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N-aralkylated Derivatives Of 1-aminobenzotriazole: Isozyme-selective Mechanism-based Inhibitors Of Guinea Pig Cytochrome P-450

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N-ARALKYLATED DERIVATIVES OF 1-AMINOBENZOTRIAZOLE: ISOZYME-SELECTIVE MECHANISM-BASED INHIBITORS OF GUINEA PIG CYTOCHROME P450

by

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ABSTRACT

1-Aminobenzotriazole (ABT) and its *N*-benzyl (BBT), *N*-α-methylbenzyl (αMB), and *N*-α-ethylbenzyl (αEB) derivatives were compared for their potency and isozyme-selectivity for mechanism-based inactivation of guinea pig hepatic and pulmonary cytochrome P450 (P450) *in vitro*, through the use of isozyme selective substrates for the guinea pig orthologs of rabbit P450 2B4, 1A1, and 4B1 (P450 2Bx, 1A1, 4Bx, respectively). αMB, αEB, and to a lesser extent, BBT, selectively inactivated P450 2Bx in pulmonary microsomes from untreated or β-naphthoflavone (βNF)-induced, and hepatic microsomes from phenobarbital (PB)-induced, guinea pigs. P450 loss caused by ABT paralleled the inhibition of enzyme activity in hepatic and pulmonary microsomes; however, P450 loss caused by BBT, αMB or αEB was never greater than 45% even when monooxygenase activity was inhibited by virtually 100%. BBT, αMB and αEB were more potent inhibitors of P450 activity in hepatic and pulmonary microsomes from untreated compared to induced guinea pigs.

The NADPH-dependent metabolism, and covalent binding to protein, of [^{14}C]ABT, *N*-benzyl-1-amino-[^{14}C]2,3-benzotriazole([^{14}C]2,3-BBT), and *N*-[^{14}C]7-benzyl-1-aminobenzotriazole ([^{14}C]7-BBT), were examined in guinea pig hepatic or pulmonary microsomes. Hepatic microsomes from βNF (vs PB) treated guinea pigs metabolized [^{14}C]ABT, [^{14}C]2,3-BBT or [^{14}C]7-BBT more extensively and to more products. NADPH-dependent covalent binding to microsomal protein of [^{14}C]2,3-BBT or [^{14}C]7-BBT was greater than that of [^{14}C]ABT in

hepatic microsomes, especially those from PB-induced animals, and the covalently modified proteins co-migrated with P450 2Bx on Western blots. Covalent binding per nmol P450 in pulmonary microsomes was 3- to 4-fold higher with [^{14}C]2,3-BBT than with [^{14}C]7-BBT or [^{14}C]ABT.

[^{14}C]BBT, at a dose which effectively inhibited pulmonary P450 2Bx, was rapidly metabolized and excreted by guinea pigs following i.v. administration, with 75% of the radiolabel excreted in the urine within 12 hr. By 48 hr, <1% of the radiolabel was present in liver, lungs, or kidneys of these animals.

In summary, BBT, *α*MB, and *α*EB are potent isozyme selective (P450 2Bx) inhibitors of guinea pig hepatic and pulmonary microsomal P450. BBT is metabolized *in vitro* to at least two reactive species capable of covalent modification of protein, and is rapidly (<12 hr) metabolized and excreted *in vivo*.

Dedicated with my deepest love and admiration

to my parents

Betty and Jack

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LIST OF ABBREVIATIONS

ABP	4-aminobiphenyl *N*-hydroxylation
ABT	1-aminobenzotriazole
[^{14}C]ABT	1-amino-[^{14}C]2,3-benzotriazole
AIA	allylisopropylacetamide
BA	benzaldehyde
BT	benzotriazole
BBT	*N*-benzyl-1-aminobenzotriazole
[^{14}C]2,3-BBT	*N*-benzyl-1-amino-[^{14}C]2,3-benzotriazole
[^{14}C]7-BBT	*N*-[^{14}C]7-benzyl-1-aminobenzotriazole
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BND	benzphetamine *N*-demethylation
βNF	β-naphthoflavone
BSA	bovine serum albumin
CCl_4	carbon tetrachloride
CH_2Cl_2	methylene dichloride
$CHCl_3$	chloroform
CH_3CN	acetonitrile
μCi	microcurie
CO	carbon monoxide
CO_2	carbon dioxide

DDC	3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine
DDEP	3,5-diethoxycarbonyl-2,6-dimethyl-4-ethyl-1,4-dihydropyridine
DMSO	dimethyl sulfoxide
*α*EB	*N*-*α*-ethylbenzyl-1-aminobenzotriazole
2EN	2-ethynylnaphthalene
ERF	7-ethoxyresorufin *O*-deethylation
EtAc	ethyl acetate
HA	hippuric acid
H_2O_2	hydrogen peroxide
HPLC	high pressure liquid chromatography
i.v.	intravenous
KH_2PO_4	monobasic potassium phosphate
LSS	liquid scintillation spectroscopy
*α*MB	*N*-*α*-methylbenzyl-1-aminobenzotriazole
3MC	3-methylcholanthrene
MeOH	methanol
MI	metabolic intermediate
$NaBH_4$	sodium borohydride
NADPH	*β*-nicotinamide adenine dinucleotide phosphate, reduced form
Na_2SO_4	sodium sulfate

NBT	*p*-nitroblue tetrazolium chloride
P450	cytochrome P450
P450 reductase	NADPH-cytochrome P450 reductase
PAH	polycyclic aromatic hydrocarbon
PB	phenobarbital
PCN	pregnenolone 16α-carbonitrile
PMS	3-(2-phenylethyl)-4-methylsydnone
PRF	7-pentoxyresorufin *O*-dealkylation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of the means
TCDD	tetrachlorodibenzo-*p*-dioxin
TEMED	tetramethylethylenediamine
TLC	thin layer chromatography
TS	Tris-saline
TTMS	3-[2-[(2,4,6-trimethylphenyl)thio]ethyl]-4-methylsydnone
TTS	Tween-Tris-saline

CHAPTER ONE

INTRODUCTION

1.1 Cytochrome P450

1.1.1 Overview

The cytochrome P450 (P450)-dependent monooxygenase enzyme system catalyzes the metabolism of a wide variety of lipophilic molecules of both endogenous and exogenous origin, including bile acids, cholesterol, steroid hormones, fatty acids, vitamins, biogenic amines, drugs, pesticides, and chemical carcinogens (Porter and Coon, 1991). P450 is widely distributed in nature, being found in animals (from mammals to invertebrates), plants and prokaryotes (Nelson *et al.*, 1993). The effect of P450-catalyzed metabolism is generally to convert lipophilic compounds into more polar forms to enable their excretion, either directly or after conjugation with molecules such as glutathione and glucuronic acid. P450 catalyzes a variety of metabolic oxidative reactions, including hydroxylation, epoxidation, oxidative deamination, desulfuration, and dehalogenation, as well as reduction. Some of these reactions are essential for life, such as the metabolism of cholesterol to corticoid and sex hormones, and biosynthesis of vitamin D_3 (Porter and Coon, 1991; Guengerich, 1991). The metabolism of xenobiotics is usually a detoxication process, but in some cases, metabolic activation of substrates occurs resulting in the formation of reactive electrophiles which are cytotoxic,

mutagenic or carcinogenic (Guengerich and Shimada, 1991). The metabolic activation of some compounds results in the formation of an active "drug" from a prodrug, for example, cyclophosphamide is converted to 4-hydroxycyclophosphamide and several other metabolites, and diazepam is metabolized to desmethyldiazepam, which is further metabolized to oxazepam (Gilman *et al.*, 1990).

P450 is a term applied to a group of hemoproteins defined by the unique spectral property of a λ_{max} of 450 nm for the ferrous (heme) iron-carbon monoxide complex, which is due to axial ligation of the heme iron with a thiolate group of a cysteine residue of the protein (Guengerich, 1991). The heme-binding cysteinyl residue, located near the carboxy terminus, is conserved in all P450s that have been sequenced (Black, 1992). The P450s are a group of isozymes belonging to a gene superfamily consisting of at least 36 gene families; twelve of these gene families, comprising 22 subfamilies, exist in all mammals examined thus far, and 221 P450 genes have currently been described in 31 eukaryotes and 11 prokaryotes (Nelson *et al.*, 1993). P450 proteins from different gene families have, by definition, <40% amino acid sequence homology, while P450s within the same subfamily have >55% sequence homology, and are found within "gene clusters" on individual chromosomes. P450s are named by an Arabic numeral denoting the gene family, followed by a capital letter indicating the subfamily, and an Arabic numeral to indicate the individual gene (eg. P450 1A1; nomenclature of Nelson *et al.*, 1993 will be used throughout). With few exceptions, each gene

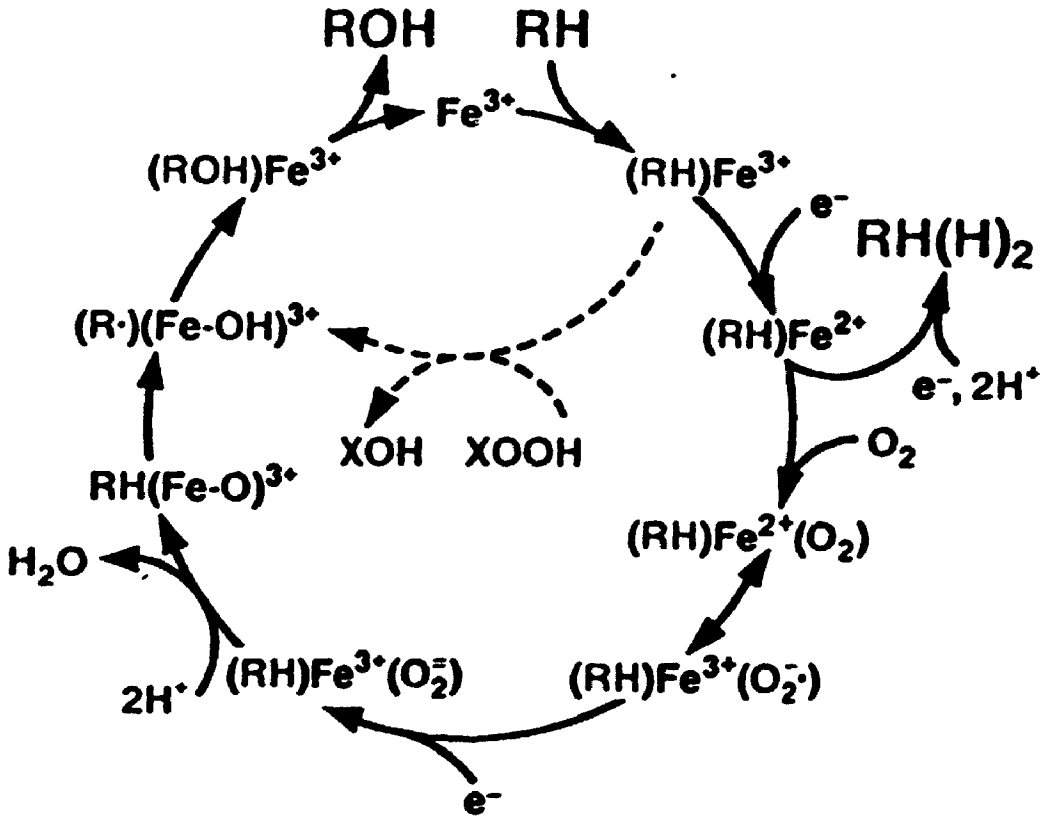
produces a single protein, or isozyme, of P450 (Nelson *et al.*, 1993), and it has been suggested that at least 25-30 different P450 genes are present in each animal species (Guengerich and Shimada, 1991). Many of these P450 isozymes are expressed constitutively, often in a development-, sex- or tissue-dependent manner, but their expression can often be increased, in an isozyme-selective manner, under the influence of an inducing chemical. There are numerous inducing agents known, including polycyclic aromatic hydrocarbons (PAHs), barbiturates, ethanol and other organic solvents, hypolipidemic drugs and steroid hormones (Gonzalez, 1989). Some P450s are only expressed constitutively [eg. hepatic P450s 2C7 (Ryan *et al.*, 1984; Gonzalez *et al.*, 1986a); and 2C11 (Ryan *et al.*, 1984)], while others are expressed only under the influence of an inducing agent [eg. hepatic P450s 2B1/2 (Omiecinski *et al.*1985); and 3A1 (Gonzalez *et al.*, 1986b)].

The P450-dependent monooxygenase system is membrane-bound, being found predominantly in the smooth endoplasmic reticulum (microsomal P450), but also in mitochondrial (Waterman *et al.*, 1986; Masumoto *et al.*, 1988) and nuclear (Fahl *et al.*, 1978; Rogan and Cavalieri, 1978) membranes, with the active site facing the cytosol (Peterson and Prough, 1986). The P450 system is comprised of an isozyme of cytochrome P450 which serves as the terminal oxidase, plus an intermediate electron acceptor which transfers electrons from the electron donor, NADPH. In the endoplasmic reticulum, the electron acceptor is the flavoprotein NADPH-cytrochrome P450 reductase (P450 reductase), containing the prosthetic groups FAD, which functions as the initial

electron acceptor from NADPH, and FMN, which reduces the P450 (Vermilion and Coon, 1978). The ratio of P450 reductase to cytochrome P450 in mammalian liver has been estimated to range between 1:10 and 1:100 (Nebert and Gonzalez, 1987). In the mitochondria the electrons are transferred to an FAD-containing ferredoxin-type reductase which subsequently transfers electrons to an iron-sulfur ferredoxin, which serves as an electron shuttle between the reductase and the P450 (Lambeth and Kamin, 1976).

P450s are termed monooxygenases because they generally catalyze the incorporation of one atom of oxygen into the substrate to form an oxidized product, while the other atom of oxygen is incorporated into water. Exceptions include catalysis of reduction reactions (Mico and Pohl, 1983), reductive cleavage of lipid hydroperoxides (Vaz and Coon, 1987; Vaz *et al.*, 1990), and the rearrangement of prostaglandin H_2 to thromboxane and prostacyclin by the P450s thromboxane synthase and prostacyclin synthase, respectively (Hecker and Ullrich, 1989). The first step of the P450 catalytic cycle (Figure 1.1) is the binding of substrate to the P450. This is followed by the reduction of the heme iron by one electron transferred via P450 reductase, and the rate of this reduction is often increased by binding of the substrate. Substrates that undergo reduction rather than oxidation, including epoxides, N-oxides, nitro and azo compounds, accept a second electron at this point along with two protons to give the reduced product. The third step in the oxidation cycle is the binding of molecular oxygen to the reduced P450 heme as the sixth ligand, *trans* to the cysteine thiolate, which results in the formation of an unstable $[Fe^{+2}O_2]$

Figure 1.1 **Schematic representation of the catalytic cycle of P450.** Fe represents the heme iron in the active site, RH the substrate, $RH(H)_2$ a reduction product, ROH a monooxygenation product, and XOOH a peroxy compound that can serve as an alternate oxygen donor. Adapted from Porter and Coon, 1991.


ROH
RH
Fe3+
(ROH)Fe3+
(RH)Fe3+
e−
RH(H)2
(R·)(Fe-OH)3+
(RH)Fe2+
e−, 2H+
XOH
XOOH
O2
RH(Fe-O)3+
(RH)Fe2+(O2)
H2O
2H+
(RH)Fe3+(O2=)
(RH)Fe3+(O2−·)
e−

complex. This is followed by transfer of the second electron from NADPH via P450 reductase. In mammalian microsomal P450 systems, cytochrome b_5 can participate as an alternative electron donor at this point, transferring an electron from NADH via NADH-cytochrome b_5 reductase or from NADPH via P450 reductase to the oxycytochrome P450 (Peterson and Prough, 1986). The next step, which is not well understood, involves the splitting of the oxygen-oxygen bond of molecular oxygen, with the concomitant uptake of two protons, resulting in the formation of H_2O and an "activated oxygen" species, comprised of the retained oxygen associated with the heme iron. Oxygen insertion into the substrate is believed to involve abstraction of a hydrogen atom (or electron) from the substrate and recombination of the resulting transient hydroxyl and carbon radicals to give the product. The oxygenated product then dissociates from the P450, restoring it to its ferric state, ready to begin another cycle. The rate-limiting step of P450 catalysis depends on the reaction being catalyzed (ie. the substrate) and the P450 isozyme involved; in some cases the reduction of P450 is limiting, while in other cases it is abstraction of the hydrogen atom or nonbonded electron (Guengerich, 1990a; Porter and Coon, 1991; Hollenberg, 1992).

P450 can also catalyze the oxidation of some substrates by the use of peroxides such as H_2O_2, lipid hydroperoxides, and xenobiotics such as *t*-butyl and cumene hydroperoxides (Kadlubar *et al.*, 1973; Rahimtula and O'Brien, 1975) as a substitute for O_2 and NADPH in a pathway known as the peroxide shunt. Lipid hydroperoxides and H_2O_2 are formed in cells as a result of lipid

peroxidation (Vaz *et al.*, 1990), and P450-catalyzed reactions can result in the formation of H_2O_2 when the oxidation of substrates is not tightly coupled to electron flow (Guengerich, 1991). However, the role of peroxide-mediated P450 catalysis *in vivo* is unknown (Guengerich, 1990a; Porter and Coon, 1991).

Many of the isozymes of P450, especially those found in the liver, have broad and overlapping substrate specificities, while others, particularly those involved in steroid metabolism, are fairly specific in their choice of substrate (Porter and Coon, 1991). Further, many P450s exhibit distinct regio- and stereo-specificity in the metabolism of various substrates (Jefcoate, 1986). Because the catalytic mechanisms by which the P450s carry out the different reactions are thought to be very similar, the substrate and catalytic specificity is thought to be due primarily to the three dimensional structure of the apoprotein. A single P450 is capable of catalyzing all types of P450-dependent reactions, and the reaction that occurs is a function of the substrate presented to the enzyme, and the fit of the substrate with the protein (Guengerich, 1991).

P450s are found in the greatest concentration in the liver, but have been detected at lower levels in all other tissues examined except erythrocytes and striated muscle (Guengerich and Shimada, 1991). Many of the P450s expressed in liver are also expressed extrahepatically [eg. P450s 1A1 (Kimura *et al.*, 1986), 2B4 (Parandoosh *et al.*, 1987), and 4B1 in some species (Vanderslice *et al.*, 1987)], while some isozymes are found in certain extrahepatic tissues but not in liver [eg. P450s 2A3 (Kimura *et al.*, 1989), 4A4

(Muerhoff *et al.*, 1987); and 4A5 (Johnson *et al.*, 1990)]. The liver is the major site of P450-dependent metabolism, due to the high concentration of total P450 and the number of isozymes present. However, extrahepatic P450-mediated metabolism can be important, both physiologically and pathobiologically.

1.1.2 Pulmonary Cytochrome P450

The lungs contain active enzyme systems, including P450, capable of metabolizing xenobiotics as well as endogenous chemicals. The lungs are a major target organ for several kinds of chemical-induced toxicity. The lungs are exposed to xenobiotics both externally by inspired air and internally by circulating blood. In the alveoli, the barrier between the epithelium and endothelium is very thin, facilitating gas exchange, but also facilitating the absorption of air-borne chemicals. The exposure of the lungs to blood-borne chemicals is significant due to the large volume of blood (venous drainage from almost the entire body) passing through the lungs (Bend *et al.*, 1985). The lungs also contain active accumulation systems for some endogenous as well as xenobiotic substances (Orton *et al.*, 1973; Smith *et al.*, 1982), and certain cell types are especially sensitive to chemical-induced toxicity due to the differential cellular distribution of bioactivating and detoxicating enzyme systems (Boyd, 1984).

Some chemicals that cause pulmonary damage are bioactivated by P450 primarily in the liver, with the toxic metabolite(s) being carried to the lungs via

the blood. An example of this type of pulmonary toxin is the pyrrolizidine alkaloid monocrotaline (Boyd, 1984; Yost *et al.*, 1989). More interesting from the viewpoint of pulmonary metabolism, however, are those compounds that are metabolized within the lung itself. These include chemicals such as 4-ipomeanol (Boyd, 1984) and 3-methylindole (Yost *et al.*, 1989), which are bioactivated by P450 in lung.

The lung contains more than forty cell types (Sorokin, 1970), but only some of these contain P450, which is found in highest concentrations in the nonciliated bronchiolar epithelial (Clara) cells and the alveolar type II cells, but has also been detected in alveolar macrophages, ciliated, goblet, and endothelial cells in a number of species (Serabjit-Singh *et al.*, 1988; Forkert *et al.*, 1989; Hall *et al.*, 1989; Strum *et al.*, 1990; Voigt *et al.*, 1990). A similar pattern of cellular distribution is seen with P450 reductase.

A number of isozymes of P450 have been identified in lung of various species, although the majority of the P450 content is comprised of three isozymes. P450s 2B4, 4B1 and 1A1 account for 85% (untreated) to 95% (2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated) of the total P450 content in rabbit lung (Domin *et al.*, 1986). P450s 2B4 and 4B1 are present in the highest concentrations, 65-70% and 30-35% of total P450, respectively (Overby *et al.*, 1992).

The orthologue of rabbit P450 2B4 is expressed in lung of rabbit (Serabjit-Singh *et al.*, 1979; Domin *et al.*, 1986), rat (Keith *et al.*, 1987; Imaoka *et al.*, 1989), guinea pig (Vanderslice *et al.*, 1987; Yamada *et al.*, 1992),

mouse (Forkert *et al.*, 1989; Chichester *et al.*, 1991), hamster (Strum *et al.*, 1990) and sheep (Williams *et al.*, 1991). The rat orthologue is P450 2B1, and because the orthologous proteins from the other species have not yet been fully sequenced and named, they will be referred to throughout as P450 2Bx. Gasser *et al.*(1988) have shown that two populations of mRNA encoding different microheterogenous forms of P450 2B4 are present in rabbit lung, but only one protein has been detected. P450 2Bx, which is inducible by treatment with phenobarbital (PB) in liver of all species examined, is not inducible in lung of these species (for example, Serabjit-Singh *et al.*, 1983; Forkert *et al.*, 1989; Imaoka *et al.*, 1989; Yamada *et al.*, 1992). The pulmonary content of 2Bx is unaffected by treatment with TCDD or other PAHs (Domin *et al.*, 1986; Forkert *et al.*, 1989). This isozyme is suspected of being one of the major isozymes involved in the pulmonary bioactivation of 4-ipomeanol (Wolf *et al.*, 1982; Slaughter *et al.*, 1983).

An orthologue of P450 4B1 has been found in lung of rabbit (Serabjit-Singh *et al.*, 1979; Vanderslice *et al.*, 1987; Gasser and Philpot, 1989), rat (Vanderslice *et al.*, 1987; Gasser and Philpot, 1989), guinea pig, mouse, hamster, monkey (Vanderslice *et al.*, 1987), and human (Nhamburo *et al.*, 1989). Because the orthologous proteins from other species have not yet been fully sequenced and named, they will be referred to throughout as P450 4Bx. Unlike P450 2Bx, which is expressed in liver as well as lung of all species examined, only rabbit and hamster have been found to express P450 4Bx in liver, and only rabbit hepatic 4B1 is inducible by phenobarbital. Similarly to

2Bx, however, pulmonary 4Bx is not induced by PB treatment in any species examined (Vanderslice *et al.*, 1987), and is not affected by treatment with PAHs (Domin *et al.*, 1986). P450 4Bx is the other major isozyme involved in the bioactivation of 4-ipomeanol in rodents (Wolf *et al.*, 1982; Slaughter *et al.*, 1983), but human 4Bx is much less active in this regard (Czerwinski *et al.*, 1991). 4Bx is also involved in the pulmonary bioactivation of aromatic amines to carcinogens in rabbits (Robertson *et al.*, 1981), but not in humans (Nhamburo *et al.*, 1989).

Cytochrome P450 1A1, but not the closely related 1A2, is expressed constitutively at low levels in lung of rabbit (Serabjit-Singh *et al.*, 1983; Overby *et al.*, 1992), rat (Keith *et al.*, 1987), guinea pig (Domin *et al.*, 1984; Philpot *et al.*, 1985), hamster (Sagami *et al.*, 1986), baboon (Wheeler *et al.*, 1990) and human (Omiecinski *et al.*, 1990; Wheeler *et al.*, 1990; Shimada *et al.*, 1992), and its content is increased in all species examined upon administration of PAHs such as TCDD, 3-methylcholanthrene (3MC), or β-naphthoflavone (βNF) [eg. 1A1 constitutes only 2-3% of total P450 in lungs of untreated rabbit, but increases to approximately 25% in rabbits treated with TCDD (Overby *et al.*, 1992)]. In human lung, higher levels of 1A1 mRNA have been noted in lungs of smokers compared to non-smokers (McLemore *et al.*, 1990). 1A1 was not found to be constitutively expressed in mouse lung when probed with a monoclonal antibody for 1A1/1A2, but was detected following treatment with 3MC (Forkert *et al.*, 1989). 1A1 is the P450 isozyme responsible for the bioactivation of PAHs to potentially carcinogenic products (DePierre and

Ernster, 1978; Ionannides and Parke, 1987).

A number of other P450 isozymes have been identified in lung at low levels or in a limited number of species. P450 4A4 is constitutively expressed at trace or non-detectable levels in rabbit lung, and is greatly induced (approximately 100-fold) during pregnancy (Williams *et al.*, 1984; Muerhoff *et al.*, 1987) or by treatment with progesterone (Yamamoto *et al.*, 1984; Matsubara *et al.*, 1987). This isozyme catalyzes the ω-hydroxylation of prostaglandins (Williams *et al.*, 1984). Orthologues of pulmonary P450 4A4 are yet to be described in other species.

P450 2F1 is constitutively expressed in human and rat lung, although its specific cellular localization is not known (Nhamburo, *et al.*, 1990). When human 2F1 was expressed in human hepatoma G2 cells using a vaccinia virus cDNA expression vector, it was very active in the bioactivation of the pulmonary toxin, 3-methylindole (Thornton-Manning *et al.*, 1991). P450 2F2 (Nelson *et al.*, 1993) is constitutively expressed in the Clara cells of mouse, but not rat, lung and is the mouse orthologue of human 2F1 (Ritter *et al.*, 1991; Chichester *et al.*, 1991). This isozyme, which bioactivates naphthalene via stereoselective oxidation to 1R,2S-naphthalene oxide, is thought to be responsible for the lung-specific (Clara cell-specific) necrosis seen in mice, but not rats or hamsters, following naphthalene exposure (Ritter *et al.*, 1991).

P450 2E mRNA is expressed in rabbit lung (Porter *et al.*, 1989), but no immunologically detectable protein has been found (Ding *et al.*, 1986; Porter *et al.*, 1989). P450 2E1 is constitutively expressed at a low level in rat (Song

et al., 1987) and human (Wheeler *et al.*, 1992) lung, and its content is increased in diabetic and acetone-treated rat lung (Song *et al.*, 1987).

P450 2A3 mRNA is expressed constitutively in lung, but not liver, of rats and is inducible by 3-MC (Kimura *et al.*, 1989). Voigt *et al.* (1990) found immunohistochemically detectable amounts of P450 3A2 in lungs of untreated rats; however, Imaoka *et al.* (1989) did not find this isozyme in rat pulmonary microsomes from untreated, phenobarbital- or 3-MC-treated rats.

Although a number of different isozymes have been described in lung of various species, some expressed constitutively at very low levels, the three quantitatively important, and most thoroughly characterized, isozymes in mammalian lung are orthologues of P450 2B4, 4B1 and 1A1.

1.2 Mechanism-Based Inhibition of Cytochrome P450

1.2.1 Overview

Mechanism-based inhibitors, also known as suicide substrates or k_{cat} inhibitors, are substrates for an enzyme which, during the course of metabolism by that enzyme, are converted to reactive intermediates that inactivate the enzyme (Rando, 1984). The parent compound is unreactive, requiring catalysis by the enzyme to form one or more reactive species responsible for its inactivation (Testa and Jenner, 1981). There are a number of criteria that characterize a compound as a mechanism-based inhibitor: a) inactivation of the enzyme should be time-dependent; b) the rate of inactivation should be slowed by competitive inhibitors or other substrates; c) the rate of inactivation should

be pseudo-first order; and d) the kinetics of inactivation should be saturable (Rando, 1984). As well, mechanism-based inactivators of P450 are dependent upon the cofactor NADPH and molecular oxygen, as these factors are required for the catalytic activity of P450.

The efficiency of a mechanism-based inhibitor can be determined by measuring the number of catalytic cycles required per inactivation event, often referred to as the partition ratio (Loosemore *et al.*, 1981). Partition ratios can range from 2 (for 10-undecynoic acid; CaJacob *et al.*, 1988) to 520 (for cyclopropyl benzyl ether; Guengerich *et al.*, 1984), and as the number of catalytic cycles increases, the inhibitor efficiency decreases (Rando, 1984). Partition ratios are normally greater than one because the enzyme can metabolize the substrate by more than one pathway, generating non-suicidal as well as suicidal metabolites, and because the reactive metabolites may be capable of diffusing away from the enzyme to react with other cellular macromolecules (De Matteis, 1987).

Following *in vivo* administration of mechanism-based inhibitors, *de novo* synthesis of P450 is normally required to restore P450-dependent metabolic activity (Murray and Reidy, 1990).

While many mechanism-based inactivators are general inhibitors, that is, they inactivate many or all of the P450 isozymes [eg. SKF 525A (Murray, 1988); 1-aminobenzotriazole (ABT, Ortiz de Montellano *et al.*, 1981a)], those which are selective for the inactivation of one or a few isozymes [eg. acetylenic fatty acids (Muerhoff *et al.*, 1989); chlorofluoroacetamides (Halpert *et al.*,

1990)] are potentially the most useful, not only as investigative probes, but also for potential clinical use as their effectiveness would be greater while undesired biological effects should be minimized (Murray and Reidy, 1990).

There are a number of uses for mechanism-based inhibitors of P450, both investigative and clinical: a) investigation of the topology of the prosthetic heme group (De Matteis *et al.*, 1983; Kunze *et al.*, 1983; Ortiz de Montellano *et al.*, 1983a) and the active site of P450s (eg. the 4A isozymes involved in fatty acid hydroxylation; CaJacob *et al.*, 1988); b) identification of amino acid residues present at or near the active site of P450 (Halpert, 1981; Halpert *et al.*, 1983); c) investigation of some of the processes involved in hepatic heme biosynthesis and the effect of different types of mechanism-based inactivation of P450 on these processes (Marks *et al.*, 1981; McCluskey *et al.*, 1986; Lukton *et al.*, 1988; Marks *et al.*, 1988; Mackie *et al.*, 1990); d) investigation of the degradation of P450 isozymes, which can be accelerated following mechanism-based inactivation of some forms of the hemoprotein (Tephly *et al.*, 1986; Correia *et al.*, 1992a, 1992b; Tierney *et al.*, 1992); e) investigation of the involvement of the P450 system and/or specific isozymes of P450 (through the use of isozyme-selective mechanism-based inhibitors) in the metabolism/bioactivation of endogenous [eg. arachidonic acid (Knickle and Bend, unpublished observations)] or exogenous [eg. 3-methylindole (Huijzer *et al.*, 1989; Nichols *et al.*, 1990)] compounds; f) investigation of the mechanisms of oxidation by P450 by examining the metabolism of various functional groups and the metabolites and reactive intermediates formed by certain isozymes from

mechanism-based inhibitors (Halpert, 1981; Ortiz de Montellano and Komives, 1985; Grab *et al.*, 1988; Decker *et al.*, 1989); g) possible clinical use to prevent the P450-mediated metabolism of a drug to inactive forms, or to prevent formation of toxic metabolites of a drug (Fleming *et al.*, 1992); and h) co-administration of mechanism-based inhibitors such as piperonyl butoxide with insecticides to decrease their rate of detoxication (Hodgson *et al.*, 1973).

Several mechanism-based inhibitors are drugs used clinically, which can lead to significant interactions if administered in conjunction with other pharmaceuticals. Macrolide antibiotics such as troleandomycin (Pessayre *et al.*, 1982) and erythromycin (Danan *et al.*, 1981) induce, and then cause the inhibition of P450 3A4, an isozyme involved in the metabolism of a wide variety of pharmaceuticals (Guengerich *et al.*, 1991). This can result in severe drug interactions, including cardiac complications in a few individuals from the effect of erythromycin on the metabolism of terfenadine, a commonly used antihistamine which is primarily, if not exclusively, metabolized by P450 3A4 (Yun *et al.*, 1993). The antibiotic chloramphenicol is known to increase the plasma levels and half-lives of other drugs (eg. tolbutamide, dicoumarol) in humans (Christensen and Skovsted, 1969) and its mechanism-based inactivation of P450 was suggested as the possible cause (Adams *et al.*, 1977).

Depending on the nature of the metabolic intermediate formed, inhibition can be reversible (formation of metabolic intermediate complex) or irreversible (covalent attachment to the prosthetic heme or the apoenzyme; Testa and

Jenner, 1981). The majority of the known mechanism-based inactivators of P450 act through the covalent modification of the enzyme. Reactive intermediates can form covalent attachments with the prosthetic heme moiety or the P450 apoprotein (De Matteis, 1987). In some instances, covalently modified heme moieties degrade into reactive products that subsequently alkylate the apoprotein (Osawa and Pohl, 1989). Some mechanism-based inhibitors act through a combination of all three of these mechanisms (Osawa and Pohl, 1989).

Mechanism-based inhibition of P450 as described above is distinct from reversible competitive inhibition of P450 (involving competition by substrates for the P450 binding site), in that mechanism-based inhibition requires metabolic activation of a P450 substrate, while competitive inhibition does not (Ortiz de Montellano and Reich, 1986; Murray and Reidy, 1990).

1.2.2 Metabolic Intermediate Complexes

Metabolic intermediates that form reversible complexes with P450 have a strong affinity for the iron of the prosthetic heme group in its reduced (Fe(II)) form, and generate complexes characterized by an absorbance maximum between 430 and 455 nm. These complexes can be formed both *in vitro* and *in vivo* (Testa and Jenner, 1981). The formation of metabolic intermediate (MI) complexes sequesters the P450 in a catalytically nonfunctional state, due to the stability of the complex; however, no physical alteration of the P450 occurs, and the *in vitro* dissociation of the MI complex through the oxidation

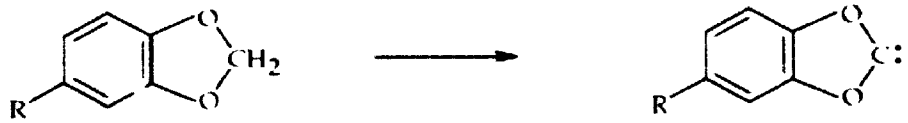
of P450 by the addition of the oxidant potassium ferricyanide (Schenkman *et al.*, 1972) results in reactivation of catalytic activity. Although activity can be recovered *in vitro*, this is unlikely to occur *in vivo*, making the formation of MI complexes with P450 a functionally irreversible inhibition (Murray and Reidy, 1990). Complexed P450 is unable to bind carbon monoxide (CO), making it undetectable by assay of the reduced, CO-bound spectrum (Testa and Jenner, 1981). MI complex formation is associated with certain chemical structures, including benzodioxoles (methylenedioxybenzene compounds), certain hydrazines, and a number of alkylamines (Murray and Reidy, 1990).

Methylenedioxybenzene derivatives (Hodgson *et al.*, 1973), including isosafrole and piperonyl butoxide, form MI complexes when the P450 heme is in the ferric, as well as the ferrous, state (Philpot and Hodgson, 1971). Consequently, the methylenedioxybenzene MI complexes cannot be dissociated by oxidation with potassium ferricyanide, reappearing immediately upon reduction of the P450 (Buening and Franklin, 1976). The ferrous P450 complexes can be dissociated by irradiation with light of a similar wavelength as the absorbance maximum (Ullrich and Schnabel, 1973). However, the complexes formed with ferric P450 are not stable, and can be dissociated by lipophilic compounds, including P450 substrates (Dickins *et al.*, 1979). Complex formation is thought to result from the formation of a reactive carbene (Figure 1.2a) through oxidation of the dioxymethylene bridge (Ortiz de Montellano and Reich, 1986).

A number of hydrazine derivatives, including 1,1-disubstituted hydrazines

Figure 1.2 **Generalized structures of proposed reactive metabolites which cause mechanism-based inactivation of P450.** Details are in text. Adapted from Ortiz de Montellano and Reich, 1986 (a,b,c); Halpert, 1982 (d); Yun *et al.*, 1992 (e); Augusto *et al.*, 1982 (f); Grab *et al.*, 1988 (g); Ortiz de Montellano and Watanabe, 1987 (h,i); Macdonald *et al.*, 1982 (j).

a) Methylenedioxy Compounds



carbene

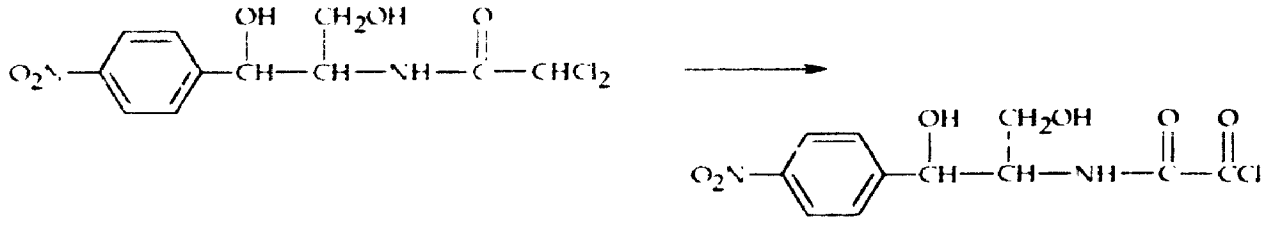
b) 1,1-Disubstituted Hydrazines

nitrene

c) Alkyl and Aromatic Amines

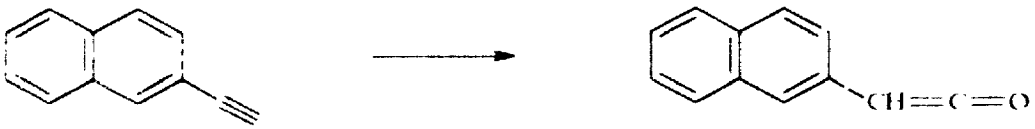
nitroso

d) Chloramphenicol



oxamyl chloride

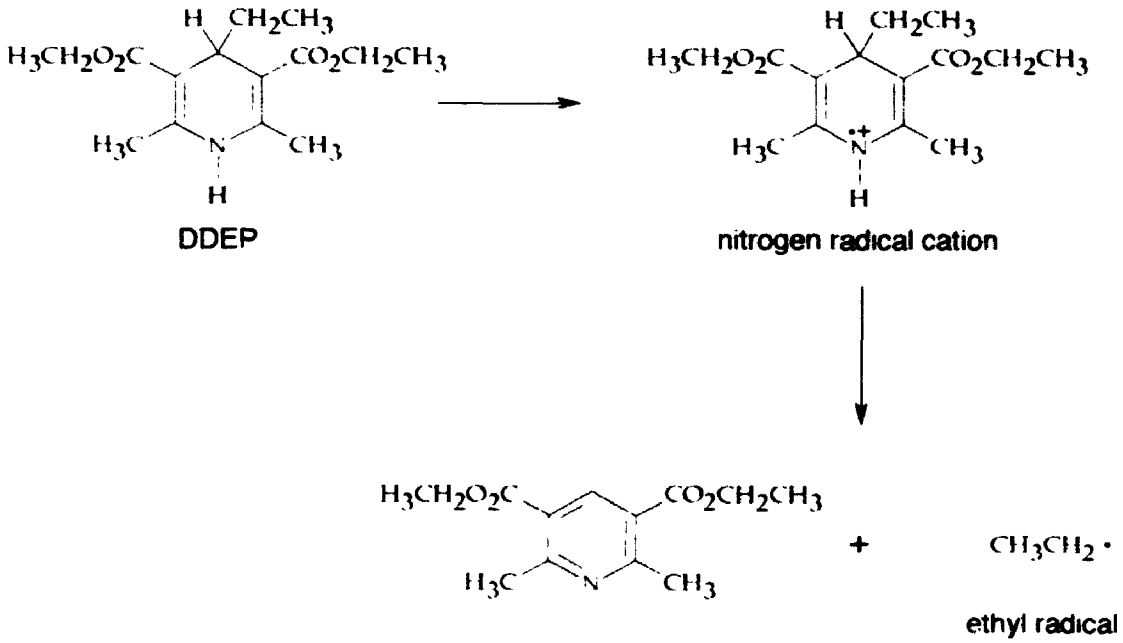
e) 2-Ethynylnaphthalene



ketene

Figure 1.2, con't.

f) Dihydropyridines



g) Sydnones

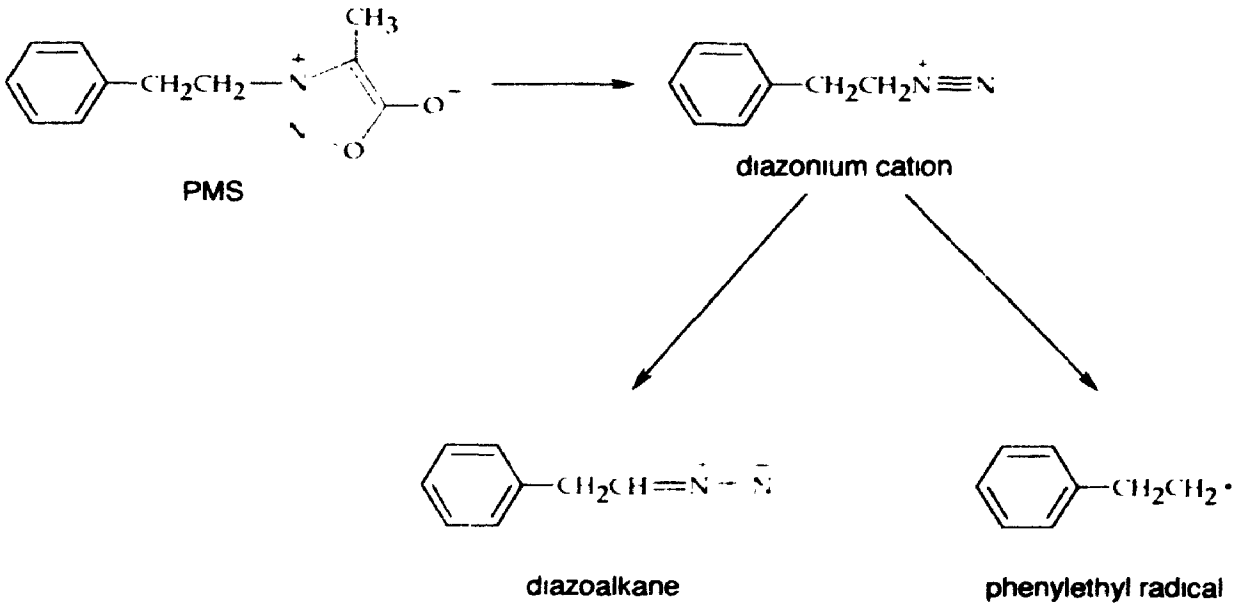
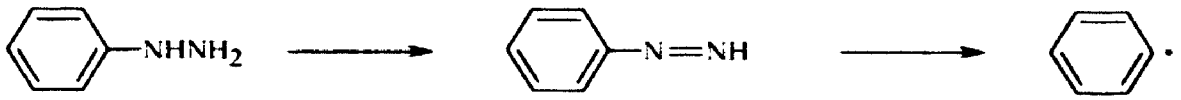


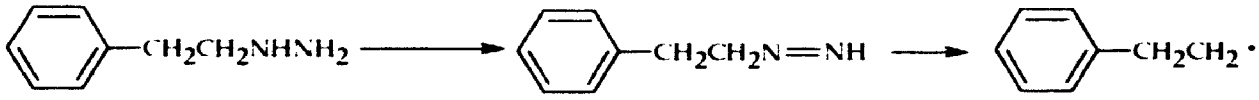
Figure 1.2, con't.

h) Phenylhydrazine



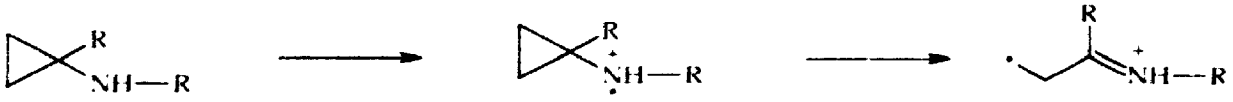
phenyldiazene phenyl radical

i) Phenelzine



phenylethyldiazene phenylethyl radical

j) Cycloalkylamines



aminium radical cation carbon-centered radical

(Hines and Prough, 1980), and monosubstituted hydrazines and hydrazides (Moloney *et al.*, 1984) also form MI complexes with both ferric and ferrous P450. Analogous to the methylenedioxybenzene derivatives, the species forming the complex is thought to be a nitrene (Figure 1.2b; Ortiz de Montellano and Reich, 1986).

