INDUCTION OF VALPROIC ACID METABOLISM IN RAT LIVER MICROSOMES BY CARBAMAZEPINE AND CARBAMAZEPINE-10,11-EPOXIDE

by

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ABSTRACT

Valproic acid (VPA) is a commonly used anticonvulsant agent that is often given in combination with carbamazepine (CBZ) to maximize seizure control. The incidence of VPA associated hepatotoxicity is increased when coadministered with other anticonvulsants. One or more of VPA's metabolites may be responsible for the hepatotoxicity.

The consequences of induction by CBZ, carbamazepine-10,11-epoxide (CBZE), phenobarbital (PB) and clofibrate (CFB) on the metabolism of VPA and (E)-2-ene VPA by rat hepatic microsomes were examined. Total hepatic cytochrome P-450 content and the isozyme(s) of cytochrome P-450 induced by these agents were determined and compared. The metabolism of VPA was monitored following induction by quantitating the microsomal formation of 4-ene VPA, 3-OH VPA, 4-OH VPA, 4-keto VPA and 5-OH VPA. For (E)-2-ene VPA, the formation of (E)-2,4-diene VPA and (E,E)-2,3'-diene VPA was monitored.

Adult male Long Evans rats (4/group) were treated intraperitoneally (*i.p.*) with one of the following: CBZ 100 mg/kg or CBZE 50 mg/kg every 12 h for 3, 7, 10 or 14 days, CFB 350 mg/kg for 7 days, or PB 75 mg/kg for 4 days. The mean hepatic cytochrome P-450 content for the CBZ treatment groups over the ten day treatment period was significantly enhanced 1.5 to 1.8 fold compared to the vehicle control group while CBZE treatment did not appear to affect total hepatic cytochrome P-450 content. PB treatment resulted in a significant 1.9 fold increase compared to the vehicle control, but a significant increase was not observed for the CFB treatment group.

Immunoblot analysis indicated that cytochrome P-450b was induced by PB and also by CBZ and CBZE. Cytochrome P-450b constituted 65 % of the total hepatic cytochrome P-450 content in the PB induced microsomes and

ranged from 31 to 66 % in the CBZ and CBZE groups over the 14 day treatment period. Pentoxyresorufin, a substrate for cytochrome P-450b, was preferentially metabolized compared to ethoxyresorufin by microsomal protein isolated from PB, CBZ and CBZE treated rats. Mean pentoxyresorufin O-dealkylation rates for the CBZ, CBZE and PB treatment groups were enhanced 12 to 53 fold when compared to their respective vehicle control groups.

The metabolism of VPA and (E)-2-ene VPA was enhanced by PB, CBZ and to a lesser extent by CBZE treatment. CFB pretreatment did not have any significant effects on the metabolism of VPA or (E)-2-ene VPA. An antibody directed against rat cytochrome P-450b was effective in completely inhibiting the metabolism of VPA to 4-ene VPA by microsomal protein isolated from PB or CBZ 3 day treated rats. The formation of 4-OH VPA and 4-keto VPA was inhibited greater than 75 % in the presence of the antibody. The metabolism of (E)-2-ene VPA to (E)-2,4-diene VPA by microsomal protein from PB or CBZ 3 day treatment groups was inhibited 89 % and 85 % respectively, in the presence of the anti-rat cytochrome P-450b antibody.

Three days of treatment with CBZ at a dose of 100 mg/kg *i.p.* every 12 h was as effective as typical PB treatment for inducing total hepatic cytochrome P-450 content, cytochrome P-450b, pentoxyresorufin O-dealkylation, and the *in vitro* metabolism of VPA and (E)-2-ene VPA. CBZE, used at an equivalent molar dose as CBZ, also may be as effective an inducer as PB. Cytochrome P-450b plays an important role in the *in vitro* metabolism of VPA and (E)-2-ene VPA to 4-ene VPA and (E)-2,4-diene VPA, respectively. These two metabolites are known hepatotoxins and their enhanced formation in the presence of enzyme inducing agents are likely responsible for VPA associated hepatotoxicity. The isozyme of cytochrome P-450 induced by CBZ or CBZE has not previously been identified.

Frank L. Abbott (Supervisor)

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(E)-2,4-diene VPA	2-propyl-(E)-2,4-pentadienoic acid
(E,E)-2,3'-diene VPA	2-[(E)-1'-propenyl)]-(E)-2-pentenoic acid
2-ene VPA	2-propyl-2-pentenoic acid
2-PGA	2-propylglutaric acid
2-PSA	2-propylsuccinic acid
3-ene VPA	2-propyl-3-pentenoic acid
3-keto VPA	2-propyl-3-oxopentanoic acid
3-OH VPA	2-propyl-3-hydroxypentanoic acid
4-ene VPA	2-propyl-4-pentenoic acid
4-keto VPA	2-propyl-4-oxopentanoic acid
4-OH VPA	2-propyl-4-hydroxypentanoic acid
5-OH VPA	2-propyl-5-hydroxypentanoic acid
AUC	area under the serum concentration *versus* time curve
Bis	*N,N'*-methylene-bis-acrylamide
BSA	bovine serum albumin
CBZ	carbamazepine
CBZD	*trans*-10,11-dihydroxy-10,11-dihydrocarbamazepine (carbamazepine diol)
CBZE	carbamazepine-10,11-epoxide
CFB	clofibrate
CO	corn oil
CoA	coenzyme A
E	trans
EDTA	ethylenediaminetetraacetic acid
g	gram(s)

GC	gas chromatography
GCMS	gas chromatography-mass spectrometry
GSH	glutathione
h	hour(s)
HEPES	*N*-[2-Hydroxyl]piperazine-*N*'-[2-ethanesulfonic acic
HPLC	high performance liquid chromatography
i.d.	internal diameter
i.p.	intraperitoneal
IgG	immunoglobulin, antibody
JVS	Jamaican Vomiting Sickness
k	thousand
kg	kilogram(s)
µ	micron(s), micrometer(s)
MCBZ	10-methoxycarbamazepine
MCPA	methylenecyclopropylacetic acid
MES	maximal electroseizures
µg	microgram(s)
mg	milligram(s)
2-MGA	2-methylglutaric acid
min	minute(s)
µL	microlitre
mM	millimolar
MSD	mass selective detector
MTBSTFA	*N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide
NAC	*N*-acetylcysteine
NADH	nicotinamide adenine dinucleotide, reduced
NADPH	nicotinamide adenine dinucleotide phosphate, reduced

ND	not detected
NS	normal saline
PA	4-pentenoic acid
PB	phenobarbital
PBS	phosphate buffered saline
PG	propylene glycol
PROD	pentoxyresorufin O-dealkylation
PTZ	pentylenetetrazole
RS	Reye's syndrome
s.d.	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGOT	serum glutamic oxaloacetic transaminase aspartate aminotransferase
SGPT	serum glutamic pyruvic transaminase alanine aminotransferase
SIM	selected ion monitoring
*t*BDMS	*tertiary*-butyldimethylsilyl
TEMED	*N*,*N*,*N*',*N*'-tetramethylethylenediamine
Vd	volume of distribution
VPA	valproic acid (2-propylpentanoic acid)

DEDICATION

To my parents for their support throughout the years.

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INTRODUCTION

Epilepsy affects approximately 20 to 40 million people throughout the world (Rall and Schleifer, 1990). The incidence of epilepsy is higher in children than in adults, with approximately 8 in 1000 children under the age of 7 years demonstrating epilepsy. Epilepsy is characterized by abnormal phenomena of motor, sensory, autonomic or psychic origin. It can be designated as primary (idiopathic) or secondary (symptomatic) epilepsy. Primary epilepsy has no known identifiable cause while secondary epilepsy may be caused by various factors including trauma, neoplasms, infection or cerebrovascular disease. The International Classification of Epileptic Seizures describes in detail the various types of epilepsy (Dreifuss, 1990).

A number of anticonvulsant agents are available for the treatment of epilepsy. Approximately 80% of patients can be effectively treated with a single anticonvulsant while the remainder require multiple anticonvulsants (Duncan, 1991). Two commonly used anticonvulsant agents are valproic acid (VPA, Depakene®) and carbamazepine (CBZ, Tegretol®). These 2 drugs are often used in combination therapy in efforts to optimize seizure control (Cloyd *et al.*, 1985).

Because treatment of epilepsy is a long term endeavour, the opportunities for undesirable interactions with other xenobiotics are immense. An interaction may result in the formation of a metabolite(s) which not only adversely affects seizure control but also proceeds to a resultant toxic reaction. Although the side effects associated with VPA usage are generally of the mild gastrointestinal type, incidents of teratogenicity, pancreatitis (Isom, 1984) and hepatotoxicity (Jager-Roman *et al.*, 1986) have also been reported. Hepatotoxicity in most cases has involved young children on multiple anticonvulsant therapy (Dreifuss

et al., 1987). The mechanism of VPA hepatotoxicity is not well understood, although one or more of its metabolites are thought to be responsible (Baillie, 1992). The metabolite, 4-ene VPA, is a minor metabolite of VPA and is similar in structure to 2 known hepatotoxic terminal olefins, 4-pentenoic acid and methylenecyclopropylacetic acid. Metabolism of 4-ene VPA *via* mitochondrial β-oxidation may result in the formation of chemically reactive intermediates which can alklyate cellular macromolecules. Thus, it is postulated that coadministration of other anticonvulsant drugs, a number of which are known to be enzyme inducing agents, will enhance the formation of the potentially toxic metabolite(s).

Because of the frequency of the combination of VPA and CBZ and the potential risks of toxicity that might result from induction of VPA metabolism, it is important to characterize this interaction completely. This study will allow us to determine if metabolic formation of the potential hepatotoxin, 4-ene VPA, is increased in the presence of an enzyme inducing agent such as CBZ. Since this thesis focuses on characterizing the effect of CBZ on the *in vitro* metabolism of VPA, a brief discussion on the metabolism of VPA and the toxicity associated with its usage will be presented. The major serum metabolite of VPA, 2-ene VPA appears to be devoid of the severe toxicities associated with VPA and thus has been considered as a potential alternative anticonvulsant agent (Loscher, 1992). Therefore, it was deemed important to also investigate the effect of CBZ induction on 2-ene VPA metabolism. A brief review on 2-ene VPA as well as a brief summary of the literature on CBZ and the interaction between VPA and CBZ will also be presented. In addition, a brief review on induction of cytochrome P-450 by phenobarbital is also presented.

VALPROIC ACID

Originally synthesized for use as a solvent over a century ago (Burton, 1881), VPA has only been available for therapeutic use in North America since 1978 although its anticonvulsant effects have been known since the early 1960's (Meunier *et al.*, 1963). VPA is effective in the treatment of a variety of seizure types including absence, myoclonic, tonic-clonic, partial (Rimmer and Richens, 1985; Duncan, 1991), infantile and photo-convulsive (Rimmer and Richens, 1985) seizures. Because of its broad effectiveness against a number of seizure types, VPA is widely used either as a single agent (monotherapy) or in combination with other anticonvulsants (polytherapy). Other uses for VPA include prophylactic treatment of febrile convulsions (Lee *et al.*, 1986), post-trauma epilepsy, status epilepticus, acute mania and alcohol withdrawal (Rimmer and Richens, 1985).

Metabolism

Since VPA shares in common the metabolic pathways of fatty acids, a brief review on fatty acid metabolism is presented.

Fatty acid metabolism

Fatty acids consist of a terminal carboxyl group and an alkyl side chain and are of the basic formula, CH_3-$(CH_2)_n$-COOH (Devlin, 1986). Fatty acids are mainly metabolized *via* β-oxidation as their coenzyme A (CoA) esters. In β-oxidation, 2 carbon fragments are sequentially removed from the carboxyl terminal after dehydrogenation, hydration, oxidation and thiolysis (Devlin, 1986; Stryer, 1981).

Mitochondrial β-oxidation of fatty acids

The first step in the β-oxidation cycle is the activation of the fatty acid to a fatty acyl CoA which occurs either in the endoplasmic reticulum or in the

outer mitochondrial membrane (Devlin, 1986; Montgomery *et al.*, 1990). The activation is performed by an acyl CoA synthase (thiokinase or acyl CoA ligase), of which at least 4 enzymes are known (Montgomery *et al.*, 1990). A short chain enzyme is specific for acetate and propionate, a medium chain enzyme specific for 4 to 10 carbon fatty acids, a long chain enzyme specific for fatty acids longer than 12 carbons and a separate enzyme for arachidonic acid. The 2 enzymes specific for short and medium chain fatty acids are mitochondrial in location whilst the other 2 enzymes are located in the endoplasmic reticulum.

Because the mitochondrial membrane is impermeable to CoA and its derivatives, a carrier is necessary for transportation of the fatty acid across the membrane (Devlin, 1986). This function is performed by carnitine which is required for the transport of activated fatty acids of chain length 12 to 18 carbons across the mitochondrial membrane. The acyl group is transferred by carnitine palmitoyl transferase I to the hydroxyl group on the carnitine molecule from the sulphur atom of CoA on the outer surface of the membrane. At the inner mitochondrial membrane, the acyl group is transferred from carnitine back to CoA by carnitine palmitoyl transferase II. However, short chain fatty acids can directly diffuse across the membrane and become activated to the CoA derivatives in the matrix compartment of the mitochondrion, *i.e.* the oxidation of short chain fatty acids is independent of carnitine.

Inside the mitochondrion, the CoA derivatives are oxidized by one of a group of acyl CoA dehydrogenases (Devlin, 1986). These enzymes are specific for a certain chain length; palmitoyl CoA dehydrogenase for medium and long chain fatty acids, while the other 3 enzymes, octanoyl CoA and 2 butyryl dehydrogenases, are specific for shorter chain fatty acids. The function of these dehydrogenases is to remove 2 hydrogen atoms to form an enoyl CoA with a trans double bond between the second and the third carbon atoms. The 2

hydrogen atoms are accepted by flavin adenine dinucleotide (FAD) and ultimately, 2 electrons are channelled into the electron transport system.

The α,β-unsaturated acyl CoA accepts a molecule of water, a reaction catalyzed by enoyl CoA hydrase to form L-β-hydroxyacyl CoA (Devlin, 1986). L-β-hydroxyacyl CoA is oxidized by β-hydroxyacyl CoA dehydrogenase to β-ketoacyl CoA which is further oxidized in the β-position by β-ketothiolase. CoA is inserted and cleavage occurs at the β-carbon to yield acetyl CoA and a saturated acyl CoA with 2 fewer carbons than the original substrate.

The steps described above are repeated until a 4 carbon butyryl CoA remains as the intermediate. Butyryl CoA is β-oxidized to yield acetoacetyl CoA and subsequently 2 molecules of acetyl CoA.

Peroxisomal β-oxidation of fatty acids

The β-oxidation of fatty acids also occurs in peroxisomes, subcellular organelles which are widely distributed in mammalian tissues including kidney, liver, muscle, intestine, heart and spleen (Lazarow, 1987).

Peroxisomal β-oxidation in mammals differs from mitochondrial ß-oxidation in 3 ways. First, carnitine is not required for fatty acid entry into the peroxisome (Lazarow, 1987). Second, the initial dehydrogenation step in peroxisomes is catalyzed by a cyanide-insensitive oxidase leading to the formation of hydrogen peroxide which is eventually eliminated. Third, the enzymes involved in the cycle differ slightly, in that 3 proteins perform the 4 reactions of peroxisomal β-oxidation whereas in mitochondrial β-oxidation, 4 proteins are involved. It appears that the role of peroxisomes is to shorten the chain length of relatively long fatty acids for β-oxidation in the mitochondria, since peroxisomal β-oxidation is unable to proceed beyond 8 carbons in the shortening of long chain fatty acids.

Omega and omega-1 oxidation of fatty acids

Metabolism of fatty acids *via* ω- and ω-1 oxidation represent minor biotransformation pathways (Devlin, 1986). Primarily, medium chain length fatty acids undergo metabolism *via* these oxidative pathways which occur in the endoplasmic reticulum of many tissues. Omega oxidation involves hydroxylation at the methyl carbon on the opposite end from the carboxyl group while ω-1 oxidation occurs at the penultimate carbon atom next to the terminal methyl group. After hydroxylation, the fatty acid may be further oxidized to a dicarboxylic acid at which stage β-oxidation can occur from either end of the molecule. The ω- and ω-1 oxidations are cytochrome P-450 mediated events (Montgomery *et al.*, 1990).

Metabolism of valproic acid

VPA, an 8 carbon, branched chain fatty acid, possesses an unique structure amongst the wide array of anticonvulsant agents. Unlike other anticonvulsant agents, VPA lacks a nitrogen moiety. Despite its simple structure, VPA undergoes extensive biotransformation (figure 1) *via* several pathways: glucuronidation, β-oxidation and ω- and ω-1 oxidation (Loscher, 1981a; Granneman *et al.*, 1984a). Very small quantities (3 to 7%) of the unchanged drug are recovered in the urine (Schobben *et al.*, 1975; Dickinson *et al.*, 1989). Glucuronidation and β-oxidation are the 2 major metabolic pathways in both man and rat (Granneman *et al.*, 1984a).

The glucuronide conjugate accounts for approximately 11 to 68% of urinary recovery in man (Granneman *et al.*, 1984a; Dickinson *et al.*, 1989). With increasing doses of VPA, glucuronidation, which occurs in the endoplasmic reticulum, increases at the expense of β-oxidation (Granneman *et al.*, 1984a; Granneman *et al.*, 1984b). Conjugation with glycine occurs but is a minor metabolic pathway.

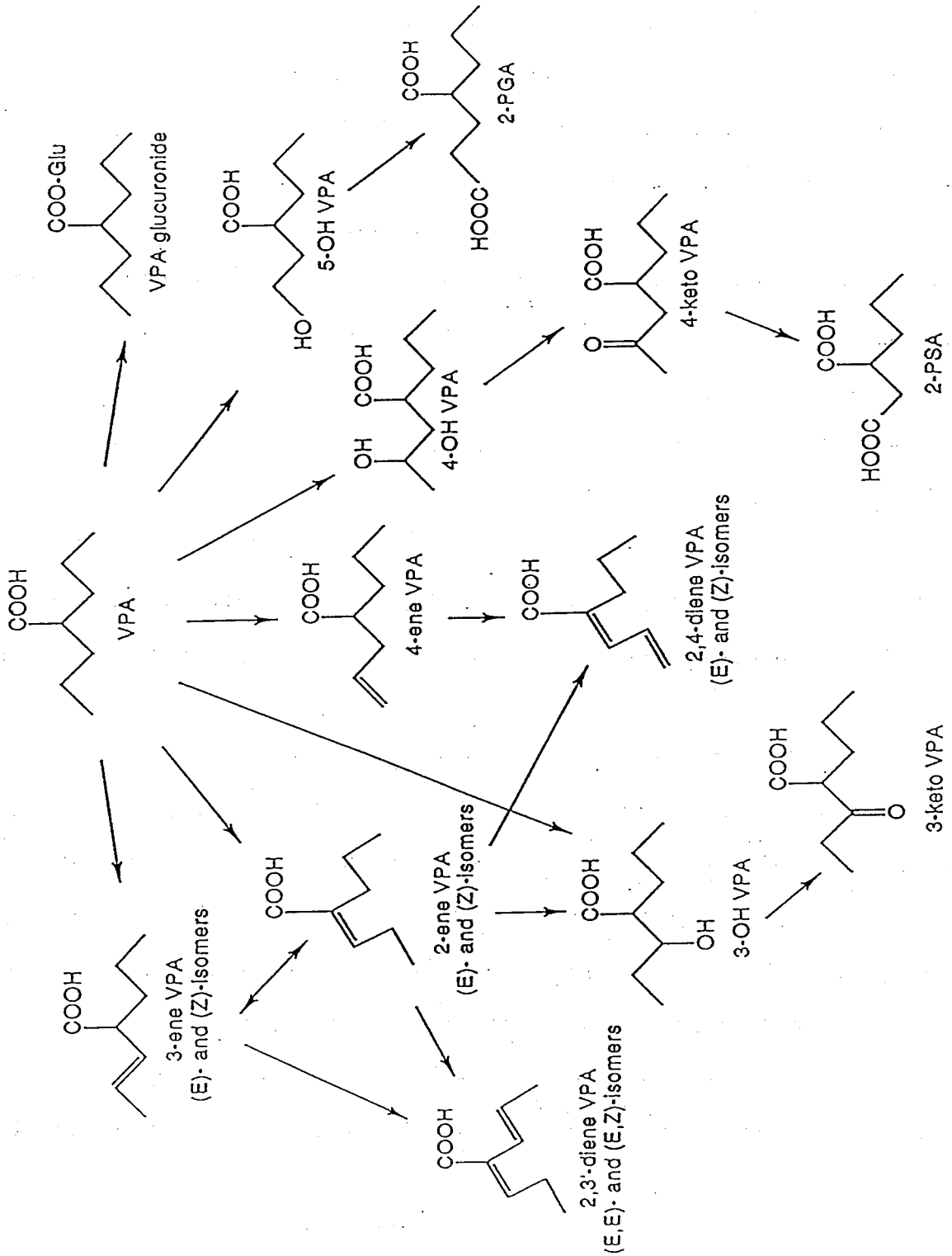

Figure 1. Summary of valproic acid metabolism (Yu *et al.*).

Mitochondrial β-oxidation of VPA results in the formation of 2-ene VPA, 3-OH VPA and 3-keto VPA (Granneman *et al.*, 1984a; Li *et al.*, 1991). In the mitochondria, VPA is activated to VPA-CoA, then dehydrogenated to 2-ene VPA-CoA by acyl-CoA dehydrogenase, then hydrated by enoyl-CoA hydrase to 3-OH VPA-CoA and finally dehydrogenated to 3-keto VPA-CoA by 3-hydroxyacyl-CoA dehydrogenase. The 3-keto VPA metabolite is the terminal product of this pathway due to prevention of thiolytic cleavage by the enzyme 3-ketoacyl CoA thiolase (Fong and Schulz, 1978) because of the presence of the 2-propyl branch (Li *et al.*, 1991). Peroxisomal β-oxidation of VPA may also occur since enhanced excretion of 3-keto VPA was observed in rats after pretreatment with clofibrate, a known peroxisomal inducer (Heinemeyer *et al.*, 1985). VPA is also hydroxylated to 3-OH VPA microsomally *via* ω-2 oxidation (Prickett and Baillie, 1984). Mean urinary recoveries of the β-oxidation pathway metabolites account for approximately 25% of the recovered dose in patients (Abbott *et al.*, 1986; Dickinson *et al.*, 1989).

Metabolism of VPA *via* ω-oxidation results in the formation of 5-OH VPA and 2-propylglutaric acid (2-PGA) (Granneman *et al.*, 1984a). The metabolite, 2-PGA, is the end product of ω-oxidation and does not undergo further metabolism *via* β-oxidation (Kuhara and Matsumoto, 1974). Oxidation of VPA at the ω-1 position leads to 4-OH VPA, 4-keto VPA and 2-propylsuccinic acid (2-PSA). The hydroxylated metabolites are not further metabolized to the unsaturated metabolites (Granneman *et al.*, 1984a). The ω- and ω-1 oxidation pathways account for approximately 20% of the recovered dose in patients (Abbott *et al.*, 1986).

Dehydrogenation of VPA at the γ-carbon results in the formation of 4-ene VPA (Granneman *et al.*, 1984a). 4-Ene VPA represents less than 1% of the recovered dose in man (Abbott *et al.*, 1986; Dickinson *et al.*, 1989). The

metabolism of VPA to 4-ene VPA is cytochrome P-450 mediated (Rettie *et al.*, 1987). Further metabolism of 4-ene VPA, 3-ene VPA and 2-ene VPA may result in the formation of diunsaturated metabolites. For example, 4-ene VPA and 3-ene VPA are metabolized *via* the β-oxidation pathway to form 2,4-diene VPA (Rettenmeier *et al.*, 1986a) and 2,3'-diene VPA (Bjorge and Baillie, 1991), respectively. The major diunsaturated metabolite in humans has been identified as 2,3'-diene VPA (Acheampong and Abbott, 1985). (E)-2-ene VPA is dehydrogenated to both diunsaturated metabolites and is β-oxidized to 3-keto VPA (Granneman *et al.*, 1984a; Loscher *et al.*, 1992).

Metabolism of 4-ene VPA

The metabolism of 4-ene VPA has been studied in rat (Granneman *et al*, 1984a; Rettenmeier *et al.*, 1985) and rhesus monkey (Rettenmeier *et al.*, 1986a). Eight metabolites of 4-ene VPA were detected by GLC and GCMS from rat bile and the perfusate medium from isolated rat livers (Rettenmeier *et al.*, 1985). The recovered metabolites were identified as 2,4-diene VPA, 3-OH-4-ene VPA, 3'-keto-4-ene VPA, 5'-OH-4-ene VPA, 5-OH VPA, 4,5-dihydroxy VPA gamma lactone, 2-PGA and the parent compound, 4-ene VPA. Most metabolites were derived *via* either β-oxidation or cytochrome P-450 mediated reactions. Six metabolic pathways for the biotransformation of 4-ene VPA were assigned: β-oxidation on the unsaturated side chain (2,4-diene VPA and 5'-OH-4-ene VPA, cytochrome P-450 mediated), β-oxidation on the saturated side chain (3'-keto), ω-hydroxylation to the primary alcohol and subsequently to the dicarboxylic acid (2-PGA), reduction followed by oxidation to the diacid, epoxidation to the gamma butyrolactone derivative and hydroxylation at the C-3 position to form 3-OH-4-ene VPA. Twenty metabolites of 4-ene VPA were identified in the urine of rhesus monkeys with 59% of the dose recovered in 24 h (Rettenmeier *et al.*, 1986a). In addition to the metabolites identified in rat liver perfusate medium,

other metabolites identified included (E)-3-ene VPA, 4-OH VPA, 4-OH VPA lactone, 4'-OH-4-ene VPA, 4'-OH-4-ene VPA lactone, 3-keto VPA and 2-PSA. Furthermore, 2 isomers of 2,4-diene VPA, a third diene metabolite and the glycine conjugate of (E)-2,4-diene VPA were also identified. Again, glucuronidation and β-oxidation were identified as the major metabolic pathways and ω- and ω-1 oxidation as minor biotransformation routes.

Metabolite activity

The metabolites (E)-2-ene VPA, 4-ene VPA (Loscher, 1981b), 3-ene VPA (Kochen and Scheffner, 1980) and (E,E)-2,3'-diene VPA (Abbott and Acheampong, 1988) possess anticonvulsant activity. (E,E)-2,3'-diene VPA is as active as (E)-2-ene VPA in mice (Abbott and Acheampong, 1988). (E)-2-ene VPA and 4-ene VPA are the most active of the metabolites, displaying approximately 60 to 90% of the potency of VPA although they are more sedating than VPA in mice (Nau and Loscher, 1984; Loscher and Nau, 1985). 2-Ene VPA was detectable in mouse plasma and brain 2 days after discontinuation of VPA and may be responsible for the elevated seizure threshold in the absence of detectable VPA brain levels (Loscher and Nau, 1984).

Adverse effects

Side effects

The majority of side effects associated with VPA are mild; nausea, vomiting, diarrhea and abdominal cramps are the most commonly observed (Bruni and Wilder, 1979). Other side effects include transient hair loss, weight gain, hyperkinesia, fine postural tremor, drowsiness and transient hallucinations (Dulac *et al.*, 1986). Tremor, weight gain, transient hair loss and limb edema are dose related side effects (Willmore *et al.*, 1991) and occur in 25% of patients on VPA therapy (Smith and Bleck, 1991). Transient and self limiting

neutropenia and thrombocytopenia have also been observed with VPA (Barr *et al.*, 1982) as well as reduced platelet adhesiveness and prolonged bleeding time (Smith and Bleck, 1991). Dementia has also been observed in patients on VPA therapy, although there was prompt remission after withdrawal of the drug (Zaret and Cohen, 1986).

Biochemical effects

Biochemical effects of VPA include hyperammonemia, inhibition of β-oxidation (Becker and Harris, 1983; Van Den Branden and Roels, 1985; Willmore *et al.*, 1991), inhibition of gluconeogenesis (Becker and Harris, 1983; Rogiers *et al.*, 1985) and hyperglycinemia (Cherruau *et al.*, 1981).

Patients on VPA are found to excrete higher amounts of dicarboxylic acids. This increased excretion is possibly due to impaired β-oxidation (Turnbull *et al.*, 1986). Increased amounts of 6 carbon dicarboxylic acids, eg. adipic acid, are also found in the urine of rats treated with VPA (Mortensen *et al.*, 1980). In a 6 year old male with Reye's syndrome, increased amounts of lactic and adipic acids as well as increased quantities of 2-PGA, the end product of VPA ω-oxidation, were recovered in the urine (Kuhara *et al.*, 1985).

In isolated rat hepatocytes VPA, (E)-2-ene VPA, 4-ene VPA, 4-OH VPA, 5-OH VPA and 2-PSA produced a concentration-dependent inhibition of gluconeogenesis from lactate (Rogiers *et al.*, 1985). The extent of toxicity in decreasing order was VPA and 4-ene VPA, 5-OH VPA, 4-OH VPA, (E)-2-ene VPA and 2-PGA.

VPA interferes with the folate-dependent one carbon enzyme responsible for glycine cleavage, resulting in hyperglycinemia in patients and animals treated with VPA (Carl, 1986). Hyperglycinemia and hyperglycinuria are observed in rats administered VPA chronically for several weeks at doses ranging from 0.3 to 1.2 mmol/kg (Cherruau *et al.*, 1981). Chronic

administration of 1% VPA to young rats resulted in significant increases in blood, liver and brain glycine levels (Martin-Gallardo *et al.*, 1985).

Hyperammonemia and hyperbilirubinemia are also associated with VPA therapy (Matsuda *et al.*, 1986; Ratnaike *et al.*, 1986). VPA inhibits urea synthesis in rat hepatocytes (Coude, 1983; Turnbull *et al.*, 1983). Coadministration of other anticonvulsants with VPA, particularly phenobarbital or phenytoin, results in increased serum ammonia levels (Warter *et al.*, 1983; Haidukewych *et al.*, 1985; Zaccara *et al.*, 1985; Ratnaike *et al.*, 1986).

VPA administration results in carnitine deficiency (Borum and Bennett, 1986; Laub *et al.*, 1986). VPA forms valproylcarnitine derivatives which themselves are not toxic but cause an increased metabolic need for carnitine to excrete the more toxic metabolites. Patients on VPA therapy display decreased plasma carnitine levels accompanied by elevated blood ammonia levels (Ohtani *et al.*, 1982). Oral administration of carnitine 50 mg/kg/day for 4 weeks was successful in correcting the VPA induced carnitine deficiency and hyperammonemia. Carnitine deficiency may be the end result of hyperammonemia. In mice, VPA exerts an immediate but transient effect on carnitine metabolism (Rozas *et al.*, 1990). Single doses of VPA in the therapeutic range for man decreased hepatic levels of free CoA, acetyl CoA and free carnitine in normal infant mice (Thurston *et al.*, 1985).

VPA at doses greater than 1 mM may uncouple mitochondrial respiration (Benavides *et al.*, 1982) due to accumulation of valproyl CoA and its further metabolites in the matrix of the hepatic mitochondria (Turnbull *et al.*, 1983) or by altering the integrity of the inner mitochondrial membrane or by actions on the substrate carriers or mitochondrial metabolites (Rumbach *et al.*, 1983). Valproyl CoA in the mitochondrial matrix is a weak inhibitor of β-oxidation (Sherratt and Vietch, 1984). Inhibition of mitochondrial β-oxidition by VPA may

be due to sequestration of CoA as valproyl-CoA (Veitch and Van Hoof, 1990).

Increased activity of the peroxisomal β-oxidation enzymes in rat liver was observed after chronic administration of VPA (Horie and Suga, 1985; Ponchaut *et al.*, 1991). Decreased cytochrome P-450 levels were observed in rats administered VPA (172.8 to 259.2 mg/kg/day) (Cotariu *et al.*, 1985).

Pancreatitis

Pancreatitis has been associated with VPA usage (Coulter *et al.*, 1980; Isom, 1984). Until 1991, 24 cases of VPA implicated pancreatitis were reported (Binek *et al.*, 1991; Asconape *et al.*, 1993). A recent survey of physicians revealed 15 additional cases of VPA induced pancreatitis (Asconape *et al.*, 1993). A further 6 cases of pancreatitis in addition to hepatic failure with VPA usage have also been reported in the literature (Binek *et al.*, 1991). Most of the cases of VPA implicated pancreatitis (77%) involved patients under the age of 20 years and 68.8% of the 39 cases occurred within the first year of VPA therapy (Asconape *et al.*, 1993). Furthermore, 76% of the cases were on VPA polytherapy. VPA pancreatitis manifests itself initially as abdominal pain and vomiting (Parker *et al.*, 1981; Wyllie *et al.*, 1984; Rosenberg *et al.*, 1987). Increased serum amylase and lipase levels have also been observed in some cases (Parker *et al.*, 1981; Wyllie *et al.*, 1984; Rosenberg *et al.*, 1987; Lott *et al.*, 1990). Although 3 patients have died from VPA induced pancreatitis, in most cases the reaction is mild with rapid resolution once VPA is discontinued (Asconape *et al.*, 1993). VPA induced pancreatitis appears to be an idiosyncratic reaction, unrelated to VPA dosage or serum concentrations.

Embryotoxicity / teratogenicity

VPA possesses the potential for teratogenicity in all species thus far investigated, including man, monkey and rodent (Cotariu and Zaidman, 1991).

Although the exact mechanism by which VPA exerts its teratogenic effects has not been established, possible mechanisms include alteration of fetal glutathione status, alteration of fetal lipid metabolism, effects on folate or zinc levels or effects on the regulation of embryonic pH (Cotariu and Zaidman, 1991).

VPA teratogenicity appears to require quite rigid structural specificities (Nau and Scott, 1987; Nau and Siemes, 1992). Structural requirements for teratogenic activity include a free carboxyl group attached to a carbon atom which is substituted with only 2 alkyl chains (Nau and Siemes, 1992). The α-H atom is quite important for teratogenic activity; substitution abolishes teratogenic activity as does the introduction of an ω-2 double bond (2-ene VPA) (Nau and Scott, 1987). The introduction of a double bond in the ω (4-ene VPA) position has no effect on the malformation rate. A decrease in teratogenicity is observed if the alkyl chain length is shortened or lengthened (Nau and Scott, 1987; Nau and Siemes, 1992). Teratogenic activity may be expressed through chiral interactions of the branched chain carboxylic acids with various embryonic constituents important in developmental processes (Nau and Siemes, 1992).

Fetal valproate syndrome includes a flat nasal bridge, an upturned nose, a long upper lip, downturned mouth, long, thin overlapping fingers and toes and hyperconvex nails (Yerby *et al.*, 1992). A consistent facial phenotype (epicanthal folds, flat nasal bridge *etc.*) was observed in 7 children who had been exposed to VPA *in utero* (DiLiberti *et al.*, 1984).

A 1 to 2% incidence in humans of VPA induced neural defects is associated with VPA administration early in pregnancy (Nau and Siemes, 1992) and may be due to interferences with zinc and other trace element metabolism (Weinbaum *et al.*, 1986). Interference with embryonic folate metabolism (Wegner and Nau, 1992) with a resultant folate deficiency has been implicated

to play an important role in the induction of neural tube defects by VPA (Dansky *et al.*, 1992). Neural tube defects are manifested mainly as spina bifida aperta, but incidents of myelomeningocele have been reported (Nau and Siemes, 1992).

The intensity of the teratogenic response as measured by neural tube defects in mice, was dependent on the concentrations of VPA achieved in the mother and fetus (Nau, 1985). In pregnant mice, both the dose of VPA and area under the serum concentration *versus* time curve (AUC) correlate with embryolethality and fetal weight retardation (Nau, 1985).

Although therapeutic doses of VPA in pregnant women do not affect fetal growth, the risk for fetal perinatal distress increases with higher doses of VPA (Jager-Roman *et al.*, 1986). VPA administration to rhesus monkeys at a human therapeutic dose of 20 mg/kg/day during organogenesis did not yield any adverse effects (Mast *et al.*, 1986). However, a dose of 200 mg/kg/day caused low birth weights, craniofacial and skeletal defects and a dose of 300 mg/kg/day was embryolethal (Mast *et al.*, 1986). A later study in rhesus monkeys (20 to 600 mg/kg) also resulted in a dose dependent developmental toxicity which manifested itself as increased embryo/fetal mortality, intrauterine growth retardation and craniofacial and skeletal defects (Hendrickx *et al.*, 1988).

In whole rat embryos, VPA at doses greater than 40 mg/kg/day caused abnormal development in 30% of embryos (Lewandowski *et al.*, 1986). In rats administered VPA 300 mg/kg daily on embryonic days 7 to 18, a decrease in maternal bodyweight and fetal weight and an increase in malformations were observed (Vorhees *et al.*, 1991).

Hepatotoxicity

Although side effects associated with VPA use are generally mild, a number of cases of fatal hepatotoxicity have been reported (Kochen *et al.*, 1984;

Jager-Roman *et al.*, 1986). Hepatotoxicity associated with VPA usage is of 2 types, either dose-related or idiosyncratic (Dreifuss *et al.*, 1987). Dose-related VPA hepatotoxicity resolves with a decrease in dosage or discontinuation of the drug. Up to 44% of patients on VPA therapy demonstrate dose related increases in liver enzyme levels (Sussman and McLain, 1979) but these increases are not predictive of hepatotoxicity since less than 0.01% of patients develop fatal hepatotoxicity (Dreifuss *et al.*, 1987). The fatal but rare idiosyncratic hepatotoxicity is irreversible and dose-independent.

A recent survey of reported cases of fatal hepatotoxicity associated with VPA usage in the United States between 1978 and 1984 concluded that age and polytherapy were the major determinants (Dreifuss *et al.*, 1987). The incidence of VPA induced hepatotoxicity decreased with increasing age. The incidence was highest in those children under 2 years of age on polytherapy (1/500) compared to the same age group on monotherapy (1/7000). The overall incidence of VPA hepatotoxicity was 1/10,000. Many of the patients who developed hepatotoxicity suffered from other medical conditions including mental retardation, developmental delay, congenital abnormalities and metabolic disorders which conspired to place them at higher risk. A follow-up study for the period 1985 to 1986 demonstrated a decline in the incidence of hepatic fatalities related to VPA from 1/10,000 to 1/49,000. This occurred despite an overall increased usage of VPA but as a single agent rather than in polytherapy (Dreifuss *et al.*, 1989).

VPA hepatotoxicity is characterized clinically by loss of appetite, nausea, vomiting, edema, abdominal distress, lethargy and malaise (Dreifuss *et al.*, 1987). The onset is usually within 90 days of initiating VPA therapy. Microvesicular steatosis is the most prominent finding with VPA hepatotoxicity (Zimmerman and Ishak, 1982) and resembles the lesions seen in Jamaican

Vomiting Sickness (JVS), Reye's Syndrome (RS) and 4-pentenoic acid (PA) toxicity (Lewis *et al.*, 1982; Nau and Loscher, 1984). Clinically, VPA hepatotoxicity shares similar manifestations with JVS, RS and PA toxicity.

Reye's syndrome was first described in 21 children (Reye *et al.*, 1963) and the etiology of RS remains unknown. RS involves encephalopathy and fatty degeneration of the liver, kidney and occasionally other organs. Other clinical features include fever, convulsions, vomiting, hypoglycemia and increased serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) levels.

Hypoglycin A (hypoglycin, L-α-amino-2-methylenecyclopropylpropionic acid), an amino acid found in unripe akee fruit and in the seeds of several varieties of maple trees, is responsible for JVS (Tanaka *et al.*, 1976). Hypoglycin is metabolized to methylenecyclopropylacetic acid (MCPA) which forms an enoyl CoA ester that inhibits fatty acid metabolism and gluconeogenesis (Kean, 1975). The toxicity results from inhibition of several acyl-CoA dehydrogenases (Kean, 1975) and is possibly worsened by sequestration of mitochondrial carnitine and coenzyme A by MCPA (Bressler *et al.*, 1969). Hypoglycin causes hyperammonemia in rats (Glasgow, 1983) and hypoglycemia is common in patients with JVS (Jelliffe and Stuart, 1954). In addition, encephalopathy and fatty degeneration of the viscera are common features. The incidence of JVS decreases after the age of 10 (Reye *et al.*, 1963).

4-Pentenoic acid is a structural analogue of hypoglycin. In rats, PA produces similar features to JVS and RS including hypoglycemia, fatty degeneration of the liver, hyperammonemia and increased SGOT levels (Glasgow and Chase, 1975). PA inhibits fatty acid β-oxidation (Hart *et al.*, 1989) *via* its β-oxidation metabolites, pent-2,4-dienoyl-CoA (Glasgow, 1983) and 3-keto-4-pentenoyl-CoA (Schulz, 1983) through inhibition of the β-oxidation

enzyme 3-ketoacyl-CoA-thiolase (Fong and Schulz, 1978; Schulz, 1983). Although 3-keto-4-pentenoyl-CoA is a minor metabolite of PA, it is a more effective inhibitor of the enzyme (Schulz, 1983). The inhibition and morphological manifestations caused by PA can be partially reversed by L-carnitine (Billington *et al.*, 1978; Sugimoto *et al.*, 1990).

