

UCSF

UC San Francisco Electronic Theses and Dissertations

Title

Aromatic heterocycles as suicide substrates of cytochrome P-450

Permalink

<https://escholarship.org/uc/item/77c813z6>

Author

Mathews, James Madison

Publication Date

1982

Peer reviewed|Thesis/dissertation

AROMATIC HETEROCYCLES AS SUICIDE SUBSTRATES OF CYTOCHROME P-450

by

James Madison Mathews

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

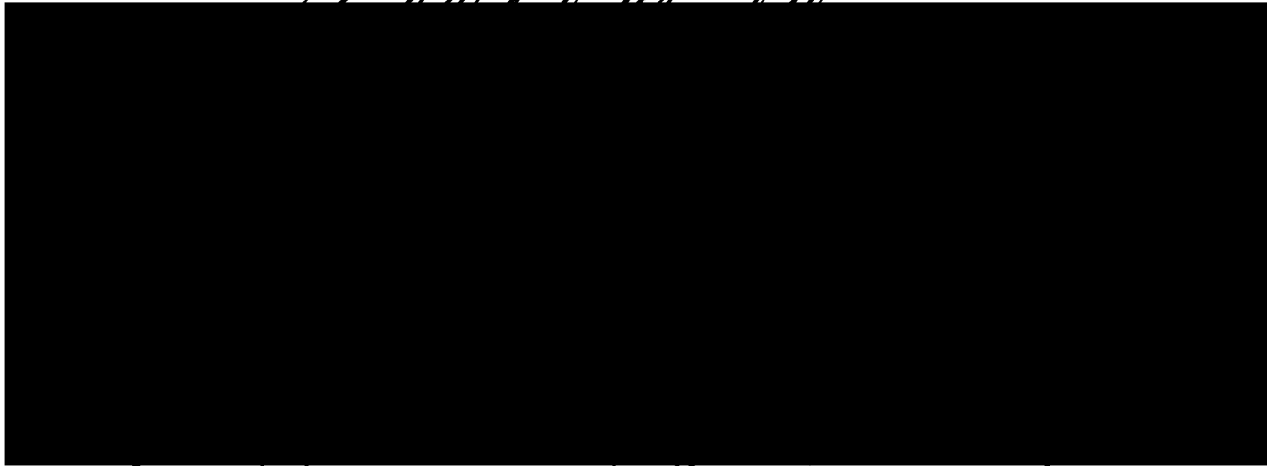
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



Date

University Librarian

DEC 31 1982

Degree Conferred:

To:

My parents, Ann and Paul Mathews

My grandmother, Josephine Eisenhower, 97 and wise

and especially to Dr. Ellen Cheung with love and appreciation.

Acknowledgement

I would like to express my sincere appreciation to Professor Paul Ortiz de Montellano for his patience, advice, and direction in the completion of my graduate studies. His enthusiasm for science and clear perspective have been an example for me which will guide my future endeavors.

I am especially grateful to Dr. Almira Correia and Dr. Neal Castagnoli for providing indispensable technical advice and for their concern for my professional development.

I would like to acknowledge the friendship and collaboration of Doctors Hal Beilan, Kent Kunze, and Bruce Mico. I wish to express my thanks to Dr. Conrad Wheeler for guiding me to my first glimpse of the "gnosis".

I thank my partners in crime, Jeff Blaney, Mark Reynolds, Richard Blatchly, Gary Pascoe, Terry Matsunaga, and Mark Watanabe for adding so very much to my life in San Francisco.

I am eternally indebted to Dr. Cheung Ngoi Yin for her heroic efforts in the preparation of this dissertation.

Abstract

The molecular and biochemical phenomena associated with the inhibition of cytochrome P-450 by 5,6-dichloro-1,2,3-benzothiadiazole (DCBT) and 1-aminobenzotriazole (ABT) have been investigated to elucidate the mechanisms involved. The applicability of these agents as selective inhibitors for different isozymes of cytochrome P-450, particularly those induced by 3-methylcholanthrene (3-MC), have been examined in vivo and in vitro.

The 1,2,3-benzothiadiazoles are known inhibitors of the cytochrome P-450 enzymes responsible for metabolism of insecticides but, despite extensive structure activity studies, little is known about their mechanism of action. In the present study DCBT has been shown to destroy microsomal cytochrome P-450 in a time-dependent process, accompanied by equimolar loss of heme, that requires oxygen and NADPH but is attenuated by the presence of carbon monoxide. These studies suggest that inhibition of the enzyme by DCBT results from oxidation of this substrate to a reactive species that reacts with the prosthetic heme group.

The known chemical oxidation of ABT to the highly reactive benzyne species inspired investigation of this agent as a potential suicide substrate for cytochrome P-450 enzymes. ABT has been found to be a uniquely potent inhibitor of mammalian and plant cytochrome P-450 enzymes. Destruction of the enzyme by ABT meets with the criteria that define the characteristics of a suicide substrate in that it is

attenuated by inhibitors of the enzyme (carbon monoxide), requires catalytic turnover of the enzyme (NADPH, NADPH reductase are required), is time-dependent, is not attenuated by the addition of exogenous nucleophiles (10 mM glutathione), and is characterized by the stoichiometric (equimolar loss of heme and cytochrome P-450 is observed) and irreversible (the prosthetic group is covalently modified) modification of the enzyme catalytic center. The plant cytochrome P-450 enzyme cinnamic acid 4-hydroxylase is likewise inactivated by ABT. Catalytic turnover of ABT by chloroperoxidase results in the formation of phenol and inactivation of the enzyme. The phenol obtained in deuterated water incorporates one deuterium into the aromatic ring, a result which implicates benzyne in the destructive process. Destructive activity against cytochrome P-450 is not abolished in ABT derivatives with methyl or acetyl substituents on the exocyclic amino group, but is lost on removal of this group or by its replacement with a hydroxyl function.

In vivo studies demonstrate that ABT and DCBT are effective inhibitors of cytochrome P-450 in phenobarbital (PB) and 3-MC pretreated rats; levels of serum transaminases, monitored as a measure of toxicity, are not concomitantly changed. The susceptibility to destruction by ABT and DCBT of the major isozymes induced by 3-MC and PB has been confirmed by experiments with reconstituted purified isozymes.

1/22 2/7/82

Paul C. S. McElroy

TABLE OF CONTENTS

CHAPTER I

THE CYTOCHROME P-450 MIXED FUNCTION OXIDASE SYSTEM

| | | |
|------|---|----|
| 1.00 | Introduction | 1 |
| 1.10 | Occurrence and Role | 2 |
| 1.20 | Components and Cofactors | 4 |
| 1.30 | Induction and Multiplicity of Cytochrome P-450 enzymes | 6 |
| 1.40 | The Catalytic Cycle | 8 |
| 1.41 | Binding of Substrate | 11 |
| 1.42 | Transfer of the First Electron | 11 |
| 1.43 | The Binding and Activation of Oxygen | 12 |
| 1.50 | Reactions Catalyzed by Cytochrome P-450; Activation of Toxins | 15 |
| 1.51 | Experimental Hepatotoxicity | 18 |
| 1.60 | Self-Catalyzed Destruction of Cytochrome P-450 | 20 |
| 1.61 | Thiono Compounds | 20 |
| 1.62 | Carbon Tetrachloride | 22 |
| 1.63 | Olefins, Acetylenes, DDC | 22 |
| 1.70 | Suicide Substrates | 26 |
| 1.80 | Proposal | 28 |

CHAPTER II

1,2,3-BENZOTHIADIAZOLES

| | | |
|-----|--|----|
| 2.1 | Introduction | 30 |
| 2.2 | Biological Activity of the 1,2,3-Benzothiadiazoles | 31 |

| | | |
|------|--|----|
| 2.3 | Chemistry of 1,2,3-Benzothiadiazoles | 32 |
| 2.4 | Results and Discussion | 34 |
| 2.41 | Destruction of Cytochrome P-450 by DCBT is a Self-catalyzed Process | 36 |
| 2.42 | Site of Inhibitory Action of DCBT on Cytochrome P-450 | 38 |
| 2.43 | Specificity of Destruction <u>In Vivo</u> and <u>In Vitro</u> of Hepatic Cytochrome P-450 Isozymes | 40 |
| 2.44 | Biochemical Toxicology of DCBT | 40 |
| 2.5 | Proposed Mechanism of Destruction | 42 |
| 2.6 | Conclusion | 47 |

CHAPTER III

1-AMINOBENZOTRIAZOLE

| | | |
|------|--|----|
| 3.10 | Introduction | 48 |
| 3.11 | Chemistry of 1-Aminobenzotriazole | 48 |
| 3.12 | The Nature of Benzyne | 49 |
| 3.13 | Proposal | 51 |
| 3.20 | <u>In Vitro</u> Interaction of 1-ABT with Hepatic Cytochrome P-450 | 53 |
| | Rate of Inactivation | 57 |
| | Extent of Destruction | 58 |
| | Specificity of Isozyme Destruction | 62 |
| 3.30 | <u>In Vivo</u> Interaction of 1-ABT with Hepatic Cytochrome P-450 | 65 |
| | Isolation and Characterization of Alkylated Porphyrin Pigments | 69 |
| 3.40 | Biological Activity of Benzotriazole Analogs | 75 |
| | Toxicity of 1-ABT and its Derivatives | 85 |
| 3.50 | Inhibition of other Oxidase by 1-ABT | 90 |

| | | |
|------|--|-----|
| 3.51 | Chloroperoxidase | 90 |
| 3.52 | Cinnamic Acid 4-Hydroxylase | 96 |
| 3.53 | Monoamine Oxidase | 97 |
| 3.60 | Mechanism of Action of 1-ABT | 98 |
| 3.70 | Conclusion | 101 |

CHAPTER IV

EXPERIMENTAL PROCEDURES

| | | |
|------|--|-----|
| 4.1 | General Experimental | 104 |
| | Reagents for Biochemical Studies | 105 |
| 4.20 | Biochemical Techniques | 106 |
| | Preparation of Rat Hepatic Microsomes | 106 |
| | Protein Determination | 106 |
| | Binding Spectrum of 1-Aminobenzotriazole | 107 |
| | Spectral Assay of Cytochrome P-450 Content in Microsomal Incubations | 107 |
| | Oxygen Requirement for Cytochrome P-450 Destruction | 108 |
| | Effect of Carbon Monoxide on the Destruction of Cytochrome P-450 | 109 |
| | Determination of Heme Content | 109 |
| | Partition Ratio between Substrate Turnover and Enzyme Destruction in the Metabolism of 1-ABT | 109 |
| | Measurement of Cytochrome b ₅ in Microsomal Incubations | 110 |
| | Determination of Cytochrome c Reductase Activity | 110 |
| | Detection of Lipid Peroxidation | 111 |
| | <u>In Vivo</u> Destruction of Cytochrome P-450 | 112 |
| | Determination of Agent Toxicity. Measurement of Serum Transaminase Levels | 112 |
| | Inactivation of Chloroperoxidase by 1-ABT | 113 |

| | | |
|-----|--|-----|
| | Isolation and Characterization of the Products resulting from Metabolism of 1-ABT by Chloroperoxidase | 113 |
| 4.3 | Isolation and Purification of Alkylated Porphyrins from Rat Liver | 115 |
| 4.4 | Synthesis | 118 |
| | 5,6-Dichloro-1,2,3-benzothiadiazole | 118 |
| | 6,7-Dichloro-1,2,3-benzothiadiazole | 118 |
| | 1-Aminobenzotriazole | 119 |
| | 2-Aminobenzotriazole | 119 |
| | 1-Diacetamidobenzotriazole | 120 |
| | 1-Acetamidobenzotriazole | 120 |
| | 1-N-methylacetamidobenzotriazole | 121 |
| | 1-N-methylaminobenzotriazole | 121 |
| | 1-Nitrobenzotriazole | 122 |
| | References | 127 |

CHAPTER ONE

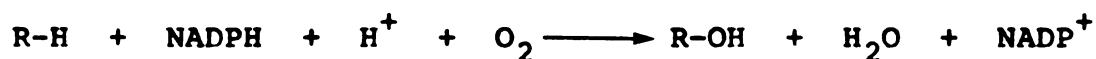
THE CYTOCHROME P-450 MIXED FUNCTION OXIDASE SYSTEM

1.00 INTRODUCTION

In this century millions of new organic compounds have been synthesized. These compounds were prepared to be used as drugs, pesticides, and solvents. Many have found their way into the biosphere where they are now environmental contaminants challenging the viability of living systems. It has become increasingly apparent that organic compounds are not inert to metabolic alteration; indeed, it is frequently the case that the metabolites rather than the parent compounds engender the more significant profile of biological activities, both beneficial and deleterious. A host of metabolic enzymes, including monoamine oxidase, the esterases, glutathione transferase, epoxide hydrase, glucuronyl transferase, and sulfotransferase, participate in the biotransformation of endogenous substrates and xenobiotics. The monooxygenase cytochrome P-450 stands at the forefront of these enzymes that ostensibly serve to terminate the action of, to detoxify, and to facilitate the excretion of, the varied agents confronting biological systems. Without such enzymes, organisms would succumb to the primary biochemical effects of chemicals and would be overwhelmed by the dissolution of lipophilic agents into membranes and adi-

pose tissues. The action of these enzymes on chemicals, however, can also inadvertently produce metabolites of greater toxicity.

Cytochrome P-450 is a hemoprotein that participates in electron transport and oxygen metabolism. As is suggested by the name, the functional core of these proteins is the heme center; the protein serves to modulate the electronic state of the ferriporphyrin and to constrain access to this core by substrate molecules. Cytochrome P-450 works in tandem with cytochrome P-450 reductase, a protein that shuttles electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH) to the terminal oxidase. In the net reaction (Scheme 1-1) dioxygen is reductively cleaved to a molecule of water and an activated oxygen atom that oxidizes the substrate.



SCHEME 1-1

This dual action of reduction and oxidation gave rise to the name "mixed function oxidase" (Mason, 1957) for the terminal oxidase.

1.10 OCCURENCE AND ROLE

The cytochrome P-450 mixed function oxidase system is widespread in nature and is present as a vital component in bacteria, fungi, plants, and animals. The enzyme complex, distributed throughout mammalian tissues, is found in lung,

skin, kidney, brain, liver, adrenals, testicles, and in the intestinal tract (Testa and Jenner, 1976). Hepatic cytochrome P-450 is mostly associated with the rough and smooth endoplasmic reticulum (Boyd et al, 1973). Vestiges of these organelles make up, after disruption and isolation by centrifugation, the vesicles of the microsomal fraction. This fraction also contains NADPH-cytochrome P-450 reductase, cytochrome b_5 , glucuronyl transferase, cytochrome b_5 reductase, glutathione transferases, esterases, phosphatases, and epoxide hydrases (Testa and Jenner, 1976).

Cytochrome P-450 catalyzes the oxidation of endogenous and xenobiotic agents (see reviews by Wislocki et al 1980, Lu and West, 1978). The cytochrome P-450 enzymes concerned with biosynthesis, such as that of steroids (Gibbons and Mitropoulos, 1973), are characterized by high specificity and efficiency in turnover of endogenous substrates. In contrast, the cytochrome P-450 enzymes responsible for the disposition of xenobiotics are by necessity of low specificity. These enzymes, which are associated with lipid membranes, function to make lipophilic compounds more polar and therefore more excretable. Toward this end the enzyme works in conjunction with enzymes such as glucuronyl transferase and sulfotransferase which add an additional hydrophilic moiety to the hydroxylated product provided by cytochrome

P-450.

1.20 COMPONENTS AND COFACTORS

The hepatic cytochrome P-450 mixed function oxidase system is an assembly of membrane bound enzymes associated with the endoplasmic reticulum. This system has been solubilized and separated into its purified constituents (White and Coon, 1980). A functional system may then be reconstituted (Lu and Coon, 1968) from the three components necessary for metabolic activity: 1) cytochrome P-450, 2) cytochrome P-450 reductase, and 3) phospholipid.

The hepatic cytochromes P-450 are a multiplicity of enzymes, the profile of which is determined by genetic, environmental, and other factors. The enzymes are hydrophobic and have molecular weights in the range of 48,000 to 58,000 daltons. One molecule of heme is retained as the prosthetic group of the enzyme.

Associated with cytochrome P-450 in the lipid matrix is NADPH-cytochrome P-450 reductase, alternately called NADPH cytochrome c reductase, which furnishes the terminal oxidase with electrons donated from NADPH. The prosthetic group of the reductase consists of one molecule each of flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) (Iyanagi and Mason, 1973). The molecular weight of the protein is in the range of 76,000 to 80,000 daltons. In contrast to cytochrome P-450, there appears to be only one form of the reductase in rat liver (Glazer et al, 1971). The

enzyme is comprised of a hydrophilic segment, which contains the catalytic site, and a hydrophobic segment which anchors the enzyme to the membrane. While the reductase is capable of supplying both of the electrons required for substrate metabolism, there is evidence (see Schenkman et al, 1976 for a review) which suggests that one of the electrons can be provided by cytochrome b_5 from NADH via cytochrome b_5 reductase.

Microsomal membranes are comprised partially of a mixture of phospholipids including a large proportion of unsaturated fatty acid residues; cholesterol is also present and accounts for about 8% of the lipid. While the presence of unsaturated chains helps to maintain membrane fluidity, it also makes the system susceptible to damage by lipid peroxidation (discussed in section 1.50). Membrane lipid plays a crucial yet poorly understood role in the mixed function oxidase system. They are most likely involved in facilitating the association of terminal oxidase and reductase and in sequestering lipophilic molecules for metabolic processing. Each of the enzyme components of the mixed function oxidase system penetrates the membrane to a depth that allows its catalytic site to assume functional proximity to the catalytic sites of the components of the system with which it reacts (Mannering, 1981). The ratio of cytochrome P-450 to reductase is approximately 20 to 1 (Estabrook et al, 1976). In reconstituted systems the lipid may be replaced with phosphatidyl choline or certain non-ionic detergents, but

the rate and extent of reduction of cytochrome P-450 by the reductase is very low in its absence (De Pierre and Dalner, 1975). The studies of Stier (1976) and others suggest that the phospholipid in the immediate vicinity of cytochrome P-450 is more highly organized, and encloses the cytochrome in a constrained spatial arrangement about the reductase. It has also been suggested that the reductase is mobile in the lipid phase and is free to meander among cytochrome P-450 molecules (Franklin, 1977). The levels of the components of the mixed function oxidase system are sensitive to the presence of lipophilic compounds, as is discussed in the next section.

1.30 INDUCTION AND MULTIPLICITY OF CYTOCHROME P-450 ENZYMES

Repeated dosing of animals with any of a variety of lipophilic chemicals results in morphological changes associated with enhanced levels of the components and cofactors of the drug metabolizing systems. Phenobarbital (PB) and 3-methylcholanthrene (3-MC) are prototypes of different groups of agents that induce distinct classes of cytochrome P-450 enzymes differing in composition as well as in catalytic and physical properties.

Following treatment of rats with PB a proliferation of the endoplasmic reticulum, restricted to the centrilobular hepatocytes, and a significant increase in liver weight, are observed. The clearance of a broad spectrum of drugs and other chemicals increases as a result of a manyfold increase

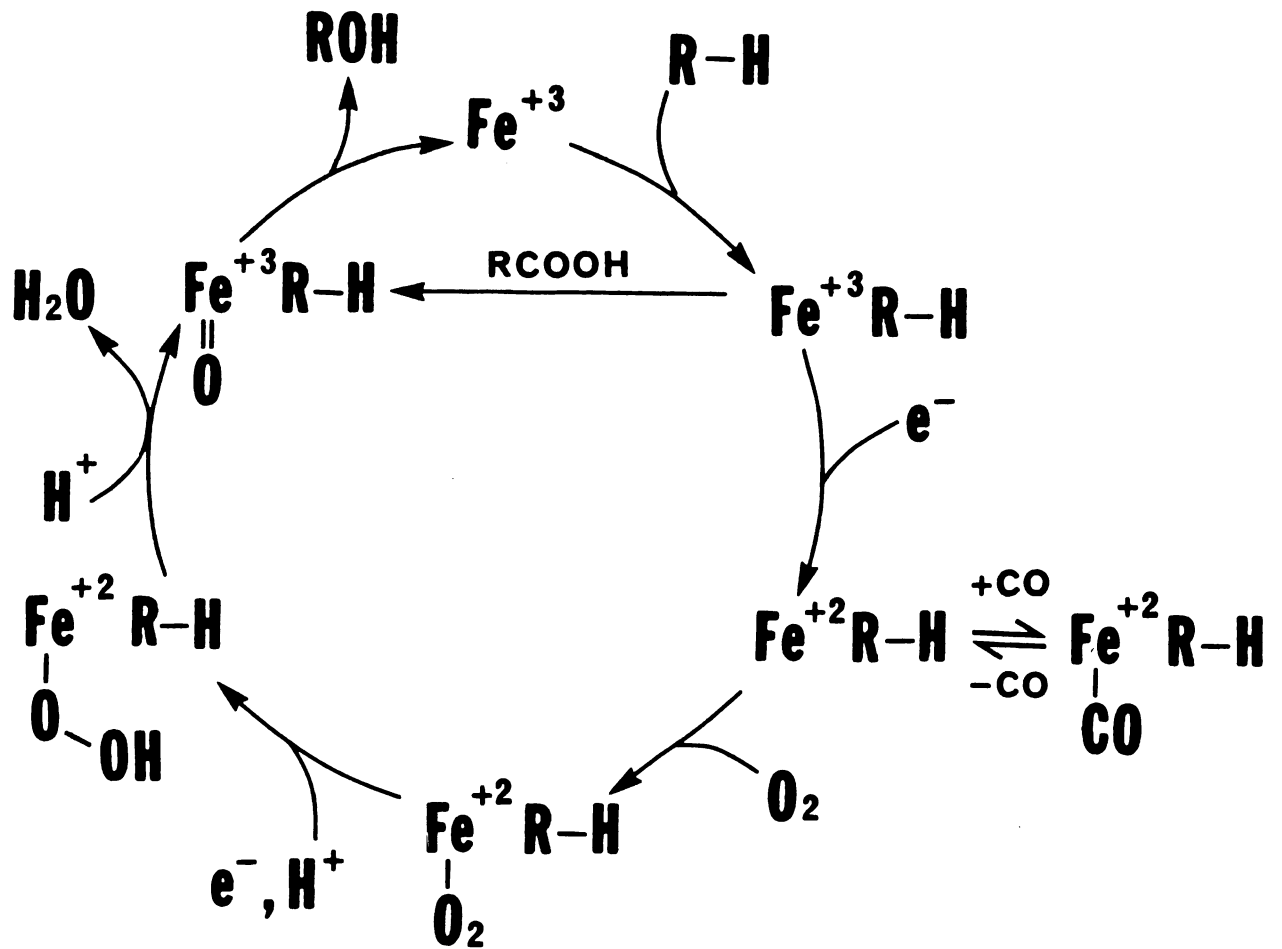
in the level of metabolic enzymes. Cytochrome P-450, cytochrome P-450 reductase, cytochrome b_5 , phospholipid, UDP-glucuronyl transferase, and heme are increased in response to induction with phenobarbital (Orrenius et al, 1969). Less overt changes occur when 3-MC is used as the inducing agent. No change in liver weight is observed, and the levels of cytochrome P-450 reductase and phospholipid are little changed. Exposure to 3-MC has a lesser effect on total levels of cytochrome P-450, but causes the synthesis of an isozyme possessing catalytic and spectral properties decidedly different from those of the isozymes in untreated or PB induced animals. The enzyme exhibits an altered electronic absorbance (hence the designation cytochrome P-448) and catalyzes the oxidation of a narrower class of substrates typified by the polycyclic aromatic hydrocarbons. As PB and 3-MC cause the synthesis of isozymes with different substrate specificities, they are useful as tools in studying the participation of the various enzyme forms in metabolic processes of pharmacological and toxicological significance. The xenobiotic-induced changes mentioned above result primarily from de novo enzyme synthesis (Omura, 1979).

The separation and purification of cytochrome P-450 from uninduced, 3-MC induced and phenobarbital induced rats has demonstrated the existence of multiple forms of the enzyme and made possible investigation of their individual properties. Uninduced rat liver contains at least four forms of the enzyme (Warner and Neims, 1979). West and

coworkers (1979) reported the isolation and purification of a single chromatographic fraction containing the major enzyme present in the livers of PB-pretreated Long-Evans male rats. An enzyme preparation, purified by this means, was used (provided by A.Y.H. Lu and G. Miwa) in the studies conducted for this thesis. Subsequent to our investigations, however, Waxman and Walsh (1982) reported the resolution of the major PB-induced enzyme (from the livers of Sprague-Dawley male rats) into two isozymes of differing catalytic activity. These authors suggested that the purified PB-induced preparation used in the studies conducted by members of this laboratory (Ortiz de Montellano et al, 1981) to determine the susceptibilities of isozymes to suicidal inactivation may have contained catalytically different forms, explaining the incomplete destruction of the PB-induced isozymes observed after incubation with suicidal substrates. The multiplicity of cytochrome P-450 isozymes has been demonstrated in studies of their electrophoretic migration patterns, visible absorption spectra, substrate selectivities, and immunological reactivities (Guengerich et al, 1981; Dent et al, 1980).

1.40 THE CATALYTIC CYCLE

The cytochrome P-450 mixed function oxidase system functions in a cycle, the intermediate steps of which (some of the postulated intermediates are still a matter of controversy) are pictured in Figure 1-1. Heme iron, initially



**Figure 1-1 Proposed Catalytic Cycle of
Cytochrome P-450**

in the Fe^{+3} state, is coordinated to the four pyrrole nitrogens of protoporphyrin IX. The fifth ligand has been shown (Cramer et al, 1978) by spectroscopic means to be a thiolate anion, probably from a cysteine residue. The porphyrin and thiolate ligands give rise to a high electron density about iron that manifests itself in the unusually long wavelength of the Soret absorbance and in an enhanced reductive strength. The nature of the sixth ligand is unclear. Evidence suggests that the sixth ligand is either a histidine, a water molecule, or an association of the two (Ullrich et al, 1977). The electronic absorption spectra of the enzyme are a function of the spin state and ligation of the iron. The ligand field strength of the coordinated molecules determines whether the iron will be low-spin ($s = 1/2$; associated with stronger ligands) or high-spin ($s = 5/2$; associated with weaker ligands). In the resting state of the enzyme the iron is predominantly hexacoordinate, low-spin, and in the plane of the porphyrin. This electronic configuration gives rise to a Soret absorbance at 418 nm, and peaks of lower intensity at 568 nm (α -band) and 535 nm (β -band).

The form of the enzyme induced by 3-methylcholanthrene (LM_4 , isolated from rabbit liver; analogous to cytochrome P_1 -450 from rats) possess either no sixth ligand or a weak one. It exists therefore in the high-spin form and exhibits a Soret band at 393 nm followed by a lower, broad absorbance at 545 nm. The iron can be converted to the low-spin hex-

acoordinate form by addition of a ligand of sufficient strength, as is accomplished by the addition of n-butanol.

1.41 Binding of Substrate

Substrate binding causes displacement of the weak endogenous sixth ligand. Upon binding of non-polar lipophilic molecules (cyclohexane, chloroform) the hexacoordinate iron shifts to a pentacoordinate, high-spin, out of plane configuration. This change gives rise to a difference spectrum with a trough at approximately 428 nm and a peak at 385 - 390 nm that is referred to as a type I spectrum. If, instead, the substrate has a strong coordinating functionality with lone-pair electrons that are accessible to the iron center (octylamine), the endogenous sixth ligand is effectively replaced. This difference spectrum, with a peak at 425 - 430 nm and a trough at 390 - 410 nm, is called a type II spectrum. Compounds possessing atoms of intermediate ligand strength (usually an oxygen atom, such as that from an alcohol) give rise to a "reverse type I" spectrum similar to that of a type II spectrum, but exhibiting a bathochromic shift in absorbance of about 5 nm, reflecting a lesser electron density contribution relative to that provided by type II substrates.

1.42 Transfer of the First Electron

The first of the two electrons comes from NADPH via the reductase, reducing the iron to the ferrous state. Ferro-

cytochrome P-450 binds carbon monoxide with great facility, producing the characteristic 450 nm difference spectrum that is used to quantitate the enzyme. Carbon monoxide competes with oxygen for binding as the sixth ligand. The inhibition of cytochrome P-450 caused thereby is exploited in in vitro studies to confirm the catalytic involvement of the enzyme in metabolic processes. Anything which disrupts the binding of the heme to the thiolate ligand of the active site converts P-450 to P-420, a denatured, inactivated form of the enzyme.

1.43 The Binding and Activation of Oxygen

Reduced substrate-bound cytochrome P-450 binds oxygen reversibly at the sixth coordination position. This ternary complex has been detected spectrophotometrically and has been shown not only to be reversible, but also to decompose to ferric cytochrome P-450 and superoxide anion (Guengerich et al, 1976). The complex can accept another electron and two protons to yield, after loss of water, a complex postulated to be the "oxene" complex. The exact nature of this "active oxygen" complex is a matter of controversy.

