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Part I. Metabolic activation of cyclic tertiary amines. Part II
Neurotoxic activation of $\beta,\beta'$-iminodipropionitrile (IDPN)

Engelhart, David Albert, Ph.D.

Case Western Reserve University, 1994
PART I. METABOLIC ACTIVATION OF CYCLIC TERTIARY AMINES.

PART II. NEUROTOXIC ACTIVATION OF BETA, BETA PRIME-IMINODIPROPIONITRILE (IDPN).

by

DAVID ALBERT ENGELHART

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Thesis Advisor: Dr. Lawrence Sayre

Department of Chemistry
CASE WESTERN RESERVE UNIVERSITY
August, 1994
CASE WESTERN RESERVE UNIVERSITY

GRADUATE STUDIES

We hereby approve the thesis of

David Albert Engelhart

candidate for the Ph.D.
degree.*

(signed) Fred L. White
(chair)

[Signatures of other committee members]

date July 11, 1994

*We also certify that written approval has been obtained for any proprietary material contained therein.
PART I. METABOLIC ACTIVATION OF CYCLIC TERTIARY AMINES.

PART II. NEUROTOXIC ACTIVATION OF \( \beta,\beta' \)-IMINODIPROPIONITRILE (IDPN).

Abstract

by

DAVID ALBERT ENGELHART

PART I. METABOLIC ACTIVATION OF CYCLIC TERTIARY AMINES.

Microsomal metabolism of cyclic tertiary amine drugs such as nicotine and 1-(phenylcyclohexyl)piperidine (PCP) is known to be associated with enzyme inactivation and/or covalent binding. While the major primary metabolites, the corresponding iminium ions, have been the major suspects for inducing enzyme inactivation, metabolism beyond the iminium stage rather than direct electrophilic adduction has been implicated, at least in the case of PCP. Using 1-benzylpiperidine (1-BP) and several of its methyl and dimethyl-substituted analogues as well as PCP, we have demonstrated that metabolism of the parent amines results in inactivation of cytochrome P-450 and that the initially formed iminiums are better metabolism-dependent inactivators than the corresponding parent amines. Since the iminiums
themselves are unlikely to be oxidized, the generation of reactive metabolites is
presumed to reflect oxidation of the enamine tautomers. The degree of inactivation
by the parent amines and the iminium/enamines reflects the pattern of endocyclic and
exocyclic substitutions made on the piperidine ring. Elimination of exocyclic N-
dealkylation coupled with the absence of ring substitutions results in maximal
inactivating potential. On the other hand, 2-methyl, 2,6-dimethyl and especially 4,4-
dimethyl substitution results in diminished inactivating potential on account of steric
inhibition of metabolic electrophile generation and/or stimulation of metabolism
through non-nitrogen-directed metabolic pathways.

Cyanide trapping of the various iminums can prevent, at least in part, the
metabolism-dependent inactivation by the parent amines, and can be used to discern
which metabolites arise from metabolism beyond the iminium/enamine stage, such
as 1-benzyl-3-piperidone from 1-benzyl-piperidine. However, cyanide levels needed
for trapping of some iminums can directly inhibit cytochrome P-450, and certain
cyanide adducts can act as metabolism-dependent inactivators. These factors must
be considered when using cyanide as a simple trapping agent.

In the pyrrolidine series, we found that 1-benzyl-3-pyrrolidinone effects a
cytochrome P-450 metabolism-dependent formation of green microsomes, which is
indicative of heme alkylation.
PART II. NEUROTOXIC ACTIVATION OF $\beta,\beta'$-IMINODIPROPIONITRILE (IDPN).

$\beta,\beta'$-Iminodipropionitrile (IDPN) is a chemical neurotoxin used to model the massive focal accumulations of neurofilaments (NF) in proximal axons seen in amyotrophic lateral sclerosis (ALS), and the behavioral excitation seen in extrapyramidal disorders. While the molecular bases of these two neurotoxic conditions have not been established, the inertness of IDPN itself suggests that a metabolic activation process is involved. We have developed a hypothesis for IDPN toxic activation based on the known urinary metabolites of IDPN and the known routes of metabolism of secondary amines. N-hydroxy-IDPN (HOIDPN), the initial product expected from metabolism of IDPN by flavin monooxygenase (FMO) was previously shown to reproduce the neurotoxic effects of IDPN at an 8-fold lower dose. We have investigated the metabolism of IDPN by rat and rabbit liver microsomes. By using the cytochrome P-450 inhibitor, metyrapone, and the FMO alternate substrate, methimazole, the metabolites $\beta$-aminopropionitrile (BAPN) and HOIDPN were identified as arising from cytochrome P-450 and FMO, respectively.

The use of these enzyme specific inhibitors in vivo demonstrates that the neurotoxicity of IDPN results from FMO-dependent metabolism and that cytochrome P-450 metabolic pathways are not involved in toxic activation.
If you want anything bad enough to go out and fight for it.
To work day and night for it.
To give up your time, your peace, and your sleep for it.
If all that you dream and scheme is about it,
And life seems useless and worthless without it.
And if you gladly sweat for it, and fret for it, and plan for it,
And loose all your terror of the opposition for it.
And if you simply go after that thing that you want
With all of your capacity, strength and sagacity,
Faith, hope, and confidence and stern pertinacity.
If neither cold, poverty, famish or gault,
Sickness or pain of body and brain
Can keep you away from the thing that you want.
If dogged and grim you besiege and beset it,
With the help of God, you'll get it.

Les Brown

This work is dedicated to my father whose support made this all possible, and to
my wife, Elizabeth, for believing in me when I doubted myself.
As anyone who has had the pleasure to work with me knows, I am a man of few words, so I will make this brief. I would like to take this opportunity to thank Balaji Venkataraman, Manoj Babu, and Dr. Durgesh Nadkarni for their time and effort in synthesizing the seemingly endless requests for authentic substituted benzylpiperidine standards. I will never forget Bala’s famous words "It’s pure Doc, I checked it by NMR".

I would also like to acknowledge Dr. Pramod Arora for his time and guidance in regards to the IDPN project and Dr. David McCoy for his inspiration, support, and laughter.

Most of all, I would like to thank Dr. Sayre for his unending dedication towards this work and for having the patience to allow me the time I needed to simply make the decision to get this thing done.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO</td>
<td>aldehyde oxidase</td>
</tr>
<tr>
<td>1-BP</td>
<td>1-benzylpiperidine</td>
</tr>
<tr>
<td>1-BP-2-CN</td>
<td>1-benzyl-2-cyanopiperidine</td>
</tr>
<tr>
<td>1-BP-2-en-4-one</td>
<td>1-benzyl-2,3-dihydro-4(1H)-pyridinone</td>
</tr>
<tr>
<td>1-BP-Im⁺</td>
<td>1-benzyl-2,3,4,5-tetrahydropyridinium</td>
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<td>1-BP-3-OH</td>
<td>1-benzyl-3-piperidinol</td>
</tr>
<tr>
<td>1-BP-4-OH</td>
<td>1-benzyl-4-piperidinol</td>
</tr>
<tr>
<td>1-BP-3-one</td>
<td>1-benzyl-3-piperidone</td>
</tr>
<tr>
<td>1-BP-4-one</td>
<td>1-benzyl-4-piperidone</td>
</tr>
<tr>
<td>1-BP-THA dimer</td>
<td>1,1′-dibenzy1-5-(2′-piperidinyl)-1,2,3,4-tetrahydropyridine</td>
</tr>
<tr>
<td>1-BPyr-3-one</td>
<td>1-benzyl-3-pyrrolidinone</td>
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<tr>
<td>α,α-DMBP</td>
<td>α,α-dimethyl-1-benzylpiperidine</td>
</tr>
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<td>α,α-DMBP-Im⁺</td>
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<td>α,α-dimethyl-1-benzyl-4-piperidone</td>
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<td>2,6-DMBP-2-CN</td>
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<td>4,4-DMBP-N-oxide</td>
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<td>4,4-DMBP-3-one</td>
<td>4,4-dimethyl-1-benzyl-3-piperidone</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>FMO</td>
<td>flavin monooxygenase</td>
</tr>
<tr>
<td>G6PD</td>
<td>glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
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<td>2-MBP</td>
<td>2-methyl-1-benzylpiperidine</td>
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<td>2-MBP-Im⁺</td>
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<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>PB</td>
<td>phenobarbital</td>
</tr>
<tr>
<td>PCP</td>
<td>1-(1-phenylcyclohexyl)piperidine</td>
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<tr>
<td>PCP-2-CN</td>
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<td>PCP-3-one</td>
<td>1-(1-phenylcyclohexyl)-3-piperidone</td>
</tr>
<tr>
<td>PCP-4-one</td>
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</tr>
<tr>
<td>SET</td>
<td>single electron transfer</td>
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xviii
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
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**PART II. NEUROTOXIC ACTIVATION OF β,β'-IMINODIPROPIONITRILE**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>BAPN</td>
<td>β-aminopropionitrile</td>
</tr>
<tr>
<td>BHT</td>
<td>butylated hydroxy toluene</td>
</tr>
<tr>
<td>dehydro-IDPN</td>
<td>3-(2-cyanoethylamino)acrylonitrile</td>
</tr>
<tr>
<td>ECC</td>
<td>excitation, circling, and choreiform head and neck movements</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>HOIDPN</td>
<td>N-hydroxy-β,β'-iminodipropionitrile</td>
</tr>
<tr>
<td>ICD</td>
<td>isocitrate dehydrogenase</td>
</tr>
<tr>
<td>IDPN</td>
<td>β,β'-iminodipropionitrile</td>
</tr>
<tr>
<td>MT</td>
<td>microtubules</td>
</tr>
<tr>
<td>NF</td>
<td>neurofilaments</td>
</tr>
</tbody>
</table>
PART I. METABOLIC ACTIVATION OF CYCLIC TERTIARY AMINES.
I.1.1 CYTOCHROME P-450

The cytochrome P-450 dependent mixed function oxygenase system is a superfamily of hemoproteins that has been found to play an integral role in the metabolism of xenobiotics. P-450s are membrane bound enzymes located predominantly in the endoplasmic reticulum of various cell types, with the liver having the highest total P-450 concentration of any organ. The P-450 system is composed of the P-450 isozymes, phospholipid, and a flavin enzyme, NADPH-cytochrome P-450 reductase, which transfers electrons from NADPH to cytochrome P-450. This process leads to the reductive activation of molecular oxygen followed by the insertion of one oxygen atom into the substrate. P-450 catalyzed reactions include hydroxylation, epoxidation, deamination, N-demethylation, desulfuration, and heteroatom oxygenation. Some of the transformations are essential for life such as the conversion of cholesterol to corticoid and sex hormones. P-450 is also involved in the detoxication of xenobiotics by converting them to more polar compounds that can be readily excreted. In some cases, this metabolic process generates products with much greater cytotoxicity, mutagenicity, or carcinogenicity.