The formation of toxic metabolites is thought to be responsible for VPA hepatotoxicity. Because of the similarities between VPA hepatotoxicity, JVS, RS and PA toxicity, a metabolite of VPA similar in structure to PA and MCPA may be responsible for VPA associated toxicity. The hepatotoxicity of VPA is thought to be caused by its mono- and/or diunsaturated metabolites (Kochen *et al.*, 1984). An increased formation of the diunsaturated metabolites appears to be characteristic in fatal hepatic failure. 4-Ene VPA was detected in the urine of 6 patients and 4,4'-diene in 3 of the patients who died of VPA related hepatic failure (Scheffner *et al.*, 1988). The metabolite, 4-ene VPA, is structurally similar to 4-pentenoic acid and MCPA and may play an important role in VPA associated hepatotoxicity. One 7 year old patient on phenobarbital and VPA therapy who died from hepatic failure resembling Reye's syndrome (Kochen *et al.*, 1983) demonstrated plasma and urine concentrations 4 to 5 times the normal levels of several unsaturated metabolites including 4-ene VPA. The 4,4'-diene VPA was also present. The β-oxidation pathway appeared to be inhibited as evidenced by the absence of 3-keto VPA, the end product of VPA β-oxidation.

In hepatotoxicity studies of VPA and metabolites in young rats, 4-ene VPA and 2,4-diene VPA caused hepatic steatosis and inhibition of β-oxidation (Granneman *et al.*, 1984c; Kesterson *et al.*, 1984). These unsaturated metabolites may be further metabolized to the chemically reactive 3-keto-4-ene VPA which could then alkylate mitochondrial proteins including enzymes involved in β-oxidation (Rettenmeier *et al.*, 1986b). This mechanism is based on

observations with 4-pentenoic acid which is transformed to a reactive intermediate that alkylates and destroys the terminal enzyme of the β-oxidation pathway (Schulz, 1983; Fong and Schulz, 1983).

In rat liver homogenates, VPA and 4-ene VPA caused inhibition of decanoic acid β-oxidation (Bjorge and Baillie, 1985). VPA depletes free CoA pools (Fears, 1985) and may cause a transient and mild inhibition of the β-oxidation pathway by sequestration of CoA (Kesterson *et al.*, 1984). 4-Ene VPA produces a more potent and prolonged inhibition by forming CoA esters which directly inhibit enzymes in the β-oxidation pathway. VPA inhibits mitochondrial β-oxidation by forming valproyl CoA which is a weak inhibitor but acts at a different site than 4-pentenoic acid and hypoglycin (Sherratt and Veitch, 1984). VPA, 4-ene VPA and 2,4-diene VPA trap intramitochondrial free CoA (Ponchaut *et al.*, 1992).

The glutathione conjugate of (E)-2,4-diene VPA was identified in the bile of rats administered either (E)-2,4-diene VPA or 4-ene VPA while the *N*-acetylcysteine conjugate of (E)-2,4-diene VPA was a major urinary metabolite (Kassahun *et al.*, 1991). Since (E)-2,4-diene VPA is a β-oxidation metabolite of 4-ene VPA, *in vivo* activation to a CoA ester which readily interacts with glutathione may explain the high urinary recovery. In patients with VPA associated hepatic failure, the levels of (E)-2,4-diene VPA recovered in the urine as the *N*-acetylcysteine conjugate were 3 to 4 times the levels observed in healthy patients (Kassahun *et al.*, 1991).

(E)-2-ENE VPA

The metabolite (E)-2-ene VPA has been touted as a potential anticonvulsant agent due to its lack of teratogenicity (Nau *et al.*, 1984; Nau and Loscher, 1986; Nau, 1986), lack of embryotoxicity (Loscher *et al.*, 1984;

Lewandowski *et al.*, 1986; Nau, 1986) and apparent lack of hepatotoxicity (Kesterson *et al.*, 1984; Schafer and Luhrs, 1984; Loscher, 1992) in experimental animals.

Anticonvulsant activity

(E)-2-ene VPA is as potent as VPA with respect to anticonvulsant activity (Loscher *et al.*, 1984). In experimental seizure models, (E)-2-ene VPA is as effective as VPA on a weight for weight basis (Loscher and Nau, 1985). In rats (E)-2-ene VPA is 2 to 3 times more potent than VPA in elevating the clonic threshold of pentylenetetrazole (PTZ) induced seizures (Semmes and Shen, 1991).

The spectrum of activity of (E)-2-ene VPA is similar to that of VPA without the potential for embryotoxicity even at doses of 600 mg/kg (Loscher *et al.*, 1984). In 4 different models of anticonvulsant activity, (E)-2-ene VPA activity was similar to that of VPA. (E)-2-ene VPA was more potent in general tonic clonic seizures in gerbils and in petit mal recurrent seizures in rats. In the maximal electroseizures (MES) and PTZ tests in mice, doses of 200 to 300 mg/kg of (E)-2-ene VPA were more sedating than VPA. However, sedation was not observed in rats or gerbils at these doses. The anticonvulsant activity of (E)-2-ene VPA is of shorter duration (2 h compared to 5 h) than VPA in mice after doses of 4 mmol/kg (Keane *et al.*, 1985).

Teratogenicity

(E)-2-ene VPA possesses very little teratogenic potential (Nau, 1986). At similar doses to VPA, (E)-2-ene VPA is not teratogenic in rats (Vorhees *et al.*, 1991). In a murine model, (E)-2-ene VPA did not affect embryonic folate metabolism at doses of 500 mg/kg *i.p.* (Wegner and Nau, 1992). In rats administered (E)-2-ene VPA at doses of 300 mg/kg daily on embryonic days 7 to

18, no increases in the percentage of resorptions or malformations were observed (Vorhees *et al.*, 1991). Increasing the dose to 400 mg/kg decreased fetal bodyweight by approximately 8% but with no change in resorptions or malformations. Abnormal embryo development or retardation of growth was not observed after administration of 2 doses of 400 mg/kg on day 10 of pregnancy in rats (Klug *et al.*, 1990). In whole rat embryos, (E)-2-ene VPA did not produce any adverse effects at doses up to 200 mg/kg/day (Lewandowski *et al.*, 1986).

Metabolism

To date, the metabolism of 2-ene VPA has only been examined in rats. After (E)-2-ene VPA administration, the major metabolites in the serum are the β-oxidation products, (E,E)-2,3'-diene VPA and 3-keto VPA and 2,4-diene VPA (Loscher *et al.*, 1992). 3-Ene VPA has also been detected in serum, most likely resulting from isomerization (Vorhees *et al.*, 1991). In addition, in urine up to 2% of the administered dose was recovered as VPA (Granneman *et al.*, 1984a).

CARBAMAZEPINE

Carbamazepine (CBZ, 5-carbamoyl-5H-dibenz[*b,f*]azepine, carbamoyl-iminostilbene), an iminostilbene derivative (Eadie and Tyrer, 1989), is structurally similar to the tricyclic antidepressants (Kutt, 1989). CBZ is effective in the treatment of a wide variety of seizure types including partial, generalized tonic-clonic and mixed seizure disorders (Rall and Schleifer, 1990).

After oral administration in man, CBZ undergoes slow and erratic absorption from the gastrointestinal tract (Rall and Schleifer, 1990). Peak plasma levels are attained in 2 to 8 h. Therapeutic plasma levels of CBZ are in the range of 3 to 14 μg/mL. CBZ has a relatively long half-life ranging from 8 to 72 h. The drug is highly plasma protein bound (75 to 90%).

Carbamazepine undergoes extensive metabolism (figure 2) *via* the liver microsomal enzyme system, with less than 1% of the parent drug excreted unchanged in the urine (Bertilsson and Tomson, 1986). The major route of metabolism is the epoxide-diol pathway where CBZ is transformed to carbamazepine-10,11-epoxide (CBZE) which is further hydrolyzed to *trans*-10,11-dihydroxy-10,11-dihydro-CBZ (*trans*-CBZ-diol, CBZD). In volunteers, approximately 22% of a single dose is recovered in the urine as CBZD (Eichelbaum *et al.*, 1984). In chronic therapy, the urinary recovery of CBZD accounts for 30 to 60% of the administered dose. Other metabolic pathways include hydroxylation in the 2, 3 and 9 positions and account for approximately 15% of the dose.

Carbamazepine induces its own metabolism as well as the metabolism of other drugs *via* induction of the hepatic microsomal enzyme system (Bertilsson and Tomson, 1986). CBZ induces the metabolism of warfarin (Hansen *et al.*, 1971; Ross and Beeley, 1980), phenytoin (Hansen *et al.*, 1971), theophylline (Rosenberry *et al.*, 1983), clonazepam (Lai *et al.*, 1978), tetracycline (Neuvonen *et al.*, 1975), haloperidol, ethosuximide, oral contraceptives (Fernandez *et al.*, 1985) and VPA (Reunanen *et al.*, 1980).

In long term treatment, CBZ induces its own metabolism (Pynnonen, 1979; Bertilsson and Tomson, 1986; Rall and Schleifer, 1990) *via* induction of the epoxide-diol pathway (Eichelbaum *et al.*, 1984). CBZ autoinduction in man has been reported to occur within 2 or 3 days (Pynnonen, 1979) although maximal CBZ autoinduction may require 3 to 4 weeks (Bleck, 1990). In a group of 77 patients, autoinduction of CBZ metabolism appeared to be complete within one week of initiating CBZ therapy or upon a change in dose (Kudriakova *et al.*, 1992). CBZ autoinduction is dose dependent in man (Rapeport *et al.*, 1983; Kudriakova *et al.*, 1992) as evidenced by dose dependent increases in antipyrine

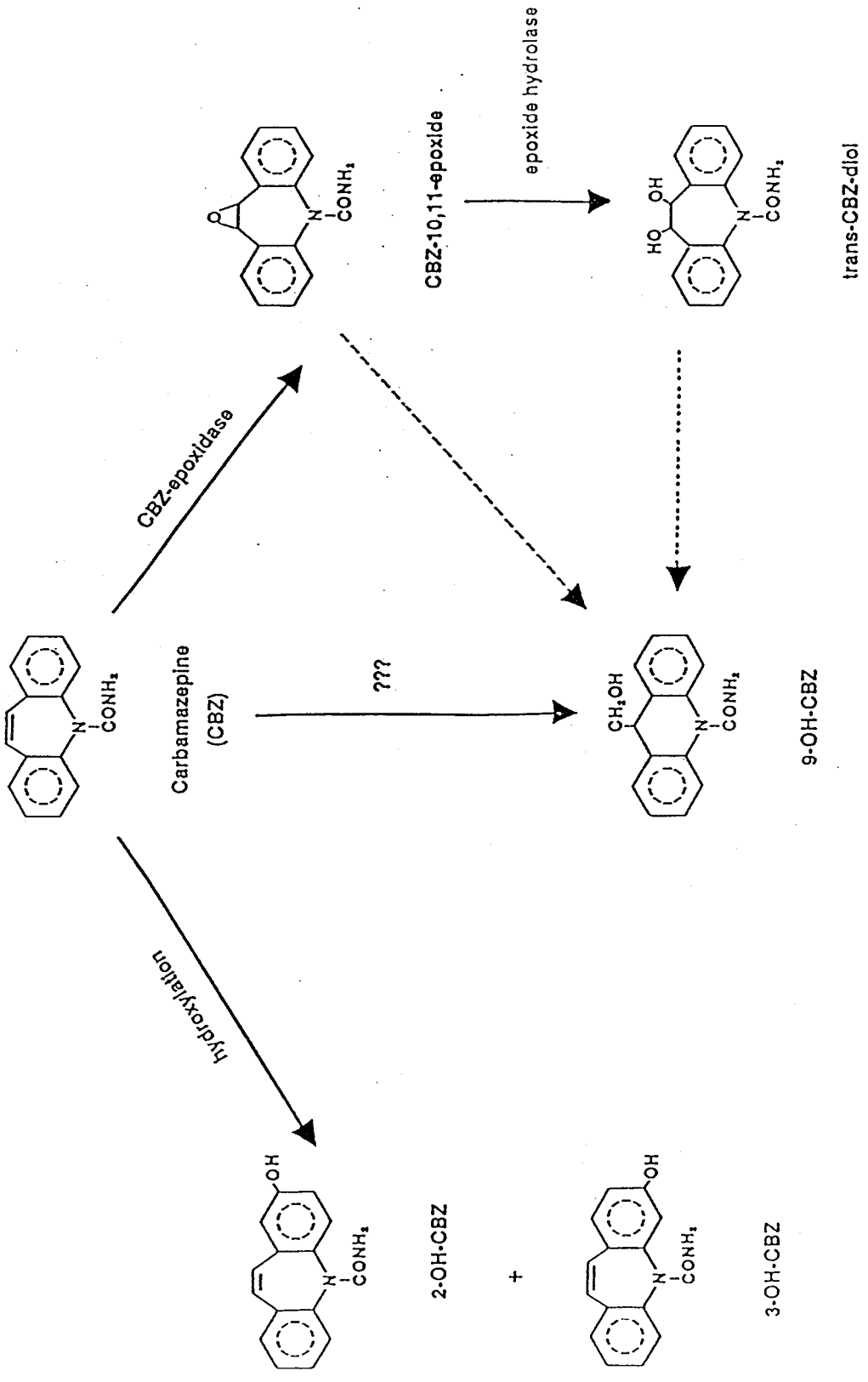

Figure 2. Summary of carbamazepine metabolism.

clearance in healthy volunteers (Rapeport *et al.*, 1983).

In rats, chronic administration of CBZ (25 mg/kg *i.p.* every 12 h for 7 days) resulted in decreased protection against maximum electroshock seizures (Farghali-Hassan *et al.*, 1976). In rats administered oral doses of 315 mol/kg (approximately 75 mg/kg/day) for 4 days, CBZ increased liver weight and induced cytochrome P-450, NADPH cytochrome P-450 reductase, aminopyrine *N*-demethylase and UDP-glucuronyltransferase (Wagner and Schmid, 1987). Regnaud *et al.* (1988) attempted to determine the dose dependency of CBZ induction in rats. CBZ was administered intraperitoneally at 60, 120 and 200 mg/kg/day for 4 days. Epoxide hydrolase activity appeared to increase with increasing dose as did aminopyrine *N*-demethylase activity. Cytochrome P-450 and aniline hydroxylase activity did not increase at doses higher than 120 mg/kg/day.

Carbamazepine-10,11-epoxide

Carbamazepine-10,11-epoxide is an active metabolite and serum levels are usually 15 to 55% and 5 to 81% of the parent compound in adults and children, respectively (Bertilsson and Tomson, 1984). CBZE possesses anticonvulsant activity similar to CBZ in various animal models (Tomson and Bertilsson, 1991). In 7 adult patients, no change was observed in seizure control when CBZE was substituted for CBZ (Tomson *et al.*, 1990). CBZE was equipotent to CBZ in controlling pain due to trigeminal neuralgia (Tomson and Bertilsson, 1984). CBZE has a shorter half-life than CBZ (approximately 6 h) in man and is metabolized almost completely to CBZD, with 67 to 100% of the epoxide dose recovered as CBZD (Tomson *et al.*, 1983; Spina *et al.*, 1988).

When CBZE was administered to rats intraperitoneally at a dose of 100 mg/kg daily for 3 and 7 days, maximal induction of epoxide hydrolase and glutathione transferase appeared to be achieved in 3 days (Jung *et al.*, 1980).

However, CBZE treatment did not alter total hepatic cytochrome P-450 levels.

INTERACTION BETWEEN VPA AND CBZ

Because VPA undergoes such extensive biotransformation in the body, coadministration of metabolic inducing agents may result in the increased formation of potentially toxic metabolites. Interactions of VPA with other anticonvulsant drugs have been studied most extensively. These interactions may be pharmaceutical, pharmacokinetic or pharmacodynamic (Smith and Bleck, 1991).

In polytherapy with other anticonvulsants, (CBZ, phenytoin and phenobarbital) shorter serum VPA half-lives are observed compared to monotherapy (Kutt, 1984). In addition, the ratio of VPA steady-state plasma levels to dose is much lower in children receiving other anticonvulsants (Sackellares *et al.*, 1981).

CBZ has been shown to decrease the plasma levels of VPA by inducing VPA metabolism (Kutt, 1984). CBZ affects VPA disposition in both pediatric and adult patients (Kutt, 1984; Baciewicz, 1986). Lower serum VPA concentrations, despite higher VPA doses, have been observed in both adult (Reunanen *et al.*, 1980) and pediatric patients (Abbott *et al.*, 1986) on VPA and CBZ compared to VPA alone. VPA serum half-life decreased and plasma clearance (Cl_p) increased when VPA was coadministered with CBZ in adult epileptic patients (Hoffman *et al.*, 1986).

Bowdle and coworkers (1979) demonstrated that administration of CBZ at a dose of 200 mg daily in healthy volunteers resulted in increased VPA clearance and decreased VPA steady state levels after 2 weeks. A change in the elimination rate constant, K_e, was not observed.

In 5 adult volunteers, the plasma clearance of VPA was significantly increased following CBZ coadministration (Panesar *et al.*, 1989). The volume of distribution (Vd) of VPA remained unchanged after CBZ, thus providing support for the induction of VPA metabolism by CBZ. *In vitro*, CBZ does not displace VPA from its plasma protein binding sites (Mattson *et al.*, 1982) so a change in the Vd was not expected. 4-Ene VPA serum levels were unchanged after CBZ administration but urinary recovery of this metabolite was increased after CBZ. The serum data suggested induction of the ω- and ω-1 oxidative pathways by CBZ. The major unsaturated metabolite, (E)-2-ene VPA, was significantly reduced in serum and urine suggesting at first that induction of β-oxidation *via* peroxisomes had occurred. The β-oxidation end product, 3-keto VPA, however was not increased suggesting that (E)-2-ene VPA metabolism was shunted into alternate pathways. Furthermore, urinary recoveries based on the assay of VPA and metabolites could not confirm an increase in VPA metabolism. Thus, it would appear that either VPA metabolite elimination occurred *via* non-renal routes or the assay failed to detect a significant proportion of the VPA metabolites. The urinary recovery of VPA and metabolites representing approximately 65% of the dose was consistent with other investigators (Pollack *et al.*, 1986).

In a study performed in our laboratory (unpublished data), significant differences were observed between the VPA metabolite profiles of 16 pediatric patients on VPA and CBZ polytherapy and the profiles of 37 pediatric patients on VPA monotherapy. VPA and unsaturated metabolites were significantly reduced in the serum of the VPA and CBZ group. The pediatric patient profiles did not show an increase in urinary 4-ene VPA elimination yet decreased serum 4-ene VPA concentrations were observed with CBZ coadministration. Induction of 4-ene VPA metabolism may have occurred, resulting in the formation of

secondary β-oxidation metabolites such as 3'-keto-4-ene VPA and the postulated reactive 3-keto-4-ene VPA which our assay did not detect. Urinary profiles were generally a reflection of the serum data. Thus, there was apparent induction of VPA metabolism *via* the ω-, ω-1 and β-oxidation pathways by CBZ. Again, the net recovery of VPA and metabolites in the urine of the VPA and CBZ group did not account for the increased VPA metabolism. An apparent induction of the ω- and ω-1 oxidation pathways of VPA metabolism was also observed in another pediatric group on combined VPA and CBZ therapy (Kassahun *et al.*, 1990).

CYTOCHROME P-450

The hepatic microsomal mixed-function oxidase systems consists of cytochrome P-450 (a hemoprotein), NADPH-cytochrome P-450 reductase and phospholipids (Lu and Levin, 1974.) Cytochrome P-450 is a fairly ubiquitous enzyme, present in virtually every tissue including lung, small intestine, liver, adrenals, testis, kidney and duodenum (Okey, 1990) with the exception of erythrocytes and striated muscle (Guengerich, 1991). Cytochrome P-450 is also present in the mitochondria and has been shown to be involved in the metabolism of carcinogens including aflatoxin B1, benzo[a]pyrene and dimethylnitrosoamine (Niranjan *et al.*, 1984). For the purposes of this study, only cytochrome P-450 in hepatic microsomes was measured.

Cytochromes P-450 represent the main group of phase 1 enzymes which are responsible for the biotransformation of hydrophobic molecules to more hydrophilic molecules which can undergo further metabolism by phase II enzymes prior to their excretion in either urine or bile (Leroux *et al.*, 1989). However, situations arise where metabolism by cytochrome P-450 results in the formation of a reactive metabolite which may ultimately cause hepatotoxicity as in the case of cocaine (Boelsterli *et al.*, 1992) and VPA (Rettie *et al.*, 1987).

The expression of the constitutive forms of cytochrome P-450 is dependent on the sex, age and strain of the animal (Soucek and Gut, 1992). In addition, levels of cytochrome P-450 may be influenced by growth hormone, the physiological status of the animal, starvation and hypertension. As an example, nutritional deficiences generally result in decreased rates of xenobiotic metabolism in rat liver microsomal fractions (Yang *et al.*, 1992).

Xenobiotics capable of inducing cytochrome P-450 can be grouped into 6 categories: 1) the polycyclic aromatic hydrocarbons (*eg.* 3-methylcholanthrene, β-napthoflavone), 2) the barbiturates (*eg.* phenobarbital), 3) steroids (*eg.* dexamethasone, pregnenolone-16α-carbonitrile), 4) simple hydrocarbons with aliphatic chains (*eg.* ethanol, acetone), 5) the hypolipidemic drugs (*eg.* clofibrate) and 6) the macrolide antibiotics (*eg.* triacetyloleandomycin) (Soucek and Gut, 1992). Induction of cytochrome P-450 can occur either *via* transcriptional activation, mRNA stabilization, or by protein stabilization.

The nomenclature of Levin and co-workers (Ryan and Levin, 1990) will be used in this thesis when referring to the isozymes of cytochrome P-450. A summary comparing their nomenclature to that of Nelson *et al.*, (1993) is provided in table 1. In humans, orthologues have been identified for rat cytochromes P-450j and P-450p (Soucek and Gut, 1992).

Induction of cytochrome P-450 by phenobarbital

Phenobarbital (PB) is an effective inducer of a number of isozymes of cytochrome P-450 in rats and other laboratory animals (Waxman and Azaroff, 1992). In addition to induction of cytochrome P-450, PB also induces a number of other enzymes which are involved in the metabolism of xenobiotics including aldehyde dehydrogenase, epoxide hydrolase, NADPH-dependent:cytochrome P-450 reductase, UDP-glucuronyltransferase and glutathione transferases. Induction is invoked by the parent compound itself as opposed to its major

Table 1. Summary comparing the nomenclature of Ryan and Levin (1990) and Nelson *et al.* (1993) for isozymes of cytochrome P-450 purified from rat liver microsomes.

Ryan and Levin	Nelson *et al.*
cytochrome P-450a	CYP2A1
cytochrome P-450b	CYP2B1
cytochrome P-450e	CYP2B2
cytochrome P-450f	CYP2C7
cytochrome P-450g	CYP2C13
cytochrome P-450h	CYP2C11
cytochrome P-450j	CYP2E1
cytochrome P-450k	CYP2C6
cytochrome P-450p	CYP3A1

Adapted from Nelson *et al.* (1993), Ryan and Levin (1990) and Soucek and Gut (1992).

metabolite, *p*-hydroxyphenobarbital (Cresteil *et al.*, 1980).

Phenobarbital induction results in proliferation of smooth endoplasmic reticulum within liver cells, increased liver weight (Remmer and Merker, 1963), liver tumour promotion and a general stabilization of liver microsomal proteins (Rees, 1979; Waxman and Azaroff, 1992). After administration of PB to rats, a rapid increase in the cytochrome P-450 levels in rough endoplasmic reticulum is observed within 3 h and maximal levels are achieved within 6 h (Ernster and Orrenius, 1965). Six hours after the administration of PB, the levels of cytochrome P-450 in the smooth endoplasmic reticulum slowly start to increase and after 12 h surpass the levels achieved in the rough endoplasmic reticulum.

Maximal induction with phenobarbital is achieved in 3 days compared to 24 h with 3-methylcholanthrene treatment (Greim *et al.*, 1981). With the barbiturates, the extent of induction in rats is directly related to plasma half-life, *i.e.* compounds possessing longer plasma half-lives are more effective inducers of cytochrome P-450 (Ioannides and Parke, 1975). Total hepatic cytochrome P-450 levels in rat liver returned to baseline levels within 5 days after discontinuation of PB injections (Ernster and Orrenius, 1965). The half lives of cytochromes P-450b and P-450e are approximately 37 h (Parkinson *et al.*, 1983).

Some isozymes of cytochrome P-450 in rats are modestly induced after PB treatment while others are more dramatically affected (Waxman and Azaroff, 1992). For example in adult rats, PB treatment induces a 2 to 4 fold increase in cytochromes P-450a (Thomas *et al.*, 1981) and P-450k (Waxman *et al.*, 1985) and modestly induces cytochrome P-450p (Heuman *et al.*, 1982). Conversely, up to 40 fold increases in cytochromes P-450e and P-450b have been reported (Thomas *et al.*, 1981; Thomas *et al.*, 1987). In addition to PB, phenothiazine, SKF-525A, Arclor 1254, isosafrole, *trans*-stilbene oxide (Thomas *et al.*, 1981), acetone and

ethanol (Ryan *et al.*, 1982) also induce cytochromes P-450b and P-450e in rats. Cytochromes P-450b and P-450e share greater than 97% amino acid sequence homology (Fujii-Kuriyama *et al.*, 1982).

Induction of cytochrome P-450 by PB in rat liver is due primarily to newly synthesized cytochrome P-450b/e protein that results from increased steady state levels of cytochrome P-450b/e mRNA (Phillips *et al.*, 1981; Waxman and Azaroff, 1992). A 20 fold induction in mRNA was observed after PB induction in rats (Phillips *et al.*, 1981). Stabilization of mRNA or proteins is not believed to be involved in the mechanism of PB induction.

Microheterogeneity of cytochromes P-450b and P-450e also exists in some strains of rats (Vlasuk *et al.*, 1982; Wilson *et al.*, 1987; Oertle *et al.*, 1991). In male Sprague-Dawley rats, PB induction resulted in the identification of 6 members of the cytochrome P-450b/e family by monoclonal antibodies and partial sequence analysis of tryptic peptides (Oertle *et al.*, 1991). Three of the 6 proteins belonged to the cytochrome P-450b family whilst the other 3 were identified as members of the cytochrome P-450e family.

Cytochrome P-450b metabolizes a wide spectrum of lipophilic drugs in addition to steroids including androgens and androstenedione (Waxman and Azaroff, 1992). Cytochrome P-450e has a similar substrate profile but is not as active as cytochrome P-450b. The differences in activity between these 2 isozymes may arise due to several factors. The cytochrome P-450e isozyme may be more susceptible to denaturation during the purification process or requires the presence of as yet unknown specific phospholipids upon reconstitution (Christou *et al.*, 1987). Alternatively, it is possible that activation of cytochrome P-450b occurs during the purification process from a membrane environment to a reconstituted system.

CLINICAL IMPLICATIONS

Enzyme induction is "the process which increases the rate of synthesis of an enzyme relative to its normal rate of synthesis in the uninduced organism" (Gelboin and Wiebel, 1971). Induction of the liver enzymes results in an enhancement in metabolic rate and thus directly affects duration and intensity of drug actions in man and animals. Induction can alter the steady state concentrations of the parent compound and its metabolites in addition to their elimination (Gillette, 1979) and possibly result in the formation of potentially toxic metabolites.

Such is the case for VPA where induction of metabolism appears to play a major role in the production of toxicity. In a retrospective study of fatal hepatotoxicity associated with VPA usage, young children on polytherapy were more susceptible to development of toxicity than those on VPA monotherapy (Dreifuss *et al.*, 1987).

In young rats, PB pretreatment was necessary for VPA to demonstrate liver toxicity in young rats (Kesterson *et al.*, 1984). At low doses of 350 mg/kg, VPA did not produce microvesicular steatosis unless rats were pretreated with PB (Lewis *et al.*, 1982). Glucuronidation and ω- and ω-1 oxidation of VPA were reported to increase following PB induction (Watkins *et al.*, 1982; Heinemeyer *et al.*, 1985).

The potential hepatotoxin, 4-ene VPA, which was produced *via* the cytochrome P-450 oxidation of VPA, was observed only with microsomes from phenobarbital treated rats (Rettie *et al.*, 1987). At low concentrations of 4-ene VPA (10 μg/mL), significant alterations to the membrane permeability of guinea pig hepatocytes were not observed unless phenytoin or phenobarbital was also present (Yu *et al.*, 1991).

Clofibrate (CFB) pretreatment in rats resulted in enhanced β-oxidation of

VPA (Heinemeyer *et al.*, 1985). Clofibrate is a known peroxisomal proliferator (Lazarow, 1987) and these observations suggested that peroxisomal β-oxidation of VPA had occurred. There was also some microsomal induction since excretion of 4-OH VPA in rats was reported to increase following CFB treatment (Heinemeyer *et al.*, 1985). In rats, 3-methylcholanthrene pretreatment also induced 3-OH VPA formation.

Increased production of 4-ene VPA by microsomal metabolism which might then be readily converted by β-oxidation to a reactive metabolite could have serious consequences regarding the risk potential of the VPA and CBZ drug combination. CBZ is not likely to be a peroxisomal inducer because it does not contain a carboxyl group, like other known peroxisome proliferators (Lundgren *et al.*, 1987). However, the major metabolite of CBZ is an epoxide which in turn is metabolized by epoxide hydrolase to CBZD (Tybring *et al.*, 1981; Eichelbaum *et al.*, 1985). Increased epoxide hydrolase activity with peroxisome proliferation has been reported (Oesch and Schladt, 1987; Moody and Hammock, 1987). Since CBZ induces its own metabolism *via* induction of the enzymes of the epoxide-diol pathway (Eichelbaum *et al.*, 1985), enhanced β-oxidation may result.

Our goal, then, is to detail the VPA and CBZ interaction based on the changes in VPA metabolism resulting from CBZ induction. The rat will be used as the model. Although CBZ is known to be an enzyme inducer, neither the time course of induction nor the extent of induction has previously been determined. The effects of CBZE on microsomal enzymes will also be investigated. VPA and (E)-2-ene VPA metabolite profiles will be compared in animals pretreated with CBZ, CBZE, PB and CFB. The hypothesis to be tested is that CBZ enhances VPA toxicity through enhanced production of toxic VPA metabolites.

SPECIFIC OBJECTIVES

1. To determine the time course and the extent of induction of hepatic microsomal enzymes in the rat by CBZ at a given dose. The effects produced by CBZ will be compared to the commonly used inducing agent PB and to CFB, a known peroxisomal inducer.
2. To determine the time course and the extent of induction of hepatic microsomal enzymes in the rat by CBZE at a given dose. These results will be compared to those obtained for CBZ, PB and CFB.
3. To determine the contribution by CBZE to the overall induction produced by CBZ.
4. To identify the isozyme(s) of cytochrome P-450 induced by CBZ and CZBE and determine if the same isozyme(s) are induced by PB.
5. To identify the metabolites from the *in vitro* metabolism of VPA using microsomal fractions from CBZ, CBZE, PB and CFB treated rats.
6. To identify the products resulting from the *in vitro* metabolism of (E)-2-ene VPA using microsomal fractions from CBZ, CBZE, PB and CFB treated rats.

EXPERIMENTAL

REAGENTS AND MATERIALS

Valproic acid and metabolites

Valproic acid (di-*n*-propylacetic acid) was obtained from K and K Fine Chemicals, ICN Biochemicals Inc. (Plainview, NY). The metabolites, (E)-2-ene VPA, 3-ene VPA, 4-ene VPA, 3-OH VPA, 4-OH VPA, 5-OH VPA, 3-keto VPA, 4-keto VPA, 2-propylglutaric acid (2-PGA) and 2-propylsuccinic acid (2-PSA) used for the preparation of the calibration curves and for *in vitro* incubations were synthesized in our laboratory as reported previously (Acheampong *et al.*, 1983). (E,E)-2,3'-diene VPA was synthesized in our laboratory as reported elsewhere (Acheampong and Abbott, 1985) as was (E)-2,4-diene VPA (Lee *et al.*, 1989).

Internal standards

The deuterated internal standards, [$^2H_7$]E-2-ene VPA, [$^2H_7$]4-ene VPA, [$^2H_7$]3-OH VPA, [$^2H_7$]5-OH VPA, [$^2H_7$]3-keto VPA, [$^2H_7$]4-keto VPA and [$^2H_7$]VPA were synthesized in our laboratory (Zheng, M.Sc. thesis, 1993). 2-Methylglutaric acid (2-MGA) was obtained from Aldrich Chemical Company.

Carbamazepine and metabolites

Carbamazepine, carbamazepine-10,11-epoxide, carbamazepine-10,11-diol and 10-methoxycarbamazepine for use as standards in the HPLC analyses were generously supplied by Ciba-Geigy Ltd. (Canada). Carbamazepine-10,11-epoxide for use in the animal studies was also supplied by Ciba-Geigy Ltd. (Canada). Carbamazepine for use in the animal studies was purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Reagents

Chemicals, solvents and reagents were obtained from the following sources:

BDH CHEMICALS (Vancouver, B.C., Canada).

Acetonitrile OmniSolve® grade, ammonium acetate, calcium chloride, citric acid anhydrous, di-potassium hydrogen orthophosphate, di-sodium hydrogen orthophosphate, dichloromethane OmniSolve® grade, ethylenediaminetetraacetic acid (EDTA), hydrochloric acid, magnesium chloride, methanol OmniSolve® grade, potassium dihydrogen orthophosphate, potasssium chloride, sodium chloride, sodium dihydrogen orthophosphate, sodium hydroxide, sodium sulphate anhydrous, sulphuric acid and trichloroacetic acid.

BIO-RAD LABORATORIES (Richmond, California, U.S.A).

Acrylamide 99.9%, ammonium persulphate 98%, *bis* (*N,N'*-methylene-bisacrylamide), 2-mercaptoethanol, SDS-PAGE 10 to 100K molecular weight standards, SDS-PAGE 40 to 250K molecular weight standards, sodium dodecyl sulphate (SDS) and TEMED (*N,N,N',N'*-tetramethyethylenediamine).

BOEHRINGER MANNHEIM CANADA LTD. (Laval, Quebec, Canada).

Bovine serum albumin, fraction V (BSA), bovine serum albumin, fatty acid free, fraction V, nicotinamide adenine dinucleotide, reduced (NADH) and nicotinamide adenine dinucleotide phosphate, reduced (NADPH).

CALEDON (Georgetown, Ontario, Canada).

Ethyl acetate distilled-in-glass grade.

FISHER SCIENTIFIC LTD. (Vancouver, B.C., Canada).

Creatinine.

INTER MEDICO (Markham, Ontario, Canada)

Goat $F(ab')_2$ anti-rabbit IgG (G+L) horseradish peroxidase conjugated IgG, affinity purified (TAGO).

J.T. BAKER CHEMICAL CO. (Phillipsburg, New Jersey, U.S.A.).

Sodium dithionite.

MOLECULAR PROBES, INC. (Eugene, Oregon, U.S.A.).

Ethoxyresorufin, pentoxyresorufin and resorufin.

PIERCE CHEMICAL COMPANY (Rockford, Illinois, U.S.A.).

N-tert-butyldimethylsilyl-*N*-methyl-trifluoroacetamide (MTBSTFA).

SIGMA CHEMICAL COMPANY (St. Louis, MO, U.S.A.).

Clofibrate, 4-chloro-1-naphthol, Folin & Ciocalteu's Phenol reagent, glycine, *N*-[2-Hydroxyethyl]piperizine-*N*'-[2-ethanesulfonic acid] (HEPES), hydrogen peroxide 30% solution, picric acid saturated solution, propylene glycol, sodium potassium tartrate, sucrose, Trizma base, Trizma HCl and Tween 20.

Primary antibodies

The primary antibodies, namely anti-rat cytochromes P-450b (P-450 2B1), P-450f (P-450 2C7), P-450g (P-450 2C13) and P-450h (P-450 2C11), were

prepared and generously provided by Dr. Stelvio Bandiera's group. The antibodies were raised in female New Zealand rabbits immunized with the electrophoretically homogeneous proteins. IgG was purified from a pool of heat-inactivated high-titer antisera obtained from multiple bleedings from several rabbits using a combination of caprylic acid precipitation followed by ammonium sulphate precipation and a final cleanup on a DEAE-Sephacel column (Bandiera and Dworschak, 1992). Antibody concentration was determined spectrophotometrically at 280 nm, $E_{1\ cm}$ = 13 for a 1% solution. Each antibody was extensively immunoabsorbed in a manner analogous to that developed for anti-rat cytochromes P-450f and P-450g to generate monospecific antibody (anti-rat cytochromes P-450f, P-450g and P-450h). The anti-rat cytochrome P-450b antibody was polyspecific.

The specificity of each antibody was assessed using Ouchterlony double diffusion analysis, noncompetitive ELISA and immunoblots. The anti-rat cytochrome P-450f, P-450g and P-450h antibodies only reacted with the antigen of immunization and did not react with any other purified cytochrome P-450. In the case of the anti-rat cytochrome P-450b antibody, it reacted with cytochrome P-450e and also with a third, noninducible member of the cytochrome P-450 2B family.

METHODS

Induction studies

Animals

Adult male Long Evans rats (190 to 225 g, Charles River, Montreal) were used for the experiments. After arrival, rats were allowed to recover for 3 to 5 days prior to commencing the studies. Rats were fed standard rat chow (Purina

5001®) *ad libitum* and allowed drinking water *ad libitum*. The rats were housed on corn cob bedding (Anderson's®) in a room with controlled light (14 h) and dark (10 h) cycles.

Treatment of solvents and compounds

Normal saline (NS), corn oil (CO) and propylene glycol (PG) were filtered *via* either a 0.2 or 0.45 μ filter (Gelman FP-Vericel™) prior to use as vehicles for phenobarbital (PB), clofibrate (CFB), carbamazepine (CBZ) and carbamazepine-10,11-epoxide (CBZE). Compounds were dissolved or suspended such that the volume of the dose administered to the animal was 0.1 mL/100 g body weight. Vehicle control animals received an equivalent volume per weight of the appropriate vehicle.

Treatment of animals with carbamazepine

Adult male Long Evans rats (4 per group) were treated *i.p.* with CBZ suspended in PG at a dose of 100 mg/kg every 12 h for 3, 7, 10 and 14 days. Control rats received an equivalent volume of PG. After administration of the last dose of CBZ, each rat was placed in a separate metabolic cage and urine collected for the 12 h period until sacrifice.

Treatment of animals with carbamazepine-10,11-epoxide

Rats (4 per group) were treated *i.p.* with CBZE suspended in PG at a dose of 50 mg/kg every 12 h for 3, 7, 10 and 14 days. Again, control rats received an equivalent volume of PG for each dose studied. After administration of the last dose of CBZE, rats were placed in metabolic cages and urine collected for the 12 h period until sacrifice.

Treatment of animals with phenobarbital

Rats were administered PB dissolved in normal saline (NS) 75 mg/kg *i.p.*

daily for 4 days. Vehicle control animals received an equivalent volume of NS.

Treatment of animals with clofibrate

Animals were administered CFB diluted in corn oil (CO) at a dose of 350 mg/kg *i.p.* daily for 7 days. Vehicle control animals received injections of CO.

Treatment of animals with valproic acid

VPA was administered at a dose of 150 mg/kg twice daily *i.p.* for 3 days. Vehicle control animals received injections of water.

ANALYSIS

Valproic acid and metabolites

Stock solutions of internal standards for GCMS

To decrease pipetting errors, the required internal standards were mixed such that the amounts required for each sample could be added simultaneously. A 200 μL aliquot contained the following amounts of each internal standard: [$^2H_7$]E-2-ene VPA 200 ng, [$^2H_7$]4-ene VPA 100 ng, [$^2H_7$]3-OH VPA 400 ng, [$^2H_7$]5-OH VPA 400 ng, [$^2H_7$]4-keto VPA 100 ng, [$^2H_7$]3-keto VPA 200 ng, [$^2H_7$]VPA 100 ng and 2-MGA 50 ng. Stock solutions were kept frozen at - 20 ^oC until needed.

Preparation of standard curves in phosphate buffer

A bulk stock solution (hereafter referred to as standard 5) was prepared in 0.2 M phosphate buffer, pH 7.4. For the calibration curve 200, 400, 600, 800 and 1,000 μL of standard 5 were made up to a final volume of 1 mL with phosphate buffer. One mL of the phosphate buffer served as the blank for the calibration curve. The stock solution was stored at - 20 ^oC until required.

Standard curve for VPA and metabolites

The concentrations of VPA and metabolites thus obtained were as follows: VPA 0, 12, 24, 36, 48 and 60 μg/mL; 3-ene VPA, 3-keto VPA, 4-ene VPA, 4-keto VPA, 2-PSA, 2-PGA, (E,E)-2,3'-diene VPA, (E)-2,4-diene VPA and (E)-2-ene VPA 0, 20, 40, 60, 80 and 100 ng/mL; 5-OH VPA 0, 40, 80, 120, 160 and 200 ng/mL; 3-OH VPA 0, 60, 120, 180, 240 and 300 ng/mL and 4-OH VPA 0, 0.24, 0.48, 0.72, 0.96 and 1.2 μg/mL.

Standard curve for (E)-2-ene VPA and metabolites

The concentrations of (E)-2-ene VPA and metabolites were as follows: VPA, 3-keto VPA, 4-ene VPA, 3-OH VPA, 3-ene VPA and (E,E)-2,3'-diene VPA 0, 19.2, 38.4, 57.6, 76.8 and 96 ng/mL; (E)-2,4-diene VPA 0, 0.8, 1.6, 2.4, 3.2 and 4 μg/mL; and (E)-2-ene VPA 0, 11.52, 23.04, 34.6, 46.08 and 57.6 μg/mL.

The calibration curves were generated by plotting the ratio of the peak area of metabolite or VPA to that of the respective internal standard *versus* the concentration of VPA or the particular metabolite. The deuterated compounds served as the internal standards for their respective undeuterated counterparts. Deuterated (E)-2-ene VPA served as the internal standard for 3-ene VPA and the diene metabolites. Deuterated VPA served as the internal standard for 4-OH VPA while 2-MGA was used as the internal standard for the 2 dicarboxylic acid metabolites. Standard curves were prepared and injected into the GCMS with each batch of samples.

