]	44.0 ± 22.3[e]
PB	Complete	19.9 ± 3.83[f]	35.9 ± 8.82[d]
βNF	Complete	15.3 + 5.30[f]	17.7 + 5.26[f]

[a]The complete incubation mixture (final volume, 3 ml) consisted of P-450 (3 μM), SPL (1.0mM), EDTA (1.5 mM), NADPH (1.5 mM), and phosphate buffer 0.1M, pH 7.4. Reactions were carried out at 37°C for 30 min, and P450 loss monitored and metabolites isolated by HPLC and quantitated as described (Scetion III). [b]P450 loss (% original). [c]Polar metabolites (nmol/incubation/15 min). All values are mean ± SD of 3-7 individual experiments, except where the mean of two experiments is given as a single value. [d,e,f,g]Statistical significance when compared with the DEX/complete system: (d) $p < .005$; (e) $p < .05$; (f) $p < .001$.

Table 18. Partition ratios for in vitro SPL polar metabolite formation and P450 destruction

Rat Pretreatment	Partition Ratio[a]
DEX	19.9
NONE	22.9
PB	20.0
βNF	12.9

[a]Partition ratio (nmol polar metabolite/nmol P450 inactivated) derived from Table 17, where nmol polar metabolite formed/ ml is divided by nmol P450 inactivated/ml after 30 min of incubation.

metabolic turnover of SPL are directly related events (Table 18). Thus, it appeared that P450IIIA was highly active in catalyzing SPL polar metabolite formation and hepatic microsomal preparations containing large P450IIIA content will exhibit a correspondingly greater formation of SPL polar metabolites and inactivation of P450.

4. Effect of superoxide dismutase and catalase on SPL polar metabolite formation

To determine whether polar metabolites were generated by externally generated hydrogen peroxide or superoxide [which are known to be formed during oxidative P450 turnover (Guengerich, 1978)], the effects of catalase (CAT) and superoxide dismutase (SOD) on polar metabolite formation were assessed in the in vitro system. Neither CAT nor SOD, alone or in combination, affected P450 inactivation, although SOD, with or without CAT, decreased polar metabolite formation (i.e. to 65 and 68%, respectively of original values). The decrease, however, was not statistically significant. These findings suggest that SPL-mediated P450 inactivation occured without hydroperoxide or superoxide involvement, but that a fraction of polar metabolite formation may have resulted from the reaction of SPL-SH with superoxide to produce the polar metabolites. Reaction of GSH with superoxide has been shown to result in both the sulfonate and disulfide derivatives of GSH (Wefers and Sies, 1983). Similar interaction of SPL-SH with superoxide thus may account for a fraction of polar metabolite formation.

5. Structural characterization of the SPL polar metabolites

To determine the structural identity of the polar metabolites, each compound (retention time of 14.9 or 16.5 min; Figure 10) was collected from the HPLC system used to separate the polar metabolites, and then subjected to further HPLC purification (Section III) to remove NaOAc, followed by lyophilization and thermospray LC/MS, FAB/MS, FAB/MS/MS, or LSIMS (M. Rashed and T. Baillie, U. of Washington, or D. Maltby, UCSF).

LC/MS analysis of each metabolite gave a molecular ion identical to that of CAN. The data suggested thermal decomposition of the metabolites under thermospray conditions. FAB/MS/MS analysis of the polar metabolites (Figure 11) yielded the spectrum of a compound consistent with the structure of the sulfonic acid derivative of SPL (SPL-SO_3H) for the relatively more hydrophilic polar metabolite [i.e. the compound eluting at 14.9 min using the original HPLC system (Figure 10)]; and a spectrum consistent with the sulfinic acid derivative of SPL (SPL-SO_2H) for the relatively less hydrophilic polar metabolite (i.e. the compound with retention time of 16.5 min).

6. Chemical syntheses of SPL-SO_3H and SPL-SO_2H

Authentic sulfonic and sulfinic acid derivatives of SPL were synthesized by oxidation of SPL-SH with *m*-chloroperoxybenzoic acid (a 2-electron oxidant, Section III). Synthetic oxidized SPL derivatives had HPLC chromatographic and mass spectral characteristics identical to those of the polar metabolites isolated from NADPH-supplemented incubations of SPL and hepatic microsomes prepared from DEX-

pretreated rats, thereby unequivocally confirming the structural identities of the polar metabolites (Figure 10), as those of the sulfonic and sulfinic acid derivatives of SPL, respectively.

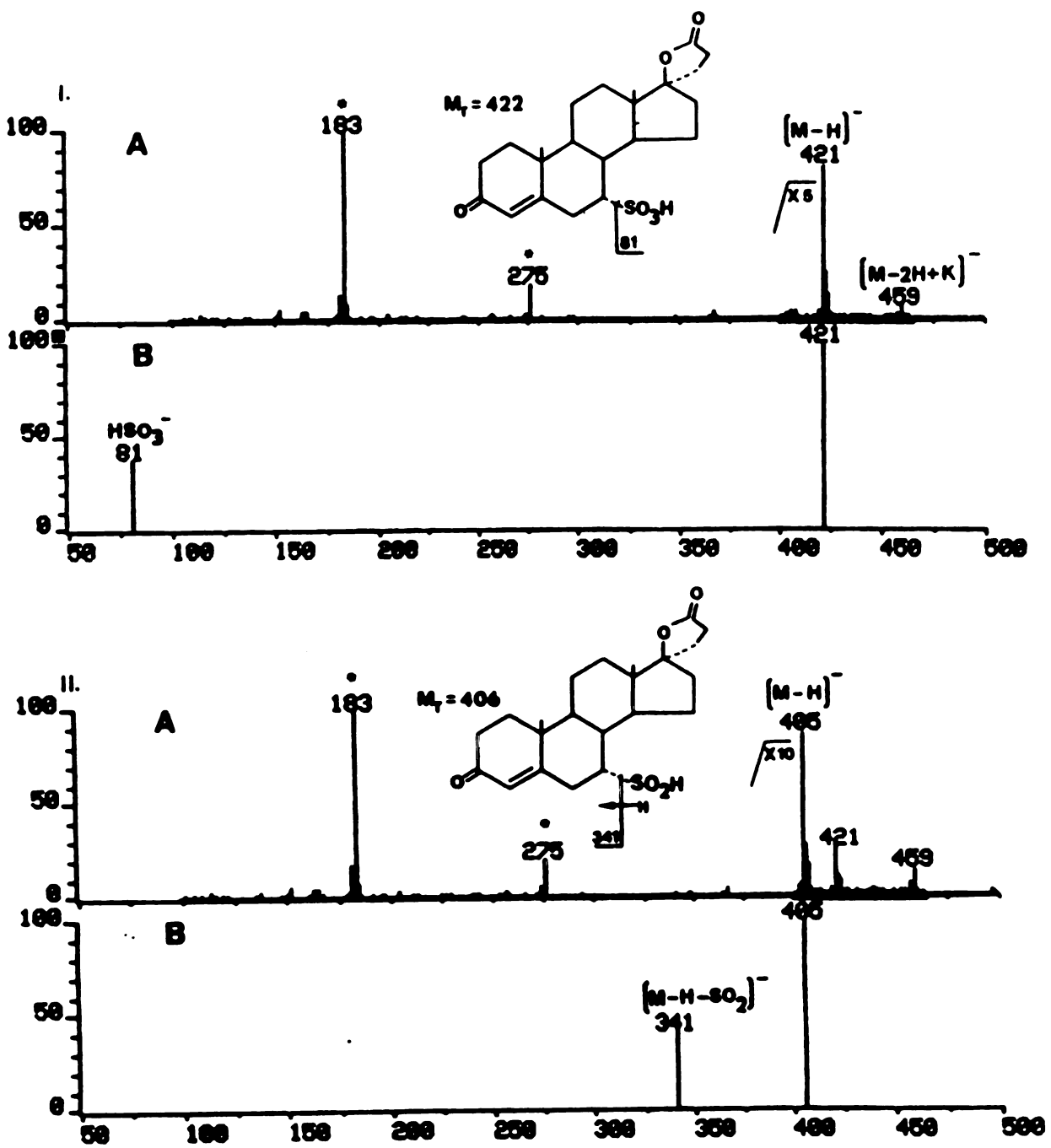
The P450-dependent formation of the SPL sulfinate and sulfonate metabolites indicated oxidation of the thiol moiety of SPL by P450. As discussed previously, the initial putative reactive intermediates of SPL generated by P450 catalysis are the thiyl radical and/or sulfenic acid derivatives. The rationale for the P450-dependent formation of these electrophilic species is derived from many studies (discussed below).