The steps in activation of oxygen can be replaced by addition of substances which can directly donate the equivalent of an electron deficient ($6 e^-$) oxygen atom. Some of the possible forms of this active oxygen complex are diagramed in Figure 1-2. In the absence of molecular oxygen and reducing equivalents, iodosobenzene (Lichtenberger et

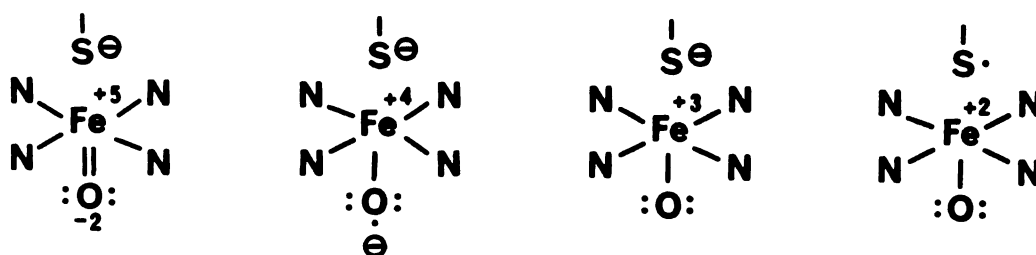


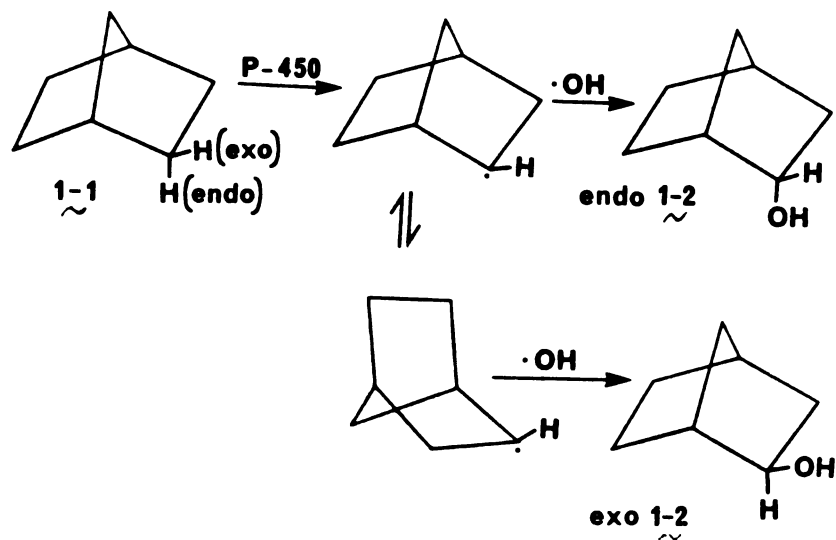
FIGURE 1-2

al, 1976) and organic peroxides (Hrycay *et al*, 1975) have been shown to support the cytochrome P-450 catalyzed oxidation of substrates, though the substrate selectivity thereby obtained is not completely independent of the oxygen donor (White and Coon, 1980). The reactions of nitrenes and carbenes parallel those of the cytochrome P-450 active oxygen in that they add across the π -bonds in benzene rings as well as simple alkenes and insert into unactivated carbon-hydrogen bonds.

The kinetic and stereochemical investigations of the aliphatic hydroxylation reaction have inspired mixed conclusions about the mechanistic details of the transfer of oxygen to various bonds. For the most part these reactions proceed with retention of configuration and are characterized by low deuterium isotope effects. This suggests a mode of oxidation in which oxygen is inserted directly into the C-H bond. Evidence for an abstraction-recombination mechanism also exists, however. The oxidation of norbornane (1-1)

to norborneol (1-2) (Groves et al, 1978) was shown to proceed with a large deuterium isotope effect ($k_H/k_D = 11.5$) and significant epimerization. These results are consistent with rate determining hydrogen abstraction to form a carbon radical that undergoes partial epimerization before combin-

Scheme 1-2



ing with a hydroxyl radical (Scheme 1-2). Cytochrome P-450 catalyzed single-electron abstractions are implicated in the destruction of the enzyme during metabolism of 3,5-dicarbethoxy-1,4-dihydrocollidine (Augusto et al, 1982a) and in the oxidation of sulfides (Watanabe et al, 1980). It may well be that oxygen transfer occurs by a spectrum of mechanisms intermediate between these two extremes.

1.50 REACTIONS CATALYZED BY CYTOCHROME P-450; ACTIVATION OF TOXINS

Cytochrome P-450 catalyzes an impressive array of redox reactions, as is shown in Figure 1-3. The catalysis of aliphatic carbon hydroxylation demonstrates the power of the oxidizing species generated by cytochrome P-450. Although the enzyme is capable of oxidizing such unactivated bonds, the reactions favor donation of oxygen to positions of high electron density on the substrate molecule. The cytochrome catalyzes such diverse reactions as the epoxidation of carbon-carbon double bonds, the desulfurization of thiono-compounds, the N-oxidation of imines and amines, the S-oxidation of sulfides and sulfoxides, and N, O, and S-dealkylation reactions. The products of these oxidation reactions tend to be more polar (and therefore more excretable) and, sometimes, more electrophilic. As a consequence cytochrome P-450 activates a number of compounds to proximate toxins that alkylate cellular macromolecules. The epoxidation of bromobenzene (Jollow et al, 1974) and the desulfurization of carbon disulfide (Neal et al, 1976) are examples of the inadvertent activation of xenobiotics to reactive molecules.

As previously mentioned, the thiolate and porphyrin nitrogen ligands lend to iron an enhanced electron density. The enzyme is capable of donating one or two electrons to substrates in reactions which are inhibited by the presence of oxygen or inhibitors of the mixed function oxidase system

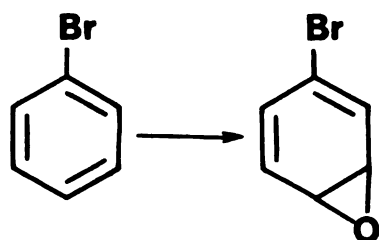
Figure 1-3 REDOX REACTIONS CATALYZED BY CYTOCHROME P-450

Oxidative Reactions

C-H Hydroxylation



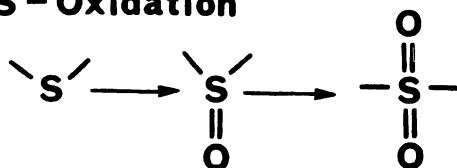
Epoxidation



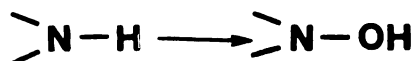
Desulfurization



S-Oxidation

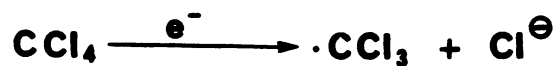


N-Oxidation

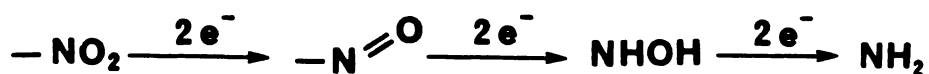


Reductive Reactions

Reduction of Halocarbons



Reduction of Nitro Compounds



(see Figure 1-3). Organic nitro compounds can be reduced to amines along a pathway yielding nitroso and hydroxyl amino compounds as sequential two-electron reduction intermediates (Mason, 1979). In contrast to the oxidation reactions, the reductive reactions tend to occur at positions on the substrate of low electron density. The toxic effects of a number of the haloalkanes are attributed to the cytochrome P-450-mediated reductive cleavage of carbon-halogen bonds to yield a halide anion and a reactive radical; the hepatotoxic potential of these compounds correlates with susceptibility to homolytic cleavage and is highest among those with the lowest bond dissociation energies. The toxic effects of carbon tetrachloride have been attributed (Sipes et al, 1977) to homolytic dehalogenation to the trichloromethyl free radical (see Figure 1-3), with subsequent lipid peroxidation, covalent binding to microsomal protein, and loss of microsomal enzyme activities.

Lipid peroxidation, the degradative oxidation of polyunsaturated fatty acids of lipids, has been associated with the aging process. The free radical intermediates formed cause disruption of the membrane structure, destruction of membrane bound enzymes, and cellular necrosis. Lipid peroxides undergo degradation to malondialdehyde, and the extent of lipid peroxidation can be determined by measurement of the formation of a colored complex with thiobarbituric acid (Asakawa and Matsushita, 1980).

1.51 Experimental Hepatotoxicity

The science of synthetic chemistry has far outdistanced the development of the field of toxicology. Consequently the synthesis and industrial use of new organic chemicals has preceded by decades knowledge of their potential toxicities. The belated experimental confirmation of the toxicities of vinyl chloride and of tetrachloroethane underline the need for reliable and convenient methods of determining beforehand the potential hazards involved in exposure to organic chemicals (for a review of this area, see Zimmerman, 1978).

In response to the need for clinical determinations of hepatotoxic responses, many diagnostic tests have been proposed and utilized with varying degree of success. Whole-animal studies can be of value in assessing the potential hazards of exposure to chemicals, but suffer from serious limitations. While there are many similarities between man and the laboratory animals employed, the differences in activating enzymes, protective mechanisms, longevity, and susceptibility often lessen the applicability of the data that is obtained. LD₅₀ values are also only a gross measure of toxicity. Histological changes brought about by these toxins may be detected by light and electron microscopy. Light microscopy has been used to detect hepatic steatosis (fatty degeneration of the liver) and necrosis, but is not capable of detecting subtle histological changes. Electron microscopy is a much more sensitive technique and does not suffer

from these limitations. Both techniques, however, require appreciable skill in the preparation and interpretation of tissue slides. Tests of liver function have also been employed in experimental heptatotoxicity. The measurement of production of urea, cholesterol, and other substances of hepatic biosynthetic origin are traditional tests of hepatic injury. The ability of the liver to excrete foreign dyes has also long been employed as a measure of hepatic function. Sulfobromophthalein (BSP) and indocyanine green (ICG) are commonly employed in these studies. The impairment of BSP excretion in chlorinated halocarbon damage correlates well with other measures of injury.

Serum enzymology has become the method of choice in the last twenty years for initial determination of the extent of liver damage. The technique exploits the higher levels of certain enzymes in hepatocytes; detection of these enzymes in the blood serum is indicative of cellular damage. The technique benefits from being sensitive, fast, and can be performed by persons with minimal training. Elevated levels of glutamic-oxalacetic transaminase, lactic dehydrogenase, isocitric dehydrogenase are observed in response to hepatic injury, but can also reflect injury to extrahepatic cells. Glutamic-pyruvic transaminase, ornithine carbamoyl transferase, sorbitol dehydrogenase, fructose 1-phosphate aldolase, arginase, and guanase are mainly found in the liver. In the foregoing study the serum level of glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transam-

inase (GPT) were selected as the marker enzymes. In mammalian systems serum GOT (SGOT) levels are a sensitive measure of acute hepatic necrosis. In the rat, the experimental animal chosen for our studies, SGPT is nearly as sensitive as SGOT, but is more specific to hepatic parenchymal cells. Caution must be exercised in the interpretation of the data from these experiments however. Substances which increase the permeability of the cells (without actually damaging them) can give rise to elevated levels of transaminases. Confirmation of hepatic damage by a combination of methods may best assure that the injury was, in fact, to the liver.

1.60 SELF-CATALYZED DESTRUCTION OF CYTOCHROME P-450

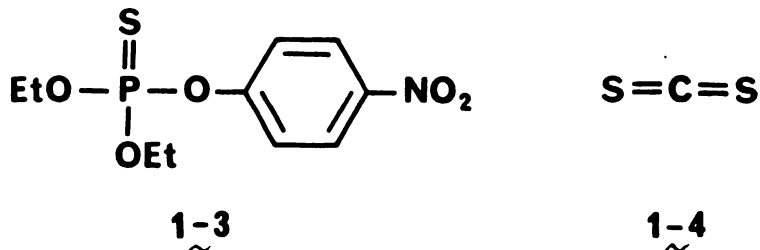
The autocatalytic destruction of cytochrome P-450 results from the metabolism of a number of organic compounds. The means of inactivation differ for these compounds, but in each case the agent is converted to a reactive species that damages components crucial to the activity of the mixed function oxidase system. In these processes the integrity of the apoprotein, the heme prosthetic group, or the associated lipids is altered in such a way as to compromise the function of cytochrome P-450.

1.61 Thiono Compounds

Compounds containing the thionosulfur group are in widespread use as drugs, industrial chemicals, and pesticides. A number of these compounds ($P=S$, $C=S$) have been

shown to decrease the levels of cytochrome P-450 in vitro and in vivo (Dalvi et al, 1975; Neal et al, 1976), and cause centrilobular necrosis (for a review see Halpert and Neal, 1981). Electrophilic sulfur, generated by the cytochrome P-450-mediated oxidative desulfurization of thiono compounds, has been implicated in the deleterious actions of such compounds.

In studies with ^{35}S radiolabelled parathion (1-3) (Kamatani and Neal, 1976) and carbon disulfide (1-4) (Castignani and Neal, 1975), it was found that the radioisotope was bound predominantly to the cytochrome P-450 in a form which could be released (to the extent of 50%) as -SCN upon



treatment by -CN. This suggests the presence of hydrodisulfide linkages, presumably formed by the attack of atomic sulfur upon cysteine residues of the apoprotein of the enzyme. The formation of hydrodisulfide linkages was accompanied by crosslinking of the cytochrome P-450, leading to the formation of high molecular weight aggregates. Destruction of the heme moiety was also detected, though quantitation of this loss showed it to account for only a portion of

the total cytochrome P-450 loss.

1.62 Carbon Tetrachloride

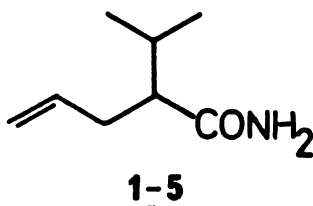
Carbon tetrachloride causes the NADPH-dependent loss of cytochrome P-450 in vitro (Smuckler et al, 1967). The agent furthermore stimulates lipid peroxidation with particular facility, and, in vivo, leads to the centrilobular necrosis associated with toxins activated by the hepatic mixed function oxidase system (Recknagel and Glende, 1973). The toxic action of carbon tetrachloride is most probably manifested through the trichloromethyl radical (Sipes et al, 1977), the product formed by reductive metabolism of one of the carbon-chlorine bonds. The presence of this radical was detected by electron spin resonance measurements (Albano et al, 1982) and is consistent with the isolation of hexachloroethane as a metabolite (Bini et al, 1975). Abstraction by this radical of an allylic hydrogen atom from lipid membranes may well initiate the sequence of events in lipid peroxidation. Whether caused by the trichloromethyl radical or subsequent products, it is clear that the destruction of components distant from the cytochrome P-450 originates from metabolism in the active site of this enzyme.

1.63 Olefins, Acetylenes, DDC

The last destructive compounds to be considered are a series of analogs which cause the autocatalytic inactivation of cytochrome P-450 primarily or exclusively by alteration

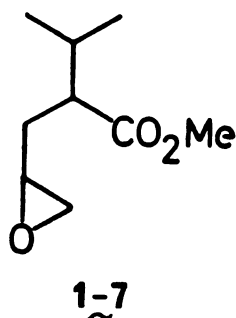
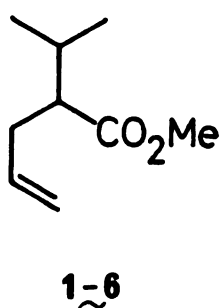
of the prosthetic heme.

The porphyrigenic agent allylisopropylacetamide (AIA, 1-5) destroys hepatic cytochrome P-450 in a time-dependent and dose-dependent process which requires NADPH and oxygen



(Ortiz de Montellano and Mico, 1981). Here, however, the molar losses of heme and of P-450 are equal. The loss of cytochrome P-450 in liver caused by in vivo treatment of rats with AIA is accompanied by accumulation of green pigment in that organ. By use of radiolabelled 6-amino-levulinic acid, a precursor of heme biosynthesis, the pigment was shown (De Matteis, 1971) to originate from the heme porphyrin. Work in our laboratory, using radiolabelled AIA, showed that the pigment also incorporated this substrate (Ortiz de Montellano et al, 1978).

AIA has in common with some other agents exhibiting this profile of destruction the presence of a double bond. Saturation of the olefinic unit of the inhibitors AIA, secobarbital (Levin et al, 1973), and fluroxene (Ivanetich et al, 1976) produces agents which are ineffective against the enzyme. The minimum required functionality was subsequently shown to be the simple carbon-carbon double bond,



illustrated by the fact that ethylene gas also destroys the enzyme and produces a pigment (Ortiz de Montellano and Mico, 1980). The presence of a double bond was shown to be a necessary but not sufficient condition for activity of analogs of this class; the olefinic unit must also be terminally situated.

The mass spectra of the pigments, demetallated and esterified during the isolation procedure, in general exhibit a parent ion of mass equal to the sum of the masses of the olefin, an atom of oxygen, and protoporphyrin IX dimethyl ester. The pigments have been analyzed by NMR and shown to be derivatives of protoporphyrin IX alkylated on a pyrrole nitrogen, the structures of which are exemplified by that produced with ethylene (Ortiz de Montellano et al, 1981a) (Figure 1-4). These findings establish the necessity for oxygen activation of the double bond for alkylation to take place. The epoxide metabolite (1-7) of methyl 2-isopropyl-4-pentenoate (1-6), an alkene known to inactivate the enzyme, binds to the active site of cytochrome P-450 but does not cause inactivation of the enzyme (Ortiz de Montel-

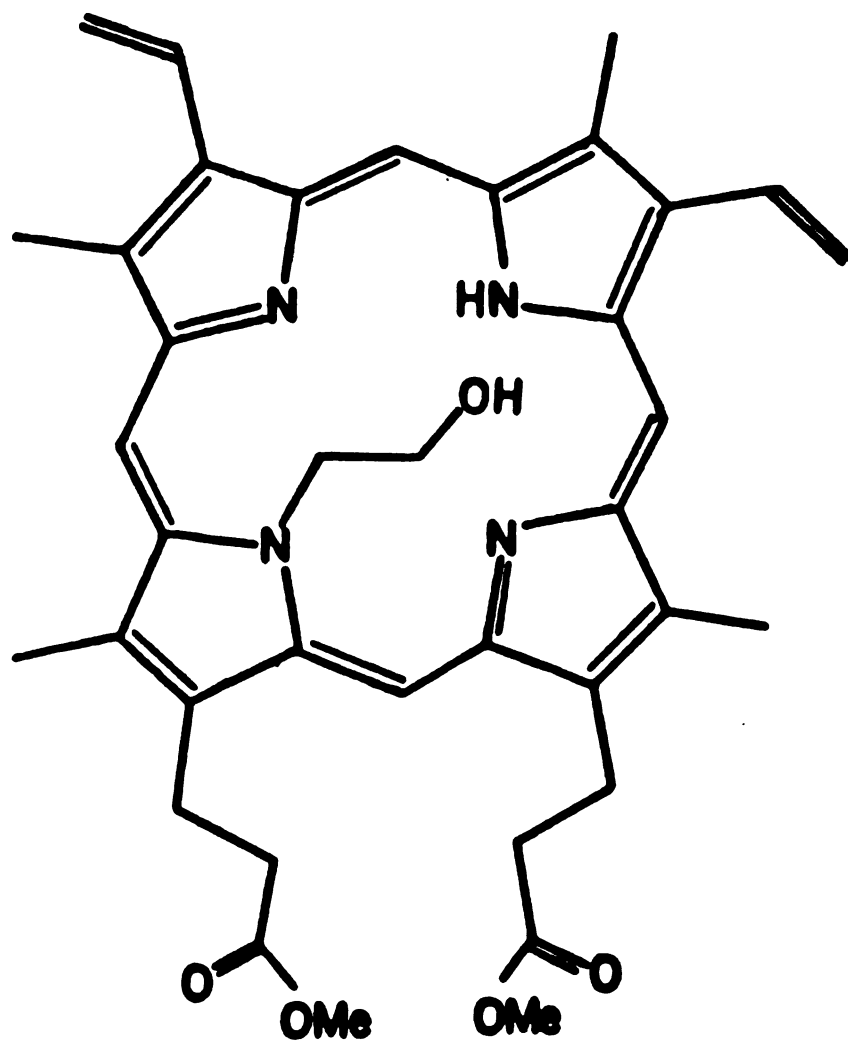


FIGURE 1-4

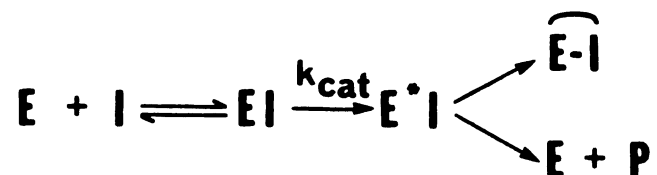
lano et al, 1979). Intervention of a destructive species intermediate in the reaction manifold from olefin to epoxide is thus indicated. Acetylenic substrates have since been shown to also inactivate the enzyme. While internal alkynes are effective against the enzyme, only the analogues possessing the functionality at the terminal position give rise to an isolable pigment (Ortiz de Montellano and Kunze, 1980a). Allenes similarly cause loss of cytochrome P-450 (Ortiz de Montellano and Kunze, 1980b), but here no pigment is isolated after in vivo treatment. 3,5-Dicarbethoxy-1,4-dihydrocollidine (DDC) destroys the enzyme in a process which alkylates the prosthetic heme group (Ortiz de Montellano et al, 1981a).

1.70 SUICIDE SUBSTRATES

Suicide substrates are molecules with functional groups which are intrinsically unreactive. The particular chemical reaction sequence of the given enzyme is required to unravel the inactivator. The inhibitor is converted to the reactive species in the active site of the enzyme and reacts there, without diffusion into the medium, to irreversibly inhibit the enzyme. Since most enzymes components are nucleophiles, the inhibitors by and large incorporate a latent electrophilic group. The olefins and acetylenes discussed above have been characterized as suicide substrates for cytochrome P-450.

Several criteria delineate a "suicidal" or "mechanism

based" inhibitory process (for a general review, see Walsh, 1982). The process should be time-dependent, saturable, and follows pseudo-first order kinetics (Maycock and Abeles, 1976).



Catalytic turnover of the inhibitor I yields an intermediate reactive species-enzyme complex E^*I which can either form the covalent complex $\widehat{E-I}$ or dissociate, releasing product P and regenerating the native enzyme E. The "partition ratio" is defined by the yield of P divided by $\widehat{E-I}$, a process of maximal efficiency having a value of 1. A plot of log activity remaining versus time should be linear (Figure

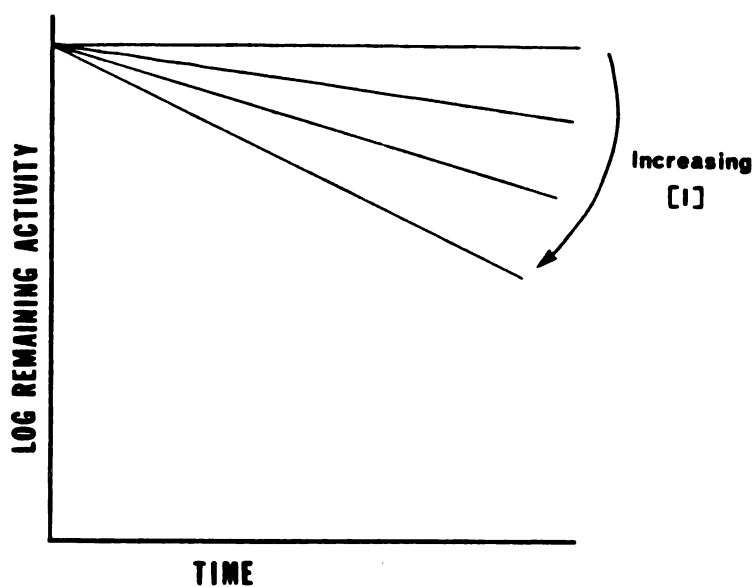


FIGURE 1-5

1-5).

A true suicidal agent works promptly at the active site; thus, exogenously added trapping agents, such as mercaptans, have no effect. The agent is said to be "specific" if it results in a 1:1 ratio of covalent adduct to dead enzyme. The destruction by these agents is competitively blocked by agents which occupy the active site of the enzyme.

1.80 PROPOSAL

It is now well established that the presence of a carbon-carbon π -bond confers upon molecules potential activity as suicide substrates for cytochrome P-450 enzymes. Acetylenes, olefins, and allenes are inhibitors of the phenobarbital inducible forms of the enzyme and, for the most part, exhibit little activity against the form(s) induced by 3-MC (Ortiz de Montellano et al, 1981). The lack of susceptibility of the 3-MC form to inactivation by these agents may be due to their rejection as substrates, as was explicitly shown for the ineffectiveness of AIA against this isozyme (Ortiz de Montellano et al, 1981). This proposal seems particularly reasonable in light of the selectivity of the 3-MC form towards catalytic oxidation of planar polycyclic molecules to the exclusion of the majority of other types of substrates. As part of a rational search for structures capable of suicidally inactivating the 3-MC form of the enzyme, which converts polycyclic aromatic hydrocarbon to proximate carcinogens, we chose to investigate the

use of planar heterocyclic moieties as structural units for the construction of appropriate suicide substrates. The insecticide synergist 5,6-dichloro-1,2,3-benzothiadiazole was first investigated because its synergistic activity appeared to result from an interaction with cytochrome P-450 enzymes. The known oxidation of the heterocycle 1-aminobenzotriazole to the highly reactive benzyne species (Campbell and Rees, 1969), and the well established ability of cytochrome P-450 to catalyze the oxidation of arylamines, made the subsequent investigation of this agent as a potential suicide substrate for cytochrome P-450 a promising pursuit.

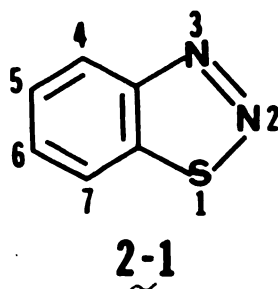
CHAPTER TWO

1,2,3-BENZOTHIADIAZOLES

2.1 INTRODUCTION

In recent years attention has been focused on the effects of pesticides on the environment. This concern has spurred interest in a class of compounds known as insecticide synergists, agents which, although not intrinsically toxic, potentiate the activity of coadministered insecticides. A mixture of an insecticide with a synergist allows application of a fraction of the dose of the insecticide required in the absence of the synergist to achieve insect control, and can restore the susceptibility of insects to agents to which they have become resistant. It is generally accepted that insecticide synergists exert their effect through inhibition of the metabolic enzymes responsible for insecticide detoxification.

It has long been known that 1,2,3-benzothiadiazole (2-1) and its analogs are effective inhibitors of the cytochrome P-450 enzymes responsible for insecticide metabolism (Philpot and Hodgson, 1974; Gil, 1973). Although in-depth studies of the structure-activity relationships of these analogs have been performed (loc. cit.), the precise mechanism by which the 1,2,3-benzothiadiazoles inhibit the action of cytochrome P-450 has not yet been ascertained. The investigations described in this thesis were conducted to determine the mechanism of action of the 1,2,3-benzo-



thiadiazoles and to examine the potential utility of the 1,2,3-benzothiadiazole moiety in the construction of in vivo agents which may inactivate specific cytochrome P-450 enzymes.

2.2 BIOLOGICAL ACTIVITY OF THE 1,2,3-BENZOTHIADIAZOLES

The 1,2,3-benzothiadiazoles (BTD) greatly increase the toxicity of insecticides whose primary mode of detoxification is oxidative metabolism by the cytochrome P-450 mixed function oxidase system. The toxicity of carbaryl (Gil and Wilkinson, 1976), pyrethrin, organophosphate, and carbamate insecticides (Felton, Jenner, and Kirby, 1970) to houseflies is greatly enhanced when combined with these compounds. Interaction of 1,2,3-Benzothiadiazoles with oxidized cytochrome P-450 is evident in that they give a Type II difference spectrum (Matthews et al, 1970). Treatment of mice with BTD brings about the in vivo loss of cytochrome P-450, increases hexobarbital sleeping time, and decreases the LD₅₀ for insecticides (Skrinjaric-Spoljar et al, 1971). The initial enzyme loss is followed by a period of enzyme induction. Inhibition of xenobiotic metabolism by BTD has also

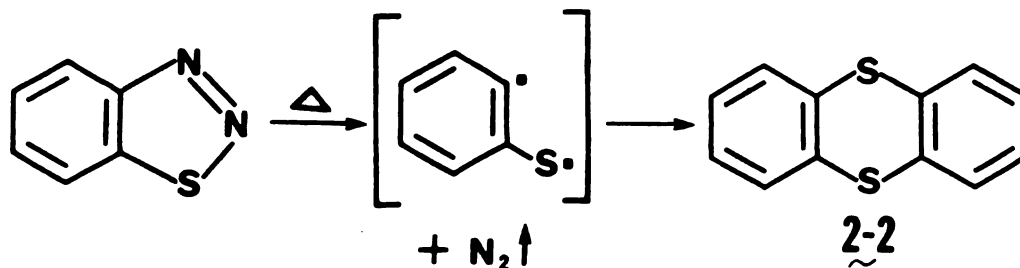
been demonstrated in vitro. The epoxidation of aldrin and hydroxylation of isodrin by rat liver and armyworm midgut microsomes, for example, is inhibited by this agent (Gil and Wilkinson, 1977). Inhibition of the oxidase system by BTD can alternatively serve a protective role; the extent of covalent protein binding observed after microsomal incubation with trichloroethylene is decreased in the presence of BTD (Bolt et al, 1977).

Structure-activity relationships for BTD as synergists for carbaryl in houseflies (Gil and Wilkinson, 1976) and as inhibitors of mammalian and insect microsomal oxidations (Gil and Wilkinson, 1977) show that activity correlates with the hydrophobic binding constant and with the homolytic free radical constant.

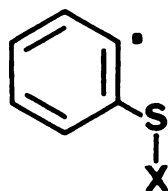
2.3 CHEMISTRY OF 1,2,3-BENZOTHIADIAZOLES

1,2,3-Benzothiadiazoles are conveniently prepared by diazotization of the appropriate 2-aminobenzenethiols available commercially or through a modification of the Herz reaction (Kirby et al, 1970). The compounds are water-insoluble solids which are stable to acid and base. Pyrolysis (200-250°C) of BTD causes the decomposition of the thiadiazole moiety to yield thianthrene (2-2). Loss of nitrogen gas, an excellent leaving group, is a common mode of decomposition of diazo compounds.

Unsubstituted 1,2,3-benzothiadiazoles undergo electrophilic substitution reactions only under extreme conditions,



but halogenated analogs react with nucleophiles (RO- and RS-) at the 4- and 6- positions in refluxing ethanol (Davies et al, 1971). The thiadiazole moiety reacts readily with radical species, however. Radical attack at the sulfur brings about the decomposition, with loss of nitrogen, of the heterocyclic moiety. The reaction of BTD with phenyl (Benati et al, 1974) and thiyl (Benati et al, 1976) radicals, as well as with triplet diphenylcarbene (Benati et al, 1981) and triplet nitrene (Benati et al, 1980), thus proceeds through intermediates of the following type:



where X=radical.