Extraction of VPA and metabolites from standard samples and incubates

The extraction procedure for VPA and metabolites is shown in figure 3. Two hundred μL of internal standard mixture were added to each tube containing the sample for analysis or standard sample (1 mL). The pH of the standard curve and incubates was adjusted to between 1.5 to 2.0. The final

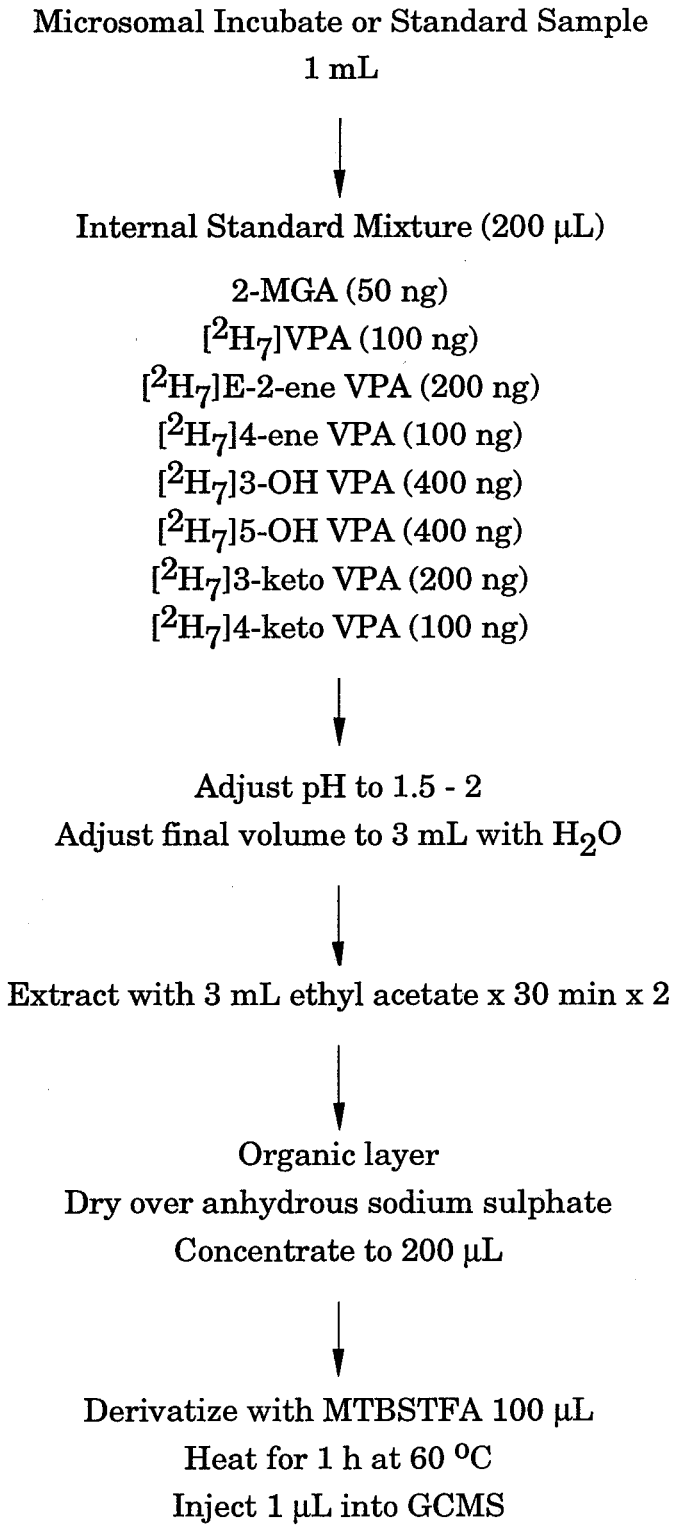

Figure 3. Summary of the extraction procedure for valproic acid and metabolites.

volume was adjusted to 3 mL with distilled water. Each sample was extracted twice with 3 mL ethyl acetate by gentle rotation for 30 min. The organic layer (total volume approximately 5 mL) was transferred to a test tube and dried over anhydrous sodium sulphate by vortexing for 1 min, waiting 10 min and centrifuging for 10 min at 3,000 rpm. The dried organic layer was transferred to another test tube, concentrated to approximately 200 µL, transferred to a 1 mL conical vial and derivatized with 100 µL MTBSTFA for 60 min at 60 °C. The derivatized samples were transferred to autosampler vials and 1 µL injected into the GCMS.

Carbamazepine and metabolites

Preparation of stock solutions for HPLC

Stock solutions of CBZ, CBZE and CBZD at a concentration of 1 mg/mL in methanol were prepared. A stock solution of 10-methoxycarbamazepine (MCBZ, internal standard) also at a concentration of 1 mg/mL was prepared in methanol and further diluted in distilled water to yield the working solution of 40 µg/mL. All stock and working solutions were stored at - 20 °C until required.

Preparation of standard curve for CBZ and metabolites

A bulk standard stock solution was prepared in rat urine from PG treated rats. Standard curves were prepared from 2, 4, 6, 8, 12, 16 and 20 µg/mL of CBZ, CBZE and CBZD. Unspiked urine served as the blank for the standard curve. Peak area ratios of CBZ, CBZE or CBZD to internal standard were plotted *versus* concentration to prepare calibration curves.

Extraction of CBZ, CBZE and CBZD from urine samples

CBZ and its 2 metabolites, CBZE and CBZD were extracted from urine using a procedure based on literature methods of Elyas *et al.* (1982) and Kumps

et al. (1985) (figure 4). A 400 µL aliquot of urine (either standard or sample) was placed into a test tube to which were added 4 µg (100 µL) of the internal standard (MCBZ) and 500 µL of 300 mM phosphate buffer, pH 6.7. The sample was extracted with 2.5 mL of ethyl acetate by gentle rotation for 10 min and centrifuged at 2,500 rpm for 10 min. The top organic layer was transferred to a second tube and evaporated under a gentle nitrogen stream in a water bath at 40 °C, reconstituted with 200 µL of acetonitrile, evaporated and reconstituted again with 200 µL of acetonitrile. The samples were transferred to autosampler vials and for each sample 20 µL were injected into the liquid chromatograph.

Instrumentation

Valproic acid and metabolites

The analyses were performed on a Hewlett-Packard 5890 Series II gas chromatograph interfaced with a Hewlett-Packard 5971A Mass Selective Detector and equipped with a 7673 autosampler. A Hewlett-Packard Vectra® 25T 486 computer, Hewlett-Packard Video Graphics Colour Display and a Hewlett-Packard Laserjet Series II printer accompanied the MSD. Operating conditions for *t*BDMS derivatives were source and injection port temperatures of 240 °C and an interface temperature of 270 °C. Helium (carrier gas) flow was 1 mL/min and the operating electron ionization energy for the mass spectrometer was 70 eV. Source temperature was 180 °C. Injection mode was splitless and column head pressure was 15 psi.

A DB 1701 (0.25 µ) bonded phase capillary column, 30 m x 0.25 mm I.D., (J & W Scientific, Folsom, California) was used for the analysis. Temperature programming for *t*BDMS derivatives was initial column oven temperature of 80 °C, increasing by 10 °C/min from 80 to 100 °C, then 2 °C/min from 100 to 130 °C and 30 °C/min from 130 to 260 °C and held at 260 °C for 8 min. Total run

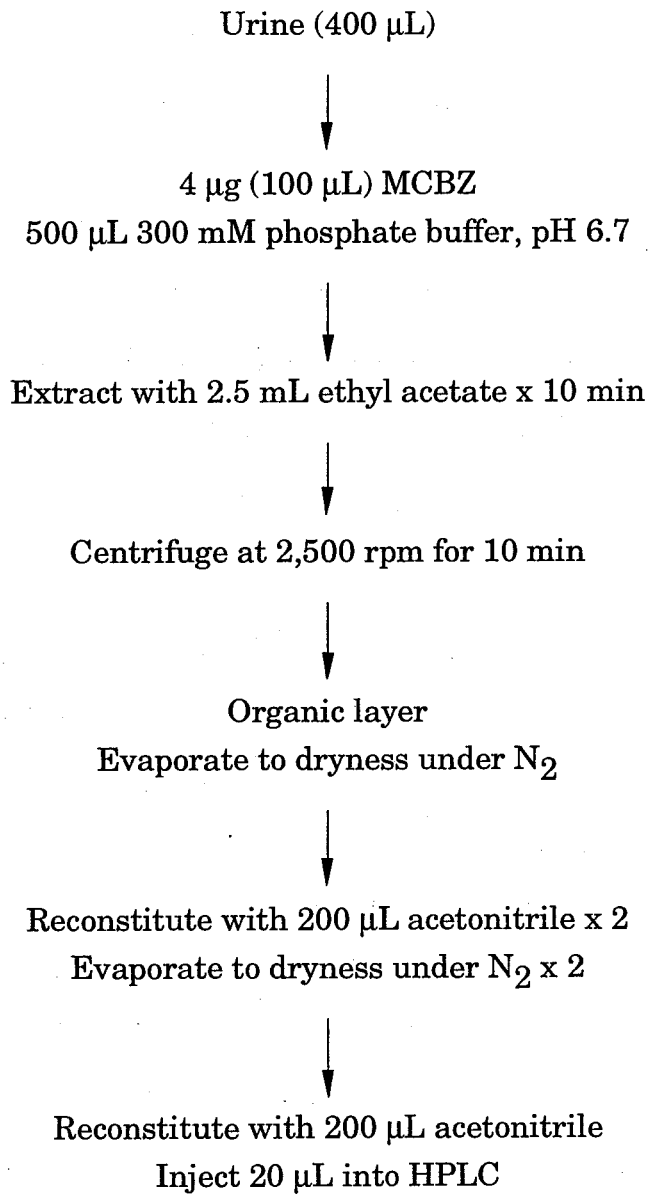

Figure 4. Summary scheme of the extraction of carbamazepine and metabolites from urine of rats.

time was approximately 29 min.

Selected ion monitoring mode was used for the analyses. The ions scanned were m/z 100 (4-OH VPA lactone), m/z 197 (dienes), m/z 199 (enes), m/z 201 (VPA), m/z 206 ([$^2H_7$]E-2-ene VPA and [$^2H_7$]4-ene VPA), m/z 208 ([$^2H_7$]VPA), (m/z 213 (3-keto-4'-ene VPA, monoderivative), m/z 215 (3- and 4-keto VPA, monoderivative), m/z 217 (3-OH VPA), m/z 222 ([$^2H_7$]4-keto VPA), m/z 224 ([$^2H_7$]3-OH VPA), m/z 317 (2-methylglutaric acid), m/z 327 (3-keto-4'-ene VPA, diderivative), m/z 329 (3-keto VPA, diderivative), m/z 331 (5-OH VPA and 2-PSA), m/z 336 ([$^2H_7$]3-keto VPA), m/z 338 ([$^2H_7$]5-OH VPA) and m/z 345 (2-PGA).

Carbamazepine and metabolites

A Hewlett-Packard Series II 1090 Liquid Chromatograph equipped with a HP3396A integrator with a Beckman Ultrasphere® ODS column, particle size 5 μm, 250 mm length, I.D. 4.6 mm was used for the analysis. Detection wavelength was 215nm and flow rate was 1 mL/min. A gradient using water (% A) and acetonitrile (% B) was formed such that the mixture was 85% A and 15% B from 0 to 2 min, 65% A and 35% B at 24 min, 65% A and 35% B at 28 min and 85% A and 15% B at 30 min. Stop time was 50 min. Ten minutes were allowed between each run for a total run time of 60 min.

Preparation of subcellular fractions from rat livers

Initially, assays were to be performed using mitochondrial, peroxisomal and microsomal fractions and thus differential centrifugation of rat liver homogenates according to the method of Cook *et al.*, (1986) was used. The livers were removed, washed in 0.9% NaCl and homogenized in 20 mL of 0.25 M sucrose/0.1% ethanol/5 mM Tris/1.15% KCl solution. The homogenate was centrifuged at 600 *g* for 10 min (4,200 rpm, J-20 rotor), the supernatant filtered

through 4 layers of cheesecloth and centrifuged at 7,500 *g* for 10 min (8,000 rpm, J-20 rotor) to obtain the mitochondrial fraction. The 7,500 *g* supernatant was centrifuged at 17,000 *g* for 10 min (12,000 rpm, J-20 rotor) to obtain the peroxisomal fraction. The 17,000 *g* supernatant was centrifuged at 100,000 *g* for 60 min (33,500 rpm, 50.2 Ti rotor) to obtain the microsomal fraction. The mitochondrial and peroxisomal fractions were each washed once with the above buffer and resuspended in either 0.25 M sucrose for the mitochondrial fraction or 0.25 M sucrose with 0.1% ethanol for the peroxisomal fraction. The microsomal pellet was washed once in buffer containing 1.15% KCl and 10 mM EDTA buffer and then resuspended in an equivalent volume of 0.25 M sucrose. The samples were stored in cryovials at - 65 ^{o}C. Eventually, only the microsomal fractions were used.

Determination of protein content of various subcellular fractions

The protein content of the various subcellular fractions was determined according to the method of Lowry *et al.* (1951). The analyses were performed on a Hewlett-Packard 8452A diode array spectrophotometer in triplicate. BSA was used as the protein standard.

Determination of cytochrome P-450 content in hepatic microsomes

Cytochrome P-450 content in the microsomal fraction from various treated groups was determined on a Hewlett-Packard 8452A diode array spectrophotometer using the method of Omura and Sato (1964). Microsomal protein was diluted 1:25 in a buffer containing 0.1 M sodium phosphate, pH 7.4, 20% glycerol and 0.1 M EDTA. Carbon monoxide was bubbled through one cuvette (sample) for 1 min and a small amount of sodium dithionite was added to each cuvette and mixed thoroughly. After one min, the sample was scanned over the range of 325 to 625 nm. The amount of cytochrome P-450 in the sample

was calculated using the millimolar extinction coefficient of 91 cm^{-1} mM^{-1} for cytochrome P-450 (Omura and Sato, 1964). The analyses were performed in duplicate.

Gel electrophoresis of microsomal protein

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) using a Hoeffer vertical slab gel unit. The separating gel was 7.5% acrylamide-bis, 0.375 M Tris-HCl, 0.1% SDS, 0.042% ammonium persulphate and 0.03% TEMED. The stacking gel was 3% acrylamide-bis, 0.125 M Tris-HCl, 0.1% SDS, 0.08% ammonium persulphate and 0.05% TEMED. Samples were diluted in sample dilution buffer (0.062 M Tris HCl, 1% SDS, 0.001% bromophenol blue, 10% glycerol and 5% β-mercaptoethanol), boiled for 2 min and 10 μg of microsomal protein were loaded per lane in a 20 μL volume. The gels were run at 12.5 mA per gel for approximately 45-50 min (time for proteins to travel through stacking gel) and then the current was increased to 25 mA per gel for approximately 2 h until the dye front reached the bottom of the gel. The gel was then fixed for 1 h in fixative (25% isopropanol and 10% acetic acid in water) to remove SDS, stained for 1 h (25% isopropanol, 10% acetic acid and 0.05% Coomassie Blue) and destained (10% isopropanol and 10% acetic acid) as required. In gels where the amount of cytochrome P-450b was to be quantitated, 5 pmol of spectrally determined microsomal cytochrome P-450 were loaded per lane.

Immunoblot

Immunoblotting was performed according to the method of Towbin *et al.* (1979) using a Hoeffer TE 52 Transphor® unit equipped with a power lid. The proteins were resolved using SDS-PAGE and electrophoretically transferred onto a sheet of 0.20 μ nitrocellulose transfer membrane (BA-S 83, Schleicher &

Schuell, Keene, NH). The transfer was performed at 0.4 mA for 2 h in a cold cabinet using a precooled buffer containing 20% methanol, 0.02 M Tris, 0.154 M glycine and 0.008% SDS at pH 8.3. After completion of the transfer, the nitrocellulose sheet was placed in an utility box containing 50 mL of blocking buffer (1% BSA and 3% skim milk powder (Carnation®) in phosphate buffered saline (PBS)) and stored at 4 °C overnight or until development.

For development of the blot, the blocking buffer was discarded and the primary antibody diluted (5 μg/mL, 1:2,500 dilution) in antibody dilution buffer (1% BSA, 3% skim milk powder and 0.05% Tween in PBS) and incubated for 2 h at 37 °C in a shaking water bath (Haake). The primary antibody was discarded and the nitrocellulose sheet washed 3 times for 10 min with wash buffer (0.05% Tween in PBS). The secondary antibody, goat anti-rabbit peroxidase conjugated antibody, was diluted (1:3,000 dilution) in antibody dilution buffer and incubated for 2 h at 37 °C in a shaking water bath. The nitrocellulose sheet was washed 3 times at 10 min each. The reaction was then visualized using a substrate solution containing 3 mL of 0.018% 4-chloro-1-naphthol in methanol, 30 μL of 30% H_2O_2 and 47 mL of PBS. The reaction was allowed to proceed until visually satisfactory and terminated by submersing the nitrocellulose sheet in a tray of distilled water.

Quantitation of cytochrome P-450b in microsomal protein from PB, CBZ and CBZE treated rats

A standard curve for cytochrome P-450b was generated by loading 0.2, 0.5, 1, 2, 3 and 4 pmol of purified cytochrome P-450b per lane. Microsomal protein equivalent to 5 pmol of spectrally determined cytochrome P-450 was loaded per lane. The conditions for gel electrophoresis and immunoblotting were as noted above. Rabbit anti-rat cytochrome P-450b antibody at a concentration of 5 μg/mL was used to probe the blot. The staining intensity of

the bands from these experiments was quantitated using the VISAGE® 110 Bio Image Analyzer (Bio Image, Ann Arbor, MI) using whole band analysis and optical density.

In vitro microsomal metabolism of VPA and (E)-2-ene VPA

In vitro metabolism of VPA and (E)-2-ene VPA was performed according to the method of Rettie *et al.* (1987). Five hundred µL 0.2 M phosphate buffer, pH 7.4, 10 µL 300 mM $MgCl_2$, 2 nmol of spectrally determined cytochrome P-450 (as microsomal protein), 10 µL 0.01 M NADPH and 10 µL 0.01 M NADH adjusted with water to a final volume of 1,000 µL were combined in glass screw capped test tubes and preincubated for 10 min at 37 °C. The reaction was initiated with either 10 µL of 0.04 M VPA or 40 µL of 0.01 M (E)-2-ene VPA and allowed to proceed for 40 min at 37 °C. The reaction was terminated with 1 mL of 10% HCl and the samples extracted as outlined above for GCMS analysis.

In vitro microsomal metabolism of VPA and (E)-2-ene VPA in the presence of anti-rat cytochrome P-450b or anti-rat cytochrome P-450h antibody

These incubations were performed as above, except that increasing amounts of one of anti-rat cytochrome P-450b antibody, anti-rat cytochrome P-450h antibody or control rabbit IgG was preincubated with the microsomal protein (2 nmol of spectrally determined cytochrome P-450) for 10 min prior to initiation of the reaction by the addition of VPA or (E)-2-ene VPA (Chang, Ph.D. thesis, 1991). The antibodies were employed at concentrations of 0, 0.5, 1, 1.5, 2 and 2.5 mg IgG/nmol of cytochrome P-450.

***In vitro* microsomal metabolism of VPA and (E)-2-ene VPA in the presence of both anti-rat cytochrome P-450b and anti-rat cytochrome P-450h antibodies.**

These incubations were performed as above, except that the microsomal protein was preincubated with both anti-rat cytochrome P-450b and anti-rat cytochrome P-450h antibodies for 10 min prior to initiation of the reaction by the addition of VPA or (E)-2-ene VPA. The antibodies were each used at a concentration of 2 mg IgG/nmol of cytochrome P-450.

Microsomal O-dealkylation of ethoxyresorufin and pentoxyresorufin

Microsomal O-dealkylation rates were determined according to the method of Burke *et al.* (1985) using either pentoxyresorufin or ethoxyresorufin as the substrate. The assays were performed on a RF-540 Shimadzu spectrofluorometer equipped with a Shimadzu DR-3 Data Recorder. Excitation wavelength was 530 nm and emission wavelength was 582 nm with a slit width of 5 nm. The reaction mixture contained 1.93 mL of 0.1 M Hepes/5 mM $MgCl_2$, pH 7.8, 10 μL of either 1 mM pentoxyresorufin or 1 mM ethoxyresorufin in DMSO and 50 μL of microsomal protein diluted in sucrose to 2 mg/mL. The reaction was initiated with the addition of 10 μL of 50 mM NADPH diluted in the above mentioned buffer. The fluorescence reading was recorded every minute for 10 min. Enzyme activity was expressed as either nmol resorufin formed/min/mg protein or nmol resorufin formed/min/nmol cytochrome P-450.

Statistical analysis

Statistical analysis was performed using one way ANOVA (Newman-Keuls test). The level of statistical significance chosen was $p \leq 0.05$.

RESULTS

The goals of this project were to identify the isozyme(s) of cytochrome P-450 induced by carbamazepine (CBZ) and its major metabolite, carbamazepine-10,11-epoxide (CBZE) and to determine the effect of these inducers on the *in vitro* metabolism of valproic acid (VPA) and its major metabolite, (E)-2-ene VPA. These effects of CBZ and CBZE on cytochrome P-450 were then to be compared to those of the classic inducer, phenobarbital (PB) and to clofibrate (CFB) which had been reported to induce VPA metabolism in rats (Heinemeyer *et al.*, 1985).

QUANTITATION AND IDENTIFICATION OF CYTOCHROMES P-450 IN HEPATIC MICROSOMES

Quantitation of total hepatic microsomal cytochrome P-450 content

PB (Remmer and Merker, 1963), CBZ (Wagner and Schmid, 1987; Regnaud *et al.*, 1988) and CFB (Gibson, 1992) have been reported to induce cytochrome P-450. The effectiveness of these compounds to induce cytochrome P-450 was compared. Total cytochrome P-450 content in rat hepatic microsomes isolated from the various treatment groups was measured according to the method of Omura and Sato (1964). The terms "untreated animals", "uninduced animals" and "control animals" are used interchangeably to describe animals which did not receive any compound or vehicle. A representative carbon monoxide sodium dithionite-reduced cytochrome P-450 difference spectrum is shown in figure 5.

A graphic representation of the total hepatic cytochrome P-450 content of microsomes (mean ± s.d.) from untreated, PB, NS, CFB and CO treated rats is shown in figure 6 and is similarly detailed for microsomes from CBZ, CBZE and PG treated rats in figure 7. The changes in total hepatic cytochrome P-450

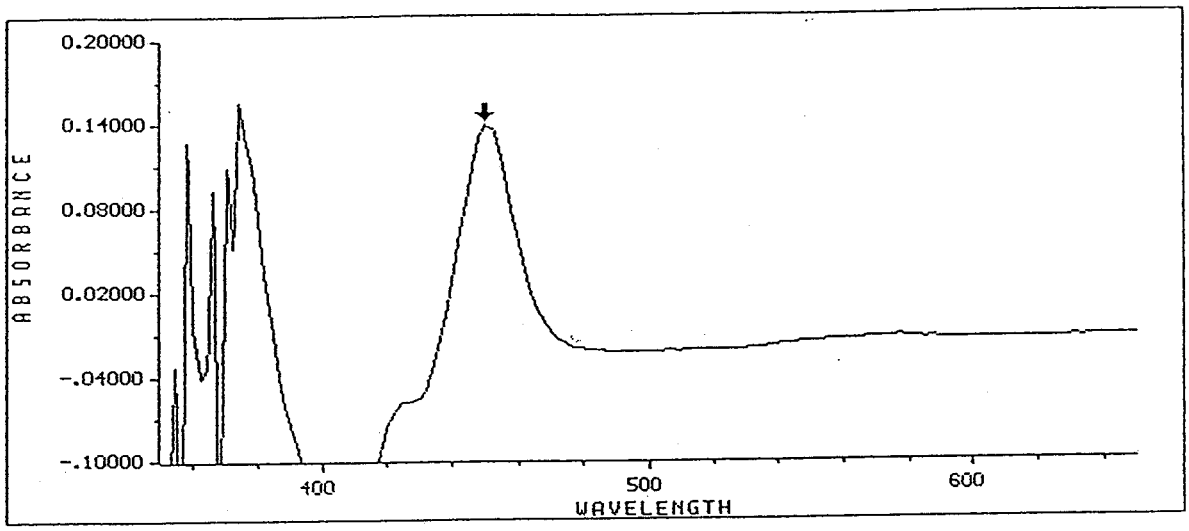

Figure 5. Carbon monoxide sodium dithionite-reduced difference spectrum of hepatic microsomal cytochrome P-450.

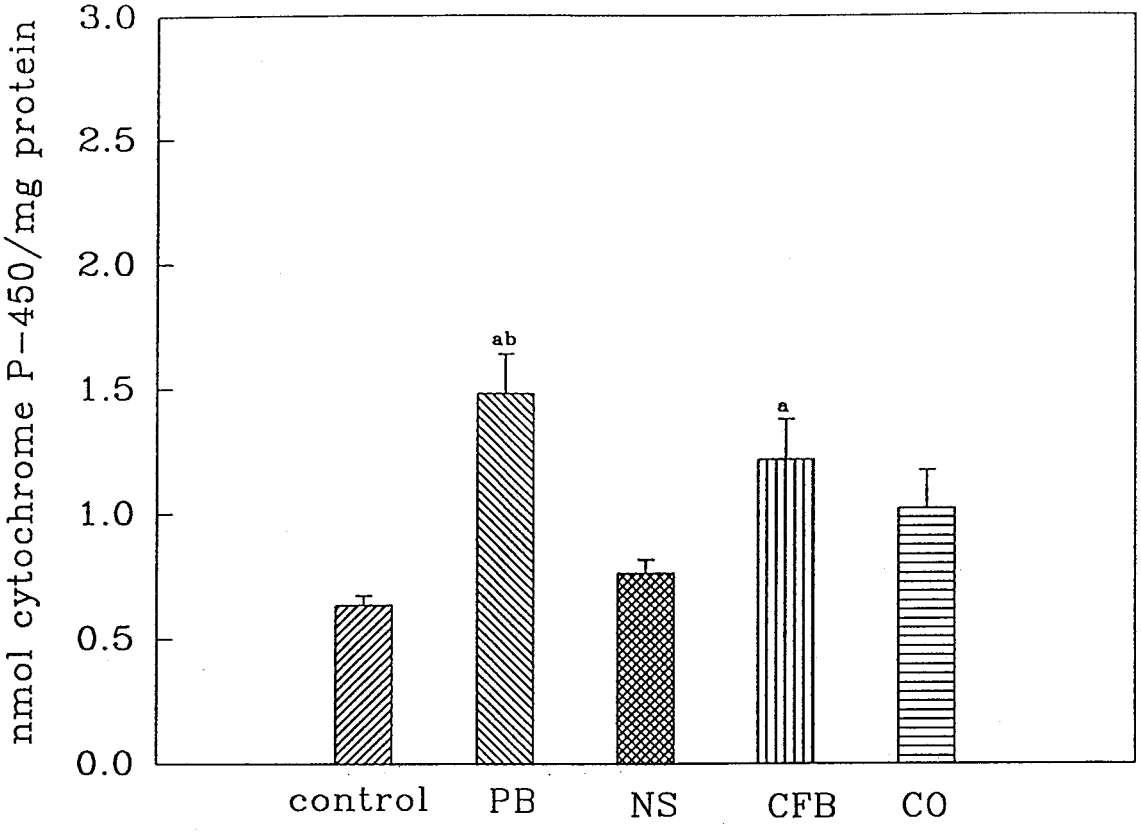

Figure 6. Cytochrome P-450 content (nmol of spectrally determined cytochrome P-450/mg protein, mean ± s.d.) of microsomes from control, PB, NS, CFB and CO treated rats (n=4). [a] significantly greater than microsomes from untreated animals ($p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals. Cytochrome P-450 was determined as outlined in the *Experimental* section.

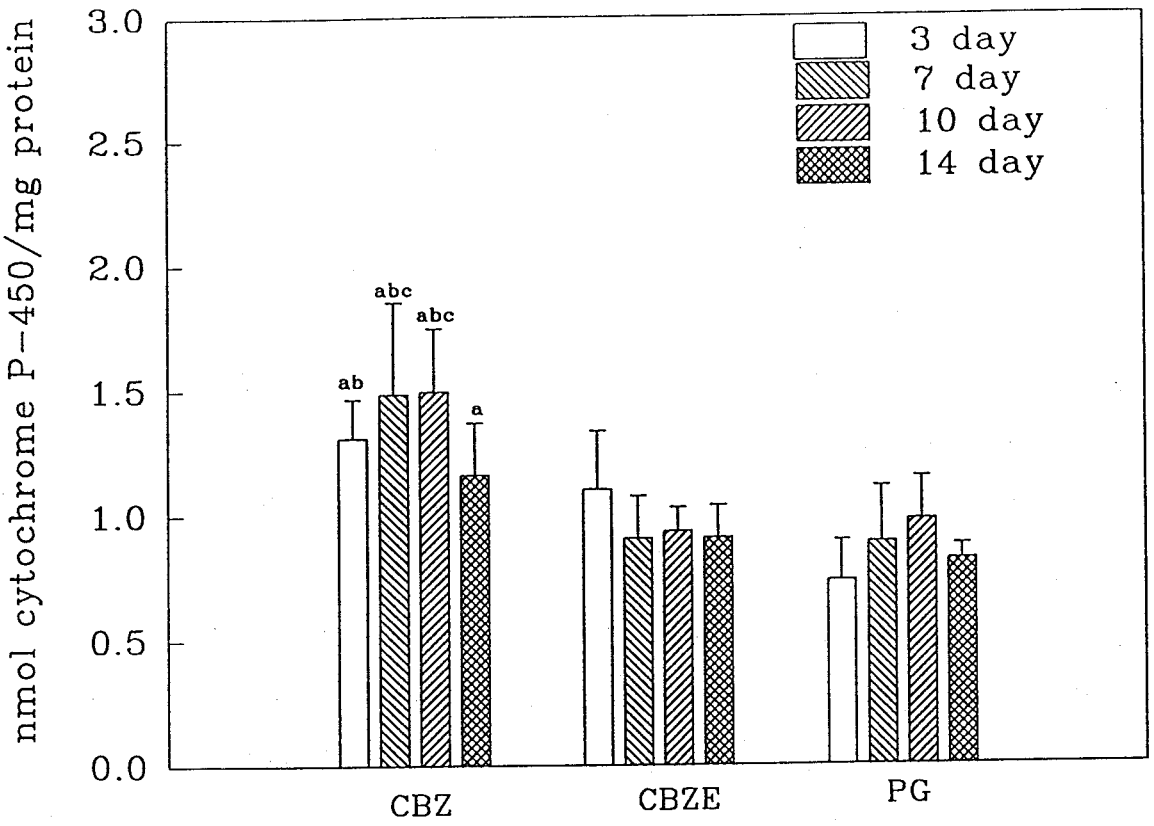

Figure 7. Cytochrome P-450 content (nmol of spectrally determined cytochrome P-450/mg protein, mean ± s.d.) of microsomes from CBZ, CBZE and PG 3, 7, 10 and 14 day treated rats (n=4). [a] significantly greater than microsomes from untreated animals (0.6 ± 0.04 nmol cytochrome P-450/mg protein, $p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period, [c] significantly greater than microsomes from CBZE treated animals over the same time period. Cytochrome P-450 was determined as outlined in the *Experimental* section.

content relative to the control group and to the respective vehicle groups for the PB, CFB, CBZ and CBZE treatment groups are summarized in table 2. The mean cytochrome P-450 content of microsomes prepared from PB (1.5 ± 0.2 nmol cytochrome P-450/mg protein, 2.3 fold increase), CFB (1.2 ± 0.2, 1.9 fold increase), CBZ 3 day (1.3 ± 0.2, 2.1 fold increase), CBZ 7 day (1.5 ± 0.4, 2.3 fold increase), CBZ 10 day (1.5 ± 0.3, 2.3 fold increase) and CBZ 14 day (1.2 ± 0.2, 1.8 fold increase) treated rats were all significantly greater when compared to the mean cytochrome P-450 content of microsomes from untreated animals (0.6 ± 0.04 nmol cytochrome P-450/mg protein). No significant differences were observed when either the NS, CO, CBZE 3, 7, 10 and 14 day or PG 3, 7, 10 and 14 day treated groups were compared to the control group.

When PB treated animals were compared to the NS (vehicle) treated group, the mean cytochrome P-450 content was significantly higher (1.5 ± 0.2 *versus* 0.8 ± 0.05 nmol cytochrome P-450/mg protein, a 1.9 fold increase, table 2). There was no statistical difference between the cytochrome P-450 content of microsomes prepared from the CFB and the CO treated rats (1.2 ± 0.2 *versus* 1.0 ± 0.2 nmol cytochrome P-450/mg protein, table 2).

The amount of cytochrome P-450 in microsomes isolated from CBZ 3, 7 and 10 day treated rats was significantly greater than the PG (vehicle) treated rats for the corresponding time period (1.5 to 1.8 fold increase, table 2). The CBZ 14 day treated group was an exception where a significant difference was not observed for the amount of total cytochrome P-450 found when compared to the corresponding PG vehicle treated group.

Hepatic microsomes from the CBZE treatment groups did not display any significant increases in the amount of total cytochrome P-450 when compared to their corresponding PG vehicle controls (figure 7, table 2).

Table 2. Summary of total hepatic cytochrome P-450 content (nmol/mg protein, mean ± s.d.) and change in cytochrome P-450 relative to the untreated group or to the respective vehicle control group for the PB, CFB, CBZ and CBZE treatment groups (n=4). Cytochrome P-450 was determined as outlined in the *Experimental* section.

Treatment	Cytochrome P-450 (nmol/mg protein)		
	nmol/mg protein mean ± s.d.	fold increase relative to untreated group	fold increase relative to vehicle group
Untreated	0.6 ± 0.04		
PB	1.5 ± 0.2[ab]	2.3	1.9
NS	0.8 ± 0.05	1.3	
CFB	1.2 ± 0.2[a]	1.9	1.2
CO	1.0 ± 0.2	1.7	
CBZ 3 day	1.3 ± 0.2[ab]	2.1	1.8
CBZ 7 day	1.5 ± 0.4[abc]	2.3	1.7
CBZ 10 day	1.5 ± 0.3[abc]	2.3	1.5
CBZ 14 day	1.2 ± 0.2[a]	1.8	1.4
CBZE 3 day	1.1 ± 0.2	1.7	1.5
CBZE 7 day	0.9 ± 0.2	1.4	1.0
CBZE 10 day	0.9 ± 0.1	1.5	1.0
CBZE 14 day	0.9 ± 0.1	1.4	1.1
PG 3 day	0.7 ± 0.2	1.2	
PG 7 day	0.9 ± 0.2	1.5	
PG 10 day	1.0 ± 0.2	1.7	
PG 14 day	0.8 ± 0.2	1.3	

($p \leq 0.05$)

a significantly greater than microsomes from untreated animals

b significantly greater than microsomes from appropriate vehicle control animals treated over the same time period

c significantly greater than microsomes from CBZE treated animals over the same time period

Comparable amounts of hepatic microsomal cytochrome P-450 were found for all 4 CBZ treatment groups as well as for the PB and CFB treated rats, with no statistical differences observed. Increases ranged from 1.8 to 2.3 fold over the untreated group (table 2).

Cytochrome P-450 levels were found to be consistent over the time course of treatment in both the CBZ and CBZE treated groups (table 2). However, the total microsomal cytochrome P-450 levels of the CBZ 7 and CBZ 10 day groups were found to be higher than the corresponding CBZE treated group.

Identification of the cytochrome P-450 isozymes induced by CBZ and CBZE using SDS-PAGE and Western blot techniques

Previous literature has suggested that CBZ may induce the same isozymes of cytochrome P-450 as PB (Wagner and Schmid, 1987). Based on this information, studies were performed to determine if cytochrome P-450b (Ryan *et al.*, 1982) (cytochrome P-4502B1, Nelson *et al.*, 1993), an isozyme known to be induced by PB, was also induced by either CBZ or CBZE. CFB served as a control since it is known to induce cytochrome P-452 (CYP4A1, lauric acid hydroxylase) (Leroux *et al.*, 1989).

The antibodies against various isozymes of cytochrome P-450 were prepared by Dr. Bandiera's group. The polyspecific antibody against rat cytochrome P-450b also reacted with cytochrome P-450e, another isozyme induced by PB.

The nomenclature of Levin and co-workers will be used throughout this thesis when referring to isozymes of cytochrome P-450 (Ryan and Levin, 1990) and a summary comparing their nomenclature to that of Nelson *et al.* (1993) is provided in table 1 in the *Introduction*.

Representative SDS-PAGE gels of microsomal protein isolated from the livers of rats treated with either CBZ or CBZE over the time course of treatment

are shown in figures 8 and 9. In both gels, lanes 2, 9 and 18 are the molecular weight standards while lane 3 is the purified cytochrome P-450b standard. Lane 5 contains microsomal protein from the PB treatment group while lanes 10, 12, 14 and 16 contain microsomal protein from either the CBZ 3, 7, 10 and 14 day treatment groups, respectively in figure 8 or from the CBZE 3, 7, 10 and 14 day treatment groups, respectively in figure 9. Lanes 11, 13, 15 and 17 contain microsomal protein from PG 3, 7, 10 and 14 day treated groups, respectively. After the separated proteins were electrophoretically transferred to a nitrocellulose membrane and probed with the antibody directed against rat cytochrome P-450b, microsomal protein from both CBZ and CBZE treated rats reacted positively, indicating that cytochrome P-450b was present (figures 10 and 11). Cytochrome P-450b could be detected in the microsomes after 3 days of treatment with either CBZ or CBZE. A second band due to reaction of the antibody with cytochrome P-450e was also observed just above the cytochrome P-450b band. Neither cytochrome P-450b nor cytochrome P-450e was detected in microsomes from untreated, NS, CO and PG treated animals. Surprisingly, a positive reaction was also observed with microsomal protein from the CFB treated group suggestive of the presence of cytochromes P-450b and P-450e.

Microsomal protein isolated from VPA treated rats did not react with the anti-rat cytochrome P-450b antibody, indicating that VPA does not influence this particular isozyme of cytochrome P-450 (data not shown).

Microsomal protein from none of the treatment groups reacted to any appreciable degree with anti-rat cytochrome P-450f or anti-cytochrome P-450g (data not shown).

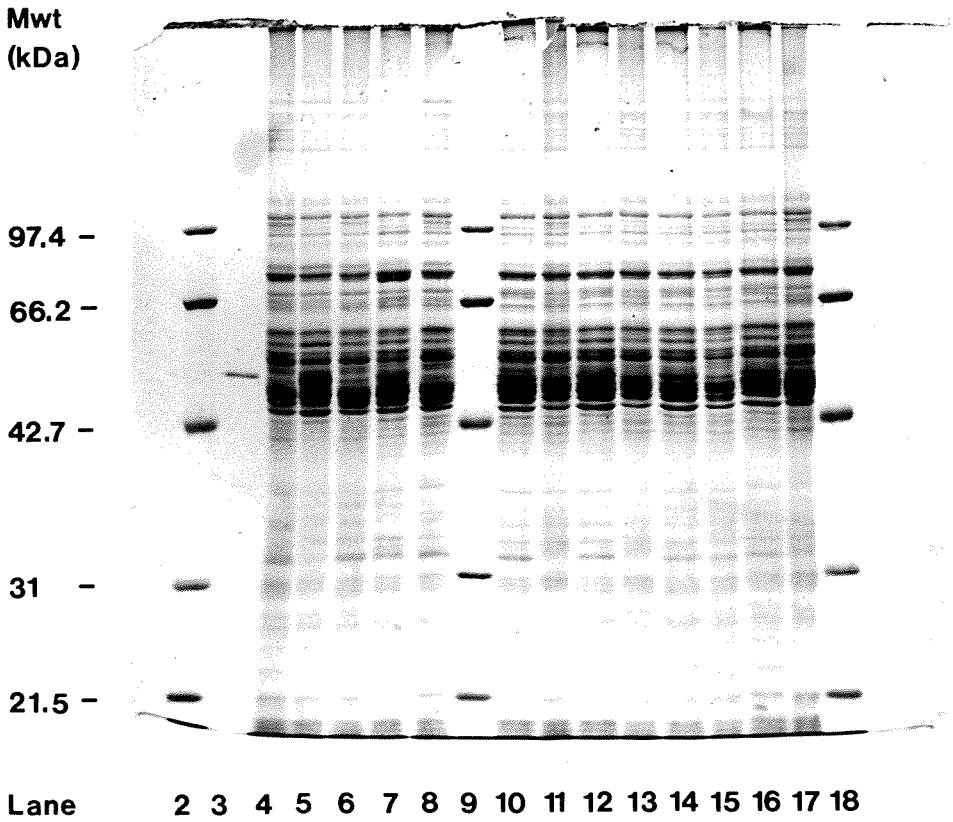

Figure 8. SDS-PAGE gel of rat liver microsomal fractions from various treatment groups. Lane 2, molecular weight standards; lane 3, purified cytochrome P-450b; lane 4, control; lane 5, PB; lane 6, NS; lane 7, CFB; lane 8, CO; lane 9, molecular weight standards; lane 10, CBZ 3 day; lane 11, PG 3 day; lane 12, CBZ 7 day; lane 13, PG 7 day; lane 14, CBZ 10 day; lane 15, PG 10 day; lane 16, CBZ 14 day; lane 17, PG 14 day and lane 18, molecular weight standards.