I.1.2 AMINE METABOLISM

The microsomal metabolism of cyclic tertiary amines has been studied extensively in the past several years, with a great deal of attention being directed towards 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), nicotine, and
phencyclidine (1-(phenylcyclohexyl)piperidine, PCP) (Figure I.1). Metabolism of these tertiary amines has been shown to be associated with low levels of covalent binding and/or inactivation of the metabolizing enzymes (Singer et al., 1985; Shigenaga et al., 1988; Hoag et al., 1984). One pathway for oxidation of tertiary

![MPTP, Nicotine, PCP structures](image)

**Figure I.1** Structures of MPTP, nicotine, and PCP.

amines in general involves oxygenation at nitrogen, converting $R_3N$ to $R_3N^+\cdot$, by microsomal flavin monooxygenase (FMO) as described by Ziegler (1980). Another pathway involves what is referred to as either N-dealkylation or C-deamination, the result of dissociation following either dehydrogenation to an iminium ion or $\alpha$-C hydroxylation to a carbinolamine. Dehydrogenation is mediated by the flavin-dependent mitochondrial monoamine oxidase (MAO), as with MPTP (Singer et al., 1985). However, structural requirements are very specific in the case of secondary and tertiary amines. Much more general is the $\alpha$-C hydroxylation by cytochrome P-450. These various pathways for microsomal metabolism of tertiary amines are shown in Scheme I.1.
Scheme I.1 Metabolism of tertiary amines.

There are two different proposed mechanisms for the oxidation of tertiary amines by P-450. Cytochrome P-450 can achieve N-dealkylation via a formal Cα hydroxylation to the carbinolamine via H· abstraction, followed by HO· transfer from iron to Cα (Scheme I.2). However, the more widely accepted mechanism is that amine oxidation involves initial single electron transfer (SET) to give an aminyl cation radical (Guengerich and Macdonald, 1990). Initial SET is followed by H⁺ transfer from Cα and then either (i) "rebound" of HO· from Fe(IV)OH to the resulting Cα radical intermediate or (ii) either H· transfer or, more likely, sequential
H⁺/e⁻ transfer, giving imine/iminium, which is converted to carbinolamine by transfer of HO⁻ from iron.

I.1.3 IMINIUM - ENAMINE EQUILIBRIUM

Iminium intermediates of acyclic tertiary amines readily hydrolyze, through the carbinolamines, to secondary amines and aldehydes or ketones. For cyclic tertiary amines, hydrolysis of the endocyclic iminium remains reversible, and the equilibrium in Scheme I.3 will be maintained. α-Aryl (or vinylogous α-aryl) amines, such as

![Scheme I.3 Equilibration of endocyclic iminium ions.](image)

MPTP, nicotine, and PCP, have pKₐ values about 1 pH unit lower than normal tertiary amines, and their corresponding enamines are thought to have pKₐ values about 1 pH unit lower than the parent amines. Thus, α-aryl cyclic enamines should have pKₐs of about 8.5, suggesting that the enamines could reach a concentration of nearly 10% that of the iminium at physiologic pH.

I.1.4 METABOLIC DETOXIFICATION

Detoxification of the endocyclic iminium metabolites is traditionally
considered to involve conversion of iminium intermediates to lactams by cytosolic aldehyde oxidase (AO). The conversion of nicotine-$\Delta^1(5')$-iminium to cotinine (Scheme I.4) is a classical example of the action of AO on iminium ions. However, this pathway is not general for metabolism of endocyclic iminiums. In the case of PCP, no lactam metabolite has ever been reported, probably on account of the steric bulk.
α to nitrogen. It is possible that the lack of AO activity in this case is responsible for the increased level of covalent binding of PCP relative to nicotine.

I.1.5 METABOLIC ACTIVATION

The observed metabolism-dependent inactivation and/or covalent binding of nicotine, PCP, and MPTP was initially thought to be due to the iminium species acting as electrophilic intermediates which could alkylate protein-based nucleophiles. Obach and Van Vunakis (1988) demonstrated the covalent non-metabolic binding of nicotine iminium ion to liver microsomes, and suggested that it is a result of electrophilic attack by the iminium on protein-based sulphydryl groups. Shigenaga et al. (1988) found that incubation of (S)-[5-3H]nicotine with rabbit liver microsomes in the presence of NADPH resulted in the covalent binding of radioactive material to microsomal macromolecules. This covalent binding was reduced by 46 % in the presence of 0.5 mM NaCN, which trapped the iminium as the corresponding α-aminonitrile derivative. Similarly, Ward et al. (1982) demonstrated that the PCP iminium ion could also be trapped as the α-aminonitrile in the presence of 0.5 mM NaCN. This reduced the metabolism-dependent covalent binding of PCP to rabbit liver microsomes by 82 percent. Cyanide has also been shown to inhibit the irreversible NADPH-dependent loss of N-demethylase activity and the reduction of microsomal cytochrome P-450 content resulting from the incubation of PCP with rabbit liver microsomes (Hoag et al., 1984).
Although all these studies demonstrated the minimal criterion of iminium ion generation in covalent binding, recent studies have provided strong evidence that metabolites formed beyond the iminium/enamine stage, rather than the iminium itself, are responsible for inactivation of microsomes and/or covalent binding to macromolecules. Incubation of the PCP iminium ion itself with microsomal fractions results in little, if any, inactivation or covalent binding to cytochrome P-450 in the absence of NADPH (Hoag et al., 1987; Osawa and Coon, 1989).

As shown in Scheme I.3 (page 6), iminium ions exist in equilibrium not only with enamines, but also with the carbinolamines which are in equilibrium with the ring-opened aldehydes or ketones. The possibility of the ring-opened aldehydes or ketones as potential metabolites or intermediates that may be responsible for the observed bioalkylation reactions of cyclic tertiary amines was investigated by Brady et al. (1987). It was found that cytochrome P-450 inactivation by PCP congeners with various heterocyclic ring sizes correlates with the tendency for maintaining the closed-ring form of the corresponding iminium. This suggests that the iminium or enamine species rather than the ring-opened aminoaldehyde or is responsible for cytochrome P-450 inactivation.

1.1.6 ISOZYME SPECIFICITY

Specific P-450 isozymes can be identified by their primary sequence hematology and characteristic tissue expression and regulation. Different drug
treatments affect the relative concentrations of P-450s. Purification and characterization of many of these isozymes has provided evidence that distinct P-450s are specific for certain types of substrates. Two isozymes demonstrated highest rates for C-oxidation and N-demethylation pathways of nicotine metabolism; these activities were associated with a phenobarbital (PB) inducible isozyme, form 2B4, as well as a constitutive isozyme, form 2C3 (McCoy et al., 1989). Shigenaga et al. (1988) found that PB-induction of rabbit liver microsomes increased both the rate of metabolism and metabolism-dependent covalent binding of nicotine to microsomal macromolecules. Phenobarbital pretreatment of rabbits and rats also enhanced the inhibitory effects of PCP on N-demethylase activity with microsomal fractions (Hoag et al., 1984). Osawa and Coon (1989) later found the mechanism-based inactivation of PCP was highly selective for P-450 form 2B4, both in rabbit liver microsomes and in the reconstituted enzyme system. The PCP iminium ion itself, however, displays metabolism-dependent inactivation of both P-450 form 2B4 and form 2C3. This result supports the distinction between the iminium formation and subsequent transformation stages of metabolism, with only the latter being associated with covalent binding potential.

I.1.7 METABOLISM BEYOND THE IMINiUM/ENAMiNE STAGE

Based on the preferential metabolism of hydrophobic rather than hydrophilic substrates by cytochrome P-450 and the low tendency of the cationic iminium species
toward further oxidation, it seems unlikely that the latter would serve as substrates for cytochrome P-450 oxidative metabolism. On the other hand, based on the ability of cytochrome P-450 to metabolize alkenes and the free base forms of amines, it seems reasonable to presume that the electron rich enamines are excellent substrates for processing by cytochrome P-450 and possibly other enzymes involved in oxidative metabolism. A plausible mechanism for cytochrome P-450 metabolism of endocyclic enamines, that is consistent with the electron transfer mechanism discussed in section I.1.2 (Scheme I.2, page 5), is shown in Scheme I.5. According to this mechanism, the endocyclic enamines should be prime candidates for giving rise to second-

Scheme I.5 Proposed pathways for the metabolism of the iminium/enamine intermediates by cytochrome P-450.
generation metabolites with reactive properties. As already stated, it appears as though metabolism beyond the iminium stage is responsible for the covalent binding associated with metabolism of cyclic tertiary amines. Therefore, characterization of metabolites or intermediates beyond the iminium stage is crucial in determining what culprits are responsible for the observed metabolism-dependent inactivation and/or covalent binding events.