There are several examples providing support for thiyl radical formation of SPL-SH by P450. P450 oxidation of hydrocarbons is generally accepted as proceeding via a one electron oxidation of the substrate followed by radical recombination to the resultant alcohol (Groves et al., 1978; Section IA2). P450 involvement in the one electron oxidation of heteroatoms is supported through spin trapping studies of released 4-alkyl substituents of 4-alkyl-1,4-dihydropyridines (Augusto et al., 1982; Section IC2b). Furthermore, oxidation (Vmax) of sulfides by P450 correlates with their one electron oxidation potentials, suggesting a one electron oxidation to the sulfide radical cation species followed by radical recombination to the sulfoxide (Watanabe et al., 1980). Thus, oxidation of the thiol moiety of SPL-SH to a thiyl radical species by P450 is plausible.

P450-dependent oxidation of methimazole to the sulfenic acid species has been suggested by the findings of Kedderis and Rickert, (1985). In these studies, GSH was found to block all FMO-dependent P450 destruction by methimazole in NT rat microsomal systems (no P450 destruction was observed when FMO was heat inactivated). PB-inducible isozymes were able to metabolize methimazole to a species that destroys P450 (independent of FMO activity, which was blocked by heat inactivation), in a process that was also inhibited by the addition of GSH. Because FMO and P450-mediated activation of methimazole to species which destroy P450 were similarly blocked by the addition of GSH (implicating a diffusible reactive intermediate), and because FMO oxidizes sulfur-containing compounds to their corresponding sulfenic acid species (Ziegler, 1980; Section IB), PB-inducible P450 isozymes were proposed to catalyze the formation of a sulfenic acid derivative of methimazole.

Metabolism of 6-thiopurine to the sulfenic acid species by P450 was proposed by Hyslop and Jardine in studies employing 6-mercapto-purine. Covalent binding of 6-mercaptopurine to rat, rabbit, and human hepatic proteins in vitro (Hyslop and Jardine, 1981a) was shown to be NADPH dependent and P450-mediated through attenuation with P450 inhibitors. Such binding was also inhibitable by the addition of GSH. Covalent binding of 6-mercaptopurine to various rabbit tissue proteins was shown to occur in vivo, with greater binding observed in the liver microsomal fractions that had been depleted

Figure 11. Mass Spectra of the Polar Metabolites



I. (A) Negative ion FAB mass spectrum of the peak material eluting at 14.9 min (Figure10), the sulfonic acid species. (B) Daughter ion spectrum following collisional activation of m/z 421 ([M -H]⁻), obtained from the same sample. II. (A) Negative ion FAB mass spectrum of the peak material eluting at 16.5 min (Figure 10), the sulfinic acid species. (B) Daughter ion spectrum following collisional activation of m/z 405 ([M -H]⁻), obtained from the same sample.Ions denoted by asterisks are from the glycerol matrix. Y and X axes denote relative abundance and mass/charge, respectively. Refer to Section III for details.

of GSH through starvation of the animals (Hyslop and Jardine, 1981b). The radiolabelled sulfenic acid derivative of 6-thiopurine was subsequently synthesized and shown to bind directly to microsomal protein (Abraham et al., 1983). Thus, two electron oxidation of SPL-SH to its sulfenic acid species by P450 appears also to be warranted.

The thiyl radical and/or sulfenic acid derivatives conceivably can further oxidize to the more stable sulfinic and sulfonic acid derivatives, either spontaneously or through further oxidation by P450. The thiyl radical has been postulated to react with molecular O_2, to form the sulfinic and sulfonic acid derivatives (Schaefer et al. 1978; Tamba et al., 1986). The sulfenic acid species has been proposed to undergo spontaneous oxidation to both the sulfinic and sulfonic acid species (Hogg and Robertson, 1979; Berger, 1963). Thus, formation of either the sulfenic acid (SPL-SOH) or thiyl radical (SPL-S·) could result in P450 destruction, or if released, could be further oxidized to the sulfinic or sulfonic acid species. Partitioning of reactive species between metabolite formation and enzyme inactivation is a feature of suicidal inactivation. Thus, P450IIIA-dependent formation of the sulfinic and sulfonic acid species implicates oxidation of SPL-SH to either the thiyl radical or sulfenic acid species, either of which can inactivate P450 in several ways (Figure 12).

The thiyl radical may be considered the direct precursor of the sulfenic acid in P450-mediated oxidation of SPL, because its one-