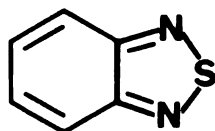
Oxidation of BTD with excess m-chloroperoxybenzoic acid (MCPBA) or peroxyacetic acid gives 1,2,3-benzothiadiazole-1,1,2-trioxide (2-3) and thianthrene tetroxide (2-4) (Braun

TABLE 2-1

IN VITRO DESTRUCTION OF CYTOCHROME P-450 BY DCBT

| <u>Conditions</u> | <u>Percent loss of cytochrome P-450</u> | | |
|----------------------------|---|----------------|----------------|
| | <u>PB-Induced</u> | | |
| | <u>10 min.</u> | <u>20 min.</u> | <u>30 min.</u> |
| complete (experiment 1) | 47 \pm 3 | 55 \pm 2 | 56 \pm 1 |
| -NADPH | ND | ND | ND |
| -O ₂ | | 14 \pm 1 | |
| complete (experiment 2) | | | 65 \pm 1 |
| +carbon monoxide | | | 49 \pm 1 |
| +10 mM glutathione | | | 41 \pm 1 |
| | <u>3-MC Induced</u> | | |
| | <u>10 min.</u> | <u>20 min.</u> | |
| complete | 27 \pm 2 | 32 \pm 1 | |
| -NADPH | ND | ND | |

DCBT was run at a nominal concentration of 10 mM (the solubility is much lower). Values are an average of determinations from 3 separate incubations, except for the O₂ and the 3-MC experiments for which 2 determinations were made, and are reported \pm the standard deviation or experimental uncertainty (1%), whichever is greater.



2,1,3-BTD

and is inactive.

2.41 Destruction of cytochrome P-450 by DCBT is a self-catalyzed process. In the absence of NADPH or oxygen, the cofactors necessary for enzyme activity, no spectroscopically measurable loss of cytochrome P-450 is observed in microsomal incubations with DCBT (Table 2-1). The requirement for substrate turnover in the inhibitory process was further demonstrated by the attenuation of enzyme destruction seen in incubations at decreased oxygen tension and with incubations performed in the presence of carbon monoxide. Loss of enzyme in the presence of NADPH and oxygen, but in the absence of DCBT, was determined in a control experiment with each microsomal preparation and was found to be less than 2%. Thus, losses through NADPH-mediated but substrate independent processes were minimal in these EDTA-containing incubation mixtures. Furthermore, lipid peroxidation was not enhanced in the presence of DCBT (Table 2-2), precluding the possibility that enzyme loss resulted from substrate-mediated lipid peroxidative damage such as that caused by carbon tetrachloride (Recknagel and Glende, 1973).

TABLE 2-2

Detection of malondialdehyde formation as a measure of lipid peroxidation in DCBT incubation with rat liver microsomes

| <u>Conditions</u> | <u>Malondialdehyde formation (micromolar)</u> |
|-------------------|---|
| -DCBT | ND |
| complete | ND |
| CCl ₄ | 4.9±0.2 |

TABLE 2-3

Correlation of heme loss to cytochrome P-450 loss

| <u>Conditions</u> | <u>Cytochrome P-450 content (nmol /mg protein)</u> | <u>Heme content (nmol /mg protein)</u> |
|-------------------|--|--|
| -DCBT | 2.4±0.1 | 3.7±0.1 |
| DCBT | 1.0±0.1 | 2.3±0.1 |
| loss | 1.4 | 1.4 |

DCBT and CCl₄ were run at 10 mM nominal concentration. Values are reported as the average of determinations from 3 separate incubations ± standard deviation.

It is interesting that the presence of 10 mM (but not 1 mM) glutathione had a protective effect on cytochrome P-450 loss in microsomes (Table 2-1). Although this protection could result from trapping of a reactive electrophilic species, it also could be due to a direct chemical reaction of glutathione with DCBT, a reaction that occurs with activated chlorobenzenes (Chasseaud, 1973). The known reactivity of DCBT with thiols (Davies et al, 1971) supports this hypothesis. Catalysis by microsomal glutathione transferase possibly could decrease the activation energy required for reaction at physiological temperatures.

In experiments with reconstituted mixed-function oxidase systems (Ortiz de Montellano et al, 1981; components provided by M.T. Miwa and A.Y.H. Lu) it was found that no cytochrome P-450 was lost in incubations containing NADPH, DCBT, and lipid; upon addition of NADPH-cytochrome P-450 reductase the losses reported in Table 2-5 were witnessed. Catalytic turnover of DCBT by the enzyme is thus required for destruction.

2.42 Site of inhibitory action of DCBT on cytochrome P-450. Workers in this laboratory have shown that the self-catalyzed destruction of cytochrome P-450 by olefinic (Ortiz de Montellano et al, 1979) and acetylenic (Ortiz de Montellano and Kunze, 1980a) substrates is a result of alkylation of the heme prosthetic group of the enzyme by a reactive intermediate formed in the catalytic oxidative process. Equimolar losses of cytochrome P-450 and heme have been

demonstrated in microsomal incubations with these and other destructive agents. Incubation of DCBT with microsomes (Table 2-3) results in a 1:1 loss of cytochrome P-450 and heme.

In contrast to other destructive agents, such as acetylene (Ortiz de Montellano and Kunze, 1980a), no abnormal porphyrin pigments could be detected on work-up, by the same procedure, of livers from DCBT treated rats. The thin layer chromatography plates employed in two attempts to isolate pigments from DCBT and from untreated rats were identical in appearance. This finding is reminiscent of the behavior of internal acetylenes and allenes (Ortiz de Montellano and Kunze, 1980a,b). Microsomal heme loss and cytochrome P-450 loss were not accompanied by the detection of pigments in hepatic tissues after in vivo treatment with these agents. The isolation and detection of pigments thus formed depends on their stability to the derivatization process, on their extractability into an organic phase, and on their visualization. The altered porphyrins must be stable to treatment with 5% sulfuric acid/methanol and to the presence of oxygen. A porphyrin adduct with a DCBT metabolite could include a benzene sulfonate residue resulting from enzymatic and/or chemical oxidation of DCBT, and thus could be very polar and be lost in the aqueous washes. Detection of the "green pigments" requires their visualization as bands which dramatically fluoresce red upon irradiation with 366 nm light. These pigments are associated with N-alkylated, but

not necessarily otherwise altered, porphyrins. Derivatization of prosthetic heme by the DCBT metabolite therefore yields products which are unstable, unisolable, or non-fluorescent.

2.43 Specificity of destruction in vivo and in vitro of hepatic cytochrome P-450 isozymes. Although no pigment could be isolated from hepatic tissues a few hours after in vivo administration of DCBT, the levels of hepatic cytochrome P-450 in PB and 3-MC pretreated rats were lowered by 29% and 34% respectively, (Table 2-4). While the activity of DCBT against cytochrome P-450 in PB and 3-MC pretreated animals suggests the differential susceptibility of the different isozymes to inactivation, the overlapping isozyme populations present in these preparations prevent a clear determination of destructive specificity. The effectiveness of DCBT against both major forms of the enzyme was demonstrated, however, (Table 2-5) in experiments with purified samples of the two principal isozymes (from rat liver) differentially induced by these agents. In contrast to the general ineffectiveness of unsaturated compounds against the 3-MC inducible isozyme, DCBT proved to be quite active (Ortiz de Montellano et al, 1981).

2.44 Biochemical toxicology of DCBT. The preceding data indicate that oxidative metabolism of DCBT brings about its transformation to a reactive species. The levels of cellular enzymes in blood serum were monitored in an effort

TABLE 2-4

In vivo loss of hepatic cytochrome P-450 in DCBT-treated rats.

| | Cytochrome P-450 content (nmoles/mg protein) | |
|--|--|------------------------|
| | <u>(% enzyme loss)</u> | |
| | <u>PB-pretreated</u> | <u>3-MC pretreated</u> |
| control | 1.60 | 1.29 |
| DCBT (100 mg/kg in propylene glycol) | 1.14 (29%) | 0.85 (34%) |

TABLE 2-5

Destruction of purified hepatic cytochrome P-450 isozyme in reconstituted systems by DCBT

| <u>Conditions</u> | <u>Percent loss cytochrome P-450, 20 min.</u> | |
|-------------------|---|--------------------|
| | <u>PB-Enzyme</u> | <u>3-MC enzyme</u> |
| complete | 44 _± 3 | 55 _± 3 |
| -NADPH | ND | ND |
| -reductase | ND | 1 |

DCBT was run at a nominal concentration of 1 mM. Values are an average of determinations from 3 separate incubations \pm standard deviation or experimental uncertainty (1%), whichever is greater.

to determine the capacity of such a species to diffuse out of the active site of cytochrome P-450 and to cause cellular damage. Glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) have been successfully employed in following necrotic processes (Zimmerman, 1978) as "marker" enzymes. However, despite the fact that cytochrome P-450 levels were greatly diminished in rats 24h after administration of DCBT, no increase in serum transaminase levels accompanied this monooxygenase destruction (Table 2-6).

2.5 PROPOSED MECHANISM OF DESTRUCTION

It is evident from the foregoing data that cytochrome P-450 is destroyed during the catalytic oxidative turnover of DCBT. No time lag is observed in this destruction; a single oxidative cycle is thus indicated for the inhibition. This differs from the multiple catalytic cycles and attendant time lag associated with inhibitory complex formation with SKF-525A. Losses of approximately 30% were observed in the first minute of the incubation, making unlikely the involvement of unstable dioxo derivatives of BTD such as the benzyne precursor 1,2,3-benzothiadiazole-1,1-dioxide (Wittig and Hoffmann, 1962) unless the substrate undergoes multiple oxidations without release from the active site. Another oxidative precursor of benzyne is discussed later and is shown to give rise to isolable porphyrin pigments.

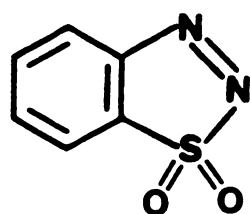
TABLE 2-6

TOXICITY OF DCBT: In vivo effect of DCBT on serum transaminase levels in PB-pretreated rats.

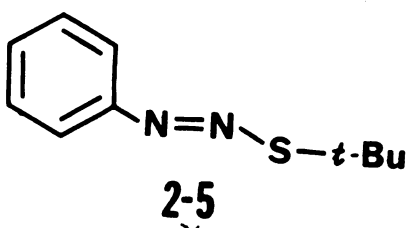
| <u>Treatment</u> | <u>SGPT (I.U./l)</u> | <u>SGOT (I.U./l)</u> |
|------------------------|----------------------|----------------------|
| Tricaprylin (N=5) | 19 <u>±</u> 1 | 60 <u>±</u> 8 |
| DCBT (N=4) | 17 <u>±</u> 2 | 56 <u>±</u> 5 |
| CCl ₄ (N=2) | >700 | >800 |

Mean values of serum glutamic-oxalacetic transaminase (SGOT) and serum glutamic-pyruvic transaminase (SGPT) levels for N rats + standard deviation, resulting from administration of: tricapyrylin (1 ml, as a vehicle control), carbon tetrachloride (1 ml/kg), and DCBT (100 mg/kg in 1 ml of tricapyrylin) respectively.

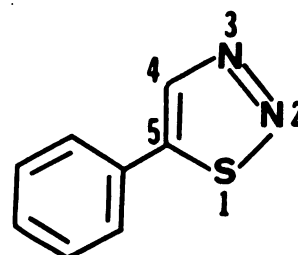
The 1,2,3-thiadiazole synergist series includes potent open chain and non ring-fused members such as 2-5 and 2-6 (Gil and Wilkinson, 1975). If the 1,2,3-thiadiazoles share a common mechanism of inhibition (as they do a spectrum of activities), proposed mechanisms must account for the activity of these analogs. The 2-oxide and 3-oxide of 2-6 are known and are stable compounds (Braun et al, 1975), although no 1-oxide of a 1,2,3-thiadiazole has ever been prepared. Oxidation of 1,2,3-benzothiadiazole with excess MCPBA gives thianthrene tetroxide (2-4). The latter process involves decomposition of the S-oxo but not the S,S-dioxo (which loses SO₂ to form benzyne) compound (Braun et al, 1973), and is best rationalized through the intermediacy of



1,2,3-BENZOTHIADIAZOLE-
1,1-DIOXIDE

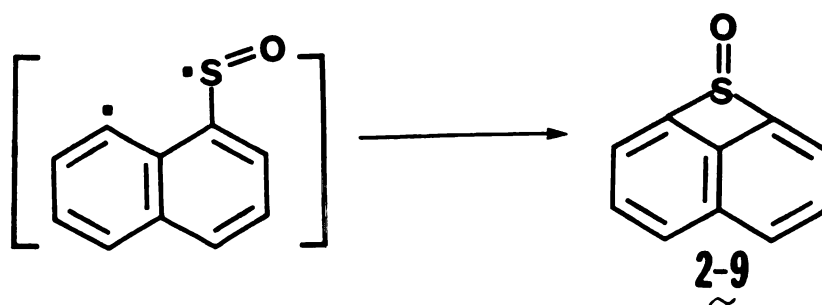
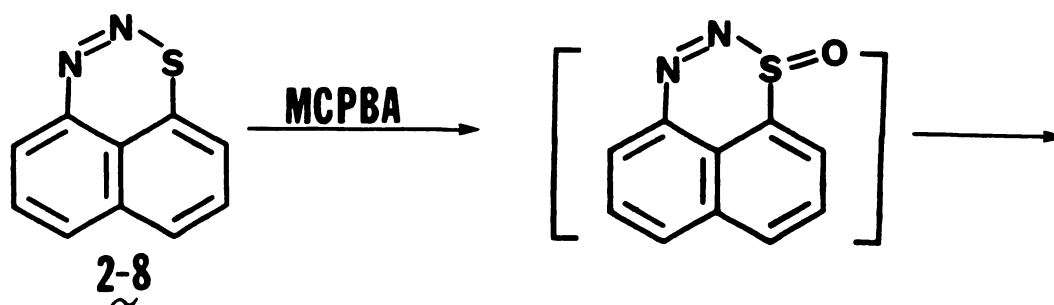
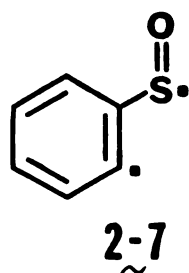


2-5



2-6

2-7. In a related system, thiadiazine 2-8 undergoes rapid decomposition, when treated with MCPBA at room temperature, to the sulfoxide 2-9 (Nakayama et al, 1979). Catalytic activation by S-oxidation of BTD is therefore postulated. The lone-pair 2p-electrons of sulfides are particularly accessible to attack by electron deficient species. Sulfides are good substrates for cytochrome P-450 and are oxidized to the corresponding sulfoxides (Watanabe et al,

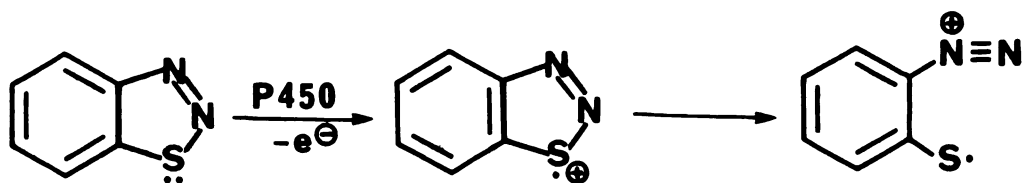


1980).

The intermediacy of the sulfinyl diradical 2-7 in the destruction of prosthetic heme is consistent with, and provides a rationale for, the structure-activity results of Gil and Wilkinson. Analogs of 2-1 with bulky groups at the 7-position (neighboring the sulfur atom) have particularly low synergistic activity, as expected if oxidation of sulfur is required for activity. Sulfinyl radicals are π -type radicals (Gilbert et al, 1977) that are stabilized by overlap

with the π -system of the benzene ring. Gil and Wilkinson found that synergistic activity correlated best with the ability of phenyl substituents to conjugatively stabilize free radicals rather than with an electron withdrawing or donating effect.

In an alternative mechanism, inactivation may occur



after removal of a single electron from the sulfur. Fragmentation of BTD by the two electron oxidant peroxy acids requires elevated temperatures, while the free radical decompositions occur at physiological temperatures. In a kinetic study of cytochrome P-450 oxidations of sulfides, Watanabe et al (1980) suggested that the oxidation of sulfides to sulfoxides proceeds via a discrete one-electron transfer from sulfur to active oxygen. Destruction of cytochrome P-450 by radicals produced in a one-electron abstraction process such as this has been demonstrated in the metabolism of a 1,4-dihydropyridine derivative (Augusto et al, 1982a).

2.6 CONCLUSION

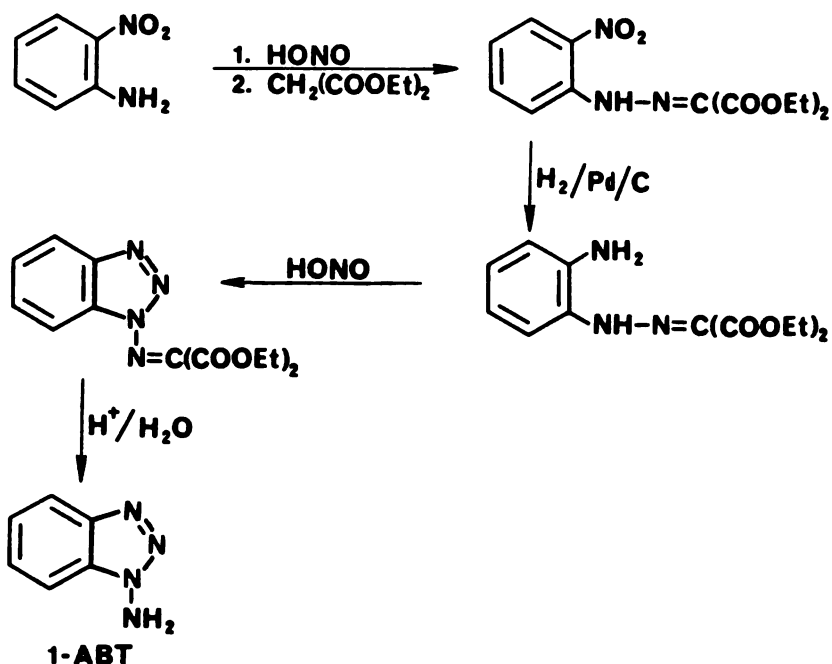
Inhibition of cytochrome P-450 by 5,6-dichloro-1,2,3-benzothiadiazole in a process catalyzed by that enzyme results, at least in part, from the destruction, presumably by a reactive DCBT metabolite, of prosthetic heme. The agent is effective against a broad spectrum of cytochrome P-450 isozymes, yet its toxic action seems to be confined to the enzyme itself. The thiadiazole moiety therefore is a good candidate for incorporation into potential 3-MC specific inactivating agents.

CHAPTER THREE

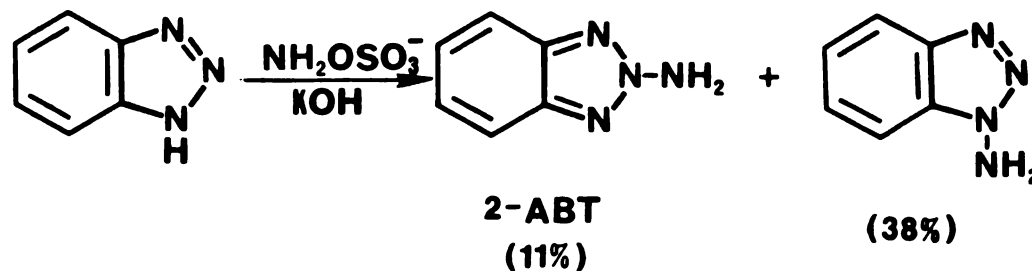
1-Aminobenzotriazole

3.10 INTRODUCTION

3.11 Chemistry of 1-Aminobenzotriazole. 1-Aminobenzotriazole (1-ABT) was first prepared by Trave and Bianchetti (1960) from 2-nitrophenylhydrazine derivatives. In a modification of this preparation Campbell and Rees (1969) synthesized the heterocycle in good yield (54%) by the following scheme:



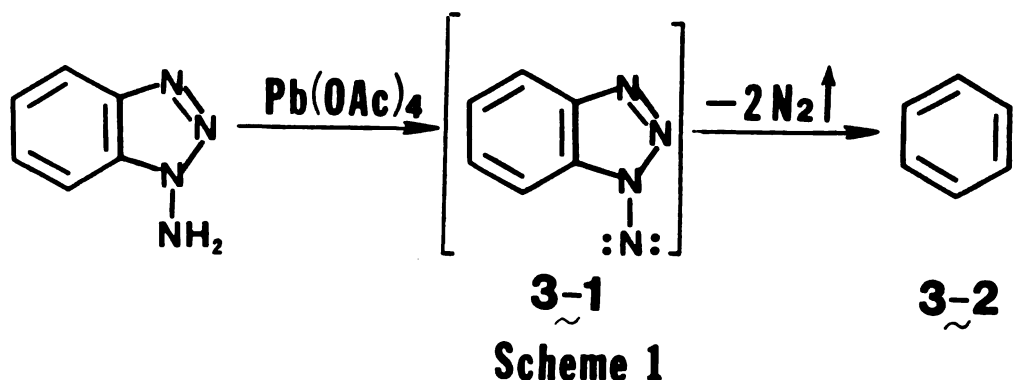
In this same study a mixture of 1- and 2-aminobenzotriazole (2-ABT) was obtained from the amination of benzotriazole with hydroxylamine-O-sulfonic acid.



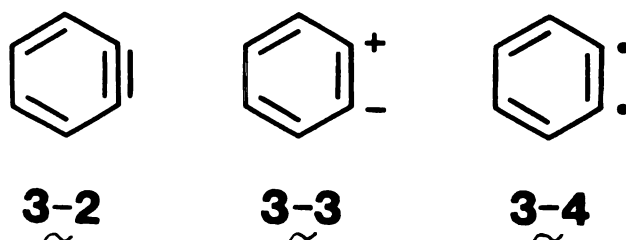
1-ABT is a solid which is freely soluble in water and stable to acid and base. Few amino-substituted analogs of 1-ABT have been reported in the literature, although in the following study the triazole was shown to be an excellent nucleophile. The 1-amino group was exhaustively derivatized in reactions with acylating and alkylating reagents. This property is possibly a consequence of an "ortho" effect produced by the presence of adjacent electron lone-pairs on the 1-amino group and on the heterocyclic nitrogen atom. The enhanced nucleophilicity of hydrazines over amines is attributed to the electron pair repulsion which manifests itself in producing this effect.

Oxidation of 1-ABT by lead tetraacetate at room temperature proceeds rapidly (Campbell and Rees, 1969) with instantaneous loss of nitrogen to generate benzyne (3-2). The reaction is believed to proceed via a two electron oxidation (Scheme 1) to the nitrene intermediate, 3-1.

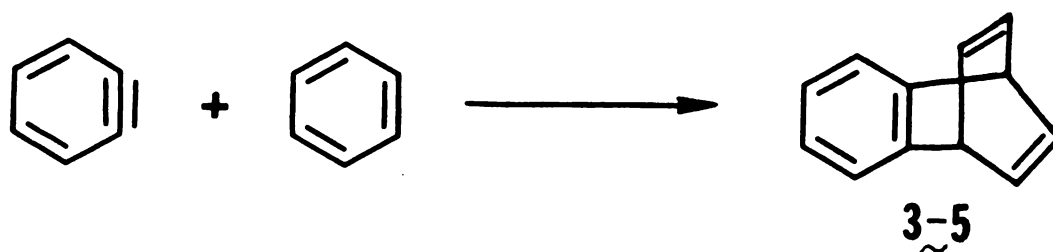
3.12 The nature of benzyne. Benzyne has never been isolated per se as it is a highly reactive species. Its existence has been established instead by numerous trapping



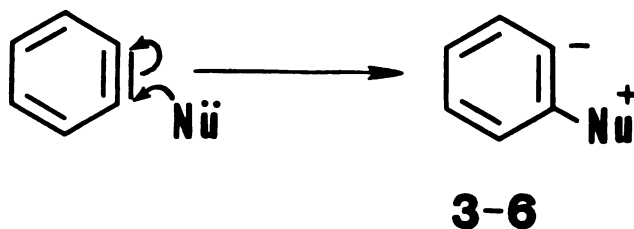
experiments. The 1,2-dehydro unit of this molecule behaves as an unsaturated bond, as in 3-2, or as the dipolar species



3-3, rather than as the diradical 3-4 (Hoffmann, 1967). In reacting with nucleophiles and with certain olefins the 1,2-dehydro unit behaves as a highly polarizable double bond. The extreme reactivity of this species is demonstrated by its low temperature reaction with benzene to yield benzobicyclo [2.2.2] octatriene (Miller and Stiles, 1963) (3-5) a reaction requiring an expenditure of approximately 36 kcal/mol in resonance energy. Reaction of benzyne with nitrogen, oxygen, and sulfur nucleophiles is a highly exothermic process that yields products resulting from a Michael-type addition. Betaines of the type 3-6 are ini-



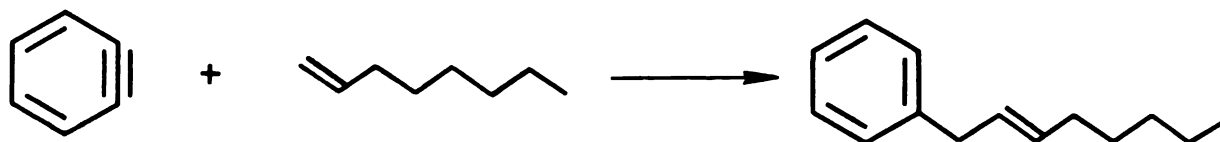
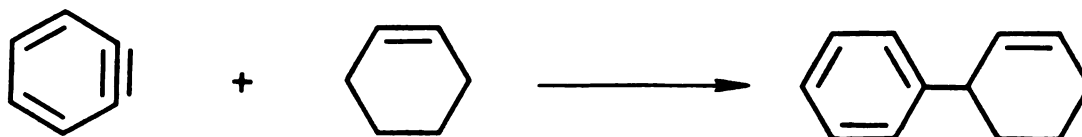
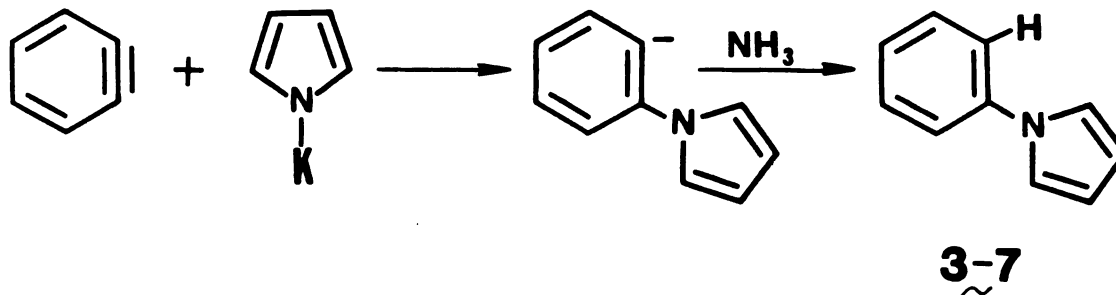
tially formed, leaving a product with a reactive phenyl anion and positively charged nucleophile that can undergo



Where $\text{N}\ddot{\text{u}} = \text{N}, \text{S}, \text{O}$, halide nucleophile

subsequent reactions. If the nucleophilic atom bears a proton the dipole 3-6 is "quenched" by intramolecular proton transfer to yield the more stable tautomer. N-phenylpyrrole (3-7) is obtained in the reaction of pyrrolylpotassium with benzyne generated at -33°C in liquid ammonia (Scardiglia and Roberts, 1958). Reaction with olefins involves abstraction of an allylic hydrogen. Where possible the double bond shifts to yield only the trans-olefin (Simmons, 1961).

3.13 Proposal. Cytochrome P-450 is known to catalyze the N-oxidation of a variety of arylamines. The known chemical oxidation of 1-ABT to the highly reactive benzyne



species inspired the ensuing study of this agent as a potential suicidal substrate for cytochrome P-450 enzymes. If benzyne is formed in the enzymatic oxidation of 1-ABT it could inhibit the enzyme by reacting with biochemical components necessary for catalytic activity. The benzyne reactions just discussed provide a model for potential disruptive reactions with the apoprotein (which contains nitrogen, oxygen, and sulfur nucleophiles), the heme prosthetic group (a pyrrole system), and the lipid membrane (which possesses olefinic constituents to which the integrity of cytochrome P-450 is sensitive).

3.20 IN VITRO INTERACTION OF 1-ABT WITH HEPATIC CYTOCHROME P-450

1-ABT was synthesized as described in the experimental section and incubated with hepatic microsomes prepared from rats pretreated with phenobarbital (PB). The agent bound to the unreduced enzyme to produce a Type-II difference spectrum (Figure 3-1). This spectrum is similar to those produced by aniline and other amines, and is characteristic of a binding interaction in which there is coordination of nitrogen to heme iron. No spectroscopically measurable loss of cytochrome P-450 occurred as a result of this interaction, however. Addition of NADPH to the incubation mixture resulted in rapid, extensive, time-dependent loss of the enzyme (Figure 3-2; Table 3-1). Since enzyme inactivation was measured here as a spectral decrease of the 450 nm peak, it could be argued that only the enzyme losses that caused a spectral perturbation were measured; 86% spectral loss, however, could not be observed if a substantial portion of the enzyme were altered in such a way as to produce inhibition without spectral loss. No enzyme loss was observed in incubations containing NADPH but lacking 1-ABT. Destruction of cytochrome P-450 was attenuated in incubations performed in the presence of carbon monoxide. Catalytic turnover of 1-ABT is thus indicated as a requirement for the destructive process.