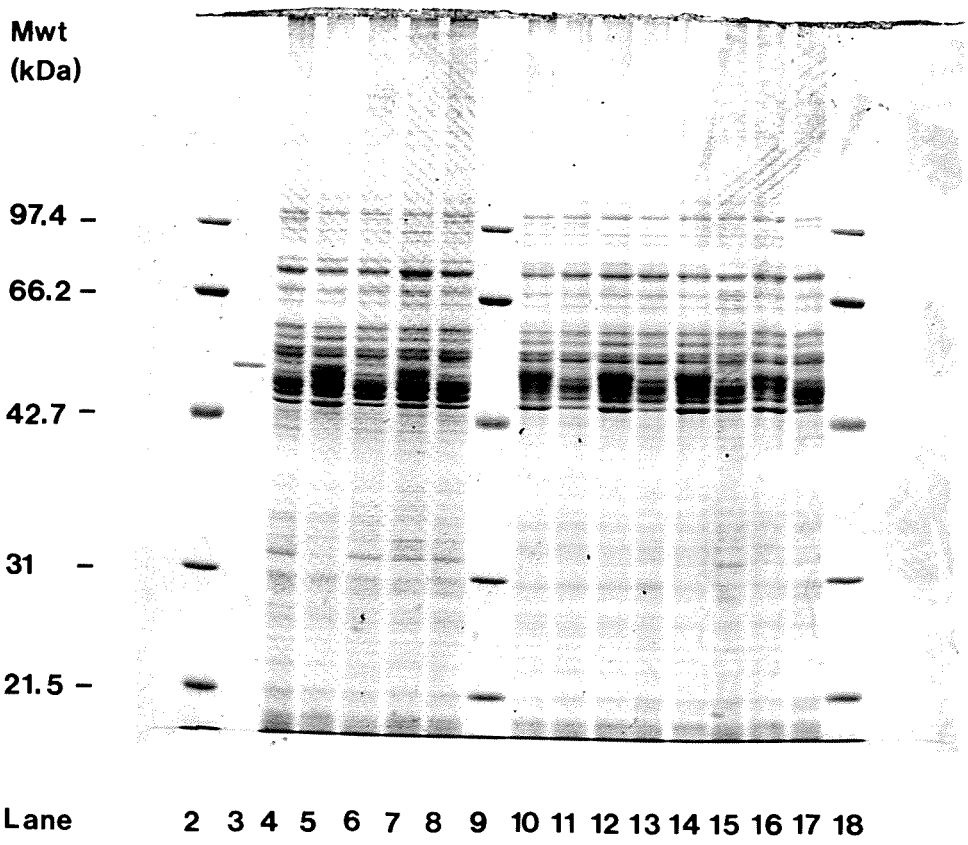

Figure 9. SDS-PAGE gel of rat liver microsomal fractions from various treatment groups. Lane 2, molecular weight standards; lane 3, purified cytochrome P-450b; lane 4, control; lane 5, PB; lane 6, NS; lane 7, CFB; lane 8, CO; lane 9, molecular weight standards; lane 10, CBZE 3 day; lane 11, PG 3 day; lane 12, CBZE 7 day; lane 13, PG 7 day; lane 14, CBZE 10 day; lane 15, PG 10 day; lane 16, CBZE 14 day; lane 17, PG 14 day and lane 18, molecular weight standards.

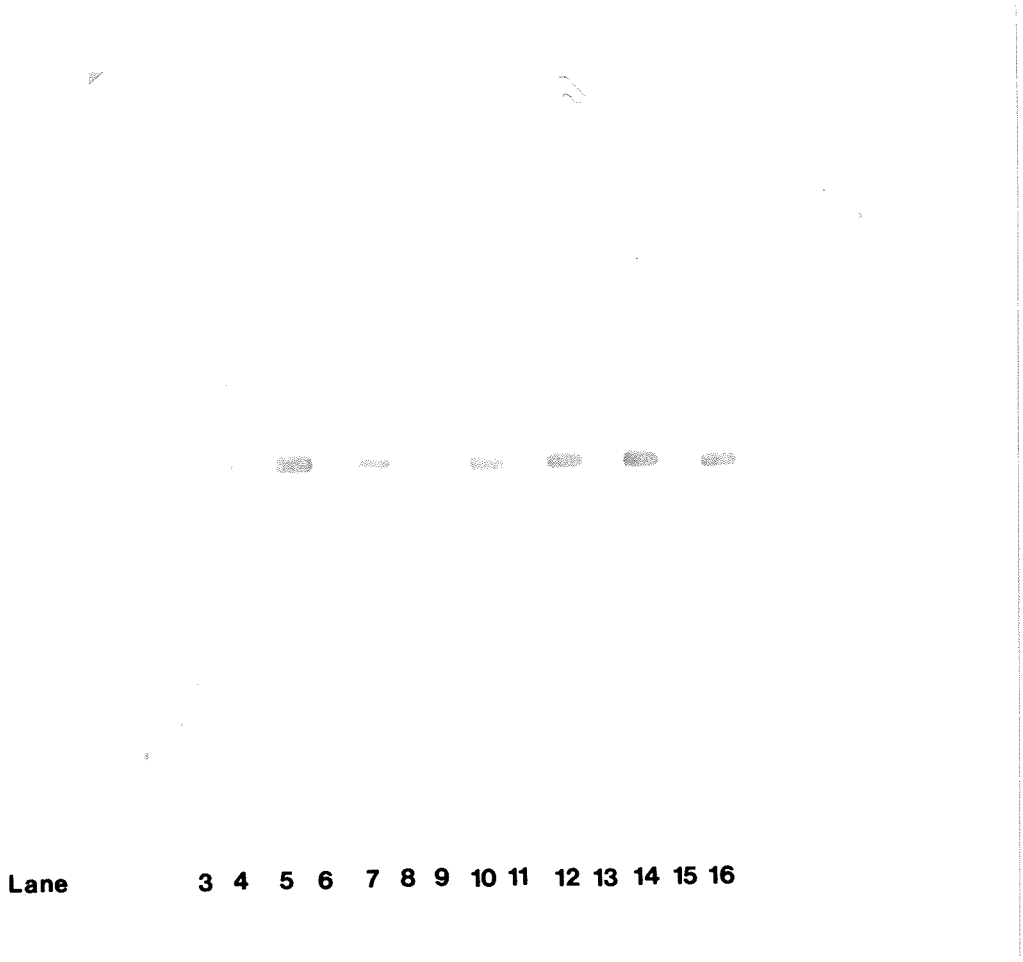

Figure 10. Immunoblot of rat liver microsomal proteins probed with anti-rat cytochrome P-450b antibody. Lane 3, purified cytochrome P-450b; lane 4, control; lane 5, PB; lane 6, NS; lane 7, CFB; lane 8, CO; lane 10, CBZ 3 day; lane 11, PG 3 day; lane 12, CBZ 7 day; lane 13, PG 7 day; lane 14, CBZ 10 day; lane 15, PG 10 day; lane 16, CBZ 14 day.

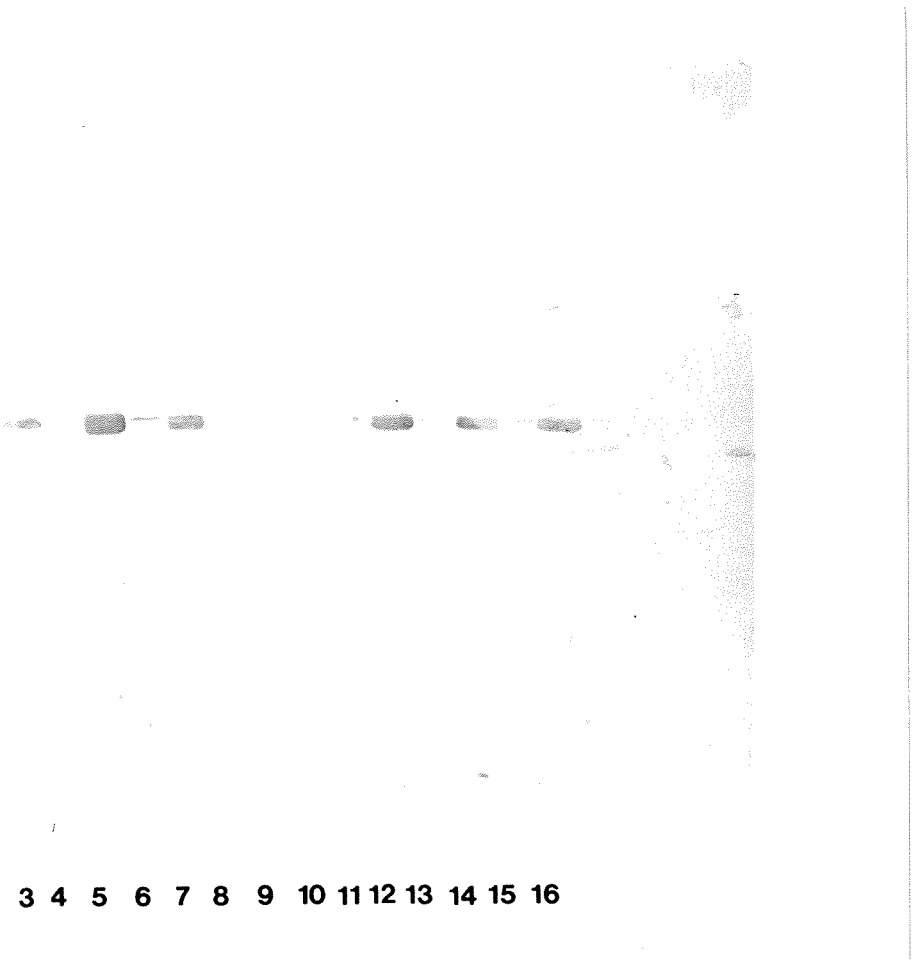

Figure 11. Immunoblot of rat liver microsomal proteins probed with anti-rat cytochrome P-450b antibody. Lane 3, purified cytochrome P-450b; lane 4, control; lane 5, PB; lane 6, NS; lane 7, CFB; lane 8, CO; lane 10, CBZE 3 day; lane 11, PG 3 day; lane 12, CBZE 7 day; lane 13, PG 7 day; lane 14, CBZE 10 day; lane 15, PG 10 day; lane 16, CBZE 14 day.

In vitro O-dealkylation of pentoxyresorufin and ethoxyresorufin catalyzed by hepatic microsomal protein from the various treatment groups

To the best of our knowledge, the isozyme(s) of cytochrome P-450 induced by CBZ and CBZE have not previously been identified. Pentoxyresorufin and ethoxyresorufin were used as substrates for the microsomal O-dealkylation reactions to confirm the identification of cytochrome P-450b isozyme of cytochrome P-450 induced by CBZ and CBZE. Pentoxyresorufin is a preferred substrate for cytochrome P-450b (PB inducible) while ethoxyresorufin is a preferred substrate for cytochrome P-450c (3-methylcholanthrene inducible) (Burke *et al.*, 1985).

The results from the O-dealkylation of ethoxyresorufin and pentoxyresorufin by microsomes from untreated, PB, NS, CFB and CO treated rats are shown in figure 12. In the untreated, CFB, or CO treated rats, ethoxyresorufin was not utilized as a substrate while O-dealkylation of ethoxyresorufin occurred only to a minor degree in the PB and NS groups. On the other hand it was readily apparent that pentoxyresorufin was utilized as a substrate to varying degrees by microsomes from all 5 treatment groups.

The microsomal O-dealkylation of pentoxyresorufin and ethoxyresorufin by microsomes from CBZ, CBZE and PG treated animals over the time course of treatment is illustrated in figures 13 and 14, respectively. Pentoxyresorufin, without question, was the preferred substrate by microsomes from all of the treatment groups. Induction of ethoxyresorufin O-dealkylation activity was observed for the CBZ 3 day treatment group (figure 14).

Changes in pentoxyresorufin O-dealkylation (PROD) for the PB, CFB, CBZ and CBZE treatment groups relative to the untreated group and to the vehicle control groups are summarized in table 3.

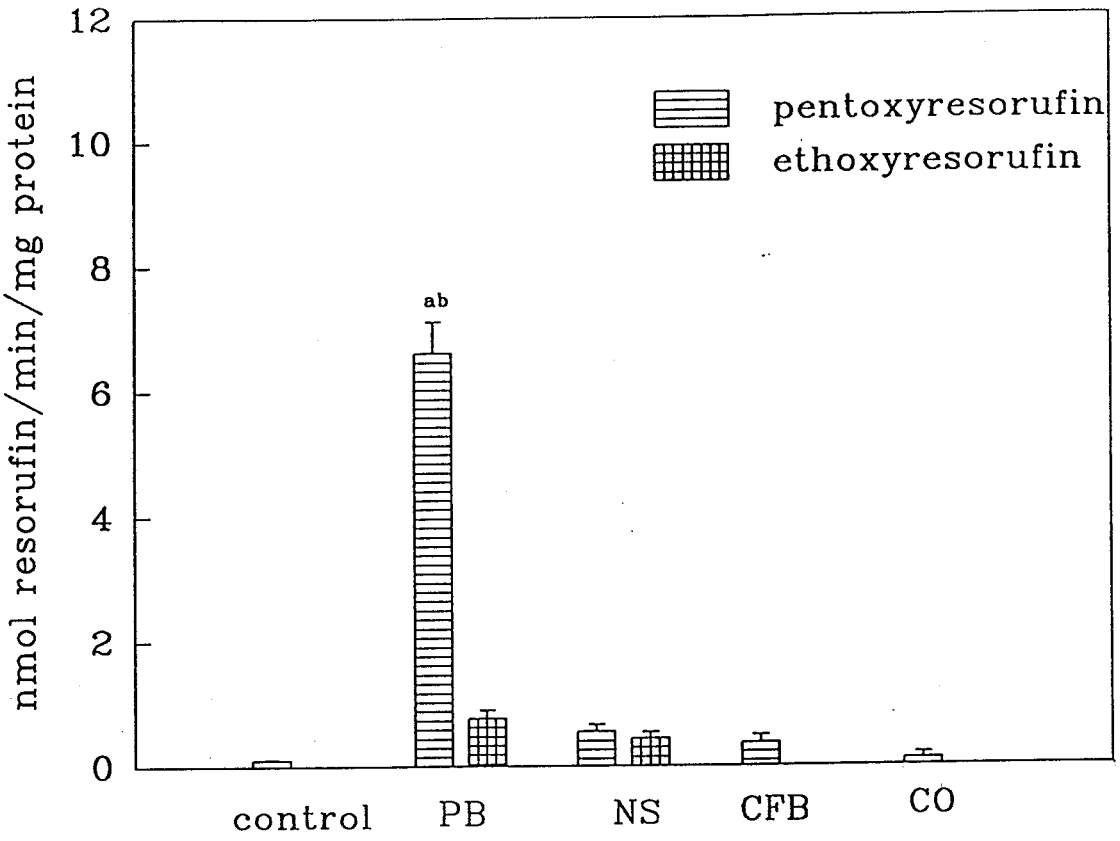

Figure 12. Microsomal O-dealkylation of pentoxyresorufin and ethoxyresorufin (nmol resorufin/min/mg protein, mean ± s.d.) by microsomes from control, PB, NS, CFB and CO treated rats (n=4). [a] significantly greater than microsomes from untreated animals ($p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period. Microsomal O-dealkylation was determined as outlined in the *Experimental* section.

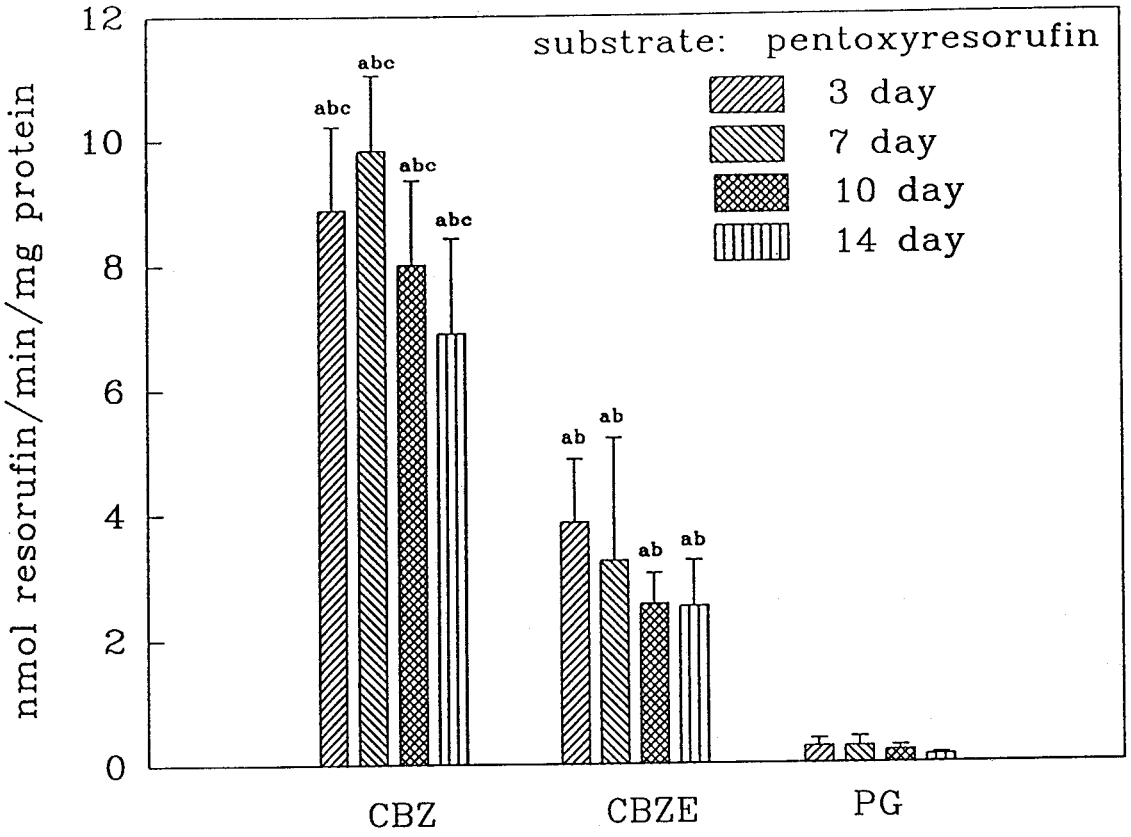

Figure 13. Microsomal O-dealkylation of pentoxyresorufin (nmol resorufin/min/mg protein, mean ± s.d.) by microsomes from CBZ, CBZE and PG 3, 7, 10 and 14 day treated rats (n=4). [a] significantly greater than microsomes from untreated animals (0.1 ± 0.01 nmol resorufin/min/mg protein, $p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period, [c] significantly greater than microsomes from CBZE treated animals over the same time period. Microsomal O-dealkylation was determined as outlined in the *Experimental* section.

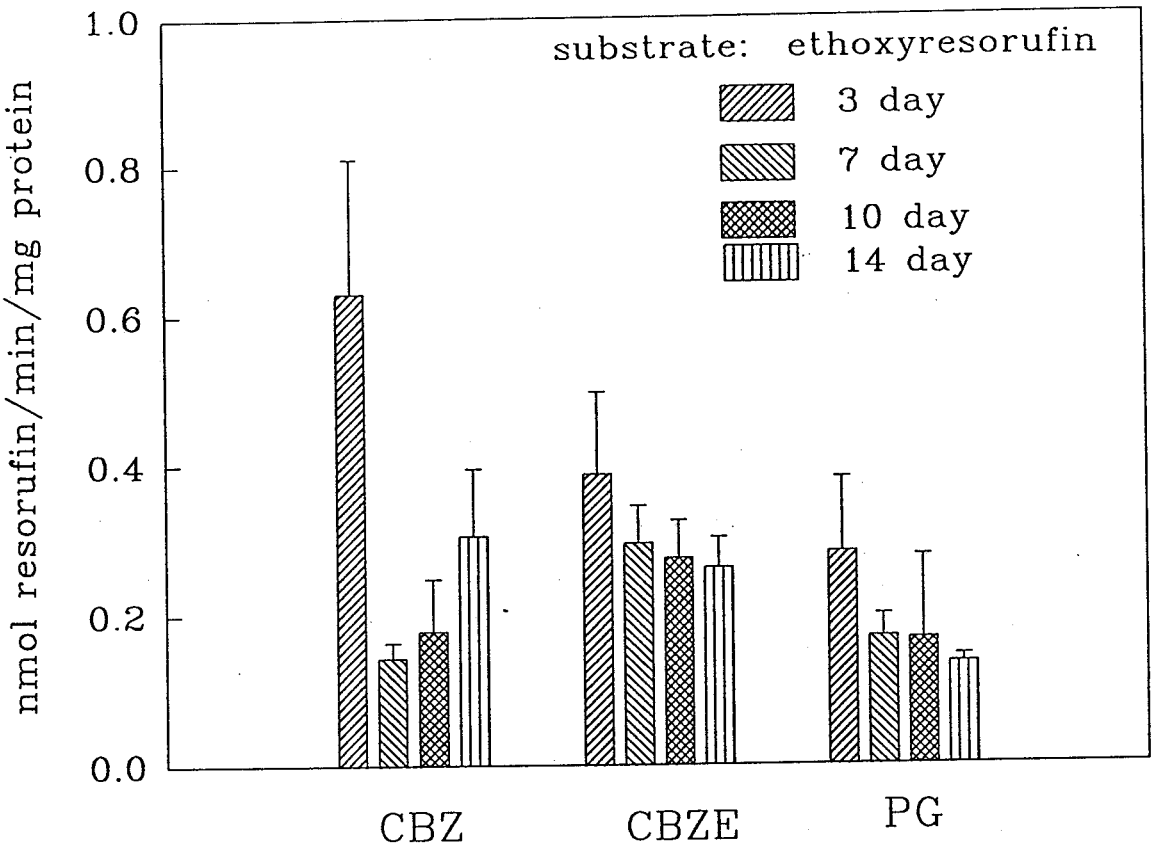

Figure 14. Microsomal O-dealkylation of ethoxyresorufin (nmol resorufin/min/mg protein, mean ± s.d.) by microsomes from CBZ, CBZE and PG 3, 7, 10 and 14 day treated rats (n=4). Microsomal O-dealkylation was determined as outlined in the *Experimental* section.

Table 3. Summary of PROD (nmol resorufin/min/mg protein) and changes in PROD relative to the untreated group or to the respective vehicle control group for the PB, CFB, CBZ and CBZE treatment groups (n=4). PROD was determined as outlined in the *Experimental* section.

Treatment	Pentoxyresorufin O-dealkylation Activity (PROD)		
	nmol resorufin formed/min/mg protein mean ± s.d.	fold increase relative to untreated group	fold increase relative to vehicle group
Untreated	0.1 ± 0.01		
PB	6.6 ± 0.5[ab]	66	12
NS	0.6 ± 0.1	6	
CFB	0.4 ± 0.1	4	3
CO	0.1 ± 0.1	1	
CBZ 3 day	8.9 ± 1.3[abc]	89	34
CBZ 7 day	9.8 ± 1.2[abc]	98	38
CBZ 10 day	8.0 ± 1.4[abc]	80	40
CBZ 14 day	6.9 ± 1.5[abc]	69	53
CBZE 3 day	3.9 ± 1.0[ab]	39	15
CBZE 7 day	3.2 ± 2.0[ab]	32	12
CBZE 10 day	2.6 ± 0.5[ab]	25	13
CBZE 14 day	2.5 ± 0.7[ab]	25	19
PG 3 day	0.3 ± 0.1	3	
PG 7 day	0.3 ± 0.2	3	
PG 10 day	0.2 ± 0.1	2	
PG 14 day	0.1 ± 0.1	1	

($p \leq 0.05$)

a significantly greater than microsomes from untreated animals

b significantly greater than microsomes from appropriate vehicle control animals treated over the same time period

c significantly greater than microsomes from CBZE treated animals over the same time period

There was no statistical difference in the O-dealkylation of pentoxyresorufin by microsomes from the CFB treated animals when compared to the CO treated group, although the rate for the CFB induced microsomes was 3 fold greater than that of the CO induced microsomes (figure 12).

The rates of pentoxyresorufin O-dealkylation by microsomes prepared from PB, CBZ and CBZE treated rats were significantly greater when compared to the microsomes from the untreated animals, with the increases ranging from a low of 25 fold for the CBZE 10 and 14 day groups to a high of 98 fold for the CBZ 7 day group (table 3).

Similarly, the rates of pentoxyresorufin O-dealkylation by microsomes from PB, CBZ or CBZE treated rats were significantly greater when compared to the O-dealkylation rates of their respective vehicle control groups with the increases ranging from 12 fold for the PB group to 53 fold for the CBZ 14 day group (table 3).

When the inducing agents were compared, the rates of pentoxyresorufin O-dealkylation by microsomes from CBZ 3 and 7 day treated animals were significantly greater than the mean pentoxyresorufin O-dealkylation rate for microsomes from PB treated rats. The higher pentoxyresorufin O-dealkylation rates compared to the PB treated group were only true for the CBZ 3 and 7 day treated groups as the rates for the CBZ 10 and 14 day treated groups appeared to decline (table 3).

Pentoxyresorufin O-dealkylation rates for microsomes from CBZE 3, 7, 10 and 14 day treated rats were not statistically different from each other over the course of treatment. When CBZE pentoxyresorufin O-dealkylation rates over the time course were compared to those of the corresponding CBZ treated group or to the PB treated group, CBZE rates were approximately 32 to 43% of the appropriate CBZ treated group and 38 to 58% of the PB treated group (table 4).

Table 4. Comparison of mean PROD activities of CBZE 3, 7, 10 and 14 day treated groups as a percent of the PROD activities of the PB and CBZ 3, 7, 10 and 14 day treatment groups. PROD was determined as outlined in the *Experimental* section.

	PROD (nmol resorufin formed/min/mg protein)			
	CBZE 3 day (%)	CBZE 7 day (%)	CBZE 10 day (%)	CBZE 14 day (%)
PB	58	49	39	38
CBZ 3 day	43	-	-	-
CBZ 7 day	-	33	-	-
CBZ 10 day	-	-	32	-
CBZ 14 day	-	-	-	36

The rates of pentoxyresorufin O-dealkylation by microsomes from CBZ 3, 7 and 10 day treated animals were not significantly different from each other. However, the rate of pentoxyresorufin O-dealkylation by microsomes from CBZ 14 day treated animals was significantly lower than the rates for microsomes from CBZ 3, 7 and 10 day treated rats.

Results similar to those discussed above for rates normalized to protein content were obtained when the rate of either pentoxyresorufin or ethoxyresorufin O-dealkylation was normalized to total cytochrome P-450 (data not shown). Pentoxyresorufin O-dealkylation rates for microsomes from PB, CBZ 3, 7, 10 and 14 day and CBZE 3, 7, 10 and 14 day treated animals were significantly increased when compared to the untreated group (17 to 42 fold) or to their respective vehicle control group (6 to 69 fold). The rates of pentoxyresorufin O-dealkylation of the CBZ treated groups were comparable to the PB treated group with the exception of the CBZ 3 day treated group which was significantly higher (6.8 ± 0.4 *versus* 4.5 ± 0.4 nmol resorufin/min/nmol cytochrome P-450). CBZE pentoxyresorufin O-dealkylation rates over the time course were approximately 61 to 80% of the PB treated group and 48 to 64% of the appropriate CBZ treated groups (data not shown).

Quantitation of cytochrome P-450b in microsomes from CBZ, CBZE and PB treated rats by SDS-PAGE and Western blot techniques

While PB is known to induce cytochrome P-450b (Waxman and Azaroff, 1992), induction of cytochrome P-450b by CBZ or CBZE has not been reported previously. Since it had been verified in the previous sections that cytochrome P-450b was inducible by CBZ, this particular isozyme was quantitated in microsomes from CBZ 3, 7, 10 and 14 day, CBZE 3, 7, 10 and 14 day and PB treated rats to determine if the inducing abilities of CBZ and CBZE were quantitatively similar to that of PB. Cytochrome P-450b content was

quantitated from Western blots based on the intensity of the bands using the Visage Bio-Image Analyser.

The amount of cytochrome P-450b quantitated in microsomes from rats treated with PB, CBZ and CBZE over the time course is shown in table 5 as pmol cytochrome P-450b/5 pmol total cytochrome P-450 (loaded per lane) and as a percentage of total hepatic cytochrome P-450. The percent of cytochrome P-450b present ranged from 31% (CBZE 14 day group) to 66% (CBZ 3 day group). Statistically equivalent amounts of the isozyme were present in the CBZ treated microsomes when compared to PB induced microsomes (table 5). The microsomes from the PB treated group contained 3.3 ± 0.1 pmol of cytochrome P-450b/5 pmol of spectrally determined cytochrome P-450, representing 65% of total hepatic cytochrome P-450 content. The microsomes from the CBZ 3 day treated group contained 3.3 ± 0.8 pmol cytochrome P-450b/5 pmol of spectrally determined cytochrome P-450 representing 66% of total hepatic cytochrome P-450. Although mean quantities of the isozyme in the CBZE induced microsomes (mean 31 to 53%, over the time course) appeared lower than in microsomes from both the PB (65%) and CBZ induced microsomes (mean 39 to 66% over the time course), no statistical differences were observed between the 3 treatment groups.

IN VITRO METABOLISM OF VPA AND (E)-2-ENE VPA

Enzyme induction plays an important role in the formation of toxic metabolites of VPA. Enzyme induction due to polytherapy is associated with a high incidence of VPA induced hepatotoxicity (Dreifuss *et al.*, 1987). The effects of PB and CBZ on the *in vitro* metabolism of VPA have been briefly investigated whilst the effects of CBZE induction have not been yet investigated. In the present work, the effects of induction by PB, CBZ, CBZE and CFB on the *in*

Table 5. Cytochrome P-450b (pmol/5 pmol of spectrally determined cytochrome P-450 or as percent of total hepatic cytochrome P-450) in microsomes from rats treated with either PB, CBZ for 3, 7, 10 or 14 days or CBZE for 3, 7, 10 or 14 days. Microsomal protein (5 pmol of spectrally determined cytochrome P-450/lane) was separated by SDS-PAGE and probed using an anti-rat cytochrome P-450b antibody as outlined in the *Experimental* section. (n=3, mean ± s.d.).

Treatment	cytochrome P-450b pmol/5 pmol P-450	cytochrome P-450b (% of total cytochrome P-450)
PB	3.3 ± 0.1	65 ± 2
CBZ 3 day	3.3 ± 0.8	66 ± 16
CBZ 7 day	2.7 ± 0.8	54 ± 16
CBZ 10 day	2.7 ± 0.5	55 ± 11
CBZ 14 day	1.9 ± 0.4	39 ± 8
CBZE 3 day	1.8 ± 0.8	35 ± 17
CBZE 7 day	2.7 ± 1.4	53 ± 29
CBZE 10 day	1.6 ± 0.7	33 ± 14
CBZE 14 day	1.6 ± 1.1	31 ± 23

vitro metabolism of VPA were investigated.

Analysis of VPA and metabolites by GCMS

The GCMS assay previously developed in our laboratory (Abbott *et al.*, 1986) was used for the analysis of reaction products extracted from the *in vitro* microsomal metabolism of VPA and (E)-2-ene VPA. It was possible to separate and detect 16 metabolites of VPA. The deuterated compounds, [$^2H_7$]E-2-ene VPA, [$^2H_7$]4-ene VPA, [$^2H_7$]3-OH VPA, [$^2H_7$]5-OH VPA, [$^2H_7$]3-keto VPA, [$^2H_7$]4-keto VPA and [$^2H_7$]VPA recently synthesized in our laboratory (Zheng, M.Sc. thesis, 1993) were used as internal standards.

***In vitro* metabolism conditions for VPA and (E)-2-ene VPA**

The *in vitro* metabolism of VPA and (E)-2-ene VPA by microsomes from the various treatment groups was investigated to determine the formation of cytochrome P-450 mediated metabolites and the effect of induction on the formation of these metabolites. The method used in this work was based on a procedure from the literature (Rettie *et al.*, 1987) and is outlined in the *Experimental* section. The amount of VPA used in the incubations was chosen such that the VPA peak in the GCMS chromatogram did not overlap extensively with that of the 4-ene VPA peak, the metabolite of primary interest. Metabolism of the major serum metabolite, (E)-2-ene VPA, was then studied at an equivalent molar dose to VPA to allow a direct comparison. Cytochrome P-450 was varied over the concentration range of 1 to 6 nmol of spectrally determined cytochrome P-450 per incubation and it was determined that 2 nmol of spectrally determined microsomal cytochrome P-450 per *in vitro* incubation were adequate to obtain quantifiable amounts of VPA or (E)-2-ene VPA metabolites as measured by our GCMS method. Over this range of cytochrome P-450 investigated, a linear relationship was not observed between the product

and the amount of protein. The use of 2 nmol of spectrally determined cytochrome P-450 per incubation avoided the use of excessive amounts of microsomal protein since the amount of product recovered did not increase. An optimal incubation time was investigated in order to allow the reaction to proceed to completion. Incubation times of 20, 30, 40, 50 or 60 min were examined. It was determined that the amount of metabolites formed did not change considerably but to be on the safe side an incubation time of 40 min was selected.

Not all 16 metabolites of VPA that could be quantitated in the GCMS assay were monitored. Firstly, not all of the metabolites are cytochrome P-450 generated products and thus were not detected. Additionally, some metabolites, for example 3-ene VPA, could not be distinguished from the background noise. Therefore, when VPA served as the substrate, the metabolites quantitated were 3-OH VPA, 4-OH VPA, 5-OH VPA, 4-ene VPA and 4-keto VPA. Only 2 metabolites, (E)-2,4-diene VPA and (E,E)-2,3'-diene VPA, were measured when (E)-2-ene VPA was used as the substrate. Additional metabolites monitored for included the formation of (E)-2-ene VPA from VPA and the formation of 3-ene VPA and 4-ene VPA from (E)-2-ene VPA. These metabolic products, however, were not detected.

In order to determine that the metabolites to be quantitated were in fact products of cytochrome P-450 mediated metabolism, the incubations were performed using boiled microsomes, or in the absence of cofactors, or in the absence of substrate to eliminate artifacts. Under these conditions, none of the expected metabolites of either VPA or (E)-2-ene VPA were detected (data not shown).

In vitro metabolism of VPA

Formation of 3-OH VPA from VPA

The amount of 3-OH VPA formed from VPA (0.4 μmol) by microsomes from control, PB, NS, CFB and CO treated animals is shown in figure 15 while that by microsomes from CBZ, CBZE and PG treated animals is illustrated in figure 16. Very small quantities of 3-OH VPA were detected for the PG and CBZE 14 day treated groups. The changes in the formation of 3-OH VPA by the PB, CFB, CBZ and CBZE treated groups relative to the untreated group and to their respective vehicle control group are summarized in table 6.

The formation of 3-OH VPA from VPA by microsomes from PB treated animals (0.40 ± 0.09 μg) was significantly increased 7 fold when compared to untreated microsomes (0.05 ± 0.02 μg) while 3 to 4 fold increases were observed for CBZ 3 day (0.20 ± 0.02 μg), 7 day (0.10 ± 0.05 μg) and 10 day (0.10 ± 0.04 μg) induced microsomes, respectively (figures 15 and 16, table 6).

A 9 fold increase in the formation of 3-OH VPA was observed for the PB treated group when compared to the NS treated group (0.40 ± 0.09 μg *versus* 0.04 ±0.01 μg) (figure 15, table 6). Increases of 4 to 20 fold were observed when the CBZ treated groups were compared to the PG treated groups (figure 16, table 6). The mean value of 3-OH VPA produced by microsomes from PB treated rats was significantly greater when compared to all other treatment groups.

Formation of 4-OH VPA from VPA

The amount of 4-OH VPA formed from VPA by microsomes from control, PB, NS, CFB and CO treated rats is shown in figure 17 while that by microsomes from CBZ, CBZE and PG treated rats is illustrated in figure 18. The changes in the formation of 4-OH VPA due to induction by PB, CFB, CBZ

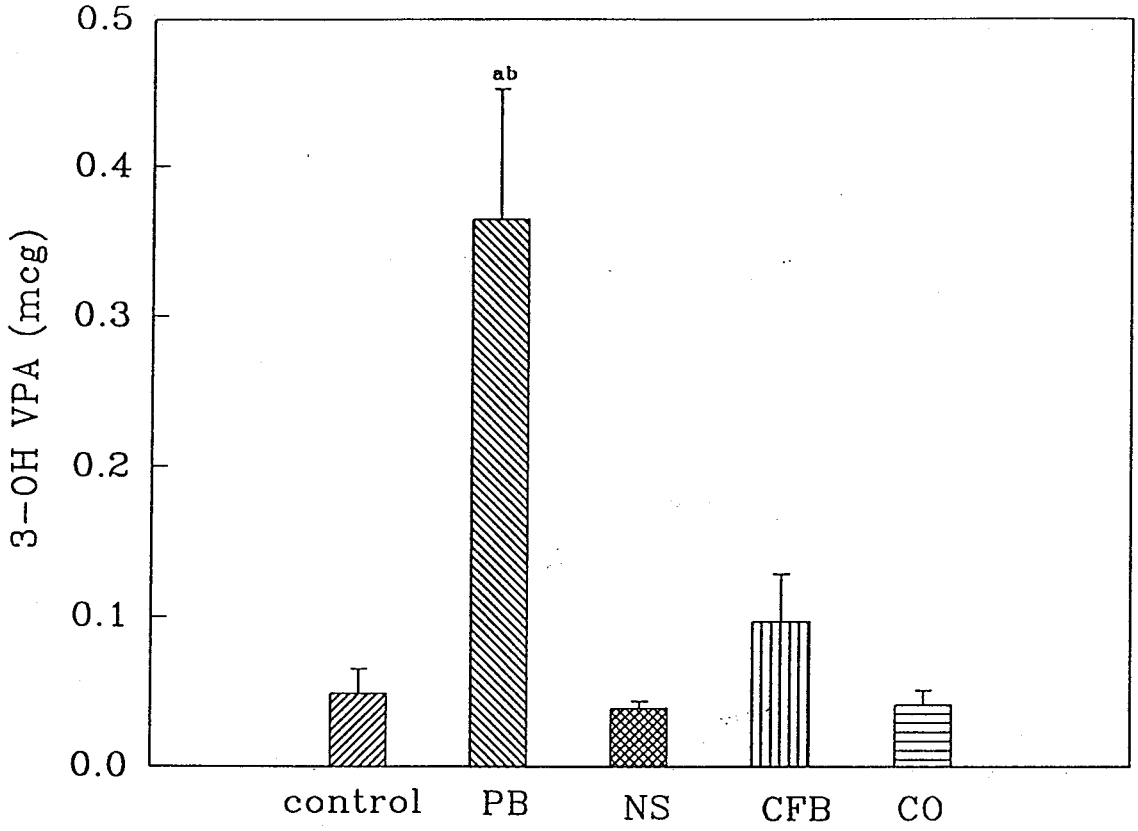

Figure 15. Formation of 3-OH VPA (μg, mean ± s.d.) from the *in vitro* metabolism of VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from untreated, PB, NS, CFB and CO treated rats (n=4). [a] significantly greater than microsomes from untreated animals ($p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period. Microsomal incubations were performed as outlined in the *Experimental* section.

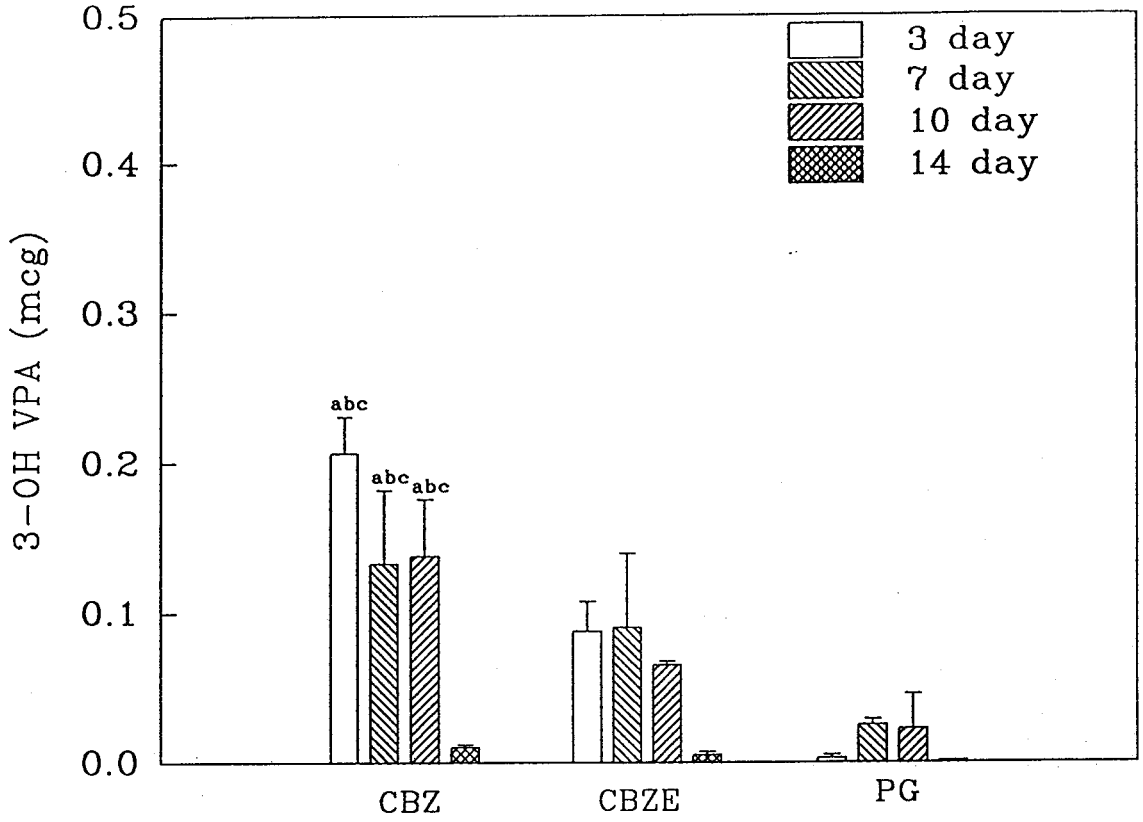

Figure 16. Formation of 3-OH VPA (μg, mean ± s.d.) from the *in vitro* metabolism of VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from CBZ, CBZE and PG 3, 7, 10 and 14 day treated rats (n=4). [a] significantly greater than microsomes from untreated animals (0.05 ± 0.02 μg, $p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period, [c] significantly greater than microsomes from CBZE treated animals over the same time period. Microsomal incubations were performed as outlined in the *Experimental* section.