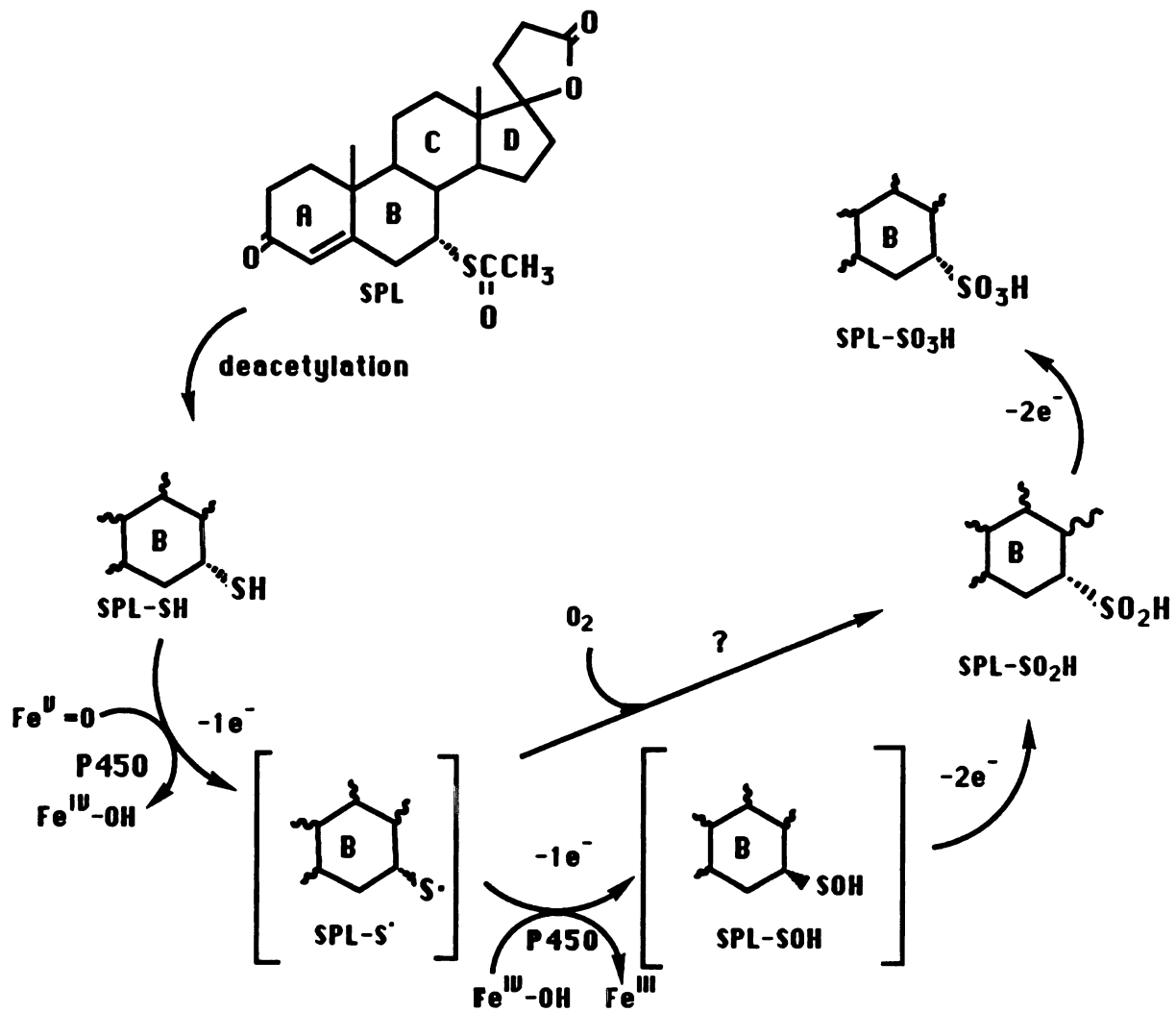
electron oxidation would concomitantly generate both the P450-heme-perferryl species (Fe^{+4}=O) and the thiyl radical, with subsequent collapse of the two to yield the sulfenic acid and Fe^{+3} [in a reaction analogous to that proposed for the heteratom nitrogen (Guengerich, 1984)]. If such collapse to the sulfenic acid occurs, it may inactivate P450 in several ways. The sulfenic acid in some ways resembles an alkyl peroxide structurally and may cause P450 inactivation (with accompanying heme binding) in a manner analogous to that of cumene hydroperoxide (Guengerich, 1986; Section IC2c). The sulfenic acid species could also inactivate P450 by reacting with protein sulfhydryls in a manner similar to that of 6-mercaptopurine (Hyslop and Jardine, 1981), or of parathion (via atomic sulfur, Neal et al., 1977). Alternatively, the sulfenic acid could react with P450 heme in the same way in which methimazole and its various analogs inactivate lactoperoxidase (Doerge, 1986).

If collapse to the sulfenic acid does not occur during P450-mediated oxidation of SPL-SH, the thiyl radical may inactivate P450 through other mechanisms. If the radical and the iron-oxo species do not collapse to form the sulfenic acid, the electron deficient iron-oxo (Fe^{+4}=O) species formed in parallel with the thiyl radical may inactivate P450, as has been proposed for other compounds believed to be oxidized by P450 via one electron oxidations (Correia et al., 1987). The thiyl radical may inactivate P450 by reacting with the heme moiety to form species which bind to the P450 apoprotein as

shown to occur with many xenobiotics (Davies et al., 1986; Section IC2c). The thiyl radical may alternatively inactivate P450 by reacting directly with the P450 apoprotein.

Thus, isolation of SPL-SO_3H and SPL-SO_2H indicates either (1) oxidation of SPL by P450 to either the thiyl radical or sulfenic species with spontaneous further oxidation; or alternatively, (2) sequential turnover of SPL-SH by P450 (i.e. three to produce the sulfonic acid species and two for the sulfinic acid derivative) wherein SPL-SOH is an obligatory intermediate. Nevertheless, isolation of these metabolites directly reflects production of either the electrophilic thiyl radical or sulfenic acid species which appears to account for the destruction of P450 caused by SPL.

Figure 12. P450-mediated formation of the sulfinic and sulfonic acid derivatives (polar metabolites) of SPL



SPL-S·: SPL-thiyl radical; SPL-SOH: SPL-sulfenic acid; SPL-SO_2H: SPL-sulfinic acid; SPL-SO_3H: SPL-sulfonic acid; Fe indicates P450 heme iron.

F. Effects of GSH on SPL-mediated inactivation of P450

GSH, an important endogenous nucleophile has been shown to protect against both free radical and sulfenic acid-mediated cellular damage (Harman et al., 1986; Krieter et al., 1984). In fact, we had shown that GSH partially attenuated SPL-elicited covalent binding to microsomal proteins (Table 16). The role of GSH in SPL-mediated inactivation of rat hepatic P450 was therefore assessed.

1. Effect of SPL on hepatic GSH levels in vivo

Hepatic GSH levels were determined (Section III) following administration of SPL (200 mg/kg) to DEX-pretreated and NT rats to determine the effect of SPL on this important cellular nucleophile, as its depletion reflects a protective mechanism occurring after the administration of many chemicals which require bioactivation to reactive electrophiles (Mannervik et al., 1988). GSH levels were decreased following SPL administration to either DEX or NT rats (Table 20). This decrease was statistically significant only in the DEX-pretreated rats, however. These findings suggested formation of a reactive electrophile in SPL-mediated inactivation of P450 (particulary P450IIIA), that is capable of depleting cellular GSH levels by either reacting directly with GSH or through the action of glutathione peroxidase.

2. Effect of GSH in vitro on P450 inactivation and polar metabolite formation

In vitro, GSH (5.0 mM) was found to attenuate P450 inactivation in systems containing liver microsomes from DEX-pretreated rats by

Table 19. Effect of SPL on hepatic P450 content and GSH levels in vivo

Rat Treatment	P450[a]	GSH[b]
DEX	1.50 ± 0.16	4.43 ± 0.68
DEX + SPL	0.59 ± 0.13[c]	3.16 ± 0.26[d]
% loss	60.7	28.7
NT	0.94 ± 0.13	5.60 ± 1.09
NT + SPL	0.87 ± 0.01	4.09 ± 0.70
% loss	10.3	27.0

DEX (as in Section III, daily for 4 days), SPL (as in Section III, 200 mg/kg, 2 h). [a]P450 (nmol/mg protein), and [b](GSH (μmol/g liver) as determined in Section III. [c]$p < .01$, [d]$p < .05$. All values represent the mean ± S.D. of n=3.

approximately 40% (Table 20). This in vitro finding narrowed the possibilities of how GSH may be functioning to rescue P450 from the effects of SPL, as glutathione peroxidase would be absent in such a microsomal system. The effect of GSH on polar metabolite formation was identical to that observed for P450 inactivation (i.e. polar metabolite levels was lowered to 60% of those in control incubations conducted in the absence of GSH). Thus, GSH appeared to diminish P450 inactivation and polar metabolite to a comparable extent, suggesting a common reactive intermediate participating in the two processes. This finding also indicated that a species responsible for ~40% of P450 inactivation and polar metabolite formation is stable enough to diffuse out of the active site of P450 to react with GSH, since GSH cannot gain access into the active site.