Cytochrome P-450 from 3-methylcholanthrene (3-MC) pretreated rats was inhibited to an extent comparable to that

FIGURE 3-1
Binding Spectrum of 1-ABT

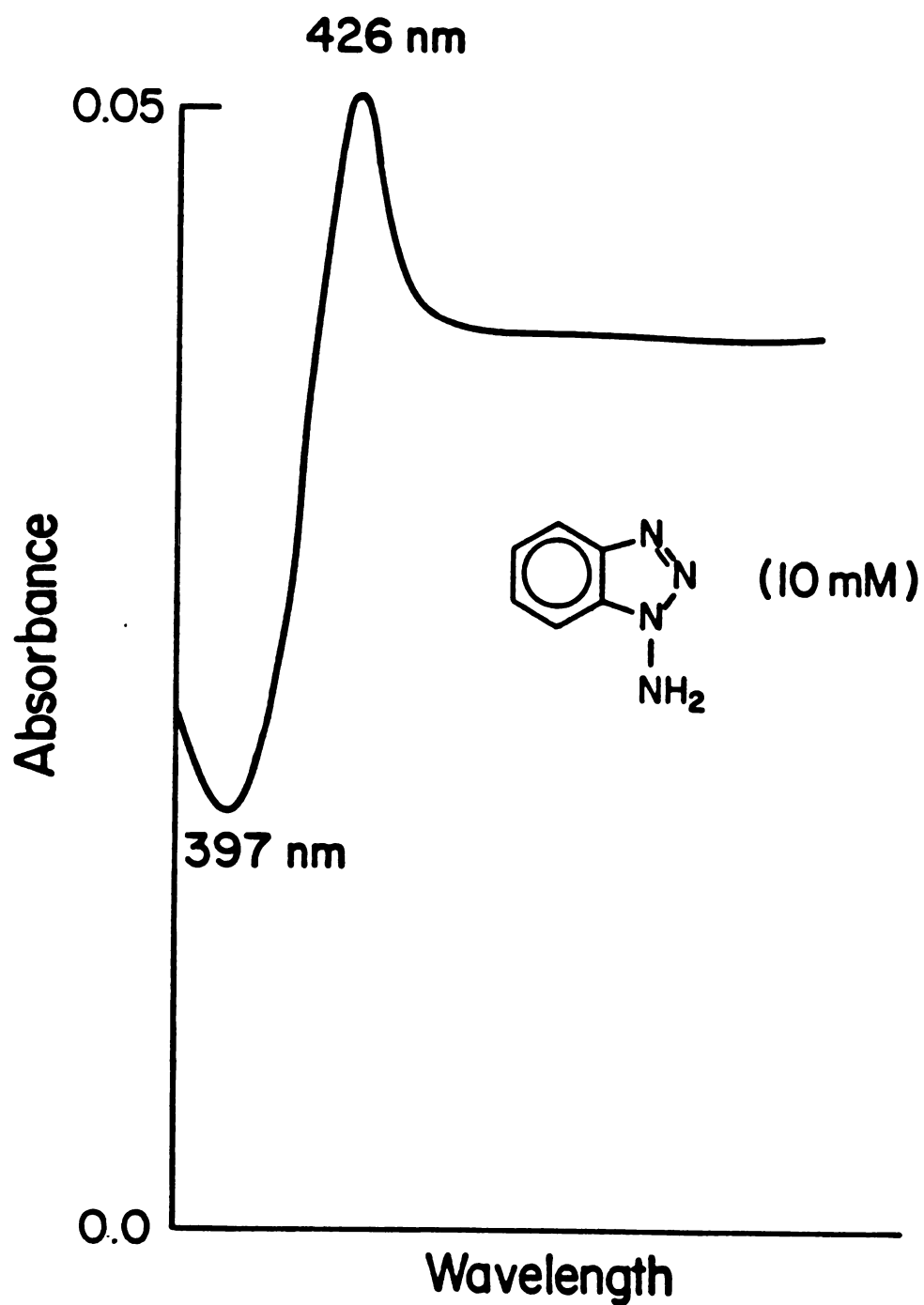


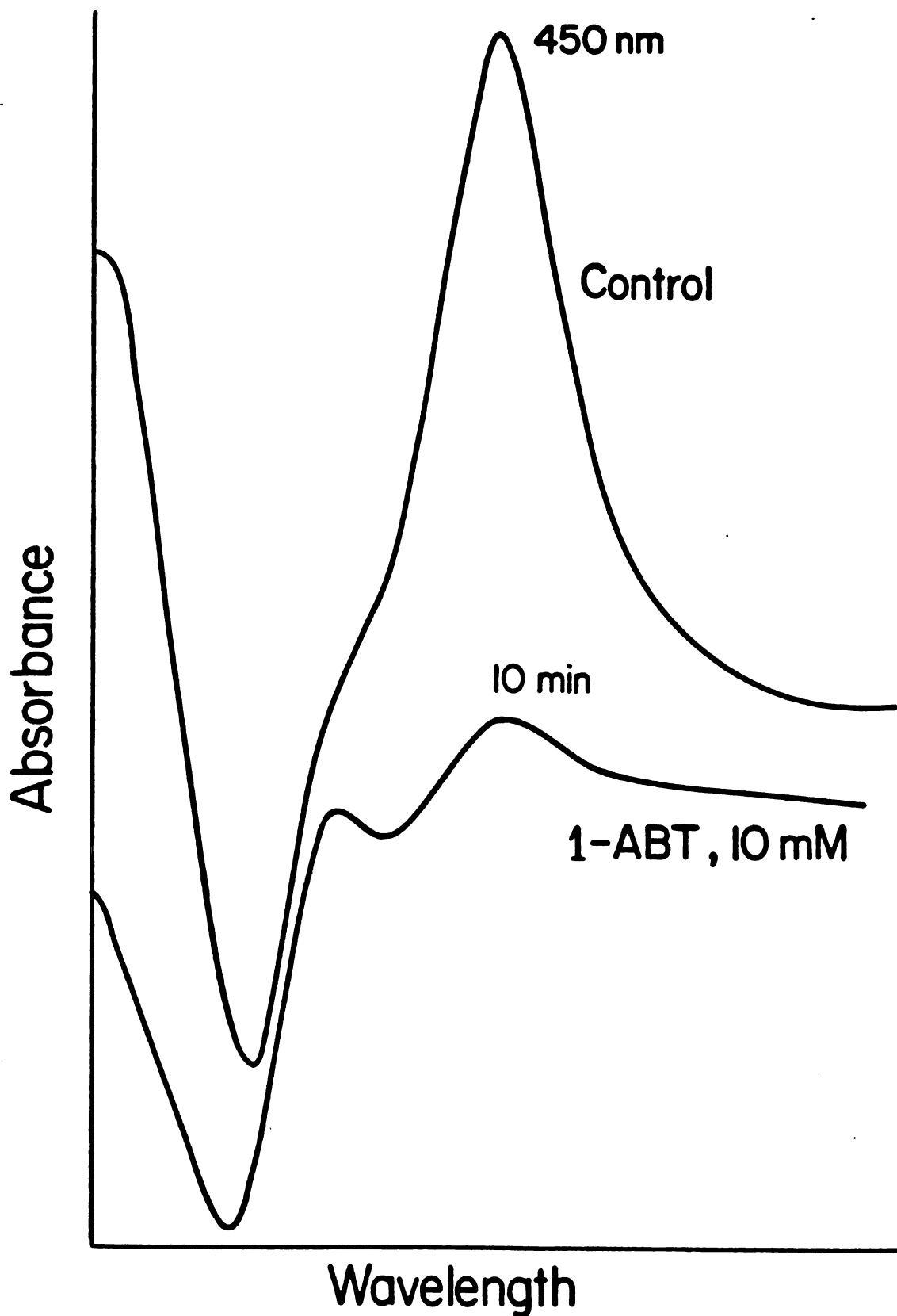
TABLE 3-1

IN VITRO DESTRUCTION OF CYTOCHROME P-450 BY 1-ABT

| <u>Conditions</u> | <u>Percent loss of cytochrome P-450</u> | | |
|---|---|----------------|----------------|
| | <u>PB-Induced</u> | | |
| | <u>10 min.</u> | <u>20 min.</u> | <u>30 min.</u> |
| complete (10 mM 1-ABT) | 81 ± 0.5 | 85 ± 0.6 | 86 ± 0.5 |
| -NADPH | ND | ND | ND |
| -ABT | ND | ND | ND |
| complete (1 mM 1-ABT) (experiment 1) | 70 ± 0.5 | 76 ± 0.5 | 77 ± 0.5 |
| complete (1 mM 1-ABT) (experiment 2) | | 65 ± 1.2 | |
| +carbon monoxide | | 49 ± 0.5 | |
| complete (0.1 mM 1-ABT) | 47 ± 0.5 | 54 ± 0.5 | 59 ± 0.5 |
| | <u>3-MC-Induced</u> | | |
| | <u>10 min.</u> | <u>20 min.</u> | |
| complete (10 mM 1-ABT) | 67 ± 3.5 | 78 ± 0.5 | |
| -NADPH | ND | ND | |

Values are averages of determinations from 3 separate incubations, except for the 3-MC experiments for which 2 determinations were made, and are reported ± the standard deviation or experimental uncertainty (0.5%), whichever is greater.

FIGURE 3-2
Spectral Determination of Cytochrome P-450
Destruction by 1-ABT



from the PB-pretreated animals. The extraordinary rate, extent, and specificity of destruction will be discussed in the following sections.

Rate of inactivation. Approximately 40% of the PB-induced enzyme was destroyed in the first minute of incubation. This rate of destruction far surpassed that observed with previously tested destructive agents. The rate of enzyme destruction by a suicidal agent is a function of the rate of substrate turnover and of the efficiency with which the reactive species that is produced inactivates the enzyme. The "partition ratio", the ratio between destructive and non-destructive outcomes of the catalytic processing of a substrate, is a measure of this efficiency. The extraordinary velocity of the destruction of cytochrome P-450 by 1-ABT could be due to a rapid rate of substrate turnover, a high efficiency of enzyme inactivation by the product, or a combination of the two factors. The partition ratio for suicidal destruction of cytochrome P-450 has been estimated in incubations with limiting amounts of substrate. This method has been used to determine the ratio between cytochrome P-450 destruction and substrate turnover in the metabolism of allylisopropylacetamide, AIA (Ortiz de Montelano and Mico, 1981). In that study 16% loss of enzyme was witnessed after the substrate, originally present at a concentration of 125 micromolar, was exhausted. This gives a calculated hit rate of approximately 230. Under similar experimental conditions 9.35 micromolar 1-ABT brought about

a 28% loss of enzyme, corresponding to a hit rate of 12.1 (Table 3-2). We cannot be certain that other microsomal enzymes, such as amine oxidase (Ziegler's enzyme), do not also consume 1-ABT. The calculated hit rate of 12.1 is, therefore, the maximum value for this process.

A rationale for the twenty-fold difference in partition ratio between AIA and 1-ABT is afforded through consideration of the reaction pathways in metabolism of the respective agents. Asymmetric addition of oxygen to the π -bond is postulated for the oxidation of olefins, yielding a reactive (cationic or radical) terminal carbon. Intramolecular capture of this reactive carbon with the newly added oxygen forms the stable epoxide and is the predominant outcome of the olefin oxidation. In approximately one out of every two hundred turnovers, however, the reactive carbon forms a covalent bond instead with the prosthetic heme group, destroying the enzyme in the process. No such intramolecular "quenching" reaction is possible for benzyne, a product offering not one but two highly reactive sites for covalent bond formation with the enzyme.

Extent of destruction. The final extent of destruction (86%) is likewise unparalleled. A maximum loss of 50% of the cytochrome P-450 in PB-induced microsomes has been observed with olefins and acetylenes, a result that suggests that not all of the spectrally detectable cytochrome P-450 is accessible to the cofactors and substrates necessary for autocatalytic inactivation of the enzyme. Indeed, asym-

TABLE 3-2

Partition Ratio for Destruction of Cytochrome P-450 by 1-ABT.

Enzyme Loss in Metabolism of Limiting Quantities of 1-ABT.

| Incubation time t (min.) | Percent Loss Cytochrome P-450 | |
|-----------------------------|-------------------------------|----------------|
| | <u>- 1-ABT</u> | <u>+ 1-ABT</u> |
| 0 | ND | ND |
| 30 | ND | 20 \pm 0.5 |
| 60 | ND | 28 \pm 1.2 |
| 90 | ND | 27 \pm 1.5 |
| 120 | ND | 28 \pm 1.0 |

Initial P-450: 55.0 nmoles

Loss P-450: 15.4 nmoles

Initial 1-ABT: 187 nmoles

Partition Ratio = $187/15.4 = 12.1$

Incubation volume 20 ml, protein concentration of 1.2 mg/ml. Fresh BHT and NADPH were added at 30 min intervals, as described in the Experimental section. Each value is the average of determinations from 3 separate incubations \pm standard deviation or experimental uncertainty, whichever is greater.

metric distribution of cytochrome P-450 in rat liver vesicles has been reported (Cooper et al, 1980). These workers concluded that the lumenally situated cytochrome P-450 was not reduced by NADPH alone, but required also superoxide anion for full reduction. Addition of superoxide dismutase prevented the complete reduction of cytochrome P-450 attained with a superoxide generating system. These data imply that the destruction of this population of cytochrome P-450 may be impaired. Addition of superoxide dismutase to microsomal incubations containing 1-ABT had little effect (Table 3-3) on the observed destruction. This result does not support the postulate that the destruction is limited in the case of olefins by differential distribution of mixed function oxidase components in vesicles. Previously, analysis of purified PB-induced cytochrome P-450 on SDS gels had revealed a single band representing the predominant enzyme form present. In a recent study by Waxman and Walsh (1982), however, this fraction has been found to consist of not one, but two enzymes. The 50% limit in the case of olefins may reflect vulnerability of only one of the isozymes present in the band previously assumed to be homogenous. As will be discussed in the next section, 1-ABT is effective against a broader spectrum of cytochrome P-450 isozymes than are the olefinic substrates. The presence of subpopulations of isozymes therefore does not manifest itself, in this case, in incomplete enzyme destruction.

TABLE 3-3

Effect of superoxide dismutase (SOD) and of glutathione on cytochrome P-450 destruction by l-ABT.

| <u>Conditions</u> | <u>Percent loss of cytochrome P-450</u> | | |
|----------------------|---|----------------|----------------|
| | <u>10 min.</u> | <u>20 min.</u> | <u>30 min.</u> |
| complete (1mM l-ABT) | 67±0.5 | 77±0.5 | 82±0.5 |
| +SOD | 67±0.5 | 75±0.5 | 79±0.5 |
| +10mM GSH | | 73±1.2 | |

Values are an average of 3 determinations and are reported ± the standard deviation or experimental uncertainty (0.5%), which ever is greater.

Specificity of isozyme destruction. While the effectiveness of 1-ABT against the PB-inducible forms of cytochrome P-450 was somewhat unexpected, perhaps more remarkable was the activity of this agent against the enzymes induced by 3-MC. The vulnerability of this form of the enzyme to inactivation by other suicide substrates tested in this and other laboratories is typically quite low. The enzyme, however, as with the PB-induced preparation, was rapidly destroyed by 1-ABT. Levels of the 3-MC induced enzymes were lowered to 22% of the initial value after 20 min of incubation with 1-ABT (Table 3-1). More definitive information about enzyme specificity was gained in experiments with purified enzymes in reconstituted mixed function oxidase systems. Here the susceptibilities of the major isozymes characteristic of PB and of 3-MC induction were ascertained. Both the PB and 3-MC forms of the enzyme were destroyed by 1-ABT in an NADPH-dependent process (Table 3-4). Deletion of NADPH-cytochrome P-450 reductase from the complete incubation mixture prevented enzyme loss, further evidence in support of the autocatalytic nature of this inhibitory process.

The active site heme group appears to be the focus of the inhibitory interaction of 1-ABT with cytochrome P-450. The ratio of cytochrome P-450 loss to heme loss in microsomal incubations was very near unity; thus destruction of the prosthetic group can account for all of the cytochrome P-450 that is lost (Table 3-5). General lipid peroxidative

TABLE 3-4

Destruction of purified hepatic cytochrome P-450 isozymes in
reconstituted systems by 1-ABT

| <u>Conditions</u> | <u>Percent loss cytochrome P-450, 20 min.</u> | |
|-------------------|---|--------------------|
| | <u>PB-Enzyme</u> | <u>3-MC Enzyme</u> |
| complete | 54±4 | 24±1 |
| -NADPH | ND | ND |
| -reductase | ND | 6 |

TABLE 3-5

Correlation of Microsomal Heme Loss to Cytochrome P-450 Loss
Caused by 1-ABT

| <u>Conditions</u> | <u>Cytochrome P-450</u> (nmole/mg protein) | <u>Heme</u> (nmole/mg protein) |
|---------------------|---|-----------------------------------|
| Experiment 1 | | |
| - 1-ABT | 2.38 ± 0.04 | 3.82 ± 0.05 |
| complete | <u>0.52 ± 0.05</u> | <u>1.70 ± 0.05</u> |
| Loss | 1.86 | 2.12 |
| | ratio 1 to 1.14 | |
| Experiment 2 | | |
| - NADPH | 2.22 ± 0.05 | 3.20 ± 0.05 |
| complete | <u>0.36 ± 0.05</u> | <u>1.40 ± 0.05</u> |
| Loss | 1.86 | 1.80 |
| | ratio 0.97 to 1 | |

damage of the enzyme, such as that caused by metabolism of carbon tetrachloride, was not responsible for the enzyme loss (Table 3-6). Cytochrome b_5 and NADPH-cytochrome P-450 reductase, other microsomal components, were unaffected during the course of the nearly complete obliteration of cytochrome P-450 (Table 3-7). Addition of high concentrations of the physiological nucleophile glutathione had little protective effect against enzyme loss (Table 3-3). These data suggest that catalytic turnover of 1-ABT produces a species so reactive that it favors, in comparison to AIA, reaction with the active site of the enzyme more than it does an innocuous result.

3.30 IN VIVO INTERACTION OF 1-ABT WITH HEPATIC CYTOCHROME P-450

The in vivo actions of 1-ABT closely paralleled those found in the in vitro studies. Intraperitoneal administration of 1-ABT to rats resulted in a precipitous decrease in hepatic cytochrome P-450 levels (Table 3-8). Three hours after this treatment the cytochrome P-450 content in PB and 3-MC-pretreated rats was decreased by 84% and 81%, respectively. Despite the powerful effect on cytochrome P-450, no overt physiological side effects on the animals were observed during the period of exposure.

Use of ethanol as a vehicle for administration of 1-ABT proved to be an inappropriate means of administering the compound but led to an interesting observation. Intraperi-

TABLE 3-6

Detection of malondialdehyde formation as a measure of lipid peroxidation in l-ABT incubation with rat liver microsomes.

| <u>Conditions</u> | <u>Malondialdehyde formation (micromolar)</u> |
|-------------------|---|
| -l-ABT | ND |
| complete | ND |
| CCl ₄ | 4.9±0.2 |

l-ABT and CCl₄ were run at 10 mM nominal concentration. Values are reported as the average of 3 determinations ± standard deviation.

Table 3-7

Effect of 1-ABT Incubation* on Microsomal Components

| <u>Component</u> | <u>initial value</u> | <u>after 30 min incubation</u> |
|--|----------------------|------------------------------------|
| Cytochrome P-450 (nmol/mg protein) | 2.22 ± 0.05 | 0.36 ± 0.05 |
| Cytochrome b ₅ (nmol/mg protein) | 1.13 ± 0.02 | 1.12 ± 0.01 |
| NADPH Cytochrome C Reductase Activity (nmol reduced/mg protein/min) | 358 ± 9 | 348 ± 10 |

* 1-ABT was present at 10 mM and NADPH at 1 mM. Values are averages of determinations from 3 separate incubations ± standard deviation or experimental error, whichever was greater.

TABLE 3-8

In vivo loss of hepatic cytochrome P-450 in 1-ABT-treated rats.

| | Cytochrome P-450 content (nmoles/mg protein) (% enzyme loss) | |
|--------------------------------------|---|-----------------|
| | PB-pretreated | 3-MC pretreated |
| control | 1.60 | 1.29 |
| 1-ABT (100 mg/kg in 1ml water) | 0.26 (84%) | 0.25 (81%) |

toneal injection of 100 mg/Kg l-ABT in 1 ml alcohol was lethal to four of six rats (PB-induced). This dose of ethanol was several times less than the reported LD₅₀ (Smyth et al, 1941). The relative importance of cytochrome P-450 in ethanol metabolism has been a subject of controversy. The observed interaction between l-ABT and ethanol suggests that cytochrome P-450 may play a larger role in ethanol metabolism than is currently assumed. Tephly and coworkers (Coffman and Tephly, 1982) have shown that ethanol inhibits the accumulation of N-alkylated porphyrins formed in the suicidal inactivation of hepatic cytochrome P-450 caused by metabolism of 3,5-diethoxycarbonyl-1,4-dihydrocollidine, DDC.

Isolation and characterization of alkylated porphyrin pigments. Abnormal pigments were isolated from the livers of rats four hours after administration of l-ABT. The details of this treatment, subsequent isolation procedure, and pigment characterization are available in the experimental section. In brief, to the extracts of the sulfuric acid/methanol-treated liver homogenates was added zinc acetate. The metal-free porphyrins tend to have a high affinity for metals; the addition of zinc prevents metalation of the porphyrins by metals other than zinc present on the preparative chromatography plates and thus insures that otherwise identical porphyrins will cochromatograph. After development of the plates, the pigments were identified as green to brown bands which fluoresce red upon irradiation

with ultraviolet light. The zinc was removed from the pigments by treatment with 5% sulfuric acid/methanol.

The major pigment isolated was of unusually high polarity relative to the great variety of other such pigments purified in this laboratory. The red fluorescing band had a very low R_f value on the preparative silica plates and was eluted from the adsorbent with great difficulty. The porphyrin-like visible spectrum of the pigment (Figure 3-3) exhibited a Soret absorbance at 414 nm followed by three peaks between 500 and 700 nm. This visible spectrum differs, however, from the nearly identical spectra of the other alkylated porphyrins of hepatic origin isolated in this and other laboratories. Treatment of this pigment with 5% sulfuric acid/methanol, or with zinc or palladium acetate, brought about no change in the visible spectrum. This property too is at variance with the behavior of the forementioned porphyrins, where conversion of metalloporphyrins to the metal-free pigment is accompanied by significant changes in the visible spectrum. The pigment, purified by high pressure liquid chromatography (HPLC), eluted from the column only after the gradient concentration of methanol in dichloromethane approached 50%. Mass spectrometric analysis of the purified pigment was performed by the field desorption technique. This technique works well for compounds of low volatility and reduces fragmentation of the parent ion. An excellent spectrum (Figure 3-4), free of impurities and fragmentation, was obtained which exhibited a molecular ion

FIGURE 3-3

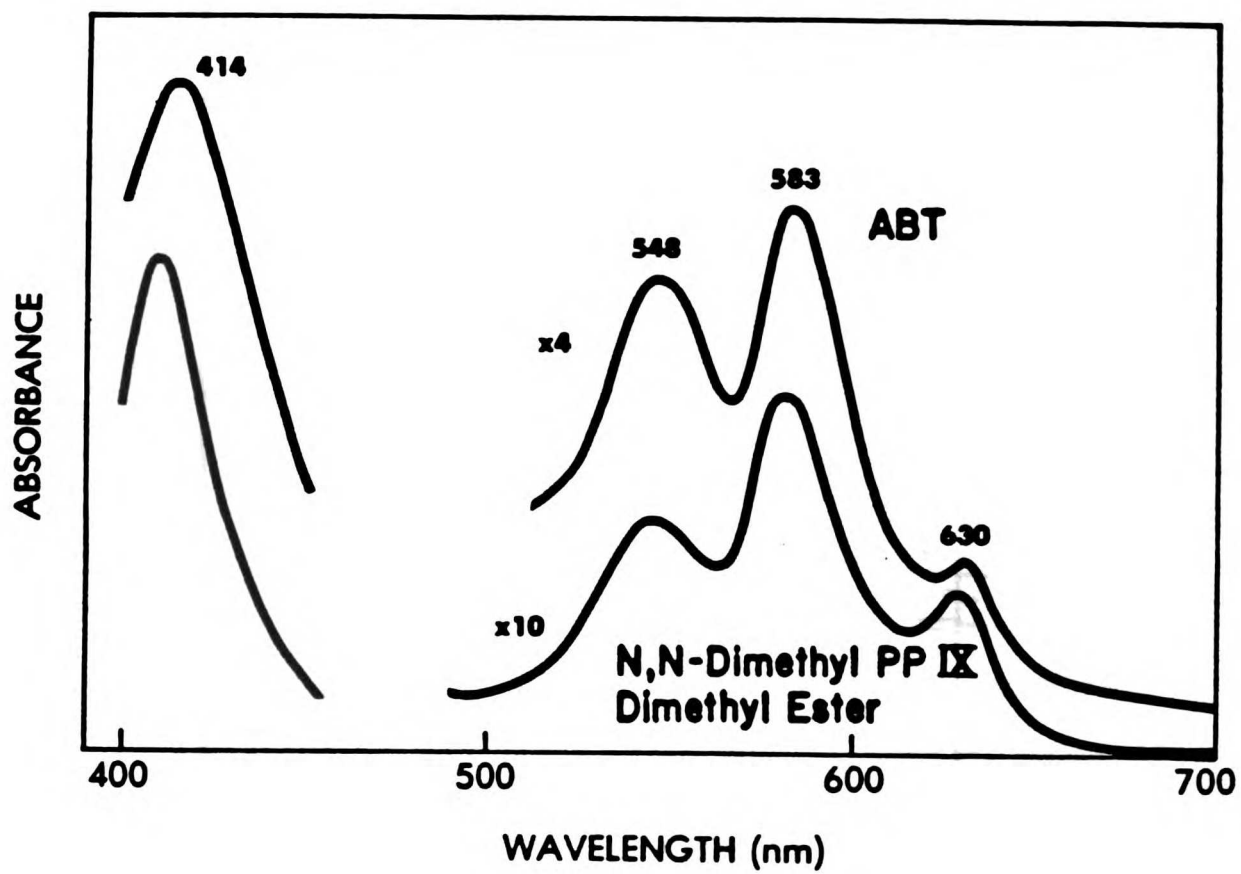
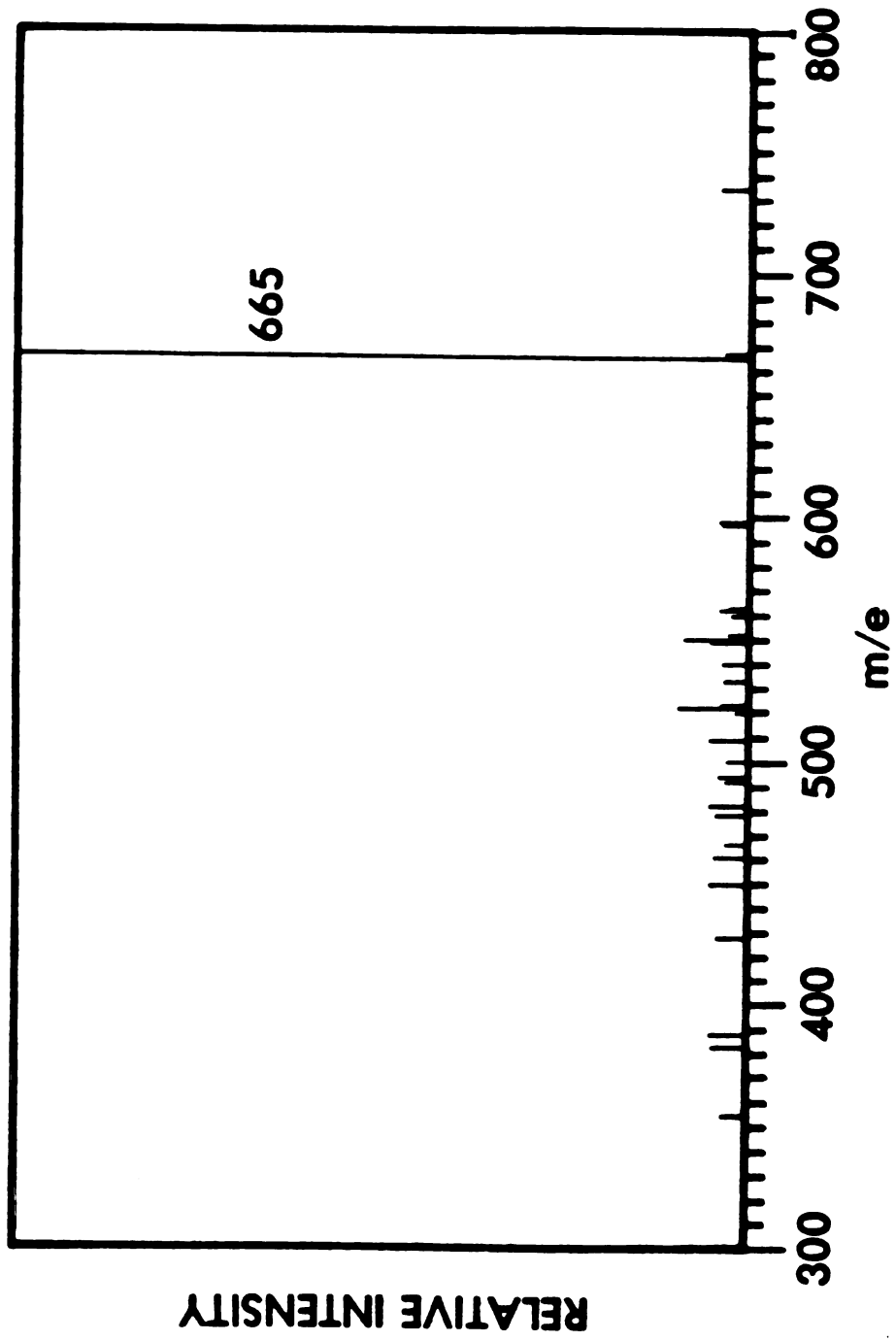
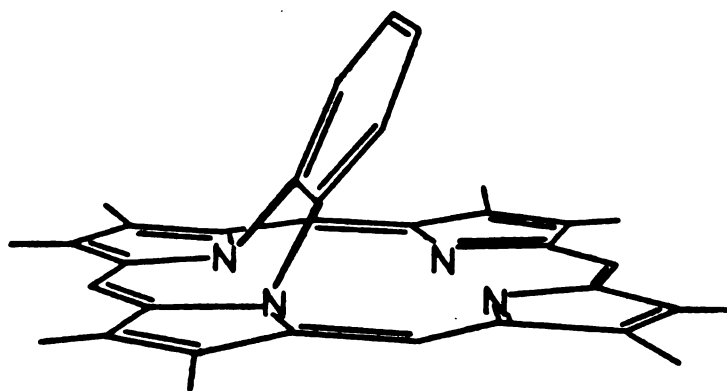
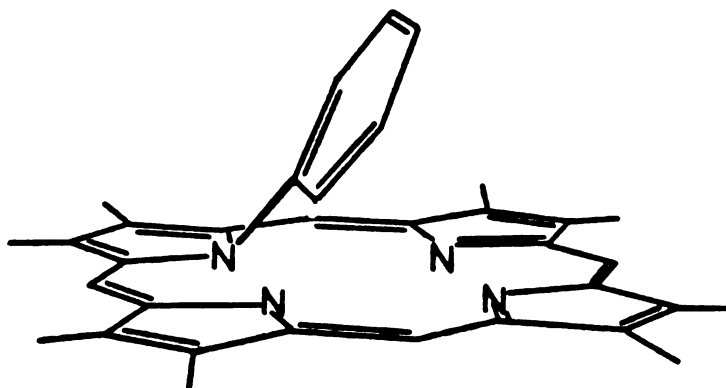


FIGURE 3-4



at 665 atomic mass units.