Alkylamine derivatives such as SKF 525-A (Schenkman *et al.*, 1972), the macrolide antibiotics troleandomycin (Pessayre *et al.*, 1981) and erythromycin (Danan *et al.*, 1981), the antiparkinsonian agent orphenadrine (Bast and Noordhoek, 1982), some amphetamines (Franklin, 1977), tricyclic antidepressants (Murray and Field, 1992), and aromatic amines including sulfanilamide and dapsone (Franklin, 1976) form MI complexes through P450-dependent oxidation of a secondary or tertiary amine to a nitroso species (Figure 1.2c) following *N*-dealkylation and *N*-hydroxylation (Ortiz de Montellano and Reich, 1986).

Administration *in vivo* of many of these complex-forming agents (eg. SKF 525-A, macrolide antibiotics and methylenedioxybenzenes) results in the induction of specific isozymes of P450, usually those involved in the metabolism of the agent to the complex-forming intermediate (Buening and Franklin, 1976; Danan *et al.*, 1981; Pessayre *et al.*, 1981; Murray *et al.*, 1983).

1.2.3 Covalent Modification of the P450 Apoprotein

There are a number of possible sites within the P450 apoprotein where

alkylation by reactive metabolites could lead to inactivation of the catalytic function of the enzyme. Covalent modification at that portion of the apoprotein where substrate binding occurs could alter the binding of substrates such that they are improperly oriented with respect to the heme, or prevent substrate binding entirely. Alternatively, irreversible binding may occur at a region of the active site that would result in a disruption of electron flow from the NADPH-cytochrome P450 reductase, or a loss of affinity for the heme group (De Matteis, 1987).

1.2.3.1 Chloramphenicol

Chloramphenicol is the best characterized mechanism-based inhibitor acting via protein modification. This antibiotic was first shown to inhibit drug metabolism *in vitro* and to be converted to reactive metabolites which covalently bind to microsomal protein by Pohl and Krishna (1978). It was subsequently demonstrated that the inactivation of P450-dependent enzyme activity in a reconstituted system purified from PB-treated rat liver was accompanied by the covalent binding of [1,2-^{14}C]chloramphenicol-derived radioactivity to cytochrome P450, with no loss of spectrally detectable P450 or heme (Halpert and Neal, 1980).

The major covalently bound adduct (accounting for approximately 50% of the bound [^{14}C]) both *in vitro* and *in vivo*, was identified as chloramphenicol oxamic acid bound to the ϵ-amino group of one or more lysine residues in the apoprotein (Halpert, 1981; Halpert *et al.*, 1983), presumably derived from an

oxamyl chloride intermediate (Figure 1.2d) formed during the oxidative dechlorination of chloramphenicol (Pohl and Krishna, 1978; Pohl *et al.*, 1978; Halpert, 1981). The remainder of the bound material was removable by hydroxylamine treatment of the protein and was released as oxalic acid but has not been fully characterized. Interestingly, prolonged incubation of the inactivated P450 led to the spontaneous release of some of this oxalic acid with the concomitant partial reactivation of P450 activity, suggesting that this adduct may also participate in the inactivation of the P450 (Halpert, 1981; 1982).

Following chloramphenicol inactivation of P450, NADPH oxidase activity and the enzymatic reduction of P450 catalyzed by P450 reductase were inhibited, but substrate binding to the P450, and monooxygenase activity supported by hydroperoxides, were not inhibited. These results led to the conclusion that inhibition of monooxygenase activity was due to the binding of chloramphenicol metabolites to amino acid residues close to the heme moiety, thereby blocking electron transport from P450 reductase to the P450 (Halpert *et al.*, 1983; Halpert *et al.*, 1985b).

The covalent adduct formation of activated chloramphenicol was selective for those isoyzmes induced by PB (vs those induced by βNF, pregnenolone 16α-carbonitrile (PCN) or clofibrate) in rat liver microsomes (Halpert *et al.*, 1983; Halpert *et al.*, 1985a), and for the major PB-inducible P450 in dog liver (Ciaccio *et al.*, 1987), but also caused inactivation of some constitutive rat liver isozymes (Halpert *et al.*, 1985a). By synthesizing

analogues of chloramphenicol with modifications to various portions of the molecule, Halpert and co-workers have generated compounds selective for the inactivation of rat P450s 1A1 [*N*-(2-*p*-nitrophenethyl)dichloroacetamide; Miller and Halpert, 1987] and 2B1 [*N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide; Halpert *et al.*, 1990].

Unlike many mechanism-based inactivators of P450, multiple doses of chloramphenicol did not induce the level of cytochrome P450 in rat liver (Halpert *et al.*, 1988).

1.2.3.2 Methoxsalen

Methoxsalen (8-methoxypsoralen) and other psoralen derivatives (Letteron *et al.*, 1986; Tinel *et al.*, 1987) are known to be bioactivated by P450 to reactive metabolite(s) that covalently modify the P450 apoprotein both *in vitro* and *in vivo*, resulting in inhibition of monooxygenase activity (Labbe *et al.*, 1989; Mays *et al.*, 1990). There was neither loss of heme, nor formation of alkylated porphyrin products accompanying the P450 inhibition, suggesting that alkylation of the P450 heme moiety did not occur (Fouin-Fortunet *et al.*, 1986). There was also no formation of MI complexes with P450 and methoxsalen (Fouin-Fortunet *et al.*, 1986; Mays *et al.*, 1990). Immunoprecipitation experiments indicated that several P450s were alkylated (Labbe *et al.*, 1989), and the level of covalent binding was increased in microsomes obtained from animals pretreated with dexamethasone, PB, or βNF (Labbe *et al.*, 1989; Mays *et al.*, 1990). Microsomal proteins other than P450 were also covalently

bound, but most of this binding could be removed by the inclusion of cysteine (Mays *et al.*, 1990) or glutathione in incubation mixtures (Fouin-Fortunet *et al.*, 1986), with no corresponding decrease in the degree of inactivation of monooxygenase activity, leading to the conclusion that a large portion (as much as 75%) of the covalent binding to microsomal protein was not involved in the irreversible inactivation of P450 (Mays *et al.*, 1990).

From experiments with ^{14}C and ^{3}H labelled methoxsalen, Mays *et al.* (1990) concluded that at least two reactive metabolites were involved in the covalent binding to microsomal proteins, and it has been suggested (Fouin-Fortunet *et al.*, 1986; Tinel *et al.*, 1987) that methoxsalen may be activated on the outer double bond of its furan ring to form a reactive radical species. This hypothesis was supported by the finding that trimethylpsoralen, in which a methyl group is present on the double bond of the furan ring, was inactive as a mechanism-based inactivator of P450 (Letteron *et al.*, 1986; Tinel *et al.*, 1987).

The site of covalent modification of P450 apoproteins has not been determined, but it has been suggested that at least a portion of the binding is at or very near the active sight, based on the observations of a reduced ability of P450 to bind CO (decrease in spectrally determined P450), and an increase in K_m values for a number of P450 substrates following methoxsalen treatment *in vitro* and *in vivo* (Fouin-Fortunet *et al.*, 1986; Labbe *et al.*, 1989; Mays *et al.*, 1990).

As is seen with many mechanism-based inhibitors of P450, repeated

administration of methoxsalen has been reported to cause P450 induction, in this case demonstrated by the increased turnover of caffeine and hexobarbital (Mays *et al.*, 1987a; Apseloff *et al.*, 1991), and methoxsalen itself (Mays *et al.*, 1987b).

1.2.3.3 2-Ethynylnaphthalene

2-Ethynylnaphthalene (2EN) is a mechanism-based inhibitor, selective for the inactivation of rat P450 1A2 (Hammons *et al.*, 1989). This compound also caused inactivation of rabbit, but not human 1A2 (Yun *et al.*, 1992). The loss of >95% of 1A2-catalyzed 2-naphthylamine *N*-oxidation in isosafrole-treated rat liver microsomes (where 1A2 constitutes approximately 40% of the P450 content; Thomas *et al.*, 1983) with a loss of only 15% of spectral P450 content indicated that alkylation of the heme could only be partially responsible for the inactivation observed, and that alkylation of the apoprotein at the active site might also contribute to the inactivation (Hammons *et al.*, 1989). Covalent binding of [^{3}H]2EN-derived radioactivity to purified rat and rabbit 1A2 led to the recovery of a single labelled tryptic peptide in each case (Yun *et al.*, 1992). The inactivation by 2EN is postulated (Hammons *et al.*, 1989; Yun *et al.*, 1992) to proceed via the formation of a reactive ketene (Figure 1.2e) or oxirene-related species as proposed for other acetylenic compounds (Ortiz de Montellano and Kimoves, 1985; CaJacob *et al.*, 1988).

The NADPH-dependent inactivation of P450 by 2EN via protein alkylation as opposed to prosthetic heme alkylation is similar to that exhibited by the

acetylenic fatty acids 10-undecynoic acid (CaJacob *et al.*, 1988) and 11-dodecynoic acid (Ortiz de Montellano and Reich, 1984), specific inhibitors of fatty acid hydroxylases, and is in contrast to a number of other compounds containing the acetylenic functional group, which inactivate P450 via alkylation of the heme moiety (Ortiz de Montellano and Kunze, 1980; Kunze *et al.*, 1983; Ortiz de Montellano and Komives, 1985).

1.2.4 Covalent Modification of the Prosthetic Heme

Mechanism-based inactivation of P450 by *N*-alkylation of the prosthetic heme group is the most common and well-characterized pathway. Chemicals known to cause inactivation of P450 by this mechanism can be grouped into two general categories: 1) unsaturated carbon compounds (olefins and acetylenes), and 2) nitrogen-containing heteroatomic substrates including dihydropyridine derivatives, sydnones, arylhydrazines, and ABT (Ortiz de Montellano and Correia, 1983).

1.2.4.1 Olefins and Acetylenes

The most intensively investigated olefin, or alkene, is allylisopropylacetamide (AIA), or 2-isopropyl-4-pentenamide (Ortiz de Montellano *et al.*, 1978; Ortiz de Montellano and Mico, 1981). Mechanism-based inhibitors in this group include ethylene (Ortiz de Montellano *et al.*, 1980; Ortiz de Montellano *et al.*, 1981b), octene (Ortiz de Montellano *et al.*, 1983b), propene (Kunze *et al.*, 1983), heptene, 3-methyl-1-octene, 4-ethyl-1-hexene,

methyl 2-isopropyl-4-pentenyl ether, methyl 2-isopropyl-4-pentenoate, 3-isopropyl-5-hexene-2-one (Ortiz de Montellano and Mico, 1980), secobarbital and other allyl-containing barbiturates (Levin *et al.*, 1972), fluroxene, vinyl fluoride, vinyl bromide (Ortiz de Montellano *et al.*, 1982), and vinyl chloride (Guengerich and Strickland, 1977).

Compounds known to inactivate P450 which contain an acetylenic functional group include acetylene, propyne, octyne (Kunze *et al.*, 1983), heptyne, decyne, tridecyne (White, 1980), phenylacetylene (Ortiz de Montellano and Kunze, 1980; Ortiz de Montellano and Komives, 1985), *p*-methylphenylacetylene, *p*-chlorophenylacetylene, *p*-nitrophenylacetylene, *o*-methylphenylacetylene (Komives and Ortiz de Montellano, 1987), 1-ethinylcyclohexanol, 1-ethinylcyclopentanol, 3-phenoxy-1-propyne, 3-(4-nitrophenoxy)-1-propyne, 3-(2,4-dichlorophenoxy)-1-propyne, 4-phenyl-1-butyne, 3-phenyl-1-propyne (Ortiz de Montellano and Kunze, 1980), and the 17-ethynyl-substituted contraceptive steroids ethinyl estradiol, norethisterone (norethindrone), norgestrel (Ortiz de Montellano *et al.*, 1979a; Blakey and White, 1986), and gestodene (Guengerich, 1990b).

Investigation of a number of compounds containing olefinic (Ortiz de Montellano and Mico, 1980), or acetylenic (Ortiz de Montellano and Kunze, 1980) groups, including the simplest alkene and alkyne, ethylene (Ortiz de Montellano and Mico, 1980; Ortiz de Montellano *et al.*, 1980) and acetylene (Ortiz de Montellano and Kunze, 1980), respectively, demonstrated that the unsaturated carbon-carbon bond is the only structural requirement for suicidal

destruction of P450 through heme *N*-alkylation. However, the presence of an unsaturated carbon-carbon bond in a substrate does not necessarily convey destructive activity (Ortiz de Montellano and Mico, 1980; White, 1980). Moreover, the unsaturated bond must be a terminal functional group (ie. monosubstituted) to give rise to *N*-alkylated porphyrin adducts (Ortiz de Montellano and Kunze, 1980; Ortiz de Montellano and Mico, 1980; White, 1980). In addition, olefins must be sterically unhindered at the allylic position for bioactivation to occur (Ortiz de Montellano and Mico, 1980). Acetylenes, on the other hand, retain P450 destructive capability even when the monosubstituted carbon is mono-, di-, or trisubstituted, although such compounds may have decreased effectiveness (Ortiz de Montellano and Kunze, 1980).

The protoporphyrin IX adducts formed from olefins and acetylenes are obtained by addition of a porphyrin nitrogen to the terminal carbon of the unsaturated bond and of an oxygen atom (as a hydroxyl group) to the internal carbon (Ortiz de Montellano *et al.*, 1981b; Ortiz de Montellano *et al.*, 1982; Kunze *et al.*, 1983), with the remainder of the substrate molecule remaining intact, unless there is a good leaving group (eg. halogen) present on the hydroxylated carbon (as in fluroxene and vinyl fluoride), in which case the leaving group is eliminated and a carbonyl derivative is obtained (Ortiz de Montellano *et al.*, 1982). The adducts formed with acetylenes are in the form of ketone or aldehyde products due to rearrangement of the second π-bond of the acetylenic group (Ortiz de Montellano and Kunze, 1981; Kunze *et al.*, 1983;

Ortiz de Montellano and Komives, 1985). *N*-Alkyl adduct formation occurs in a regiospecific manner with some, but not all, terminal olefins and acetylenes: ethylene, propene, and octene exclusively alkylate pyrrole ring D, whereas propyne and octyne alkylate pyrrole ring A (Kunze *et al.*, 1983). On the other hand, acetylene alkylates several of the pyrrole rings (Kunze *et al.*, 1983).

The catalytic mechanism that gives rise to these adducts, and the reactive intermediate(s) involved, are as yet unknown (Ortiz de Montellano and Komives, 1985; Komives and Ortiz de Montellano, 1987), but it has been demonstrated that epoxide metabolites are not involved (Guengerich and Strickland, 1977; Ortiz de Montellano *et al.*, 1979b; Ortiz de Montellano *et al.*, 1983b; Ortiz de Montellano and Komives, 1985). The involvement of a cation radical has also been ruled out because it is not possible to formulate a mechanism which rationalizes simultaneous cation radical formation (through abstraction of an electron from the triple bond) and acetylenic hydrogen migration from the terminal to the internal carbon (Komives and Ortiz de Montellano, 1987). One mechanism that has been proposed is initial oxygen transfer to the unsaturated bond by the iron-oxo complex of P450 to form either of two isomeric metallooxocyclobutene intermediates which can subsequently decompose to either *N*-alkylated porphyrin products or ketene metabolites that form ketone or aldehyde products (Ortiz de Montellano and Komives, 1985; Komives and Ortiz de Montellano, 1987).

Inactivation of P450 by some olefinic and acetylenic compounds occurs in an isozyme-selective manner. The 17α-acetylenic steroid, gestodene,

efficiently inactivates human liver microsomal P450 3A4 while causing little or no inactivation of P450s 2D6, 1A2, or 2E1 (Guengerich, 1990b). AIA, 1-ethinylcyclopentanol, and 17α-propadienyl-19-nortestosterone inactivated purified rat liver P450 2B1 but not 1A1 (Ortiz de Montellano *et al.*, 1981a). *In vivo* administration of AIA to PB-pretreated rats caused greater losses of hepatic P450 2B1, 2C11, and 3A1 compared to 2A1 or 2A4 activities (Bornheim *et al.*, 1987).

As with a number of mechanism-based inhibitors of P450 (macrolide antibiotics, methylenedioxybenzene compounds), AIA administration causes a rebound induction of P450 in liver to levels substantially above control values after causing the initial decrease (De Matteis, 1970; Ortiz de Montellano and Costa, 1986).

1.2.4.2 Dihydropyridines

3,5-Diethoxycarbonyl-1,4-dihydro-2,4,6-trimethylpyridine (DDC) and its analogues with a substitution at the 4-methyl position suicidally inactivate P450 through reactive species derived from the 4-alkyl group (Ortiz de Montellano *et al.*, 1981c; Augusto *et al.*, 1982a). The 4-ethyl (DDEP), 4-propyl, and 4-isobutyl analogues cause even greater inactivation of hepatic P450 and accumulation of green pigments (alkylated porphyrin adducts) than DDC itself (Augusto *et al.*, 1982a). Interestingly, the 4-isopropyl and 4-benzyl analogues inactivate P450 but do not result in green pigment formation, and it has been suggested that the higher stability of the 4-isopropyl and 4-benzyl radicals

could result in protein rather than heme alkylation (Augusto *et al.*, 1982a). Dihydropyridines with a 4-aryl substituent, such as nifedipine and nitrendipine, are P450 substrates but do not cause P450 inactivation or heme alkylation (Correia *et al.*, 1987; Correia *et al.*, 1989).

The 4-alkyl radical intermediates are generated by the one-electron oxidation of the dihyropyridine ring nitrogen resulting in formation of a nitrogen radical cation (Figure 1.2f), followed by aromatization of the dihydropyridine ring with the concomitant ejection of the 4-alkyl radical (Augusto *et al.*, 1982a; Lee *et al.*, 1988). It is the 4-alkyl radical that reacts with the heme of P450.

The heme adducts formed are *N*-alkylprotoporphyrins, for example *N*-ethylprotoporphyrin IX is formed from DDEP (Ortiz de Montellano *et al.*, 1981c). Although heme adduct formation with DDC analogues occurs on all four pyrrole rings, there is some degree of regioselectivity, with DDEP generating primarily the N_A and N_B regioisomers (Ortiz de Montellano *et al.*, 1981c; McCluskey *et al.*, 1989), and with the proportion of $N_{B/A}$ to $N_{C/D}$ regioisomers decreasing as the length or bulk of the *N*-alkyl group increases (McCluskey *et al.*, 1988; McCluskey *et al.*,1989). This has important implications for the porphyrinogenicity of these compounds (Marks *et al.*, 1988) due to the activity of these heme adducts as inhibitors of ferrochelatase [the enzyme that catalyzes the insertion of iron into protoporphyrin IX to make heme (Tait, 1978)], as it has been shown that $N_{B/A}$ regioisomers are much more potent inhibitors of ferrochelatase than the $N_{C/D}$ regioisomers (Ortiz de Montellano *et al.*, 1981d; McCluskey *et al.*, 1988). Heme adduct formation

from dihydropyridines also occurs in an isozyme selective manner, with DDEP generating greater quantities of the N_C regioisomer in liver of untreated rats, the N_A isomer in PB-treated rats (primarily P450 2B1), and the N_D isomer in βNF-treated (primarily P450 1A1) rats compared to the other regioisomers (De Matteis *et al.*, 1983).

Inhibition of P450-dependent catalytic activities also occurs in an isozyme-selective manner both *in vitro* (Riddick *et al.*, 1989) and *in vivo* (Tephly *et al.*, 1986; Correia *et al.*, 1987) with a number of DDC analogues. Rat hepatic P450 3A isoforms (Correia *et al.*, 1987; Riddick *et al.*, 1989; Riddick *et al.*, 1990), 2C6 (Tephly *et al.*, 1986; Correia *et al.*, 1987), 2C11 (Correia *et al.*, 1987; Riddick *et al.*, 1990), and 1A1 (Tephly *et al.*, 1986; Riddick *et al.*, 1989) are highly susceptible to inactivation, while 2A1 (Tephly *et al.*, 1986; Riddick *et al.*, 1990), and 2B1 (Tephly *et al.*, 1986; Correia *et al.*, 1987; Riddick *et al.*, 1989; Riddick *et al.*, 1990) are relatively resistant to inactivation.

1.2.4.3 Sydnones

Sydnones are another class of nitrogen heterocycles which cause suicidal inactivation of P450 via heme *N*-alkylation (Ortiz de Montellano and Grab, 1986) and result in porphyrin accumulation due to the inhibition of ferrochelatase (Sutherland *et al.*, 1986). The heme adducts have been identified as *N*-vinylprotoporphyrin IX from 3-[2-[(2,4,6-trimethylphenyl)thio]ethyl]-4-methylsydnone (TTMS; Ortiz de Montellano and Grab, 1986), and *N*-(2-phenylethyl)- and *N*-(2-phenylethenyl)protoporphyrin IX

from 3-(2-phenylethyl)-4-methylsydnone (PMS, Grab *et al.*, 1988). Interestingly, PMS, in contrast to TTMS, does not cause ferrochelatase inhibition or porphyrin accumulation, possibly due to the bulkiness of the phenylethyl and phenylethenyl groups in the *N*-alkylprotoporphyrins (Mackie *et al.*, 1990). Another possibility is a predominance of the porphyrin ring C and D regioisomers (Mackie *et al.*, 1990), which has been suggested for PMS (Grab *et al.*, 1988). The ring C and D isomers are much less effective than the ring A and B isomers in the inhibition of ferrochelatase (Ortiz de Montellano *et al.*, 1981d).

Sydnones, unlike DDC analogues, arylhydrazines (section 1.2.4.4), cycloalkylamines (section 1.2.6.3) and ABT (section 1.2.7), which are activated by oxidation of a nitrogen atom, undergo hydroxylation of the anionic ring carbon which results in fragmentation of the ring into pyruvic acid and the corresponding alkyldiazonium cation (Figure 1.2g; Ortiz de Montellano and Grab, 1986; Grab *et al.*, 1988). In the case of TTMS (Ortiz de Montellano and Grab, 1986) and the formation of *N*-(2-phenylethenyl)protoporphyrin IX from PMS (Grab *et al.*, 1988), the diazonium cation isomerizes to the corresponding diazoalkane (Figure 1.2g), and reaction of the diazoalkane with the heme results in the loss of N_2, and the insertion of the diazoalkane carbon, possibly as a carbene intermediate, into one of the iron-nitrogen bonds of the heme group to give a transient bridged Fe-CHR-N intermediate (Ortiz de Montellano and Grab, 1986). In the case of TTMS, in which a good leaving group (the arylthio moiety) is present, elimination of this leaving group produces the *N*-vinyl heme

adduct (Ortiz de Montellano and Grab, 1986). In the absence of a leaving group, as in PMS, two one-electron oxidation steps and the elimination of a proton produce the *N*-(2-phenylethenyl) adduct (Grab *et al.*, 1988). The generation of the *N*-(2-phenylethyl) heme adduct from PMS occurs through a different pathway: the diazonium cation, without the involvement of the diazoalkane intermediate, is apparently converted to the 2-phenylethyl radical (Figure 1.2g), with concomitant loss of N_2, and this carbon radical is probably responsible for the formation of the *N*-(2-phenylethyl) adduct (Grab *et al.*, 1988).

No reports of isozyme-selective P450 inactivation, or regioselective *N*-alkylprotoporphyrin IX formation have yet been made with sydnones.

1.2.4.4 Hydrazines

A number of hydrazines are mechanism-based inhibitors of P450, such as phenelzine [phenylethylhydrazine, (Muakkassah and Yang, 1981; Ortiz de Montellano *et al.*, 1983c)], 1-acetyl-2-phenylhydrazine and phenylhydrazine (Jonen *et al.*, 1982). These hydrazines undergo two-electron oxidation by P450 to unstable diazenes [Figure 1.2h and 1.2i, (Jonen *et al.*, 1982; Ortiz de Montellano and Watanabe, 1987)]. Phenylhydrazine (Itano, 1970; Tuck and Ortiz de Montellano, 1992) can also be chemically oxidized, by agents such as potassium ferricyanide, to the unstable phenyldiazene intermediate which can subsequently decompose to benzene and nitrogen (Chattaway, 1907). Phenelzine can also be chemically oxidized to the diazene intermediate

(Muakkassah and Yang, 1981).

2-Phenylethyldiazene is subsequently converted to the phenylethyl radical [Figure 1.2i, (Ortiz de Montellano *et al.*, 1983c; Ortiz de Montellano and Watanabe, 1987)], the alkylating species, resulting in the formation of *N*-(2-phenylethyl)protoporphyrin IX (Ortiz de Montellano *et al.*, 1983c). The phenyldiazene intermediate is likely converted to a phenyl radical (Figure 1.2h) as the heme alkylating species (Jonen *et al.*, 1982), analogous to the reaction of hemoglobin with phenylhydrazine (Augusto *et al.*, 1982b), and the known decomposition of phenyldiazene to benzene (Chattaway, 1907). Although an *N*-alkylated porphyrin adduct(s) formed by the P450-catalyzed oxidation of phenylhydrazine has not been unambiguously identified, the adduct formed with P450 heme from potassium ferricyanide oxidized phenylhydrazine is *N*-phenylprotoporphyrin IX (Raag *et al.*, 1990; Swanson *et al.*, 1991), and this is also the porphyrin adduct generated by the reaction of hemoglobin with phenylhydrazine (Augusto *et al.*, 1982b).

The *N*-alkyl porphyrin adduct is likely formed by initial reaction of the carbon-centered radical with the heme iron to give an alkyl-iron complex (Ortiz de Montellano *et al.*, 1983c) which is then oxidized to the *N*-alkyl porphyrin adduct. A stable phenyl-iron intermediate precedes heme *N*-alkylation in the reaction of hemoglobin with phenylhydrazine (Augusto *et al.*, 1982b), and is also formed in the potassium ferricyanide-mediated reaction of phenylhydrazine with purified P450 101 (Raag *et al.*, 1990), 2B1, 2B2 (Swanson *et al.*, 1991; Tuck and Ortiz de Montellano, 1992), 1A1 and 2E1 (Swanson *et al.*, 1991).

P450 heme pyrrole rings A and D are exclusively alkylated by intermediates formed from the potassium ferricyanide-mediated reaction of phenylhydrazine with P450 1A1, 2B1, 2B2 and 2E1 (Swanson *et al.*, 1991).

1.2.5 Covalent Binding of Heme to Protein

Covalent attachment of heme to P450 apoproteins occurs with a variety of structurally diverse compounds, many of which also cause alkylation of the heme group (Osawa and Pohl, 1989). With all compounds examined thus far, only a portion of the alkylated porphyrin becomes bound to apoprotein, ranging from 30-70% (Davies *et al.*, 1986a; Davies *et al.*, 1986b; Decker *et al.*, 1986; Correia *et al.*, 1987), with the remainder being present as intact modified porphyrin ("green pigments") and/or as other stable metabolites of heme, or as yet unidentified species (Davies *et al.*, 1986b; Decker *et al.*, 1986; Guengerich, 1986; Correia *et al.*, 1987). The precise chemical nature of the heme-derived protein adducts are unknown, but two possible pathways of adduct formation have been suggested (Osawa and Pohl, 1989). The radical intermediate could react with the heme group, resulting in an activated cation heme derivative which covalently binds to amino acid residues in the active site, or, alternatively, the radical intermediate could react initially with an amino acid residue(s) to produce an amino acid radical which then reacts irreversibly with the heme group (Osawa and Pohl, 1989).

1.2.5.1 Carbon Tetrachloride

Carbon tetrachloride (CCl_4) is metabolized by P450 via a reductive process (Manno *et al.*, 1988) to, among other products, the reactive trichloromethyl radical (Noguchi *et al.*, 1982b). This radical species is thought to be responsible for the suicidal inactivation of P450 via the alkylation of the heme group (Davies *et al.*, 1985; Davies *et al.*, 1986a; Manno *et al.*, 1988) and the attachment of a portion of the modified heme to the apoprotein both *in vitro* and *in vivo* (Davies *et al.*, 1985; Davies *et al.*, 1986a). CCl_4-mediated P450 inactivation is likely not mediated via lipid peroxidation, as was previously suggested (Slater, 1982), as it occurs in the absence of molecular oxygen, with no evidence of malondialdehyde production (Davies *et al.*, 1986a; Manno *et al.*, 1988). Studies employing immunoprecipitation demonstrated that the covalent binding of heme products to P450 protein primarily involved P450 2B1/2 (Davies *et al.*, 1985; Davies *et al.*, 1986a), which was consistent with the observation that a PB-inducible isozyme(s) selectively catalyzed the formation of the trichloromethyl radical from CCl_4 *in vivo* (Noguchi *et al.*, 1982b), although other isozymes of P450 and other microsomal proteins cannot be excluded.

1.2.5.2 DDEP

Oxidation of the heterocyclic substrate DDEP (section 1.2.4.2) results in the preferential inactivation of P450s 3A, 2C6 and 2C11 in rat (Correia *et al.*, 1987). The inactivation of 2C6 and 2C11 is thought to occur largely

through *N*-alkylation of the heme moiety (Correia *et al.*, 1987). However, the inactivation of P450 3A is accompanied by heme alkylation of P450 apoprotein both *in vitro* and *in vivo* (Correia *et al.*, 1987; Sugiyama *et al.*, 1989). The alkylation of P450 3A1/2 apoproteins by heme promotes the *in vivo* proteolytic loss of these isozymes (Correia *et al.*, 1989; Correia *et al.*, 1992b), whereas heme *N*-alkylation of P450 2C6 does not (Correia *et al.*, 1992b). The difference in the mechanisms of inactivation of these different P450 isozymes has been attributed to differences in the three-dimensional structure of the active site environment of the isozymes as it relates to the orientation of the substrate, and hence the reactive intermediate(s) formed, with the prosthetic heme vs amino acid residues of the apoprotein (Sugiyama *et al.*, 1989), similar to the manner in which different P450 isozymes direct heme *N*-alkylation onto different pyrrole nitrogens (section 1.2.4.2; De Matteis *et al.*, 1983).

A number of other mechanism-based inhibitors of P450 have been shown to cause covalent attachment of heme to microsomal proteins, although they have not yet been unequivocally identified as P450 proteins. These include spironolactone (Decker *et al.*, 1986), secobarbital (Lunetta *et al.*, 1989), norethindrone, phenylhydrazine, hydralazine (Davies *et al.*, 1986b), fluroxene, 1-octene, vinyl bromide, vinyl chloride, trichloroethylene, parathion (Guengerich, 1986), and hydroperoxides such as H_2O_2, cumene hydroperoxide (Decker *et al.*, 1986; Guengerich, 1986) and linoleic hydroperoxide (Osawa and Pohl, 1989).

1.2.6 Inactivation of P450 by a Combination of Mechanisms

Many mechanism-based inhibitors act through a combination of apoprotein alkylation, heme alkylation, and covalent binding of heme to P450 apoprotein, often in an isozyme-selective manner. However, the relative role played by each mechanism in the overall inactivation of P450 in these instances is not known.

1.2.6.1 AIA

Oxidation of the allylic substrate AIA causes inactivation of P450 not only by *N*-alkylation of the prosthetic heme (section 1.2.4.1) but also by covalent binding of heme to protein (Davies *et al.*, 1986a; 1986b; Bornheim *et al.*, 1987) and alkylation of the P450 apocytochrome (Bornheim *et al.*, 1985; Bornheim *et al.*, 1987). Immunoprecipitation experiments in microsomes from PB-treated rats demonstrated that 64% of the heme bound to microsomal protein was associated with P450 2B1/2 (Davies *et al.*, 1986b). Alkylation of P450 apoproteins likewise occurred with some rat hepatic isozymes (eg. P450 2B1/2) but not others (eg. P450 3A1; Bornheim *et al.*, 1987). Three possible reactive metabolites have been postulated to be responsible for the covalent modification of apoprotein: the AIA epoxide, its precursor, the cation radical species, and a protonated iminolactone (Bornheim *et al.*, 1987). AIA therefore inactivates P450 by potentially three mechanisms, although *N*-alkylation of the heme appears to be most prominent (Bornheim *et al.*, 1985; Bornheim *et al.*, 1987).

1.2.6.2 Secobarbital

Secobarbital, another allyl-containing substrate, also inactivates P450 by these three pathways, and it does so in an isozyme-selective manner (Lunetta *et al.*, 1989). Secobarbital preferentially inactivates P450 2B1 vs 3A1, 2C6, or 2C11 (Lunetta *et al.*, 1989). The majority of P450 2B1 inactivation is due to heme alkylation and protein alkylation, whereas the formation of heme-protein adducts contributes in a minor way (Lunetta *et al.*, 1989).

1.2.6.3 Cycloalkylamines

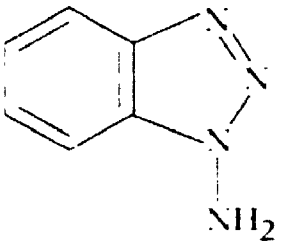
The nitrogen-containing cycloalkylamines, such as *N*-benzylcyclopropylamine, *N*-benzyl-1-methylcyclopropylamine (Hanzlik and Tullman, 1982; Macdonald *et al.*, 1982), *N*-(2-phenylcyclopropyl)benzylamine, *N*-(1-phenylcyclobutyl)benzylamine and *N*-cyclobutylbenzylamine (Bondon *et al.*, 1989), undergo one-electron oxidation of the nitrogen heteroatom to form an aminium radical cation, followed by opening of the cycloalkyl ring to form a reactive carbon-centered radical (Figure 1.2j), the presumed enzyme-inactivating species (Macdonald *et al.*, 1982; Guengerich *et al.*, 1984). *N*-Alkylation of the prosthetic heme probably occurs with cycloalkylamines as evidenced by the covalent [^{3}H] labelling of heme and the isolation of abnormal green pigments following incubation of rat hepatic microsomes with [7-^{3}H]- *N*-benzylcyclopropylamine (Hanzlik and Tullman, 1982), and the loss of microsomal heme upon incubation of PB-treated rat hepatic microsomes with *N*-benzylcyclopropylamine or *N*-benzyl-1-

methylcyclopropylamine (Macdonald *et al.*, 1982). However, Bondon *et al.*(1989) could find no evidence of *N*-alkylprotoporphyrin formation upon incubation of purified P450 2B1 or hepatic microsomes from PB-treated rats with cyclopropylamines. These compounds have also been found to cause alkylation of rat hepatic microsomal protein (Hanzlik and Tullman, 1982; Bondon *et al.*, 1989), and heme alkylation of microsomal protein (Bondon *et al.*, 1989), although the relative role of each of these pathways in the inhibition of P450 remains unknown.

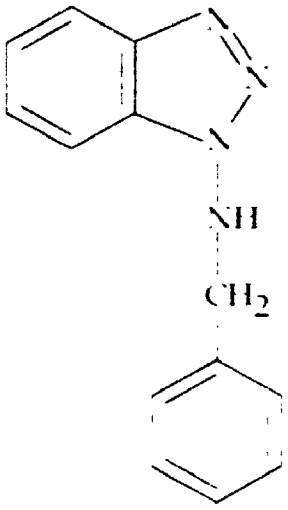
1.2.7 ABT and Its *N*-Aralkylated Derivatives

ABT (Figure 1.3), a nitrogen-containing heterocycle, is known to inactivate P450 via *N*-alkylation of the prosthetic heme moiety (Ortiz de Montellano and Mathews, 1981; Ortiz de Montellano *et al.*, 1984). ABT, which can be chemically oxidized by a variety of agents to benzyne and two molecules of nitrogen (Campbell and Rees, 1969), can also be bioactivated by P450 to form the highly reactive benzyne (Ortiz de Montellano *et al.*, 1984). Two possible routes for the bioactivation of ABT have been proposed [Figure 1.4, (Ortiz de Montellano *et al.*, 1984; Ortiz de Montellano and Reich, 1986)]. ABT could undergo one-electron oxidation of the exocyclic nitrogen, producing a nitrogen cation radical, followed by oxidation to a nitrene intermediate, followed by the loss of two molecules of nitrogen and the concomitant generation of benzyne (Figure 1.4A). Alternatively, the initial step could be hydroxylation of the exocyclic nitrogen (Figure 1.4B).

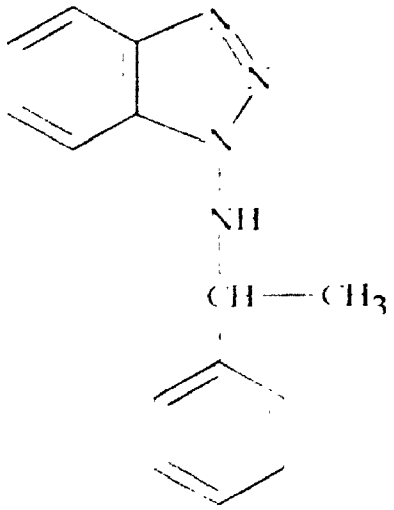
Figure 1.3 **Structures of ABT, BBT and *α*MB.**



ABT
1-aminobenzotrazole

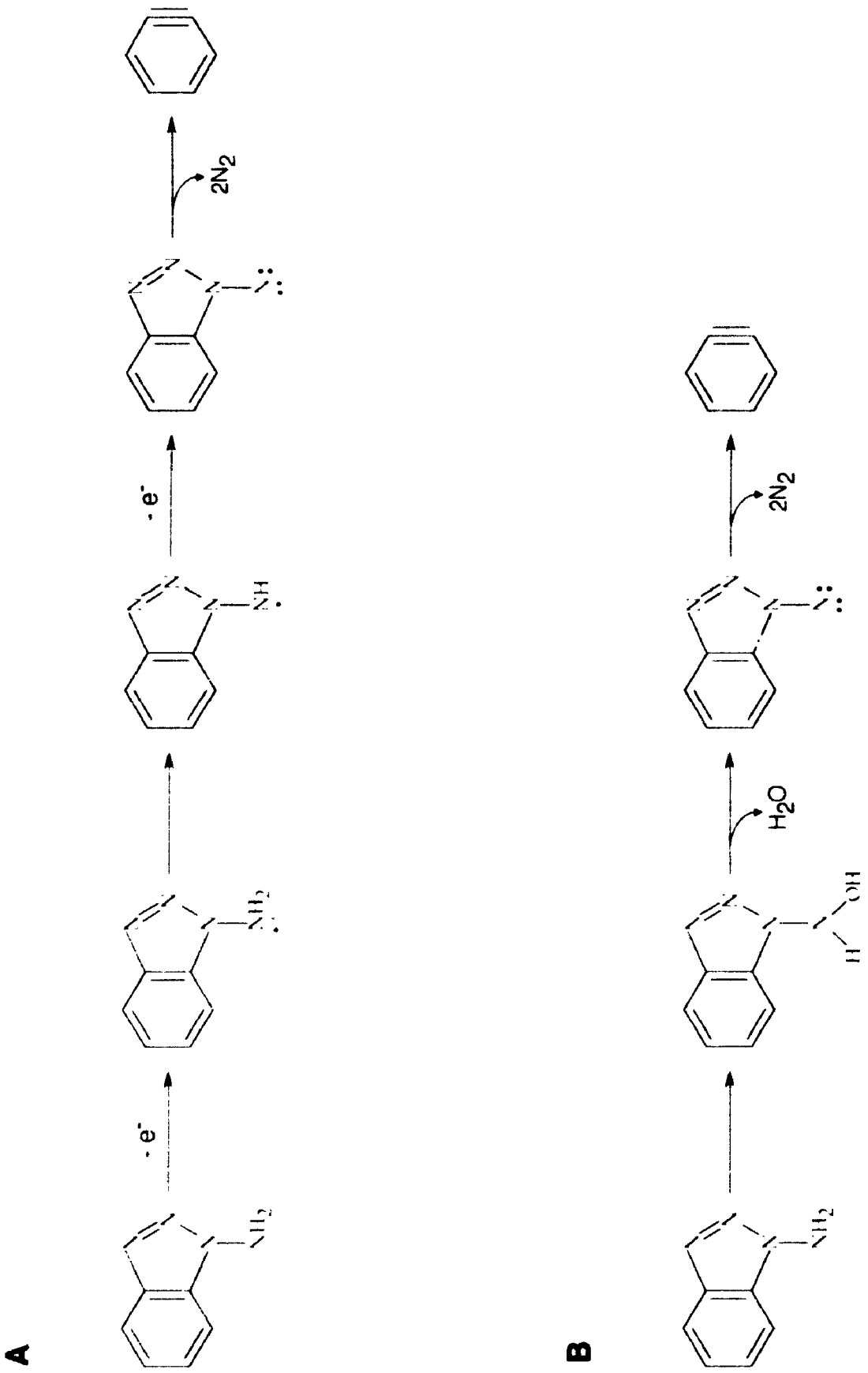


BBT
N-benzyl-1-aminobenzotriazole



αMB
N-α-methylbenzyl-1-aminobenzotriazole

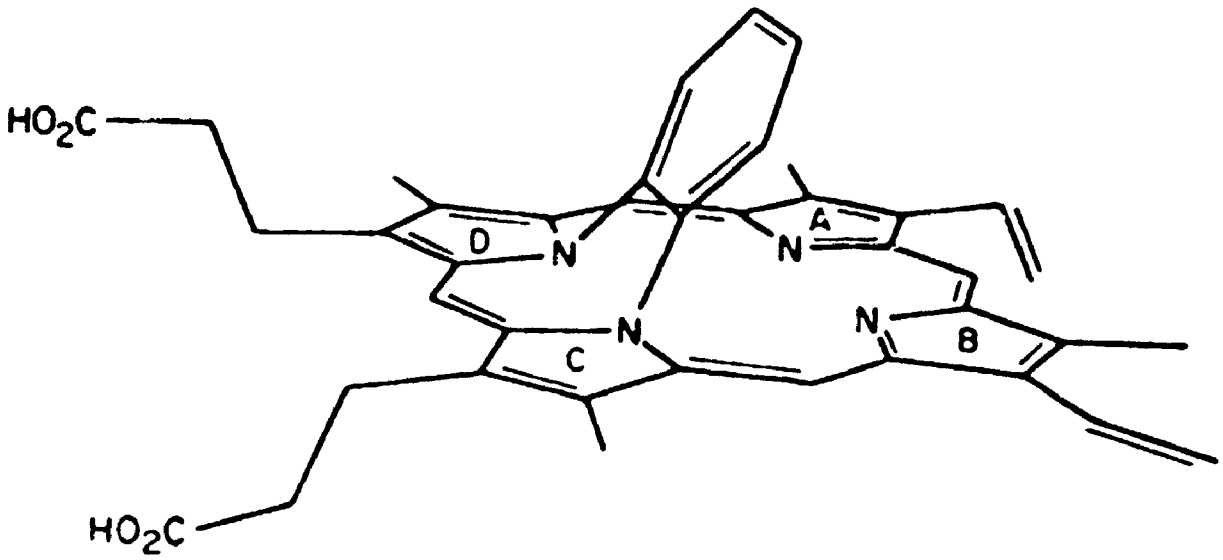
Figure 1.4 **Alternative pathways for the P450-dependent bioactivation of ABT to benzyne.** (A) Initial one-electron oxidation of the exocyclic nitrogen to produce a nitrogen cation radical, or (B) initial hydroxylation of the exocyclic nitrogen. Adapted from Ortiz de Montellano *et al.*, 1984 and Ortiz de Montellano and Reich, 1986.