3. Detection of a GSH-dependent metabolite

Conceivably, GSH could react with the thiyl radical or sulfenic acid derivative of SPL, the two electrophilic candidates implicated in SPL-mediated P450 inactivation of P450, as GSH has been proposed to react with both sulfenic acids (Krieter et al., 1984) and thiyl radicals (Quintiliani et al., 1977; Harman et al., 1984). For these reasons, in vitro NADPH-supplemented microsomal incubations of SPL and ^{3}H-GSH were analyzed for the presence of GSH adducts, by monitoring the 240 nm absorbance of the α,ß-unsaturated ketone function of SPL, as well as ^{3}H-GSH-derived radioactivity. The HPLC system used was similar to that previously employed to isolate the polar metabolites (Section III), as the proposed adduct would be expected to be quite polar relative to SPL. A peak was observed in GSH-supplemented incubations with an

Table 20. In vitro effect of GSH on P450 loss and polar metabolite formation in microsomes from DEX-pretreated rats

Incubation System[a]	P450 loss[b]	Polar metabolites[c]
Complete	51.6 ± 5.5	100.5 ± 32.0
+GSH [5mM]	34.1 ± 11.6[d]	61.9 ± 28.8
% Attenuation	39.2	38.4

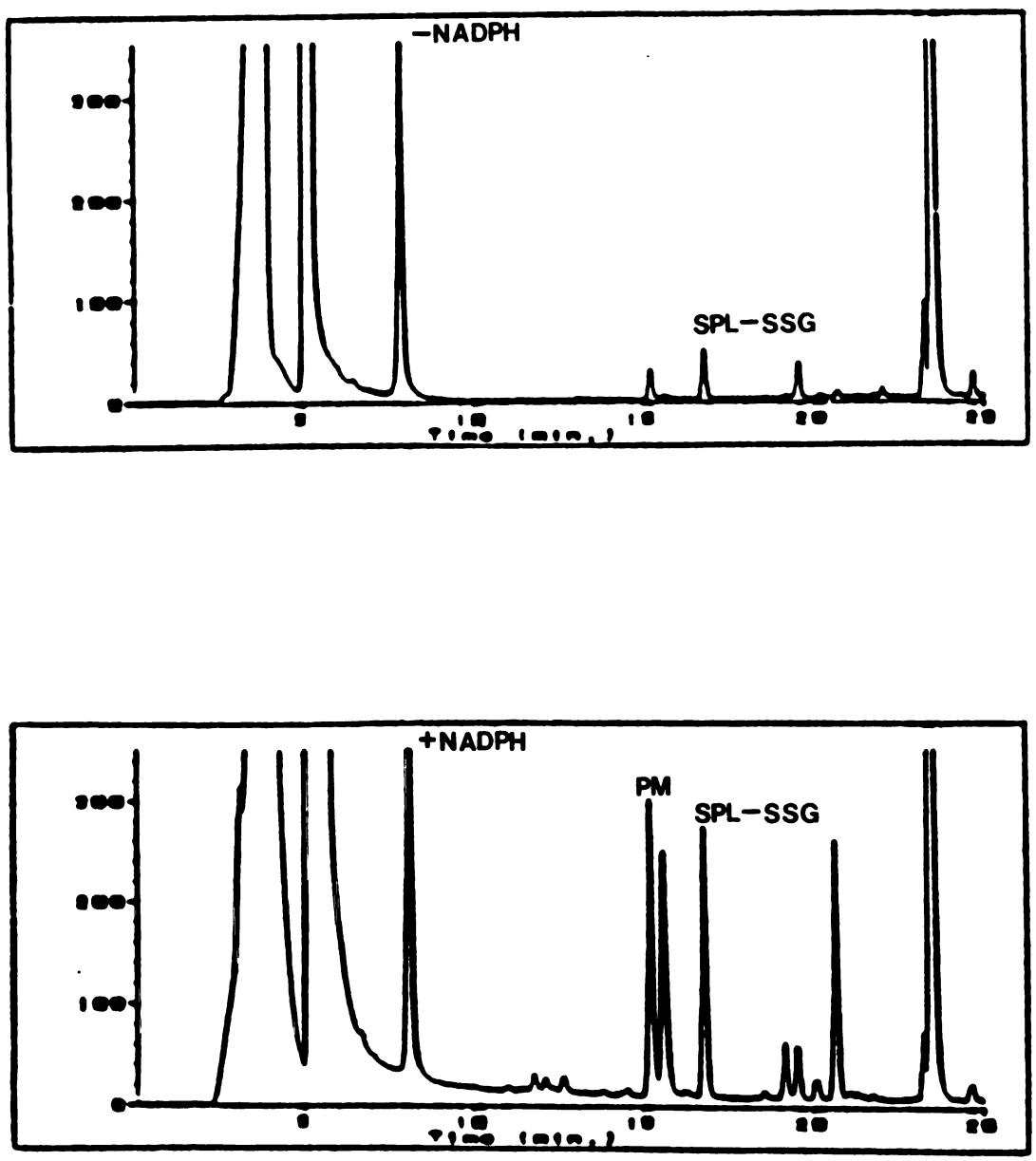
[a]The complete incubation mixture (final volume, 3 ml) consisted of P450 (3 μM), SPL (1.0 mM), EDTA (1.5 mM), NADPH (1.5 mM), and phosphate buffer 0.1M, pH 7.4. Reactions were carried out at 37°C for 30 min, and P450 loss was monitored and metabolites isolated by HPLC and quantitated as described (Section III). [b]P450 loss (% original). [c]Polar metabolites (nmol/incubation/30 min). All values are mean ± SD of 3 individual experiments. [d] $p < .02$.

absorbance maximum at 240 nm, which was increased by NADPH inclusion in the incubation mixture (Figure 13). This compound eluted at 16.5 min, approximately 1.5 min later than the polar metabolites (i.e. was more hydrophobic than the two previously characterized sulfinic and sulfonic acid metabolites of SPL, in this particular chromatographic system). Quantification of this peak using radiolabelled GSH or the integration function of the diode array detector (at 240 nm) under various conditions showed that its formation was GSH-dependent and significantly enhanced with NADPH inclusion (Table 21). Comparison of the GSH (^{3}H-GSH radiolabel) and SPL (absorbance at 240 nm) components of this compound resulted in a 1:1 ratio, strongly suggesting it to be an adduct of SPL and GSH.