PCP metabolism is complicated by the generation of many metabolites arising from hydroxylation of the cyclohexyl ring (Gole et al., 1988). In order to simplify the metabolic profile to mainly that of nitrogen-based pathways, 1-benzylpiperidine (1-BP) was chosen for comparative oxidative metabolism studies. However, work by Leonard and Hauck (1957) on the chemistry of endocyclic iminium and enamine species derived from 1-methylpiperidine led us to suspect that an iminium-enamine coupling reaction might complicate our analysis of metabolites. Since these workers found that ring methyl substituents retarded iminium-enamine coupling, we chose to include several methyl- and dimethyl-substituted analogues of 1-BP in our study: 2-methyl-1-benzylpiperidine (2-MBP), cis-2,6-dimethyl-1-benzylpiperidine (2,6-DMBP), 4,4-dimethyl-1-benzylpiperidine (4,4-DMBP), and \( \alpha,\alpha \)-dimethyl-1-benzylpiperidine (\( \alpha,\alpha \)-DMBP) were chosen, along with PCP (Figure I.2). A further advantage of these analogues was the expectation that the pattern of methyl substituents would differentially affect the course of metabolic oxidation of the corresponding enamines according to Scheme I.5. Our finding that certain analogues
might display greater or lesser degrees of enzyme inactivation would thus provide clues as to which oxidation pathway(s) are associated with reactive intermediate generation.

![Chemical structures of cyclic tertiary amines](image)

**Figure 1.2** Structures of cyclic tertiary amines.

Some information on the nature of metabolites beyond the iminium/enamine stage has been revealed in the case of the PCP iminium ion. Hoag et al. (1988) characterized 1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone (3, Scheme I.5, R = 1-phenylcyclohexyl) as an oxidative metabolite resulting from the incubation of PCP iminium ion with rabbit liver microsomes. Metabolism was proposed to proceed through oxidation of the conjugate enamine free base of the iminium ion substrate to yield an allyl alcohol intermediate (Scheme I.5, path A, page 11). This intermediate is further oxidized to yield the final product which accounts for only about 40% of
the iminium ion metabolized. Therefore, it is possible that the intermediate allyl alcohol (2, Scheme I.5, page 11) may be involved in the observed metabolism dependent inactivation and/or covalent binding of PCP and its iminium ion to microsomal macromolecules, conceivably, through the electrophilic 2,3-dihydropyridinium species (4, Scheme I.5, page 11).

Masumoto et al. (1989) reported 1-(phenylcyclohexyl)-3-piperidone (1, scheme I.5, R=1-phenylcyclohexyl, page 11) as a metabolite of PCP from incubation with rat liver microsomes in the presence of NADPH. It was found that incubation in the presence of cyanide inhibited the formation of this metabolite, which strongly suggests that it is formed as a direct result of metabolism of the iminium intermediate rather than from oxidation of the 3-ol arising directly from PCP. A plausible mechanistic pathway for the formation of this metabolite is shown in scheme I.5 (page 11, path B).
CHAPTER I.2 RESULTS
I.2.1 AMINE METABOLISM

For the purposes of presentation of the results, it is necessary to introduce the proposed metabolic pathways for the tertiary amines studied. Although some pathways are common to all the amines, other pathways and intermediates pertain to specific methyl-substituted cases. These pathways are presented in Schemes I.6 through I.10 (pages 17-20).

The metabolic rates and major product profiles for the tertiary amines studied are shown in Table I.1 (page 21). Data is shown for metabolism in the absence and presence of cyanide used to trap the initial iminium metabolite. The first three amines listed undergo NADPH-dependent oxidation principally at the N-C bond, leading to endocyclic (major) and exocyclic (minor) iminium intermediates, the latter dissociating to secondary amines and benzaldehyde. The preference for endocyclic over exocyclic oxidation was also seen previously by Ho and Castagnoli (1980) in the microsomal metabolism of 1-benzylpyrrolidine. The rates of metabolism were obtained from averaging data points from the initial stages of metabolism that were linearly dependent on both time and enzyme concentration.

1-BP and its analogues that were studied all have similar metabolic rates, although PCP and especially 4,4-DMBP are metabolized up to a rate approximately twice that of the other compounds. Cytochrome P-450 binding constants obtained for 1-BP, 2,6-DMBP, and PCP were 92, 37, and 10 μM, respectively, and appear to reflect the differences in metabolic rates. Unlike the majority of other tertiary amines
Scheme I.6 Proposed metabolic pathways for microsomal metabolism of 1-benzylpiperidine (1-BP).
Scheme I.7 Proposed metabolic pathways for microsomal metabolism of cis-2,6-dimethyl-1-benzylpiperidine (2,6-DMBP).

Scheme I.8 Proposed metabolic pathways for microsomal metabolism of α,α-dimethyl-1-benzylpiperidine (α,α-DMBP) and phencyclidine (PCP- dashed lines).
Scheme 1.9 Proposed metabolic pathways for microsomal metabolism of 2-methyl-1-benzylpiperidine (2-MBP).
Scheme I.10 Proposed metabolic pathways for microsomal metabolism of 4,4-dimethyl-1-benzylpiperidine (4,4-DMBP).
studied (Table I.1), only about 12% of the total metabolism of 4,4-DMBP was accounted for by the iminium metabolite, while 3% was represented by the exocyclic pathway. Other NADPH-dependent metabolites with retention times of 3.7 and 5.4 minutes were observed. However, neither of these corresponded to either of the potential metabolites 4,4-DMBP-3-one or 4,4-DMBP-N-oxide for which we had authentic standards. For the other 1-BP analogues, 50 to 90% of the total metabolism

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolic Rate (nmol/min/mg)</th>
<th>Major products formed during initial metabolic phasea</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-BP</td>
<td>55 (10 mM)</td>
<td>“dimer” (50%), PhCHO (1.5%)</td>
</tr>
<tr>
<td>1-BP (1mM KCN)</td>
<td>52 (10 mM)</td>
<td>α-cyano (25%), PhCHO (1.0%)</td>
</tr>
<tr>
<td>1-BP-THA Dimer</td>
<td>40 (10 mM)</td>
<td></td>
</tr>
<tr>
<td>2,6-DMBP</td>
<td>65 (10 mM)</td>
<td>iminium (71%), PhCHO (0.5%)</td>
</tr>
<tr>
<td>2,6-DMBP (1mM KCN)</td>
<td>59 (10 mM)</td>
<td>α-cyano (58%), PhCHO (0.5%)</td>
</tr>
<tr>
<td>2,6-DMBP-Im+</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>2-MBP</td>
<td>54 (10 mM)</td>
<td>n.d.</td>
</tr>
<tr>
<td>2-MBP (1mM KCN)</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>2-MBP-Im+</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>4,4-DMBP</td>
<td>112 (10 mM)</td>
<td>iminium (12%), PhCHO (3.2%)</td>
</tr>
<tr>
<td>4,4-DMBP (1mM KCN)</td>
<td>31 (10 mM)</td>
<td>PhCHO (7.1%)</td>
</tr>
<tr>
<td>4,4-DMBP-Im+</td>
<td>44 (1 mM)</td>
<td></td>
</tr>
<tr>
<td>PCP</td>
<td>89 (1 mM)</td>
<td>iminium (30%)</td>
</tr>
<tr>
<td>PCP (1mM KCN)</td>
<td>80 (1 mM)</td>
<td>α-cyano (11%)</td>
</tr>
<tr>
<td>PCP-Im+</td>
<td>80 (1 mM)c</td>
<td></td>
</tr>
<tr>
<td>α,α'-DMBP</td>
<td>37 (1 mM)</td>
<td>iminium (89%)</td>
</tr>
<tr>
<td>α,α'-DMBP (0.5 mM KCN)</td>
<td>42 (1 mM)</td>
<td>iminium (37%), α-cyano (50%)</td>
</tr>
<tr>
<td>α,α'-DMBP-Im+</td>
<td>70 (1 mM)c</td>
<td></td>
</tr>
</tbody>
</table>

a Represented as percentage of the total starting material consumed.

b Not determined.
c Estimated as a lower limit.
was accounted for by the iminium metabolites. Although the endocyclic iminium was found to be a major metabolite for 1-BP, it was not observed directly, but as the Cα-Cα'-coupled dimer (Scheme I.6, page 17). 1-BP-dimer formation is very rapid at pH 7.4 and 37 °C (incubation conditions). Dimerization of the iminium also occurred at room temperature in the HPLC aqueous buffer, 0.1 M sodium acetate, 1 % (v/v) triethylamine, pH 7.0. The dimerization of cyclic iminium species, in equilibrium with the corresponding enamines, was reported previously for substituted piperidines (Leonard and Hauck, 1957; Beeken and Fowler, 1979). Brandange and Lindblom (1979) have also reported dimer formation for the nicotine iminium ion in aqueous solutions of pH > 1. The different rate and metabolite profile for 4,4-DMBP is possibly a result of metabolism of the methyl groups at the four position, resulting in less oxidation at nitrogen. Similarly, PCP is also metabolized at a higher rate with only 30 % of metabolism represented as the iminium. This was expected, as stated in the introduction, because PCP metabolism involves the generation of many non-nitrogen-based metabolites, accounting for the lower percentage of the iminium being formed. In the case of 2-MBP, authentic standards for the two possible iminiums could not be obtained at the time these experiments were being performed, making quantification of these metabolites impossible.

Also presented in Table I.1 are the rates and profiles for the metabolism of the amines in the presence of KCN. As stated in the introduction, the purpose of adding cyanide was to trap the iminium intermediates as their corresponding α-cyano adducts as shown in Scheme I.11. However since cyanide is a potential cytochrome
Scheme I.11 Trapping of the iminium/enamine intermediates with cyanide.