Comparison of the properties of the isolated pigment with those of alkylated porphyrins led to its characterization as an N,N'-dialkylated porphyrin. The visible spectrum of the pigment is identical to that of synthetic N,N'-dimethyl protoporphyrin IX dimethyl ester (supplied by Dr. Kent Kunze of this laboratory) and is characteristic of porphyrins alkylated on two vicinal (but not distal) nitrogen atoms. These dialkylated porphyrins, like that obtained with 1-ABT, are intransigent toward complexation with Zn^{+2} and Pd^{+2} and are strong bases, existing as salts in which the unalkylated nitrogens share a "bifurcated" proton. While the monoalkyl monoprotinated porphyrins are readily deprotonated with base (with an accompanying change in the visible spectrum), deprotonation of N,N'-dialkylated porphyrins requires the action of a strong base in anhydrous conditions and results in a large change in the visible spectrum. In accordance with this, addition of triethylamine to a solution of the 1-ABT pigment in chloroform, a treatment that readily converts monoalkylated porphyrins to their free bases, causes no change in the visible spectrum. The salts of the N,N'-dialkylated porphyrins are extremely polar, a fact which rationalizes the behaviour of the isolated pigment on silica plates and on HPLC columns. The structure shown in Figure 3-5 is thus proposed for the isolated pigment. The molecular weight of the protonated porphyrin would be 665, exactly the mass found for the molecular ion

FIGURE 3-5***N,N'-DIPHENYL****N-PHENYL**

* PORPHYRIN SUBSTITUENTS NOT SHOWN

in the mass spectrometric analysis. A mechanism will be discussed later for the formation of this and the following pigment.

A minor pigment is also isolated that, as suggested by comparison with an authentic sample of this compound, most probably is N-phenylprotoporphyrin IX dimethyl ester. The visible spectra of the two pigments as the free bases and as the zinc complexes are identical (Figure 3-6), but differ significantly from the spectra of N-alkyl (as opposed to N-aryl) porphyrins. The two pigments cochromatograph with a particularly high R_f value (0.9) on silica plates.

3.40 BIOLOGICAL ACTIVITY OF BENZOTRIAZOLE ANALOGS

The analogs listed in Table 3-9 were tested to determine the structural requirements for benzotriazoles to destroy cytochrome P-450. The parent heterocycle, benzotriazole (BT), caused no NADPH-dependent destruction. At 10mM, 9% loss of the enzyme occurred regardless of NADPH inclusion; at 50mM a large P-420 peak and a 55% loss of enzyme was observed in the absence of NADPH. This peak is diagnostic of denatured cytochrome P-450 and its formation here may be related to the detergent properties of purines. None of the other analogs tested were as potent as 1-ABT in mediating NADPH-dependent destruction. Surprisingly, 2-amino-benzotriazole (2-ABT) was active as well. In appreciation of the potency of 1-ABT (which also is formed in the preparation of 2-ABT), steps were taken to rigorously purify

FIGURE 3-6

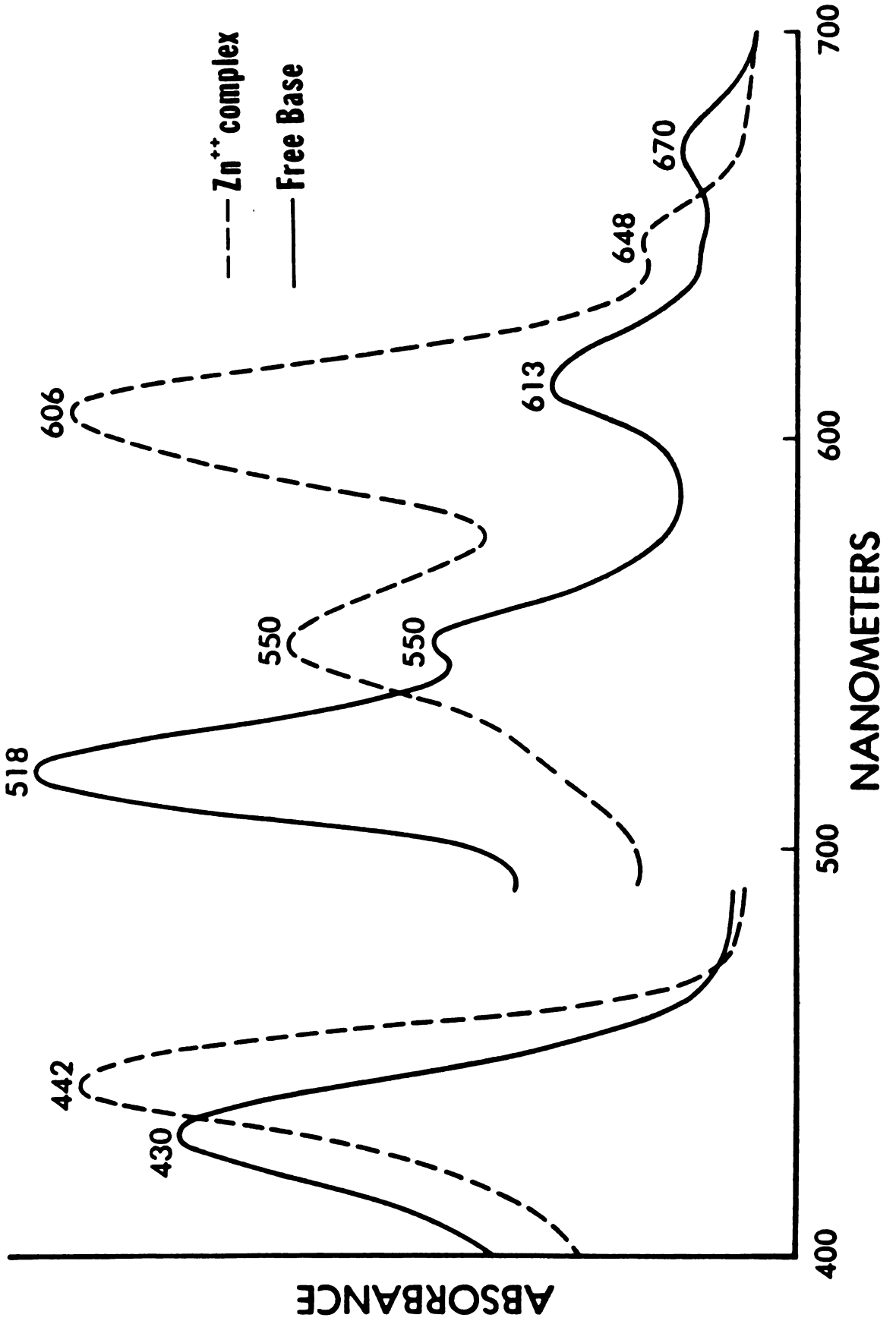


TABLE 3 - 9

IN VITRO DESTRUCTION OF HEPATIC CYTOCHROME P-450 BY
BENZOTRIAZOLE ANALOGS (*SYNTHESIZED)

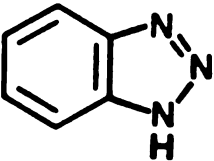
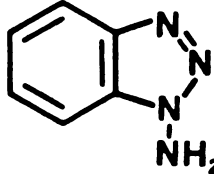
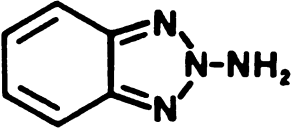
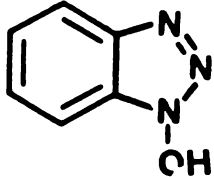
| <u>AGENT</u> | <u>PERCENT LOSS OF CYTOCHROME</u> | | <u>PIGMENT</u> | <u>FOOTNOTE</u> |
|---|-----------------------------------|----------------|----------------|-----------------|
| | <u>P-450, 30 MINUTES</u> | | | |
| | <u>1.0 mM</u> | <u>10.0 mM</u> | | |
|  | | | | |
| BT | 9 ± 1 | 9 ± 1 | - | A |
| *  | | | | |
| 1-ABT | 77.3 ± 0.5 | 86.4 ± 0.5 | Yes | |
| *  | | | | |
| 2-ABT | 34 ± 0.5 | - | No | A, B, C |
| *  | | | | |
| HBT | N. D. | - | - | A, B |

TABLE 3 - 9 (CONT'D)

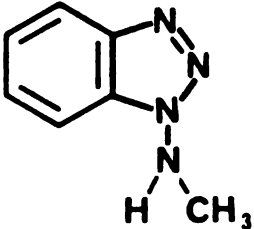
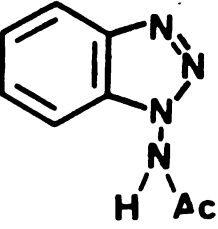
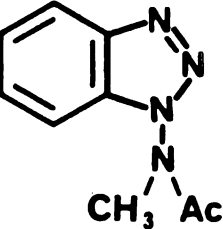
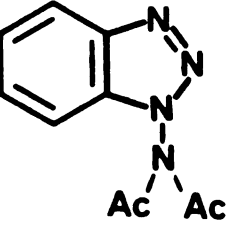
| AGENT | PERCENT LOSS OF CYTOCHROME P-450, 30 MINUTES | | PIGMENT | FOOTNOTE |
|---|---|---------------------|---------|----------|
| | 1.0 M _M | 10.0 M _M | | |
| |  | 43.6 ± 0.6 | | |
|  | 19.6 ± 1.1 | 29.7 ± 0.5 | YES | A, B, C |
|  | 10.1 ± 0.5 | - | YES | A, C |
|  | | 32.4 ± 2.1 | YES | A, C |

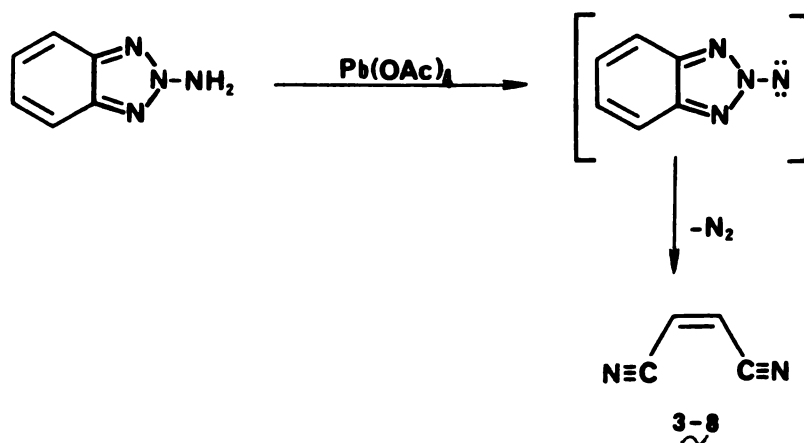
TABLE 3-9

FOOTNOTES

- A. THE AGENT WERE NOT SOLUBLE TO THE EXTENT INDICATED BY THE LISTED "CONCENTRATIONS" BUT WERE ADDED TO THE REACTION FLASKS AS DESCRIBED IN THE EXPERIMENTAL SECTION.
- B. PARTIAL SOLUBILITY OF THIS AGENT WAS NOTED. DESTRUCTION VALUES AT 30 MIN. WERE WITHIN 2% OF THE FINAL EXTENT OBSERVED.
- C. ATTEMPTED ISOLATION OF PIGMENTS FROM RATS AS DESCRIBED IN EXPERIMENTAL SECTION. ANIMALS WERE PRETREATED AS DESCRIBED FOR 1-ABT; AGENTS WERE ADMINISTERED AT A DOSE OF 1MM/KG IN 1 ML DMSO.

VALUES ARE AVERAGE OF THREE DETERMINATIONS, AND ARE REPORTED \pm THE STANDARD DEVIATION OR EXPERIMENTAL UNCERTAINTY, WHICHEVER WAS LARGER.

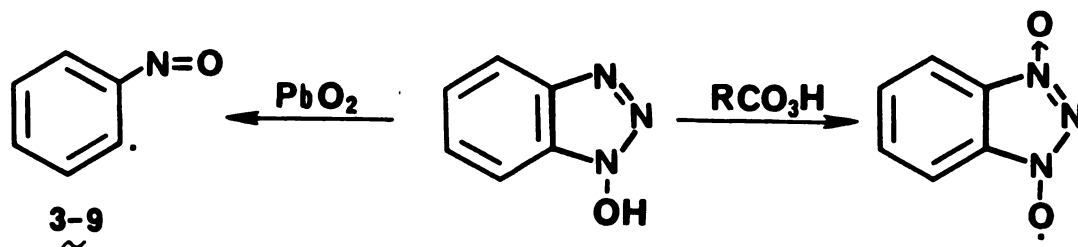
2-ABT. An impurity of only 1% 1-ABT in an incubation containing 1mM 2-ABT would of itself cause about 30% destruction. The purified 2-ABT used in the experiments reported here was analyzed for contamination and found to contain <0.1% 1-ABT, an amount capable of causing no more than a few percent destruction. While benzyne is formed by chemical oxidation of 1-ABT, oxidation of 2-ABT (Campbell and Rees, 1969) under the same conditions yields cis-muconitrile



(3-8). The mechanism by which 2-ABT inactivates the enzyme is unclear. Though cis-muconitrile is reactive by virtue of being an excellent Michael acceptor, no evidence exists that Michael acceptors can inactivate cytochrome P-450. No abnormal hepatic porphyrin pigments were detected after administration of 2-ABT to rats.

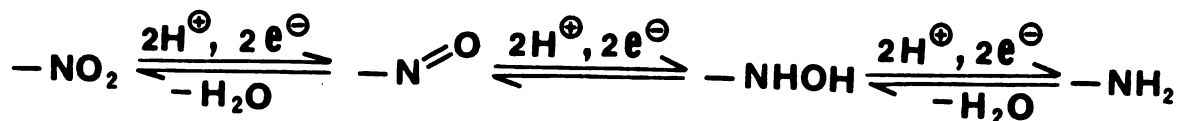
Encouraged by the success afforded by 1-ABT in developing mechanism-based inhibitors of cytochrome P-450, two other benzotriazoles were tested whose chemical reactions were within the scope of enzymatic transformations brought about by cytochrome P-450. The first of these was 1-hydroxybenzotriazole (1-HBT). Chemical oxidation of 1-HBT

by PbO_2 gives the reactive radical 3-9 (Aurich and Weiss, 1973), which can subsequently react with five and six member



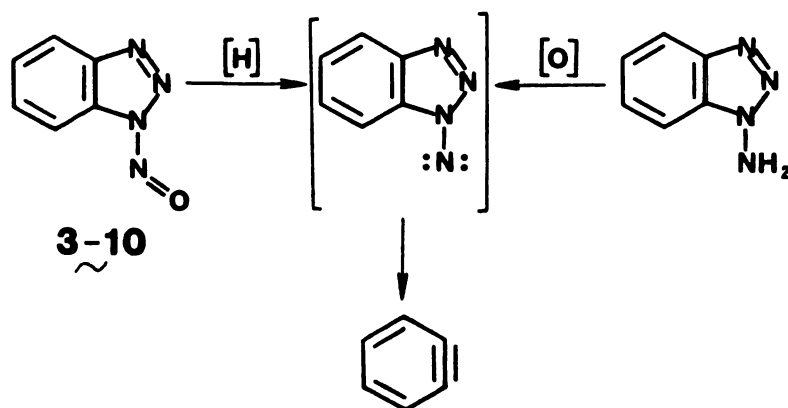
rings and undergo 1,2-additions to unsaturated bonds. The compound was completely inactive as a destructive agent, however. Oxidation of 1-HBT by peracids occurs at N-3 to yield a stable product (loc. cit.). 1-HBT possibly may not be metabolized or may be processed through the innocuous alternate oxidative pathway taken by peracids.

The strategy employed with 1-ABT and the other agents so far discussed as potential mechanism-based inhibitors of cytochrome P-450 sought to exploit the oxidative function of the enzyme to unveil a destructive species during catalytic turnover of the substrate. Cytochrome P-450 performs reductive reactions as well and can transform nitro compounds to amines along a pathway yielding nitroso and hydroxylamino compounds as sequential 2-electron reduction intermediates (Mason, 1979). Metabolic reductions are favored by hypoxic states (Sipes et al, 1980). A reductively activated suicidal agent could be of use as a biochemical tool for destruction of cytochrome P-450 in hypoxic tissues, such as those

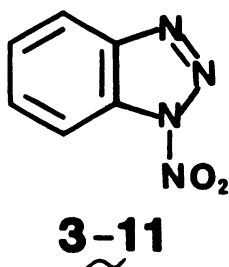


encountered in tumors. Though reductive metabolism of carbon tetrachloride and other halocarbons by cytochrome P-450 results in loss of the enzyme, the damage is not confined to the mixed function oxidase system. The biochemical disruption here is widespread, and these agents are too non-specific in their action to be of practical utility.

Benzynes are formed in the 2-electron reduction of 1-nitrosobenzotriazole (3-10) by diphenylphosphinite (Cadogan and Thompson, 1969), a reaction that proceeds through the same nitrene intermediate postulated in the oxidation of



1-ABT. Whereas 3-10 is too unstable to be of practical use, 1-nitrobenzotriazole (3-11) has been prepared as a stable compound (Cohen-Fernandes and Habraken, 1971). Four elec-



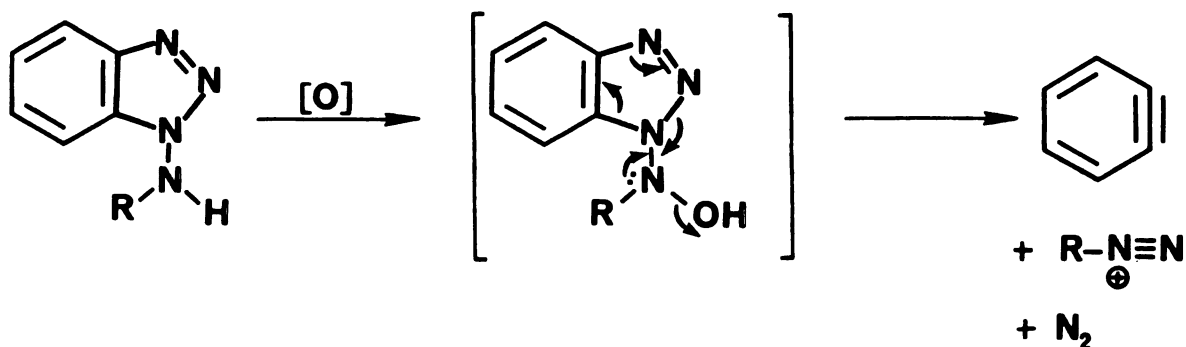
tron reduction of this nitro compound to the hydroxylamine, which is at the same oxidation state as nitrene, should yield benzyne. Unfortunately, however, rats treated with 1-nitrobenzotriazole (40 mg/Kg I.P. in 1 ml DMSO) immediately exhibited symptoms of asphyxia. Each became cyanotic, struggled to breathe, and died in a few minutes. Experiments with whole blood revealed that 3-11 causes methemoglobinemia, a property shared by other aromatic nitro compounds. The propensity of 3-11 to oxidize heme iron from Fe^{+2} to Fe^{+3} prevented spectral determination of the destructive activity of this agent by the usual in vitro assay procedure.

Derivatization of the amino group of 1-ABT yielded products which, though lower in activity, were still active. These agents could conceivably be converted to 1-ABT by dealkylation or by deacetylation by microsomal enzymes. No time lag, however, was observed for the cytochrome P-450 loss and the final extent of destruction was far less than that produced by 1-ABT. Of these 1-ABT derivatives only 1-methylaminobenzotriazole (MAB) was freely soluble in buffer.

This analog destroyed 42% of the enzyme in 20 minutes. Little additional loss of enzyme occurred thereafter, suggesting that methylation of the amino group afforded some specificity for destruction of cytochrome P-450 isozymes. 1-Acetamidobenzotriazole (AMT) was appreciably water soluble, though not to a concentration of 1mM. Here also the inactivation was considerable (29%), although only a fraction of that caused by 1-ABT. 1-Diacetamidobenzotriazole (DBT) and 1-methylacetamidobenzotriazole (MBT) were active despite their poor solubility. The activity of these disubstituted derivatives also suggests that enzymatic preprocessing to 1-ABT is not a prerequisite for cytochrome P-450 destruction.

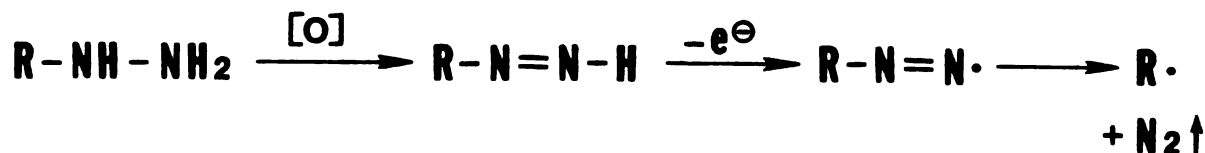
In conclusion, destruction of cytochrome P-450 is not an activity of the benzotriazole nucleus itself. It is instead a property of benzotriazole analogs with a contiguous array of four nitrogen atoms, an ensemble possessing a latent reactivity associated with the aptitude of nitrogen gas as a leaving group. Substituents on the exocyclic nitrogen atom did not prevent the enzymatic fragmentation of the triazole moiety, but did result in a decreased effectiveness against cytochrome P-450 enzymes in PB-induced microsomes. Each of the 1-ABT derivatives that caused cytochrome P-450 loss caused the hepatic accumulation of the same pigments isolated after in vivo treatment of rats with 1-ABT.

Toxicity of 1-ABT and its derivatives. The in vitro inhibition data collected for the methyl and acetyl derivatives indicates that these agents were effective inhibitors "as is". The nature of the porphyrin pigments isolated from in vivo destruction of cytochrome P-450 demonstrates that decomposition of the triazole moiety precedes the inhibitory action of these agents. In the case of 1-ABT this fragmentation gives rise to two molecules of nitrogen, but it is unclear what fragments might arise from oxidation of the substituted analogs. A possible product of the monosubstituted analogs could be an alkyl or acyl diazonium species



Scheme 2

(Scheme 2). As neither the preparation (with few exceptions) nor oxidation of nitrogen-substituted derivatives of 1-ABT has been reported, the chemistry of monosubstituted hydrazines might serve as a guide to the most probable decomposition pathways. The hydrazines are oxidized to unstable diazenes (Scheme 3); further oxidation by oxygen or by single electron acceptors proceeds, with loss of nitrogen, to yield radical products. The hepatotoxicity of acetyl hydrazine is believed to be caused by reaction of tissue



Scheme 3

nucleophiles with radicals formed from acetyl diazene, the product postulated to be formed by cytochrome P-450 catalyzed oxidation of the hydrazine. A diazo radical, such as that generated in Scheme 3, may result from the decomposition of a monosubstituted 1-ABT derivative after a one-electron oxidation. The observation that cytochrome P-450 oxidation of these analogs forms at least one (benzyne), and potentially two reactive molecules led us to measure and compare the toxicities of 1-ABT and its analogs. Serum transaminase levels were determined as a measure of the toxicity of these compounds. Elevated levels of these enzymes (accompanied by hepatic injury) have been observed in patients receiving isoniazid, a hydrazine compound. Acetylhydrazine has been implicated in this toxic response (Mitchell et al, 1975). With the exception of DABT no elevation of serum transaminase levels was detected 24 hours after administration of the triazole analogs to PB-pretreated rats (Table 3-10). This finding does not exclude the possibility that reactive fragments arise from the metabolism of these agents. Enzyme inactivation (very efficient

TABLE 3 - 10

TOXICITY OF 1-AMINOBENZOTRIAZOLE ANALOGS:
EFFECT OF IN VIVO ADMINISTRATION ON SERUM TRANSAMINASE LEVELS.

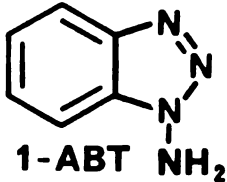
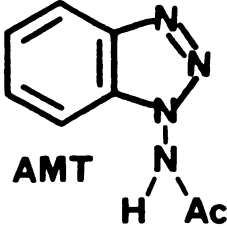
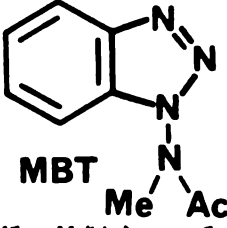
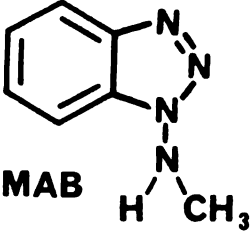
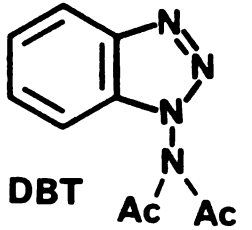
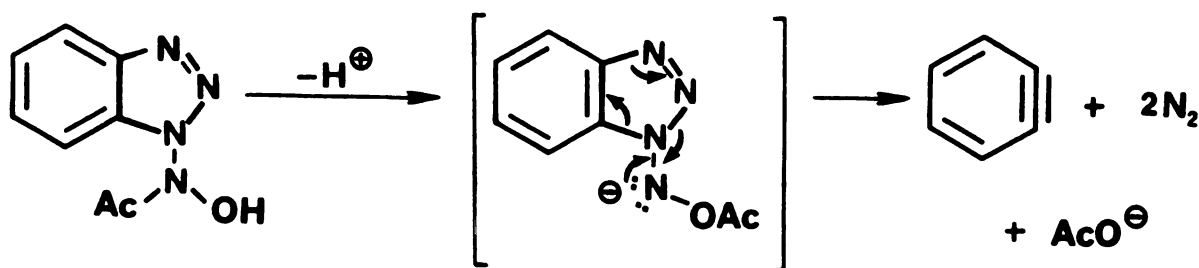
| <u>ANALOG</u> | <u># OF RATS</u> | <u>SGPT</u> | <u>SGOT</u> |
|---|------------------|-------------|-------------|
| VEHICLE CONTROL (1 ML TRICAPRYLIN) | 1 | 22 | 88 |
| SALINE | 5 | 19 ± 1 | 60 ± 8 |
| D ₂ O (1 ML) | 4 | 21 ± 2 | 64 ± 8 |
|  1-ABT NH₂ | | | |
| (1 M ¹ /KG) IN SALINE | 5 | 21 ± 4 | 53 ± 8 |
|  AMT H Ac | | | |
| (1 M ¹ /KG) IN 1 ML D ₂ O | 4 | 22 ± 3 | 57 ± 6 |
|  MBT Me Ac | | | |
| (1 M ¹ /KG) IN 1 ML D ₂ O | 4 | 20 ± 3 | 62 ± 4 |

TABLE 3 - 10 (CONT'D)

| <u>ANALOG</u> | <u># OF RATS</u> | <u>SGPT</u> | <u>SGOT</u> |
|--|------------------|-------------|-------------|
|  <p>MAB <chem>CN1N=CN=C1c2ccccc2</chem> (1 M/V/KG)</p> | 4 | 27 ± 2 | 58 ± 3 |
| <p>*</p>  <p>DBT <chem>CC(=O)N1N=CN=C1c2ccccc2C(=O)C</chem> (0.46 M/V/KG) IN TRICAPRYLIN</p> | 4 | 41 ± 5 | 164 ± 12 |
| CCl ₄ (1 ML/KG) | 2 | 7700 | 7800 |

*DOSE OF 1.38 M/V/KG LETHAL TO 4 OF 6 RATS.

with 1-ABT) accompanies metabolism and limits the amounts of such metabolites that can be formed. The species must also be of sufficient half-life to diffuse from the active site of cytochrome P-450 and cause cellular disruption. An N to O migration of the acetyl group of a hydroxylated AMT (Scheme 4) could furthermore defuse the potential cellular toxicity of this analog, permitting loss of the acetyl func-



Scheme 4

tion as the harmless acetate anion. Such a scheme affords an explanation for the singular toxicity of DBT (which would still have one remaining acetyl group) and suggests an energetically plausible mechanism by which the disubstituted heterocycles may decompose.