A
B
$2N_2$
$-e^-$
H_2O

The benzyne intermediate adds across two nitrogen atoms in the prosthetic heme moiety to generate the unique *N,N*-dialkylated porphyrin adduct, *N,N*-bridged phenylene-protoporphyrin IX [Figure 1.5, (Ortiz de Montellano and Mathews, 1981; Ortiz de Montellano *et al.*, 1984)]. NMR analysis of the *N,N*-bridged adduct indicated that alkylation occurred on two vicinal (adjacent) rather than distal nitrogens (Ortiz de Montellano *et al.*, 1984). It is unknown whether benzyne directly adds across two vicinal nitrogens, or initially reacts with a pyrrole nitrogen and the iron atom, followed by oxidative rearrangement to the *N,N*-phenylene adduct (Ortiz de Montellano *et al.*, 1984), analogous to the mechanism of adduct formation of P450 and hemoglobin heme with phenylhydrazine (section 1.2.4.4). NMR analysis of the ABT-derived *N,N*-bridged adduct also indicated the presence of two major regioisomers of *N*-alkylated porphyrin (Ortiz de Montellano *et al.*, 1984), but the identity of these isomers remains unknown.

ABT is an effective mechanism-based inhibitor of P450 both *in vitro* and *in vivo* in a number of tissues, including liver (Ortiz de Montellano and Mathews, 1981; Ortiz de Montellano and Costa, 1986), lung (Mathews *et al.*, 1985; Mathews and Bend, 1993), and kidney (Mugford *et al.*, 1992) in a variety of animals such as rat (Ortiz de Montellano *et al.*, 1984; Ortiz de Montellano and Costa, 1986), rabbit (Mathews *et al.*, 1985; Mathews and Bend, 1993), mouse (Capello *et al.*, 1990), dog (Mico *et al.*, 1988), goat (Huijzer *et al.*, 1989), and human tissues (Ruangyuttikarn *et al.*, 1991), as well as in plants (Reichhart *et al.*, 1982). ABT is a potent inhibitor, with a

Figure 1.5 **Structure of the dimethylesterified *N,N*-dialkylated porphyrin, phenylene-protoporphyrin IX, obtained with ABT.** One of the four possible regioisomers is arbitrarily shown. From Ortiz de Montellano *et al.*, 1984.


HO2C
HO2C
D
N
N
A
N
C
N
B

concentration of 10 μM being effective in perfused rabbit lung (Mathews *et al.*, 1985) and rabbit pulmonary microsomes (Mathews and Bend, 1986). A partition ratio of 12 molecules of ABT metabolically eliminated per cytochrome P450 inactivated has been estimated (Ortiz de Montellano *et al.*, 1984), indicating that ABT is a very efficient mechanism-based inhibitor.

ABT inactivates P450 in a relatively non-isozyme-selective manner (Ortiz de Montellano *et al.*, 1981a; Mathews *et al.*, 1985; Ortiz de Montellano and Costa, 1986), with at least 80% of *in vivo* (Ortiz de Montellano and Costa, 1986), and 90% of *in vitro* (Ortiz de Montellano and Mathews, 1981) rat hepatic P450, and virtually 100% of *in vitro* rabbit pulmonary P450 (Mathews and Bend, 1986) being vulnerable to destruction with high concentrations (10 mM) of ABT. ABT effectively inactivates P450 while having no effect on P450 reductase (Mugford *et al.*, 1992), cytochrome b_5 (Ortiz de Montellano and Mathews, 1981; Mugford *et al.*, 1992), or flavin-containing monooxygenase (Mathews *et al.*, 1985).

Unlike the administration of many mechanism-based inhibitors of P450 [eg. AIA (Liem *et al.*, 1983; Ortiz de Montellano and Costa, 1986), macrolide antibiotics (Danan *et al.*, 1981), methylenedioxyphenyl compounds (Murray *et al.*, 1983)], the administration of ABT (single or multiple doses) does not result in rebound induction of P450 above control levels in rat liver (Ortiz de Montellano and Costa, 1986; Mugford *et al.*, 1992) or kidney (Mugford *et al.*, 1992).

The absolute requirement of the exocyclic amino group for metabolic

activation of ABT to benzyne was demonstrated by the replacement of this functional group with a hydrogen, hydroxyl or nitro group, which led to the loss of destructive activity (Ortiz de Montellano *et al.*, 1984). On the other hand, destructive activity and heme-alkylating ability were retained when substitutions were made to the exocyclic amino group with a variety of substituents, including acetyl (Ortiz de Montellano *et al.*, 1984), methyl (Ortiz de Montellano *et al.*, 1984; Mathews and Bend, 1986), butyl, benzyl, or *α*-methylbenzyl (Mathews and Bend, 1986) groups.

In attempts to produce mechanism-based inhibitors with the high degree of effectiveness of ABT, but with greater potency and isozyme-selectivity, Mathews and Bend (1986) synthesized two *N*-aralkylated derivatives of ABT, *N*-benzyl-ABT (BBT) and *N*-*α*-methylbenzyl-ABT (*α*MB; Figure 1.3). These compounds, in addition to containing the known destructive ABT nucleus, include molecular features that mimic benzphetamine, a substrate which is specific for pulmonary P450 2B4 (Serabjit-Singh *et al.*, 1983). These features include an *N*-benzyl group, an amine group in the desired region of oxidation, and an aromatic region of similar dimension, features which increased the selectivity for destruction of rabbit pulmonary P450 2B4 vs 1A1 and 4B1 (Mathews and Bend, 1986). In addition, their greater lipophilicity increased the potency of these ABT derivatives for the inactivation of the membrane-bound P450 compared to the hydrophilic ABT (Mathews and Bend, 1986).

The *in vivo* administration of BBT to PB-treated rats resulted in the isolation of a modified porphyrin pigment (Mathews and Bend, 1986) identical

to that isolated from ABT-treated rats (Ortiz de Montellano *et al.*, 1984), indicating that the mechanism-based inactivation of P450 by BBT resulted in heme *N*-alkylation. Also analogous to ABT, a partition ratio of approximately 11 turnovers/destructive event was calculated for *α*MB (Mathews and Bend, 1986).

BBT and *α*MB were also selective for the inactivation of P450 2B4 vs 1A1 or 4B1 in lung and liver of rabbits (Mathews and Bend, 1993) and guinea pigs (Knickle and Bend, unpublished results) following i.v. administration. These compounds also displayed great selectivity *in vivo* for the inactivation of pulmonary vs hepatic P450 in rabbit (Mathews and Bend, 1993) and guinea pig (Knickle and Bend, unpublished results), possibly due to accumulation by the facilitative uptake system for lipophilic basic amines in lung (Orton *et al.*, 1973).

Both *in vitro* and *in vivo*, *α*MB displayed a much greater degree of selectivity than BBT for inactivation of rabbit pulmonary or hepatic P450 2B4 (Mathews and Bend, 1986; 1993).

CHAPTER TWO

AIMS AND APPROACHES

2.1 Aims

ABT is a known potent mechanism-based inhibitor of P450 in a number of species. Two of its *N*-aralkylated derivatives, BBT and *α*MB, are known to selectively inactivate P450 2B4 in rabbit lung (Mathews *et al.*, 1985; Mathews and Bend, 1986), and to selectively inactivate pulmonary vs hepatic P450 *in vivo* in the rabbit (Mathews and Bend, 1993), but their effects have not been characterized in any other species. A number of *N*-alkylated derivatives of ABT have been examined for their potency and isozyme selectivity in rabbit lung (Mathews and Bend, 1986). *α*MB, which is more lipophilic and more sterically hindered about the amino nitrogen (the site of oxidation by P450) than the other analogues examined, is the most potent and isozyme (2B4) selective analogue developed to date (Mathews and Bend, 1986); however, it was not known whether greater potency or selectivity could be obtained by further alterations to the structure of these compounds. In the present series of investigations, the effects of ABT, BBT and *α*MB on the hepatic and pulmonary microsomal P450 monooxygenase systems of guinea pig were characterized. In addition, the next analogue in the series, *N*-*α*-ethylbenzyl-1-aminobenzotriazole (*α*EB), was synthesized and compared for its potency and selectivity as a P450 inhibitor with the other three compounds.

The mechanism of inactivation of P450 by ABT is known to involve *N*-alkylation of the P450 prosthetic heme. However, under conditions where BBT and *α*MB inhibit virtually 100% of *β*NF-treated rabbit pulmonary P450 2B4 and 1A1 activities, which together account for up to 80% of total pulmonary P450 (Serabjit-Singh *et al.*, 1983; Domin *et al.*, 1984), loss of spectrally assayed P450 is less than 40% (Mathews and Bend, 1986), suggesting that mechanisms in addition to *N*-alkylation of the heme moiety may be involved in the inactivation of P450 by these compounds. In addition, nothing is known about the metabolism of these compounds, either *in vitro* or *in vivo*, to products other than the reactive intermediate benzyne. The possibility of alkylation of P450 apoprotein by these compounds during the inactivation of guinea pig hepatic and pulmonary microsomal P450 was examined with [^{14}C]labelled ABT and BBT, and the metabolism of these compounds was studied *in vitro* under conditions of selective alteration of the P450 isozyme content through the use of specific inducing agents, and *in vivo* in untreated guinea pigs.

2.2 Approaches

The guinea pig was chosen for these studies because its lung has substantial P450-dependent monooxygenase activity and we wanted to determine whether the potency and isozyme selectivity of BBT and *α*MB extended across species. Since orthologous P450s in different species can differ markedly in substrate specificities (Kaminsky *et al.*, 1984; Umbenhauer

et al., 1987; Ged *et al.*, 1988), and alterations to the amino acid sequence of P450 isozymes as small as substitution of one amino acid can markedly alter substrate selectivity (Lindberg and Negishi, 1989), the effectiveness and selectivity of mechanism-based inhibitors across species cannot be predicted. It was also necessary to characterize the effects of these inhibitors on guinea pig P450 *in vitro* in preparation for further study of their isozyme and tissue (lung vs liver) selectivity *in vivo*, for which the guinea pig is the species of choice due to its smaller size than the rabbit.

The guinea pig is an excellent animal model for the study of pulmonary P450 because it has a relatively high concentration of P450 in lung, second only to rabbit (Philpot *et al.*, 1977). Guinea pig lung contains orthologs of the three isozymes that constitute most of the pulmonary P450 in rabbit; 2B4 (Vanderslice *et al.*, 1987; Yamada *et al.*, 1992), 4B1 (Vanderslice *et al.*, 1987; Gasser and Philpot, 1989), and 1A1 (Domin *et al.*, 1984; Philpot *et al.*, 1985), referred to, in this thesis, as 2Bx, 4Bx, and 1A1, respectively, for the guinea pig. Similar to the rabbit, neither 2Bx (Yamada *et al.*, 1992) nor 4Bx (Vanderslice *et al.*, 1987) is induced in guinea pig lung by PB treatment, but 1A1 is induced by treatment with PAHs (Domin *et al.*, 1984). Guinea pig liver also contains P450 2Bx, which is inducible by treatment with PB (Oguri *et al.*, 1991; Yamada *et al.*, 1992), and P450 1A1 and 1A2, inducible by treatment with PAHs (Domin *et al.*, 1984; Philpot *et al.*, 1985).

The relative monooxygenase activity of individual P450 isozymes or different families or subfamilies of isozymes can be determined through the use

of isozyme selective substrates. The activity of P450 1A1, an isozyme induced by PAHs, can be measured by the *O*-deethylation of 7-ethoxyresorufin, while 7-pentoxyresorufin is a selective substrate for P450 2B4, an isozyme induced by PB (Burke and Mayer, 1974; Burke *et al.*, 1985; Lubet *et al.*, 1985; Nakajima *et al.*, 1990). Benzphetamine is also a selective substrate for isozymes induced by PB (Serabjit-Singh *et al.*, 1983), and purified guinea pig hepatic 2Bx is known to catalyze the *N*-demethylation of benzphetamine (Narimatsu *et al.*, 1990). The *N*-hydroxylation of 4-aminobiphenyl is a selective assay for P450 4B1 in lung, and is also catalyzed by P450 1A2 (primarily) and 1A1 in guinea pig liver, which does not contain P450 4B1 (Vanderslice *et al.*, 1987).

CHAPTER THREE

EFFECTS OF ABT AND THREE *N*-ARALKYLATED ABT DERIVATIVES ON GUINEA PIG HEPATIC AND PULMONARY P450 *IN VITRO*

3.1 Objective

The objectives of this study were to determine the potency and isozyme selectivity of ABT, BBT and *α*MB in hepatic and pulmonary microsomes from untreated, PB- or *β*NF-induced guinea pigs, and to compare the potency and isozyme selectivity of the *N*-*α*-ethylbenzyl derivative of ABT, *α*EB, with the other three inhibitors in hepatic and pulmonary guinea pig microsomes.

3.2 Methods

3.2.1 Materials

NADPH, bovine serum albumin (BSA), and 2,4,6-tripyridyl-*s*-triazine were obtained from Sigma Chemical Co., St. Louis, MO. *β*-naphthoflavone (*β*NF) and 4-aminobiphenyl were purchased from the Aldrich Chemical Co., Milwaukee, WI. 7-Ethoxyresorufin and 7-pentoxyresorufin were purchased from Molecular Probes, Inc., Eugene, OR, and resorufin from Pierce, Rockford, IL. Benzphetamine was obtained from The Upjohn Co., Kalamazoo, MI. Phenobarbital sodium (PE, and all other chemicals (reagent grade or better) were purchased from BDH, Toronto, Canada.

40-60% and considerably less P450 was destroyed (8 and 12% for BBT and αMB, respectively) in βNF treated animals. BBT was selective for the inactivation of PRF vs BND activity (64% vs 43%) but ABT and αMB were not. There were no marked differences in the inhibition of PRF vs ERF activities with ABT, BBT or αMB (degree of inhibition, PRF/ERF = 0.8, 1.0 and 0.9, respectively) in βNF-induced guinea pigs (Table 3.1).

The data obtained in PB-induced guinea pigs were of interest for several reasons. First, virtually all of the PRF activity was inhibited by 100 μM BBT and αMB (90 + %) whereas this concentration of ABT inhibited less than half of the PRF activity in these hepatic microsomes. Also, ABT inhibited more ERF than PRF activity whereas the reverse was observed with BBT and αMB. The amount of spectrally assayed P450 destroyed by the three compounds in microsomes of PB-induced guinea pigs was very similar (29-33%). Finally, BBT and αMB were significantly more effective as inhibitors of hepatic PRF and ERF than of BND activity after PB treatment (Table 3.1).

The inactivation of monooxygenase activity was generally greater in hepatic microsomes from untreated guinea pigs compared to either βNF- or PB-induced animals (Table 3.1). The sole exception to this was PRF activity in PB-induced animals, an activity obviously catalyzed by a P450 isozyme (or isozymes) very susceptible to inactivation by BBT and αMB (Table 3.1).

In general, the % loss of spectrally assayed P450 caused by 100 μM ABT was similar to the % loss of PRF and BND activity in all three groups of animals; however, significantly more ERF activity was inhibited than the

0.1 M potassium phosphate buffer, pH 7.4 and stored at -80°C.

Microsomal protein concentrations were determined by a modification of the method of Lowry *et al.*(1951). Following dilution of aliquots (5-50 μl) of protein samples to 0.5 ml with H_2O, 2.5 ml of the following solution were added: 0.4% NaOH in 3% anhydrous sodium carbonate:4% potassium sodium tartrate:2% cupric sulphate (100:1:1). After 10 min, 0.25 ml of Folin & Ciocalteu Phenol reagent (diluted 1:1 with H_2O) was added with vigorous vortexing. Following a 40-60 min incubation period at room temperature, absorbance was read at 660 nm on a Beckman DU-65 spectrophotometer. Protein concentrations were determined by comparison to a standard curve of bovine serum albumin (0-175 μg protein).

3.2.5 *In Vitro* Inactivation of Hepatic and Pulmonary Microsomal P450

Incubation mixtures contained hepatic or pulmonary microsomal protein (8-16 mg), 1 mM NADPH (no NADPH in controls), and various concentrations of inhibitor (no inhibitor in controls) in 0.1 M potassium phosphate buffer, pH 7.4. The total incubation volume was 2 ml. Inhibitors were dissolved in methanol, added to the incubation vessel, and the methanol was removed under a gentle stream of nitrogen at room temperature prior to addition of other incubation components. Microsomes, buffer, and the inhibitors were mixed together for 10-15 min before the addition of co-factor. Incubations were started by the addition of NADPH. Control experiments showed <5% loss of monooxygenase activities or P450 content upon incubation of hepatic or

pulmonary microsomes with NADPH in the absence of inhibitor. After a 45 min incubation at 37°C, the mixtures were cooled on ice, then centrifuged at 412,160 x g (100,000 rpm, Beckman TL-100 ultracentrifuge; TLA 100.3 rotor) for 15 min at 4°C. Subsequently, the microsomal pellets were washed by resuspension and recentrifugation to remove excess inhibitor. The microsomal pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.4, and stored at -80°C. Specific P450 content and enzyme activities (7-ethoxyresorufin *O*-deethylation, 7-pentoxyresorufin *O*-dealkylation, benzphetamine *N*-demethylation, and 4-aminobiphenyl *N*-hydroxylation) were subsequently determined in thawed samples within two weeks of freezing. Control experiments showed there is no loss of P450 content or monooxygenase activities under these conditions.

3.2.6 Enzyme Assays

3.2.6.1 P450 Content

Specific P450 content was determined by the method of Estabrook *et al.*(1972). Microsomes suspended in 0.1 M potassium phosphate buffer, pH 7.4 (1 mg protein/ml for hepatic microsomes; 1.2-1.5 mg/ml for pulmonary microsomes) were saturated by bubbling with carbon monoxide for 1-2 min and a baseline scan was performed from 500 to 400 nm with a Beckman DU-65 spectrophotometer. Following reduction of the microsomal suspension with a small amount (≈ 1 mg) sodium dithionite, the sample was scanned again, and the specific P450 content determined using an extinction coefficient of 100

$mM^{-1}cm^{-1}$ for ΔOD, 450-490 nm.

3.2.6.2 7-Ethoxyresorufin *O*-Deethylation (ERF)

ERF activity was determined fluorimetrically by the production of resorufin by a modification of the method of Burke and Mayer (1974). The reaction mixture consisted of microsomal suspension (approximately 13 μg protein/ml for hepatic microsomes; 200 μg/ml for pulmonary microsomes), 1 μM 7-ethoxyresorufin in DMSO (5 μl), in 0.1 M potassium phosphate buffer, pH 7.8. The reaction volume was 2 ml. A baseline was recorded for a few min at an excitation wavelength of 550 nm and an emission wavelength of 585 nm on a Perkin-Elmer fluorimeter (model LS-5B), and the reaction was started by addition of 100 μM NADPH. The reaction was run at 37°C, and the rate of formation of resorufin was calculated by comparison to known amounts of resorufin (15 pmol in 5 μl DMSO) added as an internal standard to the reaction mixture.

3.2.6.3 7-Pentoxyresorufin *O*-Dealkylation (PRF)

PRF activity was determined fluorimetrically by the method of Lubet *et al.*(1985). The reaction mixture consisted of microsomal suspension (50 μg protein/ml for hepatic microsomes; 400 μg/ml for pulmonary microsomes), 4 μM 7-pentoxyresorufin in DMSO (5 μl), in 0.1 M potassium phosphate buffer, pH 7.8. The reaction volume was 2 ml. A baseline was recorded for a few min at an excitation wavelength of 522 nm and an emission wavelength of 585 nm

on a Perkin-Elmer fluorescence spectrometer, and the reaction was started by addition of 100 μM NADPH. The reaction was run at 37°C, and the rate of formation of resorufin was calculated by comparison to known amounts (15 pmol in 5 μl DMSO) of resorufin added as internal standard to the reaction mixture.

3.2.6.4 Benzphetamine *N*-Demethylation (BND)

The *N*-demethylation of D-benzphetamine was determined by the colourimetric measurement of formaldehyde by the method of Nash (1953). Hepatic microsomes (1.5 mg protein) in 0.1 M potassium phosphate buffer, pH 7.4, were incubated with D-benzphetamine HCl (2 mM) in the presence of 1 mM NADPH for 15 min at 37°C. The reaction volume was 1 ml. The reaction was stopped by the addition of 9% zinc sulphate (1 ml), followed by the addition of saturated barium hydroxide (1.5 ml) and saturated sodium borate (0.5 ml). Following centrifugation of this mixture (2500 rpm for 10 min), 1.5 ml of the clear supernatant was transferred to clean test tubes and 1.5 ml of the following solution was added: 4 M ammonium acetate:0.1 M glacial acetic acid:0.04 M acetylacetone. Following incubation for 10 min at 60°C and 5-10 min at room temperature, the absorbance was read at 412 nm. Formaldehyde production was calculated using a standard curve of 0-260 nmoles formaldehyde.

3.2.6.5 4-Aminobiphenyl *N*-Hydroxylation (ABP)

The *N*-hydroxylation of 4-aminobiphenyl was determined colourimetrically by the measurement of 4-aminobiphenyl *N*-hydroxylamine by a modification of the method described for the *N*-hydroxylation of 2-aminofluorene (Vanderslice *et al.*, 1987; Belanger *et al.*, 1981). The reaction mixture consisted of 100 μM 4-aminobiphenyl, pulmonary microsomal protein (30-50 μg protein), and 2 mM NADPH in 0.1 M potassium phosphate buffer, pH 7.4 in a reaction volume of 1 ml. Following incubation for 5 min at 37°C, the reaction was stopped by the addition of 0.4 ml of the incubation mixture to 2 ml of colourimetric reagent: sodium acetate (5 M, pH 5.9), 2,4,6-tripyridyl-*s*-triazine (0.24 mM), and ferric chloride (80 μM) at 50°C. Absorbance was determined at 595 nm following 40 min of colour development at 50°C and 5-10 min at room temperature.

3.2.6.6 Statistical Analysis

Data were compared by unpaired Student's *t*-test at a level of significance of $p < 0.05$. Statistical comparisons were not performed on data from duplicate determinations.

3.3 Results

Treatment with βNF or PB resulted in a marked and selective increase of hepatic microsomal monooxygenase activities relative to those of untreated guinea pigs. βNF administration increased ERF activity six-fold, PRF activity approximately two-fold, P450 content by 1.4 fold, with a corresponding shift

of λ_{max} from 450 nm to 448 nm, and had no effect on BND activity. In PB-treated guinea pigs, PRF activity was increased 10-fold, BND and ERF activity and P450 content all about two-fold (Table 3.1).

The effects of equimolar concentrations (100 μM) of ABT, BBT and αMB as mechanism-based inhibitors were initially compared in hepatic microsomes isolated from untreated, βNF-induced or PB-induced guinea pigs (Table 3.1). The concentration of inhibitors selected was based on preliminary experiments which showed that 100 μM BBT inactivated approximately 80% of hepatic microsomal ERF and PRF in control animals. The inhibition described here was dependent on incubation with NADPH, i.e. mechanism-based inhibition. The purpose of these initial experiments was to compare the ability of the various inhibitors to inactivate P450 isozyme selective monooxygenase activities in liver of control vs induced animals *in vitro*.

All three compounds inactivated at least half of the ERF, PRF and BND activity in hepatic microsomes from untreated guinea pigs under conditions where approximately 50% (37 - 53%) of spectrally assayed P450 was lost.

There was some evidence for differences in potency and isozyme selectivity, however. BBT and αMB inhibited more of the ERF and PRF activity in these microsomes than did ABT and they also inhibited more ERF and PRF activity than BND activity (Table 3.1).

At 100 μM, all three inhibitors were less potent inactivators of the P450 monooxygenase system in hepatic microsomes isolated from βNF-induced guinea pigs (vs control): ERF, PRF and BND activities were only inhibited by

Table 3.1

Comparison of the effects of equimolar amounts (100 μM) of ABT, BBT and αMB on the P450 monooxygenase system of hepatic microsomes from untreated, βNF-induced, or PB-induced guinea pigs.

Inhibitor	% Loss from Control Values			
	ERF Activity	PRF Activity	BND Activity	P450 Content
Untreated[a]				
ABT	80 ± 4[bc]	66 ± 7	49 ± 11	53 ± 4
BBT	93 ± 3[c]	86 ± 3[c]	68 ± 7	37 ± 5
αMB	91 ± 3[c]	88 ± 4[c]	70 ± 6	38 ± 9
βNF-Induced[d]				
ABT	52 ± 6[e]	42 ± 10[e]	41 ± 11	35 ± 6[e]
BBT	61 ± 7[ce]	64 ± 8[ce]	43 ± 5[e]	8 ± 3[e]
αMB	45 ± 4[e]	39 ± 8[e]	49 ± 1[e]	12 ± 2[e]
PB-Induced[f]				
ABT	58 ± 3	40 ± 3[e]	—[g]	33 ± 1[e]
BBT	81 ± 1[ce]	93 ± 1[c]	50 ± 3[e]	32 ± 1
αMB	76 ± 4[ce]	96 ± 1[c]	49 ± 3[e]	29 ± 3

[a] Control (100%) values (means ± SEM, $n = 4$-6) for untreated microsomes were 126 ± 22 pmol/min/mg protein (ERF), 11.0 ± 2.5 pmol/min/mg protein (PRF), 4.55 ± 0.53 nmol/min/mg protein (BND), and 0.77 ± 0.05 nmol P450/mg protein (P450).

[b] Data shown are % loss from control values following incubation with inhibitor and NADPH for 45 min at 37°. Values are means ± SEM, $n = 4$-6.

[c] Significantly greater than loss of BND activity ($p < 0.05$ by unpaired Students t-test).

[d] Control (100%) values (means ± SEM, $n = 4$-6) for βNF-induced microsomes were 792 ± 151 pmol/min/mg protein (ERF), 25.9 ± 4.0 pmol/min/mg protein (PRF), 5.05 ± 0.40 nmol/min/mg protein (BND), and 1.07 ± 0.08 nmol P450/mg protein (P450).

[e] Significantly less than loss of corresponding monooxygenase activity in untreated microsomes ($p < 0.05$).

[f] Control (100%) values (means ± SEM, $n = 4$-6) for PB-induced microsomes were 225 ± 32 pmol/min/mg protein (ERF), 110.0 ± 21.4 pmol/min/mg protein (PRF), 9.94 ± 1.69 nmol/min/mg protein (BND), and 1.66 ± 0.11 nmol P450/mg protein (P450).

[g] Not determined.

40-60% and considerably less P450 was destroyed (8 and 12% for BBT and αMB, respectively) in βNF treated animals. BBT was selective for the inactivation of PRF vs BND activity (64% vs 43%) but ABT and αMB were not. There were no marked differences in the inhibition of PRF vs ERF activities with ABT, BBT or αMB (degree of inhibition, PRF/ERF = 0.8, 1.0 and 0.9, respectively) in βNF-induced guinea pigs (Table 3.1).

The data obtained in PB-induced guinea pigs were of interest for several reasons. First, virtually all of the PRF activity was inhibited by 100 μM BBT and αMB (90+%) whereas this concentration of ABT inhibited less than half of the PRF activity in these hepatic microsomes. Also, ABT inhibited more ERF than PRF activity whereas the reverse was observed with BBT and αMB. The amount of spectrally assayed P450 destroyed by the three compounds in microsomes of PB-induced guinea pigs was very similar (29-33%). Finally, BBT and αMB were significantly more effective as inhibitors of hepatic PRF and ERF than of BND activity after PB treatment (Table 3.1).

The inactivation of monooxygenase activity was generally greater in hepatic microsomes from untreated guinea pigs compared to either βNF- or PB-induced animals (Table 3.1). The sole exception to this was PRF activity in PB-induced animals, an activity obviously catalyzed by a P450 isozyme (or isozymes) very susceptible to inactivation by BBT and αMB (Table 3.1).

In general, the % loss of spectrally assayed P450 caused by 100 μM ABT was similar to the % loss of PRF and BND activity in all three groups of animals; however, significantly more ERF activity was inhibited than the

amount of P450 lost in untreated and PB-induced guinea pigs. The % loss of microsomal P450 content was always less than the loss of ERF, PRF and BND activity caused by *in vitro* incubation with BBT or αMB (Table 3.1).

The concentration dependency of the mechanism-based inactivation of the hepatic monooxygenase system of untreated, PB-induced or βNF-induced guinea pigs by ABT, BBT and αMB was subsequently determined. BBT was more potent in its inhibition of PRF activity (vs BND activity) at all concentrations studied in untreated and especially in PB-induced animals, where 2.6-fold and 1.9-fold more of the total PRF than BND was inactivated at 10 and 100 μM BBT, respectively (Figure 3.1). A similar effect was observed in βNF-induced animals, but only at 100 μM where BBT inhibited more PRF than BND activity (64% vs 43%; Table 3.2).

αMB was also very potent as an inhibitor of PRF activity in microsomes from both PB-induced and untreated guinea pigs (Figure 3.2); approximately 90% and 65% of PRF activity were inactivated by 10 μM αMB in PB-induced and control microsomes, respectively. αMB (10 μM) was much less effective in microsomes from βNF-induced animals where only 26% of PRF activity was inhibited; at 1 mM, αMB only inhibited about 50% of the total PRF activity (Table 3.2).

An interesting difference was noted in liver of control and PB-induced guinea pigs with regard to inhibition of microsomal PRF vs BND activity by αMB. Whereas induction with PB decreased the inhibition of BND activity (from about 30% to 12%) by 10 μM αMB, it increased the amount of PRF

Figure 3.1 **Percentage loss of PRF (□,■) and BND (○,●) activities in hepatic microsomes from untreated (---) or PB-induced (—) guinea pigs with BBT.** Data plotted are means ± SEM, *n* of at least 3 (except 10^3 μM BBT, untreated microsomes, $n=2$), following incubation with BBT and 1 mM NADPH for 45 min at 37°C. Each *n* represents a pool of 2-3 livers. Control (100%) values for untreated microsomes were 11.0 ± 2.5 pmol/min/mg protein (PRF) and 4.55 ± 0.53 nmol/min/mg protein (BND). Control (100%) values for PB-induced microsomes were 110.0 ± 21.4 pmol/min/mg protein (PRF) and 9.94 ± 1.69 nmol/min/mg protein (BND). *Differences between losses of PRF activity and BND activity within each treatment group were significant by unpaired Student's *t*-test ($p<0.05$). +Significantly greater loss of monooxygenase activity compared to PB-induced microsomes ($p<0.05$).

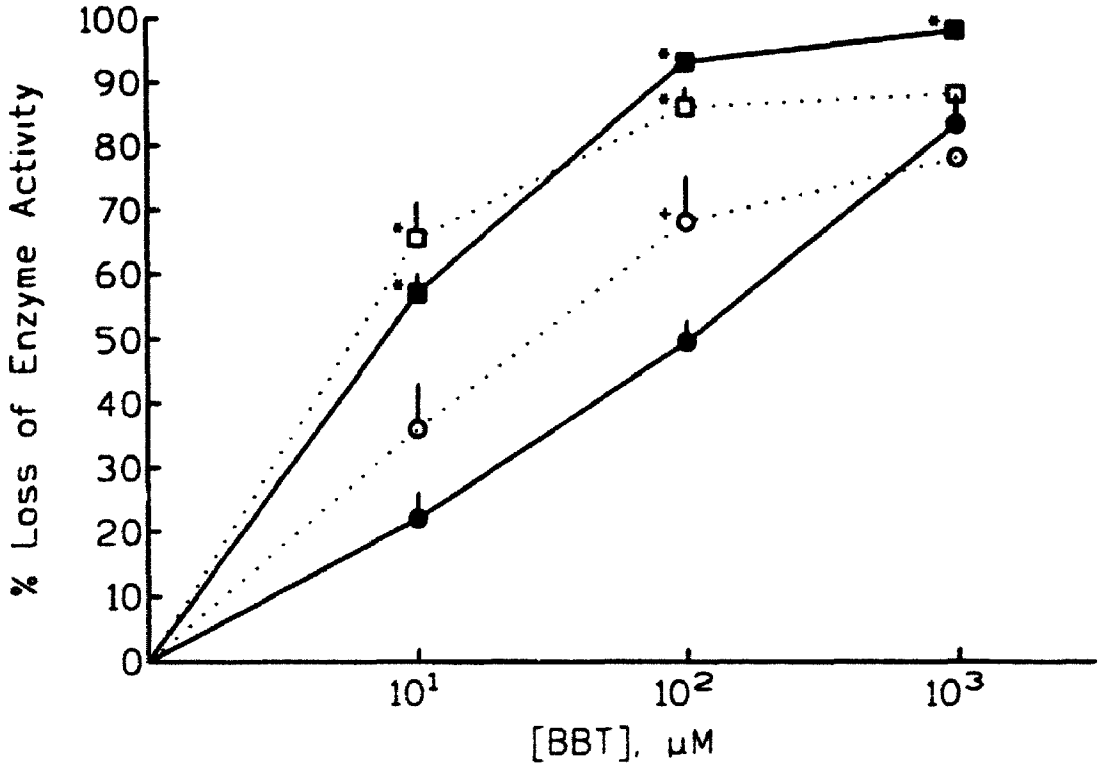

% Loss of Enzyme Activity
100
90
80
70
60
50
40
30
20
10
0
10^1
10^2
10^3
[BBT], μM

Table 3.2

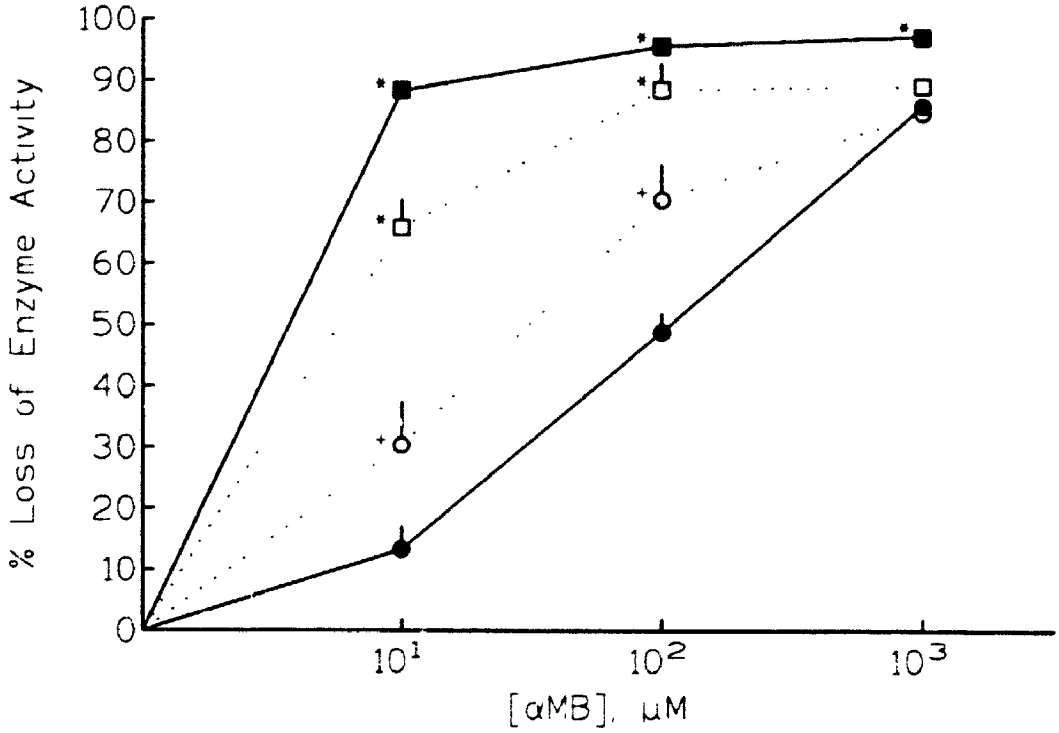
Concentration-dependence of the effects of ABT, BBT and αMB on the P450 monooxygenase system of hepatic microsomes from βNF-induced guinea pigs.

Inhibitor (mM)	% Loss from Control Values[a]			
	ERF Activity	PRF Activity	BND Activity	P450 Content
ABT				
0.01	19 ± 7[b]	25 ± 9	25 ± 10	19 ± 4
0.10	52 ± 6	42 ± 10	41 ± 11	35 ± 6
1.00	88 ± 4	79 ± 8	61 ± 20	65 ± 9
10.0	95 ±4	83 ± 7	87 ± 7	78 ± 2
BBT				
0.01	11 ± 8	28 ± 9	21 ± 4	7 ± 4
0.10	61 ± 7	64 ± 8	43 ± 5	8 ± 3
1.00	76 ± 13	83 ± 9	86 ± 3	26 ± 9
αMB				
0.01	20 ± 8	26 ± 4	26 ± 6	12 ± 6
0.10	45 ± 4	39 ± 8	49 ± 1	12 ± 2
1.00	53 ± 6	52 ± 14	80 ± 2	16 ± 5

[a] Control (100%) values for βNF-induced microsomes were 792 ± 151 pmol/min/mg protein (ERF), 25.9 ± 4.0 pmol/min/mg protein (PRF), 5.05 ± 0.40 nmol/min/mg protein (BND), and 1.07 ± 0.08 nmol P450/mg protein (P450). Values are means ± SEM, $n = 4$-6.

[b] Data shown are % loss from control values following incubation with inhibitor and NADPH for 45 min at 37°C. Values are means ± SEM, $n = 3$-5, except ABT, 10 mM, $n = 2$.

Figure 3.2 **Percentage loss of PRF (□,■) and BND (○,●) activities in hepatic microsomes from untreated (---) or PB-induced (—) guinea pigs with *α*MB.** For additional information see legend to Figure 3.1. *Differences between losses of PRF activity and RND activity within each treatment group were significant ($p < 0.05$). +Significantly greater loss of monooxygenase activity compared to PB-induced microsomes ($p < 0.05$).


% Loss of Enzyme Activity
100
90
80
70
60
50
40
30
20
10
0
10^1
10^2
10^3
[αMB], μM

inactivation (from 65 to 90%, under these conditions (Figure 3.2). This demonstrates that αMB is an effective inhibitor of the hepatic P450 isozyme(s) for which pentoxyresorufin is a selective substrate (vs benzphetamine).

Both *N*-aralkylated derivatives, BBT and αMB, (10 μM) were much more potent inhibitors of PRF activity than the parent compound ABT, causing 4.2-fold and 6.6-fold greater inactivation, respectively, in microsomes from PB-induced guinea pigs (Figure 3.3). The two ABT derivatives were also more potent inhibitors of ERF activity (3.5-fold and 4.0-fold, respectively) than ABT at this concentration in PB-induced animals although much less of the total ERF activity was inactivated (only 31% and 35%, respectively). Thus, αMB was much more selective for inhibition of PRF vs ERF activity (PRF/ERF = 2.5) than either BBT (1.8) or ABT (1.5). In microsomes from untreated animals, BBT and αMB (10 μM) were also more potent inhibitors of PRF and ERF activity than the parent compound, ABT (66% and 46% vs 23% loss of ERF activity, respectively). However, neither ABT (PRF/ERF = 0.9), BBT (1.0), nor αMB (1.4) was very selective for inactivation of PRF vs ERF activity.

The relative losses of spectral P450 content in hepatic microsomes from untreated, PB-induced or βNF-induced guinea pigs following incubation with 1 mM ABT, BBT or αMB and NADPH are shown in Figure 3.4. This concentration of inhibitor was selected because 1 mM BBT or αMB almost totally inhibits PRF and BND activity in microsomes from untreated and PB-induced guinea pigs. The amount of P450 loss due to ABT was significantly greater than that due to BBT or αMB in both untreated and βNF-induced microsomes. However, in

Figure 3.3 **Percentage loss of ERF and PRF activities in hepatic microsomes from untreated (□) or PB-induced (■) guinea pigs with 10 μM ABT, BBT or αMB.** Data shown are means ± SEM, *n* of at least 4, following incubation with inhibitor and 1 mM NADPH for 45 min at 37°C. Each *n* represents a pool of 2-3 livers. Control (100%) values for untreated microsomes were 138.5 ± 20.5 pmol/min/mg protein (ERF) and 11.04 ± 2.54 pmol/min/mg protein (PRF). Control (100%) values for PB-induced microsomes were 225.4 ± 31.5 pmol/min/mg protein (ERF) and 110.0 ± 21.4 pmol/min/mg protein (PRF). *Differences between losses of ERF activity and PRF activity within a treatment group were significant ($p<0.05$). +Significantly greater loss of monooxygenase activity compared to PB-induced microsomes ($p<0.05$).

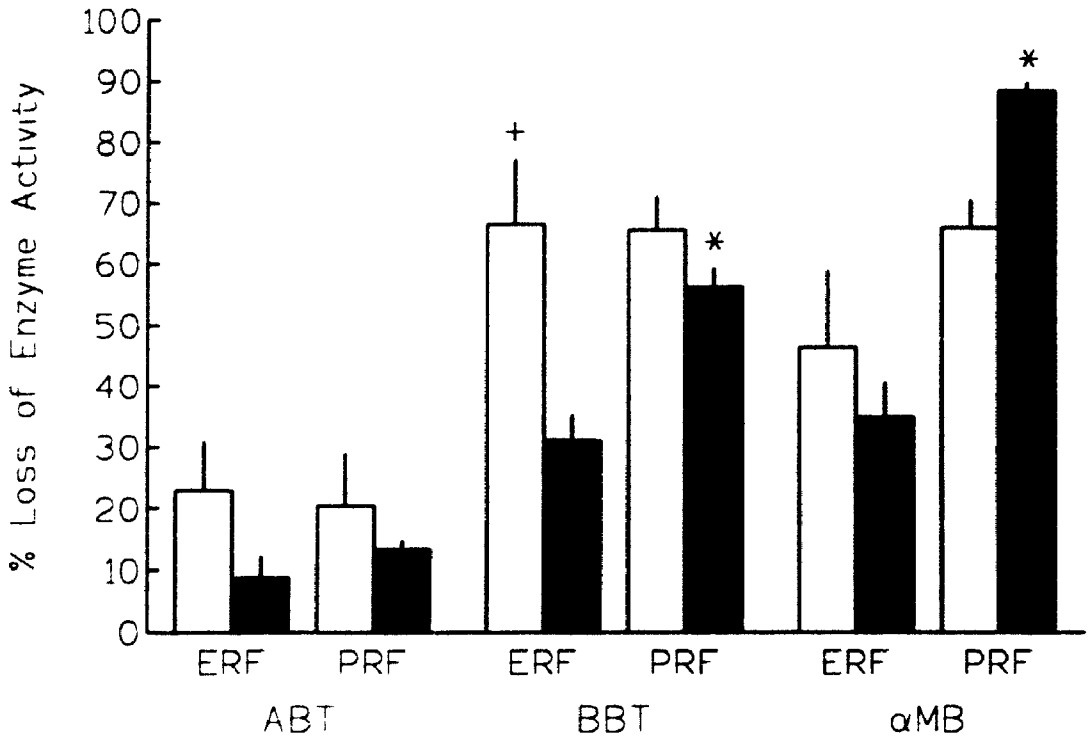
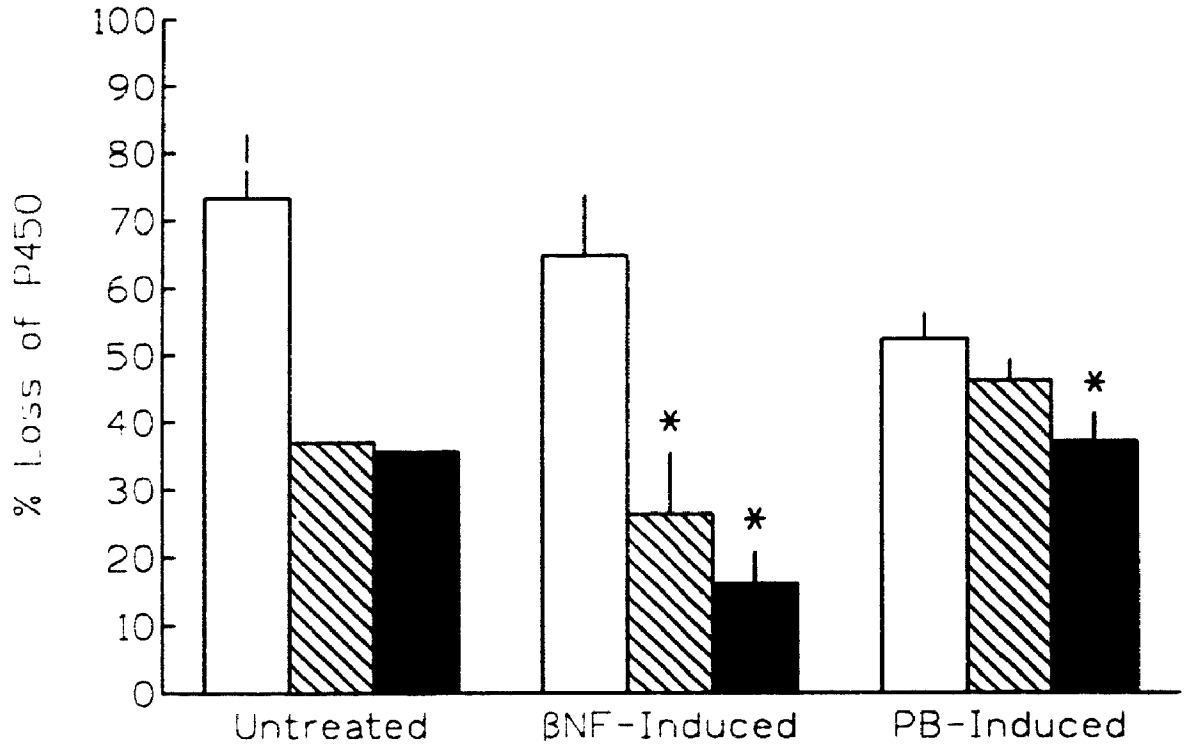

% Loss of Enzyme Activity
100
90
80
70
60
50
40
30
20
10
0
+
*
*
ERF
PRF
ERF
PRF
ERF
PRF
ABT
BBT
αMB

Figure 3.4 **Percentage loss of P450 content in hepatic microsomes from untreated, βNF-induced and PB-induced guinea pigs with 1 mM ABT (□), BBT (▨) or αMB (■).** Data shown are means ± SEM, $n = 3$ (except BBT and αMB, untreated microsomes, $n = 2$), following incubation with inhibitor and 1 mM NADPH for 45 min at 37°C. Each n represents a pool of 2-3 livers. Control (100%) values for P450 content were 0.77 ± 0.05 nmol/mg protein (untreated), 1.07 ± 0.08 nmol/mg protein (βNF-induced) and 1.66 ± 0.11 nmol/mg protein (PB-induced). *Differences between loss of P450 content with BBT or αMB compared to ABT were significant ($p < 0.05$).