Table 6. A comparison of the metabolism of VPA to 3-OH VPA by microsomes from PB, CFB, CBZ and CBZE treated rats, relative to the untreated group or to the respective vehicle control group (n=4). Microsomal incubations and quantitation of 3-OH VPA were performed as outlined in the *Experimental* section.

Treatment	Formation of 3-OH VPA		
	amount formed mean ± s.d. (μg)	fold increase relative to untreated group	fold increase relative to vehicle group
Untreated	0.05 ± 0.02		
PB	0.40 ± 0.09	7	9
NS	0.04 ± 0.01	--	
CFB	0.10 ± 0.01	2	2
CO	0.04 ± 0.01	--	
CBZ 3 day	0.20 ± 0.02	4	20
CBZ 7 day	0.10 ± 0.05	3	6
CBZ 10 day	0.10 ± 0.04	3	6
CBZ 14 day	***	--	4
CBZE 3 day	0.10 ± 0.02	2	9
CBZE 7 day	0.10 ± 0.05	2	4
CBZE 10 day	0.10 ± 0.01	--	2
CBZE 14 day	***	--	--
PG 3 day	***	--	
PG 7 day	0.03 ± 0.01	--	
PG 10 day	0.02 ± 0.02	--	
PG 14 day	***	--	

-- increase less than 2 fold

*** trace quantities detected

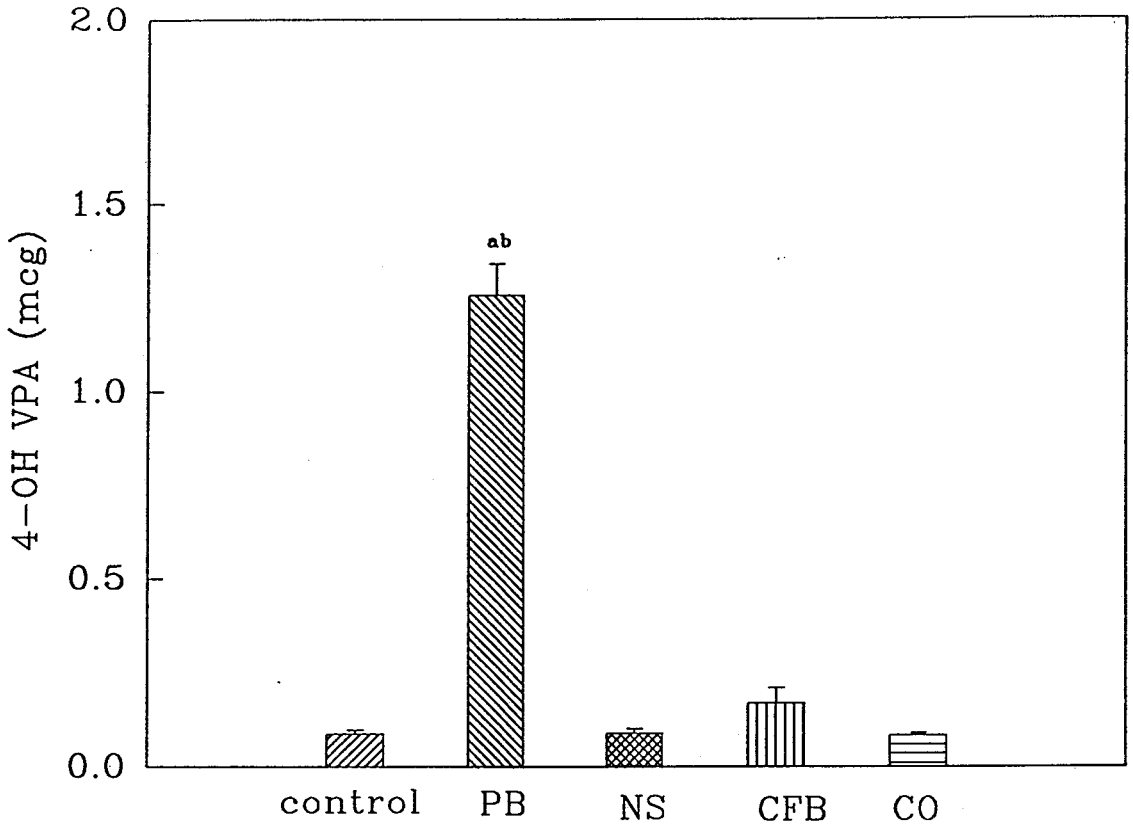

Figure 17. Formation of 4-OH VPA (μg, mean ± s.d.) from the *in vitro* metabolism of VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from untreated, PB, NS, CFB and CO treated rats (n=4). [a] significantly greater than microsomes from untreated animals ($p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period. Microsomal incubations were performed as outlined in the *Experimental* section.

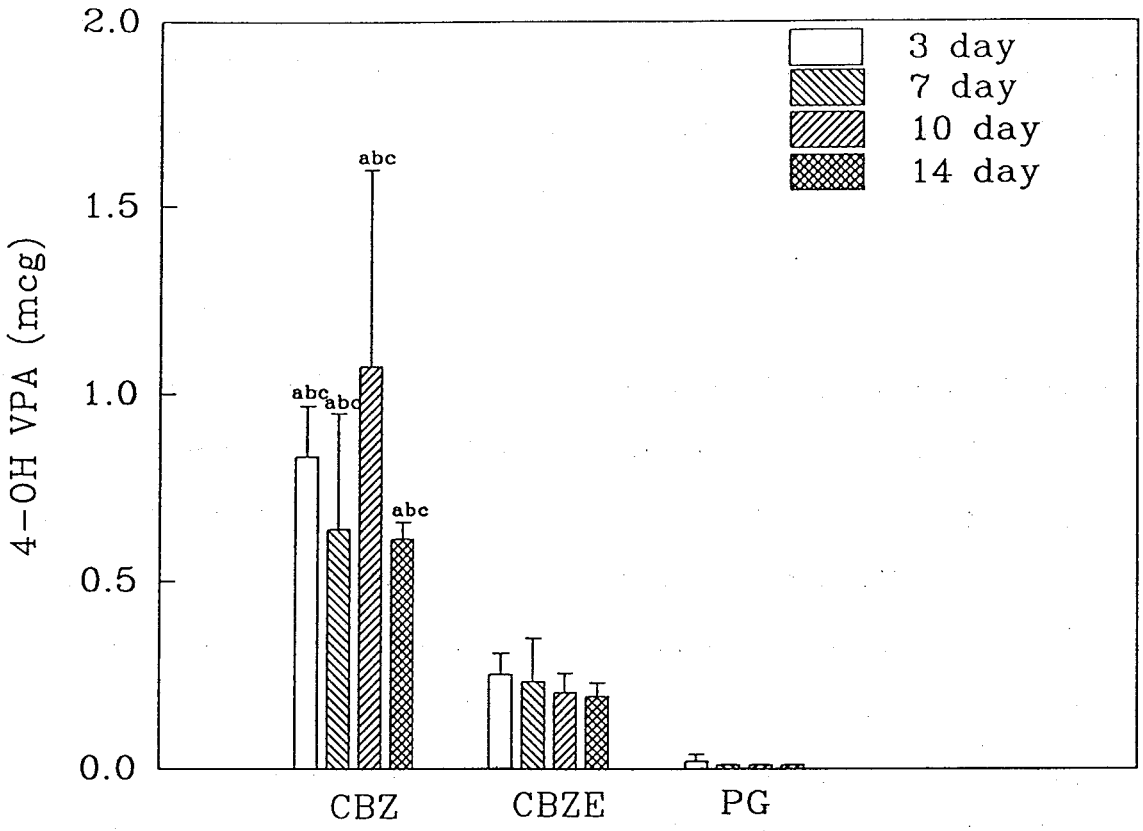

Figure 18. Formation of 4-OH VPA (μg, mean ± s.d.) from the *in vitro* metabolism of VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from CBZ, CBZE and PG 3, 7, 10 and 14 day treated rats (n=4). [a] significantly greater than microsomes from untreated animals (0.09 ± 0.01 μg, $p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period, [c] significantly greater than microsomes from CBZE treated animals over the same time period. Microsomal incubations were performed as outlined in the *Experimental* section.

and CBZE relative to the untreated group and to the appropriate vehicle control groups are noted in table 7.

When compared to microsomes from untreated animals (0.09 ± 0.01 μg, table 7), the amount of 4-OH VPA produced by PB induced microsomes was increased 15 fold (1.30 ± 0.08 μg) and was significantly greater than all other treatment groups. Increases for the CBZ treated groups ranged from 7 to 12 fold when compared to the untreated group (table 7).

Of particular note was the comparison of amounts of 4-OH VPA produced by microsomes from the CBZ and CBZE treated groups when compared to their appropriate PG control groups (figure 18). The increases ranged from a low of 9 fold for the CBZE 10 and 14 day treatment groups to a high of 52 fold for the CBZ 10 day treatment group (table 7) because very low quantities of 4-OH VPA were detected for the PG treated groups.

The CBZ treated group over the time course yielded significantly higher amounts of 4-OH VPA when compared to the appropriate CBZE treated group (figure 18).

Formation of 5-OH VPA from VPA

The amount of 5-OH VPA produced by microsomes from PB, NS, CFB, CO and untreated animals is depicted in figure 19 while that by microsomes from CBZ, CBZE and PG treated animals are shown in figure 20. A summary of the changes in the formation of 5-OH VPA by induced microsomes is given in table 8.

Compared to the untreated group (16 ± 3 ng), the amount of 5-OH VPA produced by the PB treated group (87 ± 7 ng) was significantly increased 5 fold (table 8). Significant increases ranging from 3 to 8 fold were also observed for the CFB, CBZ and CBZE 3 and 14 day treated groups when compared to the untreated group (table 8).

Table 7. A comparison of the metabolism of VPA to 4-OH VPA by microsomes from PB, CFB, CBZ and CBZE treated rats, relative to the untreated group or to the respective vehicle control group (n=4). Microsomal incubations and quantitation of 4-OH VPA were performed as outlined in the *Experimental* section.

Treatment	Formation of 4-OH VPA		
	amount formed mean ± s.d. (μg)	fold increase relative to untreated group	fold increase relative to vehicle group
Untreated	0.09 ± 0.01		
PB	1.30 ± 0.08	15	14
NS	0.10 ± 0.01	--	
CFB	0.20 ± 0.04	2	2
CO	0.10 ± 0.01	--	
CBZ 3 day	0.80 ± 0.10	10	40
CBZ 7 day	0.60 ± 0.30	7	30
CBZ 10 day	1.10 ± 0.50	12	52
CBZ 14 day	0.60 ± 0.04	7	29
CBZE 3 day	0.30 ± 0.10	3	12
CBZE 7 day	0.20 ± 0.10	3	11
CBZE 10 day	0.20 ± 0.10	2	9
CBZE 14 day	0.20 ± 0.04	2	9
PG 3 day	0.02 ± 0.02	--	
PG 7 day	0.01 ± 0.00	--	
PG 10 day	0.01 ± 0.00	--	
PG 14 day	0.01 ± 0.00	--	

-- increase less than 2 fold

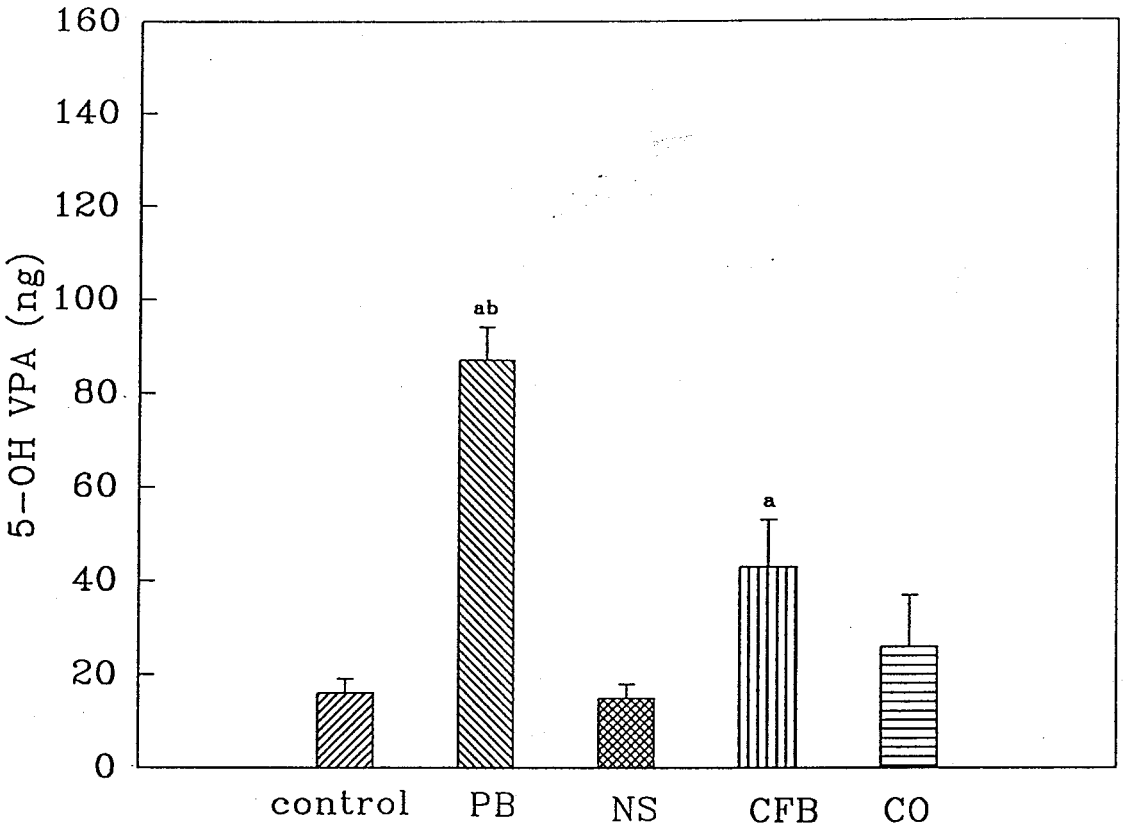

Figure 19. Formation of 5-OH VPA (ng, mean ± s.d.) from the *in vitro* metabolism of VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from untreated, PB, NS, CFB and CO treated rats (n=4). [a] significantly greater than microsomes from untreated animals ($p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period. Microsomal incubations were performed as outlined in the *Experimental* section.

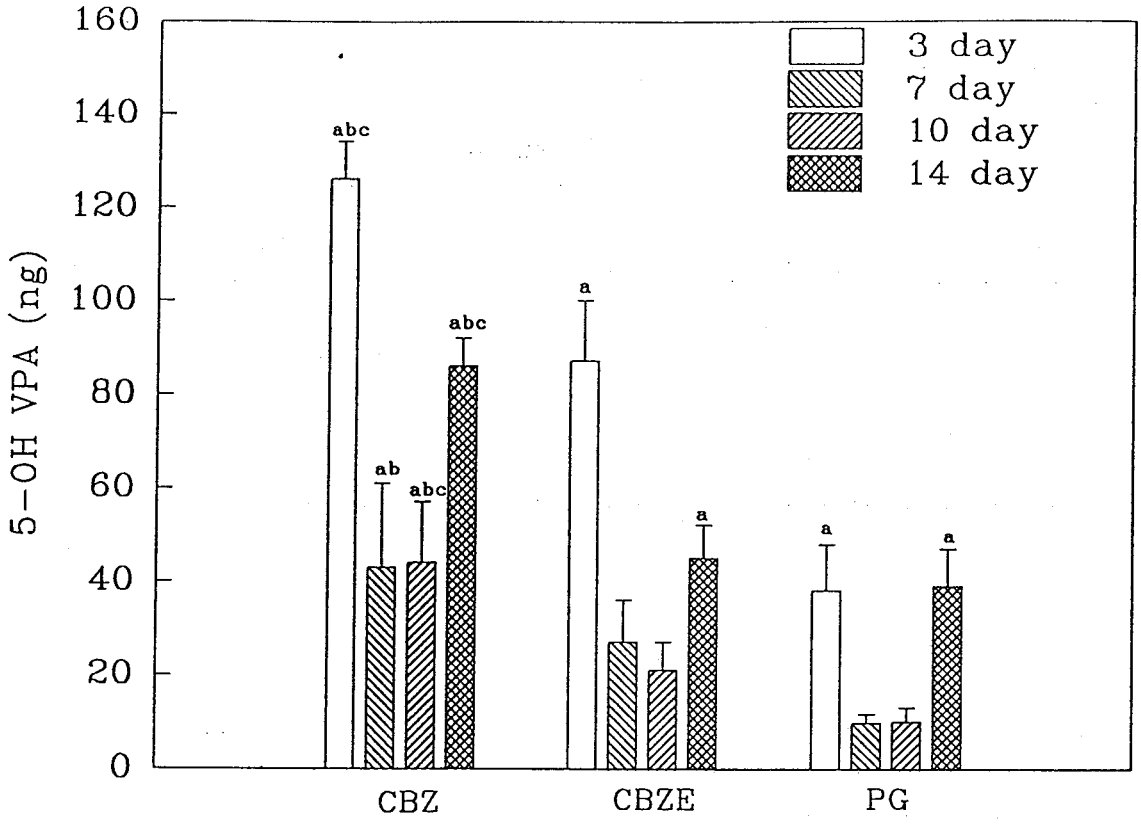

Figure 20. Formation of 5-OH VPA (ng, mean ± s.d.) from the *in vitro* metabolism of VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from CBZ, CBZE and PG 3, 7, 10 and 14 day treated rats (n=4). [a] significantly greater than microsomes from untreated animals (16 ± 3 ng, $p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period, [c] significantly greater than microsomes from CBZE treated animals over the same time period. Microsomal incubations were performed as outlined in the *Experimental* section.

Table 8. A comparison of the metabolism of VPA to 5-OH VPA by microsomes from PB, CFB, CBZ and CBZE treated rats, relative to the untreated group or to the respective vehicle control group (n=4). Microsomal incubations and quantitation of 5-OH VPA were performed as outlined in the *Experimental* section.

Treatment	Formation of 5-OH VPA		
	amount formed mean ± s.d. (ng)	fold increase relative to untreated group	fold increase relative to vehicle group
Untreated	16 ± 3		
PB	87 ± 7	5	6
NS	15 ± 3	--	
CFB	43 ± 10	3	2
CO	26 ± 11	--	
CBZ 3 day	126 ± 8	8	3
CBZ 7 day	43 ± 18	3	4
CBZ 10 day	44 ± 13	3	5
CBZ 14 day	86 ± 6	5	2
CBZE 3 day	87 ± 13	5	2
CBZE 7 day	27 ± 9	2	3
CBZE 10 day	21 ± 6	--	2
CBZE 14 day	45 ± 7	3	--
PG 3 day	38 ± 10	2	
PG 7 day	10 ± 2	--	
PG 10 day	10 ± 3	--	
PG 14 day	39 ± 8	2	

-- increase less than 2 fold

The formation of 5-OH VPA from VPA by microsomes from the PB treated rats was significantly increased 6 fold when compared to the NS group (table 8). When the CBZ treated groups were compared to the appropiate PG treated group over the time course, significant increases ranging from 2 to 5 fold were observed. A significant difference was not observed between the CBZE treated groups when compared to the PG treated groups over the time course (table 8).

With the exception of the CBZ 7 day treated group, the formation of 5-OH VPA was significantly higher when the CBZ treated groups were compared to the corresponding CBZE treated group. The amount of 5-OH VPA formed from VPA by CBZ 3 day treated microsomes was significantly greater when compared to PB microsomes and all other treatment groups (table 8).

Formation of 4-ene VPA from VPA

The amounts of 4-ene VPA produced by microsomes from the untreated, PB, NS, CFB and CO treated rats are depicted in figure 21. Values for the CBZ, CBZE and PG treated groups are illustrated in figure 22. Table 9 summarizes the increases in 4-ene VPA formation by the inducing agents when compared to the untreated and the vehicle control groups.

PB, CBZ and CBZE were capable of enhancing 4-ene VPA formation relative to the untreated group (table 9). PB gave the greatest increase (6 fold) in this regard, although the increase for CBZ was 4 to 5 fold (table 9). CBZE 3 day treatment also yielded a 2 fold increase in 4-ene formation. Very small quantities of 4-ene VPA were detected for the CBZE 7, 10 and 14 day and the PG treatment groups.

The metabolism of VPA to 4-ene VPA was induced 5 fold by PB treatment when compared to the NS group (17 ± 2 ng *versus* 3 ± 0.6 ng) while the formation of 4-ene VPA by the CBZ treated groups was increased compared to

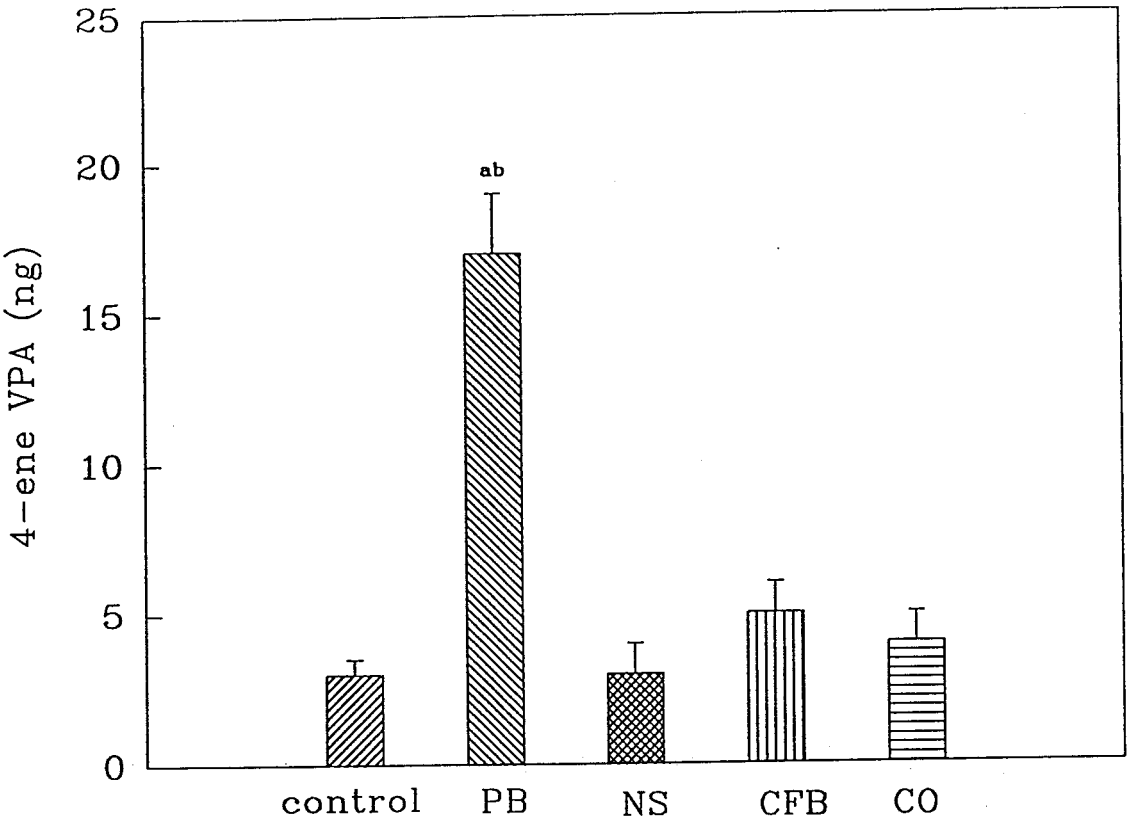

Figure 21. Formation of 4-ene VPA (ng, mean ± s.d.) from the *in vitro* metabolism of VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from untreated, PB, NS, CFB and CO treated rats (n=4). [a] significantly greater than microsomes from untreated animals ($p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period. Microsomal incubations were performed as outlined in the *Experimental* section.

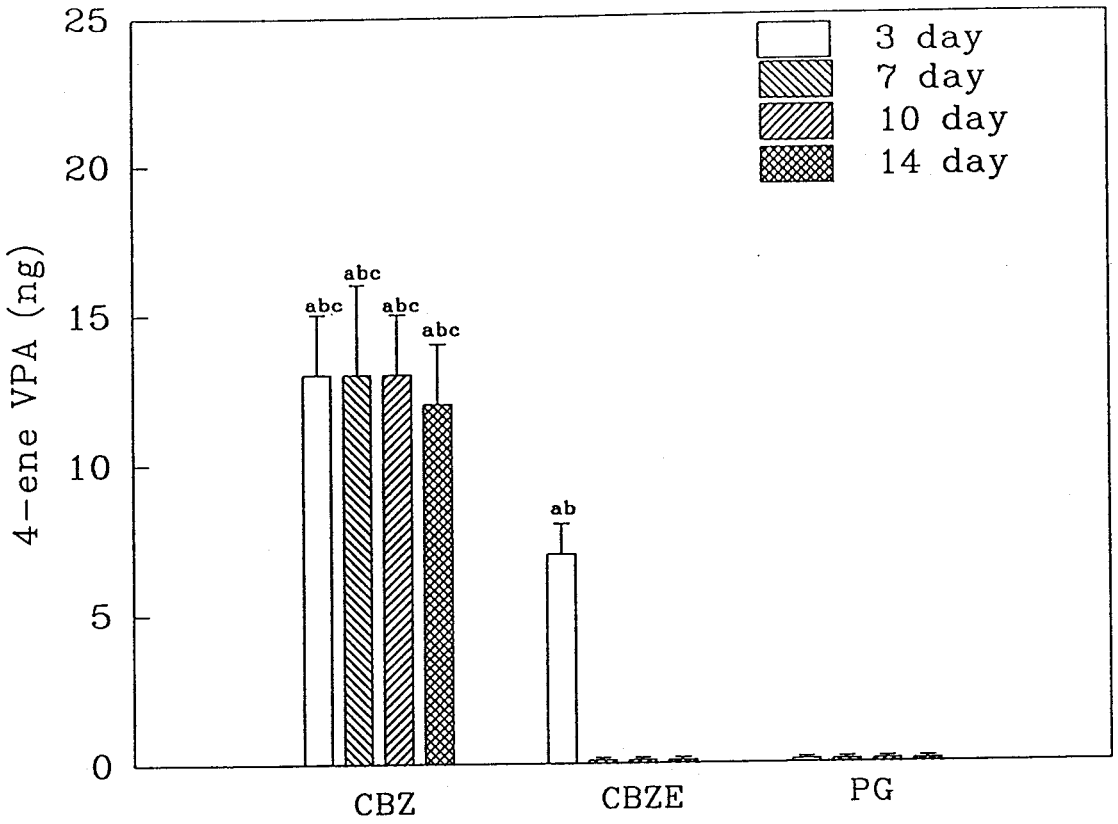

Figure 22. Formation of 4-ene VPA (ng, mean ± s.d.) from the *in vitro* metabolism of VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from CBZ, CBZE and PG 3, 7, 10 and 14 day treated rats (n=4). [a] significantly greater than microsomes from untreated animals (3 ± 1 ng, $p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period, [c] significantly greater than microsomes from CBZE treated animals over the same time period. Microsomal incubations were performed as outlined in the *Experimental* section.

Table 9. A comparison of the metabolism of VPA to 4-ene VPA by microsomes from PB, CFB, CBZ and CBZE treated rats, relative to the untreated group or to the respective vehicle control group (n=4). Microsomal incubations and quantitation of 4-ene VPA were performed as outlined in the *Experimental* section.

Treatment	Formation of 4-ene VPA		
	amount formed mean ± s.d. (ng)	fold increase relative to untreated group	fold increase relative to vehicle group
Untreated	3 ± 1		
PB	17 ± 2	6	5
NS	3 ± 1	--	
CFB	5 ± 1	2	--
CO	4 ± 1	--	
CBZ 3 day	13 ± 2	5	
CBZ 7 day	13 ± 3	4	
CBZ 10 day	13 ± 2	4	
CBZ 14 day	12 ± 2	4	
CBZE 3 day	7 ± 1	2	
CBZE 7 day	***		
CBZE 10 day	***		
CBZE 14 day	***		
PG 3 day	***		
PG 7 day	***		
PG 10 day	***		
PG 14 day	***		

-- increase less than 2 fold

*** trace quantities detected

the PG treated groups (table 9). The production of 4-ene VPA by the CBZ treatment groups was significantly higher when compared to the CBZE and PG treated groups over the time course. Only the formation of 4-ene VPA by the CBZE 3 day treated group was significantly greater when compared to the PG 3 day treated group.

Formation of 4-keto VPA from VPA

The mean quantities of 4-keto VPA produced from VPA by microsomes from the various treatment groups are displayed in figures 23 and 24. The effects of induction by PB, CFB, CBZ and CBZE on the formation of 4-keto VPA relative to the untreated and vehicle control groups are summarized in table 10.

The formation of 4-keto VPA was significantly enhanced by CBZ over the time course. Very large increases relative to the untreated group were observed. However, this increase could not be determined because only trace quantities of 4-keto VPA were formed by microsomes from the untreated group.

When compared to the vehicle controls, significant increases of 3 to 7 fold were observed for the CBZ treatment groups, plus a 4 fold increase for the CBZE 3 day treatment group (table 10). Despite much higher mean quantities of 4-keto VPA for the PB treated group, it was not significantly greater when compared to the NS treated group (figure 23). The formation of 4-keto VPA by the CBZ 3, 7 and 10 day treated groups was significantly greater when compared to the PB treated group and the corresponding CBZE treated group.

In vitro metabolism of (E)-2-ene VPA

Since (E)-2-ene VPA is the major metabolite of VPA in the serum and possesses anticonvulsant activity, it was important to study the effects of various inducing agents on its *in vitro* metabolism. The PB induced *in vitro* metabolism of (E)-2-ene VPA has only recently been reported (Kassahun and

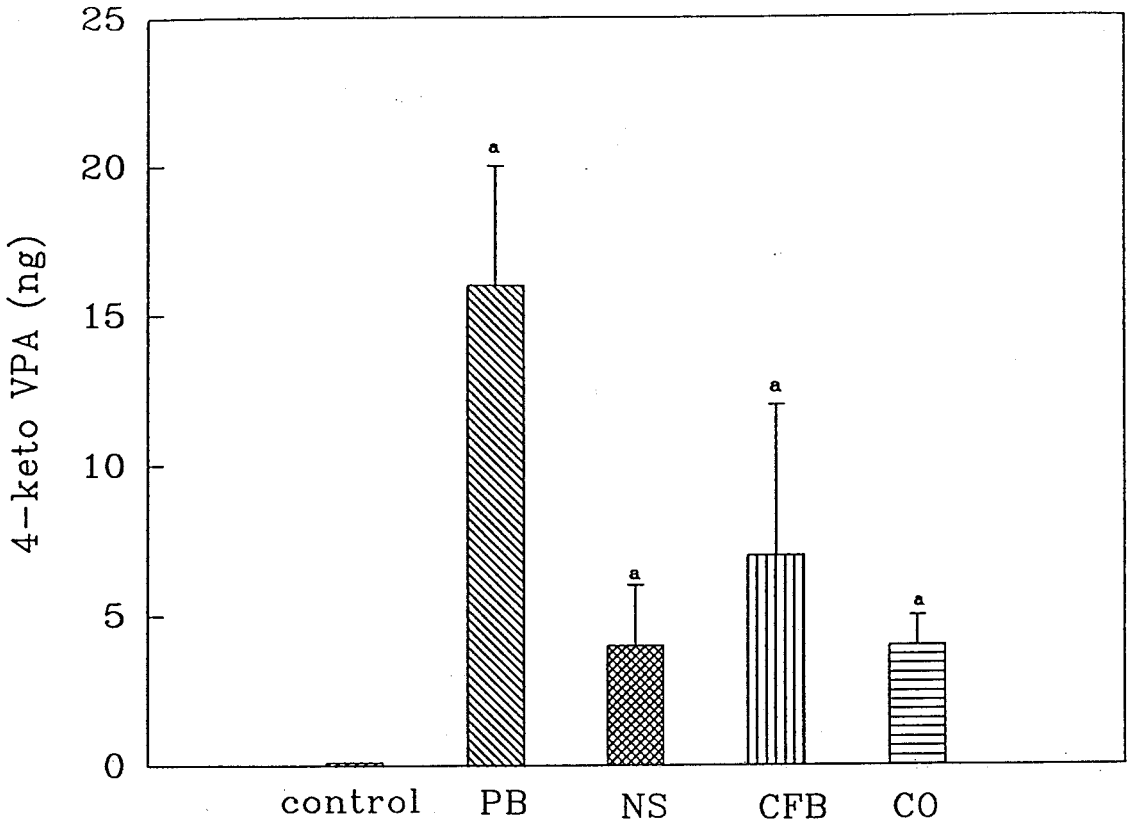

Figure 23. Formation of 4-keto VPA (ng, mean ± s.d.) from the *in vitro* metabolism of VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from untreated, PB, NS, CFB and CO treated rats (n=4). [a] significantly greater than microsomes from untreated animals ($p \leq 0.05$). Microsomal incubations were performed as outlined in the *Experimental* section.

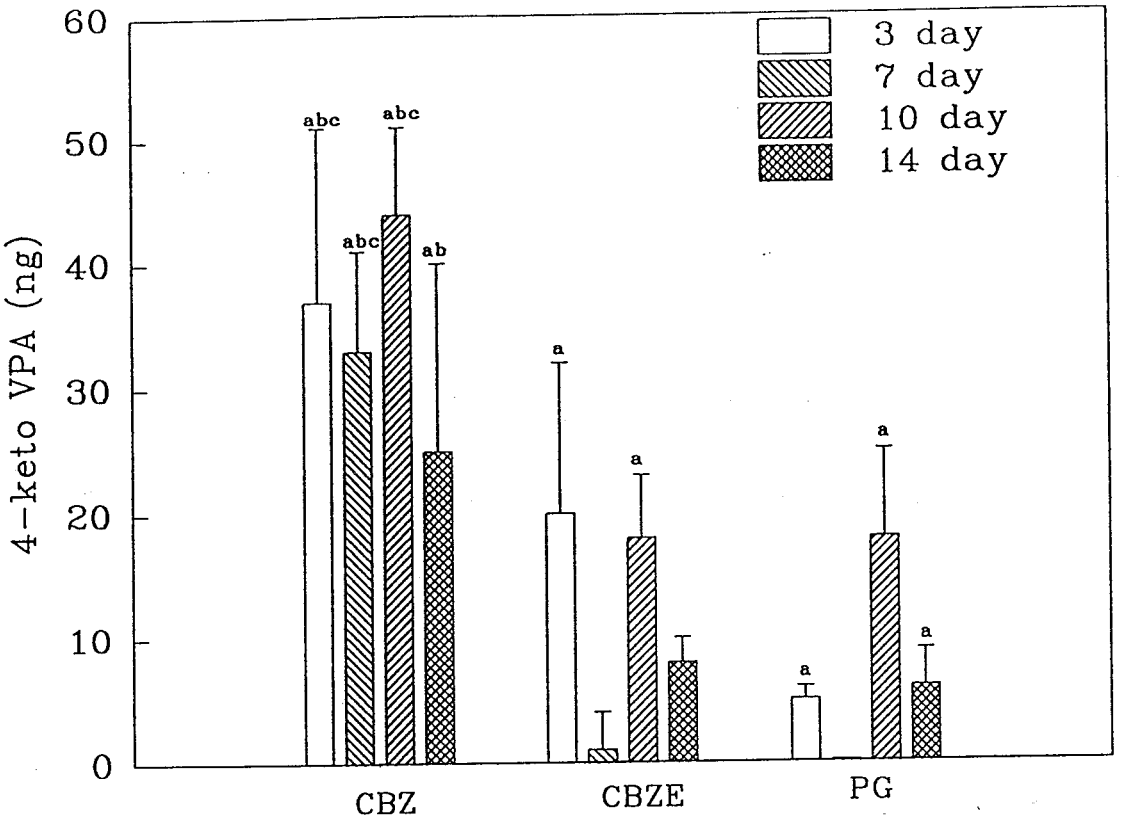

Figure 24. Formation of 4-keto VPA (ng, mean ± s.d.) from the *in vitro* metabolism of VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from CBZ, CBZE and PG 3, 7, 10 and 14 day treated rats (n=4). [a] significantly greater than microsomes from untreated animals (0.1 ± 0.01 ng, $p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period, [c] significantly greater than microsomes from CBZE treated animals over the same time period. Microsomal incubations were performed as outlined in the *Experimental* section.

Table 10. A comparison of the metabolism of VPA to 4-keto VPA by microsomes from PB, CFB, CBZ and CBZE treated rats, relative to the untreated group or to the respective vehicle control group (n=4). Microsomal incubations and quantitation of 4-keto VPA were performed as outlined in the *Experimental* section.

Treatment	Formation of 4-keto VPA		
	amount formed mean ± s.d. (ng)	fold increase relative to untreated group	fold increase relative to vehicle group
Untreated	***		
PB	16 ± 4	*	4
NS	4 ± 2	*	
CFB	7 ± 5	*	2
CO	4 ± 1	*	
CBZ 3 day	37 ± 14	*	7
CBZ 7 day	33 ± 8	*	7
CBZ 10 day	44 ± 7	*	3
CBZ 14 day	25 ± 15	*	4
CBZE 3 day	20 ± 12	*	4
CBZE 7 day	1 ± 3	*	2
CBZE 10 day	18 ± 5	*	--
CBZE 14 day	8 ± 2	*	--
PG 3 day	5 ± 1	*	
PG 7 day	***	*	
PG 10 day	18 ± 7	*	
PG 14 day	6 ± 3	*	

-- increase less than 2 fold

*** trace quantities detected

* increase > 20 fold

Baillie, 1993). If (E)-2-ene VPA is to be utilized as an anticonvulsant agent, it is likely to be used in combination with CBZ, thus necessitating investigation into the effects of induction on its metabolism. (E)-2-ene VPA is metabolized to several diunsaturated metabolites, one ((E,E)-2,3'-diene VPA) of which possesses anticonvulsant activity (Abbott and Acheampong, 1988) while another, ((E)-2,4-diene VPA), is known to produce hepatic steatosis in rats (Granneman *et al.*, 1984c).

Formation of (E,E)-2,3'-diene VPA from (E)-2-ene VPA

The production of (E,E)-2,3'-diene VPA from (E)-2-ene VPA by microsomes from control, PB, NS, CFB and CO treated rats is shown in figure 25 while that by microsomes from CBZ, CBZE and PG treated rats is illustrated in figure 26. The changes in the formation of (E,E)-2,3'-diene VPA relative to the untreated and vehicle groups are summarized in table 11.

The amount of (E,E)-2,3'-diene VPA formed from (E)-2-ene VPA significantly increased 9 fold and 2 fold, respectively for the PB and CFB treated groups when compared to the untreated group (table 11). Significant increases of 2 to 8 fold were observed for the CBZ treated groups when compared to the untreated group (table 11). The formation of (E,E)-2,3'-diene VPA by microsomes from rats treated for 3, 7 and 10 days with CBZE increased significantly 2 to 5 fold when compared to the untreated group (table 11).

The formation of (E,E)-2,3'-diene VPA from (E)-2-ene VPA by microsomes from PB treated rats was significantly greater (7 fold, 18 ± 4 ng *versus* 3 ± 1 ng) than its vehicle control, NS (figure 25, table 11). The metabolism of (E)-2-ene VPA by microsomes from CBZ treated animals over the time course were significantly greater when compared to microsomes from PG treated animals over the same time period with increases ranging from 3 to 9 fold (figure 26). The mean values observed of (E,E)-2,3'-diene VPA from the CBZE treated

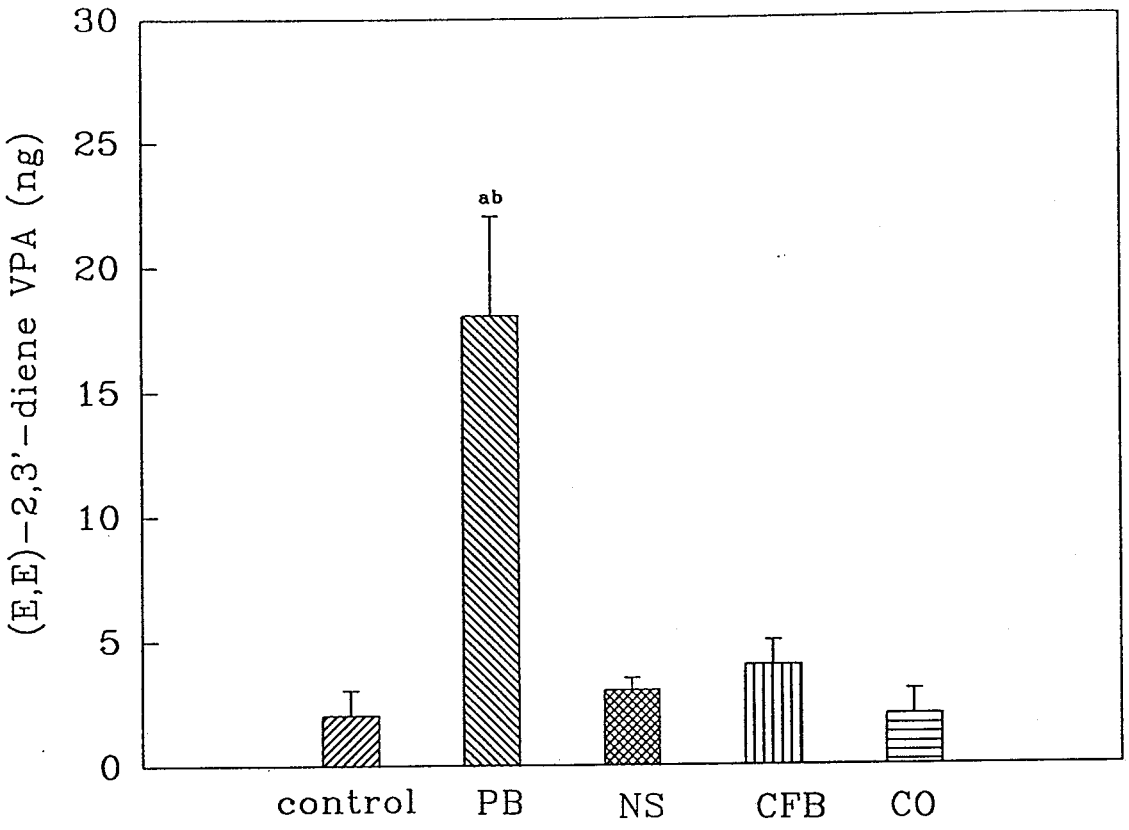

Figure 25. Formation of (E,E)-2,3'-diene VPA (ng, mean ± s.d.) from the *in vitro* metabolism of (E)-2-ene VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from untreated, PB, NS, CFB and CO treated rats (n=4). [a] significantly greater than microsomes from untreated animals ($p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period. Microsomal incubations were performed as outlined in the *Experimental* section.