A 1.38 mmol/Kg dose of DBT was lethal to four of six rats so treated, and serum transaminase levels were approximately doubled 24 hours after a 0.46 mmol/Kg dose. While the toxic response may be cytochrome P-450 mediated, DBT itself may be acting as an acetylating agent causing cellular necrosis. In chemical manipulations DBT proved to be unstable to nucleophiles. It was easily hydrolyzed to the monoacetyl compound in aqueous potassium hydroxide at room

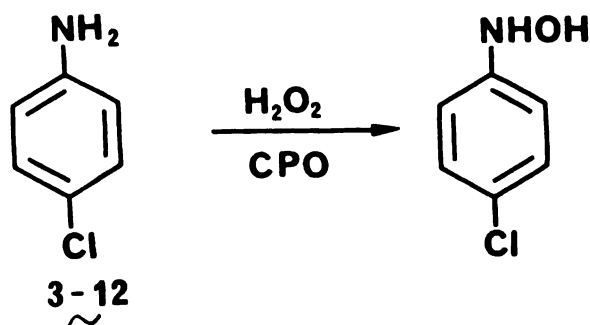
temperature and quickly reacted with refluxing ethanol to transfer an acetyl group to the alcohol.

The absence of toxic side effects, on the other hand, demonstrates the excellent target specificity of 1-ABT as an inhibitor of cytochrome P-450.

3.50 INHIBITION OF OTHER OXIDASES BY 1-ABT

The demonstrated effectiveness of the triazole against most of the isozymes of cytochrome P-450 prompted an investigation into the use of 1-ABT as an inhibitor of other oxidases capable of catalyzing the N-oxidation of amines.

3.51 Chloroperoxidase. Chloroperoxidase (CPO), a hemoprotein isolated from the mold Caldariomyces Fumago has in common with cytochrome P-450 numerous physicochemical properties (Rembra et al, 1979) that set it apart from other oxidative hemoproteins and make it an appropriate model for the study of the mechanism of cytochrome P-450 oxidations. Like cytochrome P-450, CPO readily catalyzes the N-oxidation (Corbett et al, 1980) and N-dealkylation (Kedderis et al, 1980) of arylamines. In the presence of hydrogen peroxide (the natural cofactor for the enzyme) CPO catalyzes the N-hydroxylation of arylamines, such as 4-chloroaniline (3-12), with an efficiency even greater than that of cytochrome P-450 (Corbett al, 1980). In light of this activity, the inhibitory effect of 1-ABT against CPO was investigated. The details of the experimental procedures used in the CPO studies are available in section 4.20. The incubations were



carried out in the presence of the assay substrate monochlorodimedon (MCD, 1,1-dimethyl-4-chloro-3,5-cyclohexanedione), which prevents inactivation of the enzyme by hydrogen peroxide. The catalytic activity of CPO was almost completely lost after five minutes of incubation with 1mM 1-ABT (Figure 3-7). The inactivation was time-dependent, required the presence of hydrogen peroxide, and was attenuated in rate by a ten-fold increase in the MCD concentration. In the absence of 1-ABT, or after replacement of 1-ABT with another primary amine (ethylamine), no loss of enzyme was observed. Preliminary data suggest a hit rate (using limiting amounts of hydrogen peroxide) of roughly 600. In this study the metabolic fate of the heterocycle was investigated. Catalytic turnover of 1-ABT by CPO yields one major and at least two minor metabolites as detected by gas chromatographic analysis (Figure 3-8, A). The metabolites are not formed when heat/freeze-inactivated CPO was used or when hydrogen peroxide or 1-ABT were excluded from the incubation (B). The major metabolite was identified as phenol by comparison of its retention time (C)

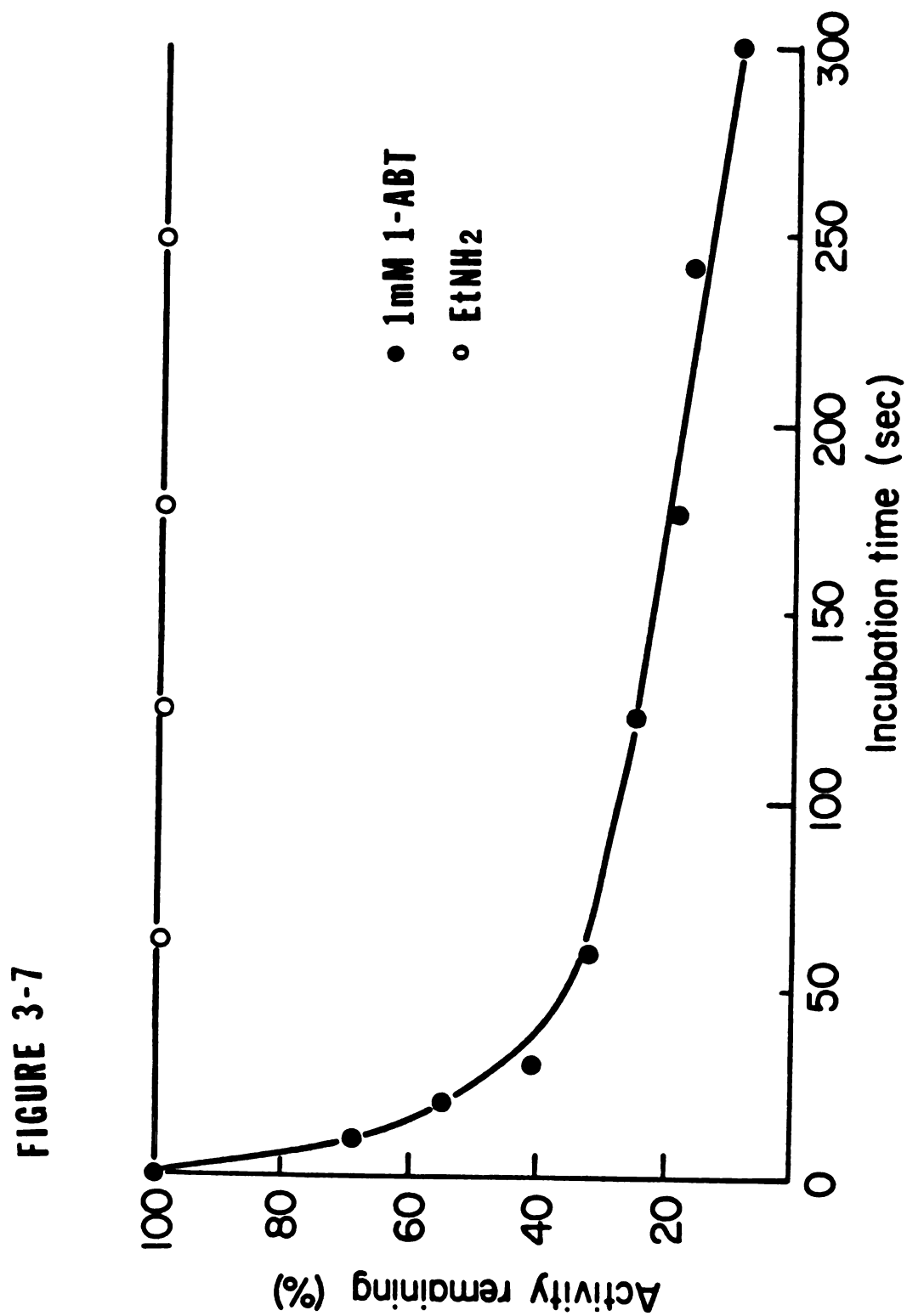
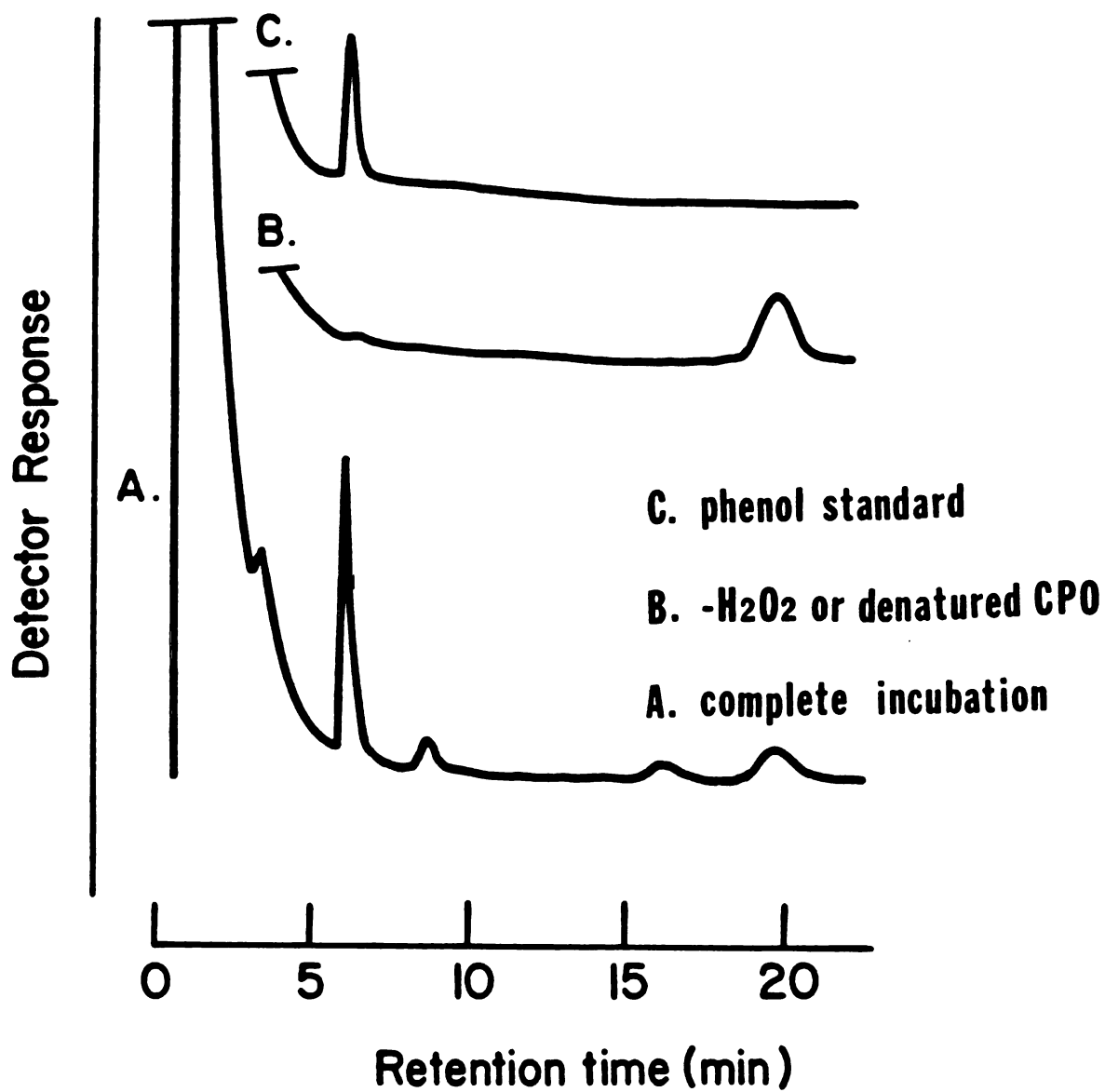
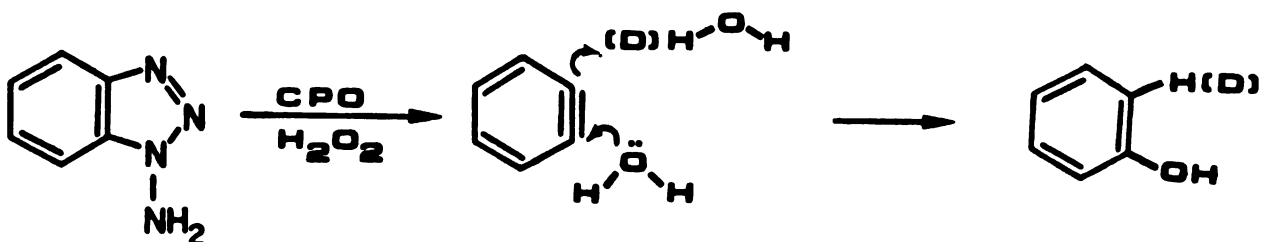


FIGURE 3-8



and mass spectrum (Figure 3-9) with that of an authentic sample of phenol. The formation of this product is consistent with the intermediacy of benzyne. Hydration of benzyne occurs readily at -20°C to yield phenol (Wittig and Hoffmann, 1962). Incubations in deuterated aqueous buffer

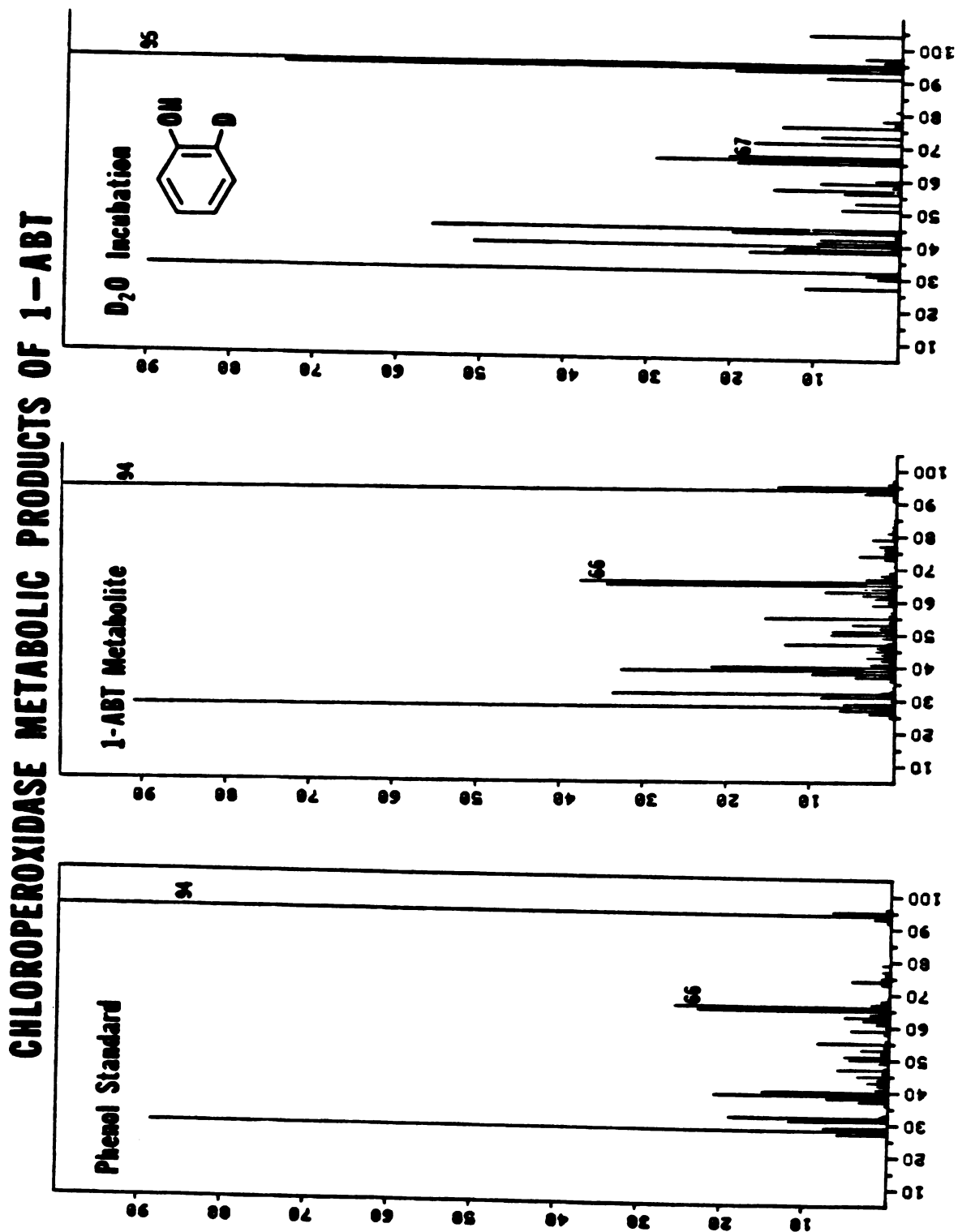


SCHEME 5

gave ortho-deuterophenol (Scheme 5; Figure 3-9). Thus, phenol was the product of the enzymatic decomposition of the triazole to yield an intermediate species bearing reactive sites for two new phenyl substituents, an organic reaction that is peculiar to benzyne.

In contrast to CPO, no phenol was isolated from incubations of 1-ABT with rat liver microsomes. Though benzyne should be capable of reacting with nucleophiles in the active site of cytochrome P-450, the hydrophobicity of this site may preclude the presence of much water. A polar binding site, on the other hand, is proposed for the cofactors in the active site of chloroperoxidase, which is 25-30% carbohydrate by weight, and includes much water of hydration. Also, the approximately fifty-fold difference in partition rate of destruction of the two enzymes by 1-ABT would allow

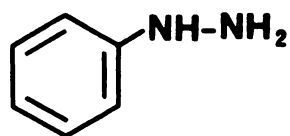
FIGURE 3-9



for the liberation of much less benzyne from the active site of cytochrome P-450, relative to that of CPO, to the aqueous medium before inactivation takes place.

3.52 Cinnamic acid 4-hydroxylase. Cytochrome P-450 enzymes have been detected in a number of plants, and are involved in the biosynthesis of components necessary for plant growth and viability. Cinnamic acid 4-hydroxylase (CA4H) and lauric acid hydroxylase are two such enzymes isolated from Helianthus Tuberosus (Jerusalem artichoke). Allylisopropylacetamide, an inhibitor of hepatic cytochrome P-450, is ineffective as an inhibitor of these plant enzymes (D. Reichhart, unpublished), a finding in accordance with the greater substrate specificity exhibited by the plant biosynthetic enzymes. In investigations performed in collaboration with Dr. Francis Durst and coworkers (Reichhart et al, 1982), 1-ABT was found to be a potent inhibitor of CA4H. NADPH was required for enzyme destruction. The time-dependent inactivation process displayed pseudo first-order kinetics and was irreversible over the experimental time period. These data are indicative of a suicidal inactivation process. Lauric acid hydroxylase activity was only slightly affected by 1-ABT demonstrating that some specificity of destruction is in fact possible with this agent. The specific inactivation of plant cytochrome P-450 achieved by 1-ABT opens the door for the use of this agent as a tool in studying the physiological role of these enzymes.

3.53 Monoamine oxidase. Monoamine oxidase (MAO) is a dioxygenase distributed widely through animal tissues, and is responsible for the oxidative deactivation of endogenous amino neurotransmitters. Many irreversible inhibitors of MAO are known (Knoll, 1976). One such inhibitor, phenylhydrazine (3-13) is chemically similar to l-ABT. Oxidative processing of phenylhydrazine by MAO results in loss of the diazo functionality of this molecule as nitrogen gas; a reactive phenyl intermediate is produced which destructively



3-13

alkylates the flavin cofactor (Kenney et al, 1980). Despite the striking chemical and biochemical parallels with phenylhydrazine, l-ABT proved to be an ineffective inhibitor of bovine MAO. Monoamine oxidase, B-type, was preincubated with 10 mM l-ABT for three minutes. MAO activity was assayed by a modification (Salach, 1979) of the method of Tabor et al (1960). Benzylamine (3.3 mM final concentration) was added to the incubations and its rate of enzymatic oxidation was measured spectrophotometrically by monitoring the increase in the UV-absorbance at 250 nm of the benzaldehyde reaction product. Even at 10 mM, a concentration

three times that of the benzylamine, the triazole could produce only 10% inhibition. Under the same conditions 0.1 mM phenylhydrazine produced about 60% inhibition. The absence of a strong effect on this crucial yet potentially vulnerable enzyme by 1-ABT was a welcome outcome in light of our goal of finding an inhibitor possessing specificity of action against oxidative enzymes.

3.60 MECHANISM OF ACTION OF 1-ABT

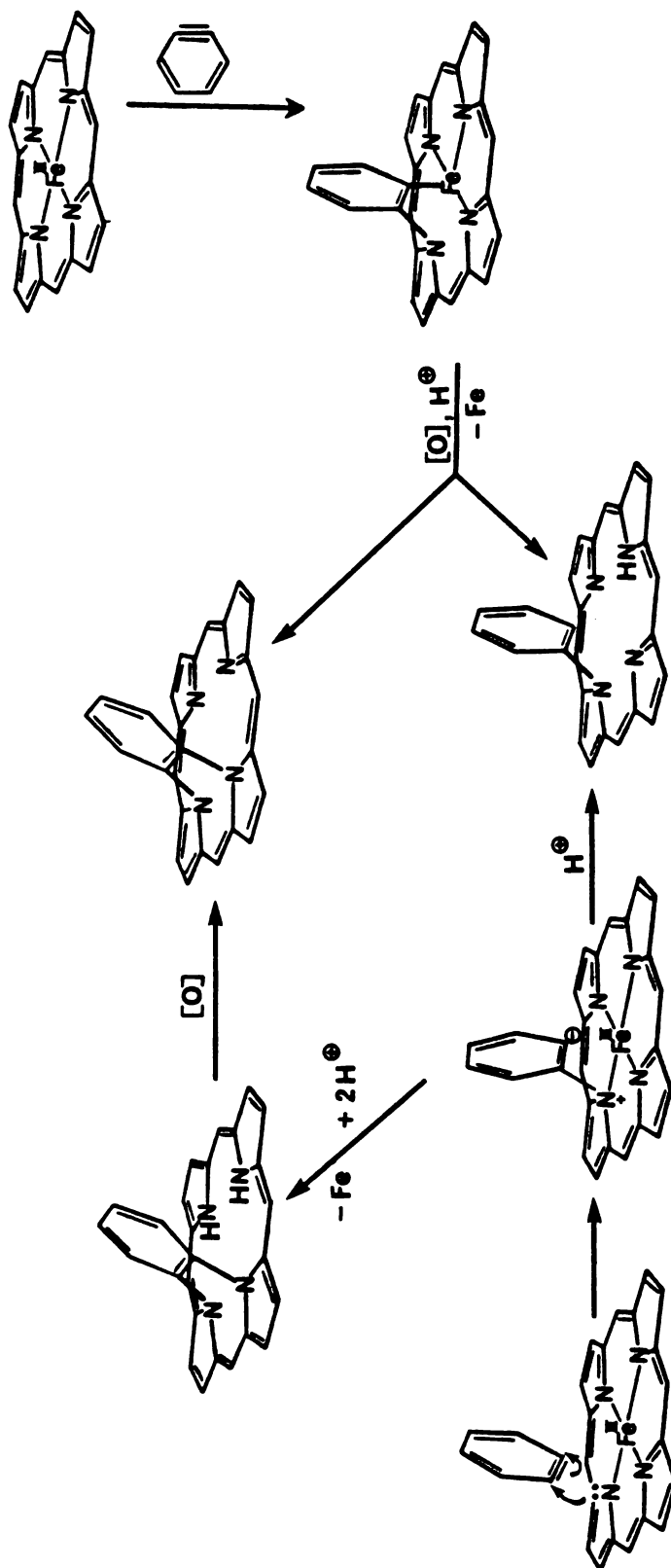
The foregoing evidence demonstrates that 1-ABT causes the mechanism-based inactivation of cytochrome P-450 process in that it:

- 1) Is a substrate of the enzyme and gives a binding spectrum.
- 2) Is inhibited by an agent (carbon monoxide) which competes for occupancy of the active site of the enzyme.
- 3) Requires catalytic turnover; NADPH and cytochrome P-450 reductase are required for enzyme loss.
- 4) Is time-dependent.
- 5) Is characterized by the irreversible and stoichiometric loss of enzyme through covalent bond formation with the catalytic center of the enzyme. There was a 1:1 ratio of enzyme loss to prosthetic heme loss, and the covalently modified prosthetic group was isolated.
- 6) The agent is intrinsically unreactive to tissue nucleophiles and is only converted to a reactive species in the active site where it reacts to inactivate only that

enzyme. Addition of trapping agents, such as thiols, has no effect on the destruction. The lack of a measurable toxic action in vivo, or effect on other microsomal components in vitro, accompanying the extensive damage to cytochrome P-450 demonstrates the active site-directed nature of the reactive species unveiled by the enzyme. Although thiols are by far the most effective nucleophiles in trapping benzyne (Hoffmann, 1967), addition of high concentrations of glutathione had little effect on enzyme destruction.

7) Displays pseudo first-order kinetics in destruction of the plant cytochrome P-450 enzyme cinnamic acid 4-hydroxylase. The heterogeneity and rapid destruction of the hepatic enzymes precluded meaningful kinetic analysis of the inactivation in the mammalian system, however.

The following scheme is proposed for the inactivation of cytochrome P-450. First, a two-electron oxidation of 1-ABT forms benzyne. Nucleophilic attack by the heme nitrogen lone pair electrons (analogous to the reaction of benzyne with metallopyrroles, section 3.12) forms the first covalent bond and a phenyl anion at the ortho position (Scheme 6). Protonation of the phenyl anion would give the isolated N-phenyl pigment. Attack of the anion at the nitrogen of the vicinal imino group could form the second covalent bond, reducing the porphyrin in the process. Analogous polar cycloaddition reactions of benzyne form benzotriazoles by bridging the two terminal nitrogen atoms of a number of organic and inorganic azides (Hoffmann, 1967).



Scheme 6 *

*Porphyrin substituents not shown

Reduced porphyrins can be air oxidized to porphyrins, a process yielding in the present case our isolated bis-alkylated porphyrin. Alternatively, iron may be involved in the shuttling of electrons and the formation of the second covalent bond. The presence of iron-carbon sigma bonds has been postulated in the inhibitory complexes formed in the metabolism of certain substrates by cytochrome P-450. The intermediacy of an N-phenyl iron-phenyl porphyrin could explain the formation of both isolated pigments. Under acidic aerobic conditions the aryl iron bond has been shown (Augusto et al, 1982; Ortiz de Montellano et al, 1982) to either break, releasing benzene, or to shift from iron to nitrogen. The equivalent migration in this system would yield the two isolated pigments.

3.70 CONCLUSION

The feasibility and utility of designing mechanism-based inhibitors of oxidative enzymes through consideration of chemical first principles was demonstrated by the successful development of 1-ABT for use as a "suicide substrate" for these enzymes. Although the chemistry summarized in Scheme 1 suggested the potential action of this agent, it is remarkable how closely the biochemistry mirrors the chemistry of the reactant and its product in this reaction scheme. The involvement of benzyne in the autocatalytic inactivation of cytochrome P-450 was evidenced by the isolation of two distinct phenylated porphyrins. The isola-

tion of phenol from incubations of 1-ABT with CPO implicated benzyne in the inhibition of that enzyme also. Thus water and porphyrin served as trapping agents in this first reported detection of benzyne in a biological process.

1-ABT possesses chemical properties well suited to its role as an efficient "suicide substrate" of cytochrome P-450 enzymes in vitro as well as in vivo. The intrinsic inertness of the water soluble triazole to the functional groups and conditions confronted in a physiological milieu teamed up with the extreme reactivity of the benzyne metabolite produce a potent site-specific inhibitor of low toxicity. As an inhibitor of general applicability, 1-ABT is superior to the olefins and acetylenes in many important respects. While strong sedative-hypnotic effects are clearly in evidence after administration to rats of most of the unsaturated alkyl inhibitors, no change in behavior was observed with 1-ABT. The epoxide metabolites of the olefins are of sufficient stability to diffuse from the active site of cytochrome P-450 and elicit the mutagenic and necrotic responses associated with alkylation of distant cellular macromolecules. The low partition ratio of destruction measured for 1-ABT was in keeping with the observed absence of toxic side effects in vivo and of disruptive effects on other microsomal components in vitro. In contrast to the majority of other inhibitors tested, the triazole was accepted as a substrate by, and caused the destruction of, most of the hepatic cytochrome P-450 enzymes induced by

3-methylcholanthrene or phenobarbital. The differing susceptibilities of the plant cytochrome P-450 enzymes isolated from Helianthus Tuberosus nevertheless demonstrate a selectivity of action. While selective destruction of the 3-MC inducible form was not attained, the aryl-fused triazole is a promising moiety for incorporation into a more discriminating structure. Mammalian monoamine oxidase was not appreciably affected by 1-ABT. Thus, 1-ABT is offered as an efficient, specific, cytochrome P-450 inhibitor of great potential use in vivo and in vitro for studying the metabolic role of these enzymes in physiological processes.

CHAPTER FOUR

EXPERIMENTAL PROCEDURES

4.1 GENERAL EXPERIMENTAL

Instrumental. Nuclear magnetic resonance (NMR) spectra were obtained on a Varian FT-80 spectrometer. Chemical ionization mass spectra (CIMS) were performed on an AEI-MS-902 spectrometer adapted to a chemical ionization mode (isobutane gas). Electron impact mass spectra (EIMS) were performed on a Kratos MS-A25S spectrometer operated in the electron impact mode. Field desorption mass spectra (FDMS) were obtained by use of a Kratos AEI-MS-902 spectrometer, as described (Ortiz de Montellano et al, 1980). Melting points were determined on a Hoover melting point apparatus and were corrected against standards of known melting point. The optical densities of microsomal samples were determined with an Aminco DW-2 double beam spectrophotometer. A Beckman Model B spectrophotometer was used in protein determinations. Ultraviolet and visible spectra were obtained on a Varian Cary 118 or on a Hewlett-Packard 8450A UV/VIS spectrophotometer. Elemental analyses were performed by the University of California Microanalytical Laboratory, Berkeley, California. A Sorvall Superspeed RC2-B centrifuge with an SC 34 rotor was used for 10,000xG centrifugations. Centrifugations at 100,000xG were performed at 4°C in a Beckman Model L-2 ultracentrifuge equipped with a type 40 rotor. High pressure liquid chromatography (HPLC) was performed

with dual Altex Model 110A pumps, a Laboratory Data Control Model 400 Solvent Programmer, and a Hitachi Model 100-40 variable wavelength spectrophotometer. Low pressure liquid chromatography was performed using a Fluid Metering, Inc. Model RPG-150 low pressure pump with Merck silica columns.