% Loss of P450
100
90
80
70
60
50
40
30
20
10
0
*
*
*
Untreated
βNF-Induced
PB-Induced

microsomes of PB-induced animals, BBT and αMB destroyed more P450 than in βNF-induced guinea pigs. The inhibition of PRF and BND (Tables 3.1, 3.2) activities following incubation of control, βNF- or PB-induced hepatic microsomes with ABT (100 μM or 1 mM) paralleled the losses of spectrally assayed P450 (Figure 3.4; Tables 3.1, 3.2) whereas maximal P450 loss mediated by BBT and αMB (1 mM) never exceeded 45% (Figure 3.4; Table 3.2) under conditions where inhibition of PRF and BND frequently exceeded 80% (Figures 3.1, 3.2; Table 3.2).

The newly synthesized compound, αEB, was also investigated for its ability to inactivate hepatic microsomal monooxygenase activity in livers from untreated or PB-induced guinea pigs (Table 3.3). The maximal loss of P450 content in either group did not exceed 52%, even at a concentration of 1 mM. αEB was more potent in the inhibition of PRF activity in microsomes from PB-induced vs control livers, reaching maximal inhibition (90%) at a concentration of 100 μM in the PB-treated group. αEB was not selective for the inhibition of PRF vs ERF activity at any concentration studied in liver of untreated guinea pigs. However, in PB-induced hepatic microsomes, αEB selectively inactivated PRF compared to either ERF or BND activities at concentrations of 10 μM or greater.

Treatment with βNF resulted in a selective increase of ERF activity (2.4-fold) in pulmonary microsomes although the other parameters monitored (PRF and ABP activities and P450 content) also showed apparent increases of approximately 1.5-fold (Table 3.4).

Table 3.3

Effect of various concentrations of αEB on the P450 monooxygenase system of hepatic microsomes from untreated or PB-induced guinea pigs.

αEB Concentration (μM)	% Loss from Control Values			
	ERF Activity	PRF Activity	BND Activity	P450 Content
Untreated[a]				
1	9[b]	26	—[c]	5
5	33	41	—	27
10	58	56	—	36
100	83	81	—	52
PB-Induced[d]				
1	34	29	9	20
10	36	81	21	12
100	53	88	39	31
1000	70	93	75	36

[a] Control (100%) values for untreated microsomes were 76.2 pmol/min/mg protein (ERF), 7.0 pmol/min/mg protein (PRF), and 0.64 nmol P450/mg protein (P450).

[b] Data shown are % loss from control values following incubation with inhibitor and NADPH for 45 min at 37°C, $n = 1$, where n = a pool of 2-3 livers.

[c] Not determined

[d] Control (100%) values for PB-induced microsomes were 83.4 pmol/min/mg protein (ERF), 35.0 pmol/min/mg protein (PRF), 9.6 nmol/min/mg protein (BND), and 1.28 nmol P450/mg protein (P450).

Table 3.4

Comparison of the effects of equimolar amounts (10 μM) of ABT, BBT, αMB and αEB on the P450 monooxygenase system of pulmonary microsomes from untreated or βNF-induced guinea pigs.

Inhibitor	% Loss from Control Values			
	ERF Activity	PRF Activity	ABP Activity	P450 Content
Untreated[a]				
ABT	59 ± 6[b]	54 ± 6	84 ± 3[c]	43 ± 5
BBT	83 ± 4[de]	96 ± 0	27 ± 3[c]	25 ± 6[c]
αMB	82 ± 4[de]	96 ± 1	19 ± 6[c]	31 ± 8[c]
αEB	65 ± 3[d]	97 ± 1	10 ± 8[c]	2 ± 9[c]
βNF-Induced[f]				
ABT	59 ± 7	60 ± 5	89 ± 3[c]	52 ± 3
BBT	67 ± 4[d]	97 ± 2	33 ± 6[c]	18 ± 5[c]
αMB	58 ± 6[d]	98 ± 1	25 ± 6[c]	30 ± 5[c]
αEB	64 ± 5[d]	98 ± 1	29 ± 2[c]	26 ± 4[c]

[a] Control (100%) values for untreated microsomes were 9.6 ± 2.6 pmol/min/mg protein (ERF), 8.8 ± 2.9 pmol/min/mg protein (PRF), 0.60 ± 0.07 absorbance units/min/mg protein (ABP), and 0.19 ± 0.02 nmol P450/mg protein (P450). Values are means ± SEM, $n = 2$-5. Each n represents a pool of 15-20 lungs.

[b] Data shown are % loss from control values following incubation with inhibitor and NADPH for 45 min at 37°C. Values are means ± SEM, $n = 2$-5.

[c] Significantly different than loss of ERF and PRF activity ($p < 0.05$ by unpaired Student's t-test).

[d] Significantly less than loss of PRF activity ($p < 0.05$).

[e] Significantly greater than loss of ERF activity in βNF-induced microsomes ($p < 0.05$).

[f] Control (100%) values for βNF-induced microsomes were 23.3 ± 6.7 pmol/min/mg protein (ERF), 15.2 ± 4.3 pmol/min/mg protein (PRF), 0.82 ± 0.04 absorbance units/min/mg protein (ABP), and 0.26 ± 0.04 nmol P450/mg protein (P450). Values are means ± SEM, $n = 3$-4.

The effects of equimolar concentrations (10 μM) of ABT, BBT, αMB and αEB as mechanism-based inhibitors were compared in pulmonary microsomes isolated from untreated and βNF-induced guinea pigs (Table 3.4). The concentration of inhibitors selected was based on preliminary experiments which showed that 10 μM BBT and αMB provided near maximal inactivation of pulmonary microsomal ERF and PRF activities in control animals.

All four compounds inhibited at least half of the ERF and PRF activity in pulmonary microsomes from untreated guinea pigs. Moreover, there was evidence for differences in potency and isozyme selectivity. BBT, αMB and αEB inhibited more of the ERF and PRF activity than did ABT, whereas ABT inhibited more ABP activity than its three *N*-aralkylated analogues (Table 3.4). BBT, αMB and αEB were also selective for the inhibition of PRF compared to ERF activity (96% vs 65-83%), and the inhibition of ABP activity by the three ABT derivatives was much less than the inhibition of either ERF or PRF activity (for example, 10 μM αMB inactivated 19, 82, and 96% of ABP, ERF and PRF activity, respectively).

Similar results were obtained with pulmonary microsomes from βNF-induced guinea pigs (Table 3.4), however, inhibition of ERF activity by 10 μM BBT and αMB was lower in microsomes from βNF-induced animals (58-67% loss of activity compared to 83% in untreated microsomes). αEB caused equivalent losses of ERF activity in both control and βNF-induced microsomes. The maximal loss of spectrally assayed P450 content with all of the compounds (10 μM) studied was less than 50% in both untreated and βNF-induced

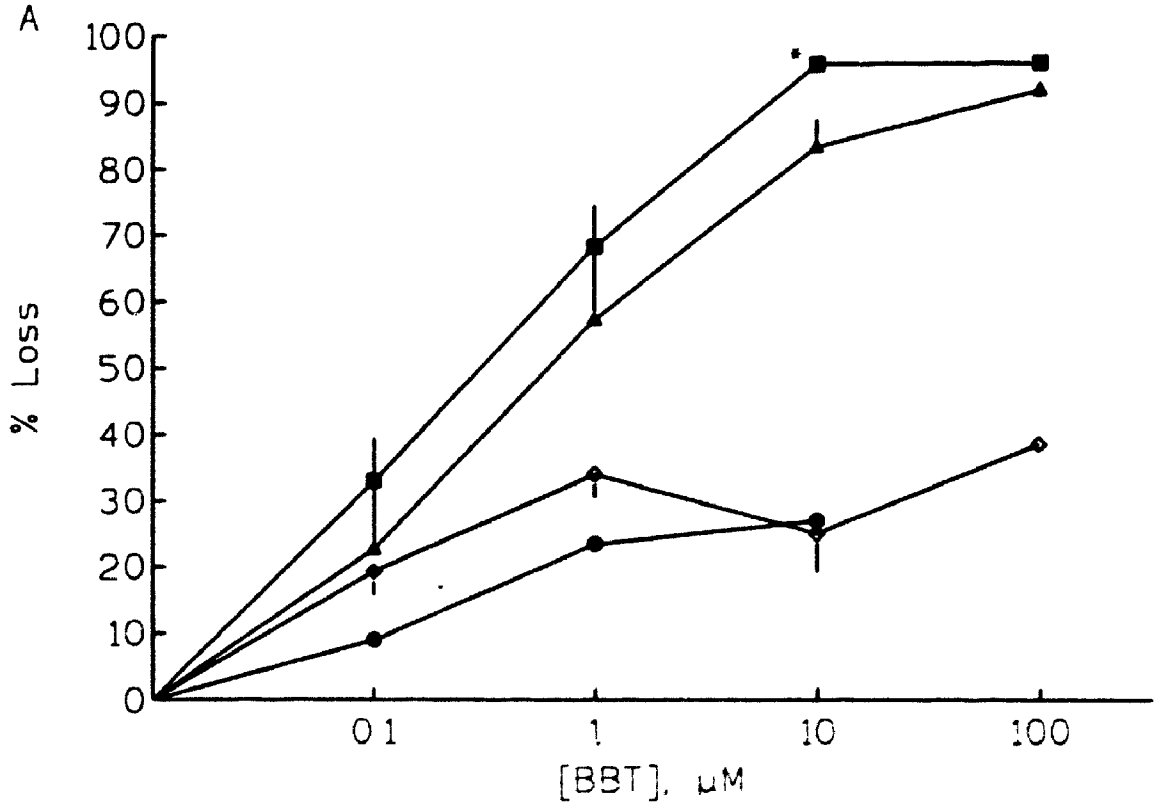
microsomes.

The concentration dependency of the mechanism-based inactivation of the pulmonary monooxygenase system of untreated or βNF-induced guinea pigs by BBT, αMB and αEB was examined next. In control animals, BBT inhibited slightly more PRF than ERF at all concentrations studied but the difference was significant only at 10 μM (Figure 3.5A). ABP activity was poorly inactivated by BBT in comparison to PRF and ERF. In microsomes from βNF-induced animals (Figure 3.5B), BBT was selective for the inhibition of PRF activity compared to ERF or ABP activity at all concentrations. At 0.1 μM, the losses of ERF and ABP activity and P450 content were less than 5%, while loss of PRF activity was greater than 30%, demonstrating the sensitivity of the P450 isozyme catalyzing this reaction to mechanism-based inhibition by BBT.

αMB was a more potent inhibitor of PRF activity than BBT in pulmonary microsomes from untreated guinea pigs; 80% vs 30% inactivation at 0.1 μM, and 95 vs 70% at 1 μM, respectively (Figures 3.5A and 3.6A). On the other hand, decreases of ERF and ABP activities and P450 content were similar in these microsomes with BBT and αMB at each concentration. αMB was also more potent than BBT in inhibiting PRF activity of microsomes prepared from lungs of βNF-induced animals (96 vs 68% at 1 μM), but was approximately equipotent to BBT in the inactivation of ERF and ABP activities and destruction of spectrally assayed P450 (Figures 3.5B and 3.6B).

αEB, like its analogue, αMB, showed a high degree of preference for the inhibition of PRF compared to ERF or ABP activity in both untreated and βNF-

Figure 3.5 **Percentage loss of PRF (■), ERF (▲), and ABP (●) activities and P450 content (◇) in pulmonary microsomes from untreated (A) or βNF-induced (B) guinea pigs with BBT.** Data plotted are means ± SEM, $n=4$ (except 100 μM BBT, 3.5A, $n=2$), following incubation with BBT for 45 min at 37°C. Each n represents a pool of 15-20 lungs. Control (100%) values for untreated microsomes were 8.8 ± 2.9 pmol/min/mg protein (PRF), 9.6 ± 2.6 pmol/min/mg protein (ERF), 0.60 ± 0.07 absorbance units/min/mg protein (ABP), and 0.19 ± 0.02 nmol P450/mg protein (P450). Control (100%) values for βNF-induced microsomes were 15.2 ± 4.3 pmol/min/mg protein (PRF), 23.3 ± 6.7 pmol/min/mg protein (ERF), 0.82 ± 0.04 absorbance units/min/mg protein (ABP), and 0.26 ± 0.04 nmol P450/mg protein (P450). *Differences between losses of PRF activity and ERF activity were significant by unpaired Student's t-test ($p<0.05$).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% Loss
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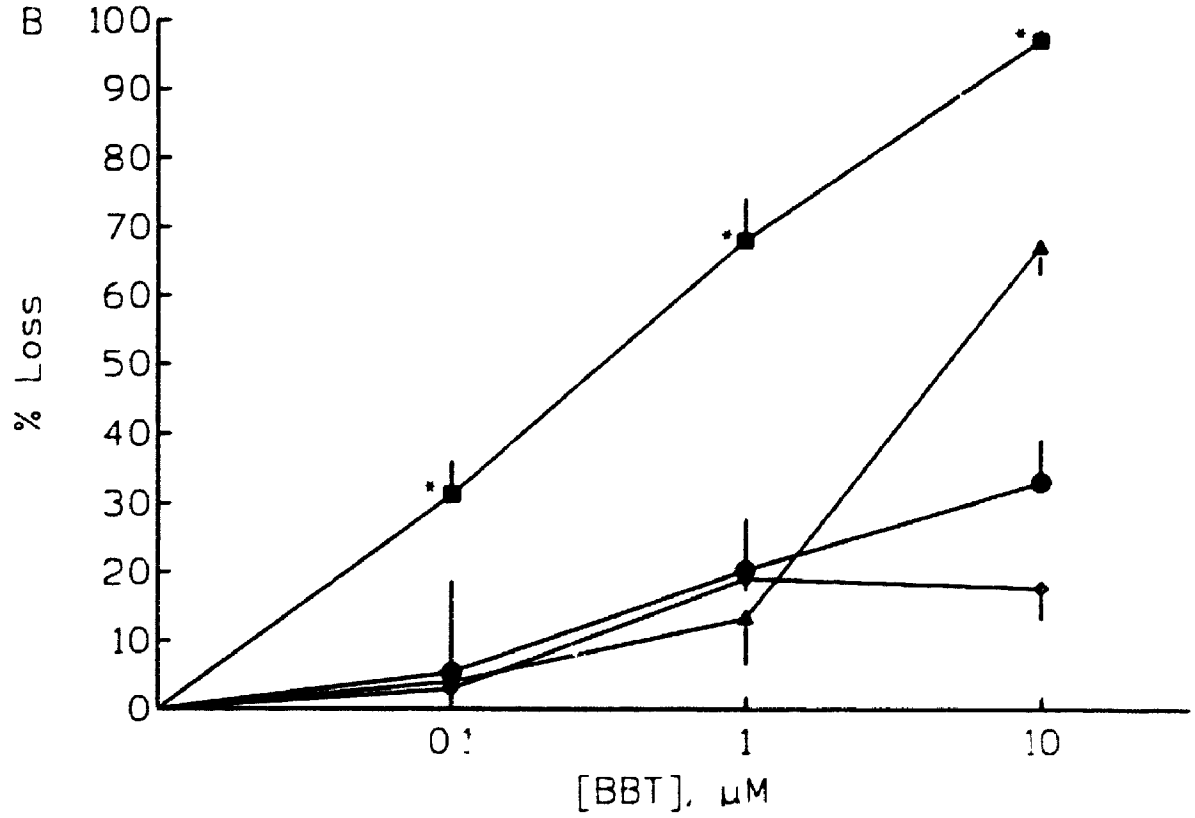
B
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% Loss
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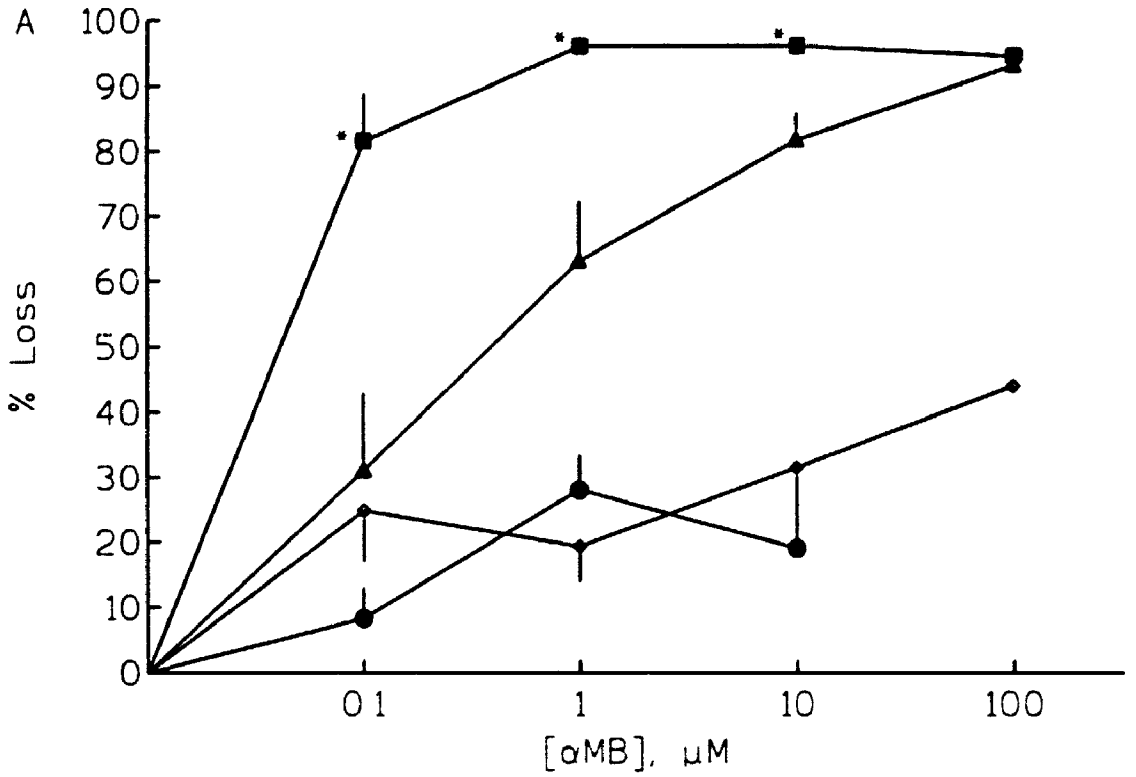
[BBT], µM

Figure 3.6 **Percentage loss of PRF (■), ERF (▲), and ABP (●) activities and P450 content (◇) in pulmonary microsomes from untreated (A) or βNF-induced (B) guinea pigs with αMB.** Other details are given in legend to Figure 3.5. *Differences between losses of PRF activity and ERF activity were significant ($p<0.05$).


A
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% Loss
0 1
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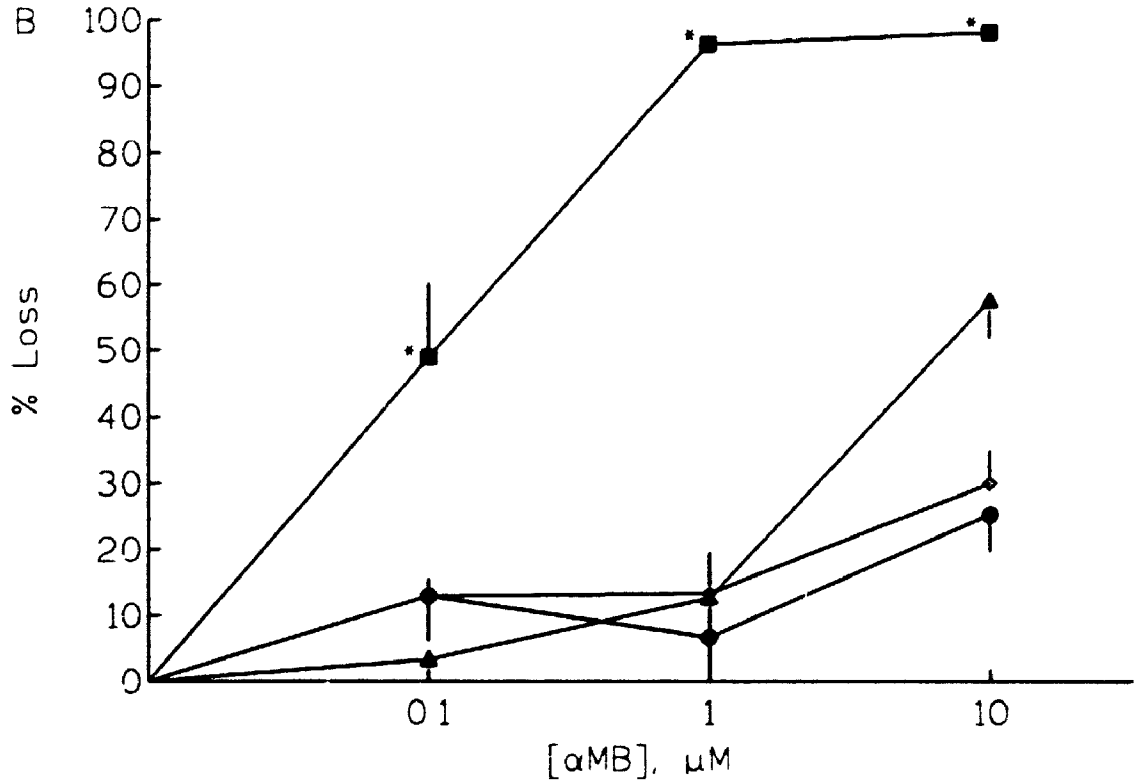
100
[αMB], μM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% Loss
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10
[αMB], μM

induced microsomes (Figure 3.7A and 3.7B, respectively).

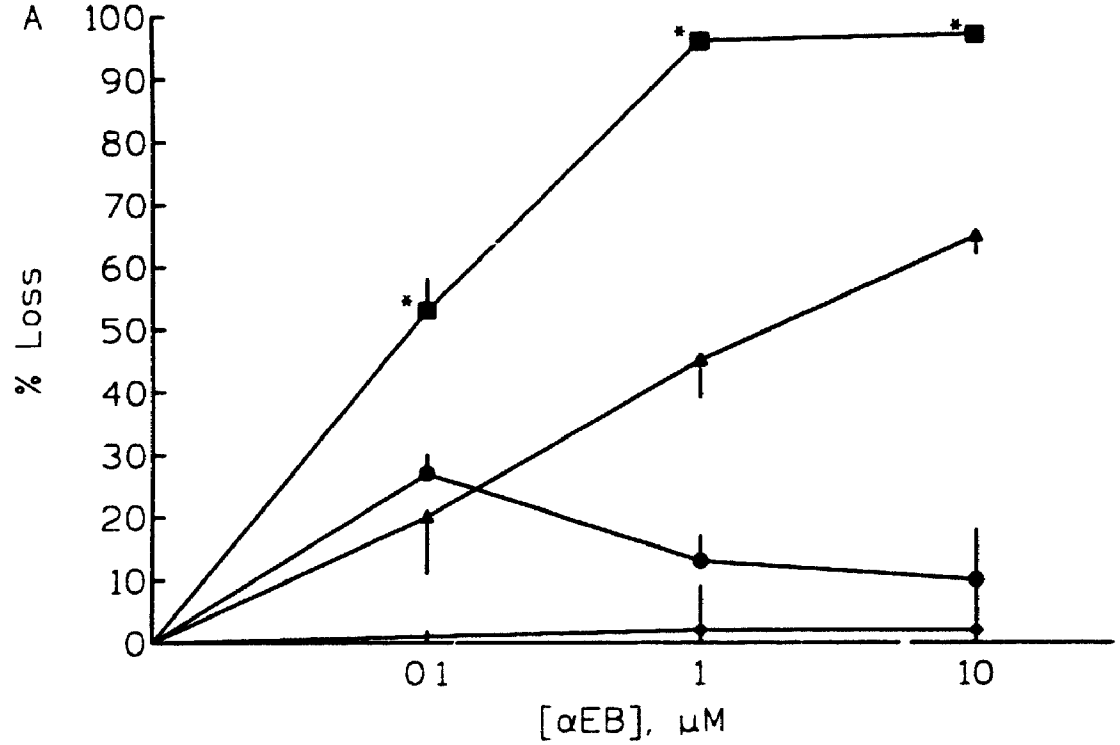
One interesting observation was made with BBT, αMB and αEB; induction with βNF decreased the amount of ERF inhibition. For example, 1 μM BBT inactivated 57% and 13% of ERF activity in pulmonary microsomes from untreated (Figure 3.5A) and βNF treated guinea pigs (Fig 3.5B), respectively; with 1 μM αMB or αEB, ERF inhibition was 63% and 13%, or 45% and 24%, respectively, with microsomes from control (Figures 3.6A; 3.7A) and induced (Figures 3.6B; 3.7B) animals. There was no effect of βNF induction on the inactivation of PRF activity by this concentration of αMB or αEB (95 + % loss of activity in both untreated and βNF-induced cases). This demonstrates that αMB and αEB are selective inhibitors of the pulmonary P450 isozyme with highest PRF activity compared to that isozyme with highest ERF activity in microsomes from βNF-induced guinea pigs. BBT, αMB and αEB are also much more selective for the inactivation of PRF vs ABP activity in microsomes from both untreated and βNF-induced animals (Figures 3.5-3.7).

Whereas αMB was more potent and selective than BBT for the inactivation of PRF, αEB was neither more potent nor more selective than αMB. The order of potency and isozyme selectivity in the inhibition of pulmonary microsomal PRF activity was found to be αMB = αEB > BBT >> ABT.

3.4 Discussion

The doses of βNF and PB used in these experiments caused the expected, selective changes in rates of alkoxyresorufin *O*-dealkylation in hepatic

Figure 3.7 **Percentage loss of PRF (■), ERF (▲), and ABP (●) activities and P450 content (◇) in pulmonary microsomes from untreated (A) or βNF-induced (B) guinea pigs with αEB.** Other details are given in legend to Figure 3.5. *Differences between losses of PRF activity and ERF activity were significant ($p < 0.05$).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% Loss
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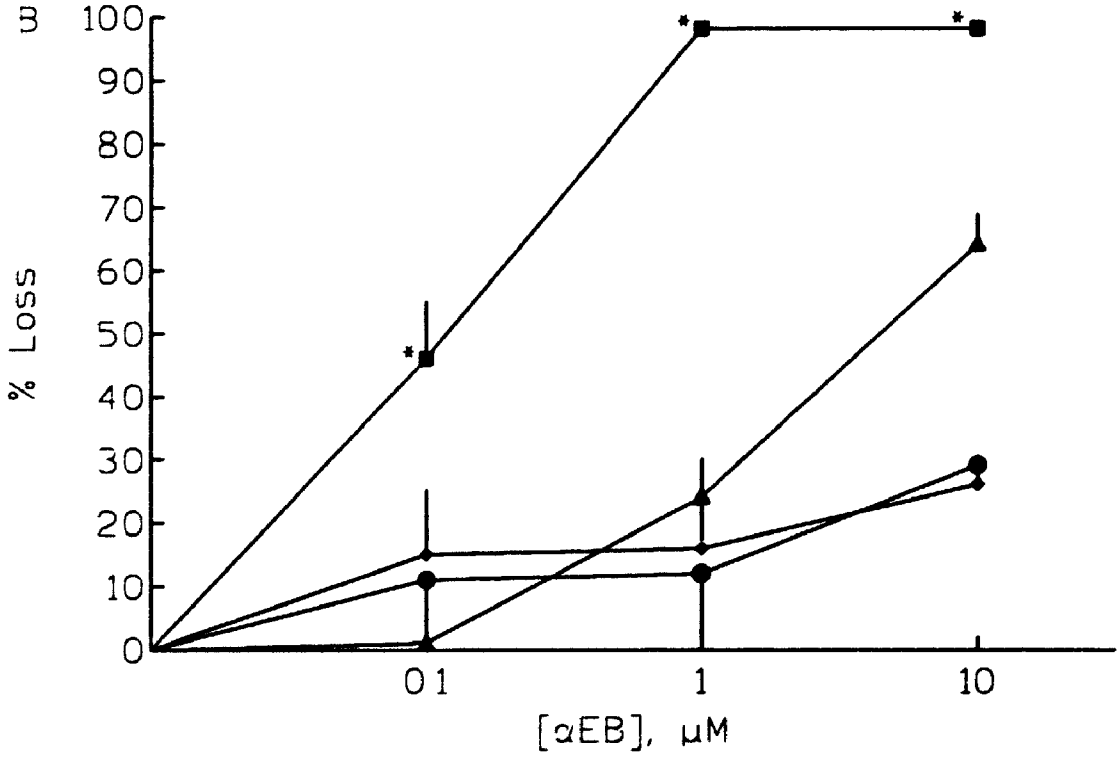
[αEB], μM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[αEB], μM

and pulmonary microsomes of induced vs untreated guinea pigs. βNF treatment enhanced hepatic ERF activity by six-fold and PB treatment increased PRF activity by ten-fold. These changes are similar to those reported in other species such as rats (Burke *et al.*, 1985; Lubet *et al.*, 1985; Nakajima *et al.*, 1990) and mice (Burke and Mayer, 1983), although the amount of induction is not as great. The lower level of induction of guinea pig (vs rat and mouse) hepatic P450 (approximately two-fold) obtained with PB in this study is in agreement with that found by Yoshimura and coworkers (Oguri *et al.*, 1991; Yamada *et al.*, 1992), and is proposed to be due to the high level of expression of hepatic P450 2Bx in untreated guinea pigs, which has been estimated to range from 19% (vs 33% after PB treatment; Yamada *et al.*, 1992) to 47% (Ohmori *et al.*, 1993) of total P450, whereas liver of untreated rats contains virtually no detectable P450 2B1/2 (Omiecinski *et al.*1985).

Pulmonary ERF activity is increased 2.4-fold by βNF treatment, consistent with previous reports of induction of P450 1A1 in guinea pig lung by PAH-type compounds including βNF (Domin *et al.*, 1984; Philpot *et al.*, 1985). It should also be noted that increases in specific activity with ethoxyresorufin and pentoxyresorufin are not specific to induction by βNF and PB, respectively. Thus, βNF treatment of guinea pigs increased the specific activity of hepatic and pulmonary microsomal PRF by approximately two-fold and 1.5-fold, respectively, and induction with PB increased hepatic microsomal ERF activity by two-fold (Tables 3.1 and 3.4). Similar effects have also been previously reported in mice and rats (Burke and Mayer, 1983; Burke *et al.*, 1985; Lubet

et al., 1985).

There are two significant differences between the examination of the inactivation of guinea pig pulmonary microsomal P450 in this study and the earlier work with ABT, BBT and *α*MB in rabbit lung; first, microsomes from lungs of both untreated and *β*NF-induced guinea pigs were compared in the present work, and second, monooxygenase activity was measured with an aromatic amine whose pulmonary *N*-hydroxylation in guinea pig is primarily, if not totally, catalyzed by P450 4B1 (Vanderslice *et al.*, 1987).

In this study ABT was found to be a mechanism-based inhibitor of guinea pig hepatic microsomal P450 with little isozyme selectivity, in agreement with previous findings in other species and tissues (Ortiz de Montellano and Mathews, 1981; Ortiz de Montellano *et al.*, 1981a; Mathews *et al.*, 1985). The ABT-mediated loss of ERF, PRF, or BND activities and P450 content were not markedly different in hepatic microsomes from PB- or *β*NF-induced, as well as untreated, guinea pigs (Tables 3.1 and 3.2; Figure 3.3).

In contrast, ABT (10 *μ*M) was found to selectively inactivate the *N*-hydroxylation of 4-aminobiphenyl in microsomes from untreated or *β*NF-induced guinea pig lungs; inhibition of ABP activity was significantly greater than that of PRF or ERF activity or spectrally assayed P450 content, although there were marked decreases (43-89% loss) in all parameters measured (Table 3.4). These data are in agreement with observations in guinea pig lung following *in vivo* ABT administration (Knickle and Bend, 1992). They also substantiate previous indirect observations in rabbit lung microsomes (Harris *et al.*, 1986), or in

perfused rabbit lung preparations (Mathews *et al.*, 1985), where the rate and stereoselectivity of styrene oxidation was used as the endpoint. In both cases, rabbit P450 4B1 was preferentially inactivated, probably reflecting the efficient oxidation of aromatic amines by this isozyme (Vanderslice *et al.*, 1987). On the other hand, equimolar concentrations of the *N*-aralkylated ABT compounds were much more potent for inactivation of PRF (P450 2Bx) and ERF (P450 1A1) than for ABP activity in microsomes from lungs of control or βNF-induced animals (Table 3.4).

ABT was also found to be a less potent mechanism-based inhibitor of hepatic and pulmonary P450-dependent monooxygenase activity than its *N*-aralkylated derivatives, BBT, αMB and αEB. These results are consistent with previous findings in pulmonary microsomes from βNF-induced rabbits where BBT and αMB were more potent and isozyme selective inhibitors of cytochrome P450 than the parent compound, ABT (Mathews *et al.*, 1985; Mathews and Bend, 1986).

The increased potency and isozyme selectivity of BBT, αMB and αEB for inactivation of guinea pig hepatic and pulmonary microsomal P450 may be related to several factors. First, the greater lipophilicity of the *N*-aralkylated derivatives may enhance their access to the microsomal P450 monooxygenase system. Second, prior to functioning as a suicide substrate for P450 a compound must be metabolized to its active form (or forms). Generally, the first step in the metabolism of a lipophilic compound by the P450 system is its binding to a specific site (the substrate binding site) in P450. In this regard,

ABT seems able to bind to most forms of P450 since it suicidally inactivates P450 with little isozyme selectivity. The increased size of the *N*-aralkylated derivatives may restrict their access to the substrate binding site of certain P450 isozymes.

An interesting aspect of this study was the high sensitivity of PRF activity in hepatic microsomes of PB-induced guinea pigs to mechanism-based inactivation by BBT, *α*EB, and especially *α*MB; the latter compound, at a concentration of 10 *μ*M, inhibited approximately 90% of total PRF activity (Figure 3.2). Pulmonary microsomal PRF activity was also highly susceptible to inactivation by the *N*-aralkyl derivatives of ABT, with a concentration of 1 *μ*M *α*EB or *α*MB inhibiting >95% of PRF activity in pulmonary microsomes from untreated or *β*NF-induced guinea pigs (Figures 3.6 and 3.7). It is known from studies in PB-induced rats that the major hepatic isozyme responsible for PRF activity is P450 2B1; an antibody to P450 2B1 was shown to inhibit PRF activity in PB-induced rat liver microsomes by more than 90% (Lubet *et al.*, 1985). Recently it has been demonstrated in our own laboratory (Knickle and Bend, 1992) that an antibody to P450 2B4, the rabbit orthologue of P450 2B1, inhibits PRF activity by greater than 90% in hepatic microsomes from PB-induced, and pulmonary microsomes from *β*NF-induced, guinea pigs. Thus, it is an ortholog (P450 2Bx) of P450 2B4 and 2B1 that is readily induced by PB in guinea pig liver (10-fold increase of PRF activity) and which is very susceptible to mechanism-based inactivation by BBT, *α*EB and *α*MB in guinea pig liver and lung microsomes. This is consistent with data we reported earlier

with the rabbit pulmonary monooxygenase system where P450 2B4-specific BND activity was shown to be very sensitive to mechanism-based inhibition by both BBT and *o*MB (Mathews and Bend, 1986).

Benzphetamine is known to be *N*-demethylated by several different hepatic P450 isozymes in rat (Guengerich, 1987) and rabbit (Schwab and Johnson, 1987) and the same is apparently true for guinea pig as shown by the preferential inactivation of PRF vs BND activity by BBT (Figure 3.1), *o*MB (Figure 3.2) and *o*EB (Table 3.3) in hepatic microsomes of PB-treated guinea pigs and a ten-fold increase in PRF activity concomitant with only two-fold increases in P450 content and BND activity in these microsomes (Table 3.1). The two-fold induction of BND activity by PB in this study is in agreement with the 2.5-fold increase in BND activity in hepatic microsomes from PB-treated guinea pigs found by Yamada *et al.*(1992). At least one other phenomenon may contribute to these data, however. Lindberg and Negishi (1989) demonstrated that single amino acid substitutions can have marked effects on the substrate selectivity of microsomal P450 isozymes. It is also known that microheterogeneous forms of various P450 isozymes exist, including rabbit P450 2B4 and 2B5 (Gasser *et al.*, 1988; Komori *et al.*, 1988). P450 2B4 and 2B5 have recently been shown to differ in their ability to catalyze the oxidation of testosterone, androstenedione, and ethoxycoumarin, and in their susceptibility to inhibition by BBT and *o*MB, with P450 2B5 being inactivated much more rapidly than 2B4 (Grimm *et al.*, 1993). Possibly guinea pig P450 2Bx exists in multiple forms in the liver, one of which is especially sensitive to inactivation

by *α*MB and very efficiently catalyzes PRF *O*-dealkylation.

In *β*NF-induced guinea pigs, high concentrations of *α*MB (1 mM) only inhibited about 50% of the hepatic microsomal PRF and ERF activity (Table 3.2). Since *β*NF treatment induced PRF activity by approximately 2-fold and ERF activity about 6-fold (Table 3.1), this observation was of interest. The data are consistent with the induction of two or more P450 isozymes in guinea pig liver by *β*NF, one of which is not inactivated by *α*MB. It is known for rat (for review see Guengerich, 1987), rabbit (for review see Schwab and Johnson, 1987) and guinea pig (Philpot *et al.*, 1985) that two major P450 isozymes are induced in liver by administration of compounds such as *β*NF, P450 1A1 and 1A2. Using immunological detection techniques and SDS-polyacrylamide gel electrophoresis, Philpot *et al.*(1985) demonstrated that two proteins, one of molecular weight 48,000 and the second of molecular weight 53,000, were specifically stained with antibodies to rabbit 1A (which react with both 1A1 and 1A2) in guinea pig liver. By analogy with what is known in rats and rabbits, the protein of molecular weight 48,000 is the guinea pig ortholog of P450 1A1 because it, and not the protein of molecular weight 53,000, was found in lung of PAH-induced guinea pig, and P450 1A2 is not present in lung (Philpot *et al.*, 1985). In reconstituted monooxygenase systems P450 1A1 from both rat (Guengerich *et al.*, 1982) and rabbit (Domin and Philpot, 1986) efficiently catalyzes the deethylation of 7-ethoxyresorufin, and 1A1-dependent ERF activity is inactivated by *α*MB in lung microsomes from *β*NF-induced rabbits (Mathews and Bend, 1986) and guinea pigs (this study). Thus, it appears that

the ERF activity not inactivated by 1 mM *o*MB in hepatic microsomes of *β*NF-induced guinea pigs is likely catalyzed by an ortholog of P450 1A2 (i.e. the 53,000 mol. wt. protein; Philpot *et al.*, 1985). Purified P450 1A2 from rat (Guengerich *et al.*, 1982) and rabbit (Norman *et al.*, 1978) catalyzes ERF activity in reconstituted systems. The PRF activity induced by *β*NF and not inactivated by *o*MB is likely catalyzed by this same isozyme (i.e. P450 1A2).

By comparison of the data obtained with pulmonary microsomes from *β*NF-induced guinea pigs (this study) with that previously reported from *β*NF-induced rabbits (Mathews and Bend, 1986), it is possible to estimate the relative potencies of BBT and *o*MB for the inactivation of rabbit P450 2B4 and guinea pig 2Bx and of P450 1A1, if all or most of the PRF and ERF activities in guinea pig lung are catalyzed by 2Bx and 1A1, respectively. At a concentration of 1 *μ*M, BBT inhibited 13% and 20% of 1A1-catalyzed activity and 68% and 35% of 2Bx-catalyzed activity in guinea pig and rabbit, respectively. At the same concentration, *o*MB inactivated 13% and 20% of 1A1 and 95% and 80% of 2Bx in guinea pig and rabbit, respectively. Thus, at equimolar concentrations, BBT and *o*MB appear to be slightly more potent and isozyme selective for inactivation of P450 2Bx in lungs of *β*NF-induced guinea pigs vs P450 2B4 in lung of PAH-induced rabbits.

All four mechanism-based inhibitors examined here were more potent inhibitors of P450-dependent ERF and BND activities (causing 20-40% greater losses of activity) in hepatic microsomes from untreated guinea pigs than in those from PB- or *β*NF-induced animals (Figures 3.1-3.3; Table 3.1-3.2).

Moreover, the inhibition of 1A1-catalyzed ERF activity was much greater (20-50% greater loss of activity) in pulmonary microsomes from untreated vs βNF-induced guinea pigs with BBT, αMB and αEB (Figures 3.5-3.7). This result could be due to the production of significant amounts of BBT, αMB and αEB metabolites which are not mechanism-based inhibitors by those isozymes induced by PB or βNF treatment. *N*-Debenzylation (of BBT) or *N*-α-methyldebenzylation (of αMB) are potential pathways of this type since the major mechanism for suicidal inactivation is expected to be oxidation at the amine nitrogen, release of benzyne and bifunctional arylation of protoporphyrin IX (Mathews and Bend, 1986). For example, a recent study with *N*-cyclopropylbenzylamines, mechanism-based inhibitors of rat P450 2B1 that are structurally related to BBT and αMB, demonstrated that *N*-debenzylation was not a relevant pathway for P450 inactivation by heme or apoprotein modification (Bondon *et al.*, 1989).