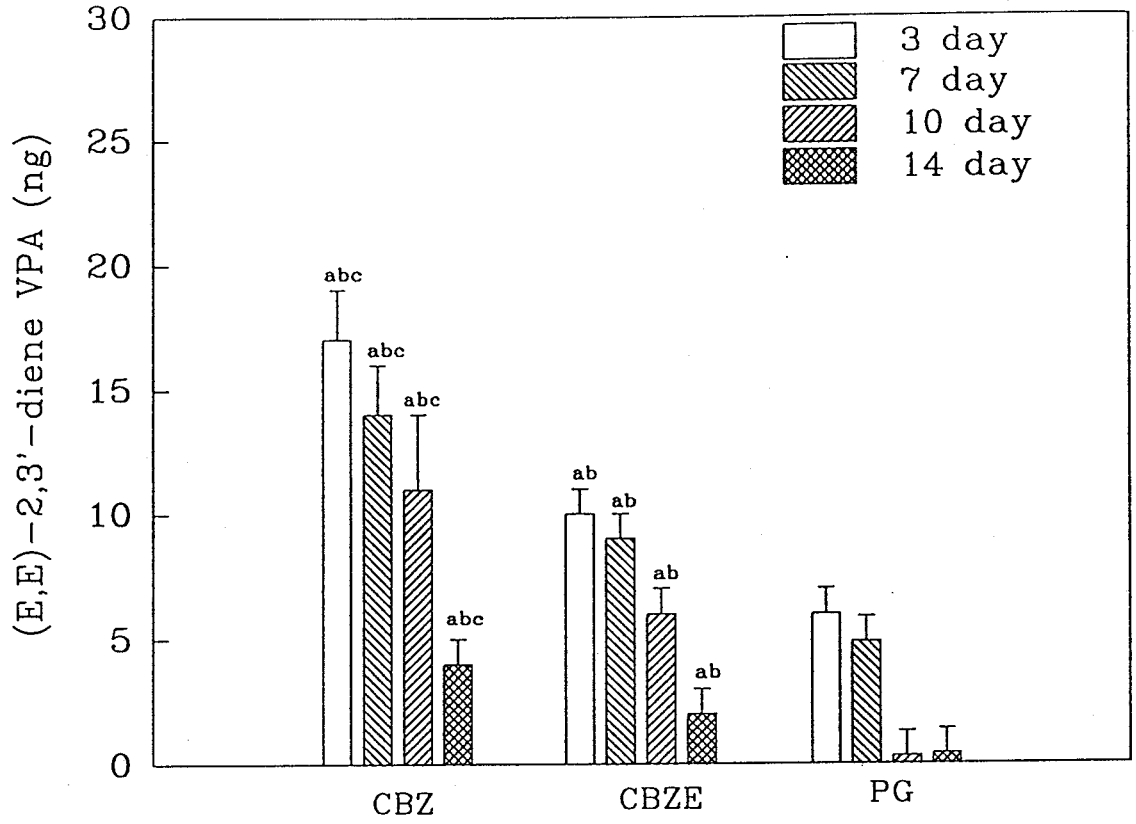

Figure 26. Formation of (E,E)-2,3'-diene VPA (ng, mean ± s.d.) from the *in vitro* metabolism of (E)-2-ene VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from CBZ, CBZE and PG 3, 7, 10 and 14 day treated rats (n=4). [a] significantly greater than microsomes from untreated animals (2 ± 1 ng, $p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period, [c] significantly greater than microsomes from CBZE treated animals over the same time period. Microsomal incubations were performed as outlined in the *Experimental* section.

Table 11. A comparison of the metabolism of (E)-2-ene VPA to (E,E)-2,3'-diene VPA by microsomes from PB, CFB, CBZ and CBZE treated rats, relative to the untreated group or to the respective vehicle control group (n=4). Microsomal incubations and quantitation of (E,E)-2,3'-diene VPA were performed as outlined in the *Experimental* section.

Treatment	Formation of (E,E)-2,3'-diene VPA		
	amount formed mean ± s.d. (ng)	fold increase relative to untreated group	fold increase relative to vehicle group
Untreated	2 ± 1		
PB	18 ± 4	9	7
NS	3 ± 1	--	
CFB	4 ± 1	2	2
CO	2 ± 1	--	
CBZ 3 day	17 ± 2	8	3
CBZ 7 day	14 ± 2	7	4
CBZ 10 day	11 ± 3	5	5
CBZ 14 day	4 ± 1	2	9
CBZE 3 day	10 ± 1	5	2
CBZE 7 day	9 ± 1	5	3
CBZE 10 day	6 ± 1	2	2
CBZE 14 day	2 ± 1	--	3
PG 3 day	6 ± 1	3	
PG 7 day	5 ± 1	3	
PG 10 day	***		
PG 14 day	***		

-- increase less than 2 fold

*** trace quantities detected

groups significantly increased 2 to 3 fold when compared to the PG treated groups (table 11, figure 26).

In addition, (E,E)-2,3'-diene VPA values for the CBZ 3, 7, 10 and 14 day treated groups were significantly higher than the CBZE treated group over the same time period (figure 26). The formation of (E,E)-2,3'-diene VPA from (E)-2-ene VPA by PB (18 ± 4 ng) was significantly greater when compared to all other groups, with the only exception being the CBZ 3 day treated group (17 ± 2 ng).

Formation of (E)-2,4-diene VPA from (E)-2-ene VPA

The amount of (E)-2,4-diene VPA produced from the biotransformation of (E)-2-ene VPA by microsomes from the various treatment groups is depicted in figures 27 and 28. The changes in the formation of (E)-2,4-diene VPA by microsomes from the PB, CFB, CBZ and CBZE treated groups relative to the untreated and vehicle control groups are summarized in table 12.

When compared to microsomes isolated from untreated control rats, the production of (E)-2,4-diene VPA was significantly increased 13 fold for the PB treated group, 6 fold for the CBZ 3 day treated group, 13 fold for the CBZ 7 day treated group, 11 fold for the CBZ 10 day treated group, 5 fold by the CBZ 14 day treated group and 9 fold for the CBZE 7 day treated group (table 12). No significant differences were observed in the formation of (E)-2,4-diene VPA when the other treatment groups were compared to the control group.

The formation of (E)-2,4-diene VPA by microsomes from PB treated animals was significantly higher than the NS treated group (12 fold increase, table 12). The amount of (E)-2,4-diene VPA produced from (E)-2-ene VPA was significantly increased for the CBZ treated groups when compared to the PG treated groups over the same time course with increases of 6 to 12 fold (figure 28, table 12). Of the CBZE treated groups, only the CBZE 7 day treated group was significantly enhanced when compared to the PG 7 day treated group (4 fold

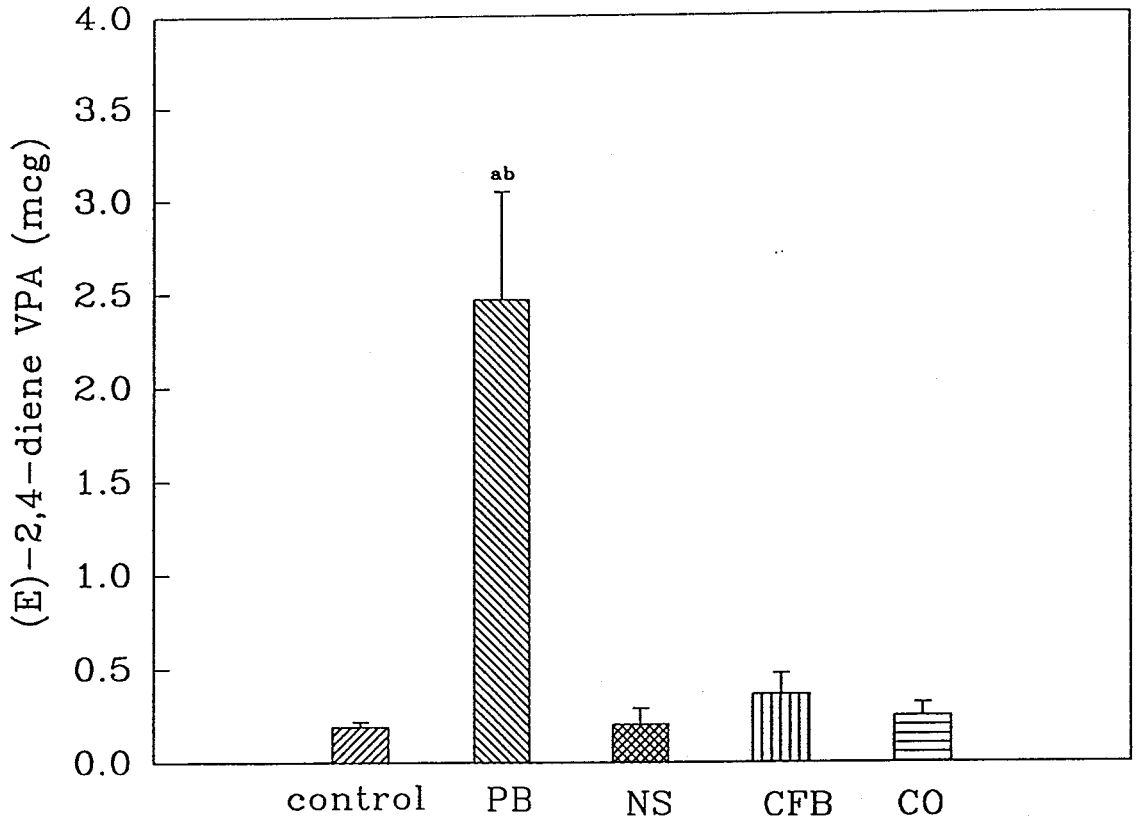

Figure 27. Formation of (E)-2,4-diene VPA (μg, mean ± s.d.) from the *in vitro* metabolism of (E)-2-ene VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from untreated, PB, NS, CFB and CO treated rats (n=4). [a] significantly greater than microsomes from untreated animals ($p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period. Microsomal incubations were performed as outlined in the *Experimental* section.

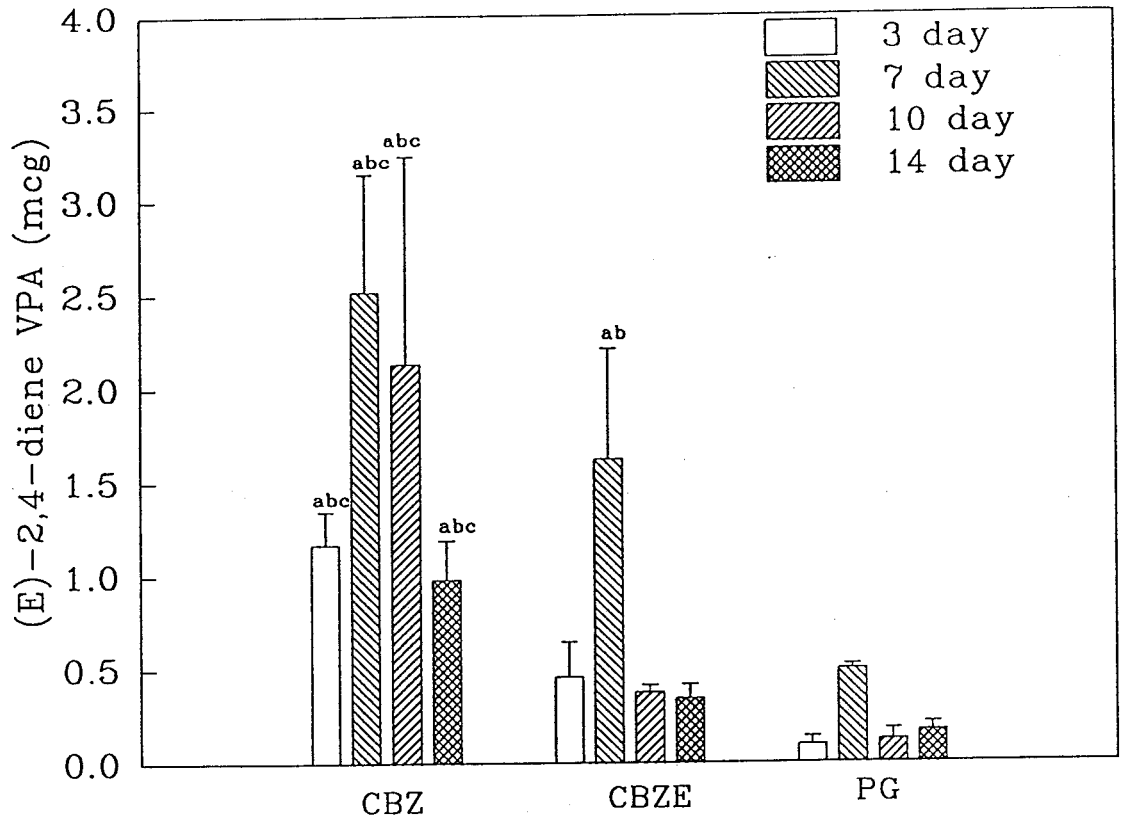

Figure 28. Formation of (E)-2,4-diene VPA (µg, mean ± s.d.) from the *in vitro* metabolism of (E)-2-ene VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from CBZ, CBZE and PG 3, 7, 10 and 14 day treated rats (n=4). [a] significantly greater than microsomes from untreated animals (0.20 ± 0.02 µg, $p \leq 0.05$), [b] significantly greater than microsomes from appropriate vehicle control animals treated over the same time period, [c] significantly greater than microsomes from CBZE treated animals over the same time period. Microsomal incubations were performed as outlined in the *Experimental* section.

Table 12. A comparison of the metabolism of (E)-2-ene VPA to (E)-2,4-diene by microsomes from PB, CFB, CBZ and CBZE treated rats, relative to the untreated group or to the respective vehicle control group (n=4). Microsomal incubations and quantitation of (E)-2,4-diene VPA were performed as outlined in the *Experimental* section.

Treatment	Formation of (E)-2,4-diene VPA		
	amount formed mean ± s.d. (μg)	fold increase relative to untreated group	fold increase relative to vehicle group
Untreated	0.20 ± 0.02		
PB	2.5 ± 0.60	13	12
NS	0.20 ± 0.10	--	
CFB	0.40 ± 0.10	--	--
CO	0.20 ± 0.10	--	
CBZ 3 day	1.2 ± 0.2	6	12
CBZ 7 day	2.5 ± 0.6	13	6
CBZ 10 day	2.1 ± 1.1	11	6
CBZ 14 day	1.0 ± 0.2	5	6
CBZE 3 day	0.5 ± 0.2	2	5
CBZE 7 day	1.6 ± 0.6	9	4
CBZE 10 day	0.4 ± 0.04	2	3
CBZE 14 day	0.3 ± 1.0	2	2
PG 3 day	0.1 ± 0.04	--	
PG 7 day	0.5 ± 0.02	2	
PG 10 day	0.1 ± 0.10	--	
PG 14 day	0.2 ± 0.05	--	

-- increase less than 2 fold

increase). In addition, a statistical difference was not observed in the amount of (E)-2,4-diene VPA formed from (E)-2-ene VPA by microsomes from PB, CBZ 7 day and CBZ 10 day treated rats. The amount of (E)-2,4-diene VPA formed by the CBZ treated groups were significantly higher than the corresponding CBZE treated groups.

Effect of anti-rat cytochrome P-450b antibody on the *in vitro* metabolism of VPA and (E)-2-ene VPA by microsomes from PB and CBZ treated rats

Cytochrome P-450b has been implicated in the formation of 4-ene VPA from VPA (Rettie *et al.*, 1988). Since cytochrome P-450b had been identified as an isozyme capable of being induced by CBZ and PB, the effect of anti-rat cytochrome P-450b antibody on the *in vitro* metabolism of VPA and (E)-2-ene VPA was investigated. Thus, the extent of involvement of cytochrome P-450b in the microsomal metabolism of VPA and (E)-2-ene VPA could be determined. Microsomal protein was incubated with either the anti-rat cytochrome P-450b antibody or control IgG for 10 min prior to initiation of the reaction by addition of the substrate, either VPA or (E)-2-ene VPA (Chang, Ph.D. thesis, 1991).

Effect of anti-rat cytochrome P-450b antibody on the in vitro metabolism of VPA

The effects of anti-rat cytochrome P-450b on the *in vitro* metabolism of VPA to 3-OH VPA, 4-OH VPA, 5-OH VPA, 4-ene VPA and 4-keto VPA by microsomes from rats treated with either CBZ for 3 days or with PB is illustrated in figures 29 to 33.

The metabolism of VPA to 3-OH VPA by microsomes prepared from PB and CBZ 3 day treated rats was inhibited 63% and 52%, respectively at the highest antibody concentration of 2.5 mg of IgG/nmol of cytochrome P-450 (figure 29).

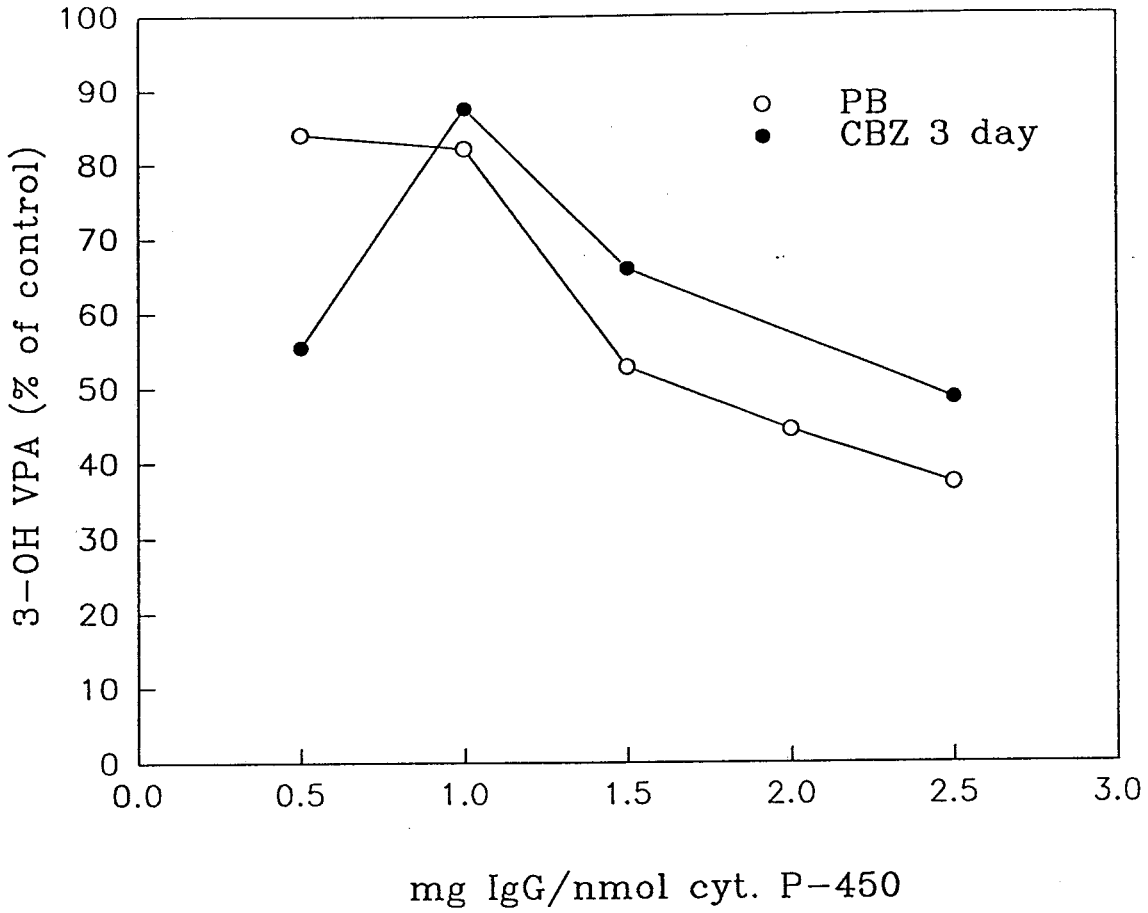

Figure 29. Effect of anti-rat cytochrome P-450b antibody on the *in vitro* metabolism of VPA to 3-OH VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from PB and CBZ 3 day treated rats. Microsomes were prepared from 4 pooled livers. Microsomal incubations were performed as outlined in the *Experimental* section.

The formation of 4-OH VPA from VPA by microsomes prepared from PB and CBZ treated rats microsomes was inhibited 81% and 88%, respectively at the highest IgG concentration investigated (figure 30).

The biotransformation of VPA to 5-OH VPA using microsomes from PB and CBZ 3 day induced rats was inhibited 60% and 45%, respectively at the highest antibody concentration investigated (figure 31).

The metabolism of VPA to 4-ene VPA by microsomes from PB and CBZ 3 day treated rats was completely inhibited in each case at an antibody concentration of 2.5 mg of IgG/nmol of cytochrome P-450 (figure 32).

The production of 4-keto VPA from VPA by microsomes from PB and CBZ 3 day induced rats was blocked 86% and 75%, respectively in the presence of the antibody at the highest concentration examined (figure 33).

Effect of anti-rat cytochrome P-450b antibody on the in vitro metabolism of (E)-2-ene VPA

The effect of anti-rat cytochrome P-450b on the *in vitro* metabolism of (E)-2-ene VPA to (E,E)-2,3'-diene VPA and (E)-2,4-diene VPA by microsomes from rats treated with CBZ for 3 days or PB is shown in figures 34 and 35, respectively. Metabolism to (E,E)-2,3'-diene VPA by microsomes prepared from CBZ treated rats was inhibited approximately 18% at the highest antibody concentration (2.5 mg of IgG/nmol of cytochrome P-450) examined while it was inhibited 48% in microsomes from PB treated rats (figure 34). Metabolism of (E)-2-ene VPA to (E)-2,4-diene VPA was inhibited 89% and 85% by microsomes from PB and CBZ treated rats, respectively at the highest antibody concentration investigated (figure 35).

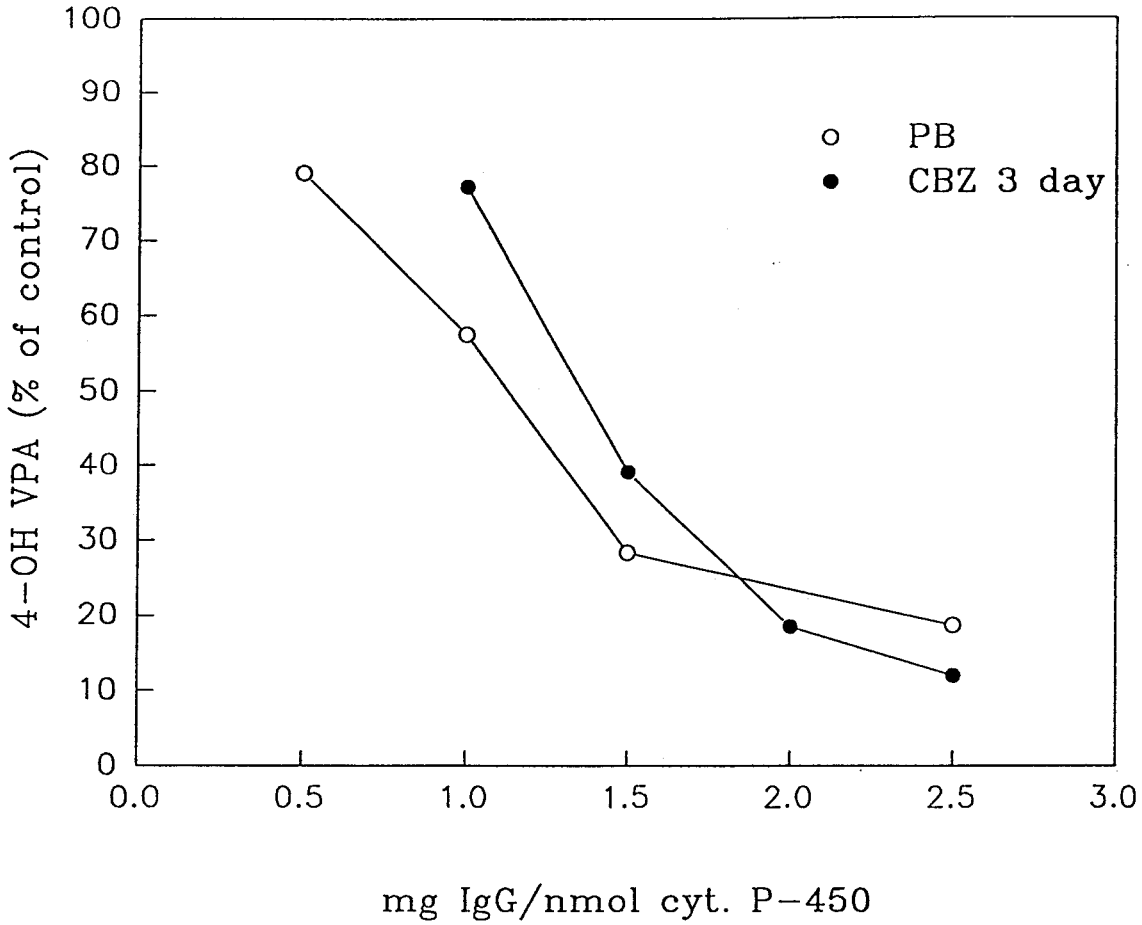

Figure 30. Effect of anti-rat cytochrome P-450b antibody on the *in vitro* metabolism of VPA to 4-OH VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from PB and CBZ 3 day treated rats. Microsomes were prepared from 4 pooled livers. Microsomal incubations were performed as outlined in the *Experimental* section.

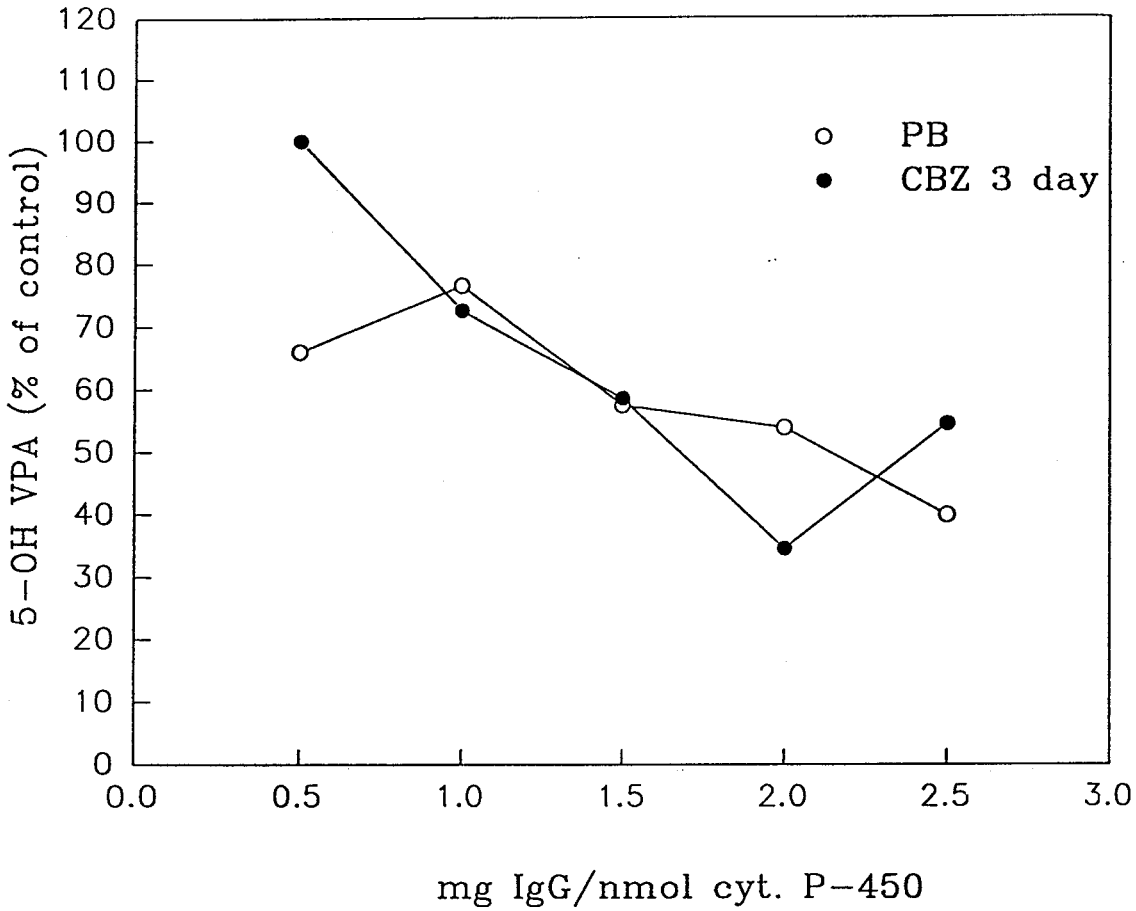

Figure 31. Effect of anti-rat cytochrome P-450b antibody on the *in vitro* metabolism of VPA to 5-OH VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from PB and CBZ 3 day treated rats. Microsomes were prepared from 4 pooled livers. Microsomal incubations were performed as outlined in the *Experimental* section.

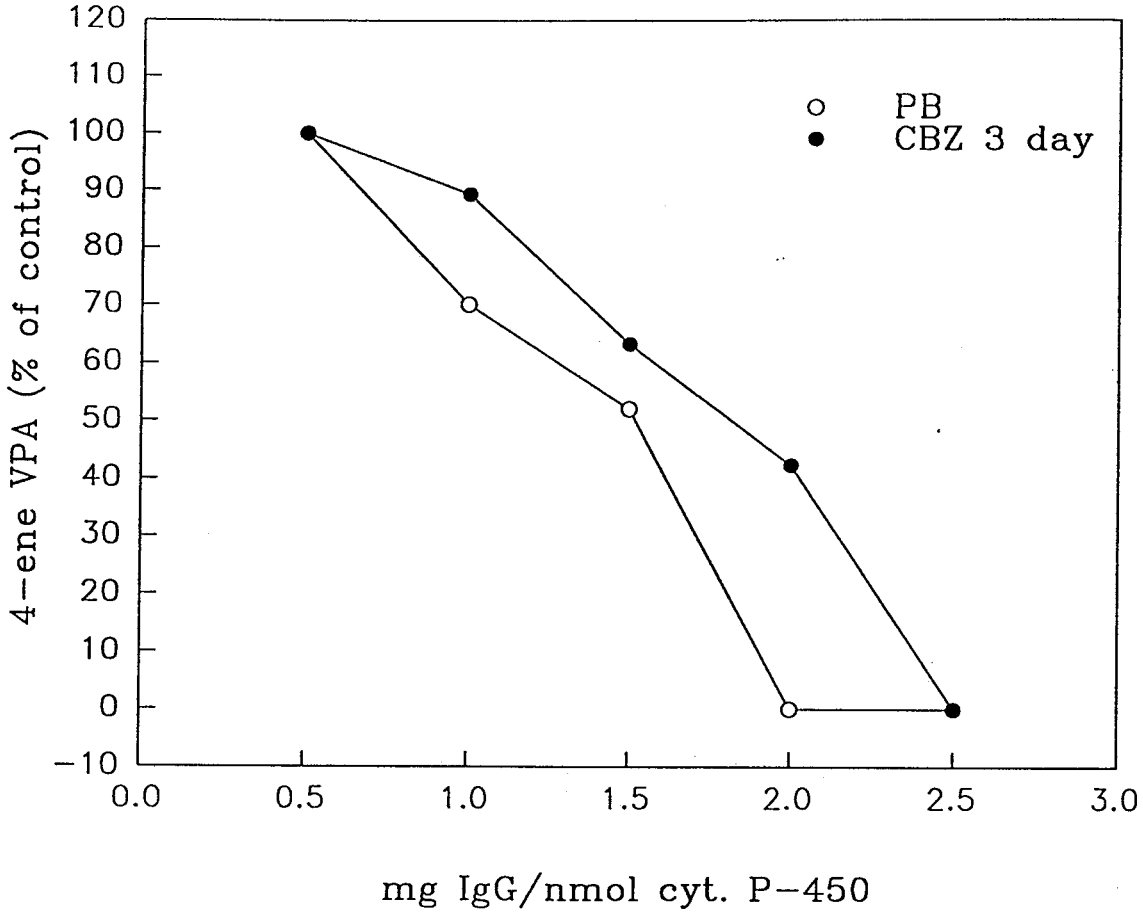

Figure 32. Effect of anti-rat cytochrome P-450b antibody on the *in vitro* metabolism of VPA to 4-ene VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from PB and CBZ 3 day treated rats. Microsomes were prepared from 4 pooled livers. Microsomal incubations were performed as outlined in the *Experimental* section.

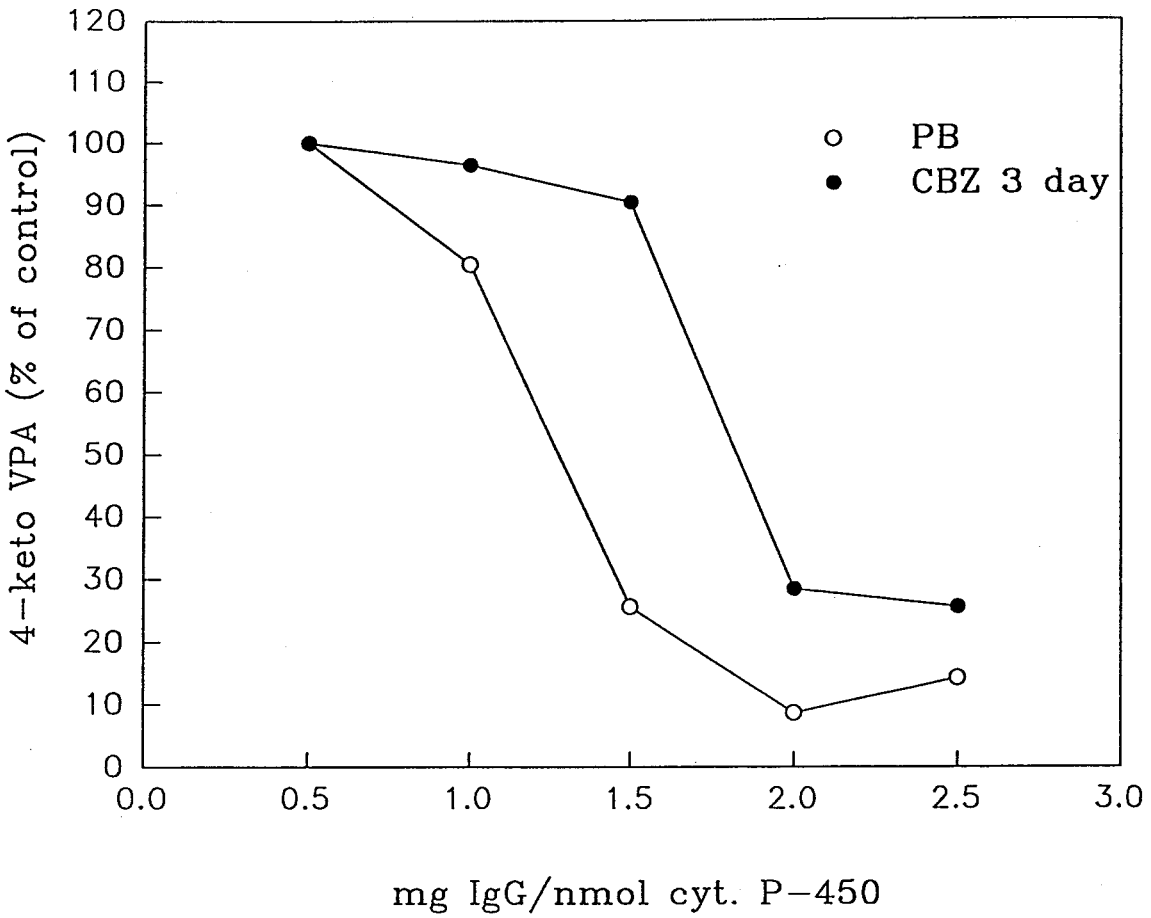

Figure 33. Effect of anti-rat cytochrome P-450b antibody on the *in vitro* metabolism of VPA to 4-keto VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from PB and CBZ 3 day treated rats. Microsomes were prepared from 4 pooled livers. Microsomal incubations were performed as outlined in the *Experimental* section.

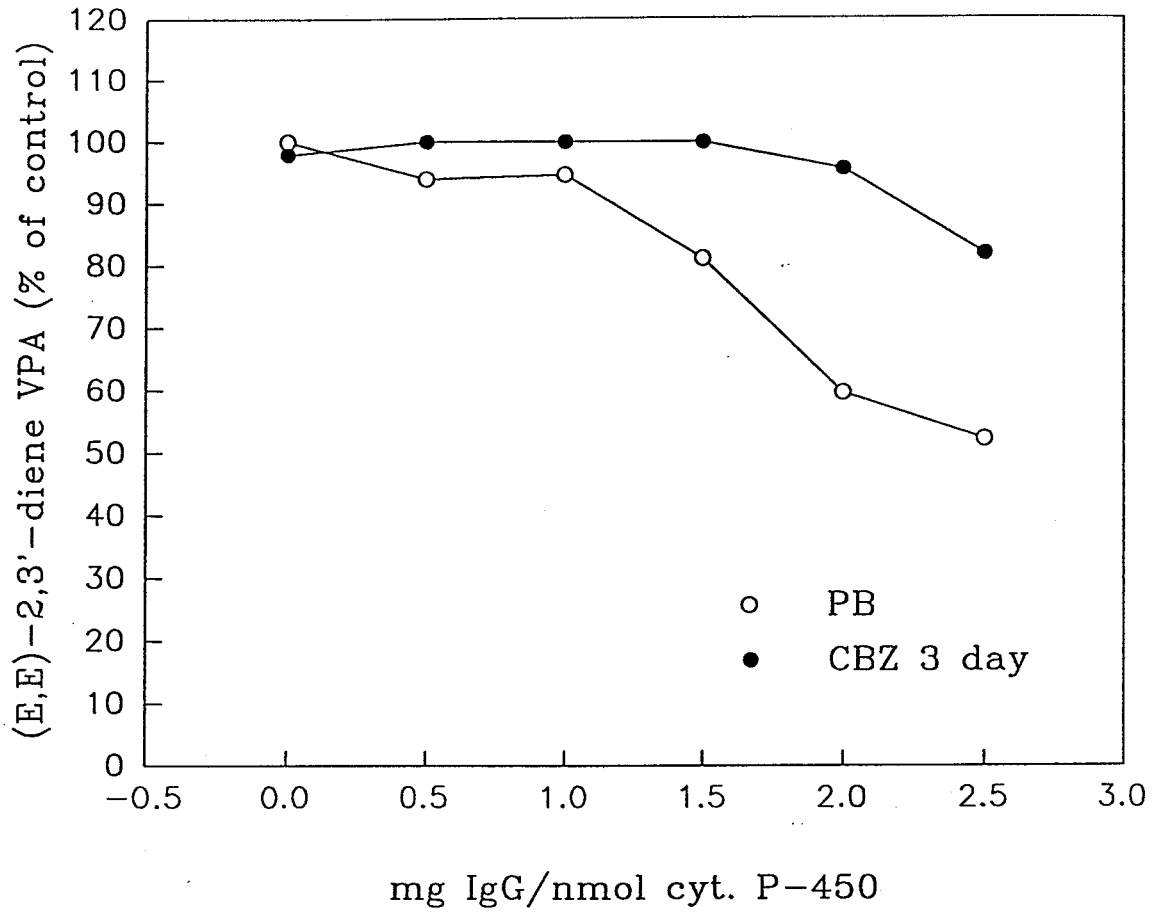

Figure 34. Effect of anti-rat cytochrome P-450b antibody on the *in vitro* metabolism of (E)-2-ene VPA to (E,E)-2,3'-diene VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from PB and CBZ 3 day treated rats. Microsomes were prepared from 4 pooled livers. Microsomal incubations were performed as outlined in the *Experimental* section.