4. Characterization of the SPL-SSG adduct

The peak material eluting at 16.5 min. was collected from the original HPLC system used to detect the adduct (Figure 13), then further purified by an additional HPLC step with H_20/MeOH, lyophilized, and subjected to positive LSIMS mass spectral analysis after hexylester derivatization (Section III). The mass spectrum (Figure 14) of this compound was consistent with all the characteristics of a disulfide adduct of SPL and GSH (i.e. SPL-SSG; Figure 15). Elucidation of the structure of this adduct provided direct evidence for the reaction of an electrophilic SPL-derived species with nucleophilic GSH, although it cannot distinguish between the SPL-thiyl radical or sulfenic acid species as the precursor, as both species are believed capable of reacting with GSH to form disulfide products (Quintiliani et al., 1977; Krieter et al., 1984). Furthermore, thiyl radicals are able to dimerize

Figure 13. HPLC chromatography of the putative SPL-SSG adduct



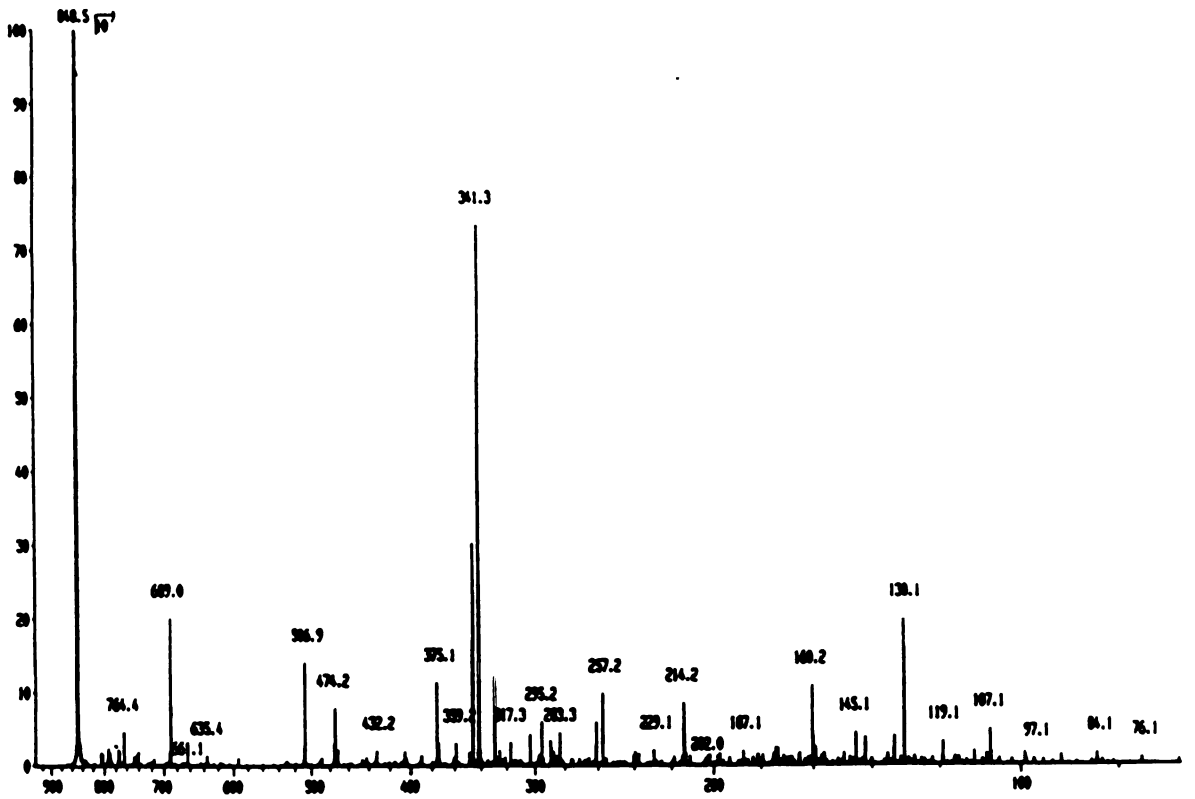
PM: polar metabolites; SPL-SSG: putative SPL-GSH adduct. Refer to Section III for HPLC conditions and experimental details.

Table 21. Quantification of the putative SPL-SSG adduct in vitro[a] using ^{3}H-GSH and UV-detection

System	SPL-SSG Adduct (nmol/3 nmol P450/15min)	
	Radiometric ^{3}H-GSH	Integration of 240 nm absorbance
Experiment 1		
- NADPH	3.60	3.62
	3.30	4.00
+ NADPH	7.80	9.70
	6.80	6.55
	3.17	4.84
Experiment 2		
- NADPH	3.31	2.73
+ NADPH	5.87	6.64
	4.11	5.72
	6.42	5.18

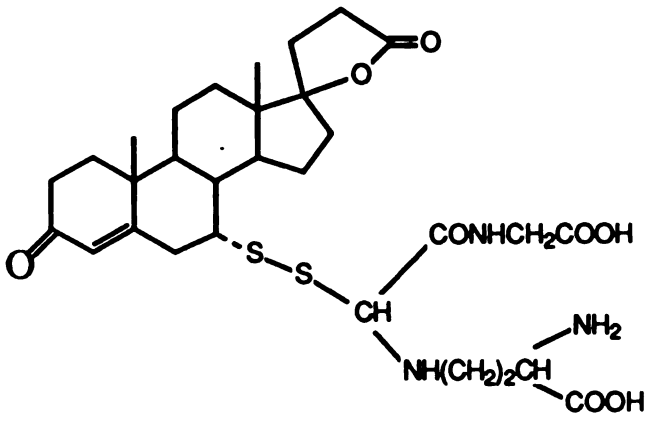
In vitro incubations as described in Section III [P450 (3 μM), GSH (5 mM), NADPH (1 mM), DTPC 1.5 mM)],which were acid precipitated for HPLC analysis of the adduct. Adduct quantified by radiometric analysis employing ^{3}H-GSH or integration of the 240 nm absorbance peak by the Hewlett Packard diode array detector. Each value represents one determination. Corresponding P450 losses were 46% in experiment 1, and 23% in experiment 2.

Figure 14. Mass Spectrum of the SPL-SSG Adduct



Liquid SIMS MS/MS spectrum of the hexylated adduct. The 848.5 (MH^+) peak denotes the dihexylated disulfide adduct; the 764.4 peak the monohexylated disulfide adduct; the 506.9 peak the dihexylated/SSG fragment; and the 341.3 peak the CAN fragment.
Y and X axes denote relative abundance and mass/charge, respectively. Refer to Section III for details.

Figure 15. Structure of the SPL-SSG adduct



to disulfides (Hoffman and Hayan, 1972), thus reaction of a glutathione thiyl radical [GS·, formed after donating an electron to a SPL thiyl radical (SPL-S·)] with yet another SPL-S· molecule could also produce the SPL-SSG adduct (albeit through an indirect route). If SPL-S· rapidly extracts electrons from GSH, the formation of GS· will be favored (in the presence of excess GSH), resulting in the ultimate formation of GSSG. However, when GSSG was monitored in in vitro incubations identical to those used for adduct isolation, no NADPH-dependent increase in GSSG levels were observed, thus providing no evidence for this sequence of reactions. This finding also indicates that the SPL-SSG adduct does not undergo disulfide exchange reactions with GSH in vitro as such reactions would also produce GSSG (Krieter et al., 1984). Thus, isolation and structural characterization of the disulfide adduct of SPL and GSH provides a mechanistic explanation for the attenuation of both P450 inactivation and polar metabolite formation by GSH, via the reaction of P450 with either the SPL thiyl radical and/or sulfenic acid species (Figure 16).

Because rat liver microsomes contain FMO, and FMO can also oxidize thiol compounds to their corresponding sulfenic acids [which may subsequently react with GSH (Ziegler, 1980; Krieter et al., 1984)], its role in the formation of the SPL-SSG adduct was investigated.