With ABT as inhibitor, the loss of spectral P450 correlated fairly well with the loss of monooxygenase activity in hepatic or pulmonary microsomes from all three guinea pig groups; however, with either BBT, αEB or αMB as substrate, the loss of hepatic or pulmonary P450 content was never greater than 50% even when the combined loss of ERF, PRF, and BND or ABP activity was much higher (Figures 3.1-3.7; Tables 3.1-3.4). These data suggest that some isozymes in guinea pig liver and lung are relatively immune to destruction by BBT and αMB (e.g. hepatic 1A2 and lung 4B1). It was previously shown (Mathews and Bend, 1986) that treatment of PB-induced rats with BBT resulted

in the formation of a modified heme pigment in liver identical to that obtained after ABT treatment, suggesting that at least one mechanism for P450 inactivation by BBT is alkylation of protoporphyrin IX at the active site of P450 by the reactive metabolite, benzyne. It has also been demonstrated (Mathews and Bend, 1986) that both BBT and *α*MB caused equimolar losses of P450 and heme in rat liver, suggesting that heme alkylation is a mechanism which occurs during P450 inactivation of P450 2B1 by BBT and *α*MB. However, there are other mechanisms that could also contribute to the potent mechanism-based inhibition of *α*MB and BBT. For example, in experiments in perfused rabbit lung, one molar equivalent of ABT-derived radioactivity became tightly bound to microsomal protein for each equivalent of P450 inactivated (Mathews *et al.*, 1985). The radiolabel was in the benzo ring of ABT, and would be liberated as [^{14}C]benzyne during P450-dependent oxidation. Covalent binding of reactive metabolites to the apoprotein of P450 with no or minimal loss of heme or spectrally assayed P450 is known for P450 inactivation by chloramphenicol (Halpert and Neal, 1980; Halpert *et al.*, 1985b) and methoxsalen (Fouin-Fortunet *et al.*, 1986; Mays *et al.*, 1990). There are also suicide substrates of P450, such as secobarbital, that are known to alkylate not only the prosthetic heme group, but the P450 apoprotein as well (Lunetta *et al.*, 1989), with both mechanisms contributing to the loss of monooxygenase activity. Heme, covalently modified by mechanism-based inhibitors, can subsequently fragment to reactive metabolites which covalently bind to P450 and inhibit monooxygenase activity. This is known to occur with hepatic P450 3A1 after

administration of DDEP (Correia *et al.*, 1987; Sugiyama *et al.*, 1990). Any or all of these alternative mechanisms of inactivation might occur with BBT and *α*MB.

In summary, we have demonstrated that ABT and three of its *N*-aralkylated derivatives synthesized in this laboratory, BBT, *α*EB, and *α*MB, are potent mechanism-based inhibitors of hepatic and pulmonary microsomal P450 of untreated, PB-induced, or *β*NF-induced guinea pigs. The order of potency of these inhibitors is *α*MB = *α*EB > BBT > ABT. BBT (at low concentrations) is more selective than ABT, and *α*EB and *α*MB are even more selective for the inactivation of the major hepatic isozyme catalyzing the *O*-dealkylation of pentoxyresorufin (P450 2Bx) in microsomes from PB-induced guinea pigs. On the other hand, BBT is slightly more potent than *α*MB or *α*EB in its ability to inhibit the guinea pig hepatic ortholog of P450 1A1, the major *β*NF inducible isozyme catalyzing ERF activity. Likewise, BBT, *α*EB and *α*MB are selective for the inactivation of pulmonary 2Bx in microsomes from untreated, and especially *β*NF-induced, guinea pigs. Conversely, ABT is selective for the inhibition of pulmonary P450 4Bx in microsomes from both untreated and *β*NF-induced animals. This study emphasizes the similarities between the pulmonary P450 monooxygenase systems of the guinea pig and rabbit. Isozyme-specific or highly selective mechanism-based inhibitors appear to be an efficient method for the comparison of P450 monooxygenases of hepatic and extrahepatic tissues, across species.

CHAPTER FOUR

METABOLISM, AND COVALENT BINDING TO MICROSOMAL PROTEIN, OF [^{14}C]ABT, AND TWO FORMS OF [^{14}C]BBT, IN GUINEA PIG HEPATIC AND PULMONARY MICROSOMES

4.1 Introduction

A phenylene-protoporphyrin IX adduct, indistinguishable from that of ABT, was isolated from PB-treated rats administered BBT (400 mg/kg) *in vivo* (Mathews and Bend, 1986), suggesting that *N*-debenzylation of BBT to ABT and P450-dependent oxidation of ABT to benzyne preceded P450 inactivation by BBT under these conditions. However, near complete NADPH-dependent inhibition of P450 2Bx activity in guinea pig hepatic and pulmonary microsomes (Chapter 3) and in guinea pig lung *in vivo* (Knickle and Bend, unpublished results) by BBT and *α*MB can occur under conditions where little or no loss of spectrally assayed P450 is noted, whereas the loss of monooxygenase activity closely parallels P450 loss with ABT *in vitro* (Chapter 3) and *in vivo* (Knickle and Bend, 1992). Consequently, it was hypothesized that BBT and *α*MB might be acting through reactive-intermediate modification of P450 apoprotein in addition to *N*-alkylation of P450 heme.

The extent of inhibition of monooxygenase activity with equimolar ABT, BBT or *α*MB is greater in hepatic microsomes from untreated and PB-induced than *β*NF-induced guinea pigs (Chapter 3). This led to the hypothesis that

those P450 isozymes induced by βNF treatment might generate greater quantities of stable metabolites that are not mechanism-based inhibitors of P450 than constitutive isozymes or those induced by PB treatment.

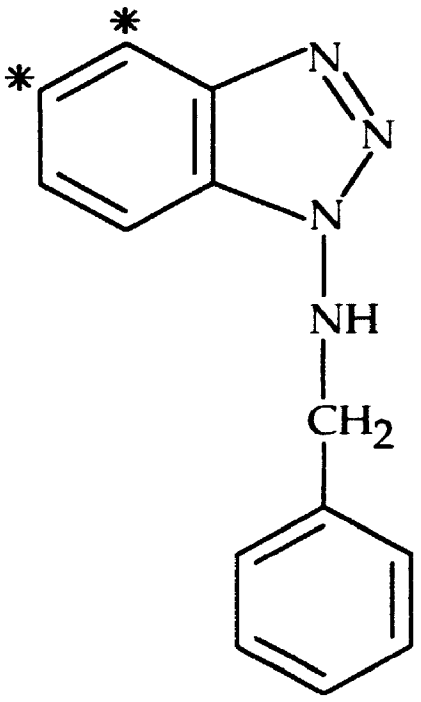
The objectives of this study were to investigate the mechanisms by which BBT and ABT inactivate P450, and to compare the metabolism of these compounds by guinea pig hepatic and pulmonary microsomes *in vitro*. To achieve these objectives two radiolabelled forms of BBT, *N*-benzyl-1-amino-[^{14}C]2,3-benzotriazole ([^{14}C]2,3-BBT), with the label in the 2,3- positions of the benzotriazole ring, and *N*-[^{14}C]-7-benzyl-1-aminobenzotriazole ([^{14}C]7-BBT), with the label on C-7 of the *N*-benzyl substituent (Figure 4.1), were synthesized.

4.2 Methods

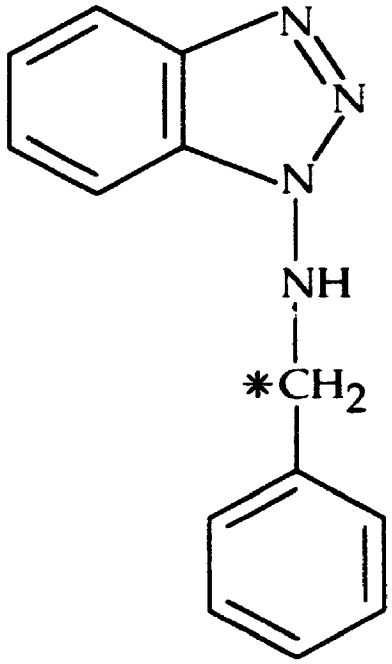
4.2.1 Materials

Benzaldehyde, benzotriazole, *N,N*-carbonyldiimidazole and lithium aluminum hydride were purchased from the Aldrich Chemical Co., Milwaukee, WI. All electrophoresis reagents, gelatin, goat anti-rabbit IgG alkaline phosphatase conjugate, *p*-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were purchased from Bio-Rad Laboratories Ltd., Mississauga, Canada. Schleicher and Schuell nitrocellulose was obtained from Mandel Scientific Co., Guelph, Canada. Rabbit anti-goat IgG was purchased from Sigma Chemical Co., St. Louis, MO. Ready Protein and Ready Organic scintillation cocktails and BTS 450 tissue solubilizer were obtained

Figure 4.1 **Structures of [^{14}C]2,3-BBT and [^{14}C]7-BBT.** [^{14}C]2,3-BBT (*N*-benzyl-1-amino-[^{14}C]2,3-benzotriazole), and [^{14}C]7-BBT (*N*-[^{14}C]-7-benzyl-1-aminobenzotriazole). * Indicates radiolabelled carbons.



[^{14}C] 2,3-BBT



[^{14}C] 7-BBT

from Beckman Canada, Mississauga, Ontario. Sodium borohydride ($NaBH_4$), benzoic acid, and HPLC grade organic solvents were purchased from BDH, Toronto, Ontario. All other materials were obtained as described in section 3.2.1. Goat antibodies to rabbit P450 2B4, as previously described (Serabjit-Singh *et al.*, 1979) were generously provided by Dr. R.M. Philpot of the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

[^{14}C]7-Benzoic acid (32.3 mCi/mmol) was purchased from ICN, Mississauga, Canada, and 1-amino[^{14}C]2,3-benzotriazole ([^{14}C]ABT; 19.75 mCi/mmol) from Pathfinder Laboratories Inc., St. Louis, MO. Due to its instability, [^{14}C]ABT was purified to >97% chemical and radiochemical purity (analysis in HPLC system A or C as described below) before each experiment by low pressure chromatography (LPC) on a Lobar 310x25 mm LiChroprep Si 60 column (40-63 μm; Beckman Canada, Mississauga, Ontario) by elution with ethyl acetate (EtAc):$CHCl_3$, 3:1 at 3 mL/min.

4.2.2 Synthesis of [^{14}C]BBT

[^{14}C]BBT, with the radiolabel in two different positions, was synthesized by minor modifications of the procedure used initially to prepare BBT (Mathews and Bend, 1986). At that time the product was extensively characterized, including by exact mass spectrometry.

Formation and analysis of chemical and radiochemical purity of [^{14}C]BBT was monitored using two HPLC systems. Normal phase isocratic HPLC on a

Waters Resolve C_{18} Radial-Pak column, 5 μm eluted with 1 mL/min hexane:EtAc, 6:1, gave a BBT retention time of 7.5 min (HPLC system A). Reverse phase gradient HPLC on a Waters μBondapak C_{18} Radial-Pak column, 10 μm, using the solvent system, 5 mM aqueous KH_2PO_4, pH 3.2:CH_3CN (95:5, v/v) to aqueous KH_2PO_4:CH_3CN (60:40, v/v) from 0 to 25 min in a hyperbolic gradient, followed by a linear gradient to 100% CH_3CN from 25 to 45 min at a flow rate of 1.5 mL/min, based on the report of Sidhu et al (1987), gave a BBT retention time of 36 min (HPLC system B). The chemical and radiochemical purity of both forms of [^{14}C]BBT used in these experiments was >97%. No single radiochemical peak other than BBT accounted for >1% of the radiolabel.

N-Benzyl-1-amino-[^{14}C]2,3-benzotriazole ([^{14}C]2,3-BBT; Figure 4.1A) was synthesized in this laboratory by Dr. E.W. Szczepan. To a solution of [^{14}C]ABT (2 mg, 14.9 μmol, 300 μCi) in glacial acetic acid (0.5 mL), excess benzaldehyde (10 μL, 98 μmol) was added and the solution was stirred overnight under a nitrogen atmosphere at room temperature. The mixture was evaporated *in vacuo* to leave an oily residue which was dissolved in 7 mL CH_2Cl_2:MeOH, 95:5 and washed three times with 5 mL 5% sodium bicarbonate and then with three 4 mL portions of water. The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated *in vacuo*. This impure imine product (a Schiff's base) was dissolved in 10 mL MeOH:CH_2Cl_2, 2:1 and reduced with $NaBH_4$ (220 mg, 5.81 mmol). After stirring for 1 h at room temperature, the solvent was evaporated to leave a white solid, which was

dissolved in 10 mL CH_2Cl_2:MeOH, 95:5. The organic phase was washed with three 150 mL portions of water, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude extract was purified by LPC on a Lobar 310x25 mm LiChroprep Si 60 column by elution with EtAc:$CHCl_3$, 3:1 at 3 mL/min. The elution time for product from the column was approximately 40 min. The purified product (>97% by HPLC analysis) had identical retention times with authentic BBT in HPLC systems A and B. The yield of [^{14}C]2,3-BBT, 19.75 mCi/mmol, was 36.7%.

N-[^{14}C]-7-benzyl-1-aminobenzotriazole ([^{14}C]7-BBT; Figure 4.1B) was synthesized in this laboratory by Mr. C.D. Webb. To a solution of [^{14}C]7-benzoic acid (1.0 mg, 8.2 μmol, 250 μCi) and cold benzoic acid (9.0 mg, 73.8 μmol) in 4 mL ether, *N,N*-carbonyldiimidazole (14 mg, 86 μmol) was added and the solution was stirred at room temperature under a nitrogen atmosphere for 24 hr. The solution was warmed gently under slight vacuum before being cooled to $-20°C$. Reduction was accomplished by the dropwise addition of lithium aluminum hydride (2 mg, 52.6 μmol) in 4 mL anhydrous ether over 30 min with vigorous stirring under a nitrogen atmosphere. The ether was removed under vacuum, and 2 mL of ice-cold MeOH were added to the vessel and the solution was acidified to pH 4 with 10% H_2SO_4. The MeOH was removed under a gentle stream of nitrogen and the residue, containing [^{14}C]7-benzaldehyde, was dissolved in 2 mL glacial acetic acid and reacted with excess ABT (10.4 mg, 77.6 μmol) for three days under nitrogen at room temperature with constant stirring. Extraction of the Schiff's base, reduction

of this imine with $NaBH_4$, and final product purification was completed as described above for [^{14}C]2,3-BBT. The yield of purified radioactive product (3.23 mCi/mmol) was 30.16 μCi (12.1%).

4.2.3 Animal Treatment

Animals were treated as described in section 3.2.3.

4.2.4 Preparation of Microsomes

Microsomes were prepared as described in section 3.2.4.

4.2.5 *In Vitro* Incubation of Hepatic and Pulmonary Microsomes with [^{14}C]Labelled Mechanism-Based Inhibitors

Incubation mixtures contained hepatic or pulmonary microsomal protein (14-16 mg), and [^{14}C]ABT, [^{14}C]2,3-BBT or [^{14}C]7-BBT (100 μM, 0.225 μCi) in 0.1 M potassium phosphate buffer, pH 7.4. The total incubation volume was 2 mL. The reaction was started by the addition of 1 mM NADPH (no NADPH in controls). [^{14}C]ABT, [^{14}C]2,3-BBT or [^{14}C]7-BBT was dissolved in EtAc and ABT or BBT was dissolved in MeOH, added to the incubation vessel, and the solvents removed under a gentle stream of nitrogen at room temperature. The residue was dissolved in 10 μL DMSO prior to addition of other components. Incubations including NADPH were performed in duplicate, while controls were single incubations. After incubation for 45 min at 37°C, the mixtures were immediately centrifuged at 412,160 x g (Beckman TL-100 ultracentrifuge; TLA

100.3 rotor) for 15 min at 4°C to sediment the microsomes. The resulting supernatant fraction was saved for extraction of metabolites. The microsomal pellets were washed by resuspension and recentrifugation to remove excess inhibitor. This microsomal wash supernatant was stored at −80°. The resulting microsomal pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.4) to a final concentration of 3-5 mg protein/mL. The ^{14}C content of all fractions was determined by liquid scintillation spectroscopy (LSS) of small aliquots.

4.2.6 Extraction of Microsomal Supernatant and Microsome Fractions

The microsomal supernatant was extracted repeatedly with 2 mL of EtAc at neutral pH until no further radioactivity could be extracted into the organic phase (6-10 extractions). The aqueous phase was then adjusted to pH 10 with 1N NaOH, and re-extracted with EtAc (as above). The aqueous phase was subsequently adjusted to pH 2 with 1N HCl, and re-extracted with EtAC.

Prior to extraction of the microsomal fraction, the microsomal protein concentration was determined by the method of Lowry *et al.*(1951) as described in section 3.2.4, and spectral P450 content was determined by the method of Estabrook *et al.*(1972) as described in section 3.2.6.1. Microsomes were exhaustively extracted at neutral pH, then acidic pH (2.0, adjusted with H_3PO_4) with EtAc. The residual acidic aqueous phase was neutralized with 5% potassium bicarbonate. The ^{14}C content of all extracts was determined by LSS, organic extracts of like pH were pooled, dried over anhydrous Na_2SO_4

overnight, filtered, and the EtAc was removed under a gentle stream of nitrogen. The pooled extracts were stored at −80° in EtAc for subsequent analysis by HPLC.

4.2.7 Covalent Binding of [^{14}C] Radiolabel to Microsomal Protein

The neutralized extracted microsomal protein was dialyzed against distilled H_2O overnight and then lyophilized to dryness. Small aliquots (approx. 2.5-5 mg) of lyophilized microsomal protein were solubilized in 3% sodium dodecyl sulfate (SDS) with gentle heating, followed by determination of protein concentration by the method of Lowry described in section 3.2.4 and analysis of [^{14}C] content by LSS. Separate aliquots of lyophilized protein (equivalent to 0.8-1.2 mg solubilized protein) were solubilized in 3% SDS, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels (3 mm thick) according to the method of LaemmLi (1970), using a BioRad Protean II electrophoresis apparatus. Lanes containing [^{14}C]labelled protein were sliced into 2 mm slices, solubilized with Beckman BTS-450 tissue solubilizer and [^{14}C] content determined by LSS, following addition of 10 mL ReadyProtein® scintillation cocktail.

4.2.8 Localization of [^{14}C] Radiolabel on Western Blots

Aliquots of lyophilized hepatic microsomal protein (equivalent to approximately 1 mg solubilized protein) from PB- or βNF-treated guinea pigs following incubation with [^{14}C]2,3-BBT and EtAc extraction as described in

sections 4.2.5 and 4.2.6 were solubilized in 3% SDS, and subjected to SDS-PAGE as described in section 4.2.8 on 9% gels (3 mm thick), as this has been reported to provide a better separation of proteins in the 50 kD region (Labbe *et al.*, 1989). Following electrophoresis, the gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) for 1 hr prior to electrophoretic transfer of the proteins onto a 0.2 μm nitrocellulose membrane by the method of Towbin *et al.*(1979) in a BioRad Trans Blot Cell at 30 V, 100 mA overnight.

The nitrocellulose was blocked with 3% gelatin in Tris-saline (TS; 20 mM Tris-HCl, 0.9% NaCl, pH 7.4) for 1-2 hr, followed by incubation with antibodies to rabbit P450 2B4 diluted 1:5000 in antibody buffer [1% gelatin in Tween-Tris-saline (TTS; 0.05% Tween-20 in TS)] for 1 hr. This was followed by incubation with rabbit anti-goat IgG diluted 1:1000 in antibody buffer for 20 min, and goat anti-rabbit IgG alkaline phosphatase conjugate diluted 1:3000 in antibody buffer for 20 min. Each incubation step was followed by three 5-10 min washes with TTS prior to addition of the next antibody. Following a final wash with TS for 5-10 min, the immunoreactive protein bands were visualized with the phosphatase substrates NBT and BCIP. BioRad prestained low molecular weight standards were used to determine approximate molecular weight regions on the Western blot.

Following development of the blots, the lanes were cut into successive 0.2 mm strips, each of which was cut into three pieces. Each strip was placed in 10 mLs ReadyProtein®scintillation cocktail for determination of [^{14}C] content

by LSS.

4.2.9 HPLC Analysis of Neutral Organic Extracts

ABT and its neutral metabolites (dissolved in MeOH) were separated by reverse phase gradient HPLC on a Waters Resolve C_{18} Radial-Pak column, 5 μm. Metabolites were eluted with a linear gradient of MeOH:H_2O (5:95, v/v) to MeOH:H_2O (25:75, v/v) from 0 to 20 min, followed by isocratic MeOH/H_2O (25:75, v/v) for 20 min at a flow rate of 1 mL/min (HPLC system C). Column effluent was monitored at 280 nm and fractions (1 min) were collected and analysed for [^{14}C] content. HPLC standards used were ABT and benzotriazole.

BBT and its neutral metabolites (dissolved in CH_3CN) were separated by reverse phase gradient HPLC with system B as described in section 4.2.2. Column effluent was monitored at 280 nm and fractions (0.75 min) were collected and analyzed for [^{14}C] content. HPLC standards used were ABT, benzotriazole, benzaldehyde and BBT. Gradients in HPLC systems B and C were operated by a Waters Maxima 820 Chromatography Workstation

4.3 Results

NADPH-dependent covalent binding of [^{14}C]ABT-, [^{14}C]2,3-BBT- and [^{14}C]7-BBT-derived radioactivity to microsomal protein occurred with all hepatic and pulmonary microsomes examined (Table 4.1).

Covalent binding of [^{14}C]2,3-BBT- or [^{14}C]7-BBT-derived radioactivity (per mg protein) was similar in PB- and βNF-induced hepatic microsomes (Table 4.1). However, binding per nmol P450 (or per nmol P450 lost) was greater in

Table 4.1

Covalent binding of [^{14}C]ABT-, [^{14}C]2,3-BBT- and [^{14}C]7-BBT-derived radioactivity to microsomal protein following incubation with 100 μM [^{14}C]-labelled ABT or BBT.

Inhibitor	Microsome Source	Covalently Bound ^{14}C Equivalents (nmol/mg protein)	
		-NADPH	+NADPH
[^{14}C]ABT	Liver	0.03[a]	0.34 ± 0.01[b]
	PB Liver	0.21	0.72 ± 0.02
	βNF Liver	0.07	0.90 ± 0.03
	Lung	0.06	0.14 ± 0.01
[^{14}C]2,3-BBT	PB Liver	0.09	1.12 ± 0.02
	βNF Liver	0.08	1.06 ± 0.01
	Lung	0.07	0.40 ± 0.01
[^{14}C]7-BBT	PB Liver	0.04	1.12 ± 0.01
	βNF Liver	0.04	1.24 ± 0.03
	Lung	0.03	0.15 ± 0.01

[a]Values are from a single control incubation (-NADPH) for each inhibitor.
[b]Values are means ± SEM of duplicate incubations in the presence of 1 mM NADPH.

Table 4.1, con't.

Loss of Spectral P450 Content[c] (nmol/mg protein)	NADPH-dependent nmol ^{14}C equivalents bound/mg protein	NADPH-dependent nmol ^{14}C equivalents bound/nmol P450[g]
ND[d]	0.31	0.48
0.37 ± 0.01[e] (26%)[f]	0.51	0.36 (1.38)[h]
0.19 ± 0.01 (20%)	0.83	0.86 (4.37)
0.06 ± 0.01 (55%)	0.08	0.71 (1.33)
0.38 ± 0.04 (27%)	1.03	0.73 (2.71)
0.23 ± 0.04 (24%)	0.98	1.02 (4.26)
0.03 ± 0.01 (29%)	0.33	2.91 (10.97)
0.46 ± 0.04 (32%)	1.08	0.77 (2.35)
0.17 ± 0.01 (17%)	1.20	1.25 (7.06)
0.05 ± 0.01 (40%)	0.11	1.00 (2.20)

[c]nmoles of initial spectrally detectable P450 lost/mg microsomal protein. Initial P450 content of hepatic microsomes was: untreated, 0.64; PB, 1.41; βNF, 0.96 nmol/mg protein. Initial P450 content of pulmonary microsomes was 0.11 nmol/mg protein.

[d]ND = not determined

[e]Values are means ± SE of duplicate determinations from duplicate incubations.

[f]Numbers in brackets are % loss of P450 content compared to control (-NADPH) incubation.

[g]nmol [^{14}C]labelled inhibitor equivalents bound/nmol initial spectrally detectable P450.

[h]nmol [^{14}C]labelled inhibitor equivalents bound/nmol P450 lost

βNF- vs PB-induced microsomes (approximately 1.5-fold with [^{14}C]2,3-BBT and 1.6- and 3-fold greater per nmol P450 and per nmol P450 lost, respectively, with [^{14}C]7-BBT). This corresponds to a lower initial content of P450 (0.96 vs 1.41 nmol P450/mg protein), and a smaller loss of P450 (0.20 vs 0.42 nmol P450 lost/mg protein) in βNF-induced compared to PB-induced hepatic microsomes, respectively (Table 4.1).

Covalent binding of [^{14}C]7-BBT-derived radioactivity was greater than that of [^{14}C]2,3-BBT in βNF-liver microsomes (7.1 vs 4.3 nmol/nmol P450 lost, respectively) but similar in PB-liver microsomes (2.4 vs 2.7 nmol/nmol P450 lost, respectively; Table 4.1).

The rank order of covalent binding to pulmonary microsomal protein was [^{14}C]2,3-BBT (2.9; 10.9) > [^{14}C]7-BBT (1.0; 2.2) > [^{14}C]ABT (0.7 nmol/nmol P450; 1.3 nmol/nmol P450 lost, respectively; Table 4.1). Covalent binding (per mg protein) was much lower for pulmonary than hepatic microsomes for all[^{14}C] inhibitors, whereas binding per nmol of P450 was equal to or greater in lung vs liver.

The combined covalent binding of both forms of [^{14}C]BBT (Table 4.1) was 1.5 (5.1), 2.3 (11.3), and 3.9 (13.2) nmol/nmol P450 (nmol/nmol P450 lost) in PB-liver, βNF-liver, and lung microsomes, respectively.

The rank order for covalent binding of [^{14}C]ABT-derived radioactivity was βNF liver (0.83 nmol) > PB liver (0.50 nmol) > liver (0.31 nmol) >lung (0.08 nmol/mg microsomal protein), respectively (Table 4.1).

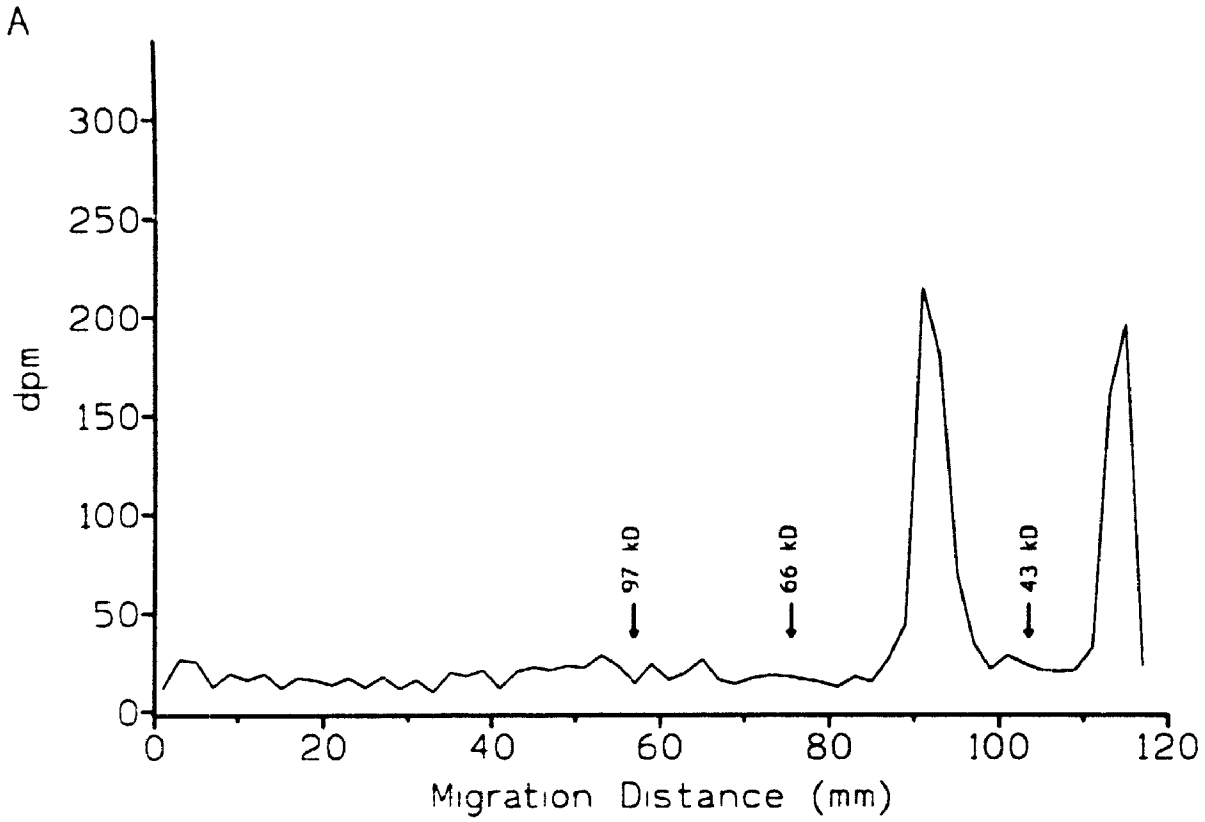
NADPH-independent binding also occurred with all microsomes and [^{14}C]

inhibitors examined (Table 4.1) but this was normally < 10% of P450-mediated binding. However, NADPH-independent binding of [^{14}C]ABT was about 40% of that found in the presence of NADPH in pulmonary microsomes and 30% in hepatic microsomes from PB-induced animals.

Upon SDS-PAGE, [^{14}C]2,3-BBT- (Figure 4.2) and [^{14}C]7-BBT- (Figure 4.3) derived radioactivity covalentiy bound to hepatic microsomal protein of PB- or βNF-treated guinea pigs migrated (selectively) to the molecular weight region corresponding to isozymes of P450. Similar results were obtained on SDS-PAGE analysis of pulmonary microsomes from untreated animals following incubation with [^{14}C]2,3-BBT or [^{14}C]7-BBT (Figure 4.4), and of hepatic microsomes from PB- or βNF-treated guinea pigs following incubation with [^{14}C]-ABT (Figure 4.5), in the presence of NADPH. Insufficient amounts of radioactivity were bound to hepatic microsomal protein in the absence of NADPH to permit similar analysis.

Immunoblotting with antibodies to rabbit P450 2B4 following transfer to nitrocellulose of hepatic microsomal protein from PB- or βNF-induced guinea pigs following incubation with [^{14}C]2,3-BBT in the presence of NADPH produced a single immunoreactive band (Figures 4.6; 4.7). Upon analysis of the Western blots for [^{14}C] content, radioactivity was localized to the region corresponding to the P450 2B4 immunoreactive band with hepatic microsomes from PB-induced guinea pigs (Figure 4.6). With hepatic microsomes from βNF-treated animals (Figure 4.7), radioactivity was also localized to a region containing the P450 2B4 immunoreactive band, as well as regions of slightly

Figure 4.2 **Radioactivity profile from SDS-PAGE of hepatic microsomes incubated with [^{14}C]2,3-BBT.** Hepatic microsomes from (A) PB-induced or (B) βNF-induced guinea pigs were incubated with [^{14}C]2,3-BBT (100 μM, 0.225 μCi) and 1 mM NADPH. Location of molecular weight standards is indicated by arrows.


A
300
250
200
150
100
50
0
dpm
97 kD
66 kD
43 kD
0
20
40
60
80
100
120
Migration Distance (mm)

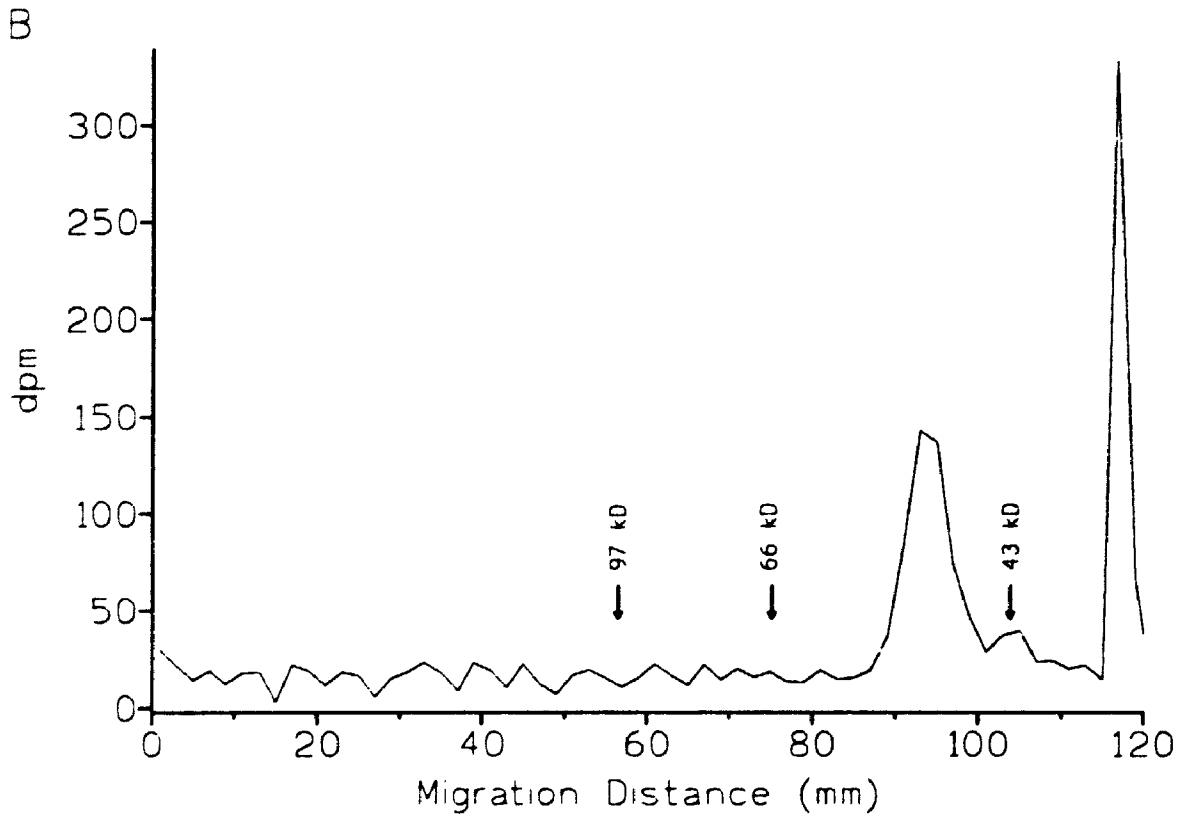

B
300
250
200
150
100
50
0
dpm
97 kD
66 kD
43 kD
0
20
40
60
80
100
120
Migration Distance (mm)

Figure 4.3 **Radioactivity profile from SDS-PAGE of hepatic microsomes incubated with [^{14}C]7-BBT.** Hepatic microsomes from (A) PB-induced or (B) βNF-induced guinea pigs were incubated with [^{14}C]7-BBT (100 μM, 0.225 μCi) and 1 mM NADPH. Location of molecular weight standards is indicated by arrows.

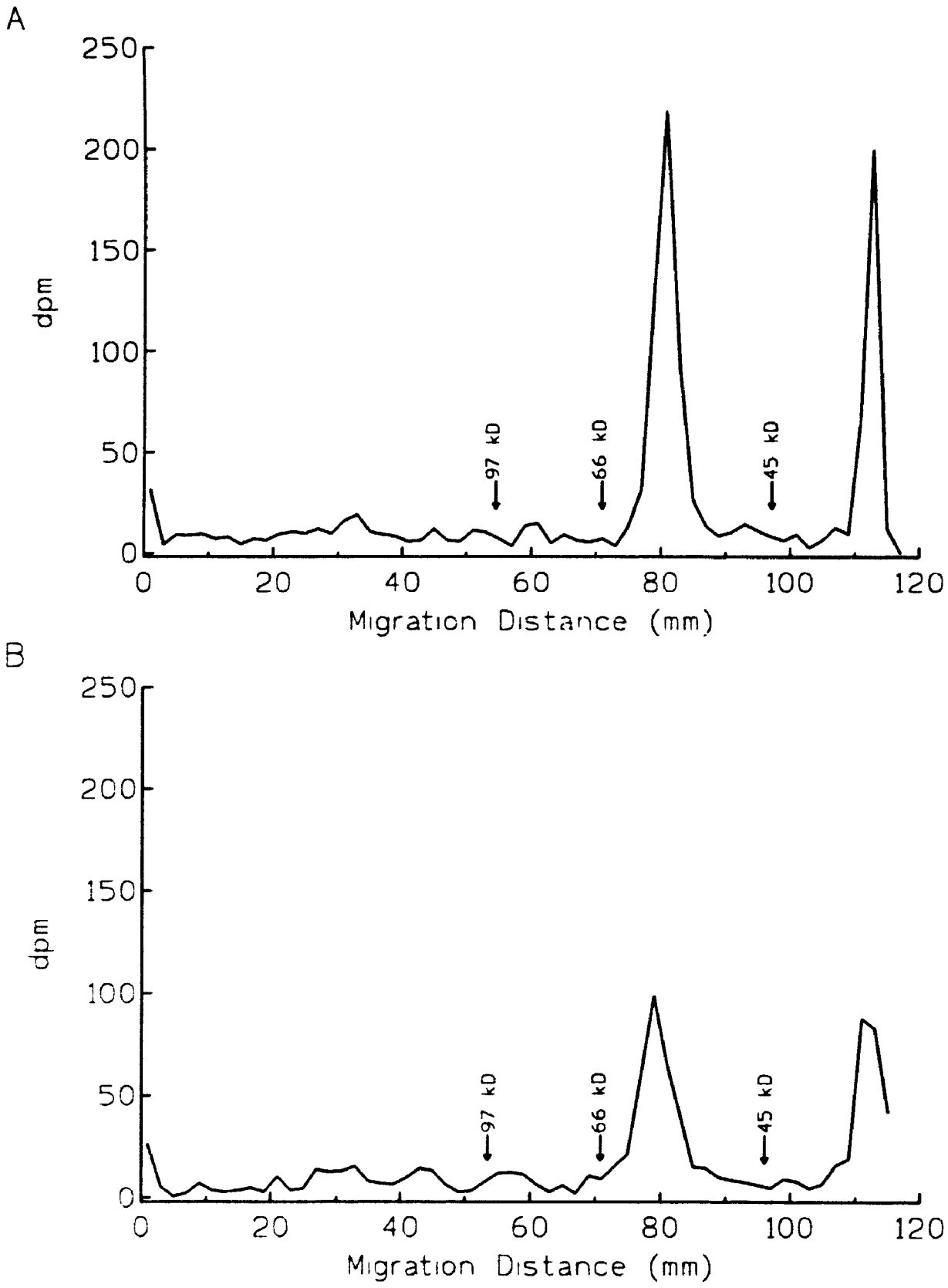

A
250
200
150
100
50
0
dpm
97 kD
66 kD
45 kD
0
20
40
60
80
100
120
Migration Distance (mm)
B
250
200
150
100
50
0
dpm
97 kD
66 kD
45 kD
0
20
40
60
80
100
120
Migration Distance (mm)

Figure 4.4 **Radioactivity profile from SDS-PAGE of pulmonary microsomes incubated with [^{14}C]2,3-BBT or [^{14}C]7-BBT.** Pulmonary microsomes from untreated guinea pigs were incubated with (A) [^{14}C]2,3-BBT or (B) [^{14}C]7-BBT (100 μM, 0.225 μCi) and 1 mM NADPH. Locations of molecular weight standards are indicated by arrows.

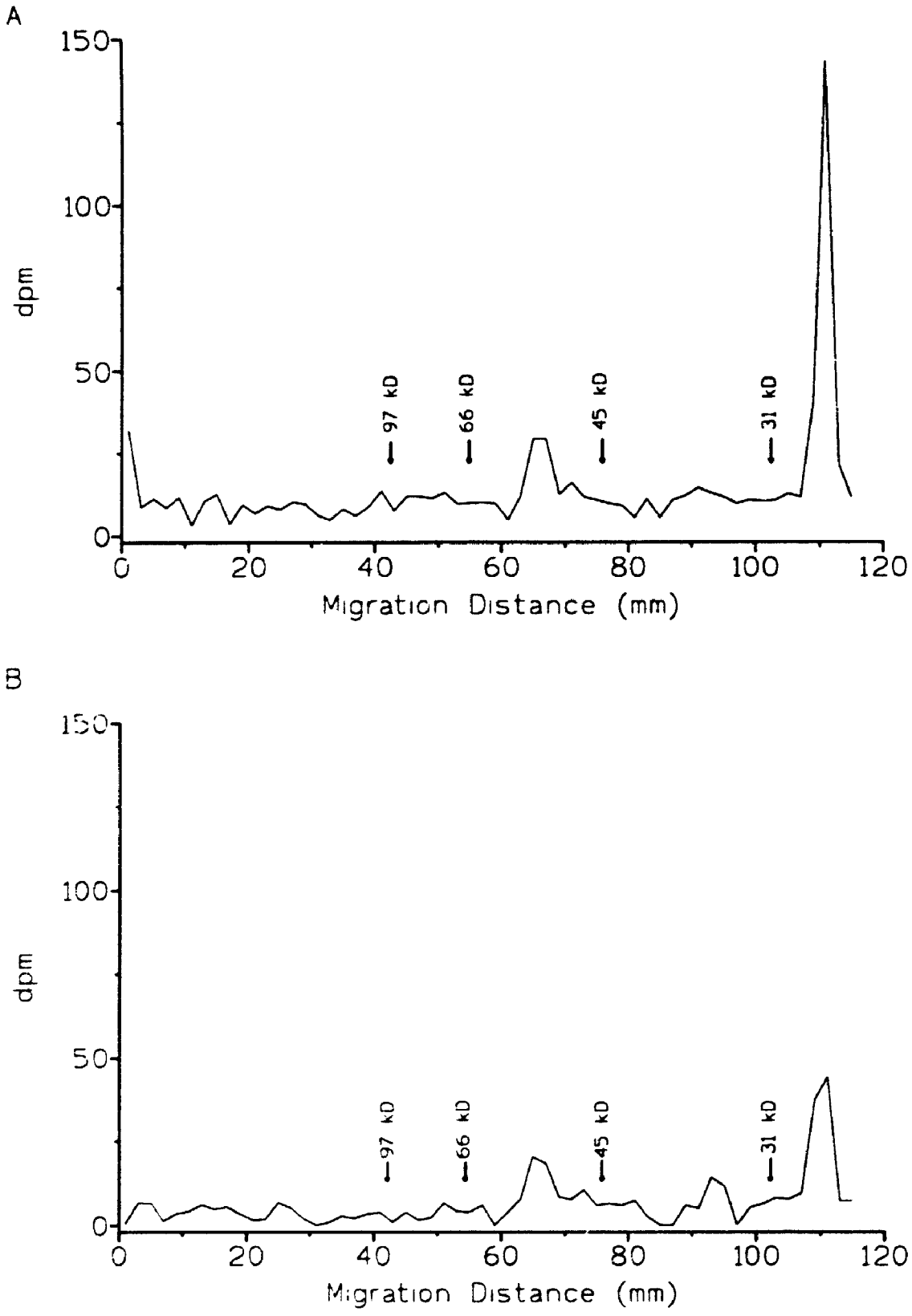

A
150
100
50
0
dpm
97 kD
66 kD
45 kD
31 kD
0
20
40
60
80
100
120
Migration Distance (mm)
B
150
100
50
0
dpm
97 kD
66 kD
45 kD
31 kD
0
20
40
60
80
100
120
Migration Distance (mm)

Figure 4.5 **Radioactivity profile from SDS-PAGE of hepatic microsomes incubated with [^{14}C]ABT.** Hepatic microsomes from (A) PB-induced or (B) βNF-induced guinea pigs were incubated with [^{14}C]ABT (100 μM, 0.225 μCi) and 1 mM NADPH. Locations of molecular weight standards are indicated by arrows.