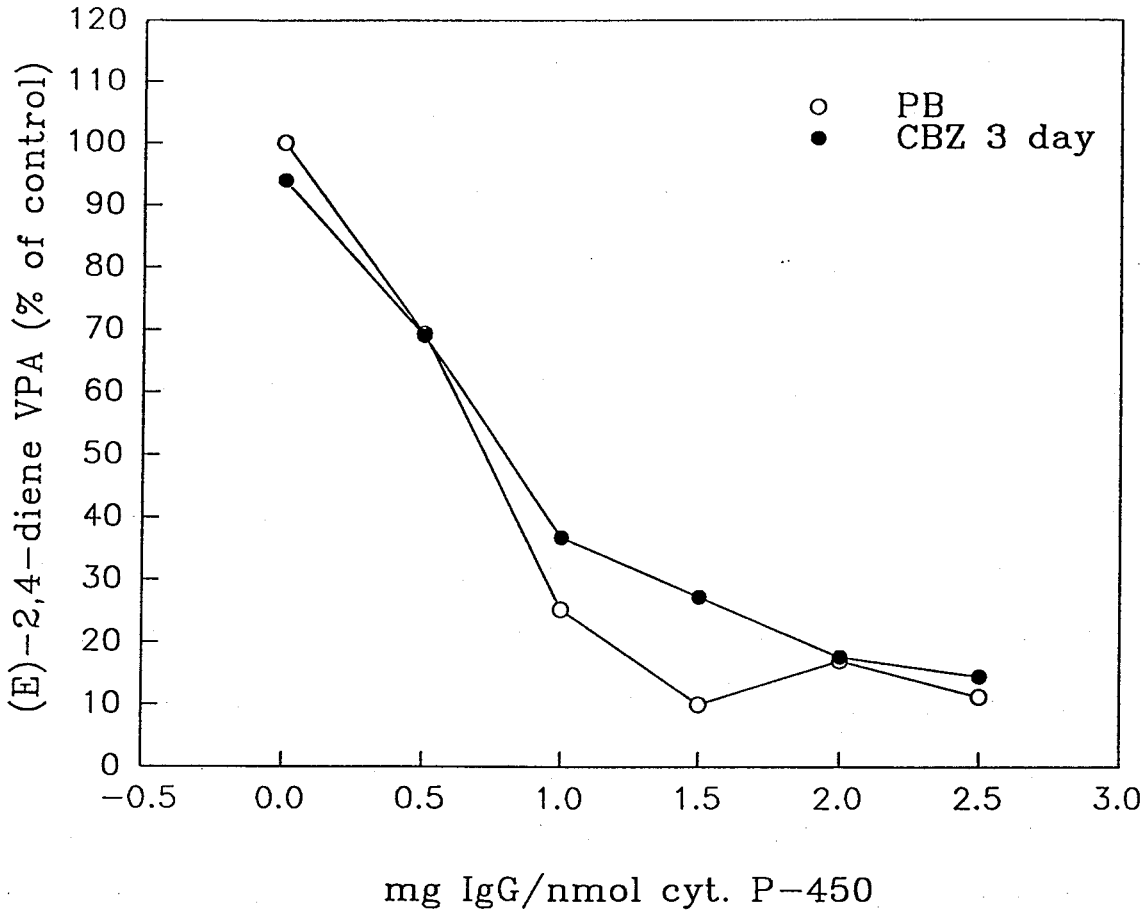

Figure 35. Effect of anti-rat cytochrome P-450b antibody on the *in vitro* metabolism of (E)-2-ene VPA to (E)-2,4-diene VPA by microsomes (2 nmol of spectrally determined cytochrome P-450) from PB and CBZ 3 day treated rats. Microsomes were prepared from 4 pooled livers. Microsomal incubations were performed as outlined in the *Experimental* section.

Effect of anti-rat cytochrome P-450h on the *in vitro* metabolism of VPA and (E)-2-ene VPA by untreated microsomes

Cytochrome P-450h, a male specific isozyme, catalyzes a number of metabolic reactions including progesterone and testosterone 2α- and 16α-hydroxylation (Ryan and Levin, 1990). The effect of an antibody specific for cytochrome P-450h was investigated to determine if this isozyme was also involved in the metabolism of VPA or (E)-2-ene VPA, particularily to the metabolites whose formation was not completely inhibited in the presence of the anti-rat cytochrome P-450b antibody.

Anti-rat cytochrome P-450h antibody did not exhibit any significant effects on the metabolism of VPA by control microsomes (data not shown). In order to examine the possibility of an additive effect on VPA metabolism, the anti-rat cytochrome P-450h antibody (2 mg of IgG/nmol of cytochrome P-450) was added in the presence of the anti-rat cytochrome P-450b antibody (2 mg of IgG/nmol of cytochrome P-450) to microsomes prepared from CBZ 3 day or PB treated rats. No differences were observed in the inhibition profiles of 3-OH VPA, 4-OH VPA and 5-OH VPA (data not shown). The results obtained were similar to those obtained with the anti-rat cytochrome P-450b antibody alone.

In an effort to test that the isozyme(s) of cytochrome P-450 responsible for (E)-2-ene VPA metabolism were the same as those for VPA, the effects of the antibody against rat cytochrome P-450h on the *in vitro* metabolism of (E)-2-ene VPA to (E,E)-2,3'-diene VPA and (E)-2,4-diene VPA by microsomes prepared from untreated animals was investigated. As in the case of VPA, the antibody did not exhibit any significant inhibitory effects on the biotransformation of (E)-2-ene VPA to its 2 diunsaturated metabolites.

When the additive effects of anti-rat cytochrome P-450h antibody (2 mg/nmol cytochrome P-450) and anti-rat cytochrome P-450b antibody (2

mg/nmol cytochrome P-450) were examined in either CBZ 3 day or PB microsomes, no significant inhibitory actions were observed.

QUANTITATION OF CBZ, CBZE AND CBZD

CBZ is known to induce its own metabolism *via* the epoxide-diol pathway (Eichelbaum *et al.*, 1985). The metabolites of CBZ and CBZE were quantitated in 12 h rat urine collections over the 14 day time course to confirm if this pathway was induced with chronic administration.

Analysis of CBZ and metabolites in rat urine by HPLC

The methods of Elyas *et al.* (1982) and Kumps *et al.* (1985) were combined to provide the methodology for CBZ and metabolite analysis by HPLC. Ethyl acetate was found to be a more efficient extraction solvent than dichloromethane for CBZD. The isocratic method of Elyas *et al.* (1982) was found to be incapable of adequately separating CBZD from the endogenous compounds present in rat urine so a gradient method was developed. Representative chromatograms of CBZ and metabolite standards, with comparison to CBZ and metabolites extracted from a rat urine sample and a spiked urine sample are shown in figure 36. The assay was linear over the concentration range examined.

Urinary recoveries of CBZ, CBZE and CBZD after dosing with CBZ

The 12 h urinary recoveries of CBZ and metabolites after dosing rats with CBZ at 100 mg/kg are shown in table 13. Very small quantites of CBZ were recovered in all 12 h collections, ranging from mean quantities of 17.3 μg for the CBZ 3 day group to 44.4 μg for the CBZ 7 day group. The recoveries of CBZE (mean 267 to 339 μg) and CBZD (337 to 602 μg) were an order of magnitude higher than the CBZ levels.

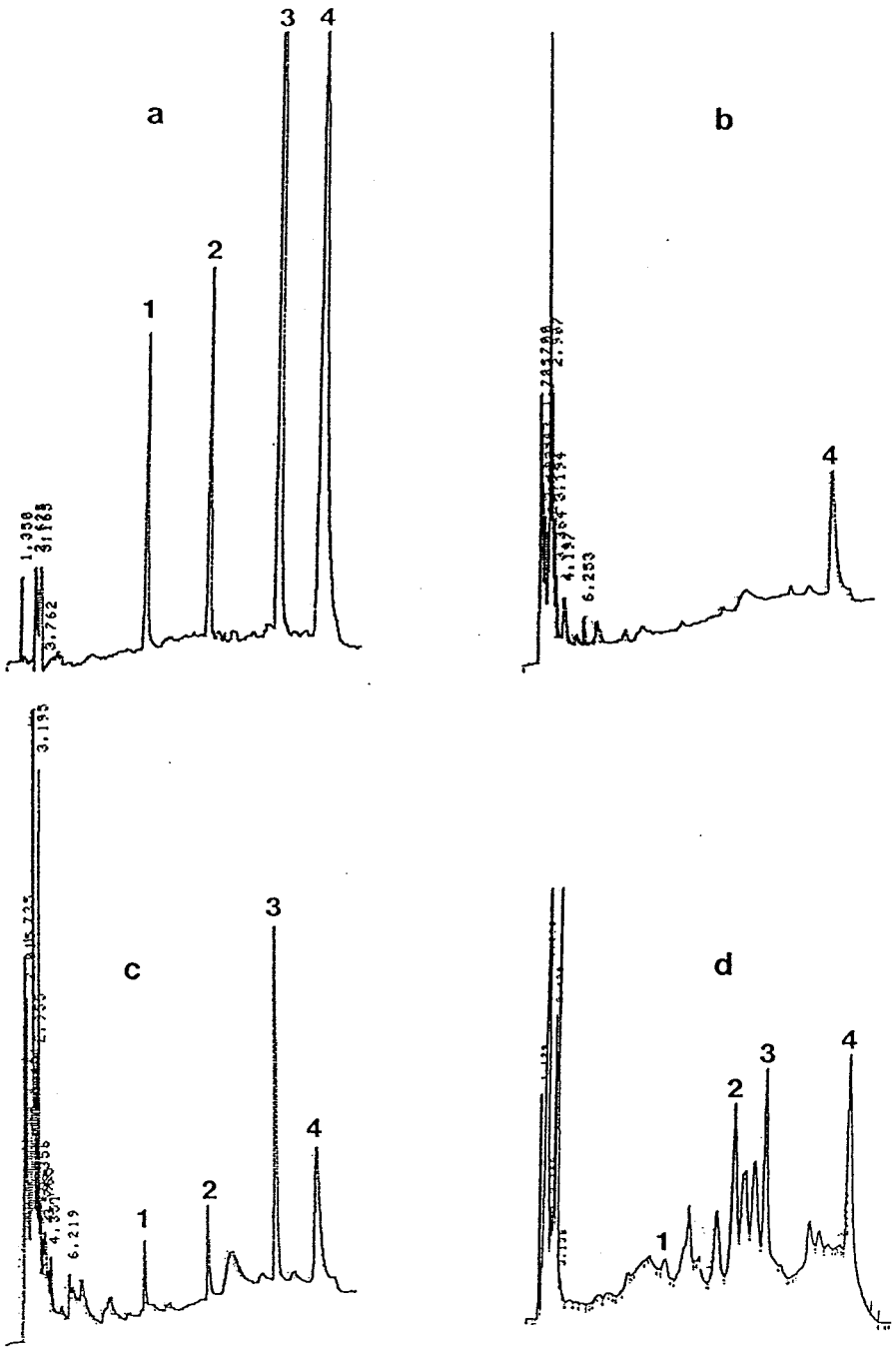

Figure 36. HPLC chromatograms of a) standards of CBZ, CBZE, CBZD and MCBZ, b) extracted blank rat urine sample, c) extracted spiked rat urine sample and d) extracted rat urine sample. Peak 1, CBZD, peak 2, CBZE, peak 3, CBZ and peak 4, MCBZ. Chromatography conditions were as described in the *Experimental* section.

Table 13. Total 12 h urinary recoveries of CBZ, CBZE and CBZD (μg) from rats treated with CBZ 100 mg/kg every 12 h. (n=4, mean ± s.d.). CBZ, CBZE and CBZD were quantitated as described in the *Experimental* section.

Treatment	CBZ (μg)	CBZE (μg)	CBZD (μg)
CBZ 3 day	17.3 ± 15.5	317 ± 256	602 ± 461
CBZ 7 day	44.4 ± 25.3	270 ± 138	337 ± 152
CBZ 10 day	35.9 ± 19.7	267 ± 94	551 ± 279
CBZ 14 day	38.4 ± 8.3	339 ± 195	365 ± 110

The recoveries of CBZ, CBZE and CBZD expressed as a percent of the dose of CBZ administered are shown in table 14. Only 0.1 to 0.2% of the dose administered was recovered as CBZ. The recoveries of CBZE and CBZD represented 1.1 to 1.6% and 1.5 to 3.0% of the dose administered, respectively. Approximately 2.9 to 4.6% of the dose was recovered in the urine in the 12 h following administration of the last dose.

No significant differences were observed in the urinary recoveries over the 14 day treatment period investigated.

Urinary recoveries of CBZE and CBZD after dosing with CBZE

The 12 h urinary recoveries of CBZE and CBZD after administration of CBZE at a dose of 50 mg/kg are summarized in table 15. The mean 12 h recovery of CBZE ranged from 121 to 504 μg while the mean recovery of CBZD ranged from 177 to 523 μg over the same time period.

The recoveries of CBZE and CBZD expressed as a percent of the dose of CBZE administered are shown in table 16. The mean recovery of CBZE as a percent of the dose administered ranged from 0.4 to 2.0% while the recovery of CBZD ranged from 0.6 to 2.0% over the same time period. The total dose recovered as CBZE and CBZD ranged from 1.5 to 4.0% of the dose administered.

As with the CBZ treatment groups, no significant differences were observed over the 2 week period of CBZE administration.

Table 14. Urinary recoveries (12 h) of CBZ, CBZE and CBZD as percent of dose administered from rats treated with CBZ 100 mg/kg every 12 h. (n=4, mean ± s.d.). CBZ, CBZE and CBZD were quantitated as described in the *Experimental* section.

Treatment	CBZ (% of dose)	CBZE (% of dose)	CBZD (% of dose)	total (% of dose)
CBZ 3 day	0.1 ± 0.1	1.6 ± 1.3	3.0 ± 2.4	4.6 ± 3.6
CBZ 7 day	0.2 ± 0.1	1.2 ± 0.6	1.5 ± 0.7	2.9 ± 1.1
CBZ 10 day	0.2 ± 0.1	1.1 ± 0.4	2.3 ± 1.1	3.5 ± 1.5
CBZ 14 day	0.2 ± 0.1	1.4 ± 0.8	1.5 ± 0.5	3.0 ± 1.3

Table 15. Total 12 h urinary recoveries of CBZE and CBZD (μg) from rats treated with CBZE 50 mg/kg every 12 h. (n=4, mean ± s.d.). CBZE and CBZD were quantitated as described in the *Experimental* section.

Treatment	CBZE (μg)	CBZD (μg)
CBZE 3 day	247 ± 83	447 ± 494
CBZE 7 day	504 ± 174	523 ± 139
CBZE 10 day	121 ± 83	319 ± 256
CBZE 14 day	249 ± 134	177 ± 83

Table 16. Urinary recoveries (12 h) of CBZE and CBZD as percent of dose from rats treated with CBZE 50 mg/kg every 12 h. (n=4, mean ± s.d.). CBZE and CBZD were quantitated as described in the *Experimental* section.

Treatment	CBZE (% of dose)	CBZD (% of dose)	total (% of dose)
CBZE 3 day	1.1 ± 0.4	2.0 ± 2.2	3.1 ± 2.6
CBZE 7 day	2.0 ± 0.6	2.0 ± 0.5	4.0 ± 1.1
CBZE 10 day	0.4 ± 0.3	1.1 ± 0.9	1.6 ± 1.1
CBZE 14 day	0.9 ± 0.5	0.6 ± 0.3	1.5 ± 0.5

DISCUSSION

CHOICE OF EXPERIMENTAL CONDITIONS

Animal model

Previous work suggested the rat should provide a reasonably good model to study the interaction between VPA and CBZ since the metabolism of both CBZ (Faigle and Feldmann, 1982) and VPA (Granneman *et al.*, 1984a) in the rat are quite similar to that of man. CBZ undergoes biotransformation in rat and human by the same major metabolic pathways: epoxidation of the 10,11 double bond, hydroxylation of the 6-membered aromatic rings, *N*-glucuronidation of the carbamoyl side chain and substitution on the 6 membered rings by sulphur containing groups (Faigle and Feldmann, 1982; Faigle and Feldmann, 1989). In both rat and man, the 2 major metabolic pathways of VPA metabolism are glucuronidation and β-oxidation (Granneman *et al.*, 1984a).

The male Long Evans rats used in this study weighed from 195 to 230 g and were approximately 7 to 9 weeks of age and, thus, are considered to be adults. In male, untreated Wistar rats, total hepatic cytochrome P-450 content remained unchanged from 1 week to 24 months of age and low levels of cytochromes P-450b and P-450e were detectable (Imaoka *et al.*, 1991).

Choice of vehicle for CBZ and CBZE

CBZ possesses very low solubility in water (72 mg/L in phosphate buffer) but is quite soluble in organic solvents including chloroform, dichloromethane (Kutt, 1989), alcohol, acetone and propylene glycol (The Merck Index, 1989). CBZE also has very limited solubility in aqueous solutions but like CBZ, is readily soluble in organic solvents (Kerr and Levy, 1989). Some organic solvents such as acetone and ethanol are known to induce cytochrome P-450 in rats

(Soucek and Gut, 1992). Thus, propylene glycol was chosen as the suspending vehicle for CBZ and CBZE because in humans it did not produce changes in the antipyrine test at an oral dose of 55 mL (Nelson *et al.*, 1987). Although there has been one report in the literature of PG causing appetite suppression in rats (Carl and Smith, 1989), the doses used in the current study (0.1 mL/kg twice daily) were much lower than in the 1989 study (8 mL/kg 3 times daily *via* gastric intubation).

GCMS analysis of VPA and metabolites

A modified version of the gas chromatograph-mass spectrometric assay previously developed in our laboratory was employed for the analysis of VPA and its metabolites in the microsomal incubates (Abbott *et al.*, 1986). The samples were analyzed on a gas chromatograph equipped with a mass selective detector. With this assay, it was possible to simultaneously quantitate VPA and 16 metabolites using the selected ion monitoring mode. Briefly, the method employed a capillary column and the extracted samples were derivatized with MTBSTFA to yield the *t*BDMS derivatives. The $[M-57]^+$ ion which was formed from the loss of the *t*-butyl group was monitored. The modified assay utilized 2-MGA as the internal standard for the 2 diacid metabolites of VPA, 2-PSA and 2-PGA. In addition, heptadeuterated analogues of VPA and some of the VPA metabolites, $[^2H_7]$4-ene VPA, $[^2H_7]$2-ene VPA (E and Z), $[^2H_7]$3-keto VPA, $[^2H_7]$4-keto VPA, $[^2H_7]$3-OH VPA and $[^2H_7]$5-OH VPA, were used as internal standards. Correlation coefficients of the calibration curves were 0.990 or better. The assay has been described at a recent conference (Yu *et al.*, 1992) and a manuscript is currently in preparation (Yu *et al.*).

Choice of metabolites monitored from the *in vitro* microsomal metabolism of VPA and (E)-2-ene VPA

Initially upon injection of the derivatized extracts of the microsomal incubates, all possible metabolites of VPA and (E)-2-ene VPA were monitored. For VPA, the additional metabolites monitored included 3-keto VPA, 2-ene VPA, 3-ene VPA, 2-PSA and 2-PGA. Signals from these metabolites could not be distinguished from background and subsequently only 3-OH VPA, 4-OH VPA, 5-OH VPA, 4-keto VPA and 4-ene VPA were monitored and quantitated. In extracts of incubates where (E)-2-ene VPA was employed as the substrate, neither 3-ene VPA nor VPA peaks were detectable above the background noise although these metabolites have been reported as products of 2-ene VPA metabolism in rats (Granneman *et al.*, 1984a; Lee, 1991; Vorhees *et al.*, 1991; Loscher *et al.*, 1992). The 3-OH VPA and 3-keto VPA metabolites of (E)-2-ene VPA, that arise from mitochondrial β-oxidation were not detected from the *in vitro* microsomal metabolism of (E)-2-ene VPA. Consequently, only the (E)-2,4-diene VPA and (E,E)-2,3'-diene VPA metabolites were monitored as the products of rat hepatic microsomal metabolism of (E)-2-ene VPA.

HPLC analysis of CBZ and metabolites

The HPLC assay used for the analysis of CBZ, CBZE and CBZD from rat urine samples was a combination of 2 procedures from the literature (Elyas *et al.*, 1983; Kumps *et al.*, 1985). Kumps and co-workers (1985) were able to separate a number of anticonvulsants and their metabolites (16 compounds in total) in serum. The extraction of CBZD was greatly enhanced by changing the extraction solvent from dichloromethane to ethyl acetate. Many attempts to separate the CBZD peak from endogenous compounds extracted from the urine, including varying the composition of the mobile phase, changing columns and employing various gradient systems did not yield the extent of separation

claimed in the literature (Kumps *et al.*, 1985). Urine may contain more endogenous compounds compared to serum, leading to a more complicated chromatographic separation.

A COMPARISON OF THE INDUCTION OF RAT HEPATIC MICROSOMAL CYTOCHROME P-450 CONTENT BY PB, CBZ, CBZE AND OTHER INDUCING AGENTS

Cytochrome P-450 content in hepatic microsomes from untreated rats

Cytochromes P-450b and P-450e were not detected on the immunoblots of hepatic microsomal protein from untreated rats. This was not surprising since these isozymes are present in very low quantities in untreated rat liver (Waxmann and Azaroff, 1992). In untreated rats, cytochrome P-450b content is reported to be highest in the lung in comparison to liver, kidney, adrenal or small intestine while cytochrome P-450e content is highest in the liver (Christou *et al.*, 1987). With PB induction, the highest levels of both cytochromes P-450b and P-450e are found in the liver. Sex differences in rats have been observed in the induction of these 2 isozymes (Yamazoe *et al.*, 1987).

Cytochromes P-450b and P-450e are minor constituents of the cytochrome P-450 pool, representing 5% or less of the total cytochrome P-450 content of hepatic microsomes from untreated male or female Long Evans rats (Thomas *et al.*, 1981). In uninduced Long Evans, male immature and adult rats, cytochrome P-450b content was 4% and 2%, respectively, of the total hepatic cytochrome P-450 content (Thomas *et al.*, 1981). The expression of cytochrome P-450b in uninduced male rats from 5 different strains (Sprague-Dawley, Long Evans, Wistar, Brown Norway and Fischer F344) varied considerably (< 2 to 9 pmol/mg protein) (Wilson *et al.*, 1987). However, the expression of cytochrome P-450e exhibited very little interstrain variability (17 ± 5 pmol/ mg protein) with

the exception of the Brown Norway strain (8.5 pmol/mg protein).

Effect of VPA on cytochrome P-450 content

VPA is generally not considered to be an enzyme inducing agent although there are conflicting reports in the literature. For instance, VPA did not affect total rat hepatic microsomal cytochrome P-450 content when administered at doses of 80 mg/kg or 120 mg/kg *i.p.* twice daily for 3 days (Sapeika and Kaplan, 1975). In another study, when VPA was administered to rats at doses of 100 mg/kg or 200 mg/kg *i.p.* daily for 30 days, a dose dependent increase in mitochondrial carnitine acetyltransferase activity occurred but no changes were observed in the total hepatic cytochrome P-450 content (Singh *et al.*, 1987). Cotariu *et al.* (1985) observed a decrease in cytochrome P-450 content when VPA was administered to male rats whereas Rogiers *et al.* (1988) observed a significant increase in total cytochrome P-450 content when VPA was administered to rats at a dose of 100 mg/kg *i.p.* daily for 10 days. In the current study, VPA administration did not result in increased total hepatic cytochrome P-450 content. Furthermore, anti-rat cytochrome P-450b antibody did not react with microsomal protein isolated from the livers of rats treated with VPA. The effects of VPA on cytochrome P-450, thus, remain unclear. Perhaps a longer treatment with VPA is necessary to elicit changes in cytochrome P-450. We treated rats for only 3 days similar to the study of Sapeika and Kaplan (1975) who observed no changes while Rogiers *et al.* (1988) noted increased cytochrome P-450 content with administration of VPA over 10 days at a lower dose.

Effect of CFB on hepatic cytochrome P-450 content

Corn oil is commonly used as a vehicle for CFB and other compounds not readily soluble in aqueous solutions and when administered as such it has not been reported to affect hepatic cytochrome P-450 (Thomas *et al.*, 1981). In this

study, corn oil itself, appeared to cause an increase in the total hepatic cytochrome P-450 content. Increased levels of cytochromes P-450d, P-450e, P-450j and P-450p have been observed when CO (20%) was administered as a dietary source (Yoo *et al.*, 1992).

CFB treatment appeared to induce total hepatic cytochrome P-450 content when compared to the untreated group. However, when compared to the CO treated group, the increase was not statistically significant. A higher dose of CFB was initially used, but due to the loss of 2 animals with an "apparent" drug toxicity, the dose of CFB was decreased to 350 mg/kg *i.p.* daily from 500 mg/kg *i.p.* daily. Based on reports in the literature, a wide range of CFB doses have been utilized for induction experiments in rats. Bachmann and co-workers (1988) used doses of 200 mg/kg of CFB *i.p.* daily for 3 days and Heinemeyer and co-workers (1985) used doses of 500 mg/kg *i.p.* daily for 7 days. A dose of 250 mg/kg daily for 3 days *via* gastric intubation resulted in significantly increased (33.6%) cytochrome P-450 levels when the CFB treated group was compared to a peanut oil control group (Sharma *et al.*, 1988a). In the current study, only a 22% increase was observed when the CFB treated group was compared to the CO control group. The assumption then, is that either the dose of CFB that we employed and/or the time interval of CFB treatment was inadequate to achieve maximal induction of total hepatic cytochrome P-450 content.

Cytochrome P-452 (lauric acid hydroxylase, cytochrome P-450 4A1), is the isozyme of cytochrome P-450 that is induced by CFB (Soucek and Gut, 1992). Cytochrome P-452 did not display any cross reactivity with the isozymes of cytochrome P-450 induced by PB or by polycyclic hydrocarbons (Tamburini *et al.*, 1984). Thus, the reaction of anti-rat cytochrome P-450b antibody with microsomal protein from CFB treated rats on some Western blots was somewhat

surprising (figures 10 and 11). This reaction was not observed on all Western blots and may be due to carryover.

Cytochrome P-452 constituted approximately 6% of the total cytochrome P-450 pool in untreated male Long Evans rats and after CFB administration increased to 11% (Chinje and Gibson, 1991). Although specific cytochrome P-452 was not quantitated in this study, it has been reported to increase in a dose dependent manner when CFB (50 to 250 mg/kg daily) was administered to rats for 3 days *via* gastric intubation (Sharma *et al.*, 1988b).

Ethoxyresorufin, the preferred substrate for cytochrome P-450c, which is induced by 3-methylcholanthrene, was not utilized as a substrate by microsomes from CBZ treated rats (Burke *et al.*, 1985). Although the microsomal protein from the CFB treated rats appeared to cross react with cytochrome P-450b, pentoxyresorufin was utilized as a substrate only to the same extent as microsomal protein from untreated rats (figure 12) further demonstrating the differences between cytochrome P-452 and PB inducible cytochrome P-450b. This finding agrees with the results of Tamburini *et al.* (1984).

A comparison of the effects of PB, CBZ and CBZE on hepatic cytochrome P-450 content in rats

To the best of our knowledge, there are no reports in the literature where the time dependency of cytochrome P-450 induction by CBZ in animal models has been examined. Thus, the time period tested for the induction of cytochrome P-450 by CBZ was based on the assumption that 2 weeks of induction in rats should be comparable to the reported autoinduction of CBZ metabolism in humans of 4 to 5 weeks (Bertilsson *et al.*, 1980; Pynnonen *et al.*, 1980; Moreland *et al.*, 1982). In the case of CBZE, treatment of rats for either 3 or 7 days did not demonstrate any differences in total hepatic cytochrome P-450 content between the 2 time periods of treatment (Jung *et al.*, 1980). Therefore,

rats were treated for 3, 7, 10 or 14 days in order to determine the time course for induction by CBZ and CBZE.

When the CBZ or CBZE treated groups were compared over the 14 day treatment period, statistical differences were not observed in total cytochrome P-450 content. This would indicate that 3 days of treatment with CBZ or CBZE were sufficient to yield maximal induction of cytochrome P-450 at the doses employed in this study.

The dose of CBZ employed in the current study was based on reports that an oral dose of 400 mg/kg in rats yielded serum levels of CBZ equivalent to the 8 to 10 μg/mL levels observed in man (Morselli *et al.*, 1971) and that the oral absorption of CBZ is reported to range from 58 to 86% in monkeys (Morselli and Frigerio, 1975). Carbamazepine-10,11-epoxide was dosed at half the CBZ dose based on reported serum CBZE concentrations after dosing with CBZ to be usually 10 to 50% those of the parent drug in humans (Bertilsson and Tomson, 1986). Carbamazepine and carbamazepine-10,11-epoxide were suspended in propylene glycol at a concentration of 100 mg/mL and 50 mg/mL, respectively.

While not specifically investigated in this work, the induction properties of CBZ and presumably also of CBZE, suggest dose dependency in humans. CBZ invoked dose dependent induction of antipyrine clearance which returned to control values within 2 weeks after discontinuation of CBZ therapy in adult volunteers (Rapeport *et al.*, 1983). At higher doses of CBZ in patients, increases in dosage resulted in disproportionately low elevations of CBZ plasma concentrations as a result of dose dependent induction (Tomson *et al.*, 1989). In humans, CBZ demonstrated dose dependent induction as evidenced by a curvilinear relationship between dose and steady state concentrations (Kudriokova *et al.*, 1992). In rats, however, a statistical difference was not observed between a 60 mg/kg and a 100 mg/kg twice daily dose when the

induction of various enzyme activities was examined (Regnaud *et al.*, 1988).

The observed increase in hepatic microsomal cytochrome P-450 content in the PB and CBZ treated groups when compared to the untreated control group were not unexpected since 2 to 3 fold increases in cytochrome P-450 content have been observed after PB treatment in rats (Conney, 1967; Phillips *et al.*, 1981). Regnaud and co-workers (1988) observed significant increases in total hepatic cytochrome P-450 content when CBZ treated rats were compared to control rats. A 48% increase in total hepatic cytochrome P-450 content by CBZ treatment over 4 days in rats compared to the control group was reported by Wagner and Schmid (1987).

The total hepatic cytochrome P-450 content of microsomes from each of the CBZ 3, 7 and 10 day treated groups was significantly increased when compared to their corresponding PG treated group while there was no difference in the total hepatic cytochrome P-450 content of the CBZ 14 and PG 14 day treated groups (figure 7). A plateau may have been reached whereby the continued presence of CBZ did not result in a further increase in cytochrome P-450 content; *i.e.* tolerance to induction by CBZ had developed. Another consideration is that since the inducer must be present in high concentrations to produce induction (Conney, 1967) and because CBZ metabolism is autoinducible, lower concentrations of CBZ can be expected at the site of action when studied at the longest time point. In order to invoke induction, the inducing agent must be able to maintain adequate intracellular concentrations after repeated administration (Conney, 1967). In one case of PB induction in rats, a plateau in cytochrome P-450 levels was achieved after 5 days possibly as the result of the formation of a repressor as opposed to any increase in metabolism of PB (Ernster and Orrenius, 1965; Orrenius *et al.*, 1965).

In order to investigate the possibility of autoinduction of CBZ and CBZE

metabolism over the time course, rat blood samples were collected at sacrifice after 3, 7, 10 and 14 days of treatment. Unfortunately, the concentrations of CBZ and metabolites that were present 12 to 13 h after administration of the last dose were extremely low and thus unquantifiable. However, CBZ and metabolites were quantitated in urine collected for 12 h following the last dose of either CBZ or CBZE. The urinary recoveries of CBZ, CBZE and CBZD representing the expoxide-diol metabolic pathway did not increase over the 2 week time period and ranged from 2.9 to 4.6% of the administered dose. In a previously reported rat study, the recovery of compounds in the epoxide-diol pathway (CBZ, CBZE and CBZD) in a 24 h urine collection accounted for 7 to 10% of the dose of CBZ administered (Regnaud *et al.*, 1988). Our recoveries are therefore significantly lower and may be due to differences in our assay and/or the strain of rat used. The low recoveries observed for metabolites in the epoxide-diol pathway make it difficult to use such data to conclude if autoinduction had in fact occurred. It is possible that other metabolites of CBZ were formed that we did not or could not measure. A possible clue to this is the study by Regnaud and co-workers (1988) where 50% of the CBZ dose was recovered in the urine in the form of thioethers. These thioethers were not specifically identified but suggest an increased formation of mercapturate metabolites after repeated administration of CBZ to rats. We were not able to pursue the identification and quantitation of such metabolites in this investigation.

The lack of statistical differences apparent between the observed total hepatic cytochrome P-450 values for the CBZE and PG treated groups may be due to the lower dose of CBZE used compared to CBZ. Previously, CBZE administration to Sprague-Dawley rats at a dose of 100 mg/kg daily *i.p.* for either 3 or 7 days did not affect total hepatic cytochrome P-450 when compared

to the control group (Jung *et al.*, 1980). Assuming that CBZE induction is a dose dependent phenomenon as reported for CBZ, the CBZE even at lower serum concentration could play an important role in the induction of cytochrome P-450. If plasma half-life plays an important role in achieving maximal induction for the barbiturates (Ioannides and Parke, 1975), perhaps this is also true for CBZ and CBZE. Repeated administration of CBZ enhances its own elimination and this may also be true for CBZE (Faigle and Feldmann, 1982). The mean plasma half-life of CBZ after a single dose was 35.6 ± 15.3 h and decreased to 20.9 ± 5.0 h after chronic administration in patients (Eichelbaum *et al.*, 1985). The plasma half-life of CBZE in humans was approximately 6 to 8 h after single doses (Tomson *et al.*, 1983; Spina *et al.*, 1988; Tomson and Bertilsson, 1991). The shorter plasma half-life of CBZE could also be responsible for limiting its effectiveness at inducing total hepatic cytochrome P-450.

The mean recoveries of CBZE and CBZD in the 12 h urine following administration of CBZE was only 1.5 to 4% of the administered CBZE dose. By comparison, it was reported that 15% and 1% of the dose was recovered as CBZE and CBZD, respectively, in urine collected for 5 days from Sprague-Dawley rats dosed with 4 mg of CBZE *i.v.* (Kerr and Levy, 1989). In man, after single doses of CBZE, the urinary recovery of CBZD varies from 67 to 90% of the dose with urine collections over 3 (Spina *et al.*, 1988) or 5 days (Tomson *et al.*, 1983). Our results and that of Kerr and Levy (1989) implicate a species difference in the metabolism of CBZE to CBZD with rats being much less efficient than humans in producing the diol metabolite.

A comparison of the effects of PB, CBZ and CBZE on the induction of cytochrome P-450b

Previous reports in the literature speculated that CBZ should induce the same isozyme(s) of cytochrome P-450 as does PB (Faigle and Feldmann, 1982;

Wagner and Schmid, 1987). The results of our study would appear to support that speculation. After treatment for just 3 days with either CBZ or CBZE, cytochrome P-450b was clearly detectable as was cytochrome P-450e. The rat anti-cytochrome P-450b antibody used in the present study reacts with cytochrome P-450e, also inducible by PB (Dutton and Parkinson, 1989). Cytochromes P-450b and P-450e are 2 of the major PB inducible isozymes and may be induced up to 40 fold in rats (Thomas *et al.*, 1981; Thomas *et al.*, 1987). Cytochrome P-450e migrates in SDS-PAGE just above the cytochrome P-450b band and has a slightly higher apparent molecular weight of 52,500 daltons *versus* 52,000 daltons for cytochrome P-450b (Ryan and Levin, 1990). Rat liver cytochromes P-450b and P-450e share greater than 97% amino acid sequence homology as determined from amino acid and cDNA analysis (Fujii-Kuriyama *et al.*, 1982). Cytochromes P-450b and P-450e are not immunochemically separable when using polyclonal antibodies unless first separated by SDS-PAGE (Ryan *et al.*, 1982).

Cytochrome P-450b was found to comprise 65% of the total hepatic cytochrome P-450 isolated from the PB treated group of rats (table 5). Over the 14 day time course, microsomal cytochrome P-450b content varied from 39 to 66% of the total cytochrome P-450 for the CBZ treated groups and 31 to 53% for the CBZE treated groups. Mean quantities of cytochrome P-450b in the CBZE treated groups appeared to be lower than both the PB and CBZ induced groups but did not test statistically different. The specific content of cytochrome P-450b in the cytochrome P-450 protein of the PB and CBZ 3 day treatment groups was very similar to literature values. After PB treatment of male Long Evans rats, microsomal cytochrome P-450b content ranged from 43 to 57% of the total hepatic cytochrome P-450 content (Thomas *et al.*, 1981). In PB induced adult, male Sprague-Dawley rats, cytochrome P-450b represented 51% of the total

hepatic cytochrome P-450 (Wilson *et al.*, 1987). Thus, the values for cytochrome P-450b obtained after PB induction in our study are very close to the reported literature values. Furthermore, our results suggest that at the doses used in this study, CBZ is equally as effective as PB in inducing cytochrome P-450b. CBZE, if utilized at an equivalent molar dose to CBZ, also may have provided comparable induction of cytochrome P-450b.

Although the induction of cytochrome P-450b in the PB, CBZ and CBZE treatment groups was confirmed by the use of antibodies, quantitation of the monooxygenase enzyme activities by fluorimetric assays can provide further corroboration of the identity of the isozymes induced. Fluorometric assays include O-dealkylation of coumarins (umbelliferones), phenoxazones (resorufins), fluorescein (Mayer *et al.*, 1989) and quinolones (Mayer *et al.*, 1990). Of these, pentoxyresorufin and ethoxyresorufin have been demonstrated to be specifically metabolized by the isozymes of cytochrome P-450 induced by PB and 3-methylcholanthrene, respectively (Mayer *et al.*, 1989). Although we did not study the effects of antibodies on microsomal dealkylation, an antibody against cytochrome P-450b inhibited pentoxyresorufin dealkylation more than 90% in hepatic microsomes from PB induced rats (Dutton and Parkinson, 1989).

In the present study, pentoxyresorufin was found to be the preferential substrate for the O-dealkylation of reactions catalyzed by cytochromes P-450 induced not only by PB but by CBZ and CBZE as well (figures 12 to 14). The rates of pentoxyresorufin O-dealkylation on a per mg of protein basis by microsomes from the PB treated group were increased 12 fold over the vehicle control group (table 3). Induction with PB is reported to result in an enhancement of pentoxyresorufin O-dealkylation activity over a wide range of 20 to 283 fold in rats (Burke *et al.*, 1985; Dutton and Parkinson, 1989; Lubet *et al.*, 1990; Mayer *et al.*, 1990). The values obtained in this study for the PB

treated group were slightly lower when compared to the literature values. Pentoxyresorufin O-dealkylation rates for microsomal protein from the CBZ (34 to 53 fold increase) and the CBZE (12 to 19 fold) treatment groups were also in the reported range for PB.

Thus, it appears that 3 days of treatment with CBZ at 100 mg/kg *i.p.* twice daily will provide induction of total hepatic cytochrome P-450, cytochrome P-450b content and enhancement of pentoxyresorufin O-dealkylation rates that are very similar to that obtained with typical PB induction in rats. Although we did not examine the following enzyme activities, CBZ administration to rats is reported to result in increased activities of UDP-glucuronyltransferase, NADPH-cytochrome P-450 reductase (Faigle and Feldmann, 1982; Wagner and Schmid, 1987), 4-nitroanisole O-demethylase (Wagner and Schmid, 1987), aminopyrine *N*-demethylase and aniline hydroxylase (Wagner and Schmid, 1987; Regnaud *et al.*, 1988). The increases in enzyme activities reported for CBZ induction, albeit of lower magnitude, were similar to those observed with PB administration.

The rates for pentoxyresorufin O-dealkylation for the CBZE treated rats ranged from 32 to 43% that of the CBZ treatment groups and appear to reflect the decreased dose of CBZE that was tested. Furthermore, these rates also reflect the lower levels of cytochrome P-450b quantitated in microsomal protein from the CBZE treatment groups, with the exception of the CBZE 7 day treatment group. An example in the literature that was similar to these findings is the reported induction of microsomal ethoxycoumarin O-dealkylation by 115% in rats after CBZE administration for 3 days at a dose of 100 mg/kg (Jung *et al.*, 1980). No further increases in ethoxycoumarin O-dealkylation were observed when CBZE was administered for an additional 4 days.

Effect of CBZ, CBZE and PB treatment on cytochromes P-450f and P-450g

The microsomal protein isolated from the CBZ, CBZE and PB treatment groups did not react to any appreciable degree with anti-rat cytochrome P-450f or anti-rat cytochrome P-450g antibody. These results are consistent with what is known about these particular isozymes of cytochrome P-450. Cytochrome P-450f is a constitutive isozyme present at higher concentrations in females than in males and is regulated by age (Leroux *et al.*, 1989). Cytochrome P-450g is a constitutive, male specific isozyme which is regulated by both sex and age (Soucek and Gut, 1992). These isozymes are normally present in small quantities in untreated rat hepatic microsomes. Cytochromes P-450f and P-450g represent approximately 1.4% and 0.8%, respectively of the total hepatic P-450 content of hepatic microsomes from untreated immature male Long Evans rats (Bandiera *et al.,* 1986). In adult rats, the amounts of cytochromes P-450f and P-450g increase to approximately 7% and 17% of total hepatic cytochrome P-450, respectively (Bandiera *et al.,* 1986). Furthermore, cytochromes P-450f and P-450g are relatively refractory to induction by all common classes of P-450 inducers (Bandiera *et al.*, 1986).

In summary, hepatic cytochrome P-450b in rats was significantly induced by PB and CBZ and appeared also to be induced by CBZE administration. Three days of induction with CBZ at a dose of 100 mg/kg *i.p* twice daily was found to be sufficient to yield induction comparable to the usual PB dose of 75 mg/kg *i.p.* for 4 days. CBZE at a dose of 50 mg/kg *i.p.* twice daily was approximately 50% as effective as CBZ for both the induction of cytochrome P-450b and the enhancement of pentoxyresorufin O-dealkylation and is attributed to the lower dose used. The induction of cytochrome P-450b and pentoxyresorufin O-dealkylation by CBZ and CBZE has not previously been

reported. We attempted to confirm this induction by examination of the metabolic profile of CBZ and CBZE in rats, but the urinary recoveries of metabolites did not provide substantial evidence for the induction of the epoxide-diol metabolic pathway.