5. pH dependence of SPL-SSG adduct formation in vitro from rat hepatic P450IIIA isozymes

To assess the relative contributions of FMO and P450 in SPL-mediated P450 destruction, polar metabolite formation and adduct formation, incubations containing microsomes from DEX-pretreated rats were conducted at either pH 9.0 or pH 7.4, the optimal pH for FMO or P450 activity, respectively (Poulsen and Ziegler 1979; Sato and Omura, 1978). At pH 7.4, P450 inactivation and polar metabolite formation were higher than those observed at pH 9.0 (Table 22). At pH 9.0, P450 inactivation and polar metabolite formation decreased, but SPL-SSG adduct formation was increased (in the absence and presence of NADPH) over that observed at pH 7.4. Higher basal levels of the disulfide adduct were anticipated at pH 9.0 due to the increased spontaneous oxidation of thiols with the alkalinity of the solution (Wallace et al., 1963). At pH 9.0, the difference between the +NADPH and -NADPH values for adduct formation also increased (i.e. Δ=3.78 at pH 9.0, vs. Δ=1.85 at pH 7.4), indicating FMO involvement in adduct formation. These findings suggested that P450 inactivation and polar metabolite formation (which behave similarly in all studies, Section IVE) are primarily P450-dependent processes, and that although adduct formation does occur under optimal P450 conditions, it is enhanced under conditions favorable for FMO activity.

6. Formation of the SPL-SSG adduct by Purified FMO

Incubation of purified FMO with SPL-SH resulted in exclusive formation of a compound with the HPLC and spectral characteristics of

the SPL-SSG disulfide adduct with no detectable SPL-sulfinic or sulfonic acid derivative formation. Although the incubations were terminated by acid precipitation rather than by centrifugation at 105,000 xg (as is the usual procedure for analysis of polar metabolite formation when using microsomes), polar metabolites were still detectable in acid precipitated incubations (Figure 13). Thus polar metabolites if produced by FMO oxidation would have been detectable in this system. Formation of the adduct was dependent on active enzyme, SPL-SH and GSH (Table 23). Adduct levels were also significantly enhanced by NADPH inclusion. Furthermore, adduct formation was much greater at pH 9.0 (the pH optimum for FMO) than at pH 7.4, indicating a role of FMO in catalysis of adduct formation (Table 23).

7. Chemical Synthesis of a SPL-GSH disulfide adduct

Chemical synthesis of the metabolite was undertaken to obtain structural confirmation. A compound was synthesized by reaction of GSSG and SPL-SH at pH 9.0 (Section III, as disulfide exchange would occur at high pH values), which possessed an absorbance maximum at 240 nm, and chromatographed identically (i.e. 16.5 min retention time) to the biologically dervived material using the same HPLC system (Figure 13). The chromatographic and spectral characteristics of the synthesized material supported the structural identity of the FMO-SPL-SH derived metabolite as that of the SPL-GSH disulfide adduct.

Unfortunately, mass spectral analyses of the FMO-derived or synthetic material were initially unsuccessful in elucidating its structure due to Na^+ ion contamination. More recently, Drs. J. R. Cashman, D. Maltby (Dept. Pharmaceutical Chemistry, UCSF) and K. Sugiyama (Dept. Pharmacology, UCSF) have obtained mass spectral data for both the synthetic and FMO-generated adduct which unequivocally confirmed its structure as that of SPL-SSG (Figure 15).

These studies distinguish the ways in which SPL-SH was metabolized by FMO and P450. P450-dependent metabolic activation favored the formation of the sulfinic and sulfonic acid derivatives of SPL, whereas FMO catalysis produced only the SPL-SSG adduct when GSH was included in the system. SPL-SOH is the expected metabolite of FMO-mediated metabolism of SPL-SH, as FMO is a fairly well established exclusive 2 electron oxidant, (Ziegler, 1980). The lack of sulfinic or sulfonic acid metabolites of SPL in incubations containing FMO suggested that FMO (at the 15 min time point examined) does not further oxidize the SPL-sulfenic acid to the sulfinic acid or sulfonic acid derivatives, as has been previously postulated for formanidine sulfenic acids (Poulsen et al., 1974; 1979), as well as that the sulfenic acid does not spontaneously oxidize to the sulfinate and sulfonate (Hogg and Robertson, 1979) under these conditions.

The data suggested that in the P450 system, the polar metabolites were formed either from further oxidation of the thiyl radical or from successive turnovers of SPL-SOH by P450, but not from either spontaneous oxidation of SPL-SOH or from FMO-mediated oxidation of

Table 22. pH dependence of polar metabolite formation[a], adduct formation[b] and % P450 loss[c] in vitro[d] using microsomes from DEX-pretreated rats

pH addition	polar metbolites[a]	Adduct[b]	% P450 loss
7.4			
NADPH	16.6	ND	36
	16.5	ND	49
mean	16.6		43
7.4			
GSH	ND	2.34	0
	ND	1.21	0
mean		1.78	
7.4			
NADPH + GSH	14.3	4.46	26
	8.79	2.78	16
mean	11.5	3.62	21
9.0			
NADPH	10.7	ND	26
	13.0	ND	37
mean	11.9		32
9.0			
GSH	ND	3.16	0
	ND	1.43	0
mean		2.30	
9.0			
NADPH +GSH	6.02	7.89	0
	6.33	4.26	16
mean	6.18	6.08	8

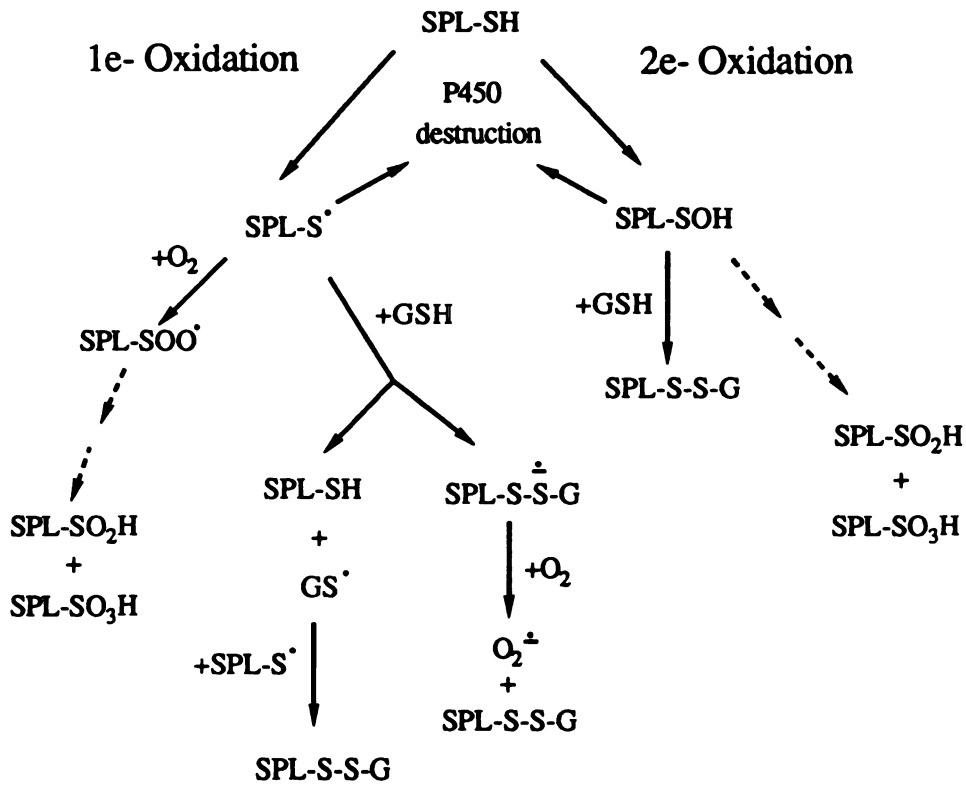
[a]Polar metabolite formation assay performed as described in Section III (nmol metabolite/3 nmol P450/15 min). [b]Adduct formation (nmol/3 nmol P450/15 min) as in Section III from acid-precipitated incubations. [c]P450 loss determined and [d]in vitro incubations performed with liver microsomes from DEX-pretreated rats as in Section III. Each value represents the mean of two determinations in one experiment. ND: not detectable.