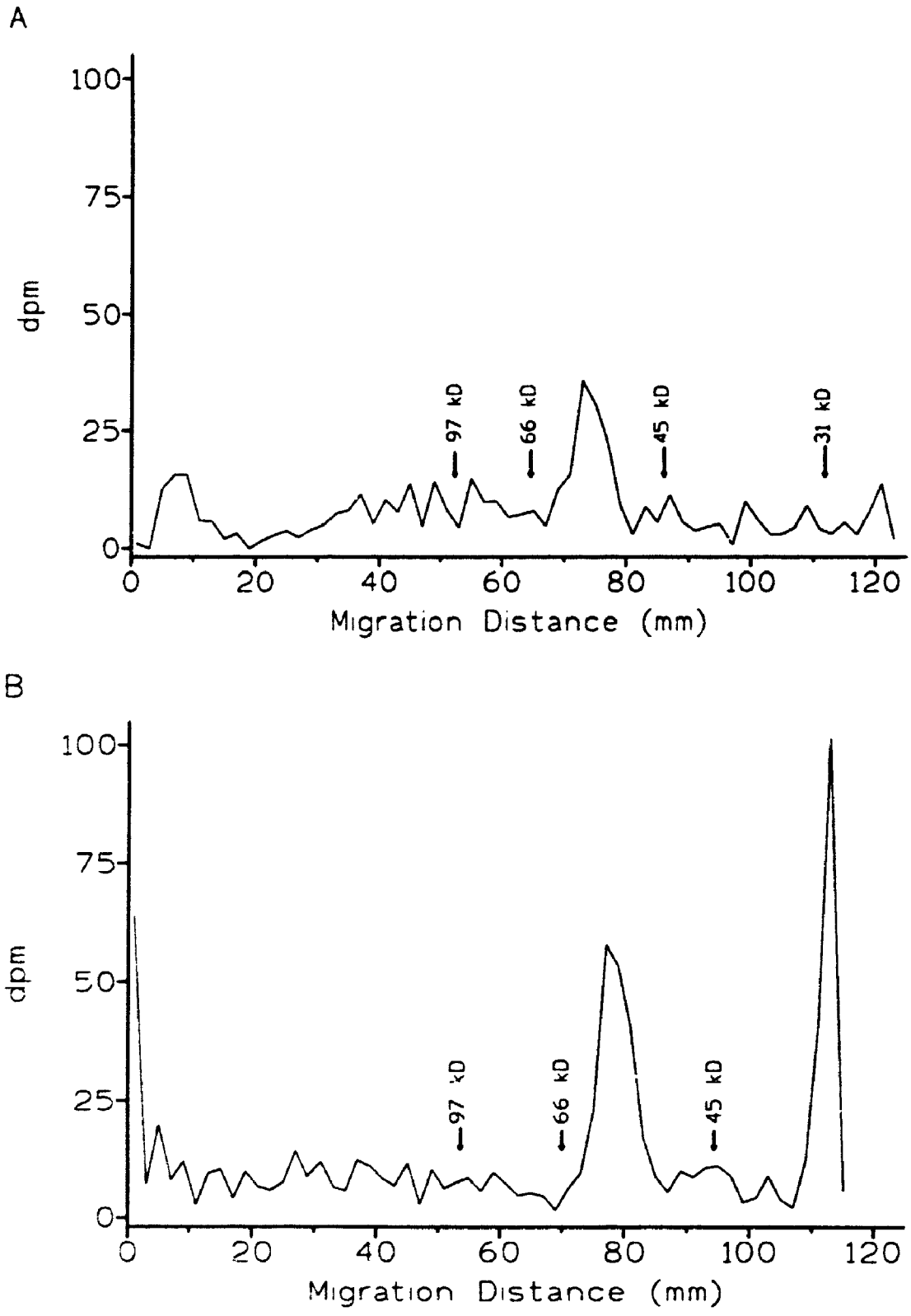
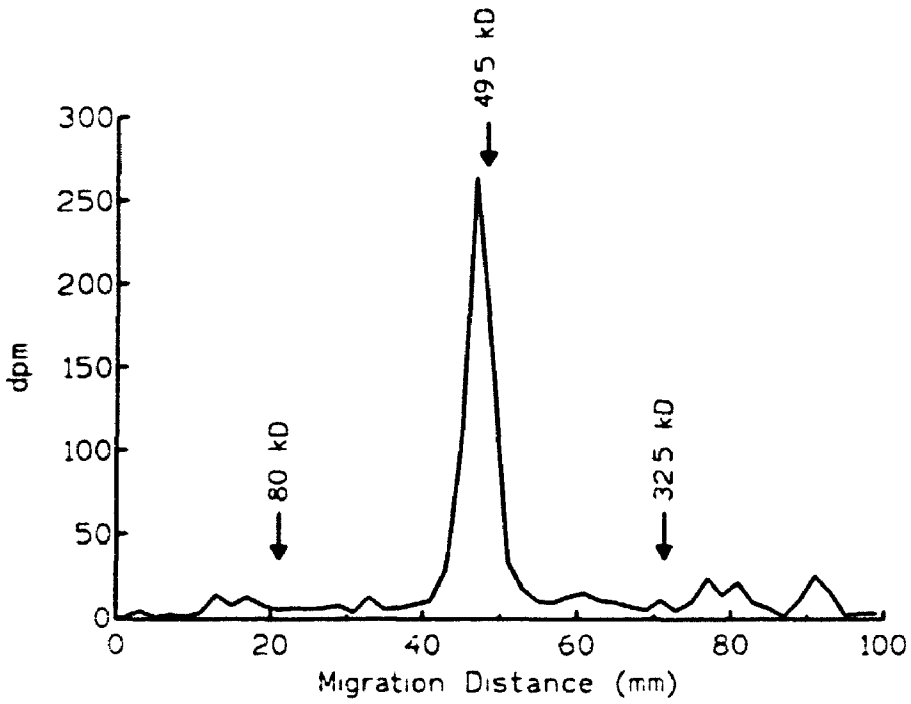
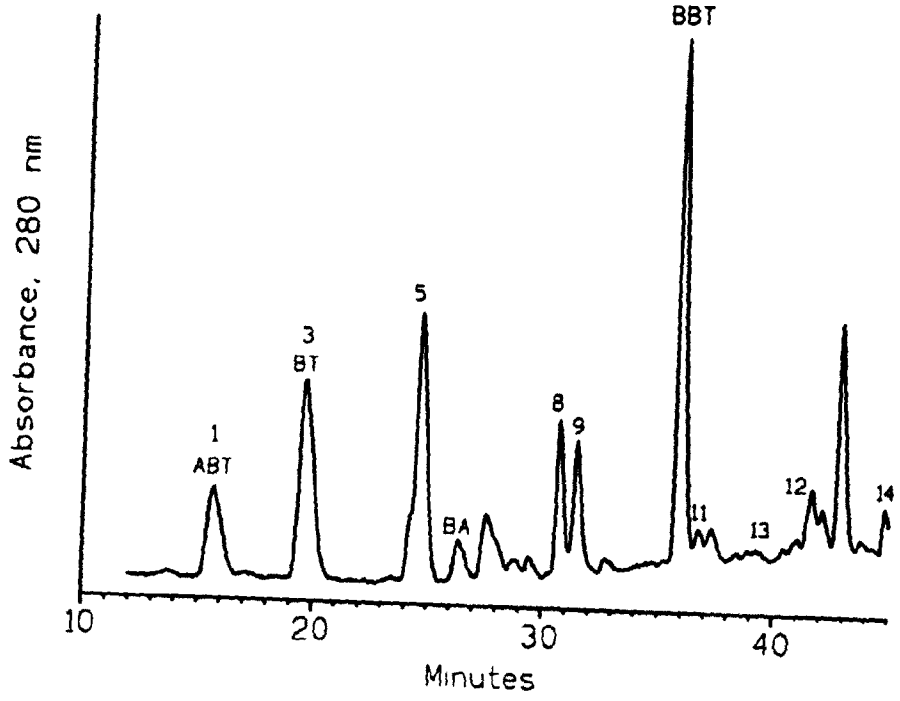

A
100
75
50
25
0
dpm
97 kD
66 kD
45 kD
31 kD
0
20
40
60
80
100
120
Migration Distance (mm)
B
100
75
50
25
0
dpm
97 kD
66 kD
45 kD
0
20
40
60
80
100
120
Migration Distance (mm)

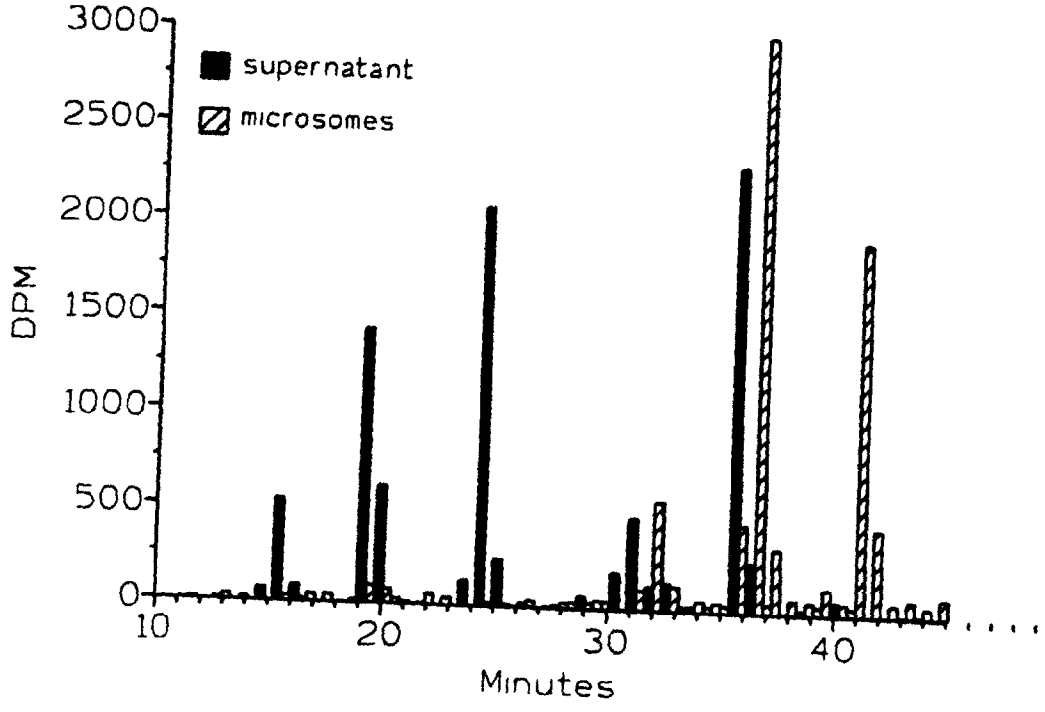
Figure 4.6 **Western blot for P450 2B4 and corresponding radioactivity profile of hepatic microsomes from PB-induced guinea pigs incubated with [^{14}C]2,3-BBT.** Hepatic microsomes from PB-treated animals were incubated with [^{14}C]2,3-BBT (100 μM, 0.225 μCi) and 1 mM NADPH, subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with antibodies to rabbit P450 2B4. Approximate locations of prestained molecular weight standards are indicated by arrows.

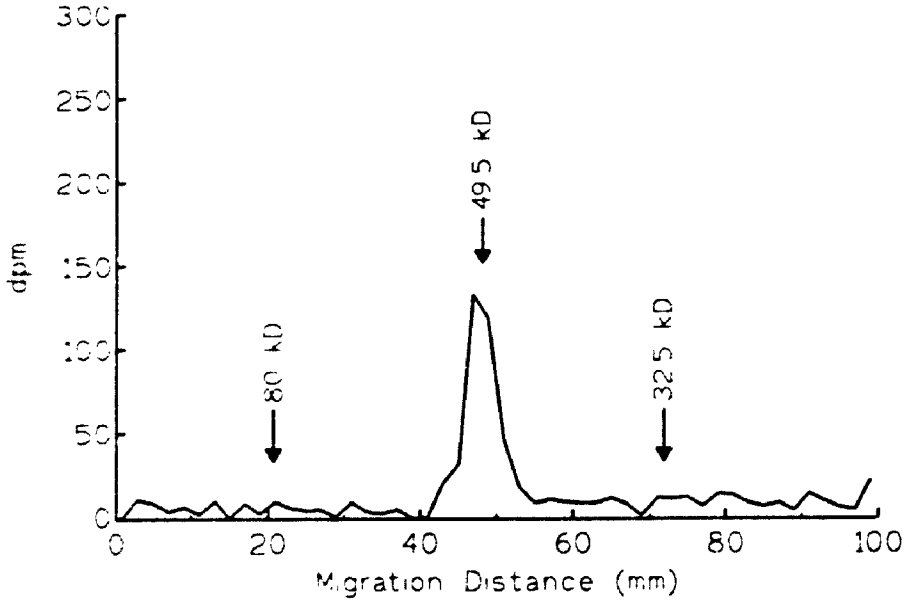

495 kD
300
250
200
150
100
50
0
dpm
80 kD
325 kD
0
20
40
60
80
100
Migration Distance (mm)

A


Absorbance, 280 nm
1
ABT
3
BT
5
BA
8
9
BBT
11
13
12
14
10
20
30
40
Minutes

B


3000
2500
2000
1500
1000
500
0
DPM
supernatant
microsomes
10
20
30
40
Minutes


300
250
200
150
100
50
0
dpm
80 kD
49.5 kD
32.5 kD
0
20
40
60
80
100
Migration Distance (mm)

lower and higher molecular weight than P450 2Bx.

[^{14}C]2,3-BBT was converted to multiple radiolabelled metabolites by hepatic microsomes from βNF-induced (Figure 4.8) and PB-induced (Figure 4.9) guinea pigs in the presence of NADPH. Four metabolites were formed exclusively by microsomes from βNF-induced (metabolites 2, 4, 7, 11) and one from PB-induced (metabolite 8) animals. Metabolites 1 and 3 were identified as [^{14}C]ABT and [^{14}C]benzotriazole, respectively, on the basis of co-elution of the radioactive material with authentic standards.

In the presence of NADPH, [^{14}C]7-BBT was also converted to multiple metabolites by microsomes from βNF-liver (Figure 4.10) and PB-liver (Figure 4.11). One metabolite (10) was formed exclusively by microsomes from βNF-induced guinea pigs, while highly lipophilic metabolites 13 and 14 were not found in these microsomes. Metabolite 6 was identified as [^{14}C]benzaldehyde on the basis of co-elution with authentic standard.

Pulmonary microsomes, in the presence of NADPH, formed very small amounts (<5 nmol) of five radiolabelled metabolites from [^{14}C]2,3-BBT (Figure 4.12; Table 4.2), including [^{14}C]benzotriazole. Each of these metabolites, with the exception of [^{14}C]benzotriazole, was also generated from [^{14}C]7-BBT (Figure 4.13; Table 4.2). None of these metabolites were unique to pulmonary microsomes. Unchanged BBT accounted for 50% of the [^{14}C]2,3-BBT, and 60% of the [^{14}C]7-BBT initially added to the incubation mixtures (Table 4.2), and no ABT was found.

The metabolite profile of [^{14}C]2,3-BBT and [^{14}C]7-BBT differed

Figure 4.8 **Representative HPLC chromatogram of [^{14}C]2,3-BBT metabolites and corresponding elution profiles of radioactivity formed by hepatic microsomes from βNF-induced guinea pigs.** (A) Chromatogram of [^{14}C]2,3-BBT metabolites present in neutral EtAc extracts of microsomal supernatant and (B) corresponding elution profiles of [^{14}C] obtained by HPLC analysis (HPLC system B) of neutral EtAc extracts of microsomal supernatant (■) and microsomes (▨) from incubation mixtures of hepatic microsomes from βNF-induced guinea pigs with [^{14}C]2,3-BBT (100 μM, 0.225 μCi) and 1 mM NADPH. Radiolabelled metabolites are indicated by Arabic numerals. BT = benzotriazole, BA = benzaldehyde.

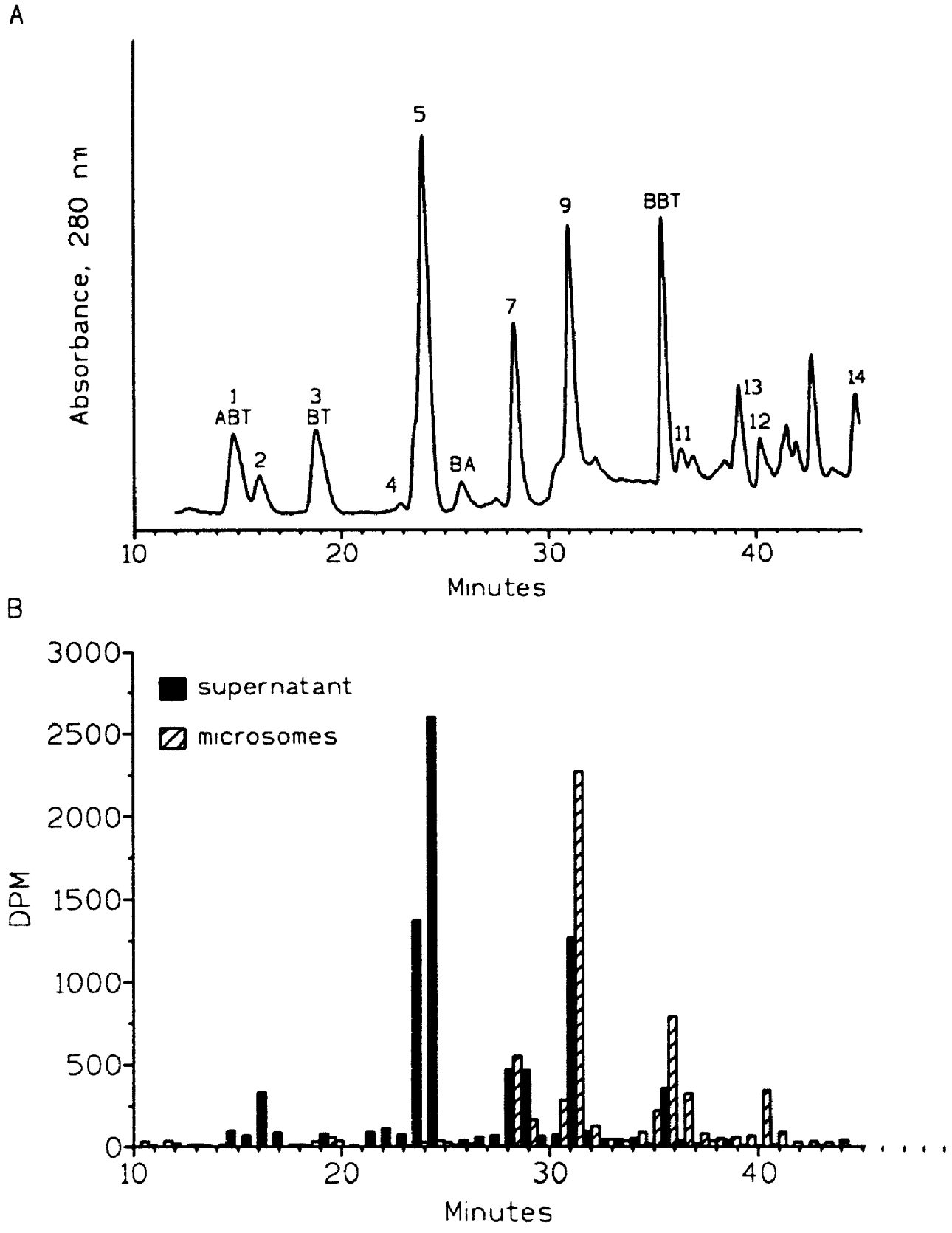

A
Absorbance, 280 nm
1
ABT
2
3
BT
4
5
BA
7
9
BBT
11
12
13
14
10
20
30
40
Minutes
B
3000
2500
2000
1500
1000
500
0
DPM
supernatant
microsomes
10
20
30
40
Minutes

Figure 4.9 **Representative HPLC chromatogram of [^{14}C]2,3-BBT metabolites and corresponding elution profiles of radioactivity formed by hepatic microsomes from PB-induced guinea pigs.** (A) Chromatogram of [^{14}C]2,3-BBT metabolites present in neutral EtAc extracts of microsomal supernatant and (B) corresponding elution profiles of [^{14}C] obtained by HPLC analysis (HPLC system B) of neutral EtAc extracts of microsomal supernatant (■) and microsomes (▨) from incubation mixtures of hepatic microsomes from PB-induced guinea pigs with [^{14}C]2,3-BBT (100 μM, 0.225 μCi) and 1 mM NADPH. Radiolabelled metabolites are indicated by Arabic numerals. BT = benzotriazole, BA = benzaldehyde.

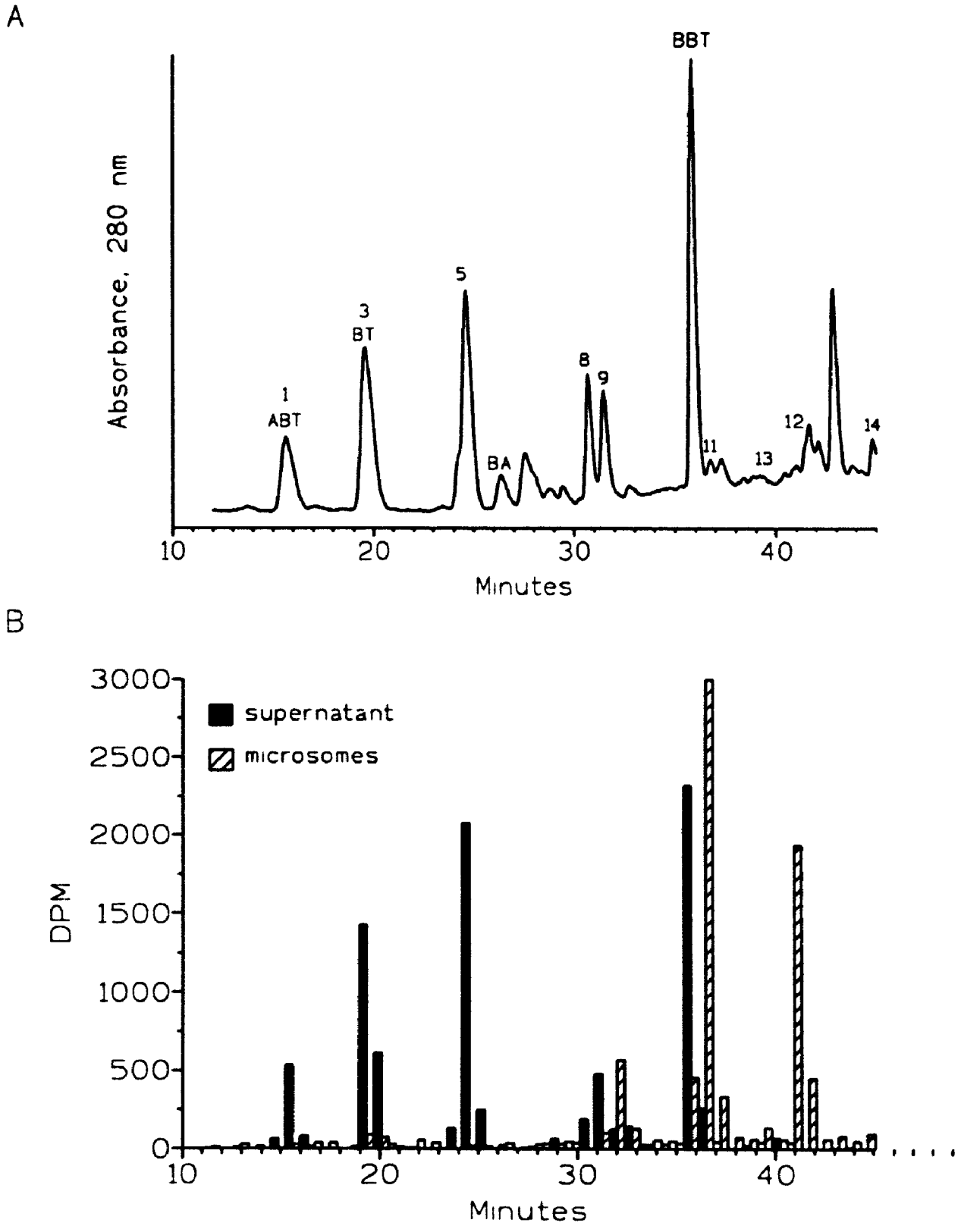

A
Absorbance, 280 nm
1
ABT
3
BT
5
BA
8
9
BBT
11
12
13
14
10
20
30
40
Minutes
B
3000
2500
2000
1500
1000
500
0
DPM
supernatant
microsomes
10
20
30
40
Minutes

Figure 4.10 **Representative HPLC chromatogram of [^{14}C]7-BBT metabolites and corresponding elution profiles of radioactivity formed by hepatic microsomes from βNF-induced guinea pigs.** (A) Chromatogram of [^{14}C]7-BBT metabolites present in neutral EtAc extracts of microsomal supernatant and (B) corresponding elution profiles of [^{14}C] obtained by HPLC analysis (HPLC system B) of neutral EtAc extracts of microsomal supernatant (■) and microsomes (▨) from incubation mixtures of hepatic microsomes from βNF-induced guinea pigs with [^{14}C]7-BBT (100 μM, 0.225 μCi) and 1 mM NADPH. Radiolabelled metabolites are indicated by Arabic numerals. BT = benzotriazole, BA = benzaldehyde.

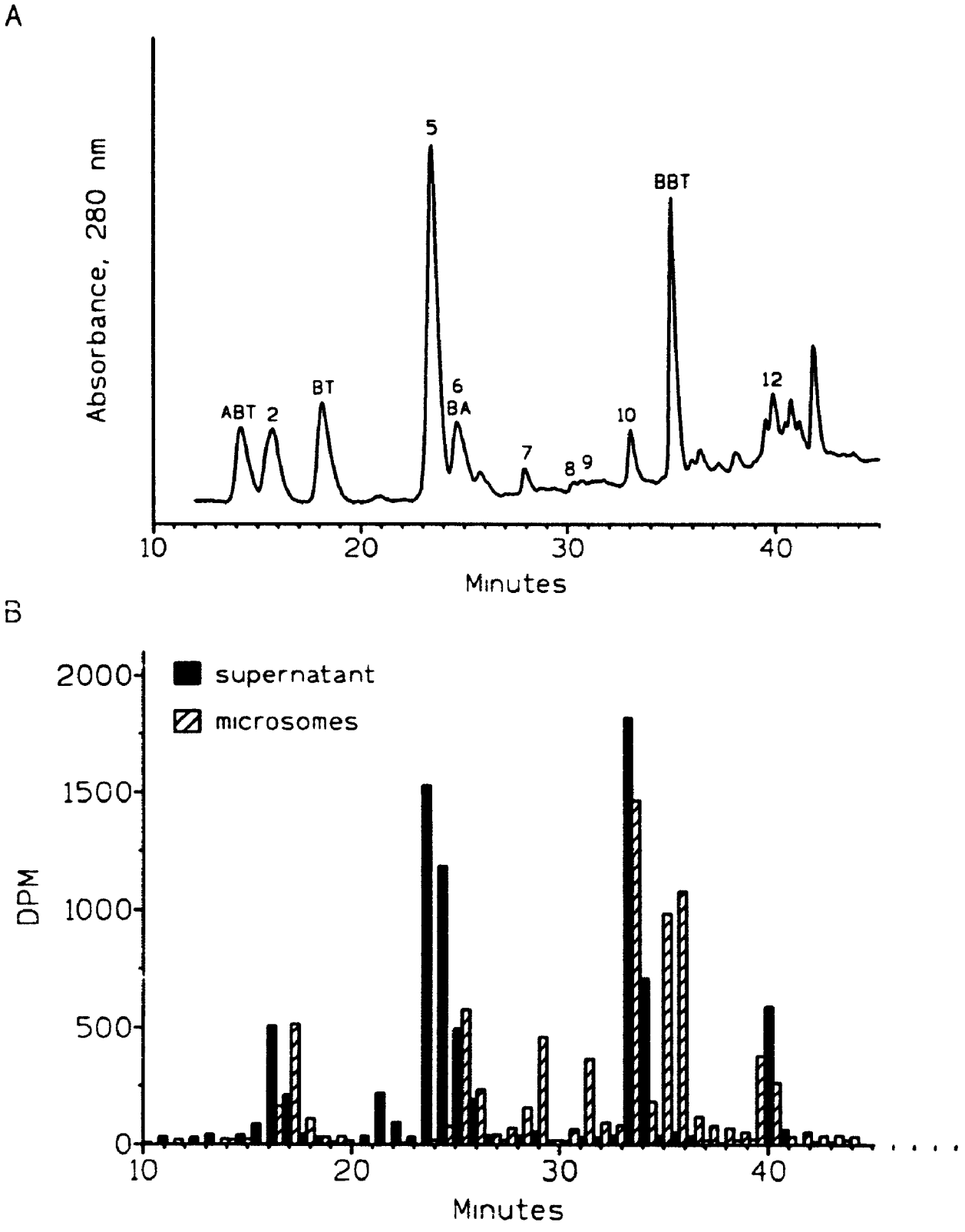
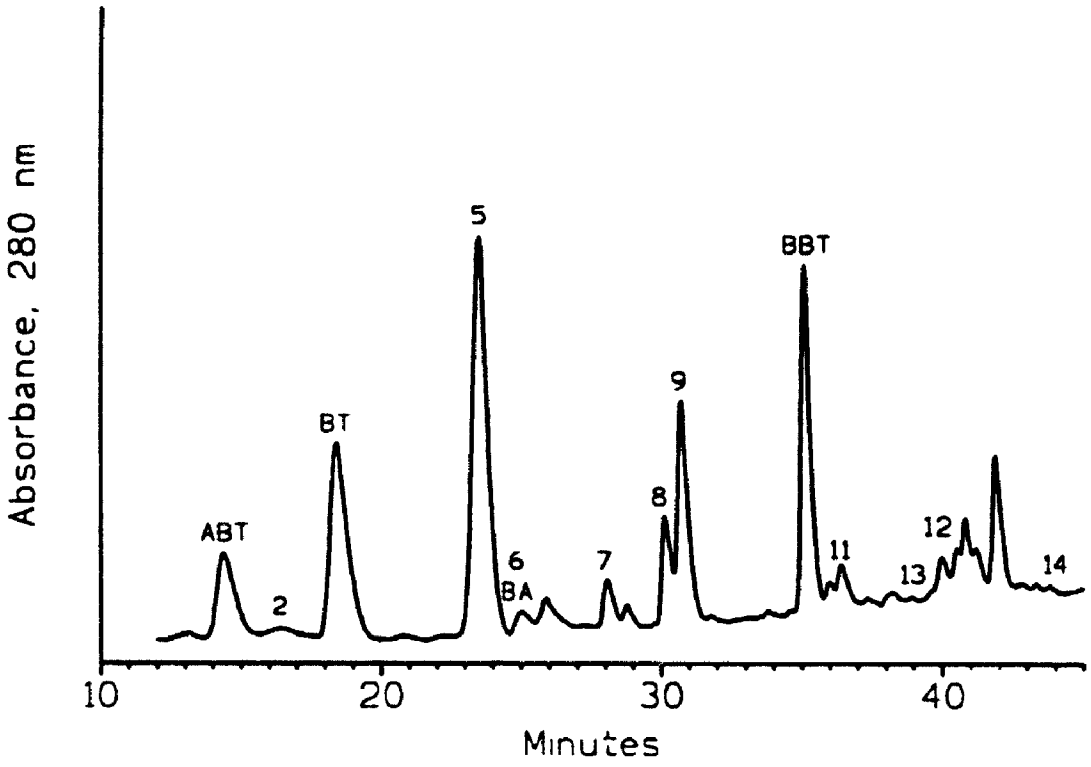

A
Absorbance, 280 nm
ABT
2
BT
5
6
BA
7
8
9
10
BBT
12
10
20
30
40
Minutes
B
supernatant
microsomes
2000
1500
1000
500
0
DPM
10
20
30
40
Minutes

Figure 4.11 **Representative HPLC chromatogram of [^{14}C]7-BBT metabolites and corresponding elution profiles of radioactivity formed by hepatic microsomes from PB-induced guinea pigs.** (A) Chromatogram of [^{14}C]7-BBT metabolites present in neutral EtAc extracts of microsomal supernatant and (B) corresponding elution profiles of [^{14}C] obtained by HPLC analysis (HPLC system B) of neutral EtAc extracts of microsomal supernatant (■) and microsomes (▨) from incubation mixtures of hepatic microsomes from PB-induced guinea pigs with [^{14}C]7-BBT (100 μM, 0.225 μCi) and 1 mM NADPH. Radiolabelled metabolites are indicated by Arabic numerals. BT = benzotriazole, BA = benzaldehyde.

A


Absorbance, 280 nm
ABT
2
BT
5
6
BA
7
8
9
BBT
11
12
13
14
10
20
30
40
Minutes

B

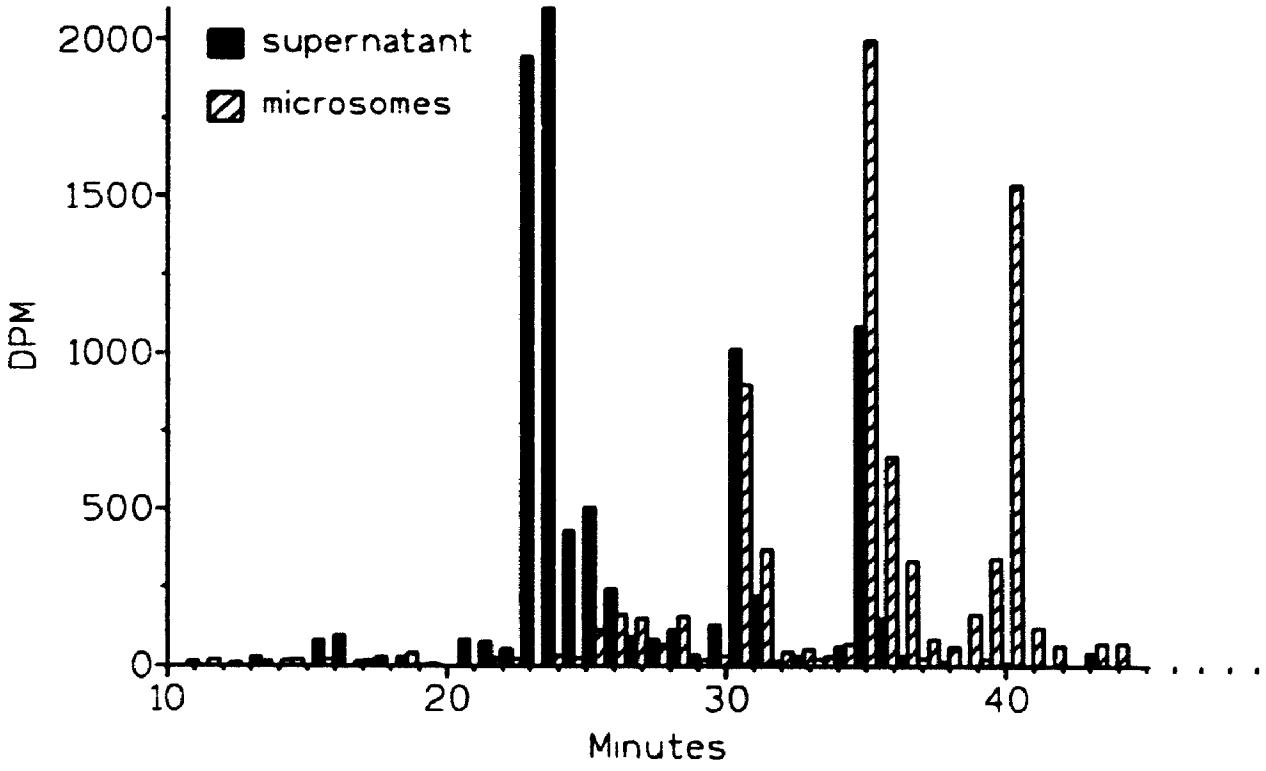
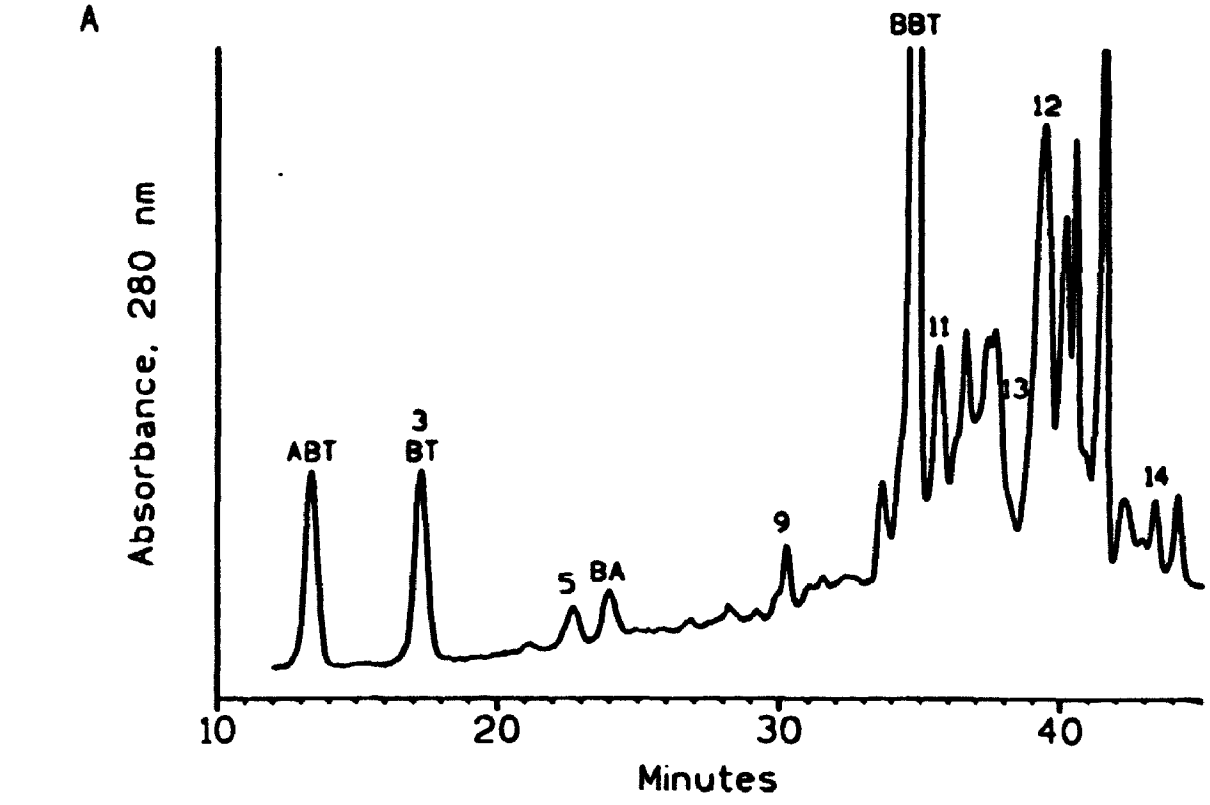

supernatant
microsomes
DPM
2000
1500
1000
500
0
10
20
30
40
Minutes

Figure 4.12 **Representative HPLC chromatogram of [^{14}C]2,3-BBT metabolites and corresponding elution profiles of radioactivity formed by pulmonary microsomes from untreated guinea pigs.** (A) Chromatogram of [^{14}C]2,3-BBT metabolites present in neutral EtAc extracts of microsomal supernatant and (B) corresponding elution profiles of [^{14}C] obtained by HPLC analysis (HPLC system B) of neutral EtAc extracts of microsomal supernatant (■) and microsomes (▨) from incubation mixtures of pulmonary microsomes from untreated guinea pigs with [^{14}C]2,3-BBT (100 μM, 0.225 μCi) and 1 mM NADPH. Radiolabelled metabolites are indicated by Arabic numerals. BT = benzotriazole, BA = benzaldehyde.


A
Absorbance, 280 nm
ABT
3
BT
5
BA
9
BBT
11
12
13
14
10
20
30
40
Minutes

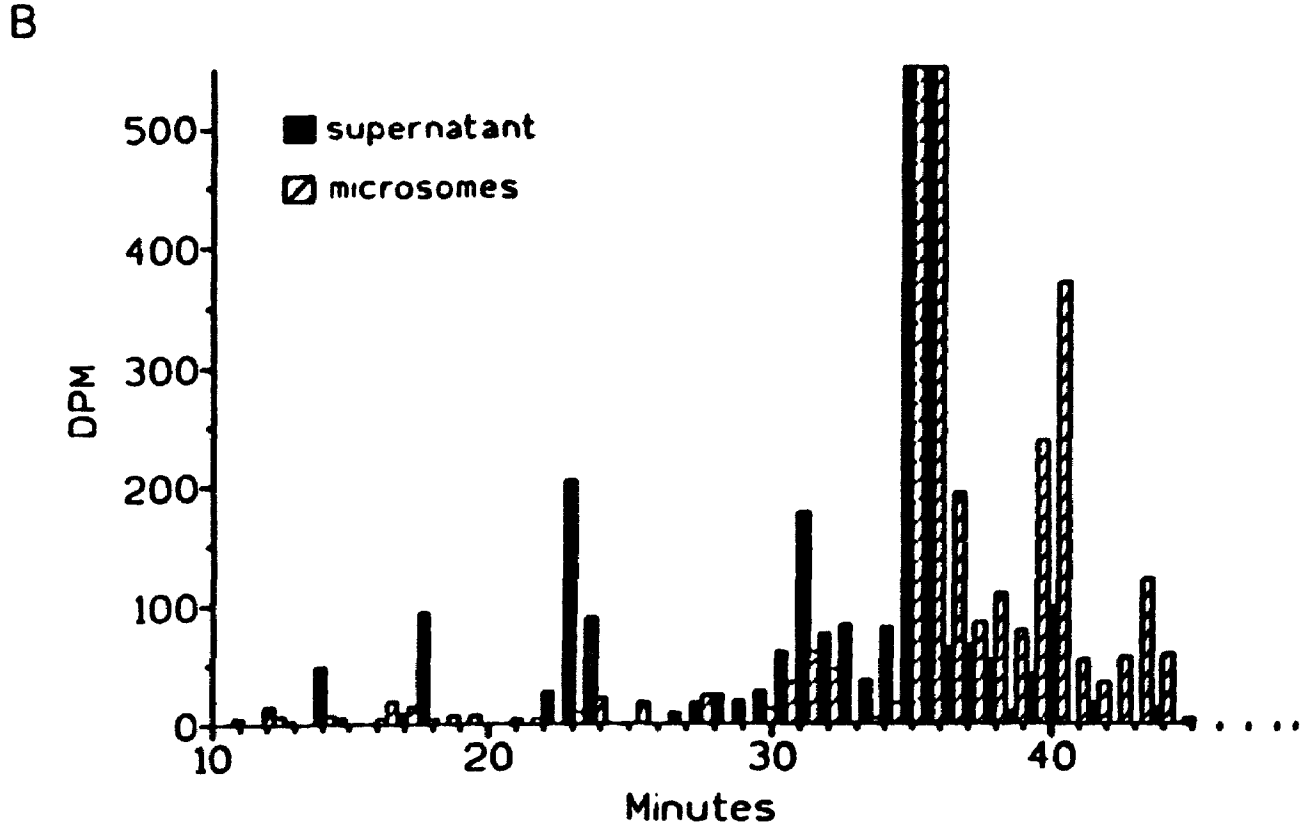
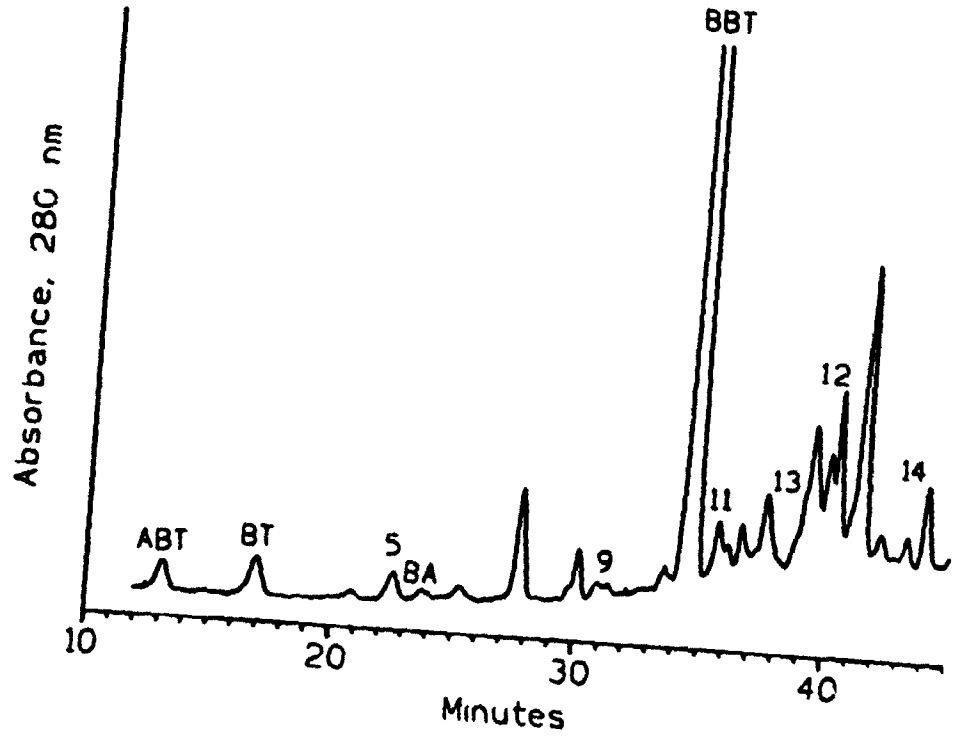

B
supernatant
microsomes
DPM
500
400
300
200
100
0
10
20
30
40
Minutes

Figure 4.13 **Representative HPLC chromatogram of [^{14}C]7-BBT metabolites and corresponding elution profiles of radioactivity formed by pulmonary microsomes from untreated guinea pigs.** (A) Chromatogram of [^{14}C]7-BBT metabolites present in neutral EtAc extracts of microsomal supernatant and (B) corresponding elution profiles of [^{14}C] obtained by HPLC analysis (HPLC system B) of neutral EtAc extracts of microsomal supernatant (■) and microsomes (▨) from incubation mixtures of pulmonary microsomes from untreated guinea pigs with [^{14}C]7-BBT (100 μM, 0.225 μCi) and 1 mM NADPH. Radiolabelled metabolites are indicated by Arabic numerals. BT = benzotriazole, BA = benzaldehyde.