P-450 inhibitor, it was important to determine that the levels of KCN used to trap the iminiums would not substantially inhibit amine metabolism. Enzyme inhibition would be avoided altogether by adding cyanide only at the end of the incubation. However this was not possible owing to the instability of iminiums toward dimerization and further metabolism. Based on other literature reports (Ho and Castagnoli, 1980) a level of 0.5 - 1.0 mM was chosen as a compromise between efficient trapping of the iminiums and cytochrome P-450 inhibition. An excess of cyanide was still added at the end in order to assure efficient completion of the iminium to α-cyano conversion. 1-BP and PCP rates in the presence of 1.0 mM KCN were reduced only by 5 -10%, and the amount of metabolism represented as the α-cyano adduct from trapping of the iminium accounted for about half of the iminium formed in the absence of KCN.

Initial studies on 2,6-DMBP also demonstrated a 5 -10% reduction in the metabolic rate in the presence of 1.0 mM KCN, with 58 % of the total metabolism being accounted for as the corresponding α-cyano adduct. However, later experiments done with this compound in the presence of cyanide gave some
conflicting results. While the metabolic rate in the absence of cyanide was consistent with the earlier results, the rate in the presence of 1.0 mM cyanide was now found to be reduced by 50%. This discrepancy caused us to investigate the inhibitory effect of cyanide more carefully, as discussed in section 1.2.3 (page 27).

The metabolic rate for α,α-DMBP was not altered by the presence of 0.5 mM KCN. Fifty percent of the total metabolites was the α-cyano adduct while the iminium accounted for another 37% (the samples were not quenched with excess cyanide in this case). The total of these two percentages was equivalent to that of the iminium alone in the absence of KCN.

Unlike the other amines studied, the 4,4-DMBP metabolic rate was significantly reduced in the presence of both 1.0 mM and 0.1 mM KCN, from 112 to 31 and 34 nmol/min/mg respectively. The α-cyano adduct could not be quantitated for this compound due to the lack of an authentic standard, but no iminium was observed when cyanide was present at either concentration. The amount of metabolite formed by the exocyclic pathway, i.e. PhCHO, was enhanced by both levels of cyanide to an amount over twice that found without KCN present. An unidentified metabolite (retention time of 5.4 min) was reduced by 27% and 54% by 0.1 and 1.0 mM KCN, respectively. While another unidentified metabolite (retention time of 3.7 min) was not affected by 0.1 mM KCN, it was reduced by 51% by 1.0 mM cyanide. Thus, for 4,4-DMBP, cyanide reduces the overall rate and promotes the formation of two unidentified metabolites, while increasing the percentage of metabolism.
through the exocyclic pathway by a factor of two.

Large scale incubations (10.0 - 20.0 mL) were done on 1-BP in an effort to identify secondary metabolites, e.g., those resulting from further oxidation of iminium/enamine. The "dimer" still represents the major product (36%), with small amounts of the 3-hydroxy (1-BP-3-OH), 4-hydroxy (1-BP-4-OH), and the 3-one (1-BP-3-one) derivatives being formed (Scheme I.12). The 3- and 4-hydroxy compounds presumably arise from direct monoxygenation of 1-BP, similar to the generation of the 3- and 4-hydroxy compounds in the case of PCP (Gole et al., 1988). While the 3-one was quantitated by derivatization to its 2,4-DNP adduct and also by sodium borohydride reduction to the 3-hydroxy metabolite (measuring the increase in peak integration). This was required due to our inability to obtain HPLC peak resolution of the 3-one from other analytes. In the presence of 1.0 mM KCN, 1-BP-3-OH and 1-BP-4-OH were still formed, while the formation of 1-BP-3-one was inhibited.
Thus, the 3-one does not appear to result from further oxidation of the 3-hydroxy metabolite. The fact that the 4-one was not observed, which would likely be a metabolite formed from the 4-hydroxy if such alcohol dehydrogenation were occurring, is consistent with this interpretation. These results are similar to those obtained by Masumoto et al. (1989) who also observed the 3- and 4-hydroxy compounds, as well as the 3-one, but not the 4-one for the microsomal metabolism of PCP.

A preliminary study performed with the purified cytochrome P-450 isozyme 2B4, the major phenobarbital-inducible isozyme, demonstrated efficient generation of 1-BP-3-one from 1-BP. In this purified system, the 3-one was detected by HPLC analysis directly, eliminating the need for 2,4-DNP derivatization or NaBH4 reduction to the alcohol.

### 1.2.2 IMINIUM/ENAMINE METABOLISM

Since we hypothesize that enzyme inactivation by and/or covalent binding of cyclic tertiary amines is a consequence of metabolism beyond the iminium/enamine stage, determination of the metabolic rates for the corresponding iminiums (independently prepared) was attempted. The PCP and α,α'-DMBP iminiums (PCP-Im+ and α,α'-DMBP-Im+) displayed non-linear NADPH-dependent metabolism with respect to time, along with substantial turnover in the absence of a NADPH regenerating system. Since the metabolic rate decreased with time, only a lower limit
could be estimated from the initial slopes. As listed in Table I.1 (page 21), rates of 80 and 70 nmol/min/mg were calculated for the PCP and \( \alpha,\alpha\)-DMBP iminiums, respectively. Since the 1-BP-Im\(^+\) could not be studied directly due to rapid dimerization, the dimer, which itself represents an iminium/enamine pair, was screened as a potential substrate for cytochrome P-450. Not surprisingly, it was found to be a relatively good substrate with an initial metabolic rate of 40 nmol/min/mg, which is very similar to the rates obtained for most of the amines presented earlier. Likewise, the 4,4-DMBP-Im\(^+\) was also processed by cytochrome P-450 with an initial rate of 44 nmol/min/mg. While metabolism dependent HPLC peaks were found after incubation with microsomes, none of them were identified.

The 2-MBP and 2,6-DMBP iminiums did not display any metabolic activity according to the HPLC analysis performed upon samples incubated with microsomes. However, since both of these compounds are known to be metabolism-dependent inactivators of cytochrome P-450 (data will be presented later), they must be metabolized to some extent (see Discussion section I.3.1, page 45).

I.2.3 CYANIDE INHIBITION OF CYTOCHROME P-450 METABOLISM

Based on literature precedent (Ho and Castagnoli, 1980), we initially chose to use 1.0 mM KCN in incubations intended to trap the iminium metabolites as their \( \alpha\)-cyano adducts. The metabolic rates we determined for PCP, 1-BP, and 2,6-DMBP in the presence of 1.0 mM KCN were found to be only slightly compromised by this
level of KCN. However, the rate of metabolism of 4,4-DMBP was found to be reduced by 72% in the presence of 1.0 mM KCN. In fact, even 0.1 mM KCN had a large inhibitory effect on the rate of 4,4-DMBP metabolism. At this point, the rate for 2,6-DMBP in the presence of KCN was reetermined as a control experiment. In the absence of cyanide the rate was the same as previously reported, but the rate in the presence of 1.0 mM KCN was reduced by 50%. One explanation for these conflicting results could be that the flavin reductase (supplying electrons to cytochrome P-450) level in these particular microsomes was very low compared to the ones used for the earlier studies. If the reductase level was low, then these microsomal preparations could display a more pronounced inhibition by cyanide if cyanide also inhibited the reductase. However, control studies done with cytochrome c as the acceptor, demonstrated that concentrations of cyanide up to 1.0 mM did not inhibit reductase activity in the microsomes in question. Thus, the discrepancy in extent of cyanide metabolism is not due to possible variations in reductase levels between the different microsomal preparations.

In order to determine if the observed effect of KCN was dependent upon the individual batches of microsomes used, microsomes prepared from ten different rabbits (5 from PB-induced and 5 from control rabbits) were incubated for 10 minutes with 0, 0.2, and 1.0 mM KCN and checked for benzphetamine N-demethylase activity. The results indicate that approximately 17% of the P-450 activity was inhibited by 0.2 mM KCN for both PB-induced and control microsomes, while 49
and 39% inhibition was found for PB-induced and control microsomes, respectively, in the presence of 1.0 mM KCN. The results were consistent for all the microsomes tested with a standard deviation of less than 5%. These results, along with those obtained with other concentrations of KCN are listed in Table I.2 (see next page) and plotted in Figure I.3.

Figure I.3 Cyanide inhibition of benzphetamine N-demethylation with PB-induced rabbit liver microsomes. Benzphetamine (1.0 mM) was incubated at 37 °C, for 10 minutes, with varying concentrations of KCN. Values are an average of at least 3 determinations.
TABLE I.2 Cyanide inhibition of benzphetamine N-demethylation with PB-induced rabbit liver microsomes. Benzphetamine (1.0 mM) was incubated at 37 °C, for 10 minutes, with varying concentrations of KCN.

<table>
<thead>
<tr>
<th>[KCN] (mM)</th>
<th>% Activity Direct Assay&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Activity After Pelleting/Resuspension&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>98 ± 1</td>
<td>103</td>
</tr>
<tr>
<td>0.10</td>
<td>94 ± 1</td>
<td>100</td>
</tr>
<tr>
<td>0.20</td>
<td>83 ± 3</td>
<td>n.d.&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.20</td>
<td>84 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>n.d.</td>
</tr>
<tr>
<td>0.25</td>
<td>87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>103</td>
</tr>
<tr>
<td>0.50</td>
<td>78 ± 2</td>
<td>106</td>
</tr>
<tr>
<td>1.00</td>
<td>51 ± 4</td>
<td>105</td>
</tr>
<tr>
<td>1.00</td>
<td>61 ± 2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values represent percent of control activity ± S.E.M. of at least 3 determinations.