Reagents for biochemical studies. Beta-nicotinamide adenine dinucleotide phosphate, enzymatically reduced form (NADPH), tetrasodium salt, type III, 98-99%; beta-nicotinamide adenine dinucleotide, enzymatically reduced (NADH), grade III, 98%; glutathione, reduced form free acid, 98-100%; ethylenediaminetetraacetic acid (EDTA), tetrasodium salt, practical grade, 95%; tricaprylin (glyceryl tri-caprylate), Grade II, 90+%; monochlorodimedon; chloroperoxidase; glucose oxidase; and butylated hydroxytoluene (BHT) were obtained from Sigma Chemical Co. 2-Thiobarbituric acid and ethylamine hydrochloride were purchased from Eastman Kodak Co. D-Glucose, monobasic sodium phosphate, dibasic potassium phosphate, and potassium chloride were obtained from Mallinckrodt, Inc. and were of analytical reagent grade. Carbon monoxide was provided by Matheson Gas. Mazola corn oil was used as a vehicle for injections of 3-methylcholanthrene. Pierce Chemical Co. was the source of 1-hydroxybenzotriazole; deuterium oxide, 99.8%; benzotriazole (99+%) and 2,1,3-benzothiadiazole were purchased from Aldrich Chemical Co.

A Hewlett-Packard 33C calculator was used in calculating standard deviations.

4.20 BIOCHEMICAL TECHNIQUES

Preparation of rat hepatic microsomes. Male Sprague-Dawley rats, 200-250 g, were injected once a day for four days with sodium phenobarbital (80 mg/ml in water) at a dose of 80 mg/kg, or with 3-methylcholanthrene (20 mg/kg, 4 mg/ml in corn oil free of preservatives). On the fifth day the animals were sacrificed by decapitation and their livers were immediately perfused in situ with ice-cold isotonic KCl (1.15% w/v in distilled water) by cannulation of the inferior vena cava. The livers were homogenized in isotonic KCl (v=1.5 x gram weight liver) with a Dounce homogenizer (10 strokes loose pestle, 10 strokes tight pestle). The homogenate was centrifuged at 10,000 x G for 20 min. at 4°C. The supernatant was centrifuged at 100,000 x G for 60 min. to form the microsomal pellet and the supernatant was discarded. The pellet was gently washed away from the lower glycogen layer and rehomogenized in a volume of KCl equal to that used before. The microsomal suspension was recentrifuged at 100,000 x G for 30 min. and resuspended to a protein concentration of about 10 mg/ml in a 0.1 M sodium-potassium phosphate buffer containing 20 mM NaH₂PO₄, 80 mM K₂HPO₄, 150 mM KCl, and 1.5 mM Na₄EDTA in deionized water.

Protein determination. Protein concentrations were determined by the method of Lowry et al (1951). A sample of the microsomal suspension (approximately 10 mg/ml) was diluted one hundred fold with buffer. A 0.5 ml aliquot of

this microsomal suspension was transferred to a 15 ml test tube and to it was added 5 ml of a solution of 0.01% copper sulfate pentahydrate, 0.02% sodium-potassium tartarate, and 2% sodium carbonate in 0.1 N aqueous sodium hydroxide. After 10 min. 0.5 ml of commercial Folin reagent, diluted with equal parts distilled water, was added to each tube. The mixture was allowed to stand an additional 30 min. before the absorbance at 750 nm was measured. Protein determinations were made in triplicate; protein concentration was determined by reference to a standard curve obtained with human serum albumin.

Binding spectrum of l-aminobenzotriazole. The method of Estabrook et al (1972) was used. Hepatic microsomes were prepared from phenobarbital pretreated rats, as previously described, and diluted to 1 mg/ml protein. To a sample of microsomes in an Erlenmeyer flask was added solid l-ABT to a concentration of 10 mM. The flask was shaken in a Dubnoff metabolic shaker at room temperature for five minutes and a 1 ml aliquot was withdrawn. A difference spectrum of the aliquot versus a reference sample free of l-ABT was recorded from 370 to 450 nm using an Aminco DW-2a spectrophotometer.

Spectral assay of cytochrome P-450 content in microsomal incubations. Parallel incubations (20 ml in 50 ml Erlenmeyer flasks) containing the microsomal suspension (1mg/ml) in 0.1 M phosphate buffer, pH 7.4 (as prepared above), and substrate were preincubated at 37°C for 3 min.

in a Dubnoff metabolic shaker. The reaction was initiated by addition of NADPH dissolved in buffer (final concentration 1 mM) to one of the flasks. Aliquots of approximately 3 ml were withdrawn from each sample at the designated times and were quickly chilled in an ice-water bath. The cytochrome P-450 content of the aliquots was determined by a modification of the method of Estabrook et al (1972). Carbon monoxide (bubbled through an oxygen scrubber containing 0.5% sodium dithionite and 0.05% sodium anthroquinone-2-sulfate) was gently bubbled for 10 sec. through the chilled microsomal aliquot. An aliquot of the incubation with NADPH was transferred into one of the cuvettes and a baseline was recorded relative to the corresponding NADPH-free reference cuvette. A milligram of sodium dithionite was added to one of the cuvettes and a difference spectrum from 400 to 550 nm was recorded. The difference in absorbance between 450 nm and the asymptotic part of the curve at about 520 nm and a millimolar extinction coefficient of 100 were used to calculate the cytochrome P-450 concentration. Sparingly soluble substrates were added to the empty flasks in ether and dispersed about the sides of the container; the ether was removed under a stream of nitrogen gas before addition of microsomes.

Oxygen requirement for cytochrome P-450 destruction. A stream of argon was directed over a stirred microsomal incubation in a septum sealed flask at 0°C for 30 min. An aqueous solution of NADPH was purged of oxygen in tandem with

the incubation flask. The microsomes were then warmed to 37°C and NADPH solution was added (final concentration 1mM) by syringe transfer. Aliquots were removed by syringe and the cytochrome P-450 content was determined as before.

Effect of carbon monoxide on the destruction of cytochrome P-450. Carbon monoxide (scrubbed of oxygen as before, flow 10 ml/min) was admitted to the sealed incubation flask under the conditions described above and the cytochrome P-450 content of the aliquots was determined as previously described.

Determination of heme content. The method of Omura and Sato (1964) was used. Microsomal suspensions were added to 2 volumes of 0.1 N NaOH solution containing 20% pyridine, mixed, and the mixture divided between two cuvettes. The cuvettes were placed in an Aminco DW-2a double beam spectrophotometer and a few milligrams of dithionite were added to the sample cuvette. The difference spectrum between 500 and 600 nm was promptly recorded. The difference in absorbance between 558 and 600 nm and a millimolar extinction coefficient of 34.2 cm^{-1} were used to calculate the heme concentration.

Partition ratio between substrate turnover and enzyme destruction in the metabolism of 1-ABT. The method of Ortiz de Montellano and Mico (1981) was used. Microsomes were prepared from phenobarbital pretreated rats and diluted to a cytochrome P-450 concentration of 2.75 nmol/ml. To these

incubations were added 9.35 nmol/ml of l-ABT (no l-ABT in controls) and BHT (1 mM). This concentration of l-ABT was chosen to produce measureable yet sub-maximal inactivation of the enzyme. The reaction was initiated by addition of NADPH (1 mM). At 30 min. intervals the incubations were assayed for enzyme destruction and fresh NADPH and BHT (1 mM increments of each) were added. No further enzyme loss was observed after 90 min. of incubation. All the substrate was assumed to be consumed at this point. The partition ratio was calculated by dividing the initial moles of substrate present by the moles of the enzyme destroyed.

Measurement of cytochrome b_5 in microsomal incubations.

The method of Omura and Sato (1964) was used. Aliquots from the microsomal incubations were withdrawn and divided into two cuvettes. To one of the cuvettes was added NADH (100 micromolar final concentration), and the reduced versus oxidized difference spectrum was recorded. The cytochrome b_5 concentration was calculated from the absorbance difference between 425 and 410 nm using a millimolar extinction coefficient of 185.

Determination of cytochrome c reductase activity.

The method of Williams and Kamin (1962) was used. Solutions of cytochrome c (0.15 mM oxidized cytochrome c, Sigma Type III, 1.42 mg/ml), potassium cyanide (19.5 mg/100 ml), and NADPH (5 mM, Sigma Type III) were prepared in 0.05 M K_2HPO_4 - KH_2PO_4 buffer, pH 7.4. Equal volumes of cytochrome c solution,

potassium cyanide solution, and microsomal sample (0.2 mg/ml protein) were mixed and 1 ml of the mixture was transferred into a cuvette. To the cuvette was added 50 microliters of the NADPH solution; the solution was quickly mixed by inversion and the increase in absorbance at 550 nm was measured as a function of time. The NADPH-cytochrome c reductase activity was determined from the initial rate of cytochrome c reduction. A millimolar extinction coefficient of 19.1 cm^{-1} was used to calculate the reductase activity in units of nmol cytochrome c reduced/mg microsomal protein/min.

Detection of lipid peroxidation. The method of Asakawa and Matsushita (1980) was used. To 0.1 ml of microsomal sample were added 0.1 ml of ferric chloride solution (ferric chloride hexahydrate, 270 mg in 100 ml water), 0.1 ml of BHT solution (220 mg BHT in 100 ml ethanol), 1.5 ml of 0.2 M glycine-hydrochloric acid buffer, pH 3.6, and 1.5 ml of thiobarbituric acid reagent (0.5 g of thiobarbituric acid and 0.3 g of sodium dodecyl sulfate in 100 ml water). The tube was heated momentarily in a boiling water bath, capped with a septum, and heated for an additional 15 min. After it was cooled by immersion in ice water, 1 ml of glacial acetic acid and 2 ml of chloroform were added to the tube. The mixture was shaken and centrifuged at low speed for a few minutes. The optical density of the supernatant was determined at 532 nm using a 1 cm cuvette.

A reagent blank was run simultaneously. A molar extinction coefficient of $156,000 \text{ cm}^{-1}$ was used to calculate

the amount of malondialdehyde formed.

In vivo destruction of cytochrome P-450. Sprague-Dawley male rats (240-260 g) were injected intraperitoneally once a day for four days with sodium phenobarbital (80 mg in water) or with 3-methylcholanthrene (20 mg in corn oil). On the fifth day the agents to be tested were administered intraperitoneally in vehicles and at doses specified in the results sections. The rats were sacrificed by decapitation three hours later and microsomes were prepared from their livers as previously described. The microsomal protein and cytochrome P-450 contents were determined as before. Each value represents a determination of the P-450 content in two pooled rat livers.

Determination of agent toxicity. Measurement of serum transaminase levels. Sprague-Dawley male rats (230-270 g) were injected intraperitoneally once a day for four days with sodium phenobarbital (80 mg/kg in water). On the fifth day the agents were administered in vehicles and at doses specified in the results sections. The rats were sacrificed by decapitation twenty-four hours later. Approximately 5 ml of blood was collected separately from each rat and was allowed to stand for 30 min. in a 37°C bath to promote clot aggregation and retraction. The samples were centrifuged at low speed, and the clear yellow serum was collected and filtered through glass wool to remove any remaining red blood cell aggregates. Care was taken to avoid hemolysis, as red

blood cells contain significant levels of transaminases. The serum concentrations of glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) were determined using Sigma Kit No. 505.

Inactivation of chloroperoxidase by l-ABT. Incubations contained chloroperoxidase (43300 units/ml), monochlorodimedon (MCD, 1 mM), l-ABT (1 mM), glucose (15 mg/ml), and glucose oxidase (3.0 µg/ml). In control incubations l-ABT or hydrogen peroxide was deleted from the complete system or ethylamine hydrochloride (1 mM) replaced l-ABT as the substrate. Incubations of 100 µl volume were initiated by addition of glucose oxidase and were performed at 25°C. Aliquots of 10 µl were removed at the designated timepoints and quenched into 2.50 ml of 0.1 M potassium phosphate buffer (pH 2.75) containing 0.1 mM MCD. Enzyme activity was measured by the method of Hager et al (1965). The change in absorbance at 278 nm (the λ_{\max} of MCD) with time was determined over the period from 0 to 60 sec. The reported value for each time point was an average of determinations from three separate measurements differing by less than 10%.

Isolation and characterization of the products resulting from metabolism of l-ABT by chloroperoxidase. The incubations were performed at 25°C and contained, in a total volume of 1.35 ml, chloroperoxidase (477,000 units), l-ABT (9.26 mM), and hydrogen peroxide (7.96 mM) in 0.1 M potassium phosphate buffer (pH 2.75). The hydrogen peroxide was

added in 17 aliquots at 15 second intervals. Control incubations deviated from the complete system in alternately deleting hydrogen peroxide or chloroperoxidase, or in use of enzyme which had been first denatured by four cycles of freezing in a dry ice/acetone bath followed by 5 minutes of heating at 90°C. After the final addition of hydrogen peroxide the incubations were extracted with ether (4 X 1 ml, freshly distilled over CaH₂ to remove peroxides). The ether extracts were combined and dried by passage through short columns of anhydrous sodium sulfate. The extracts were concentrated at room temperature under a stream of nitrogen gas and then purified by gas chromatography on a 5 X 0.25 inch 10% carbowax 20M on 120/140 Chrom Q column maintained at 200°C in a Varian Model 920 instrument fitted with a thermal conductivity detector. Fractions were collected over a liberal period centered about the retention time of phenol, condensing in a glass loop immersed in a dry ice/acetone bath. The fractions were analyzed both by gas chromatography on a 6 foot 3% OV 225 glass column maintained at 100°C in a Varian Model 2100 instrument equipped with a flame ionization detector, and by GC/MS on a system consisting of a Supelco SP 2330 column connected to a Kratos MS 25 mass spectrometer operating in electron impact mode (70 eV). In other experiments the incubations were performed instead in deuterated aqueous buffer (pD=2.65) made by dissolving 136 mg of KH₂PO₄ and 25 µl of H₃PO₄ in 13 ml of 99.8% deuterium oxide. The ether extracts were combined and washed

with nondeuterated potassium phosphate buffer (0.1 M, pH 2.75, 2 X 3 ml) to replace any exchangeable deuterons with protons. The samples were then dried and analyzed as before.

4.3 ISOLATION AND PURIFICATION OF ALKYLATED PORPHYRINS FROM RAT LIVER

A modification of the method of Unseld and De Matteis (1976) was used. Male Sprague-Dawley rats, 200-400 g, were injected once a day for four days with an intraperitoneal dose of sodium phenobarbital (80 mg/kg in water). On the fifth day a 100 mg/kg dose of 1-ABT in 1 ml of water (pigment was isolated also from rat livers following administration of other triazoles in the vehicles and at the doses listed in Table 3-10, Chapter 3; no pigment was isolated after administration of 100 mg/kg DCBT in 1 ml tricapyrin) was administered by intraperitoneal injection. The rats were sacrificed four hours later. Their livers were perfused in situ with cold isotonic 0.9% NaCl to flush out the remaining blood, homogenized, and added to a stirred solution of cold 5% H₂SO₄/methanol (1:10 v/v). In all subsequent procedures the pigments were protected from light and were stored under nitrogen in a freezer. The mixture was allowed to stand overnight at 4°C in the dark, filtered, and a half volume of distilled water added. This solution was extracted with three 1 volume portions of dichloromethane. The organic layers were combined, washed with distilled

water (3 x 0.5 volumes), dried over anhydrous sodium sulfate, filtered, and concentrated. Zinc acetate (approx. 5 ml of 0.5% zinc acetate in methanol) was added to the residue. The residue was again concentrated, dissolved in a minimum volume of dichloromethane, applied to an Analtech silica gel G preparative thin layer plate (2000 micron thickness, solution from 5 rat livers/plate), and eluted with chloroform/acetone/methanol, 7:3:1. Two major bands that fluoresced red under 366 nm light were observed at R_f values of approximately 0.3 and 0.8, respectively.

The less polar band was eluted from the silica gel with methanol and was rechromatographed on a silica gel G plate (Analtech 500 micron plate, chloroform/acetone 5:1). A comparison of the R_f of this pigment (and its free base) with that of authentic N-phenylprotoporphyrin IX dimethyl ester (and its zinc complex, provided by Dr. K.L. Kunze) were made using the latter chromatographic conditions. The pigment was demetalated by treatment with 5% sulfuric acid/methanol. After 5 min. one volume of chloroform and one volume of water were added, and the porphyrin was extracted into the organic phase. The organic layer, washed three times with distilled water, was dried over anhydrous sodium sulfate. Electronic absorbance spectra were obtained for the pigments dissolved in chloroform. A spectrum was obtained for the pigment free base after addition of 10 microliters of triethylamine (used as a base) to 1 ml of the chloroform solution in a cuvette.

The more polar band ($R_f=0.3$) was scraped from the silica plate and 50 ml of methanol and 50 ml of saturated aqueous sodium bicarbonate were added to it. A stir bar was added and the suspension was stirred for 30 min. to deactivate the silica gel. Dichloromethane (50 ml) was then added; the silica floated on this layer. After 30 min. the organic layer was removed and dried over anhydrous sodium sulfate. A visible spectrum was recorded of this and subsequent 50 ml organic layers to determine the extent of recovery; approximately 90% of the pigment was recovered after the third extraction. The pigment was applied to preparative plates (Analtech silica gel G, 500 micron, chloroform/acetone/methanol 7:3:1) that had been washed by development in methanol five successive times. One major band (approximately 80%) and three minor bands of lower R_f were eluted from the silica gel as before and found to have identical visible spectra. A sample suitable for mass spectral analysis was purified by high pressure liquid chromatography on a 4.6 x 250 mm Whatman Partisil 10 PAC column using a 15 min. exponential gradient of from 0-100% methanol in dichloromethane (flow rate of 2 ml/min., detector set at 417 nm). The pigment eluted as one peak at approximately 60% methanol. The pigment, after concentration, was analyzed by FDMS.

4.4 SYNTHESIS

All reactions were performed under a nitrogen atmosphere. Solvents were removed by rotary evaporation. Nuclear magnetic resonance spectra were obtained in deuteriochloroform unless otherwise indicated. Peak positions are reported in parts per million (ppm) downfield from tetramethylsilane with the following in parenthesis: proton integration, multiplicity, assignment.

5,6-dichloro-1,2,3-benzothiadiazole (1) and 6,7-dichloro-1,2,3-benzothiadiazole (2). The isomers were prepared by the method of Kirby *et al* (1970). A solution of 2-chloroaniline (42 g, 0.33 mole) in 50 ml of glacial acetic acid was added to 180 ml of sulfur monochloride with stirring in an ice-water bath. After the addition the mixture was stirred for 2h at room temperature, then for 4h at 65°C. Benzene (200 ml) was added and the mixture was cooled and filtered. The product was washed with benzene (3 X 30 ml) and allowed to air dry. The crude material was dissolved in 50% sulfuric acid (300 ml) at 75°C, filtered through glass wool, cooled to between -5 and 0°C in a rock salt-ice-water bath, and stirred while adding dropwise a solution of sodium nitrite (0.46 mole) in 50 ml water. The mixture, after stirring for an additional 1h, was poured into 1.6 kg of ice. After the ice melted the mixture was extracted with ether (3 X 100 ml). The ether extracts were combined, washed with 5% aqueous sodium hydroxide solution (2 X 50

ml), dried over anhydrous magnesium sulfate, and evaporated, leaving a crude yellow solid containing 1, 2, and much sulfur. The solid was purified by low pressure chromatography (chloroform-hexanes, 3:1), 1 being eluted first, (23% yield), m.p. 119-120°C. NMR: 8.33 (1H,s), and 8.84 ppm (1H,s). CIMS (m/e): 205 (M+1), 207 (1Cl³⁷ M+1), 209 (2Cl³⁷ M+1). Analysis calculated for C₆H₂Cl₂N₂S: C,35.14; H,0.98; Cl,34.58; N,13.66; S,15.63. Found: C,35.18; H,1.05; Cl,34.30; N,13.67; S,15.62. The less mobile isomer, 2, was then eluted (18% yield), mp. 86-87°C. NMR: 7.70-7.85 (1H,d) and 8.45-8.60 ppm (1H,d). CIMS (m/e): 205 (M+1), 207 (1Cl³⁷ M+1), 209 (2Cl³⁷ M+1). Analysis calculated for C₆H₂Cl₂N₂S: C,35.14; H,0.98; Cl,34.58; N,13.66; S,15.63. Found: C,35.23; H,1.00; Cl,34.49; N,13.72; S,15.56.

1-Aminobenzotriazole (3) was prepared from 2-nitroaniline according to the procedure of Campbell and Rees (1969). The agent was purified by sublimation (70°C, 0.5 mm Hg) to yield white crystals, m.p. 83-84°C. NMR: 7.95-8.10 (1H,m,phenyl), 7.33-7.79 (3H,m,phenyl), and 5.50-5.85 ppm (1H,s,amino, exchangeable with D₂O). EIMS (m/e): 135 (M+1). Analysis calculated for C₆H₆N₄: C,53.72; H,4.51; N,41.77. Found C,53.91; H,4.27; N,42.07.

2-Aminobenzotriazole (4) was prepared along with 3 from benzotriazole following a literature procedure (loc. cit.). The crude mixture (600 mg), separated by low pressure chromatography (chloroform-ethyl acetate, 3:1), yielded 80

mg of white crystals (13%) of 4, m.p. 121-122°C. NMR: 6.54-6.58 (2H,s,amino), 7.30-7.42 (2H,m,phenyl), and 7.73-7.85 ppm (2H,m,phenyl); EIMS (m/e): 135 (M+1). Analysis calculated for C₆H₆N₄: C,53.72; H,4.51; N,41.77. Found: C,53.52; H,4.57; N,42.11.

1-Diacetamidobenzotriazole (5, see Figure 4-1). Acetic anhydride (10.0 g) was added to a stirred solution of 3 (1.00 g, 7.46 mmoles) and potassium carbonate (3.0 g) and the mixture was stirred for approximately 12 hr at room temperature. The mixture was then filtered, the solvent was removed on a rotary evaporator, and the crude residue was dissolved in 100 ml of dichloromethane. The solution, washed with three 100 ml portions of water, dried over anhydrous magnesium sulfate, and concentrated, gave the diacetamide, 5 (1.51 g, 94%). Purification by low pressure chromatography (chloroform-ethyl acetate, 3:1) provided an analytical sample, white needles, m.p. NMR: 2.36 (6H,s,acetyl methyl), 7.29-7.65 (3H,m,phenyl), and 8.09-8.22 ppm (1H,m,phenyl); EIMS (m/e): 219 (M+1), 218 (M+); Analysis calculated for C₁₀H₁₀N₄O₂: C,55.04; H,4.62; N,25.67. Found: C,54.68; H,4.97; N,25.72.

1-Acetamidobenzotriazole (6, see Figure 4-2). A suspension of 5 (1.00 g, 4.59 mmoles) in 100 ml of absolute ethanol was heated to reflux for 8h. The resulting solution was cooled to room temperature and concentrated to give 6 (799 mg, 99%), white crystals m.p. 159.5-161°C. EIMS (m/e):

177 (M+), 176 (M+). NMR (CD₃OD) : 2.30 (3H,s,acetyl methyl), 7.30-7.59 (3H,m,phenyl), 7.90-8.04 (1H,m,phenyl), and 9.4 ppm (CDCl₃, but not CD₃OD,1H,s,amide nitrogen). Analysis calculated for C₈H₈N₄O: C,54.54; H,4.58; N,31.80. Found: C,54.87; H,4.32; N,31.66.

1-N-methylacetamidobenzotriazole (7, see Figure 4-3).

To a suspension of 1.57 g potassium carbonate in 25 ml acetone was added 6 (1.00 g, 5.68 mmoles). The solution was heated to reflux and methyl iodide (1.00 g, 7.82 mmoles) in 10 ml acetone was added in five portions over a period of one hour. After 2h the solution was cooled, filtered, and concentrated. The residue, purified by low pressure chromatography (hexane-ethyl acetate, 1:1), yielded 0.745 g 7 (69% yield) as white crystals, m.p. 74.5-76.0°C. NMR: 1.81 (3H,s,acetyl methyl), 3.57 (3H,s,N-methyl), 7.35-7.66 (3H,m,phenyl), and 8.05-8.20 ppm (1H,m,phenyl). EIMS (m/e): 190 (M+). Analysis calculated for C₉H₁₀N₄O: C,56.83; H,5.30; N,29.46. Found C,56.81; H,5.25; N,29.36.

1-N-methylaminobenzotriazole (8, see Figure 4-4). To 50 ml of 10 M aqueous potassium hydroxide was added 7 (510 mg, 2.68 mmoles). The suspension was heated to reflux and stirred vigorously for 2h, then cooled and extracted with dichloromethane (5 X 25 ml). The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and concentrated. The oily yellow residue, purified by low pressure chromatography (hexanes-THF, 1:1), gave 8 (309 mg, 78%)

as white crystals, m.p. 69.5-71.0°C. NMR: 3.20 (3H,s,N-methyl), 4.83 (1H,s,amino, exchangeable with D₂O), 7.31-7.65 (3H,m,phenyl), and 7.94-8.08 ppm (1H,m,phenyl); EIMS (m/e): 149 (M+1). Analysis calculated for C₇H₈N₄: C,56.74; H,5.44; N,37.81. Found C,56.75; H,5.63; N,37.62.

1-Nitrobenzotriazole (9). The triazole was prepared by the method of Cohen-Fernandes and Habraken (1971). To a stirred solution of 1.19 g (10.0 mmoles) of benzotriazole in 8 ml of acetic acid was added 0.5 ml of concentrated nitric acid. After 30 minutes 1 ml of acetic anhydride was added. The solution was poured into 20 ml of ice water and extracted with dichloromethane (3 X 20 ml). The extracts were combined, washed twice with 25 ml of 10% sodium bicarbonate solution, dried (MgSO₄), filtered, and concentrated. The residue, purified by low pressure chromatography (chloroform-ethyl acetate, 3:1), gave 1.28 g of 9 (78%) as white crystals, m.p. 69-70°C. NMR: 7.45-7.87 (2H,m,phenyl), and 8.06-8.21 ppm (2H,m,phenyl); CIMS (m/e): 165 (M+1). Analysis calculated for C₆H₄N₄O₂: C,43.91; H,2.46; N,34.14. Found: C,44.03; H,2.31; N,34.42.