A


Absorbance, 280 nm
ABT
BT
5
BA
9
11
BBT
12
13
14
10
20
30
40
Minutes

B

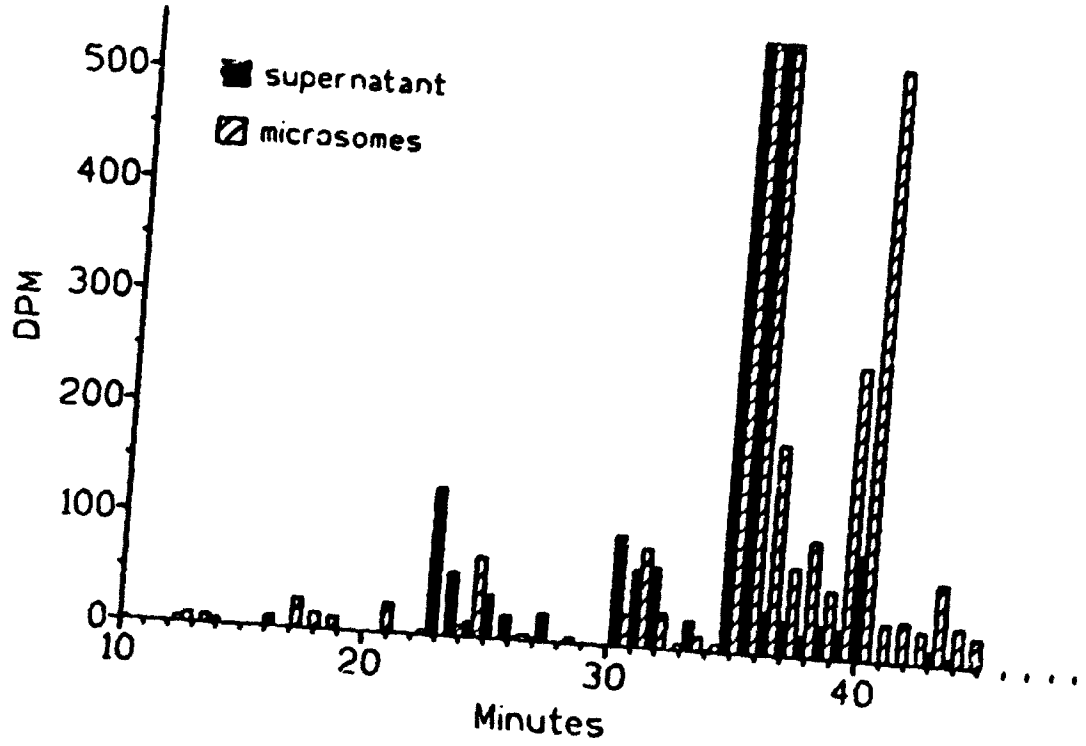

supernatant
microsomes
DPM
500
400
300
200
100
0
10
20
30
40
Minutes

Table 4.2

Radiolabelled [^{14}C]2,3-BBT or [^{14}C]7-BBT metabolites (nmol) in EtAc extracts of hepatic microsomal incubation mixtures from PB- or βNF-induced guinea pigs or pulmonary microsomes from untreated guinea pigs.*

	Microsome Source	Metabolite					
		1 (ABT)	2	3 (BT)	4	5	6 (BA)
2,3-BBT	PB Liver	3.9		12.4		12.9	
	βNF Liver	1.4	3.6	1.5	2.8	37.5	
	Lung			1.4		1.8	
7-BBT	PB Liver		0.9			33.2	4.2
	βNF Liver		7.0			20.7	5.6
	Lung					2.0	

*Values shown are means of duplicate analyses from duplicate incubations of [^{14}C]2,3-BBT or [^{14}C]7-BBT, 1 mM NAPDH, and microsomes from the indicated source. Duplicate values differed by <10%. Metabolite numbers correspond to those in Figures 4.1-4.6. BT = benzotriazole, BA = benzaldehyde.

Table 4.2, con't.

HPLC Peak No.								
7	8	9	10	11	12	13	14	BBT
	2.0	7.3			11.7	0.9	0.4	40.2
12.2		23.4		1.6	1.8	0.4	0.4	6.9
		3.2		1.8	3.6	0.6	0.5	96.6
2.6	2.5	13.5		1.7	9.1	0.9	0.6	23.6
0.7	0.8	1.0	21.1		5.8			3.2
		2.0		2.2	4.7	0.5	0.3	121.3

quantitatively between hepatic microsomes from PB- and βNF-induced animals (Table 4.2). Of the common metabolites, 5 and 9 were produced in greater quantities from [^{14}C]2,3-BBT by βNF- than PB-microsomes (38 vs 13 and 23 vs 7 nmol, respectively), while the reverse was true for [^{14}C]7-BBT (33 vs 21 and 14 vs 1 nmol, PB- vs βNF-induced, respectively). ABT, benzotriazole and metabolite 12 from [^{14}C]2,3-BBT were produced in greater quantities by PB-microsomes (4 vs 1, 12 vs 2, and 12 vs 2 nmol, PB- vs βNF-induced, respectively), while metabolite 2 from [^{14}C]7-BBT was formed in greatest quantity by microsomes of βNF-induced animals (7 vs 1 nmol). Slightly greater amounts of [^{14}C]benzaldehyde from [^{14}C]7-BBT were also formed by βNF-liver microsomes (6 vs 4 nmol).

The amount of unchanged BBT remaining was greater following incubation with hepatic microsomes from PB- than βNF-induced guinea pigs (40 vs 7 nmol [^{14}C]2,3-BBT and 24 vs 3 nmol [^{14}C]7-BBT, respectively; Table 4.2).

In the presence of NADPH, [^{14}C]ABT was converted to [^{14}C]benzotriazole (co-chromatography with authentic compound in HPLC systems B and C) by all hepatic and pulmonary microsomal preparations examined (Table 4.3). The rank order for benzotriazole formation was βNF liver > PB liver > liver > lung, determined by HPLC analysis of [^{14}C]benzotriazole formed (Table 4.3). The amount of unchanged [^{14}C]ABT present was found to have the reverse rank order (lung > liver > PB liver > βNF liver; Table 4.3). Benzotriazole was the only metabolite formed in microsomes from lung, liver and PB-liver. However, small amounts (2 nmol) of a second unknown stable metabolite, retention time

Table 4.3

[^{14}C]ABT and metabolites (nmol) in EtAc extracts of hepatic or pulmonary microsomes from untreated, PB– or βNF–induced guinea pigs.[a]

Microsome Source	Benzotriazole	Unknown	ABT
Liver	8.2		108
PB Liver	12.4		96
βNF Liver	16.5	2.3	84
Lung	4.5		124

[a]Values shown are means of duplicate analyses from duplicate incubations of [^{14}C]ABT, 1 mM NADPH and microsomes from the indicated source. Duplicate values differed by <10%.

< ABT (HPLC systems B and C) were also formed by hepatic microsomes from βNF-induced guinea pigs.

More than 70% (>140 nmol) of [^{14}C]ABT-derived radioactivity partitioned into the supernatant fraction upon centrifugation of hepatic microsomal incubation mixtures following incubation in the presence or absence of NADPH. With pulmonary microsomes, >80% of the radiolabel was present in the supernatant fraction.

In contrast, approximately 40% of [^{14}C]2,3-BBT- or [^{14}C]7-BBT-derived radioactivity was retained in the microsomes following incubation of hepatic microsomes from PB- or βNF-induced animals in the absence of NADPH. Upon incubation with NADPH, a much larger fraction (up to 70%) of total radioactivity was in the supernatant fraction. On the other hand, in incubation mixtures containing pulmonary microsomes, 70-80% of the initial [^{14}C]BBT content was found in the supernatant, with little variation between incubations with or without NADPH, or between [^{14}C]2,3-BBT and [^{14}C]7-BBT.

EtAc extraction of basified (pH 10) or acidified (pH 2) aqueous residues of EtAc extracted microsomal supernatant or microsomal fractions removed less than 1.5% of the total radiolabel, except extraction of acidified hepatic microsomal supernatants, which removed up to 7.5% (0.5-15 nmol) of the total radiolabel.

4.4 Discussion

The inhibitor concentration chosen for this study was based on our

previous finding that 100 μM BBT inhibits >80% of PRF and ERF activities in hepatic microsomes from PB-induced or untreated guinea pigs with <40% loss of P450 (Chapter 3), a situation where significant amounts of P450 inactivation occur by a mechanism other than covalent heme modification.

The covalent binding and metabolic data demonstrate conclusively that BBT is activated by P450 by at least two routes, each with one or more reactive metabolites. *N*-Debenzylation of BBT occurs as shown by the formation of [^{14}C]ABT and [^{14}C]benzotriazole from [^{14}C]2,3-BBT, and [^{14}C]benzaldehyde from [^{14}C]7-BBT (Figures 4.8-4.11; Table 4.2). *N*-Debenzylation of BBT could result from either initial benzylic carbon hydroxylation, or the oxidation of the exocyclic amino nitrogen (Figure 4.14). Some of the metabolically generated ABT is further metabolized to benzyne; [^{14}C]2,3-BBT-derived radioactivity became covalently bound to hepatic and pulmonary microsomal protein (Table 4.1), and a phenylene-modified porphyrin adduct indistinguishable from that of ABT was previously isolated from livers of PB-induced rats treated with BBT (Mathews and Bend, 1986).

Those metabolites that were common to both radiolabelled forms of BBT were not further characterized as they were not involved in the suicidal mechanism of BBT.

Based on NADPH-dependent covalent binding of [^{14}C]7-BBT to hepatic and pulmonary microsomal proteins, the formation of an additional reactive metabolite from BBT, possibly benzyl radical, also occurs. Two possible routes of formation of such a radical (Figure 4.15) are the generation of unstable

Figure 4.14 **Possible pathways for *N*-debenzylation of BBT.** P450-dependent *N*-debenzylation of BBT could proceed from initial oxidation at the benzylic carbon or the exocyclic amino nitrogen. Adapted from Lindeke and Cho (1982).

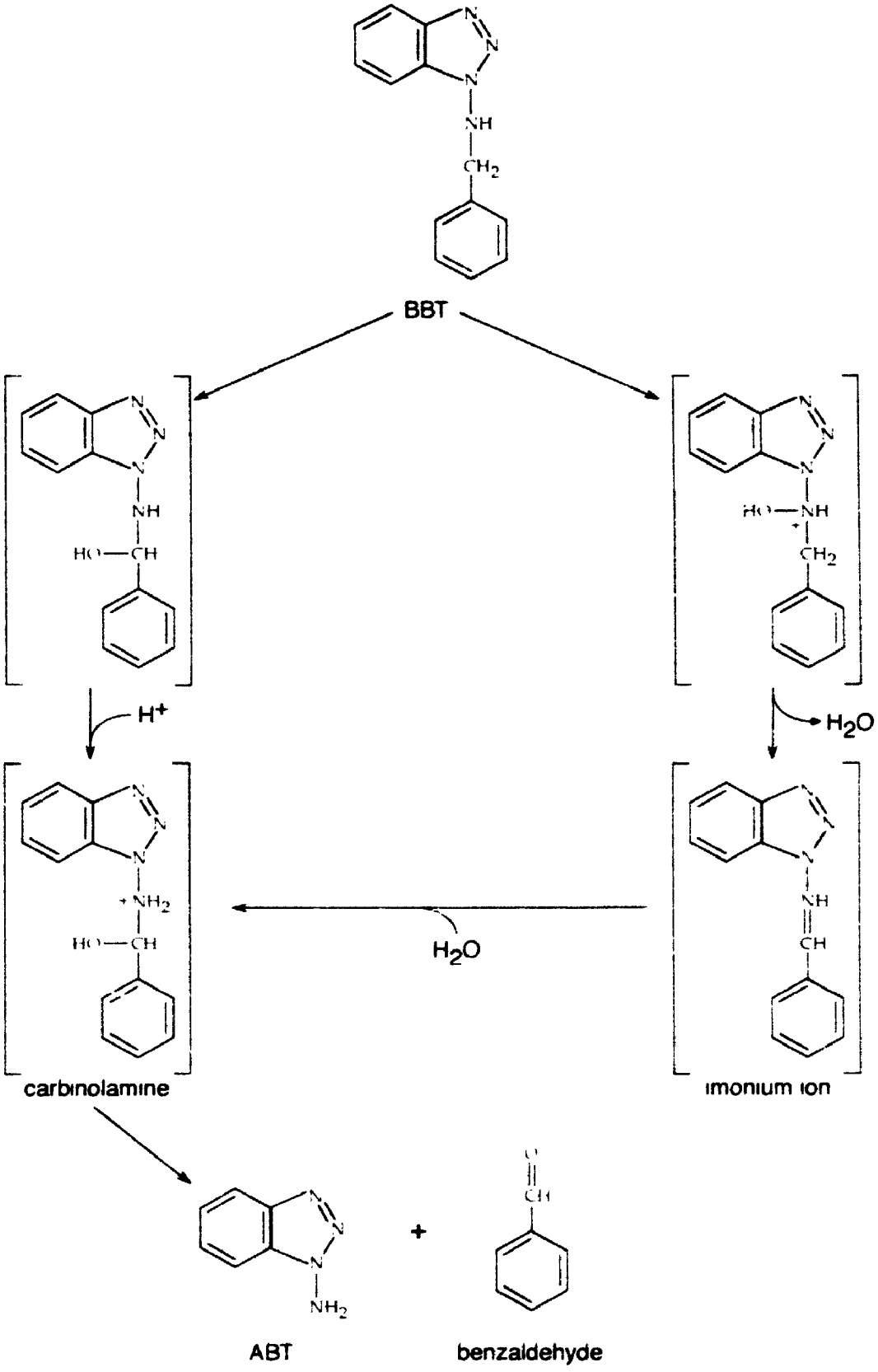
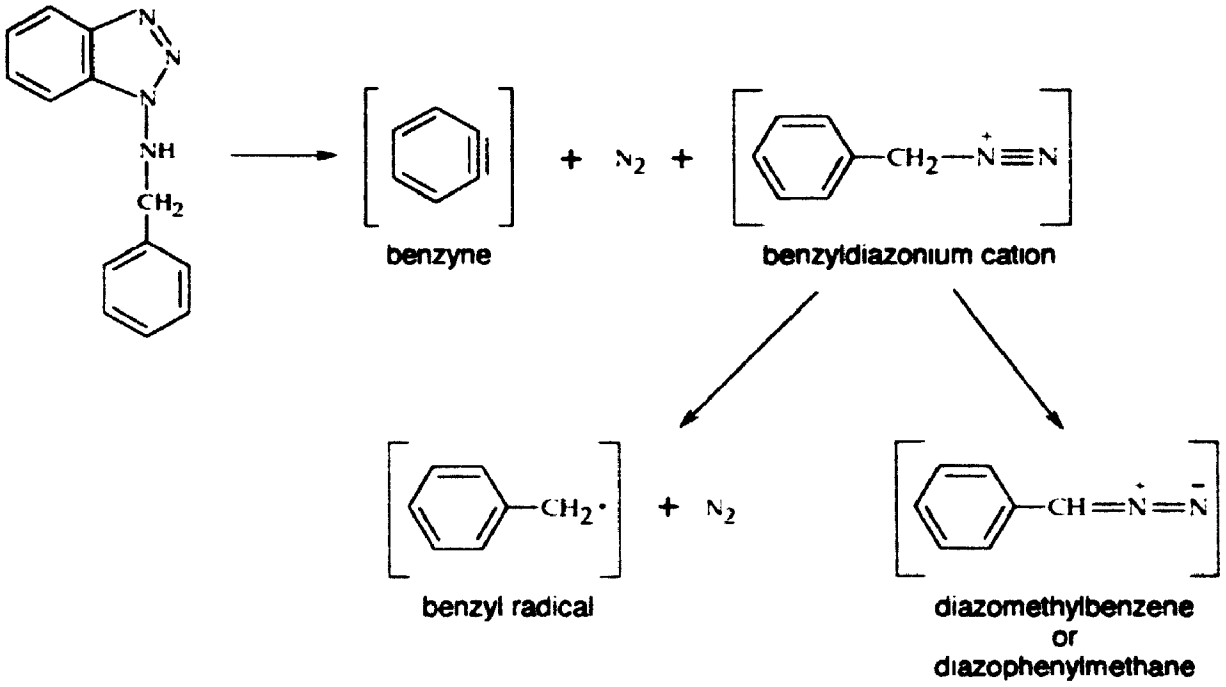
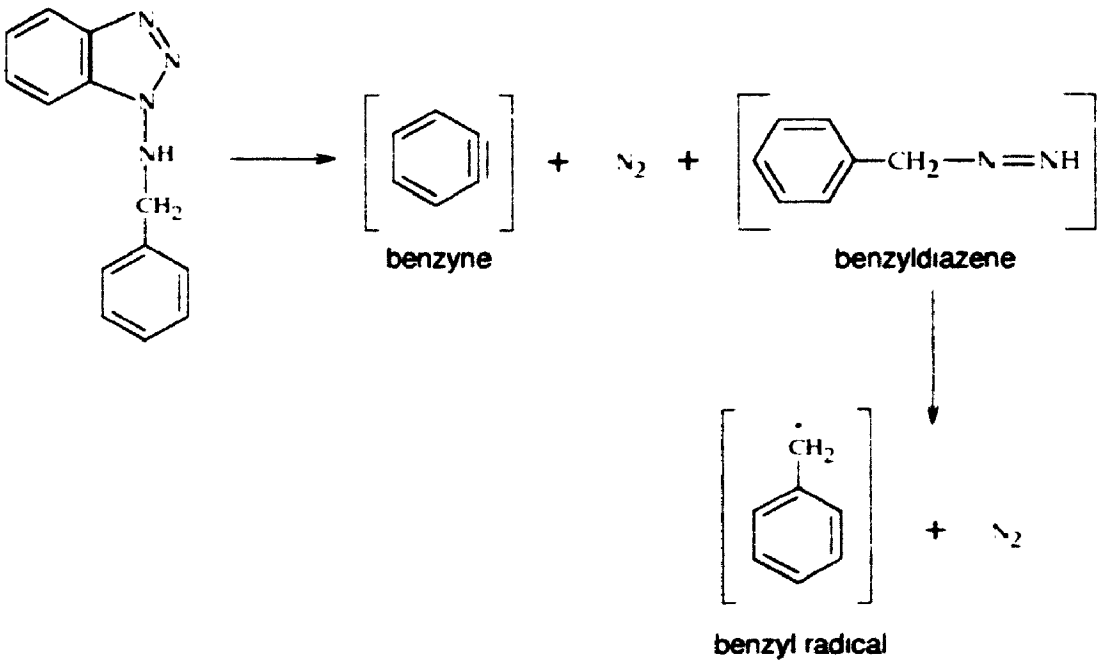

BBT
H+
H2O
H2O
carbinolamine
imonium ion
ABT
benzaldehyde

Figure 4.15 **Possible routes of cytochrome P450-dependent bioactivation of BBT to a reactive intermediate.** Following oxidation of the exocyclic nitrogen and fragmentation of the BBT molecule, benzyl radical formation might proceed via a diazonium cation (A) or diazene (B) intermediate. Adapted from (A) Grab *et al.* (1988) and (B) Ortiz de Montellano and Watanabe (1987).

A)


benzyne
benzyldiazonium cation
benzyl radical
diazomethylbenzene
or
diazophenylmethane

B)


benzyne
benzyldiazene
benzyl radical

benzyldiazonium cation or benzyldiazene species formed upon fragmentation of the BBT following oxidation of the exocyclic amino group. Analogous to the formation of alkyldiazonium cations from sydnones (section 1.2.4.3; Figure 1.2g), the benzyldiazonium cation could be converted to the benzyl radical with the concomitant release of N_2, or it could isomerize to the corresponding diazoalkane, as suggested for *N*-substituted ABT derivatives by Ortiz de Montellano *et al.*(1984). Diazoalkanes formed in this manner are also capable of causing covalent modification of P450, as with the sydnones, PMS and TTMS, although the reactive species involved is not yet known (Grab *et al.*, 1988). Benzyldiazene could also be converted to the benzyl radical by a pathway analogous to the formation of phenylethyl and phenyl radicals from phenelzine and phenylhydrazine, respectively (section 1.2.4.4; Figure 1.2h; 1.2i). Whether or not direct oxidation of the exocyclic nitrogen of BBT occurs to release benzyne (Ortiz de Montellano *et al.*, 1984) is unknown; however, one novel (still unidentified) metabolite was formed from [^{14}C]7-BBT (metabolite 10) consistent with such a possibility.

NADPH-dependent covalent binding of radiolabel from [^{14}C]7-BBT (*vs* [^{14}C]2,3-BBT) was slightly higher in hepatic microsomes from βNF-induced animals (Table 4.1), and more [^{14}C]7-benzaldehyde was present in incubations of liver microsomes from βNF- than PB-treated guinea pigs (Table 4.2), suggesting that P450 1A isozymes may preferentially activate the *N*-benzyl portion of the BBT molecule (vs benzyne formation). Covalent modification of P450 apoprotein by a reactive intermediate (eg. benzyl radical), could occur

directly, or indirectly by release of the radical from benzyl-modified heme, due to the instability of *N*-benzyl-porphyrins in the presence of alternative nucleophilic sites (Schauer *et al.*, 1987; Sugiyama *et al.*, 1989).

Higher amounts of [^{14}C]ABT and [^{14}C]benzotriazole were produced from [^{14}C]2,3-BBT by hepatic microsomes from PB- vs βNF-induced guinea pigs (Table 4.2), suggesting that P450 2Bx preferentially activates the benzotriazole ring of BBT. This is consistent with the pulmonary data where NADPH-dependent covalent binding of [^{14}C] from [^{14}C]2,3-BBT was 3-fold greater than from [^{14}C]7-BBT (Table 4.1), and where P450 2Bx was preferentially inactivated compared to P450 4Bx by BBT (Chapter 3).

Total covalent binding of [^{14}C]BBT metabolites to pulmonary protein is 11-fold greater than the loss of P450, indicating that apoprotein modification could be a major mechanism of pulmonary P450 inactivation by BBT. The extent of covalent reaction on a nmole per nmole P450 basis exceeds the amount of P450 present in pulmonary microsomes. Thus, either P450 has been modified by reaction with multiple amino acid residues (not necessarily all of them being at or near the active site), or significant amounts of covalent binding occur to other proteins.

Covalent binding of [^{14}C]ABT-derived radioactivity to hepatic microsomal protein occurred in the order βNF-induced > PB-induced > untreated guinea pigs (Table 4.1), the inverse of inhibition of P450 activities and P450 loss (Chapter 3). It is possible that some of the covalent binding of [^{14}C]ABT-derived radioactivity is to microsomal proteins other than P450, or at sites on

the P450 proteins other than the active site such that the covalent modification does not interfere with catalytic function.

The low level of NADPH-dependent covalent binding of [^{14}C]ABT to microsomal protein, its inverse correlation with P450 inactivation, and a good correlation between P450 loss and inhibition of monooxygenase activities in guinea pig liver and lung *in vitro* (Chapter 3) and *in vivo* (Knickle and Bend, 1992; Knickle and Bend, unpublished observations), demonstrate that heme adduct formation is the major mechanism of P450 inactivation by ABT in guinea pig liver and lung.

Autooxidation of ABT may account for the NADPH-independent covalent binding of [^{14}C]ABT-derived radioactivity to hepatic microsomes from PB-induced and pulmonary microsomes from untreated animals.

The radiolabelled metabolites of [^{14}C]ABT and both chemical forms of [^{14}C]BBT covalently bound to hepatic and pulmonary microsomal protein migrated to the molecular weight region corresponding to P450 on SDS-PAGE (Figures 4.2-4.5), demonstrating that protein binding was not random. Further analysis of these radiolabelled proteins by immunoblotting with antibodies to rabbit P450 2B4 indicated that the covalently bound radioactivity derived from [^{14}C]2,3-BBT incubated with hepatic microsomes from PB-induced guinea pigs corresponded precisely to the region immunostained for P450 2B4 (Figure 4.6). On the other hand, [^{14}C]2,3-BBT-derived radioactivity bound to hepatic microsomal protein from βNF-induced animals (Figure 4.7) was associated not only with the immunoblot of 2B4, but also with regions of slightly lower and

higher molecular weight than 2Bx (reported as 52 kD by Oguri *et al.*, 1991). This radioactivity likely represents binding to other P450s, possibly 1A1 or 1A2 (molecular weights of 48 kD and 53 kD, respectively; Philpot *et al.*, 1985), both of which are selectively increased by βNF treatment.

The inactivation of P450 by covalent binding of reactive metabolites to its apoprotein occurs with the mechanism-based inhibitors chloramphenicol (section 1.2.3.1), methoxsalen (section 1.2.3.2) and 2-ethynylnaphthalene (section 1.2.3.3), while inactivation of P450 through covalent modification of both the heme moiety and P450 apoprotein, similar to what is occurring with BBT, has been found with the suicide substrate secobarbital (section 1.2.6.2). Other possible routes of inactivation also exist; P450 heme, covalently modified by mechanism-based inhibitors, can become covalently attached to P450 apoprotein, thus inhibiting monooxygenase activity, a process known to occur with DDEP (section 1.2.5.2) and spironolactone (1.2.5.2). Several mechanism-based inhibitors of P450 act through the formation of metabolic intermediate complexes with the P450 heme iron (section 1.2.2). The formation of such complexes from BBT has recently been examined in our laboratory (Sinal and Bend, unpublished results) and it was found that under the conditions employed in this study and that described in Chapter 3 (45 min incubation with inhibitor followed by two centrifugation steps) no BBT-derived MI complex was found with hepatic or pulmonary microsomes.

An alternative explanation for the inhibition of P450 by BBT is the formation of benzaldehyde. A number of aldehydes have previously been shown to

differential ultracentrifugation. The homogenate was centrifuged at 700 x g (Beckman J2-21M centrifuge, JA-20 rotor) for 10 min at 4°C to obtain the nuclear fraction. The resulting supernatant was centrifuged at 10 000 x g (Beckman J2-21M centrifuge, JA-20 rotor) for 20 min at 4°C to isolate the mitochondrial fraction. The cytosolic and microsomal fractions were isolated by ultracentrifugation of the mitochondrial supernatant at 100,000 x g (Beckman L8-55 ultracentrifuge, 50.2 Ti rotor) for 50 min at 4°C. All subcellular fractions were washed by an additional centrifugation step and resuspended in 0.1 M potassium phosphate buffer, pH 7.4. Aliquots (500 μl) of each fraction were solubilized with 1.5 mL BTS-450 tissue solubilizer overnight at room temperature, and a further 2-3 hr at 50°C. [^{14}C] content was determined after the addition of 10 mL ReadyOrganic scintillation cocktail.

The microsomal and mitochondrial fractions were extracted repeatedly with EtAc (5 mL) at neutral, basic (pH 10), and acidic (pH 2) pH. The remaining aqueous phase was neutralized, dialysed overnight vs H_2O, and lyophilized to dryness. Aliquots (10-40 mg) were solubilized in 3% SDS with gentle heating, followed by determination of protein concentration by the method of Lowry described in section 3.2.4, and analysis of [^{14}C] content by LSS.

5.2.5 Extraction of 12 Hr Urine for HPLC Analysis of Metabolites

Aliquots of 12 hr urine (4.0-7.5 mL; 160-200 x 10^3 dpm) were adjusted to pH 7 (from initial pH of 7.8-8.7) with 1N HCl, then extracted repeatedly with

BBT is a more potent inhibitor of P450 activities in hepatic microsomes from PB- vs βNF-induced guinea pigs (Chapter 3). The metabolism of BBT to a greater number and amount of stable metabolites by βNF-induced hepatic microsomes (Table 4.2) suggests that increased detoxication of BBT (i.e. conversion to non-suicidal metabolites) by those isozymes induced by βNF treatment is responsible, at least in part.

[^{14}C]ABT was metabolized to benzotriazole by hepatic and pulmonary microsomes, slightly more being produced by liver microsomes from PB- or βNF-induced guinea pigs (Table 4.3). Recent findings by Town *et al.*(1993), in which the *N*-glucuronide of benzotriazole was found as a urinary metabolite following oral administration of [^{14}C]-ABT to rats, confirm the metabolic formation of benzotriazole from ABT. Formation of benzotriazole from ABT could result from hydroxylation of the exocyclic amino group, to form *N*-hydroxy-ABT which decomposes to yield benzotriazole and hydroxylamine.

Small amounts of a second metabolite, more polar than ABT, were produced only by hepatic microsomes from βNF-induced animals, suggesting that P450 1A1 and/or 1A2 is responsible. P450 1A1 and 1A2 are known to catalyze the ring oxidation of other primary aromatic amines (Hammons *et al.*, 1985; Butler *et al.*, 1989), suggesting this also occurs with ABT. The inefficient metabolism of [^{14}C]ABT is likely due, at least in part, to its hydrophilicity; > 70% of [^{14}C]ABT was localized in the microsomal supernatant (vs microsomal) fraction of incubation mixtures. The inefficient metabolism of [^{14}C]ABT observed in this study supports the previous findings of Ortiz de

Montellano *et al.*(1984) where no stable metabolites were isolated following incubation of unlabelled ABT with hepatic microsomes from PB-induced rats.

In summary, BBT is bioactivated by P450 to two reactive metabolites; benzyne from the benzotriazole portion of the molecule, as shown in this study by the covalent binding to protein of [^{14}C]2,3-BBT-derived radioactivity and the ability of P450 to generate [^{14}C]ABT and [^{14}C]benzotriazole from [^{14}C]2,3-BBT; and an additional intermediate, possibly the benzyl radical, from the *N*-benzyl substituent, as evidenced by the covalent binding to protein of a [^{14}C]7-BBT-derived metabolite(s) and the formation of [^{14}C]benzaldehyde from [^{14}C]7-BBT. [^{14}C]2,3-BBT-, and [^{14}C]7-BBT-derived radioactivity becomes covalently bound to hepatic and pulmonary microsomal protein via an NADPH-dependent mechanism, and this covalently modified protein(s) comigrates with P450 2B4 on Western blots, indicating that covalent modification of P450 apoprotein is a mechanism of inactivation of P450 by BBT, in addition to the well-known mechanism of heme alkylation by benzyne.

CHAPTER FIVE

METABOLISM AND DISTRIBUTION OF [^{14}C]2,3-BBT AND [^{14}C]7-BBT IN THE GUINEA PIG *IN VIVO*

5.1 Introduction

There is currently no information available regarding the *in vivo* metabolism, excretion, or tissue distribution, of BBT or *α*MB, *N*-aralkylated derivatives of ABT, except for the formation of a phenylene-modified heme pigment identical to that obtained with ABT, following the administration of a high dose of BBT (400 mg/kg) to PB-induced rats (Mathews and Benu, 1986). This information was intended to complement studies of the mechanism(s) of inactivation of P450 by BBT (Chapter 4), as well as studies performed in this laboratory of the *in vivo* inactivation of pulmonary, hepatic, and renal P450 by BBT and *α*MB following i.v. administration to guinea pigs (Knickle and Bend, unpublished results). This information will also be useful for future *in vivo* studies which make use of these inhibitors to examine isozyme-selective, tissue-selective P450 dependent metabolism of endogenous and exogenous chemicals.

The objective of this study was to examine the time-course of elimination of BBT and its metabolites, and the distribution of BBT, employing the two radiolabelled forms of BBT described in Chapter 4, [^{14}C]2,3-BBT and [^{14}C]7-BBT.

5.2 Methods

5.2.1 Materials

Methoxyflurane (Metofane®) was obtained from M.T.C. Pharmaceuticals, Mississauga, Canada. Emulphor® EL-620 was purchased from GAF Corporation, New York, New York. Silica gel G thin layer chromatography (TLC) plates (0.5 mm thick) were purchased from BDH, Toronto, Canada. All other materials were obtained as described in sections 3.2.1 or 4.2.1.

[^{14}C]2,3-BBT and [^{14}C]7-BBT were synthesized as described in section 4.2.2.

5.2.2 Animal Treatment

Male guinea pigs (350-400 g; Charles River Ltd., St. Constant, Quebec) were anaesthetized with the recoverable inhalable anaesthetic methoxyflurane. The jugular vein was exposed under sterile conditions and [^{14}C]2,3-BBT or [^{14}C]7-BBT (0.75 μmol/kg [0.168 mg/kg]; 0.85 μCi; 0.78 mL volume) dissolved in BSA:Emulphor®:dimethyl sulfoxide (1:0.05:0.025) was slowly injected. Three animals were used in each treatment group. The incision was closed with 4-5 stitches and the guinea pigs were placed in metabolic cages to allow separate collection of urine and feces at 12, 24, and 48 hr. Recovery from anaesthesia was complete within 30-45 min. Animals were allowed free access to food and water throughout. Animals were killed by CO_2 asphyxiation at 48 hr, bile was removed from the gall bladder, and livers, lungs, and kidneys were removed to ice-cold 50 mM potassium phosphate buffer, pH 7.4,

containing 1.15% KCl.

5.2.3 Analysis of [^{14}C] Content in Urine and Feces

Aliquots (200 μl) of urine at each time point were counted directly in ReadyProtein® scintillation cocktail, and the remaining urine was stored at -80°C. Feces were dessicated, ground with a glass mortar and pestle, weighed, and aliquots (17-25 mg) were solubilized in 1.5 mL Beckman BTS-450 tissue solubilizer for two days at 50°C. Solubilized feces were decolourized with 100 μl 50% H_2O_2, neutralized with 50 μl glacial acetic acid, and 15 mL ReadyOrganic® scintillation cocktail were added for determination of [^{14}C] content.

5.2.4 Analysis of Tissue [^{14}C] Content

Organs were homogenized immediately in ice-cold 50 mM potassium phosphate buffer (pH 7.4), 1.15% KCl, using a Potter-Elvejhem tissue homogenizer, and aliquots (50 mg tissue equivalent) of tissue homogenate were solubilized in 1 mL Beckman BTS-450 tissue solubilizer overnight at 50°C. Samples were decolourized with 100 μl 50% H_2O_2, 10 mL ReadyOrganic® scintillation cocktail were added, and the [^{14}C] content determined by LSS. Aliquots (200 μl) of bile were placed directly into 10 mL ReadyProtein® cocktail and left at room temperature overnight prior to determination of [^{14}C] content.

Subcellular fractionation of livers from [^{14}C]2,3-BBT-treated animals into nuclear, mitochondrial, cytosolic, and microsomal fractions was performed by

differential ultracentrifugation. The homogenate was centrifuged at 700 x g (Beckman J2-21M centrifuge, JA-20 rotor) for 10 min at 4°C to obtain the nuclear fraction. The resulting supernatant was centrifuged at 10 000 x g (Beckman J2-21M centrifuge, JA-20 rotor) for 20 min at 4°C to isolate the mitochondrial fraction. The cytosolic and microsomal fractions were isolated by ultracentrifugation of the mitochondrial supernatant at 100,000 x g (Beckman L8-55 ultracentrifuge, 50.2 Ti rotor) for 50 min at 4°C. All subcellular fractions were washed by an additional centrifugation step and resuspended in 0.1 M potassium phosphate buffer, pH 7.4. Aliquots (500 μl) of each fraction were solubilized with 1.5 mL BTS-450 tissue solubilizer overnight at room temperature, and a further 2-3 hr at 50°C. [^{14}C] content was determined after the addition of 10 mL ReadyOrganic scintillation cocktail.

The microsomal and mitochondrial fractions were extracted repeatedly with EtAc (5 mL) at neutral, basic (pH 10), and acidic (pH 2) pH. The remaining aqueous phase was neutralized, dialysed overnight vs H_2O, and lyophilized to dryness. Aliquots (10-40 mg) were solubilized in 3% SDS with gentle heating, followed by determination of protein concentration by the method of Lowry described in section 3.2.4, and analysis of [^{14}C] content by LSS.

5.2.5 Extraction of 12 Hr Urine for HPLC Analysis of Metabolites

Aliquots of 12 hr urine (4.0-7.5 mL; 160-200 x 10^3 dpm) were adjusted to pH 7 (from initial pH of 7.8-8.7) with 1N HCl, then extracted repeatedly with

2 mL EtAc until no further radioactivity could be extracted into the organic phase (6 extractions). The aqueous phase was adjusted to pH 10 with 1N NaOH, and re-extracted with EtAc as above. The aqueous phase was then adjusted to pH 2 with 1N HCl, and re-extracted with EtAC (12 extractions). The remaining aqueous phase was analysed for [^{14}C] content, and stored at -80°C. The [^{14}C] content of all extracts was determined, organic extracts of like pH were pooled, dried over anhydrous Na_2SO_4 overnight, filtered, and the EtAc was removed under a gentle stream of nitrogen. The pooled extracts were stored at −80°C in EtAc for subsequent analysis by HPLC.

HPLC analysis of EtAc extracts of neutral or acidified urine was performed by reverse phase gradient HPLC with system B as described in section 4.2.2. Column effluent was monitored at 280 nm and fractions (0.75 min) were collected and analyzed for [^{14}C] content. HPLC standards used were hippuric acid, ABT, benzotriazole, benzaldehyde and BBT. The solvent gradient was operated by a Waters Maxima 820 Chromatography Workstation.

5.2.6 Extraction of 12 Hr Urine for Alkylated Porphyrin

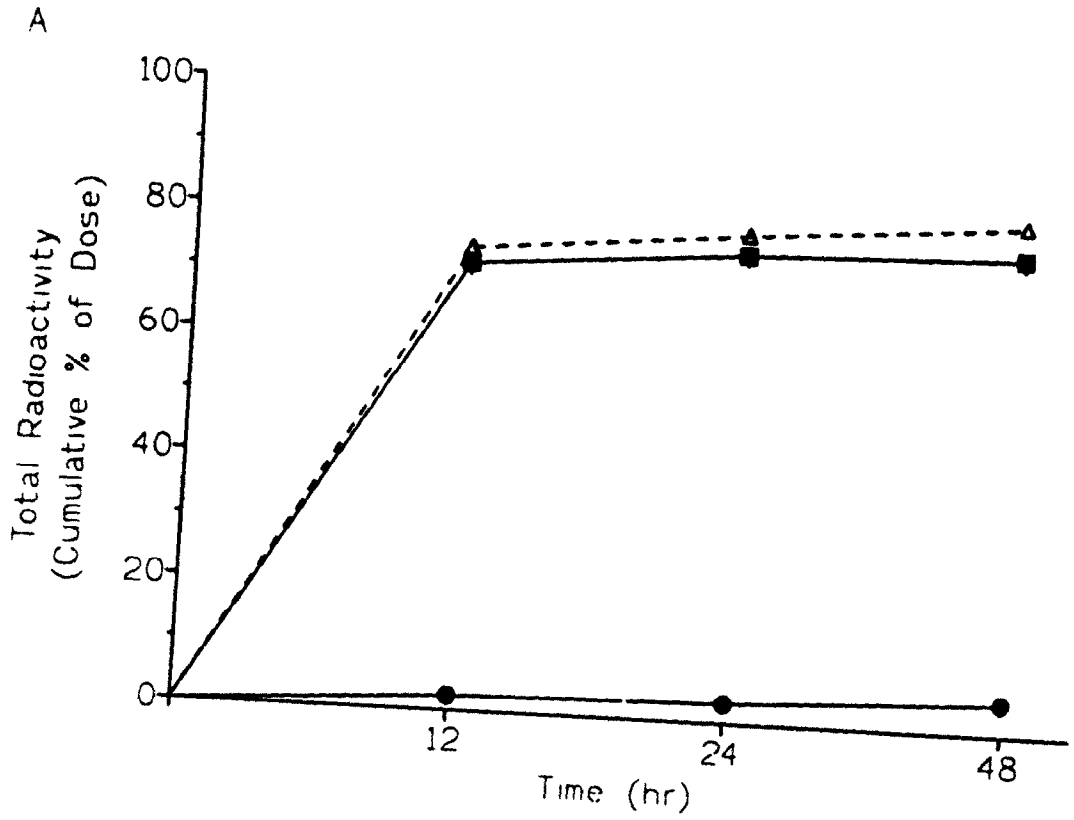
Samples of 12 hr urine from [^{14}C]2,3-BBT-treated guinea pigs were extracted for alkylated porphyrin as described by Ortiz de Montellano *et al.*(1984). To aliquots (3-4 mL; 140-170 x 10^3 dpm) of urine was added 10 mL 5% H_2SO_4 in MeOH. The mixture, after standing overnight at 4°C, was vacuum filtered and the filtrate was diluted with equal volumes of CH_2Cl_2 and H_2O. The aqueous phase was extracted with CH_2Cl_2 until no further

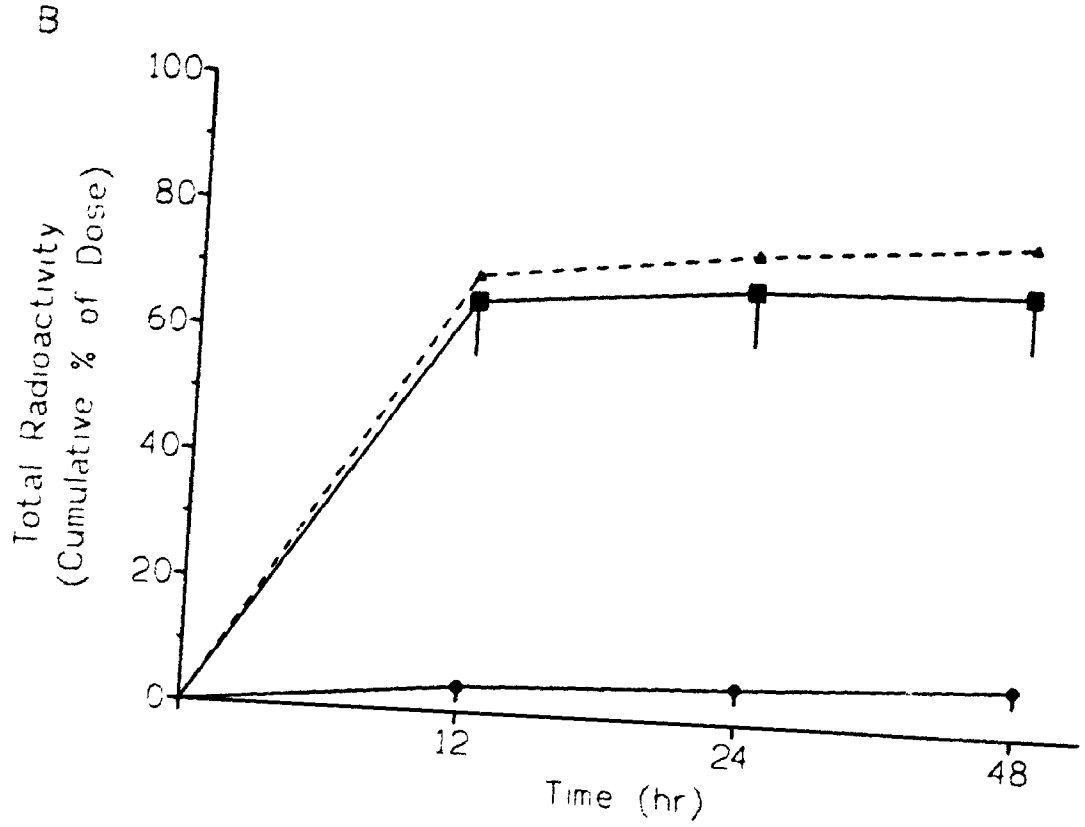
radioactivity could be extracted (4 extractions), and all organic extracts were combined. The organic phase was washed several times with H_2O until the aqueous phase was no longer acidic. The washed organic phase was then dried over Na_2SO_4, filtered, and the solvent was removed *in vacuo* on a rotary evaporator at 35°C. The residue was dissolved in a small volume of MeOH and was chromatographed on 20 x 20 cm TLC plates with a 0.5 mm thick layer of silica gel G, developed with 5% (v/v) MeOH in CH_2Cl_2. Silica was scraped from the TLC plates in 5 mm strips and extracted with 5% (v/v) MeOH in CH_2Cl_2, followed by analysis of [^{14}C] content and electronic absorption spectrum (300-700 nm) on a Beckman DU-65 spectrophotometer.