<sup>b</sup>Data represents an average of two determinations

<sup>c</sup>Control microsomes were used

<sup>d</sup>Not determined

In order to determine if inhibition by cyanide is reversible or irreversible, cytochrome P-450 assays were conducted before and after pelleting/resuspension, which allows for removal of any reversibly bound agents. In these cases, Lowry protein determinations must be done on each sample to correct for the loss of microsomal material when performing this procedure. Since full activity was seen after pelleting/resuspension, the inhibition of cytochrome P-450 by cyanide is reversible.

Nonetheless, it is clear that the 0.5 - 1.0 mM level of KCN causes substantial inhibition of benzphetamine N-demethylase activity and thus cannot be considered an innocent level of KCN for trapping iminium intermediates. Furthermore, the extent of cyanide inhibition appears to be greater for certain amine substrates. It thus seems
wise to determine cyanide inhibition for each potential amine substrate before
deciding on an appropriate KCN concentration to use in iminium trapping
experiments.

1.2.4 INHIBITION OF BENZPHE TAMINE N-DEMETHYLASE ACTIVITY

The ability of the various amines and metabolic intermediates to inhibit and/or
inactivate cytochrome P-450 was investigated by assessing microsomal benzphetamine
demethylase activity. The results are presented in Table I.3 (page 33). This is the
standard assay for the phenobarbital-inducible P-450 isofrom (2B4) that is most active
in aliphatic amine metabolism. No inhibition was seen for any of the amines studied
in the absence of NADPH. However, all of the compounds exhibited some level of
metabolism-dependent inhibition of cytochrome P-450, which was predominantly
irreversible, as shown by comparing the inhibitory potency after
pelleting/resuspension of the microsomes. The rank order of irreversible inhibitory
potency was α,α-DMBP > PCP ≥ 2-MBP > 1-BP > 2,6-DMBP > 4,4-DMBP.

In comparison to the parent amines 2,6-DMBP, 4,4-DMBP, and PCP, the
corresponding iminiums, especially for PCP, exhibited a more pronounced
metabolism-dependent inhibition of enzyme activity, which again was mostly
irreversible. These finding are consistent with the iminiums being on the pathway of
metabolism-dependent inactivation of microsomal P-450 by the parent amines.

In the case of 1-BP, the effect of the iminium could not be observed directly
due the rapid dimerization discussed earlier. However, the dimer, which is an iminium/enamine pair, irreversibly inhibited cytochrome P-450 in a metabolism-dependent manner at an efficiency which fell in the mid-range of that seen for the monomeric iminiums. The $\alpha,\alpha$-DMBP-Im$^+$ also caused a large level of irreversible microsomal P-450 inactivation. However, for this compound, the level of inactivation for the iminium was identical to that of the parent amine itself.

In contrast to the other iminiums studied, the 2-MBP-Im$^+$ demonstrated much less irreversible metabolism-dependent inhibition of cytochrome P-450 activity than the corresponding parent amine. As stated previously, there is the possibility of generating two different endocyclic iminium intermediates (Scheme I.9, page19) for 2-MBP and only the more substituted iminium was purified and used for these studies. The results obtained, therefore, suggest that the level of inactivation observed for the parent amine is a consequence of metabolism through both iminiums, but may reflect predominantly the less substituted iminium, which was not tested by us. The rank of irreversible inhibitory potency for the iminiums listed was PCP $>$ $\alpha,\alpha$-DMBP-Im$^+$ $>$ 1-BP-dimer $\geq$ 2,6-DMBP-Im$^+$ $>$ 4,4-DMBP-Im$^+$ $>$ 2-DMBP-Im$^+$.

Of all the iminiums screened, only PCP-Im$^+$ produced any significant inactivation of cytochrome P-450 in the absence of added G6PD, and that inactivation was found to be irreversible. These results confirm those obtained by Hoag et al. (1987) who suggest that PCP-Im$^+$ possesses a direct covalent binding capability. However, this iminium did not produce any inactivation of purified cytochrome P-450
<table>
<thead>
<tr>
<th>Substrate (1 mM)</th>
<th>% Activity Direct Assay</th>
<th>% Activity After Pelleting/Resuspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCP, + G6PD</td>
<td>58 ± 6</td>
<td>57 ± 9</td>
</tr>
<tr>
<td>PCP, - G6PD</td>
<td>98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>104 ± 4</td>
</tr>
<tr>
<td>PCP-Im&lt;sup&gt;+&lt;/sup&gt;, + G6PD</td>
<td>36 ± 2</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>PCP-Im&lt;sup&gt;+&lt;/sup&gt;, - G6PD</td>
<td>80 ± 2</td>
<td>78 ± 4</td>
</tr>
<tr>
<td>PCP-2-CN, + G6PD&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60 ± 9</td>
<td>94 ± 9</td>
</tr>
<tr>
<td>PCP-2-CN, - G6PD&lt;sup&gt;c&lt;/sup&gt;</td>
<td>77 ± 1</td>
<td>105 ± 8</td>
</tr>
<tr>
<td>1-BP, + G6PD</td>
<td>57 ± 5</td>
<td>65 ± 7</td>
</tr>
<tr>
<td>1-BP, - G6PD</td>
<td>99&lt;sup&gt;b&lt;/sup&gt;</td>
<td>99 ± 2</td>
</tr>
<tr>
<td>1-BP-THA dimer, + G6PD</td>
<td>51 ± 2</td>
<td>60 ± 1</td>
</tr>
<tr>
<td>1-BP-THA dimer, - G6PD</td>
<td>100 ± 4</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>1-BP-2-CN, + G6PD</td>
<td>80 ± 9</td>
<td>92 ± 4</td>
</tr>
<tr>
<td>1-BP-2-CN, - G6PD</td>
<td>87 ± 10</td>
<td>95 ± 2</td>
</tr>
<tr>
<td>1-BP-3-one, + G6PD</td>
<td>86 ± 8</td>
<td></td>
</tr>
<tr>
<td>1-BP-3-one, - G6PD</td>
<td>96 ± 5</td>
<td></td>
</tr>
<tr>
<td>2,6-DMBP, + G6PD</td>
<td>71 ± 3</td>
<td>70 ± 2</td>
</tr>
<tr>
<td>2,6-DMBP, - G6PD</td>
<td>98&lt;sup&gt;b&lt;/sup&gt;</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>2,6-DMBP-Im&lt;sup&gt;+&lt;/sup&gt;, + G6PD</td>
<td>56 ± 2</td>
<td>62 ± 1</td>
</tr>
<tr>
<td>2,6-DMBP-Im&lt;sup&gt;+&lt;/sup&gt;, - G6PD</td>
<td>96 ± 4</td>
<td>108 ± 2</td>
</tr>
<tr>
<td>2,6-DMBP-2-CN, + G6PD</td>
<td>54 ± 2</td>
<td>63 ± 1</td>
</tr>
<tr>
<td>2,6-DMBP-2-CN, - G6PD</td>
<td>104 ± 1</td>
<td>102 ± 3</td>
</tr>
<tr>
<td>4,4-DMBP, + G6PD</td>
<td>85 ± 11</td>
<td>93 ± 4</td>
</tr>
<tr>
<td>4,4-DMBP, - G6PD</td>
<td>95 ± 2</td>
<td>104 ± 3</td>
</tr>
<tr>
<td>4,4-DMBP-Im&lt;sup&gt;+&lt;/sup&gt;, + G6PD</td>
<td>75 ± 6</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>4,4-DMBP-Im&lt;sup&gt;+&lt;/sup&gt;, - G6PD</td>
<td>99&lt;sup&gt;b&lt;/sup&gt;</td>
<td>102 ± 3</td>
</tr>
<tr>
<td>α,α-DMBP, + G6PD</td>
<td>43 ± 3</td>
<td>45 ± 5</td>
</tr>
<tr>
<td>α,α-DMBP, - G6PD</td>
<td>95 ± 3</td>
<td>104 ± 3</td>
</tr>
<tr>
<td>α,α-DMBP-Im&lt;sup&gt;+&lt;/sup&gt;, + G6PD</td>
<td>37 ± 4</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>α,α-DMBP-Im&lt;sup&gt;+&lt;/sup&gt;, - G6PD</td>
<td>91 ± 7</td>
<td>97 ± 4</td>
</tr>
<tr>
<td>2-MBP, + G6PD</td>
<td>57 ± 5</td>
<td>58 ± 2</td>
</tr>
<tr>
<td>2-MBP, - G6PD</td>
<td>99 ± 1</td>
<td>105 ± 3</td>
</tr>
<tr>
<td>2-MBP-Im&lt;sup&gt;+&lt;/sup&gt;, + G6PD</td>
<td>85 ± 3</td>
<td>89 ± 8</td>
</tr>
<tr>
<td>2-MBP-Im&lt;sup&gt;+&lt;/sup&gt;, - G6PD</td>
<td>96 ± 3</td>
<td>101 ± 3</td>
</tr>
<tr>
<td>2-MBP-2-CN, + G6PD</td>
<td>70 ± 7</td>
<td>79 ± 8</td>
</tr>
<tr>
<td>2-MBP-2-CN, - G6PD</td>
<td>96 ± 6</td>
<td>103 ± 4</td>
</tr>
</tbody>
</table>

<sup>a</sup> incubated for 30 minutes, 37 °C, pH 7.4 with microsomes from PB-induced rabbits. Values are percent of control activity ± S.E.M. of at least 3 determinations.

<sup>b</sup> single determination

<sup>c</sup> 0.5 mM concentration
isozyme systems as reported by Osawa and Coon (1989). This suggests that the inactivation observed for the PCP-Im⁺ without added G6PD represents either a background metabolism arising from adventitious reducing equivalents present in this crude system, a complication not apparent with the weaker inactivators studied, or represents a separate enzymatic pathway present in the microsomal system that is not NADPH-dependent.