Table 23. Formation of the SPL-SSG adduct in vitro[a] with purified FMO[b]

	SPL-SSG Adduct (nmol/nmol FMO/15 min)		
	pH 9.0 Exp. 1	pH 9.0 Exp. 2	pH 7.4 Exp. 2
complete	19.2	40.0	5.66
-NADPH	2.12	9.43	2.73
-GSH	ND		
-FMO	2.77		
-FMO, -NADPH		8.76	1.31
-SPL-SH		ND	

[a]Incubations as in Section III. [b]FMO purified as described in Section III. All values represent the mean of 2 determinations. ND: not detected.

Figure 16. P450-mediated formation of the polar metabolites and SPL-SSG



SPL-S$^{\bullet}$: SPL-thiyl radical; SPL-SOH: SPL-sulfenic acid; SPL-SO_2H: SPL-sulfinic acid; SPL-SO_3H: SPL-sulfonic acid; GS$^{\bullet}$: glutathione thiyl radical; SPL-SOO$^{\bullet}$: SPL-peroxyl radical; SPL-S-S-G: SPL-GSH disulfide adduct; SPL-S-$\dot{S}$-G: SPL-GSH disulfide anion radical; O_2: molecular oxygen; $O_2^{\bullet-}$: superoxide.

SPL-SOH. Although the adduct was indeed formed in the P450 system, it was formed to a lesser extent than from that of FMO, revealing that if it arose from the sulfenic acid generated by P450, the sulfenic acid either is formed and released from the active site of P450 to a much lesser extent than from that of FMO; or that the sulfenic acid is oxidized yet another time by P450, more readily than it reacts with GSH. Formation of the SPL-SSG adduct in the P450 system may still occur via the thiyl radical pathway (Figure 16), but these studies are incapable of elucidating this process. Thus, P450 may generate both SPL-SOH and SPL-S· in the P450 microsomal system, and each of these reactive species may not only inactivate P450, but also serve as progenitors of the disulfide adduct as well as polar metabolites (Figure 16).

The finding that FMO metabolized SPL-SH indicates that SPL-mediated P450 inactivation may in part be caused by FMO-dependent generation of SPL-SOH. This non-suicidal inactivation (i.e. produced by an enzyme other than that inactivated) could reflect the 40% of total inactivation which is attenuated by the addition of GSH (Section IVF2). Thus, a component of SPL metabolism may parallel that of the thiocarbamide drug methimazole, which is metabolized by both P450 and FMO to species capable of inactivating P450 isozymes (Section IC2ai; Kedderis and Rickert, 1985).

These results distinguish between SPL-SH oxidative pathways (i.e. the P450/polar metabolite vs. the FMO/disulfide adduct) and provide

new examples of how thiol-containing compounds are oxidized by P450 and FMO, two critical biotransformation enzymes of xenobiotics.

G. Effect of SPL on human P450 forms

1. Studies employing human liver microsomes

a. SPL-SH mediated P450 inactivation and polar metabolite formation

Polar metabolite formation and P450 inactivation were assessed in vitro using human liver microsomes (Section III) to ascertain the clinical significance of the previous findings (Sections IVA; IVB; IVE). SPL-SH was found to inactivate human hepatic P450s and also to produce polar metabolites (with a partition ratio comparable to that of rat hepatic P450 isozymes; Table 24). Thus, SPL-SH inactivated human hepatic P450, to an extent comparable to that of NT male rat hepatic isozymes (Section IVB4), in a process that also afforded polar metabolites.

b. Effect of SPL-SH in vitro on testosterone hydroxylation

Testosterone hydroxylase activities were assessed following SPL-SH mediated inactivation of human P450 in vitro. There appeared to be some interference of SPL-SH in the assay despite washing the incubations with albumin prior to assessment of the testosterone hydroxylation activities (Section III). Testosterone 6ß-hydroxylase activity in samples incubated with SPL-SH in the absence of NADPH, was nearly 89% of that in control samples (not exposed to SPL-SH), and thus may still provide a control value for P450 activity. Because the liver used for these studies was obtained from a male patient who had received Dexadron, the

P450-dependent values obtained may reflect an induced state. Testosterone 6ß- and 15ß-hydroxylase activities [which as in the rat are also associated with P450IIIA forms in humans (Waxman et al., 1988b)], were markedly inactivated (Table 25), indicating the inactivation of human P450IIIA isozymes by SPL. These findings paralleled the SPL-mediated loss of P450IIIA-dependent testosterone hydroxylase activities that were previously observed in intact DEX-pretreated rats (Section IVA5). Testosterone 2ß-hydroxylase activity was unaffected by SPL, since this activity also is believed to be catalyzed by P450IIIA isozymes in human liver, the implication of this finding was unclear. Testosterone 2α-hydroxylase activity [a P450IIC11 functional marker in the rat; (Waxman, 1988a)] was decreased 55% by SPL (Table 25), as in the DEX-pretreated rat (Figure 5). A human P450 isozyme similar in its N-terminal sequence to rat P450IIC11 has been purified and characterized (Shimada et al., 1986), suggesting that SPL may inactivate the human P450 isozyme related to P450IIC11. Thus, in addition to rat hepatic P450s IIIA and IIC11, SPL-SH inactivated orthologous human liver P450 isozymes, thereby revealing its potential for human drug-drug interactions since SPL is widely prescribed.

2. P450 inactivation and polar metabolite formation in human adrenal microsomes

Because SPL had been found to destroy several adrenal P450 isozymes in guinea pigs and dogs (Menard et al., 1974b;1976), polar metabolite

Table 24. SPL-SH-mediated P450 inactivation and polar metabolite formation by human liver microsomes

Hepatic Microsomal System	% P450 Loss[b]	polar metabolites[c]	partition ratio[d]
human	22.6 ± 6.42	5.59 ± 0.45	8.24
DEX-pretreated rat	54.6	13.9	8.47

Human hepatic microsomes were prepared and incubated as described in Section III. [a]P450 content (nmol/mg microsomal protein). [b]%P450 loss as determined in Section III. [c]Polar metabolite formation (nmol/3 nmol P450/15 min). [d]Partition ratio (nmol polar metabolite formed/nmol P450 inactivated). P450 content (nmol/mg protein) were human (0.68) and DEX-pretreated rat (1.43). Values represent the mean ± S.D. of three determinations in a single experiment (human), or the mean of two determinations in a single experiment (DEX-pretreated rat). The DEX-pretreated rat data are relatively low for this experiment (Tables 17 and 18), thus values obtained for the human liver may actually have been underestimated under these experimental conditions.