IN VITRO METABOLISM STUDIES OF VPA AND (E)-2-ENE VPA / EFFECTS OF INDUCING AGENTS

In order to detail the effects of CBZ induction on VPA metabolism that is apparent from patient interaction studies and from *in vivo* studies in rats, it was important to investigate *in vitro* effects of CBZ induction on VPA metabolism and one of its major metabolites, (E)-2-ene VPA. Induction by CBZ and CBZE was compared to the classic inducer, PB and to CFB. Since maximal induction of total cytochrome P-450 content, cytochrome P-450b and microsomal pentoxyresorufin O-dealkylation appeared to be achieved after 3 days of CBZ treatment, only the group treated with CBZ for 3 days will be compared to the PB, CFB and CBZE 3 day treatment groups.

Interaction between VPA and CBZ

As the number of medications that a patient is prescribed increases, so does the possibility of adverse drug interactions (McInnes and Brodie, 1988). CBZ induces the metabolism of VPA in healthy subjects and in epileptic patients (Levy and Pitlick, 1982). In epileptic patients on VPA and CBZ, steady state VPA levels were 37 to 64% lower than predicted from single dose studies of VPA (Levy and Pitlick, 1982). In one volunteer study, CBZ caused increased clearance of VPA accompanied by decreased steady state plasma concentrations (Bowdle *et al.*, 1979). The effect of CBZ on VPA clearance does not result from competition for protein binding sites because CBZ plasma levels are low compared to the amount of albumin present and thus, CBZ should not act as a

displacing agent (Levy and Pitlick, 1982).

In a volunteer study conducted in this laboratory, the volume of distribution of VPA did not change upon CBZ administration suggesting that enzyme induction and not a competition for plasma protein binding sites was responsible for the increased clearance of VPA and the resulting decreased serum levels, AUC and half-life values (Panesar *et al.*, 1989). The major unsaturated metabolite, (E)-2-ene VPA, was significantly decreased in serum and urine, suggesting that it too was being cleared as a result of induced metabolism. The serum levels of 4-ene VPA were unchanged after CBZ administration but urinary recoveries, mainly as the glucuronide conjugate, were increased after CBZ. When the urinary recoveries of VPA and its metabolites were compared before and after CBZ administration, an increase in metabolism elicited by CBZ could not be confirmed. Either VPA metabolites were eliminated *via* non-renal routes or the GCMS assay failed to detect a significant proportion of the VPA metabolites. Thus, in order to verify this apparent *in vivo* induction of VPA metabolism, it was deemed important to examine the effects of CBZ on the metabolism of VPA at the *in vitro* level.

Effect of anti-rat cytochromes P-450b and P-450h antibodies on VPA metabolite profiles from rat liver microsomes

Anti-rat cytochrome P-450b and P-450h antibodies were employed to determine the extent of involvement of cytochromes P-450b and P-450h in the *in vitro* microsomal metabolism of VPA and (E)-2-ene VPA. These antibodies, prepared and made available to us by Dr. Bandiera's research group, were used in a concentration range of 0.5 to 2.5 mg of IgG/nmol of spectrally determined cytochrome P-450.

The metabolism of VPA to 4-ene VPA by microsomal protein from PB and CBZ 3 day treated rats was completely inhibited in the presence of the anti-rat

cytochrome P-450b antibody while the formation of 3-OH VPA and 5-OH VPA was only partially inhibited. The formation of 4-OH VPA and 4-keto VPA was inhibited by 75% or greater. These results suggest that cytochrome P-450b is the major isozyme of cytochrome P-450 involved in the metabolism of VPA to 4-ene VPA, 4-OH VPA and 4-keto VPA while other isozymes of cytochrome P-450 participate in the formation of 3-OH VPA and 5-OH VPA.

The results obtained for the microsomal metabolism of VPA to 4-ene VPA demonstrate that formation of this putative hepatotoxin occurs directly from VPA and not *via* dehydration of either 4-OH VPA or 5-OH VPA (Kochen and Scheffner, 1980; Granneman *et al.*, 1984a). Metabolic studies of stable isotope analogues of VPA had indicated that the origin of 4-ene VPA was different from that of 3-ene VPA and (E)-2-ene VPA (Rettenmeier *et al.*, 1987).

Thus, induction of a particular isozyme of cytochrome P-450, namely cytochrome P-450b, may play an important role in VPA induced hepatotoxicity. The formation of 4-ene VPA occurs *via* cytochrome P-450b catalyzed oxidation of a nonactivated alkyl substituent to the corresponding olefin (Baillie, 1988). This cytochrome P-450 isozyme functions as a desaturase (figure 37) oxidizing certain alkanes to olefins without an intermediate alcohol due to partitioning between hydroxylation and desaturation reactions (Guengerich, 1990). Initial abstraction of a hydrogen atom generates a transient free-radical intermediate that partitions between recombination (alcohol formation) and elimination (olefin production). Based on the observed metabolism of deuterium labeled VPA, the carbon-centered radical was found to be located at C-4 (Rettie *et al.*, 1988). This partitioning mechanism is similar to the cytochrome P-450 mediated formation of 17β-hydroxy-4,6-androstadien-3-one from testosterone *via* dual hydrogen abstraction (Nagata *et al.*, 1986). The 6β-hydrogen is abstracted forming a transient radical, followed by abstraction of the 7β-hydrogen to form

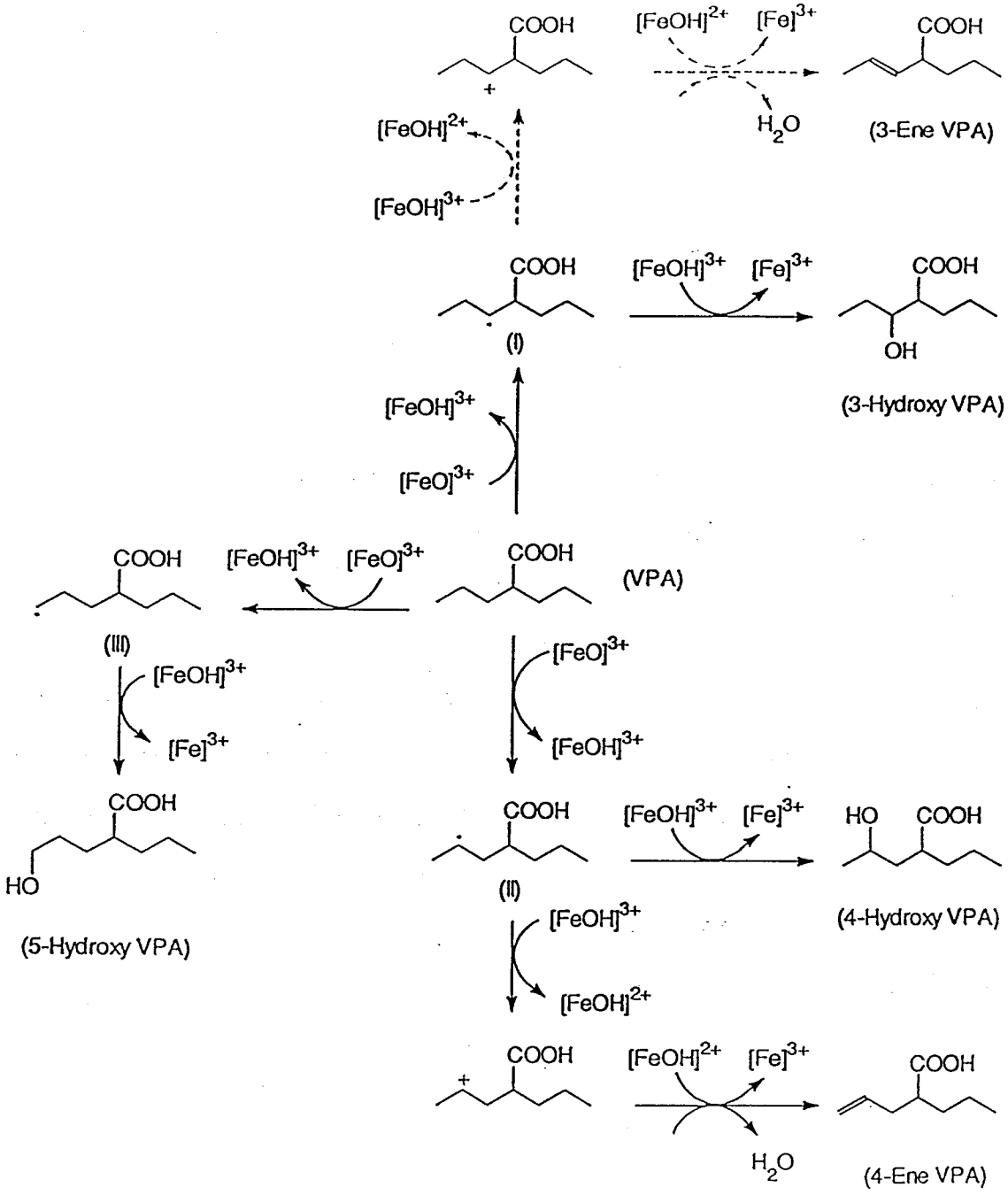

Figure 37. Cytochrome P-450 catalyzed metabolism of VPA to 3-OH VPA, 4-OH VPA, 5-OH VPA, 3-ene VPA and 4-ene VPA. (Based on Rettie *et al.*, 1988).

the resultant double bond. The free radical intermediate generated solely by cytochrome P-450b which partitions between alcohol and olefin formation explains our results in that the antibody to cytochrome P-450b not only completely blocked 4-ene VPA formation but also the formation of 4-OH VPA and the derived 4-keto VPA (figures 30, 32 and 33).

In order to further affirm the specificity of cytochrome P-450b in the metabolism of VPA to 4-ene VPA and 4-OH VPA, one other antibody to cytochrome P-450 was tested. Cytochrome P-450h is a male specific isozyme of cytochrome P-450 which catalyzes a number of metabolic reactions (Ryan and Levin, 1990). It is non-detectable in newborn rats but rapidly increases at 4 to 6 weeks of age and then plateaus (Waxman *et al.*, 1985). The anti-rat cytochrome P-450h antibody did not exhibit any significant effects on the metabolism of VPA by microsomes from untreated, PB or CBZ 3 day treated groups, evidence that VPA or (E)-2-ene VPA are not substrates for cytochrome P-450h.

Since the antibody directed against cytochrome P-450b was unable to completely inhibit the metabolism of VPA to 3-OH VPA and 5-OH VPA, other isozymes of cytochrome P-450 may be involved. Another isozyme of cytochrome P-450 inducible by PB is cytochrome P-450p (Soucek and Gut, 1992). Induction of cytochrome P-450p significantly increased the biotransformation of CBZ to CBZE in mouse hepatic microsomes and this reaction was inhibited by gestodene, a cytochrome P-450p inhibitor (Pirmohamed *et al.*, 1992). CBZ and CBZE may also induce cytochrome P-450p. To determine whether cytochrome P-450p is involved in the metabolism of VPA to 3-OH VPA and 5-OH VPA, future inhibition experiments with gestodene or anti-cytochrome P-450p antibody are required.

A comparison of the effects of PB, CBZ and CBZE induction on the *in vitro* metabolism of VPA

Phenobarbital pretreatment resulted in enhanced metabolism of VPA to 3-OH VPA, 4-OH VPA, 5-OH VPA, 4-ene VPA and 4-keto VPA. Similar results were obtained with the CBZ treated groups.

Formation of 4-ene VPA

Granneman and co-workers (1984a) were one of the first groups to report that PB induction increased 4-ene VPA formation in VPA treated rats. Our work confirms that CBZ and CBZE are capable of this action as well. Rettie and co-workers (1987) were the first to report the *in vitro* formation of 4-ene VPA from VPA as a result of a cytochrome P-450 mediated reaction. The 4-ene VPA metabolite could only be observed in the presence of PB induced rat liver microsomes. Similar results were obtained for liver microsomes from PB induced rabbits, mice and humans with 2.5 to 8.4 fold increases in the formation of 4-ene VPA, depending upon the species (Rettie *et al.*, 1988). A comparative study using microsomes from CBZ, phenytoin and PB induced rats (80 mg/kg for 4 days) indicated that PB was the more effective inducer of this desaturation pathway leading to 4-ene VPA (Rettie *et al.*, 1987). In our study, the formation of 4-ene VPA was increased 6 fold and 5 fold respectively, by the PB and CBZ 3 day treated groups when compared to the untreated group. Only a 2 fold increase was observed with the CBZE 3 day treated group.

The 4-ene VPA metabolite is not easily detected because of the low serum levels. For example, in a survey of 49 patients, 4-ene VPA was only detected in the serum of patients receiving VPA in combination with CBZ (Tennison *et al.*, 1988). This was probably due in part to increased doses of VPA in the VPA and CBZ group plus the induction effects of CBZ in forming increased 4-ene VPA. Extremely sensitive assays are required in order to reliably quantify significant

changes in 4-ene VPA serum concentrations. Using a GCMS assay, the formation clearance of 4-ene VPA was determined to increase 2 fold in patients who were on combined therapy with VPA and either CBZ or phenytoin (Levy *et al.*, 1990).

Significance of 4-ene VPA formation to the mechanism of VPA hepatotoxicity

There are currently 2 major hypotheses regarding the mechanism(s) of VPA induced hepatotoxicity. The first proposed mechanism for VPA toxicity entails the depletion of free CoA due to sequestration by VPA to form valproyl CoA and derivatives (Becker and Harris, 1983; Thurston and Hauhart, 1992). The reduced concentrations of free CoA result in increased levels of fatty acids and decreased β-oxidation. A second proposed mechanism that our laboratory favours, is that hepatotoxicity of VPA is mediated through the formation of a toxic metabolite and 4-ene VPA has been implicated as the most likely candidate (Zimmerman and Ishak, 1982; Rettenmeier *et al.*, 1985; Rettenmeier *et al.*, 1986b). The 4-ene VPA metabolite is similar in structure to the hepatotoxin 4-pentenoic acid (PA) and to MCPA, the metabolite of hypoglycin (figure 38). VPA hepatotoxicity shares many similar manifestations with Jamaican Vomiting Sickness (JVS), Reye's Syndrome (RS) and PA toxicity (Lewis *et al.*, 1982; Nau and Loscher, 1984). PA is bioactivated to the reactive electrophilic species, 3-keto-4-pentenoic acid, which alkylates 3-ketoacyl CoA thiolase, the terminal enzyme of mitochondrial β-oxidation (Schulz, 1983; Fong and Schulz, 1983). It has been postulated that analogous to PA, 4-ene VPA undergoes β-oxidation to (E)-2,4-diene VPA which is ultimately bioactivated to the electrophilic metabolite, 3-keto-4-ene VPA (Rettenmeier *et al.*, 1985). This metabolite has just recently been identified in rats although it is present in very trace quantities and specialized techniques were required for its identification (Kassahun *et al.*, 1993).

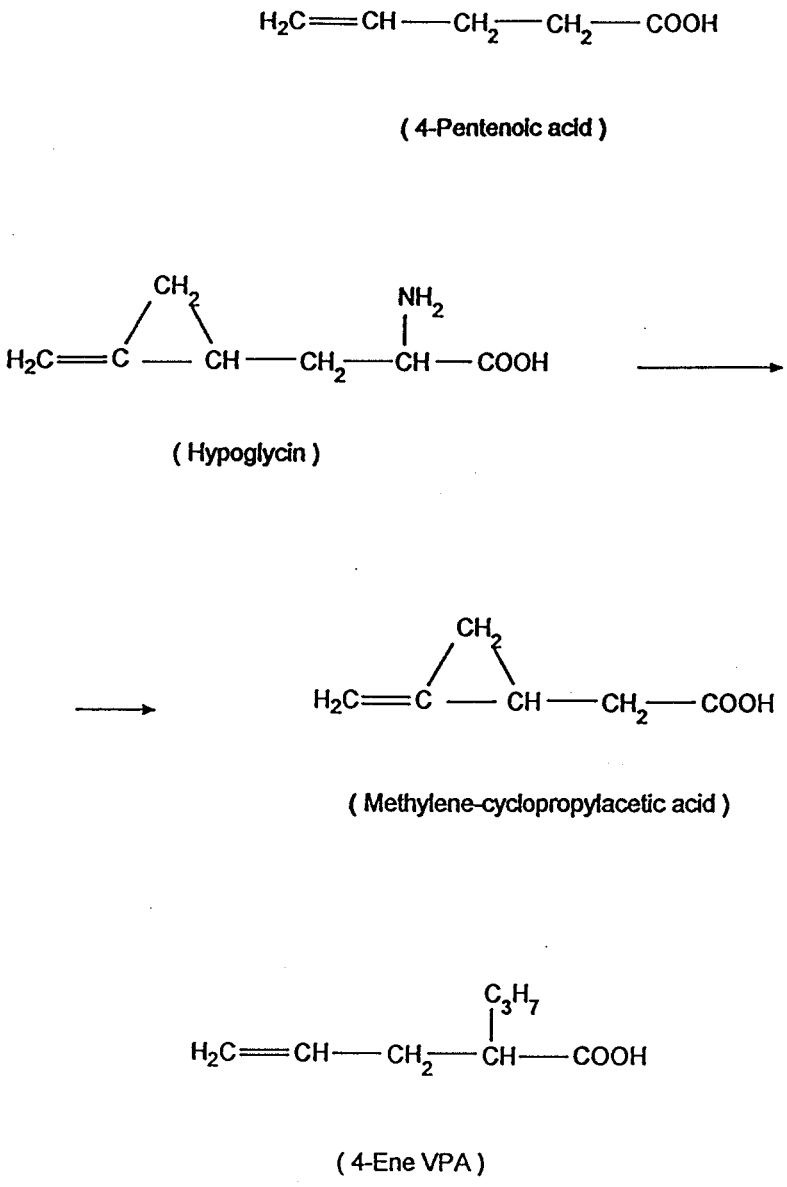

Figure 38. Structural similarity amongst hypoglycin, 4-pentenoic acid and 4-ene VPA.

It has been established that VPA and 4-ene VPA undergo metabolic activation in rat liver, both *in vivo* and *in vitro*, to electrophilic intermediates that bind covalently to cellular macromolecules (Porubek *et al.*, 1989). In support of a reactive metabolite mechanism, Kassahun *et al.* (1991) found that in rats administered 4-ene VPA, the major metabolite recovered in bile was the gluthathione (GSH) conjugate of (E)-2,4-diene VPA. GSH is a tripeptide which reacts with a variety of electrophilic compounds aiding in the minimization of cellular injury (Reed, 1990; DeLeve and Kaplowitz, 1991). Urinary *N*-acetylcysteine (NAC) conjugates are the end products of GSH conjugation. The NAC conjugate of (E)-2,4-diene VPA was a prominent urinary metabolite in rats that had received 4-ene VPA and was detected in the urine of patients receiving VPA. It was proposed that the toxic effect exerted by (E)-2,4-diene VPA could be due to localized mitochondrial depletion of GSH, leading to oxidative stress and subsequent cellular injury (Kassahun *et al.*, 1991). Thus, patients who are subjected to increased serum levels of 4-ene VPA and/or have deficiencies in their glutathione defense mechanisms could be at an increased risk to VPA induced hepatotoxicity. In the retrospective study by Dreifuss and co-workers (1987), VPA polytherapy, especially when in combination with CBZ, was implicated as a major factor in the increased incidence of VPA hepatotoxicity.

Formation of the hydroxy metabolites of VPA

The formation of the hydroxy metabolites was significantly enhanced by CBZ and PB induction while smaller increases were observed with CBZE treatment. The hydroxy metabolites of VPA (3-OH VPA, 4-OH VPA and 5-OH VPA) have been demonstrated in human embryo homogenates of liver, lung, brain and adrenals (Rettie *et al.*, 1986) and rat liver microsomes (Prickett and Baillie, 1984). The formation of 3-OH VPA from VPA was thought to be mediated primarily *via* the β-oxidation pathway (Bjorge and Baillie, 1991; Li *et*

al., 1991) but evidence suggests that it may partly arise *via* cytochrome P-450 mediated ω-2 oxidation (Prickett and Baillie, 1984). The ω-2 oxidation pathway, inducible by PB, is a minor metabolic route for the rat hepatic microsomal metabolism of simple alkanes, such as *n*-hexane (Frommer *et al.*, 1972) and *n*-heptane (Frommer *et al.*, 1974).

Phenobarbital treatment in rats was reported to primarily enhance the ω-1 oxidation pathway of VPA metabolism with the urinary recoveries of 4-OH VPA, 4-keto VPA and 2-PSA being increased by a factor of 3.8 (Granneman *et al.*, 1984a). The urinary excretion of 5-OH VPA was also increased. In one pediatric patient who died from hepatic failure associated with VPA and PB polytherapy, increased quantities of 2-PGA, the endproduct of the ω-oxidation of VPA, were recovered in the urine (Kuhara *et al.*, 1990). In the present study, PB treatment was more effective at inducing ω-1 (4-OH VPA) and ω-2 (3-OH VPA) hydroxylation while CBZ was more efficient for the induction of ω-oxidation (5-OH VPA). This could, therefore, have implications regarding the incidence of VPA induced hepatic failure depending on the associated drugs and the metabolic pathway responsible for the ultimate hepatotoxic response.

Despite the presence of 3-OH VPA, 3-ene VPA was not detected in the microsomal incubates from the metabolism of VPA. In rats, 3-ene VPA was not observed as a metabolite of 3-OH VPA (Granneman *et al.*, 1984a). These results indicate that 3-ene VPA probably arises through a different mechanism than the 4-ene VPA/4-OH VPA pathway described in figure 37. Alternatively, the degree of 3-ene VPA formation may have been below the level of detection of our assay.

The oxidation of fatty acids involving hydroxylation at the ω, ω-1 or ω-2 positions occurs microsomally and can be succeeded by further biotransformation *via* alcohol or aldehyde dehydrogenases to the dicarboxylic

acids or keto acids (Bjorkhem, 1972a). Dehydrogenation reactions can occur in either the soluble or microsomal fraction, although the microsomal dehydrogenase activity is not as efficient as that of the soluble fraction (Bjorkhem, 1972b). This may explain why 4-keto VPA was detected in the *in vitro* microsomal metabolism of VPA although it was not expected, since the formation of 4-keto VPA is the second step in the ω-1 oxidation pathway. Based on the theory that partitioning occurs between desaturation and hydroxylation as illustrated in figure 37, if larger quantities of the alcohol are formed (*i.e.* 4-OH VPA) then a larger concentration of substrate is present for alcohol dehydrogenases to form 4-keto VPA. CBZ was a more effective inducer of the formation of 4-keto VPA than was PB which may also relate to the lower quantities of 4-ene VPA observed with the CBZ treated group.

In a discussion of the effects of induction by CBZ and CBZE on the metabolism of VPA and (E)-2-ene VPA, the vehicle, PG, needs to be considered. PG appeared to invoke an inhibitory effect on the microsomal metabolism of VPA to some metabolites. There is some precedence in the literature for this inhibition by PG from studies in mice. Administration of PG (4 mL/kg *i.p.*) to mice within 3 h of receiving acetaminophen, provided protection against acetaminophen induced liver toxicity (Hughes *et al.*, 1991). This protection by PG was perhaps afforded by inhibition of the formation of the toxic metabolite *N*-acetyl-*p*-benzoquinoneimine by cytochrome P-450, a mechanism by which ethanol also provides protection against acetaminophen toxicity (Wong *et al.*, 1980; Tredger *et al.*, 1985). In rats PG (4 mL/kg twice daily *i.p.* for 3 days) increased hexobarbital sleeping time from 30 to 53 min (Dean and Stock, 1974). In microsomes prepared from rats treated with PG (4 mL/kg twice daily *i.p.* for 3 days), aminopyrine demethylase activity was observed to decrease. This depressant effect of PG on aminopyrine demethylase activity was abolished

when PB was co-administered. We cannot be certain that induction by CBZ and CBZE produced the same effects as PB. Alternate vehicles which lack the inhibitory effects may need to be considered for further studies of CBZ and CBZE induction.

Effect of anti-rat cytochrome P-450b and P-450h antibodies on (E)-2-ene VPA metabolite profiles from rat liver microsomes

The major serum metabolite of VPA, (E)-2-ene VPA, has been touted as a possible anticonvulsant agent (Nau *et al.*, 1984). (E)-2-ene VPA possesses anticonvulsant activity similar to VPA without exhibiting the toxicities (teratogenicity, embryotoxicity and hepatotoxicity) associated with the parent compound (Honack *et al.*, 1992). In rats, (E)-2-ene VPA is metabolized to several diunsaturated metabolites, (E,E)-2,3'-diene VPA and 2,4-diene VPA, as well as 3-keto VPA (Loscher *et al.*, 1992). (E,E)-2,3'-diene VPA, the major diunsaturated metabolite in the serum also possesses anticonvulsant activity (Acheampong and Abbott, 1985). However, the second diunsaturated metabolite, (E)-2,4-diene VPA, produces hepatic steatosis in rats (Granneman *et al.*, 1984c; Kesterson *et al.*, 1984).

The effect of the anti-rat cytochrome P-450b antibody on the metabolism of (E)-2-ene VPA was investigated to determine the extent of this isozyme's involvement. The metabolism of (E)-2-ene VPA to (E)-2,4-diene VPA in microsomes from PB and CBZ 3 day treated rats was inhibited by almost 90% while the formation of (E,E)-2,3'-diene VPA was partially inhibited in the presence of the anti-rat cytochrome P-450b antibody. The anti-rat cytochrome P-450h antibody did not exert any inhibitory effects on the metabolism of (E)-2-ene VPA. These results clearly indicate that cytochrome P-450b plays an important role in the metabolism of (E)-2-ene VPA to (E)-2,4-diene VPA analogous to the formation of 4-ene VPA from VPA. However, other cytochrome

P-450 isozymes appear to be responsible for the formation of (E,E)-2,3'-diene VPA. Further details of the *in vitro* metabolism of (E)-2-ene VPA and the significance of our findings with respect to the prospective use of this compound as an anticonvulsant agent are described below.

A comparison of the effects of PB, CBZ and CBZE induction on the *in vitro* metabolism of (E)-2-ene VPA in rat liver microsomes

The production of (E,E)-2,3'-diene VPA by microsomes isolated from the PB, CBZ 3 day and CBZE 3 day treatment microsomes was significantly enhanced over controls as was the formation of (E)-2,4-diene VPA by the PB and CBZ 3 day treatment groups. The extent of induction by CBZ and PB as measured by the degree of formation of these 2 metabolites was very similar.

In our *in vitro* studies of (E)-2-ene VPA metabolism, the 4-ene VPA metabolite was not detected. We were also unable to distinguish the peaks for VPA or 3-ene VPA from the background in the SIM chromatograms of the extracted incubates. VPA, albeit in small quantities, has been detected in rats after administration of (E)-2-ene VPA (Granneman *et al.*, 1984a; Loscher *et al.*, 1992). The metabolite 3-ene VPA was detected in rat plasma after administration of 300 mg of (E)-2-ene VPA daily for 12 days (Vorhees *et al.*, 1992). Thus, our results suggest that microsomal cytochrome P-450 enzymes are not responsible for the reduction of (E)-2-ene VPA to VPA nor for the isomerization of (E)-2-ene VPA to 3-ene VPA. After dosing with (E)-2-ene VPA (250 mg/kg *i.p.* for 7 days), VPA, (E) and (Z)-2-ene VPA, (E,E)-2,3'-diene VPA, (E)-2,4-diene VPA and 3-keto VPA were detected in rat plasma (Loscher *et al.*, 1992). The major urinary metabolite of (E)-2-ene VPA detected in rats was the β-oxidation product 3-keto VPA (Granneman *et al.*, 1984a).

The detection of (E)-2,3'-diene VPA as a microsomal metabolite of (E)-2-ene VPA in our study was unusual because its formation has been attributed to

β-oxidation mechanisms in mitochondria (Bjorge and Baillie, 1991) that are summarized in figure 39. The (E)-2-ene VPA metabolite reversibly isomerizes to 3-ene VPA, that in turn is metabolized by the β-oxidation pathway to (E,E)-2,3'-diene VPA (Bjorge and Baillie, 1991). However, our *in vitro* results indicate that similiar to the formation of 3-OH VPA from VPA, (E,E)-2,3'-diene from (E)-2-ene VPA also appears to arise as a product of cytochrome P-450b metabolism, although in very small quantities.

The induced formation of (E)-2,4-diene VPA by microsomes from the CBZ 3 day treated group was comparable to that achieved by the PB treated group, with increases of 12 fold compared to vehicle controls and confirms that CBZ is as effective as PB for the induction of this desaturation pathway. A significant increase in the formation of (E)-2,4-diene VPA occurred only in the CBZE 7 day treatment group which also corresponded to the maximal increase observed for cytochrome P-450b by CBZE. Similar results have recently been reported for the effects of PB on the induction of (E)-2-ene VPA metabolism to (E)-2,4-diene VPA *in vitro* (Kassahun and Baillie, 1993). The (E)-2,4-diene VPA was a major metabolite and was increased approximately 3 fold by PB.

The metabolite, (E)-2,4-diene VPA, is a known to cause steatosis, although doses as high as 100 mg/kg daily must be administered in order to invoke hepatotoxicity in rats (Granneman *et al.*, 1984c; Kesterson *et al.*, 1984). Elevated plasma and urinary levels of (E)-2,4-diene VPA were observed in 6 cases of fatal VPA hepatotoxicity (Kochen *et al.*, 1984). In patients with VPA associated hepatic failure, the urinary recovery of (E)-2,4-diene VPA as the NAC conjugate was increased 3 to 4 fold (Kassahun *et al.*, 1991). Therefore, the significant enhancement of (E)-2,4-diene VPA formation by CBZ and PB on the metabolism of (E)-2-ene VPA raises important questions regarding the potential

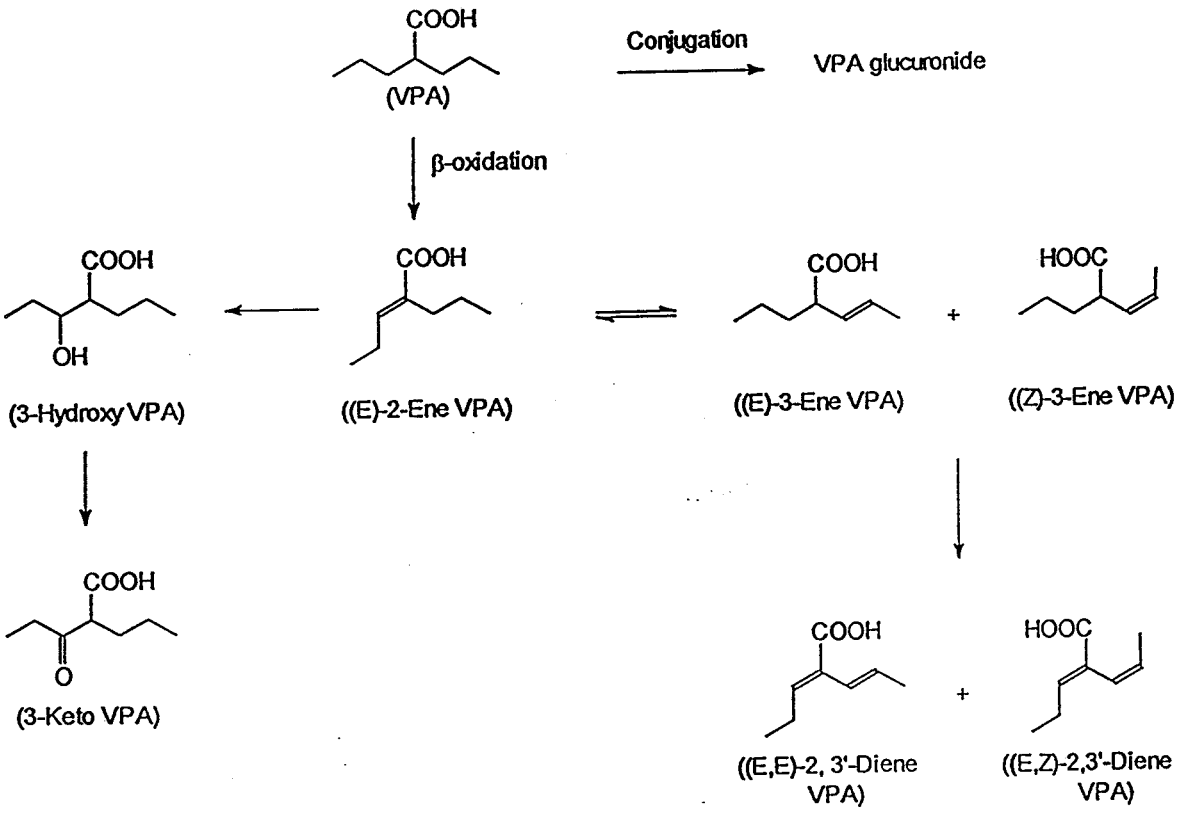

Figure 39. The β-oxidation pathway of VPA metabolism in mitochondria.

safety of this drug. This is especially true should (E)-2-ene VPA be used in situations of polytherapy.

Consideration should also be given to the relative amounts of (E)-2,4-diene VPA that is formed from either VPA or (E)-2-ene VPA administration. A comparison of the amounts of (E)-2,4-diene VPA and 4-ene VPA formed from (E)-2-ene VPA and VPA, respectively, revealed that the formation of (E)-2,4-diene VPA greatly exceeded that of 4-ene VPA. For example, with the PB treatment group, 17 ng of 4-ene VPA were formed compared to 2.5 μg of (E)-2,4-diene VPA. For the CBZ treatment groups over the 2 week treatment period, 11 to 13 ng of 4-ene VPA were produced in comparison to 1 to 2.5 μg of the (E)-2,4-diene VPA. Thus, the desaturation catalyzed by cytochrome P-450b in (E)-2-ene VPA metabolism appears to be considerably more efficient than for VPA. One possible explanation is that because of the conjugated structure of the (E)-2,4-diene VPA product, radical intermediates are being stabilized by the double bond during the metabolism of (E)-2-ene VPA.

The *in vitro* results from our study indicating the enhanced formation of (E)-2,4-diene VPA from (E)-2-ene VPA by CBZ and PB induction suggest that the relative lack of toxicity reported for (E)-2-ene VPA should be perceived with caution. If (E)-2,4-diene VPA is hepatotoxic in rats as reported (Kesterson *et al.*, 1984), then it is surprising that (E)-2-ene VPA is claimed to be less hepatotoxic than VPA. One rationale for such a hypothesis may be related to the organelle in which the (E)-2,4-diene VPA is formed. With (E)-2-ene VPA, the desaturation to (E)-2,4-diene VPA occurs microsomally in comparison to mitochondrial formation from 4-ene VPA after the administration of VPA. In mitochondria, the site of initial liver toxicity, the (E)-2,4-diene is readily conjugated by GSH (Kassahun *et al.*, 1991) and may cause depletion of GSH while (E)-2,4-diene VPA produced in microsomes can react with the much larger pool of cytosolic

GSH (Reed, 1990).

Effect of CFB treatment on the metabolism of VPA and (E)-2-ene VPA

CFB treatment did not appear to have any significant effects on the metabolism of either VPA or (E)-2-ene VPA although the CO vehicle may have exerted an effect which overshadowed or minimized any CFB invoked effects. When olive oil, was used as a vehicle for CFB, an increased urinary excretion of 4-OH VPA, 2-PGA and 3-keto VPA after VPA administration to CFB treated rats was observed (Heinemeyer *et al.*, 1985).

The cytochrome P-450 isozymes inducible by CFB possess the unique ability to ω-oxidize fatty acids (Bains *et al.*, 1985). The mechanism by which CFB affects fatty acid metabolism is thought to be as follows. Administration of CFB elicits an increase in cytochrome P-452, leading to increased ω-hydroxylation of fatty acids. These fatty acids undergo further cytosolic oxidation to long chain dicarboxylic acids which then enter the peroxisomes. The resultant increased peroxisomal β-oxidation provides an increased load of shorter chain fatty acids for mitochondrial β-oxidation (Sharma *et al.*, 1988a). In rats, CFB treatment was reported to increase the excretion of 4-OH VPA and the β-oxidation metabolite 3-keto VPA (Heinemeyer *et al.*, 1985.) However, in a more recent study, CFB pretreatment in rats did not significantly affect the metabolism of VPA, suggesting a minimal role for peroxisomal mediated β-oxidation (Bachmann *et al.*, 1988). Based on the results of our study, neither VPA nor (E)-2-ene VPA were substrates for cytochrome P-452.

CLINICAL RELEVANCE

Cytochrome P-450b was the major isozyme in rats induced by PB, CBZ and to a lesser extent by CBZE. The *in vitro* microsomal formation of 4-ene VPA from VPA and (E)-2,4-diene VPA from (E)-2-ene VPA was enhanced by

pretreatment with all 3 drugs and was inhibited completely in the presence of the anti-rat cytochrome P-450b antibody. Thus, both of these metabolites appear to be formed by a common desaturation mechanism. Both of these metabolites are capable of producing hepatotoxicity in rats (Granneman *et al.*, 1984c; Kesterson *et al.*, 1984). Elevated levels of (E)-2,4-diene VPA and 4-ene VPA have been observed in cases of VPA associated hepatotoxicity (Kochen *et al.*, 1983; Scheffner *et al.*, 1988). The demonstrated inducibility of (E)-2-ene VPA to (E)-2,4-diene VPA suggests that the 'toxicity free' nature of (E)-2-ene VPA needs to be re-evaluated, particularly when the drug is administered in combination with inducing agents such as CBZ. Alternatively, if (E)-2-ene VPA proves to be free of hepatotoxicity, the proposed role of (E)-2,4-diene VPA in the hepatotoxicity of VPA will need reassessment.

Future studies in rats should examine the induction of cytochrome P-450 by an equivalent molar dose of CBZE to that of CBZ and thus establish the apparent dose dependent properties of CBZE. In previous studies of VPA metabolism in patients, CBZ significantly reduced serum (E)-2-ene VPA concentrations but not those of (E)-2,4-diene VPA. The results from this study suggest that enhanced metabolism of (E)-2-ene VPA to (E)-2,4-diene VPA or inhibition of β-oxidation occurs. Any further studies must include mitochondrial and peroxisomal effects of CBZ and CBZE, in light of the evidence that mitochondrial cytochromes P-450 are involved in the activation of aflatoxin B_1 (Niranjan *et al.*, 1984; Shayiq and Avadhani, 1989) and may also play a role in VPA metabolism. In addition, the effects of induction on phase II metabolism of VPA and metabolites, including glutathione conjugates should be investigated.

SUMMARY AND CONCLUSIONS

1. CBZ was compared to PB with respect to induction of total hepatic cytochrome P-450, cytochrome P-450b and the catalysis of pentoxyresorufin O-dealkylation in rats. For maximal induction of these parameters, an *i.p.* dose of 100 mg/kg twice daily for 3 days of CBZ appeared to be equivalent to the normal protocol for PB of 75 mg/kg *i.p.* for 4 days.
2. Except for an apparent dose-related effect, CBZE produced a similar induction profile to that of CBZ. Cytochrome P-450b and pentoxyresorufin O-dealkylation values for the CBZE treatment groups were approximately 50% of the CBZ treatment groups.
3. Evidence for autoinduction of CBZ metabolism was sought by examining the urinary recoveries of metabolites that constitute the epoxide-diol pathway. No differences in metabolite recoveries were found after chronic administration of CBZ.
4. Studies with anti-rat cytochrome P-450b antibody indicated that cytochrome P-450b was the primary isozyme that catalyzes the biotransformation of VPA to 4-ene VPA, 4-OH VPA and 4-keto VPA. In addition, cytochrome P-450e may also be involved. Results from the inhibition experiments indicated that all 3 metabolites probably arise from a common intermediate.
5. Inhibition studies with anti-rat cytochrome P-450b antibody on the formation of (E)-2,4-diene VPA from (E)-2-ene VPA strongly suggest that cytochrome P-450b is the primary isozyme responsible for this biotransformation.

6. The amount of (E)-2,4-diene VPA formed from (E)-2-ene VPA per nmol of cytochrome P-450 was approximately 100 fold greater than the conversion of VPA to 4-ene VPA as measured in microsomes from either CBZ 3 day or PB treated rats. The enhanced production of (E)-2,4-diene VPA, a metabolite known to be hepatotoxic in rats, could have severe consequences regarding the relative safety of (E)-2-ene VPA if used therapeutically and in combination with other drugs.
7. Anti-rat cytochrome P-450h antibody did not inhibit the metabolism of VPA or (E)-2-ene VPA in microsomes from CBZ 3 day or PB treated rats. This result further illustrates the specificity of cytochrome P-450b in the metabolism of both VPA and (E)-2-ene VPA to potentially hepatotoxic metabolites.
8. A major metabolite of VPA, (E,E)-2,3'-diene VPA, recently shown to be a mitochondrial metabolite of 3-ene VPA, was identified in this study as a microsomal metabolite of (E)-2-ene VPA. Inhibition studies indicated cytochrome P-450b to be only partially responsible for the conversion of (E)-2-ene VPA to (E,E)-2,3'-diene VPA.
9. In addition to cytochrome P-450b, other isozymes are likely to be involved in the metabolism of VPA to 3-OH VPA and to 5-OH VPA by microsomes from CBZ 3 day and PB induced animals.
10. The treatment of rats with CFB produced no obvious effects on the *in vitro* microsomal metabolism of VPA or (E)-2-ene VPA. Thus, the reported effects of CFB on the *in vivo* metabolism of VPA likely occur *via* non-cytochrome P-450 catalyzed mechanisms. Alternatively, the corn oil vehicle and the lower dose of CFB used in this study may have minimized the observed effects.

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