Based on the ability to trap the various iminium metabolites with cyanide (to a greater extent in some cases than in others), the presence of cyanide in the incubation medium would be expected to inhibit the loss of cytochrome P-450 activity if the iminium-enamine species were an obligatory intermediate in the inactivation process. Table I.4 (page 36) lists the ability of various concentrations of cyanide to protect against inactivation. For the compounds PCP, 1-BP, α,α-DMBP, and 2-MBP, partial protection was observed in the presence of 0.5 mM (or less) KCN. This is consistent with the partial protection previously observed in the case of PCP (Hoag et al., 1984; Brady et al., 1987).

Studies done in the presence of 1.0 mM KCN resulted in either no apparent protection from inactivation, or an actual enhancement of the inactivation by the parent amines. These results run contrary to the expectation that higher cyanide concentrations should afford greater protection against inactivation. The inability to completely protect against inactivation by trapping of the iminium with cyanide can be explained, at least in part, by the finding that the corresponding α-cyano adducts
(independently synthesized) are themselves rather potent inhibitors of the enzyme (data shown in Table I.3, page 33).

In order to evaluate the possibility that the greater cytochrome P-450 inhibition seen at higher KCN levels might be due to an added inhibitory effect of cyanide itself, pelleting/resuspension experiments were performed since previous studies (Table I.2, page 30) demonstrated that cyanide inhibition of cytochrome P-450 is reversible. The results are presented in Table I.4 (see next page). For the most part, there is not a significant difference in the values obtained before and after pelleting/resuspension, showing that the enhanced inhibitory effects of cyanide are irreversible. Thus, high levels of cyanide appear to induce a greater sensitivity of cytochrome P-450 to inactivation by amines.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>[KCN] mM</th>
<th>% Activity Direct Assay</th>
<th>% Activity After Pelleting/Resuspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCP</td>
<td>-</td>
<td>58 ± 6</td>
<td>57 ± 9</td>
</tr>
<tr>
<td>PCP</td>
<td>0.5</td>
<td>78 ± 5</td>
<td>88 ± 4</td>
</tr>
<tr>
<td>PCP</td>
<td>1.0</td>
<td>57 ± 4</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>1-BP</td>
<td>-</td>
<td>57 ± 5</td>
<td>65 ± 7</td>
</tr>
<tr>
<td>1-BP</td>
<td>0.05</td>
<td>81 ± 1</td>
<td></td>
</tr>
<tr>
<td>1-BP</td>
<td>0.1</td>
<td>78 ± 4</td>
<td></td>
</tr>
<tr>
<td>1-BP</td>
<td>0.5</td>
<td>73 ± 4</td>
<td>77 ± 3</td>
</tr>
<tr>
<td>1-BP</td>
<td>1.0</td>
<td>41 ± 10</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>2,6-DMBP</td>
<td>-</td>
<td>71 ± 3</td>
<td>70 ± 2</td>
</tr>
<tr>
<td>2,6-DMBP</td>
<td>1.0</td>
<td>25 ± 1</td>
<td>42 ± 1</td>
</tr>
<tr>
<td>4,4-DMBP</td>
<td>-</td>
<td>85 ± 11</td>
<td>93 ± 4</td>
</tr>
<tr>
<td>4,4-DMBP</td>
<td>0.01</td>
<td>84 ± 1</td>
<td></td>
</tr>
<tr>
<td>4,4-DMBP</td>
<td>0.05</td>
<td>86 ± 2</td>
<td></td>
</tr>
<tr>
<td>4,4-DMBP</td>
<td>0.1</td>
<td>84 ± 1</td>
<td></td>
</tr>
<tr>
<td>4,4-DMBP</td>
<td>0.5</td>
<td>83 ± 7</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>4,4-DMBP</td>
<td>1.0</td>
<td>63 ± 2</td>
<td></td>
</tr>
<tr>
<td>α,α-DMBP</td>
<td>-</td>
<td>43 ± 3</td>
<td>45 ± 5</td>
</tr>
<tr>
<td>α,α-DMBP</td>
<td>0.5</td>
<td>75 ± 2</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>2-MBP</td>
<td>-</td>
<td>57 ± 5</td>
<td>58 ± 2</td>
</tr>
<tr>
<td>2-MBP</td>
<td>0.01</td>
<td>56 ± 2</td>
<td></td>
</tr>
<tr>
<td>2-MBP</td>
<td>0.05</td>
<td>60 ± 5</td>
<td></td>
</tr>
<tr>
<td>2-MBP</td>
<td>0.1</td>
<td>61 ± 5</td>
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<td>2-MBP</td>
<td>0.25</td>
<td>68 ± 1</td>
<td></td>
</tr>
<tr>
<td>2-MBP</td>
<td>0.5</td>
<td>75 ± 9</td>
<td>81 ± 2</td>
</tr>
<tr>
<td>2-MBP</td>
<td>1.0</td>
<td>47 ± 8</td>
<td></td>
</tr>
</tbody>
</table>

*incubated for 30 minutes, 37 °C, pH 7.4 with microsomes from PB-induced rabbits, with G6PD added. Values are percent of control activity ± S.E.M. of at least 3 determinations.
I.2.5 1-BENZYL-3-PYRROLIDINONE METABOLISM

In the very initial stages of this work, we attempted to reproduce the results obtained by Ho and Castagnoli (1980) who reported characterization and quantification of 1-benzylpyrrolidine metabolism with PB-induced rabbit liver microsomes. We did this as a learning experience and to insure that the incubation and HPLC assay conditions that we chose would be acceptable for the types of experiments that we wanted to conduct with the 1-benzylpiperidine analogs. During this process, microsomal metabolism was carried out on the potential iminium/enamine oxidation product, 1-benzyl-3-pyrrolidinone (1-BPyr-3-one). Incubation of this compound with microsomes resulted in a NADPH-dependent formation of green microsomes, which is indicative of heme alkylation (Ortiz de Montellano et al., 1981, 1983). Later studies demonstrated that incubation of 1-BPyr-3-one with microsomes also resulted in an irreversible loss of activity as monitored by benzphetamine N-demethylation (Table I.5, see next page). These results were consistent with our hypothesis that metabolism beyond the iminium/enamine stage, in part giving 3-one, might generate reactive species capable of covalent binding and/or enzyme inactivation.

In order to determine whether or not the green microsome formation represented a cytochrome P-450 dependent pathway, the effects of P-450 and FMO inhibitors was investigated. The results are presented in Table I.5. The presence of the FMO competitive substrate, methimazole, resulted in an enhancement of the
observed inactivation, and no effect upon green microsome formation. Thus FMO activity appears not to be involved in the generation of green microsomes. Metyrapone, which is a P-450 inhibitor, provided partial protection against inactivation, but had no effect on the formation of green microsomes. On the other hand, SKF 525-A and Triton X-100, which are also P-450 inhibitors, provided complete inhibition of green microsome formation. SKF 525-A also displayed partial protection from inactivation. Cyanide is not only a potential P-450 inhibitor (discussed earlier), but it is also capable of trapping reactive electrophilic intermediates. Interestingly, cyanide showed no protection from inactivation, yet it inhibited the formation of green microsomes. This differential effect of cytochrome P-450 modulators suggests that the inactivation and green microsome formation are resulting from two different cytochrome P-450 isozyme systems.

### TABLE I.5 Effects of cytochrome P-450 and FMO inhibitors on the NADPH-dependent 1-benzyl-3-pyrrolidinone inhibition of benzphetamine N-demethylation with microsomes from PB-induced rabbits.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% Activity Direct Assay</th>
<th>Formation of green microsomes(^a)</th>
<th>% Activity After Pelleting/Resuspension</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>57 ± 5</td>
<td>yes</td>
<td>55 ± 4</td>
</tr>
<tr>
<td>KCN (0.5mM)</td>
<td>60 ± 7</td>
<td>no</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>Methimazole (1mM)</td>
<td>45 ± 6</td>
<td>yes</td>
<td>41 ± 3</td>
</tr>
<tr>
<td>Metyrapone (2mM)</td>
<td>87 ± 9</td>
<td>yes</td>
<td>79 ± 5</td>
</tr>
<tr>
<td>SKF 525-A (1mM)</td>
<td>70(^b)</td>
<td>no</td>
<td>n.d.</td>
</tr>
<tr>
<td>Triton X-100 (1% -v/v)</td>
<td>n.d.(^c)</td>
<td>no</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

\(^a\) when determined by pelleting/resuspension, inhibition of green microsome formation was irreversible (data not shown)
\(^b\) average of two determinations
\(^c\) not determined
CHAPTER 1.3 DISCUSSION
I.3.1 AMINE METABOLISM

While it is well established that the metabolism of cyclic tertiary amines such as PCP and nicotine by cytochrome P-450 results in enzyme inactivation and/or covalent binding, what role the major metabolite, the iminium ion, plays in this process is not clear. In the case of PCP, evidence has been provided that the observed inactivation and/or covalent binding is a result of metabolism beyond the iminium/enamine stage. We demonstrated in our studies that 1-BP and several of its methyl- and dimethyl-substituted analogues cause inactivation of cytochrome P-450 upon incubation with microsomal fractions, and that the iminium/enamine intermediates are the major metabolites formed. We then attempted to establish that these intermediates play an crucial role in enzyme inactivation by showing (i) that the iminium itself induces a higher level of enzyme inactivation than the parent amine and (ii) that cyanide protects against enzyme inactivation by trapping the iminium as the α-cyano adduct. Due to rapid dimerization of the iminium/enamine pair of 1-BP, the first approach was clearly not possible with this compound. Also, we found that cyanide offered only partial protection against enzyme inactivation.