Table 25. Hepatic testosterone hydroxylase activities of human liver microsomes following SPL-SH-mediated P450 inactivation in vitro

	Testosterone hydroxylase activity (nmol/mg protein/min)									
	6α	15β	15α	7α	6β	16α	16β	2α	2β	AND
System[a]										
-NADPH -SPL-SH	0.65	0.26	0.01	0.13	7.53	0.00	0.31	0.16	0.91	0.46
-NADPH +SPL-SH	0.00	0.12	0.01	0.08	6.67	0.04	0.14	0.11	0.65	1.13
+NADPH +SPL-SH	0.26	0.03	0.02	0.07	0.52	0.00	0.00	0.06	0.65	0.27
% original[b]	100	25	100	88	7.8	0	0	55	100	24

[a]Microsomes were isolated from in vitro incubations for testosterone hydroxylase activity analysis and testosterone hydroxylase activities were determined as described in Section III. [b]% original [(+NADPH +SPL-SH) values divided by corresponding (-NADPH +SPL-SH) values x 100]. All values represent the mean of two individual determinations. P450 content was 0.68 nmol P450/mg protein. AND: androstenedione.

formation and P450 inactivation were examined in human adrenal microsomes to determine the relationship, if any between the two events in this system, and to compare it to our previously characterized rat hepatic system. SPL-SH (0.5 mM) was found to destroy ~25% of human adrenal P450 in vitro (Table 19). Polar metabolites were isolated in the same manner previously detailed, and yielded HPLC chromatographic and mass spectral data identical to those observed in the in vitro system containing microsomes from DEX-pretreated rats. Polar metabolites were quantitated as described previously (Section III) and a partition ratio (nmol metabolite/nmol P450 inactivated) of 11.8 was obtained (Table 26). Thus, SPL-mediated destruction of non-hepatic adrenal P450 isozymes was also found to be associated with polar metabolite formation, further linking the two processes. Humans produce cortisol rather than corticocosterone because they possess 17α-OHase (Hall, 1984), which is susceptible to SPL-SH-mediated inactivation. The destruction of adrenal P450 enzymes thus rationalizes such effects as reduced cortisol production following SPL administration to humans (Greiner et al., 1976). The formation of polar metabolites in conjunction with adrenal P450 inactivation indicated that these processes were mechanistically similar to those observed with rat hepatic P450IIIA and human liver P450 (Sections IVE2; IVG1a). Recently, differential responses to SPL-SH-mediated inactivation have been reported in different regions of the guinea pig adrenal

Table 26. SPL-SH-mediated P450 inactivation and polar metabolite formation by human adrenal microsomes

	P450 content[a]	% P450 Loss[b]	partition ratio[c]
Adrenal # 1	0.68	26.5	11.2
Adrenal # 2	0.69	23.2	13.7
Adrenal #3	0.83	22.2	10.5
mean ± S.D.	0.73 ± 0.08	24.0 ± 2.30	11.8 ± 1.7

Incubations containing P450 (0.5 nmol/ml) were performed as described in Section III. Adrenal microsomes were prepared as described in Section III. [a]P450 content (nmol/mg microsomal protein). [b]%P450 loss as determined in Section III. [c]Partition ratio (nmol polar metabolite formed/nmol P450 inactivated). All values represent the mean of at least two determinations. Corresponding values for P450 loss (48.5 ± 5.0%) and partition ratio (15.6 ± 5.5) for DEX-pretreated rats (n=5) in experiments run in parallel.

cortex (Sherry et al., 1989). Regions containing low levels of 17α-OHase (such as the outer zone) were found less susceptible to SPL-SH-mediated inactivation of P450 than those of the inner zone. In the present studies, no distinction between individual regions of the cortex were made and microsomes were prepared from the whole adrenal cortex. Thus, it is possible that the extent of P450 inactivation by SPL-SH may actually have been underestimated and that it might have been higher, were regions of the inner cortical zone specifically examined.

V. Conclusions

These studies establish the mechanism-based inactivation of rat hepatic cytochrome P450IIC11 and IIIA isozymes by SPL both in vivo and in vitro, and provide novel mechanisms by which this process occurs. Central to this inactivation mechanism is the catalytic oxidation of thiol compounds by P450. The mechanism by which SPL produces such inactivation is not fully understood in that although heme is lost stoichiometrically in parallel to P450, only a fraction (approximately, 30%) can be accounted for as being covalently bound to microsomal proteins. Covalent binding of SPL species to P450 apoproteins also occurs, although its role in P450 inactivation is unclear.

Oxidative metabolism of the sulfhydryl moiety of SPL by P450 results in the formation of the sulfinic and sulfonic acid metabolites of SPL. Formation of these metabolites also parallels P450 inactivation, suggesting that electrophilic species such as the thiyl radical or sulfenic acid might initially be produced by P450 oxidation. The partitioning of the reactive intermediate (thiyl radical and/or sulfenic acid) between inactivation (loss of P450 heme chromophore with subsequent covalent binding of heme fragment and/or steroid to P450 apoprotein) and turnover (sulfinic acid/sulfonic acid formation) pathways satisfies the criteria for a true suicide inactivator.

GSH partially attenuates both polar metabolite formation and P450 inactivation to a comparable extent indicating its quenching of a common reactive intermediate. This implicates a SPL-derived species in both P450 inactivation and polar metabolite formation that is stable enough to diffuse out from the active site of P450 to react with GSH. Isolation and characterization of a SPL-SSG adduct (whose concentration is enhanced by NADPH inclusion) provides direct evidence that the observed attenuation results from GSH-mediated electrophile (sulfenic acid and/or thiyl radical) scavenging. Exclusive formation of the SPL-SSG adduct (and its enhancement by NADPH inclusion), without detectable polar metabolite formation from incubations of purified hog liver FMO and GSH distinguish the way in which these important microsomal xenobiotic-biotransformation enzymes (i.e. P450 and FMO) oxidize SPL.

Inactivation of human hepatic and adrenal P450 isozymes by SPL rationalizes observations of clinical side effects associated with the drug's use such as decreased cortisol levels (Greiner et al., 1976). More importantly, these findings signal a potential hazard for adverse drug-drug interactions. Furthermore, such studies may have considerable bearing on the porphyrinogenic potential of SPL, which has been documented (Anderson, 1978), although the rationale for this was not fully understood.

The elucidation of P450 inactivation pathways is crucial for the understanding of potential adverse drug interactions which may occur as a consequence of coadministration of therapeutic agents. SPL, a widely prescribed diuretic, is often co-administered with drugs that require P450 metabolism for their elimination. It is thus plausible that SPL could alter drug metabolism by inactivating the very enzymes required for their metabolism. In addition, the induction of human P450IIIA isozymes by commonly used drugs such as DEX, PB and TAO followed by SPL administration could result in a dramatic loss of total P450 (as compared to the uninduced individual) resulting in the severe impairment of the metabolism of endogenous substrates, drugs or other xenobiotics in vivo. A recent report (Pichard et al., 1990a) documents the effects of various agents on human P450IIIA-dependent oxidation of cyclosporin A. Although they did not assess SPL, similar drug interactions are probable. The studies detailed in this thesis clearly demonstrate that SPL inactivates hepatic P450IIIA isozymes, thus contributing to the growing list of drugs which have a potential to interfere with the metabolism of cyclosporin, and numerous other drugs which require P450IIIA oxidation.

SPL may additionally affect the metabolism of other agents through its depletion of GSH levels. Thus, the detoxification of compounds which require GSH conjugation (such as acetominophen) conceivably also may be compromised when co-administered with SPL. The recognition and understanding of

these potential consequences of SPL administration may thus assist in preventing future therapeutic misadventure.

VI. References

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