5.3 Results

The cumulative recovery of radioactivity in urine and feces following the i.v. administration of 0.17 mg/kg [^{14}C]2,3-BBT or [^{14}C]7-BBT to guinea pigs is illustrated in Figure 5.1. Renal elimination was the major route of excretion of BBT, with 50-75% of the injected dose of [^{14}C] being recovered in the urine during the first 12 hr, and an additional 3-7% over the next 36 hr, for a total urinary recovery of 55-80% of the initial dose. Excretion via the feces accounted for 3-13% of the initial dose over the 48 hr period. Over 48 hr, 65-85% of the administered radioactivity was recovered in the urine and feces. There was no difference in urinary or fecal excretion of [^{14}C] between animals administered [^{14}C]2,3-BBT or [^{14}C]7-BBT. One guinea pig in the [^{14}C]7-BBT-treated group had a very low recovery of [^{14}C] in the 12 hr urine compared to

Figure 5.1 **Cumulative excretion of [^{14}C]2,3-BBT- or [^{14}C]7-BBT-derived radioactivity in urine and feces over 48 hr.** Data plotted are [^{14}C] content as a cumulative percentage of initial dose (0.75 μmol/kg; 0.85 μCi) of (A) [^{14}C]2,3-BBT or (B) [^{14}C]7-BBT present in urine (■), feces (●), and urine plus feces (Δ) at 12, 24, and 48 hr following i.v. administration of [^{14}C]BBT. Values are means ± SEM, $n = 3$ for each treatment group.


A
100
80
60
40
20
0
Total Radioactivity
(Cumulative % of Dose)
12
24
48
Time (hr)


B
100
80
60
40
20
0
Total Radioactivity
(Cumulative % of Dose)
12
24
48
Time (hr)

the other five animals (50% vs 70-75% of initial [^{14}C], respectively), but this animal also had a higher than average recovery of radioactivity in the 12 hr feces sample (9% vs 1-4% of initial [^{14}C], respectively) due to the contamination of this feces sample by a portion of the urine. The urinary and fecal excretion of [^{14}C] by this animal at the 24 and 48 hr time points was no different than the other five animals.

Livers taken from the [^{14}C]2,3-BBT-treated guinea pigs at 48 hr contained approximately 1% of the administered dose of [^{14}C], while those from [^{14}C]7-BBT-treated animals contained 0.4% of the initial radiolabel (Table 5.1). Lungs and kidneys from animals in either treatment group contained <0.1% of the initial radiolabel, and virtually no radioactivity was recovered in the bile (<0.01% of administered [^{14}C]; Table 5.1). Further analysis of the livers from [^{14}C]2,3-BBT-treated animals by subcellular fractionation showed that the [^{14}C] present in these livers (67 ± 7.0 x 10^{-4} μCi) was roughly equally distributed among the nuclear (15%), mitochondrial (28%), cytosolic (27%), and microsomal (29% of total liver [^{14}C]) fractions. EtAc extraction of the microsomal and mitochondrial fractions at neutral, basic, and acidic pH recovered <2% of the radioactivity present in these fractions. Upon lyophilization and solubilization of the remaining aqueous phase of these EtAc extracted subcellular fractions, approximately 14 ± 3, and 5 ± 1 dpm/mg of solubilized protein was found in the microsomal and mitochondrial fractions, respectively.

EtAc extraction of 12 hr urine at neutral pH normally extracted only

Table 5.1

Recovery of [^{14}C] (μCi x 10^{-4}) in liver, lung, kidney and bile 48 hr after i.v. administration of [^{14}C]2,3-BBT or [^{14}C]7-BBT (0.75 μmol/kg; 0.85 μCi) to guinea pigs.*

Inhibitor	Liver	Lung	Kidney	Bile
[^{14}C]2,3-BBT	67 ± 7.0	2.7 ± 0.5	7.3 ± 0.1	1.0 ± 0.5
[^{14}C]7-BBT	35 ± 1.2	1.4 ± 0.2	3.8 ± 0.4	0.4 ± 0.3

*Values are means ± SEM, $n = 3$ for each treatment group.

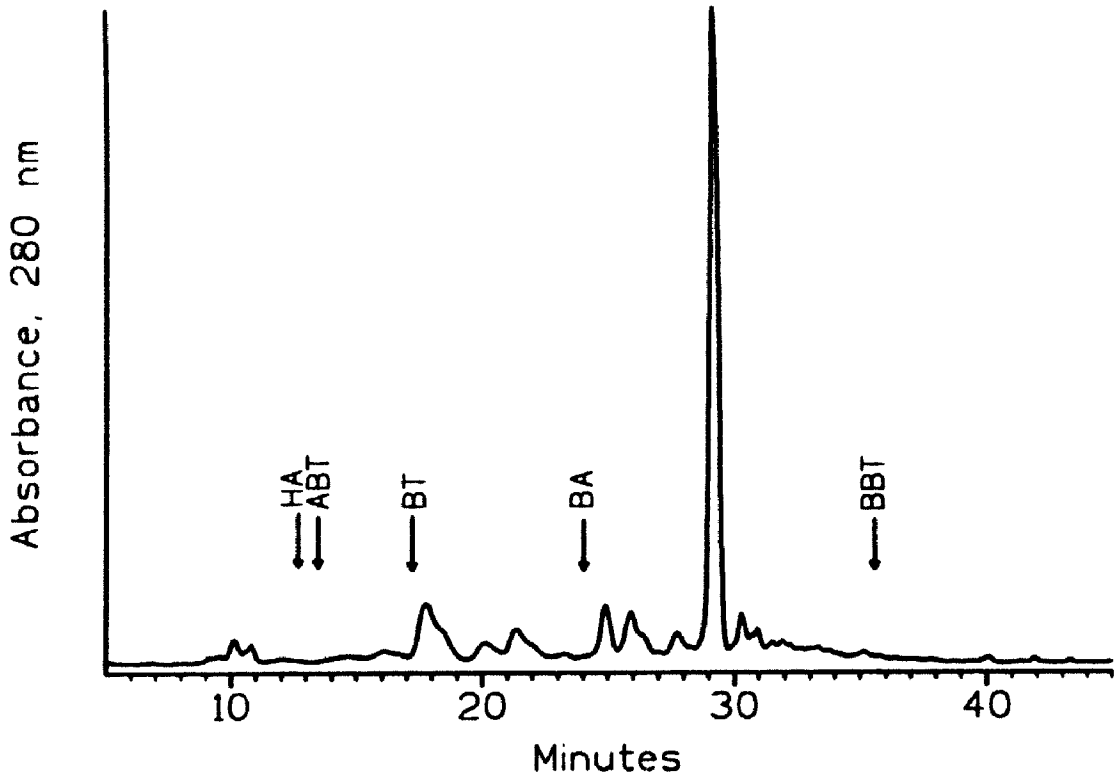
3-6% of the radioactivity present, except with one [^{14}C]7-BBT-treated animal, where approximately 18% of the urinary [^{14}C] was extracted. Extraction at basic pH removed < 2% of the ^{14}C from the urine of any animal. However, 65-80% of the radioactivity was extracted into EtAc upon acidification of the urine of [^{14}C]2,3-BBT- or [^{14}C]7-BBT-treated guinea pigs. The remaining aqueous phase following EtAc extraction contained 20-30% of the urinary [^{14}C].

Both radiolabelled forms of BBT were extensively metabolized *in vivo* as demonstrated by the absence of unchanged [^{14}C]BBT in 12 hr urine samples upon HPLC analysis (Figures 5.2-5.4). [^{14}C]7-BBT was converted *in vivo* to three major [^{14}C]labelled metabolites found in extracts of acidified urine (Figure 5.2; peaks 1, 2, 3), only one of which was present in extracts of neutral urine from one [^{14}C]7-BBT-treated animal (Figure 5.3; peak 3). Metabolite peak 3 in Figure 5.3 may actually be two overlapping peaks, but the distribution of [^{14}C] among these peaks is unknown. [^{14}C]2,3-BBT was converted to two major [^{14}C]labelled metabolites (Figure 5.4; peaks 2, 3) which correspond in retention time to those generated from [^{14}C]7-BBT (Figure 5.2). None of the radiolabelled metabolite peaks corresponded to ABT, benzotriazole, or benzaldehyde. No [^{14}C] hippuric acid was excreted by guinea pigs treated with [^{14}C]7-BBT.

Following acidic MeOH precipitation, extraction, and TLC separation of 12 hr urine from [^{14}C]2,3-BBT-treated guinea pigs, no evidence was found for the presence of *N*-alkylated porphyrin adducts in the urine (data not shown). An *N*-alkylated porphyrin adduct was previously isolated by the same technique from livers of PB-induced guinea pigs treated intraperitoneally with ABT (100

Figure 5.2 **Representative HPLC chromatogram and corresponding elution profile of radioactivity of acidic urinary [^{14}C]7-BBT metabolites.** (A) Chromatogram and (B) elution profile of [^{14}C]7-BBT metabolites obtained by HPLC analysis of EtAc extracts of acidified 12 hr urine from [^{14}C]7-BBT (0.75 μmol/kg; 0.85 μCi)-treated guinea pigs. Radiolabelled metabolites are indicated by Arabic numerals. Locations of HPLC standards are indicated by arrows. HA = hippuric acid, ABT = 1-aminobenzotriazole, BT = benzotriazole, BA = benzaldehyde, BBT = *N*-benzyl-ABT.

A


Absorbance, 280 nm
HA
ABT
BT
BA
BBT
10
20
30
40
Minutes

B

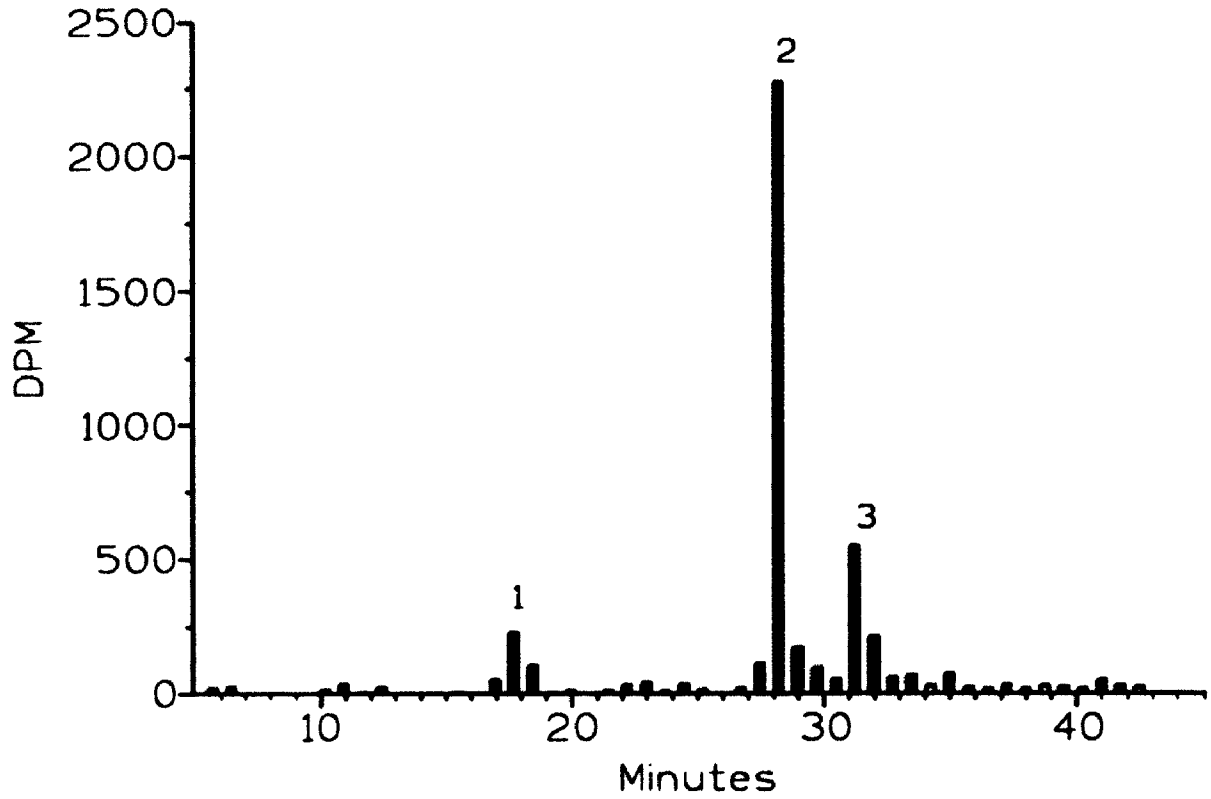

DPM
2500
2000
1500
1000
500
0
1
2
3
10
20
30
40
Minutes

Figure 5.3 **Representative HPLC chromatogram and corresponding elution profile of radioactivity of neutral urinary [^{14}C]7-BBT metabolites.** (A) Chromatogram and (B) elution profile of [^{14}C]7-BBT metabolites obtained by HPLC analysis of EtAc extracts of neutral 12 hr urine from [^{14}C]7-BBT (0.75 μmol/kg; 0.85 μCi)-treated guinea pigs. Radiolabelled metabolites are indicated by Arabic numerals. Locations of HPLC standards are indicated by arrows. HA = hippuric acid, ABT = 1-aminobenzotriazole, BT = benzotriazole, BA = benzaldehyde, BBT = *N*-benzyl-ABT.

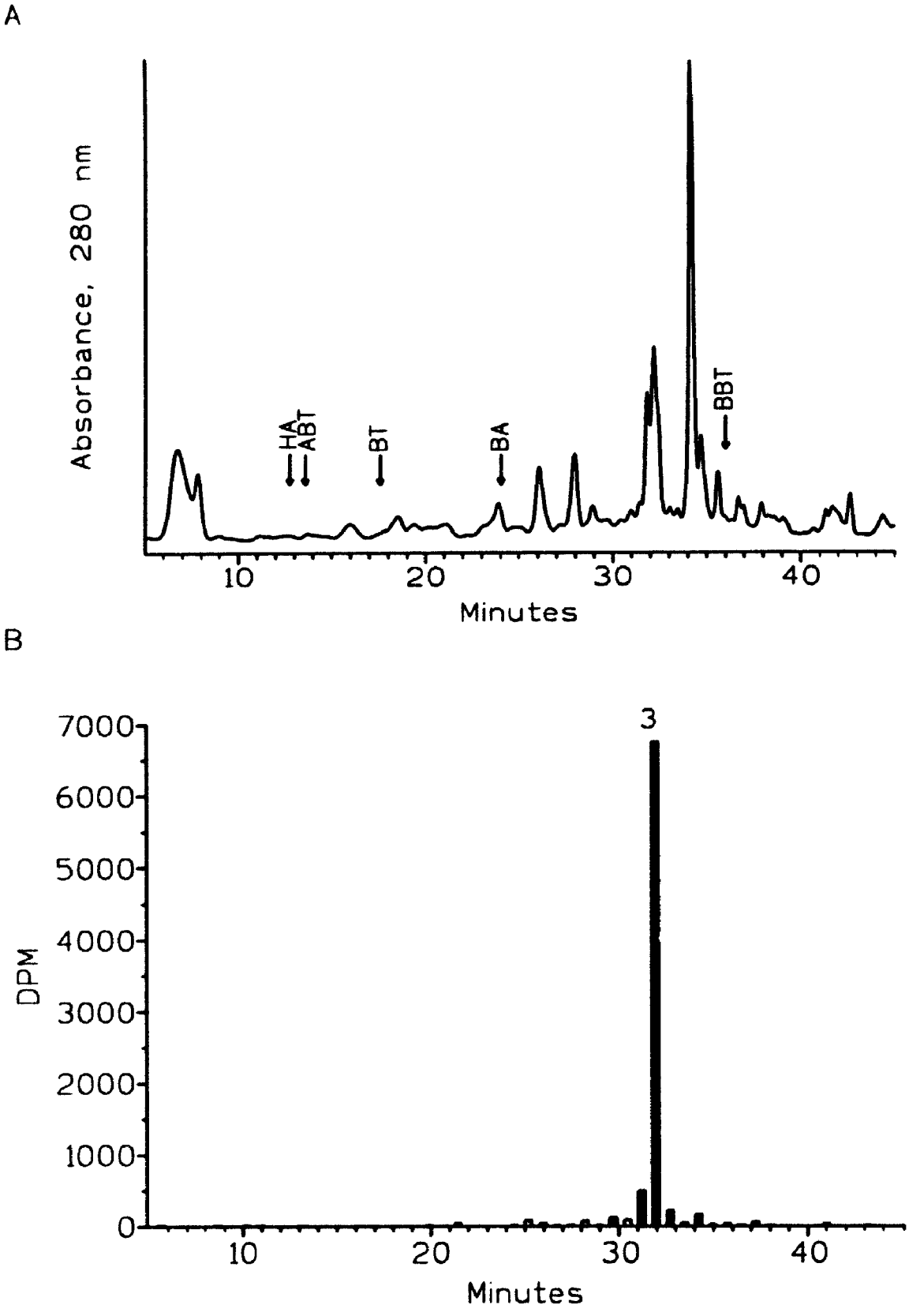
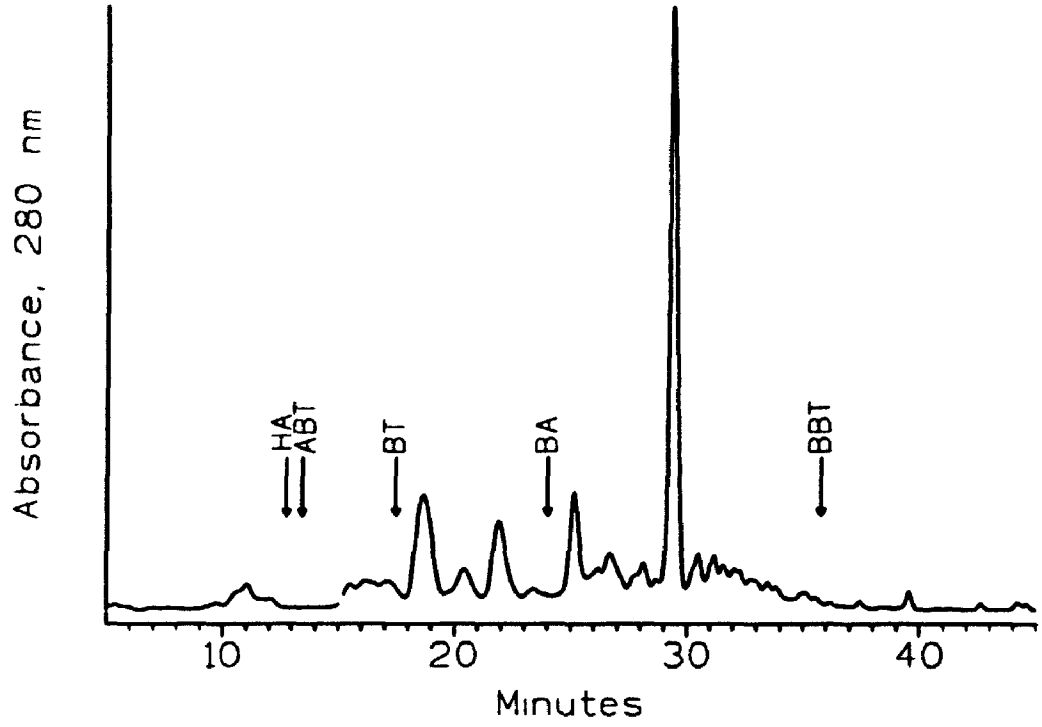
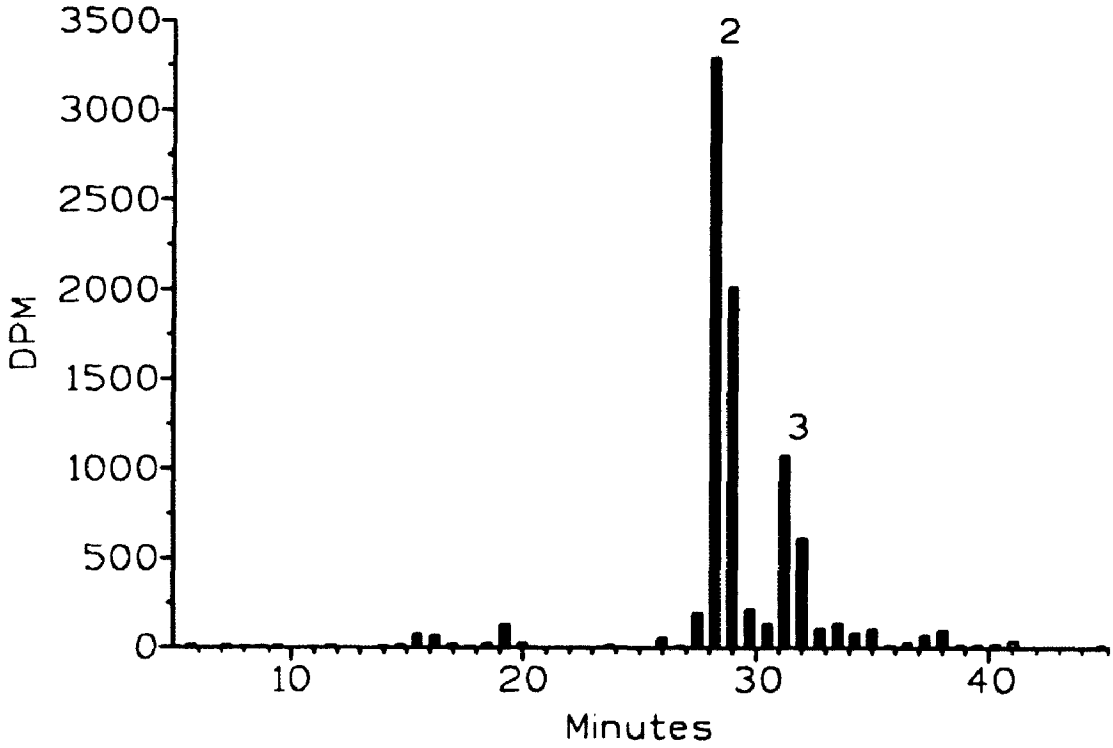

A
Absorbance, 280 nm
HA
ABT
BT
BA
BBT
10
20
30
40
Minutes
B
DPM
7000
6000
5000
4000
3000
2000
1000
0
3
10
20
30
40
Minutes

Figure 5.4 **Representative HPLC chromatogram and corresponding elution profile of radioactivity of acidic urinary [^{14}C]2,3-BBT metabolites.** (A) Chromatogram and (B) elution profile of [^{14}C]2,3-BBT metabolites obtained by HPLC analysis of EtAc extracts of acidified 12 hr urine from [^{14}C]7-BBT (0.75 μmol/kg; 0.85 μCi)-treated guinea pigs. Radiolabelled metabolites are indicated by Arabic numerals. Locations of HPLC standards are indicated by arrows. HA = hippuric acid, ABT = 1-aminobenzotriazole, BT = benzotriazole, BA = benzaldehyde, BBT = *N*-benzyl-ABT.

A


Absorbance, 280 nm
HA
ABT
BT
BA
BBT
10
20
30
40
Minutes

B


DPM
3500
3000
2500
2000
1500
1000
500
0
2
3
10
20
30
40
Minutes

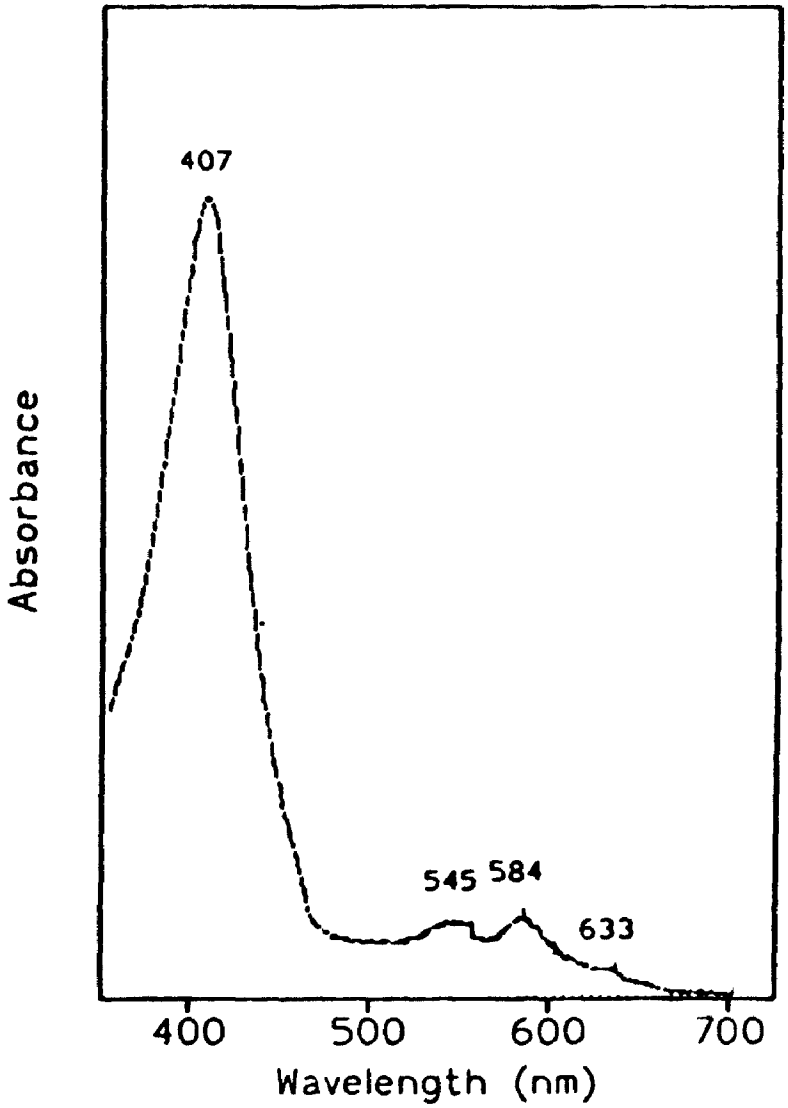
mg/kg). This adduct, following isolation by TLC and analysis of its visible absorption spectrum, had identical absorption spectrum characteristics (Figure 5.5) to the alkylated porphyrin isolated from ABT-treated rats (Ortiz de Montellano and Mathews, 1981).

5.4 Discussion

The dose of BBT chosen for this study was based on the previous finding (Knickle and Benc, unpublished results) that the i.v. administration of 0.75 μmol/kg BBT to guinea pigs resulted in the greatest selectivity for the inactivation of pulmonary P450 2Bx vs 1A1 or 4Bx, and the greatest selectivity for the inactivation of pulmonary, but not hepatic, P450 2Bx, at 4 hr following administration. This dose therefore represents the most appropriate for studying relationships between metabolism and inactivation of pulmonary P450 2Bx *in vivo* in guinea pigs. The 48 hr time point employed in this initial study of the metabolism and distribution of [^{14}C]BBT was chosen to provide ample time for [^{14}C]-porphyrin adducts to be excreted and for near-complete elimination of BBT metabolites.

At this low dose, BBT is rapidly metabolized and eliminated by guinea pigs following i.v. administration, with as much as 75% of the initial radiolabel appearing in the urine in the first 12 hr after dosing (Figure 5.1). The lipophilic BBT was completely metabolized by guinea pigs prior to excretion in the urine (Figures 5.2-5.4). This is in contrast to the more hydrophilic parent compound, ABT, a substantial portion of which was excreted unchanged in the urine of

Figure 5.5 **Absorption spectrum of the modified heme pigment isolated from livers of PB-treated guinea pigs following intraperitoneal administration of ABT.** Guinea pigs were treated once daily for 4 days with PB (80 mg/kg) prior to administration of ABT (100 mg/kg) 24 hr later. Animals were killed 4 hr following ABT administration. The modified porphyrin was isolated from the liver and chromatographed as described in the text.


407
545
584
633
Absorbance
400
500
600
700
Wavelength (nm)

rats administered ABT orally (Town *et al.*, 1993). The excretion of substantial amounts of unchanged ABT in rat urine may also reflect differences in metabolism between rats and guinea pigs.

The possibility that unmetabolized BBT associated with membrane lipids could account for the small fraction (<1%) of the administered radiolabel found in the liver, lungs and kidneys after 48 hr (Table 5.1) was examined by organic extraction of the microsomal and mitochondrial fractions of the livers of animals administered [^{14}C]2,3-BBT, which resulted in virtually no radioactivity being extracted into EtAc. As the radiolabel present in these livers was equally distributed among the subcellular fractions (nuclear, mitochondrial, cytosolic, microsomal), could not be extracted into EtAc, and was found to be tightly associated with the mitochondrial and microsomal proteins, this radioactivity may represent non-specific binding of reactive BBT metabolite(s) to cellular proteins. It could also be a result of debenzylation of [^{14}C]benzyl heme adducts, which are known to be unstable (Schauer *et al.*, 1987), and reaction of the [^{14}C]benzyl moiety with tissue proteins.

An estimate of the potential amount of covalently bound [^{14}C] in lung of [^{14}C]BBT-treated guinea pigs, if 50% of the pulmonary P450 (P450 2Bx) were susceptible to apoprotein alkylation, and 3 nmol [^{14}C]2,3-BBT, or 1 nmol [^{14}C]7-BBT, equivalents became bound per nmol P450 (the degree of NADPH-dependent covalent binding *in vitro* with pulmonary microsomes; Chapter 4), is 40-80 and 13-26 x 10^{-4} μCi for [^{14}C]2,3-BBT and [^{14}C]7-BBT, respectively. Because the half-life of many P450 proteins, including P450 2B1, is 24 hr or

less (Shiraki and Guengerich, 1984), the small amount of [^{14}C]BBT-derived radioactivity remaining in guinea pig lung 48 hr after administration (Table 5.1) was not surprising.

There was no direct evidence for the *N*-debenzylation of BBT *in vivo*, as neither [^{14}C]ABT, [^{14}C]benzotriazole (from [^{14}C]2,3-BBT) nor [^{14}C]benzoic or hippuric acids via [^{14}C]benzaldehyde (from [^{14}C]7-BBT) was excreted in the urine (Figures 5.2-5.4). As well, no glucuronide or acetate conjugates of ABT, reported to be formed from ABT *in vivo* in rats (Town *et al.*, 1993), were apparently present in extracts of acidified urine of [^{14}C]BBT-treated guinea pigs (these conjugates should have retention times less than that of ABT [13-14 min] in the HPLC system used in this study). No radiolabelled metabolites were detected with retention times less than 15 min (Figures 5.2-5.4). However, one urinary metabolite (peak 1, Figure 5.2) was excreted which contained only the [^{14}C]7-benzyl portion of BBT (the other two metabolites contained both radiolabelled portions of BBT). This metabolite may be a product of BBT *N*-debenzylation. It is possible that ABT conjugates, and other metabolites (including conjugates of other *N*-debenzylation products), may not have been extractable into EtAc from acidified urine, and may have remained in the aqueous phase, which retained 20-30% of the urinary [^{14}C] following extraction.

The urinary metabolite profiles of [^{14}C]2,3-BBT and [^{14}C]7-BBT seen in this study (Figures 5.2-5.4) may have been affected by the anaesthetic, methoxyflurane, which is metabolized primarily via P450s 2B4 and 4B1 in

rabbit liver and lung (Waskell *et al.*, 1986). As guinea pig hepatic and pulmonary P450 2Bx is selectively inactivated by BBT both *in vitro* (Chapter 3) and *in vivo* (Knickle and Bend, unpublished results), methoxyflurane may have inhibited the P450 2Bx-dependent metabolism of [^{14}C]BBT in a competitive manner, causing BBT to be metabolized by alternate pathways. This possibility is supported by the finding that BBT administered i.v. to guinea pigs anaesthetized with methoxyflurane resulted in no greater inactivation of P450 compared to vehicle-treated controls at a time point of 6 hr following BBT administration (Knickle and Bend, unpublished results), in contrast to the very efficient inactivation of pulmonary 2Bx by i.v. BBT administered to guinea pigs anaesthetized with the non-recoverable anaesthetic, urethane (Knickle and Bend, unpublished results).

There was no evidence for the presence of *N*-alkylated porphyrin pigments in the urine of [^{14}C]2,3-BBT-treated guinea pigs. It is likely that the dose of BBT used in this study (0.75 μmol/kg), which causes only a 25% loss of spectral P450 in guinea pig lung (estimate of 0.2-0.4 nmoles P450), and no loss of hepatic P450 4 hr following i.v. administration (Knickle and Bend, unpublished results), would not generate large enough quantities of the modified porphyrin to be detected, even if they were excreted in the urine. Studies of the fate of alkylated porphyrin adducts formed in norethindrone-treated rats (White, 1982b), however, found these adducts (admittedly of higher molecular weight than the adducts suspected from BBT) were excreted unchanged in the bile, but not the urine, over a 48 hr period. [^{14}C]2,3-BBT- and

[^{14}C]7-BBT-derived radioactivity (up to 7% of the initial [^{14}C]) was excreted in the feces in the present study, and may be partially comprised of BBT-derived porphyrin adducts, although due to the low dose of BBT used, it is unlikely these adducts would be present in identifiable quantities.

In summary, BBT is rapidly metabolized and excreted (in the first 12 hr after dosing), primarily via the urine, by guinea pigs following i.v. administration. Three major [^{14}C]labelled metabolites formed from [^{14}C]7-BBT, and two from [^{14}C]2,3-BBT (with retention times identical to two of the metabolites from [^{14}C]7-BBT), were detected in urine by HPLC. By 48 hr following administration of [^{14}C]BBT, less than 1% of the radiolabel was present in liver, lungs, or kidneys of these animals. It is clear from this study that time points of less than 12 hr, and [^{14}C]BBT of higher specific activity, should be employed to examine the tissue distribution of [^{14}C]BBT. At short enough time points following administration of BBT, it may be possible to discern whether there is selective accumulation of BBT by the lungs via the facilitative uptake system for lipophilic basic amines in lung (Orton *et al.*, 1973), which may be one reason for the lung-selective (vs liver) P450 inactivation seen with BBT in guinea pigs *in vivo* (Knickle and Bend, unpublished results).

CHAPTER SIX

CONCLUDING REMARKS AND FUTURE CONSIDERATIONS

6.1 Concluding Remarks

The work presented in this thesis examined the mechanism-based inactivation of guinea pig hepatic and pulmonary P450 *in vitro* by ABT, and three of its *N*-aralkylated derivatives, BBT, αMB, and αEB. We have shown that the *N*-aralkyl derivatives of ABT selectively inactivate P450 2Bx in hepatic microsomes, especially those from PB-induced guinea pigs. These compounds also inactivate P450 2Bx in pulmonary microsomes from untreated or βNF-induced animals, with even greater selectivity and potency than in hepatic microsomes. This work demonstrated that αMB is more potent and isozyme-selective (for P450 2Bx) than BBT, and that further increasing both the lipophilicity and steric hindrance about the site of oxidation (the amino nitrogen), as in the αEB analog, does not further increase either potency or isozyme selectivity.

We have also demonstrated that these mechanism-based inhibitors are effective across species, retaining the same relative order of potency and isozyme selectivity as found earlier in rabbit pulmonary microsomes (Mathews and Bend, 1986). However, the effectiveness of these compounds for P450 inactivation may vary among species, as we found a greater degree of inactivation of P450-dependent monooxygenase activities at equimolar

concentrations of BBT and αMB in guinea pig vs rabbit (Mathews and Bend, 1986) and of αMB in guinea pig vs goat (Huijzer *et al.*, 1989) pulmonary microsomes.

BBT and αMB have also been shown, by work done in this laboratory (Knickle and Bend, unpublished results), to be highly selective (at low doses) for the inactivation of pulmonary (vs hepatic) P450 2Bx *in vivo*. These compounds should prove to be very effective probes of the *in vitro* and *in vivo* pulmonary P450 2Bx-dependent metabolism/bioactivation of both exogenous and endogenous chemicals. αMB has been employed to demonstrate that the pulmonary toxin, 3-methylindole, is bioactivated to its alkylating intermediate(s) in goat pulmonary microsomes primarily by the goat orthologue of rabbit P450 2B4 (Huijzer *et al.*, 1989), and BBT and αMB have been used to demonstrate that P450 2Bx is solely responsible for the formation of epoxyeicosatrienoic acids (EETs) from arachidonic acid in guinea pig pulmonary microsomes (Knickle and Bend, unpublished results). These mechanism-based inhibitors have the advantage over inhibiting antibodies as isozyme-selective probes as they can be used in intact cell systems such as whole cells, perfused organs, and *in vivo*, in addition to broken cell preparations, to which antibodies are limited because of their inability to penetrate cell membranes. BBT and αMB may also prove useful in the differentiation of microheterogenous forms of P450 2Bx. The differential inactivation of guinea pig hepatic microsomal PRF and BND activities reported in this thesis suggest that microheterogenous forms of 2Bx may exist in the guinea pig, as have previously been reported in rabbit (Gasser

et al., 1988; Komori *et al.*, 1988) and human (Yamano *et al.*, 1989) liver. In this regard, BBT and *α*MB have recently been found to differentially inhibit the microheterogenous rabbit liver forms P450 2B4 and 2B5 (Grimm *et al.*, 1993).

ABT is known to inactivate P450 by *N*-arylation of its prosthetic heme moiety with benzyne (Ortiz de Montellano and Mathews, 1981), and the same major phenylene substituted heme pigment has previously been isolated from BBT-treated rats (Mathews and Bend, 1986). Novel data presented in this thesis demonstrate that BBT is bioactivated by P450 to at least two reactive intermediates (one containing the benzotriazole ring of [^{14}C]2,3-BBT and one containing the *N*-benzyl group of [^{14}C]7-BBT) which also cause covalent modification of microsomal protein, likely P450 apoprotein. This shows that mechanism-based inhibitors can be designed that contain two (or more) functional groups capable of being bioactivated to reactive species, both of which can participate in the inactivation of P450. This study emphasizes that alterations to the structure of a known mechanism-based inhibitor can affect not only the potency and/or isozyme selectivity of the inhibitor, but also its mechanism of inactivation, by affecting its orientation in the active site of P450. A similar observation was reported with a series of dichloromethyl compounds (chloramphenicol analogs), in which alterations to the hydrophobic side chain length resulted in P450 inactivation through heme, as well as apoprotein, alkylation (Halpert *et al.*, 1986). This study also emphasizes that the ability of a chemical to inactivate P450 in a mechanism-based manner should not be assessed on the basis of P450 loss alone, as there may be

mechanisms in addition to heme alkylation responsible for the enzyme inactivation.

Finally, we have demonstrated that small doses of BBT, effective for the selective inhibition of pulmonary P450 2Bx, are rapidly and completely metabolized by the guinea pig following i.v. administration and are excreted primarily in the urine. As well, by 48 hr after administration, virtually none of the compound remained in the liver, lungs, or kidneys. It is apparent from this study that time points of less than 12 hr must be used to determine if selective tissue accumulation (lung/liver) of BBT is responsible for the selective inactivation of pulmonary vs hepatic P450 2Bx in guinea pigs. Moreover, BBT of much higher specific activity will be required to determine the amount of [^{14}C] covalently bound to P450 2Bx apoprotein in lung vs liver after *in vivo* administration.

6.2 Future Considerations

As research should, the work presented in this thesis produced as many questions as it did answers. A number of lines of research logically stem from these questions, as suggested below:

1) Determine whether there is any NADPH-dependent non-specific covalent binding to microsomal proteins (ie. other than the P450 responsible for metabolite formation) upon incubation of [^{14}C]BBT in hepatic and pulmonary microsomes by the inclusion of glutathione, cysteine, or BSA as reactive intermediate scavengers.

2) Determine whether covalent binding of inhibitor-modified porphyrin to protein is also a mechanism involved in the inactivation of P450 by ABT and its *N*-aralkylated derivatives.

3) Identify the P450 isozymes which undergo covalent apoprotein modification by BBT by immunoprecipitation following incubation of [^{14}C]BBT of high specific activity, or [^{3}H]BBT, in a microsomal system, or at a short time point (eg. 4 hr) following i.v. administration to guinea pigs.

4) Examine the mechanism(s) of inactivation by [^{14}C]ABT and [^{14}C]BBT of single recombinant P450 isozymes in reconstituted monooxygenase systems.

5) Identify the quantitatively significant metabolites formed *in vitro* and *in vivo* from [^{14}C]2,3-BBT and [^{14}C]7-BBT.

6) Identify the reactive metabolite(s) formed from [^{14}C]7-BBT, possibly through free radical spin-trapping.

7) Determine the *in vivo* tissue distribution of [^{14}C]BBT at shorter time points (eg. 2, 4, 12 hr) following i.v. administration.

8) Determine the *in vivo* toxicity of BBT and *α*MB in guinea pig lung at multiple time points after administration of the minimal dose required to totally inactivate pulmonary P450 2Bx.

9) Use BBT and *α*MB as *in vitro* and *in vivo* probes of the pulmonary P450 2Bx-dependent metabolism/bioactivation of endogenous and exogenous chemicals.

10) Examine the inactivation of microheterogenous forms of P450 2Bx by BBT and *α*MB.

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