We were however, able to achieve a more complete identification of products arising from microsomal metabolism by carrying out large scale incubations. As reported in section I.2.1, the dimer still is the major product in the large scale incubations, but small amounts of the 3- and 4-hydroxy derivatives of 1-BP are formed, as well as a small amount of the 3-one (Scheme I.12, page 25). We propose
that formation of the 3-one is a consequence of metabolism of the enamine. Consistent with this hypothesis, the 3-one was not produced from 1-BP in the presence of 1 mM cyanide, which traps the enamine as the α-cyano adduct.

A mechanism which explains the formation of 1-BP-3-one from the enamine was presented in section I.1.7 (Scheme I.5, page 11). An alternative pathway, also shown in Scheme I.5, could lead to a 2-en-4-ol, which might be further oxidized to a 2-en-4-one (3, a vinylogous lactam). The latter pathway is preceded by the isolation of the PCP-derived 2-piperidein-4-one from metabolism of the PCP iminium by Hoag et al. (1988). In the case of 1-BP, however, the 2-en-4-one was not produced in levels sufficient for our detection.

In order to confirm the metabolic generation of an endocyclic iminium/enamine by observing it directly, and to enable independent evaluation of its ability to induce enzyme inactivation in either a metabolism-independent or -dependent manner, we had to rely on our studies on the methyl and dimethyl analogs of 1-BP, where methyl substitution was known to sterically prevent or retard iminium/enamine coupling (Leonard and Hauck, 1957). Our control studies on the independently prepared iminium salts established their relatively long term stability under both HPLC assay and incubation conditions.

The predominant metabolites for the methyl-substituted 1-BP analogues were indeed the "undimerized" iminiums as reported in Table I.1 (page 21). In the presence of 1 mM cyanide, formation of the iminiums was suppressed, with the α-
cyano adducts being observed instead. By monitoring the cytochrome P-450 activity with a benzphetamine N-demethylase assay, we were able to evaluate the role of these iminium/enamine species on the enzyme inactivation process.

Studies performed on PCP and 2,6-DMBP demonstrated that these compounds were metabolized at rates similar to that of 1-BP, and that metabolism resulted in a NADPH-dependent irreversible inhibition of microsomal benzphetamine N-demethylase activity. In comparison to these two parent amines, the corresponding iminium species (Schemes I.7 and I.8, page 18) exhibited more pronounced enzyme inhibition, which again was mostly irreversible. The key finding is that inactivation by 2,6-DMBP-Im+ or PCP-Im+ was completely or mostly metabolism dependent (for PCP-Im+, we observed 70% and 20% inactivation in the presence and absence of G6PD, respectively). Thus, the major enzyme inactivating ability of these two iminiums stems not from their direct covalent binding capacity but from that of more advanced metabolites.

On the other hand, 2-MBP-Im+ exhibited less metabolism-dependent irreversible inhibition of cytochrome P-450 than the corresponding parent amine. However, as stated earlier, there is the possibility of generating two different endocyclic iminium intermediates (Scheme I.9, page 19) for 2-MBP and only the more substituted iminium was used for these studies. The results obtained, therefore, suggest that the level of inactivation observed for the parent amine may be a consequence of metabolism through both iminiums, but presumably reflects
predominantly the less substituted iminium, which we did not evaluate. This is consistent with the results obtained with 2,6-DMBP, which can only generate a substituted iminium. In this case metabolism resulted in less irreversible inactivation than either 2-MBP or 1-BP. Even though 2,6-DMBP is efficiently metabolized to its iminium, the iminium itself, like 2-MBP-Im\(^+\), is a weaker inactivator than 1-BP-Im\(^+\). Thus, it appears as though substitution at C-2 results in a decrease in the inactivating potential of the iminium/enamine species. This may be a result of steric hindrance by the methyl group in the enzyme alkylation event or the metabolic activation step. Another possible explanation may be a shift in the iminium-enamine-carbinolamine equilibrium due to the substitution at C-2. For the C-2-methyl-substituted compounds, the ring-opened form is a ketone as opposed to an aldehyde in the unsubstituted cases. Since the ketone is more stable, the ring-opened form may be more prominent in the substituted cases, resulting in less of the closed-ring iminium/enamine species. Since we propose that inactivation results from metabolism of the closed-ring form, this would account for the lower rates of inactivation for these iminiums.

Studies performed on 4,4-DMBP suggest that although it is a better substrate for microsomal metabolism than the other amines studied, production of the iminium is actually lower. One explanation for this observation may be that the 4,4-dimethyl groups result in a different substrate binding orientation for 4,4-DMBP which directs metabolism away from nitrogen, possibly to the 4,4-dimethyl groups. Two unidentified metabolites were found and their formation was only partially inhibited
by the presence of cyanide at levels sufficient to completely trap the iminium. This suggests that they are not advanced metabolites of the iminium/enamine species. Furthermore, while the corresponding iminium is indeed a better inactivator than the parent amine, the degree of inactivation is very weak compared to the other iminiums. If inactivation involves one of the two pathways in Scheme I.5 (page 11), then 4,4-dimethyl substitution would not only preclude functionalization at C-4 but would also sterically hinder enzymatic oxygenation at C-3. The decreased inactivation in this case would then be a consequence of decreased oxidative metabolism of the corresponding enamine.

In contrast, while α,α-DMBP, a close analogue of PCP (Scheme I.8, page 18), was found to have the lowest rate of microsomal metabolism of any of the amines studied, it showed the highest level of metabolism-dependent inactivation. Our interpretation of this increased inactivation is that blocking of the exocyclic C-N dehydrogenation pathway in this case results in increased endocyclic C-N dehydrogenation. This would, in turn, give rise to greater levels of endocyclic enamine-derived metabolism. Also, whereas α,α-DMBP is a more potent inactivator than PCP, PCP-Im⁺ is a more potent inactivator than α,α-DMBP-Im⁺. This may mean that a greater fraction of α,α-DMBP metabolism proceeds to the endocyclic iminium, perhaps because alternative cyclohexyl ring metabolic pathways found in the case of PCP are not possible for α,α-DMBP.

An important objective of our study on the methyl-substituted 1-BP analogs
was to directly investigate the metabolism of the iminiums which was feasible because iminium/enamine coupling is minimal for these analogues. 4,4-DMBP-Im\(^+\), \(\alpha,\alpha\)-DMBP-Im\(^+\), PCP-Im\(^+\), and the 1-BP-dimer were all metabolized at rates that were very similar to the parent amines. In the cases of 4,4-DMBP-Im\(^+\) and \(\alpha,\alpha\)-DMBP-Im\(^+\), NADPH-dependent metabolites were observed; however, none of them could be identified. In the case of \(\alpha,\alpha\)-DMBP-Im\(^+\), we had at least expected to find the corresponding 3-one and 2-en-4-one metabolites observed in the case of PCP-Im\(^+\) (Scheme I.5, page 11) because of the close structural similarity; however these were not found (based on HPLC co-injection of authentic standards). While large scale incubations were performed in attempts to isolate and characterize the metabolites of \(\alpha,\alpha\)-DMBP-Im\(^+\), we were unable to obtain sufficient quantities for characterization. Further studies, using GC-MS analysis, are needed to elucidate the nature of these metabolites.

Attempted metabolism of 2-MBP-Im\(^+\) and 2,6-DMBP-Im\(^+\) resulted in no apparent metabolic activity. However, since both of these iminiums demonstrated metabolism-dependent inactivation of cytochrome P-450, they must be metabolized to some extent, albeit undetected. In these cases, it is possible that enzymatic processing always results in inactivation, without release of a turnover product. This corresponds to a "partition ratio" of 1, a phenomenon which could be verified using a pure enzyme preparation. Although unlikely, it must be noted that an apparent lack of metabolism could arise if products co-elute on the HPLC analysis with the iminium
reactant, with comparable extinction coefficients at the detection wavelength.

While we did not determine the exact agent(s) responsible for the observed metabolism-dependent inactivation of microsomal cytochrome P-450, we did obtain evidence which points to an advanced metabolite beyond an iminium/enamine intermediate in the metabolism of cyclic tertiary amines, as a general phenomenon. We demonstrated, in the case of 1-BP, that the 3-one is generated, at least in small quantities, from metabolism beyond the iminium/enamine stage. This metabolite was also reported for PCP by Masumoto et al. (1989). We proposed the 3-one formation channel as a possible pathway associated with enzyme inactivation. However, while the 3-one was metabolized by microsomes, no loss of cytochrome P-450 activity was observed. Nonetheless, it is possible that an intermediate species preceding formation of the 3-one (path B, Scheme I.5, page 11) is responsible for the observed inactivation. Alternatively, the observed cytochrome P-450 inactivation by PCP could reflect path A in this scheme, i.e. that involving formation of the 2-en-4-ol precursor 2 to the 2-ene-4-one metabolite 3 observed in the case of PCP (Hoag et al., 1988).

It should be noted here that the 3-one of 1-benzylpyrrolidine did, however, demonstrate rather potent metabolism-dependent inactivating capabilities which will be discussed later. This suggests that the ability of the 3-one species to generate an enzyme inactivating agent may be dependent upon ring size.
I.3.2 EFFECTS OF CYANIDE ON AMINE METABOLISM

Based on our ability to trap, at least in part, the various iminium metabolites as their cyanide adducts, we would expect that the presence of KCN in the incubation medium would afford protection of cytochrome P-450 activity if the iminium/enamine species were obligatory intermediates in the inactivation process. As presented in Table I.4 (page 36), the presence of 0.5 mM KCN offered only partial protection against enzyme inactivation, whereas 1.0 mM KCN resulted in either no apparent protection or an actual enhancement of inactivation due to the parent amines. These results run contrary to the expectation that higher cyanide concentrations should afford greater protection against inactivation.