Figure 4-1

NMR and mass spectrum of 1-diacetamidobenzotriazole (5)

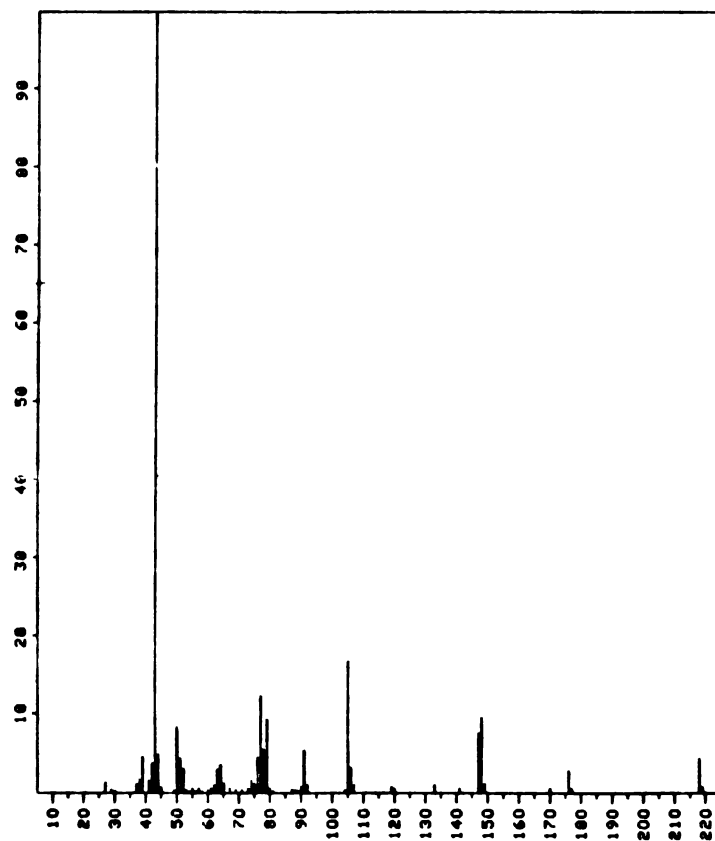
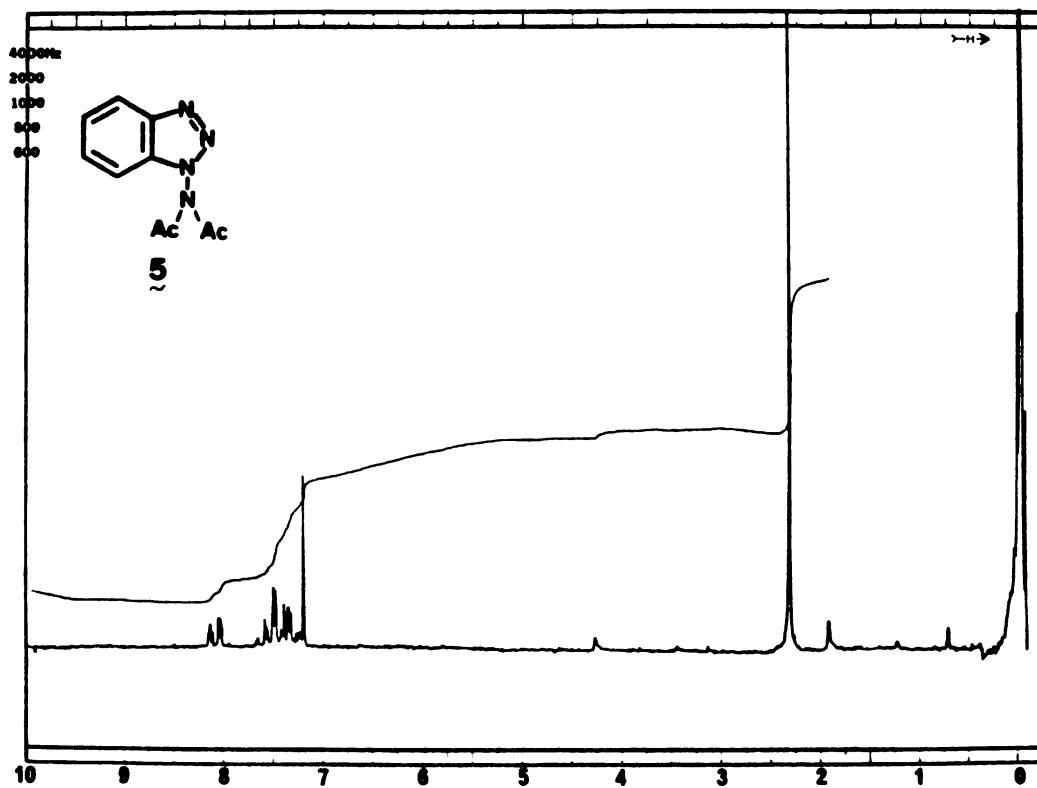


Figure 4-2

NMR and mass spectrum of 1-acetamidobenzotriazole (6)

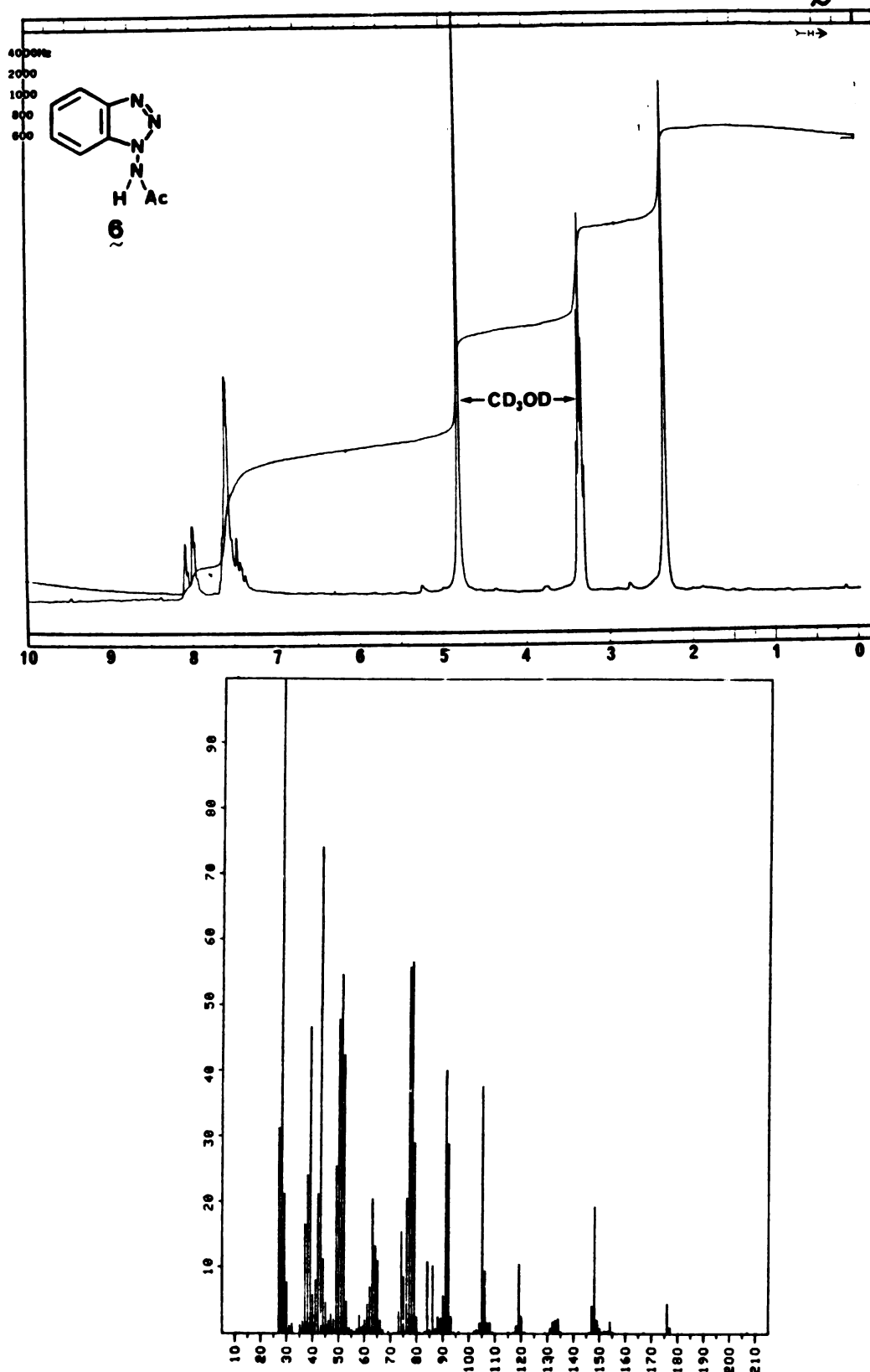


Figure 4-3

NMR and mass spectrum of 1-N-methylacetamidobenzotriazole (7)

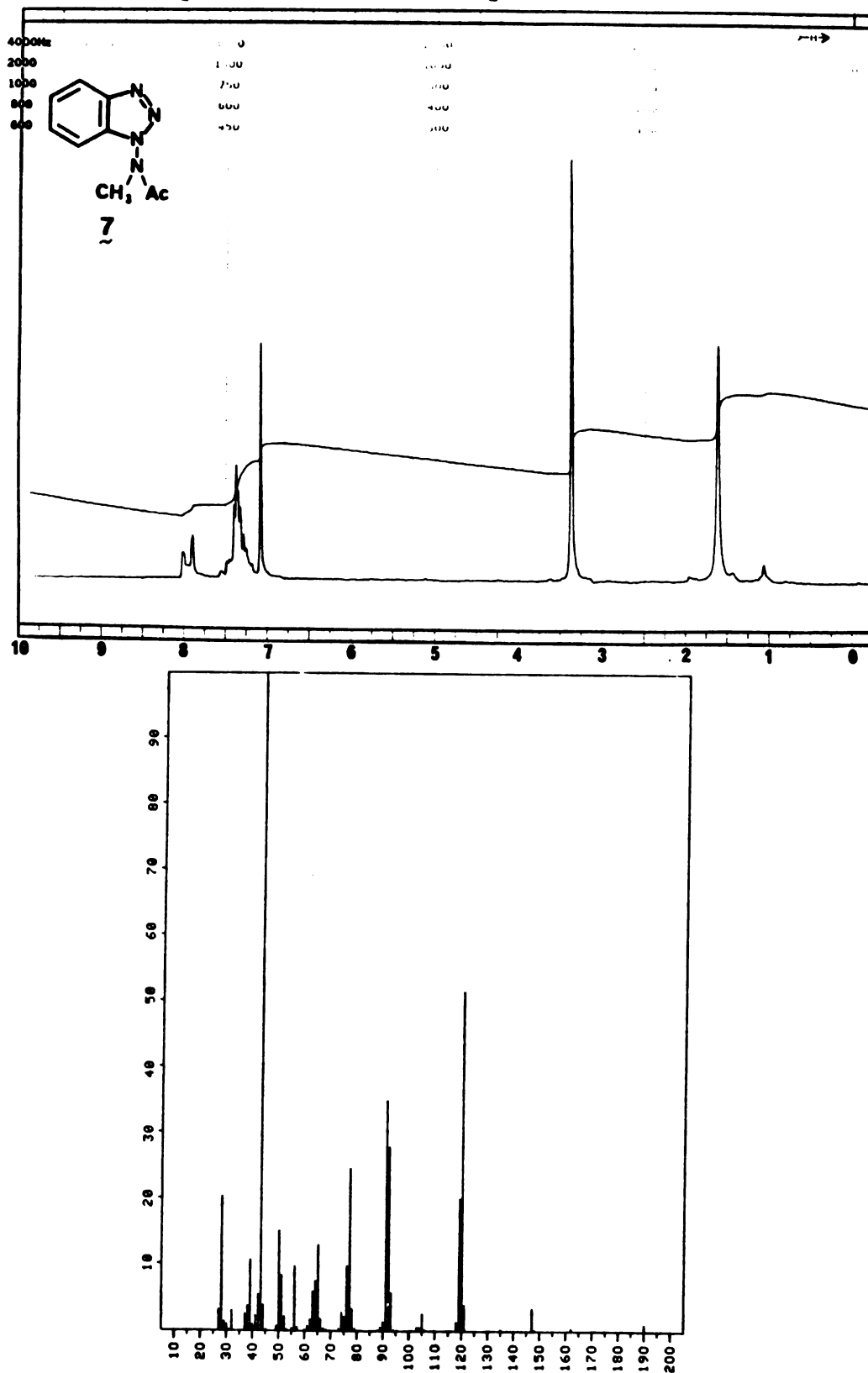
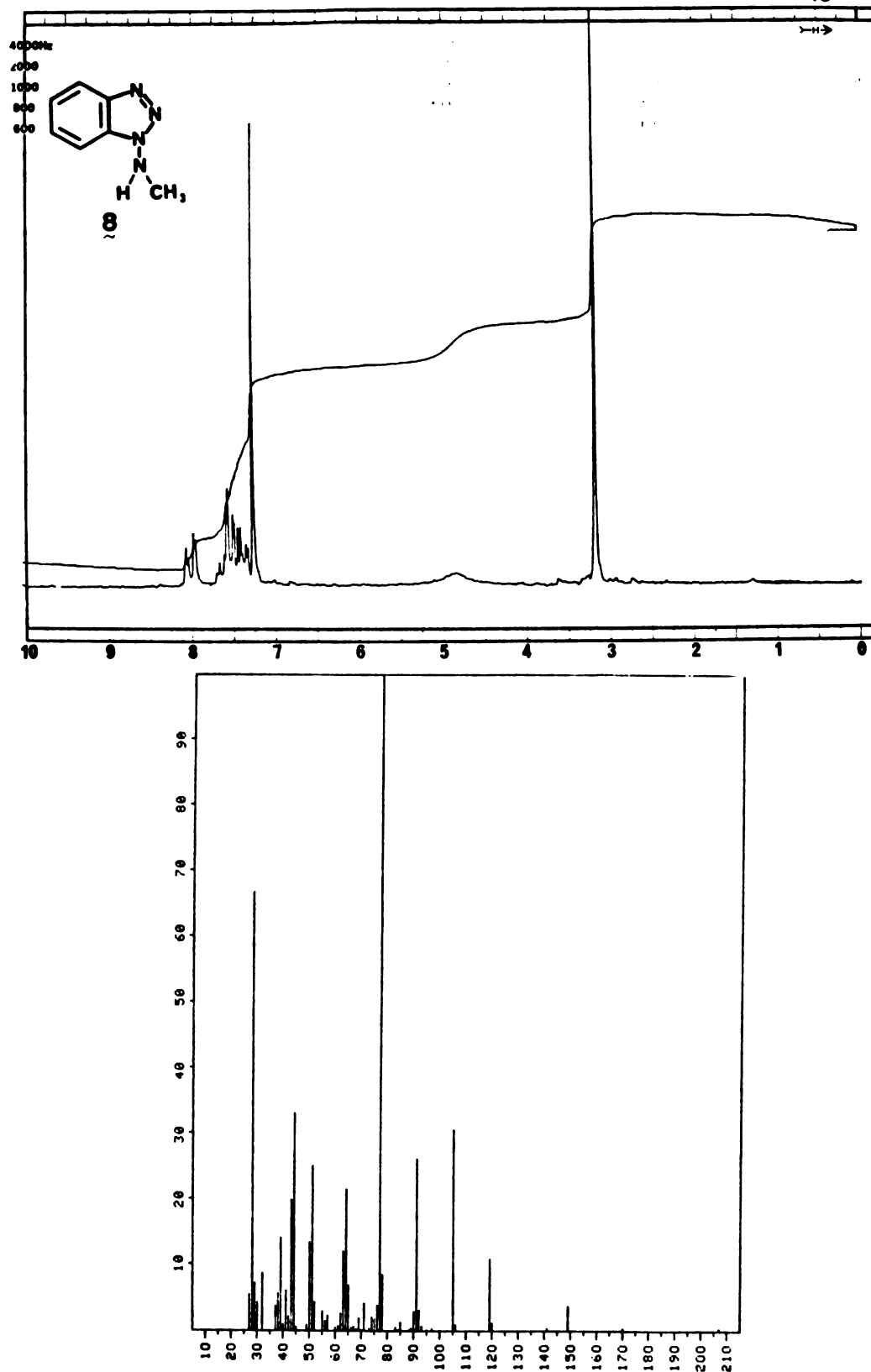


Figure 4-4

NMR and mass spectrum of 1-N-methylaminobenzotriazole (8)



REFERENCES

- Albano, E., Lott, K.A.K., Slater, T.F., Stier, A., Symons, M.C.R., and Tomasi, A. (1982), Biochem. J. 204, 593.
- Asakawa, T. and Matsushita, S. (1980), Lipids 15, 137-140.
- Augusto, O., Beilan, H.S. and Ortiz de Montellano, P.R. (1982a), J. Biol. Chem. 257, 11288-11295.
- Augusto, O., Kunze, K.L., and Ortiz de Montellano, P.R. (1982b), J. Biol. Chem. 257, 6231.
- Aurich, H.G. and Weiss, W. (1973), Chem. Ber. 106, 2408.
- Benati, L., Montevecchi, P.C. and Spagnolo, P. (1980), J. Chem. Soc. Chem. Commun., 715.
- Benati, L., Montevecchi, P.C., Spagnolo, P. and Tundo, A. (1981), J. Chem. Soc., Perkin Trans. I, 1544.
- Benati, L., Montevecchi, P.C., Tundo, A. and Zanardi, G. (1974), J. Chem. Soc., Perkin Trans. I, 1276.
- Benati, L., Montevecchi, P.C., Tundo, A. and Zanardi, G. (1976), J. Org. Chem., 41(8), 1331.
- Bini, A., Vecchi, G., Vivoli, G., Vannini, V., and Cessi, C. (1975), Pharmacol. Res. Comm. 7, 143.
- Bolt, H.M., Buchter, A., Wolowski, L., Gil, D.L. and Bolt, W. (1977a), Int. Arch. Occup. Envir. Hlth. 39, 103.
- Bolt, H.M., Kappus, H., Buchter, A. and Bolt, W. (1977b), Arch. Toxic. 7, 153.
- Boyd, G.S., Grimwade, A.M., and Lawson, M.E. (1973), Eur. J. Biochem. 37, 334.
- Braun, V.H.P., Zeller, K. and Meier, H. (1973), Chem. Zeitung 97, 567-568.
- Braun, H.P., Zeller, K. and Meier, H. (1975), Liebigs Ann. Chem., 1257-1263.
- Cadogan, J.I.G. and Thompson, J.B. (1969), Chem. Comm., 770.
- Calligaro, A., and Vannini, V. (1975), Pharmacol. Res. Comm. 1, 323.
- Campbell, C.D. and Rees, C.W. (1969), J. Chem. Soc. (C), 742-756.

- Castignani, G.L. and Neal, R.A. (1975), Biochem. Biophys. Res. Comm. 65, 629.
- Chasseaud, L.F. (1973), Drug. Metab. Rev., 2(2), 185-220.
- Coffman, B.L., and Tephly, T.R. (1982), in abstracts of the 66th Annual Meeting of the Federation of American Societies for Experimental Biology, New Orleans, Louisiana, April, 1982, p.1209.
- Cohen-Fernandes, P. and Habraken, C.L. (1971), J. Org. Chem. 36, 3084-3086.
- Coon, M.J., Haugen, D.A., Guengerich, F.P., Vermillion, J.L., and Dean, W.L. (1976), in "The Structural Basis of Membrane Function" (Y. Hatefi and L. Djavadi-Ohanian, Eds.). Academic, New York, pp. 409-427.
- Cooper, M.B., Craft, J.A., Estall, M.R. and Rabin, B.R. (1980), Biochem. J. 190, 737-746.
- Corbett, M.D., Chipko, B.R., and Batchelor, A.E. (1980), Biochem. J. 187, 893-903.
- Cramer, S.P., Dawson, J.H., and Hager, L.P. (1978), J. Am. Chem. Soc. 100, 7282.
- Dalvi, R.R., Hunter, A.L., and Neal, R.A. (1975), Chemico-Biol. Interactions 10, 347.
- Davies, J.H., Haddock, E., Kirby, P. and Webb, S.B. (1971), J. Chem. Soc. (C), 2843.
- De Matteis, F.A. (1971), Biochem. J. 124, 767.
- De Pierre, J.W., and Dalner, G. (1975), Biochim. Biophys. Acta. 415, 411.
- Dent, J.G., Graichen, M.E., Schnell, S., and Lasker, J. (1980), Toxicol. Appl. Pharmacol. 52, 45-53.
- Estabrook, R.W., Peterson, J., Baron, J. and Hildebrandt, A. (1972), in Methods in Pharmacology, Vol. 2, C.F. Chignell, ed., Appleton-Century-Crofts, New York, 303-350.
- Estabrook, R.W., Werringloer, J., Masters, B.S.S., Jonen, H., Matsubara, T., Ebel, R., O'Keefe, D., and Peterson, J.A. (1976), in "The Structural Basis of Membrane Function" (Y. Hatefi and L. Djavadi-Ohanian, Eds.). Academic, New York, pp. 429-445.
- Felton, J.C., Jenner, D.W. and Kirby, P. (1970), J. Agric. Food Chem. 18, 671.

- Franklin, M.R. (1977), Pharmacol. Ther., (A) 2, 227.
- Gibbons, G.F., and Mitropoulos, K.A. (1973), Eur. J. Biochem. 40, 269.
- Gil, D.L. (1973), Ph.D Thesis, Cornell University, Ithaca, New York.
- Gil, D.L., Ferreira, J. and Reynafarje, B. (1980), Xenobiotica 10, 7.
- Gil, D.L. and Wilkinson, C.F. (1976), Pestic. Biochem. Physiol. 6, 338.
- Gil, D.L. and Wilkinson, C.F. (1977), Pestic. Biochem. Physiol. 7, 183.
- Gilbert, B.C., Kirk, M.C., Norman, R.O.C. and Laue, H.A.H. (1977), J. Chem. Soc. Perkin II, 497.
- Glazer, R.I., Schenkman, J.B., and Sartorelli, A.C. (1971), Mol. Pharmacol. 7, 683-688.
- Griffin, B.W. (1978), Arch. Biochem. Biophys. 190 (2), pp. 850-853.
- Grigg, R., Shelton, G. and Sweeney, A. (1972), J. Chem. Soc. Perkin I, 1789.
- Groves, J.T., McClusky, G.A., White, R.E., and Coon, M.J. (1978), Biochem. Biophys. Res. Comm. 81, 154-160.
- Groves, J.T., Nemo, T.E., and Meyers, R.S. (1976), J. Amer. Chem. Soc. 101, 1032.
- Guengerich, F.P., Ballou, D.P., and Coon, M.J. (1976), Biochem. Biophys. Res. Comm. 70, 951-956.
- Guengerich, F.P. and Strickland, T.W. (1977), Mol. Pharmacol. 13, 993.
- Guengerich, F.P., Wang, P., Mason, P.S., and Mitchell, M.B. (1981), Biochem. 20, 2370.
- Hager, L.P., Morris, D.R., Brown, F.S., and Eberwein, H. (1965), J. Biol. Chem. 241, 1769.
- Halpert, J., and Neal, R.A. (1981), Drug Metab. Rev. 12, 239-259.
- Hildebrandt, A., and Estabrook, R.W. (1971), Arch. Biochem. Biophys. 143, 66.
- Hoffmann, R.W. (1967), "Dehydrobenzene and Cycloalkynes".

Weinheim/Borgstr., Verlag Chemie; New York, London, Academic Press.

Hrycay, R., Gustafsson, J.A., Ingelman-Sundberg, M., and Ernster, L. (1975), Biochem. Biophys. Res. Comm. 66, 209.

Ivanetich, K.M., Bradshaw, J.J., Marsh, J.A., Harrison, G.G., and Kaminsky, L.S. (1976), Biochem. Pharmacol. 25, 773.

Iyanagi, T., and Mason, H.S. (1973), Biochem. 12, 2297.

Jollow, D.J., Mitchell, J.R., Zampaglione, N., and Gillette, J.R. (1974), Pharmacology 11, 151.

Kamataki, T., and Neal, R.A. (1976), Mol. Pharmacol. 12, 933.

Kedderis, G.L., Koop, D.R., and Hollenberg, P.F. (1980), J. Biol. Chem. 255(21), 10174-10182.

Kenney, W., Nagy, J., Salach, J., and Singer, T., Enzyme Inhibitors (Edited by U. Brodbeck), p.25 Verlag, Weinheim (1980).

Kirby, P., Soloway, S.B., Davies, J.H. and Webb, S.B. (1970), J. Chem. Soc. (C), 2250.

Knoll, J. (1976), in "Monoamine Oxidase and Its inhibition", Ciba Foundation Symposium 39, pp. 135-161, Elsevier, Amsterdam.

Lichtenberger, F., Nastainczyk, W., and Ullrich, V. (1976), Biochem. Biophys. Res. Comm. 70, 939.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951), J. Biol. Chem. 193, 265-275.

Lu, A.Y.H., and Coon, M.J. (1968), J. Biol. Chem. 243, 1331.

Lu, A.Y.H., and West, S.B. (1978), Pharmacol. Ther. Part A Chemother. Toxicol. Metab. Inhibitors 2, 337-358.

Mannering, G.J. (1981), in "Concepts in Drug Metabolism" Part B, (P. Jenner and B. Testa, Eds.). Marcel Dekker, Inc., New York, pp. 53-166.

Mason, H.S., (1957), Science 125, 1185.

Mason, R.P. (1979), Rev. in Biochem. Toxicol., vol.1, p.151; E. Hodgson, J.R. Bend, and R.M. Philpot editors; Elsevier, New York.

Mathews, J.M., and Ortiz de Montellano, P.R. (1982).

"Oxidative Metabolism of 1-Aminobenzotriazole to a Benzyne Intermediate", in abstracts of the 66th Annual Meeting of the Federation of American Societies for Experimental Biology, New Orleans, Louisiana, April, 1982, p.1541.

Matthews, H.B., Skrinjaric-Spoljar, M. and Casida, J.E. (1970), Life Sci. 9, 1039.

Maycock, A. and Abeles, R. (1976), Accts. Chem. Res. 9, 313.

Miller, R.G. and Stiles, M. (1963), J. Amer. Chem. Soc. 85, 1798.

Mitchell, J.R., Thorgeirson, U.P., Black, M., Timbrell, J.A., Snodgrass, W.R., Potter, W.Z., Jollow, D.J. and Keiser, H.R. (1975), Clin. Pharmacol. Ther. 18, 70-79.

Nakayama, J., Fukushima, T., Seki, E. and Hoshino, M. (1979), J. Am. Chem. Soc., 101, 7684.

Neal, R.A., Kamataki, T., Hunter, A.L., and Catignani, G. (1976), Hoppe-Seyler's Z. Physiol. Chem. 357, 1044.

Nelson, S.D., Hinson, J.A., and Mitchell, J.R. (1976), Biochem. and Biophys. Res. Comm., 69, 900.

Omura, T. (1979), in "The Induction of Drug Metabolism" (R.W. Estabrook and E. Lindenlaub, Eds.) pp. 161-175, F.K. Schattauer Verlag, New York.

Omura, T. and Sato, R. (1964), J. Biol. Chem. 239, 2370-2378.

Orrenius, S., Das, M., and Gnesspelius (1969), in "Microsomes and Drug Oxidations" (J.R. Gillette, A.H. Conney, G.J. Cosmides, R.W. Estabrook, J.R. Fouts, and G.J. Mannering, Eds.) pp.251-277, Academic Press, New York.

Ortiz de Montellano, P.R., Beilan, H.S., and Kunze, K.L. (1981a), Proc. Natl. Acad. Sci. USA 78, 1490.

Ortiz de Montellano, P.R., Beilan, H.S., Kunze, K.L., and Mico, B.A. (1981b), J. Biol. Chem. 256, 4395.

Ortiz de Montellano, P.R. and Kunze, K.L. (1980a), J. Biol. Chem. 255, 5578.

Ortiz de Montellano, P.R. and Kunze, K.L. (1980b), Biochem. Biophys. Res. Comm. 94, 443.

Ortiz de Montellano, P.R., Kunze, K.L., and Augusto, O. (1982), J. Amer. Chem. Soc. 104, 3545.

Ortiz de Montellano, P.R., Kunze, K.L. and Mico, B.A.

(1980), Mol. Pharmacol. 18, 602-605.

Ortiz de Montellano, P.R., Mico, B.A., Yost, G.S., and Correia, M.A. (1978), in "Enzyme-Activated Irreversible Inhibitors" (N. Seiler, M.J. Jung and J. Koch-Weser, Eds.). Elsevier/North-Holland Biochemical Press, Amsterdam, pp. 337-352.

Ortiz de Montellano, P.R. and Mico, B.A. (1980), Mol. Pharmacol. 18, 128-135.

Ortiz de Montellano, P.R. and Mico, B.A. (1981), Arch. Biochem. Biophys. 206, 43-50.

Ortiz de Montellano, P.R., Mico, B.A., Mathews, J.M., Kunze, K.L., Miwa, G.T., and Lu, A.Y.H. (1981), Arch. Biochem. and Biophys. 210, 717.

Ortiz de Montellano, P.R., Yost, G.S., Mico, B.A., Dinizo, S., Correia, M.A. and Kambara, H. (1979), Arch. Biochem. Biophys. 197, 524.

Philpot, R.M. and Hodgson, E. (1974), Drug Metab. Rev., 3, 231.

Rando, R.R. (1977), Methods in Enzymology 46, 28.

Recknagel, R.D. and Glende, E.A. (1973), CRC Crit. Rev. Toxic. 2, 263.

Reichhart, D., Simon, A., Durst, F., Mathews, J.M. and Ortiz de Montellano, P.R. (1982), Arch. Biochem. Biophys. 216(2), 522-529.

Reitman, S. and Frankel, S. (1957), Am. J. Clin. Path. 28, 56.

Rembra, R.D., Champion, P.M., Fitchen, D.B., Chiang, R., and Hager, L.P. (1979), Biochemistry 18, 2280-2290.

Salach, J.I. (1979), Arch. Biochem. Biophys. 192, 128.

Scardiglia, F. and Roberts, J.D. (1958), Tetrahedron 3, 197.

Schenkman, J.B., Jansson, I., and Robie-Suh, K.M. (1976), Life Sci. 19, 611.

Simmons, H.E. (1961), J. Amer. Chem. Soc. 83, 1657.

Sipes, I.G., Gandolfi, A.J., Pohl, L.R., Krishna, G. and Brown, B.R. (1980), J. Pharmacol. Exp. Ther., 214, 716.

Sipes, I.G., Krishna, G., and Gillette, J.R. (1977), Life Sci. 20, 1541.

- Skrinjaric-Spoljar, M., Matthews, H.B., Engel, J.L. and Casida, J.E. (1971), Biochem. Pharmacol. 20, 1607.
- Smuckler, E.A., Arrenius, E., and Hultin, T. (1967), Biochem. J. 103, 55.
- Smyth, H.F. Jr., Seaton, J., and Fischer, L. (1941), J. Ind. Hyg. Toxicol. 23, 259.
- Stier, A. (1976), Biochem. Pharmacol. 25, 109.
- Strobel, H.W., Lu, A.Y.H., Heidema, J., and Coon, M.J. (1970), J. Biol. Chem. 245, 4851.
- Tabor, C.W., Tabor, H., and Rosenthal, D.M. (1960), J. Biol. Chem. 208, 645-661.
- Takahashi, T., Fukushima, D., Fujimori, K., Iyanagi, T. and Oae, S. (1978), Heterocycles, 10, 229.
- Testa, J., and Jenner, P. (1976), in "Drug Metabolism: Chemical and Biochemical Aspects", Marcel Decker Inc., New York, pp. 419-453.
- Trave, R. and Bianchetti, G. (1960), Atti. Accad. Naz. Lincei, Rend. Classe Sci. Fis. Mat. Nat., 28, 652.
- Ullrich, V., Ruf, H.H., and Wende, P. (1977), Croatica Chem. Acta. 49, 213.
- Unsel, A. and De Matteis, F. (1976), in Proceedings of the International Porphyrin Meeting, Freiburg, May 1-4, 1975.
- Walsh, C. (1982), Tetrahedron 38(7), 871-900.
- Warner, M. and Neims, A.H. (1979), Drug Metab. Dispos. 7, 188.
- Watanabe, Y., Iyanagi, T. and Oae, S. (1980), Tetrahedron Letters 21, 3685.
- Waxman, D.J., and Walsh, C. (1982), J. Biol. Chem. 257, pp. 10446-10457.
- West, S.B., Huang, M.K.T., Miwa, G.T., and Lu, A.Y.H. (1979), Arch. Biochem. Biophys. 193, 42-50.
- White, R.E., and Coon, M.J. (1980), Ann. Rev. Biochem. 49, 315-356.
- Williams, C.H. and Kamin, H. (1962), J. Biol. Chem. 237, 587-595.
- Wislocki, P.G., Miwa, G.T., Lu, A.Y.H. (1980) in "Enzymatic

Basis of Detoxification" (W.B. Jakoby, Ed.) Vol.1, pp. 135-182, Academic Press, New York.

Wittig, G. and Hoffmann, R.W. (1962), Chem. Ber. 95, 2718.

Wittig, G. and Reichel, B. (1963), Chem. Ber. 96, 2851.

Zimmerman, H.J. (1978), "Hepatotoxicity", Ch. 8, Appleton-Century-Crofts, New York.