Further investigation revealed several factors which could explain the observed inability of cyanide to protect against inactivation. One finding was that the α-cyano adducts themselves are rather potent irreversible inhibitors of cytochrome P-450 (Table I.3, page 33). Thus, trapping the iminium intermediates as their corresponding α-cyano adducts merely replaces one cytochrome P-450 inactivator with (or by) another.

The concentration of cyanide required to trap the iminiums is another factor which could affect the results obtained in the presence of KCN. For the amines 1-BP, PCP, and 4,4-DMBP, complete trapping of the iminiums was accomplished at 0.5 mM cyanide. In these cases, protection from inactivation should not be dependent upon the concentration of cyanide used for our studies. However, for 2,6-DMBP,
\(\alpha,\alpha\)-DMBP, and 2-MBP, a much higher concentration of cyanide (10 mM) was needed to efficiently trap the iminiums, presumably for steric reasons. Since this concentration of cyanide would inhibit enzymatic activity (see below), 10 mM KCN was added only at the end of the incubation period (conducted with 0.5 and 1.0 mM KCN) to induce complete conversion to the \(\alpha\)-cyano adduct of whatever iminium was present at that time. In these cases, the lack of cyanide protection against inactivation by the parent amines can be explained in part by the fact that there is a substantial amount of iminium present during the incubation process. Coupling this with the fact that the \(\alpha\)-cyano adducts also have inactivating potential, may account for the inability to achieve complete protection by cyanide for these compounds.

A third factor which needs to be addressed is the finding that cyanide, at the concentrations used for the trapping studies, inhibits cytochrome P-450-dependent benzphetamine N-demethylase activity. As shown in Table I.2 (page 30), 0.5 and 1.0 mM KCN \textit{reversibly} inhibit benzphetamine N-demethylation by 22 and 49 percent, respectively. Since the incubation assays were aliquoted into the benzphetamine N-demethylase assays, resulting in a 1:20 dilution, the concentrations of cyanide we used should not affect the rate of benzphetamine N-demethylation in our studies. However, since benzphetamine is metabolized principally by the same cytochrome P-450 isozyme (2B4) as the parent amines we studied, the question arises as to whether or not 0.5 and 1.0 mM KCN would inhibit amine metabolism to the same extent as it inhibited benzphetamine N-demethylation.
Interestingly, cyanide inhibition of amine metabolism was found to be substrate dependent. For 1-BP, α,α-DMBP, and PCP, cyanide demonstrated little or no inhibition on the rate of metabolism (Table I.1, page 21). Cyanide did, however, reduce the rate of metabolism in the cases of 2,6-DMBP and 4,4-DMBP. The results obtained for 2,6-DMBP varied, with cyanide inhibiting anywhere from 10-50 percent of metabolism. Cyanide had a large effect on the rate of 4,4-DMBP metabolism, reducing it by approximately 70 percent with both 0.5 and 1.0 mM KCN. Interestingly, the amount of metabolism represented by the exocyclic pathway was enhanced by both levels of cyanide to an amount over twice that found without cyanide present. As discussed earlier, the formation of two unidentified metabolites of 4,4-DMBP which were only partially reduced in the presence of cyanide is consistent with them resulting from metabolism of the 4,4-dimethyl groups.

Overall, it is clear that the notion of using cyanide as solely an iminium trapping agent is an oversimplification. Its use must take into account the various factors pointed out here which can result in significant perturbation of metabolism and enzyme inactivation profiles.

I.3.3 1-BENZYL-3-PYRROLIDINONE METABOLISM

Microsomal metabolism of 1-benzyl-3-pyrrolidinone (1-BPyr-3-one) was found to result in a NADPH-dependent formation of green microsomes, which is indicative of heme alkylation, and irreversible inactivation of cytochrome P-450 as
monitored by benzphetamine N-demethylation. Therefore, it is possible that the metabolism-dependent inactivation observed upon microsomal incubation of 1-benzylpyrrolidine (determined from previous studies in our laboratory) is a result of advanced metabolism through the 3-one species.

By using specific cytochrome P-450 and FMO inhibitors we obtained evidence that the inactivation of cytochrome P-450 and the formation of green microsomes reflect independent cytochrome P-450 isozymes. Metyrapone, which inhibits the PB-inducible 2B4 isozyme, and SKF 5025-A, an inhibitor of multiple isoforms, exhibited partial protection against inactivation, but only SKF 525-A had an inhibitory effect on the formation of green microsomes. While it is unclear as to which isozyme(s) are responsible for generating green microsomes, it appears that the inactivation is resulting mainly from metabolism through the 2B4 isozyme. Further studies with purified isozymes are needed to distinguish which isozymes are responsible for the two independent metabolic pathways.

It is possible that the formation of green microsomes is not due to alkylation of the cytochrome P-450 heme but merely due to a metabolite which becomes incorporated into the microsomal fraction making them appear green. Several different techniques need to be employed to distinguish between these two plausible explanations of the apparent green microsome formation. There are standard methods for extraction and isolation of microsomal cytochrome P-450 heme. It thus should be possible to distinguish between either the "green" metabolite or the modified heme
by appropriate extraction and/or chromatographic experiments. If heme alkylation is indicated, it would be important to isolate and structurally characterize the alkylated heme.

1.3.4 CONCLUSIONS AND FUTURE DIRECTIONS

Our work established some aspects of how the extent of metabolism-dependent covalent binding of cyclic tertiary amines depends on the structural effect of substituents. Thus, although unsubstituted N-alkylpiperidines are expected to undergo cytochrome P-450-dependent covalent binding, especially when exocyclic dehydrogenation is blocked, the presence of methyl substituents at the 2- and 4- position significantly retards reactive electrophile generation. Our results are compatible with the enamine being responsible for metabolism-dependent covalent binding, with the effect of methyl substituents being that which would be predicted on the basis of steric inhibition of cytochrome P-450 oxidation. Alternatively, the lower degree of inactivation caused by 2-methyl substitution could reflect an increase in the ring-opened form of the iminium (i.e. aminoketone) and a decrease in the enamine form.

One benefit of our research findings relates to the large variety of amine-based drugs currently in development. It is very common in structure activity work to prepare tertiary amine versions of a parent primary amine drug using dimethylamino, piperidino, pyrrolidino, and morpholino groups in place of NH₂. This is done without
regard to possible consideration of covalent binding events which may accompany metabolism of the drug. In the case where the piperidino version, for example, may exhibit the best pharmacologic activity, it would be useful to utilize a pharmacologically equivalent piperidino analog which was devoid of covalent binding potential. Our results suggest that this goal may be easily met by using ring-methylated piperidinos.

We initially chose not to study the effect of 3-methyl substitution because this would a priori exclude generation of the 3-one, which was of interest to us, as well as the potential 3-one-derived metabolites. However, based on our findings using 2,6-DMBP and 4,4-DMBP, it is important to verify that 3-methyl substitution inhibits (or eliminates) metabolism-dependent covalent binding. For this study, 1-benzyl-3,5-dimethylpiperidine would be used, so that both sides of the ring are equivalent.

Another important direction for future research is to extend our studies of microsomal metabolism to individual cytochrome P-450 isozymes. Our studies were performed with liver microsomes isolated from phenobarbital (PB)-induced rabbits, and therefore would tend to reflect action principally of the PB-inducible 2B4 isozyme that is known to be the major amine-metabolizing P-450 isoform. However, it is important to verify our most interesting data with the purified enzyme (using a reconstituted system). We did in fact observe a "cleaner" generation of the 3-one from I-BP in a preliminary study using the purified 2B4 isozyme. Any differences
found between the pure isozyme and the microsomal enzyme mixture would reflect the involvement of other microsomal isozymes, and any suspected isozymes could be individually studied. For example, the constitutive 2C3 isozyme could be involved in certain instances (Osawa and Coon, 1989).
CHAPTER 1.4 MATERIALS AND METHODS
1-Benzylpiperidine (1-BP), 1-benzyl-2,3,4,5-tetrahydropyridinium (1-BP-Im⁺), 1-benzyl-3-piperidinol (1-BP-3-OH), 1,1’-dibenzyl-5-(2’-piperidinyl)-1,2,3,4-tetrahydropyridine (1-BP-dimer), 1-benzyl-2-cyanopiperidine (1-BP-2-CN), 1-benzyl-2,3-dihydro-4(1H)pyridinone (1-BP-2-en-4-one), 2,6-dimethyl-1-benzylpiperidine (2,6-DMBP), 2,6-dimethyl-1-benzyl-2,3,4,5-tetrahydropyridinium hydrochloride (2,6-DMBP-Im⁺), 2,6-dimethyl-1-benzyl-2-cyanopiperidine (2,6-DMBP-2-CN), 1-(1-phenylcyclohexyl)piperidine (PCP), 1-(1-phenylcyclohexyl)-2,3,4,5-tetrahydropyridinium perchlorate (PCP-Im⁺), and 1-(1-phenylcyclohexyl)-2-cyanopiperidine (PCP-2-CN) were prepared and characterized as described by Sayre et al. (1991).

The compounds 1-benzyl-2-piperidone (1-BP-2-one), 1-benzylpiperidine N-oxide (1-BP-N-oxide), 2,6-dimethyl-1-benzylpiperidine N-oxide (2,6-DMBP-N-oxide), 1-(1-phenylcyclohexyl)-2-piperidone(PCP-3-one), 1-(1-phenylcyclohexyl)-2,3-dihydro-4(1H)-pyridinone (PCP-2-en-4-one), 1,1’-di(phenylcyclohexyl)-5-(2’-piperidinyl)-1,2,3,4-tetrahydropyridine (PCP-THA-dimer), 4,4-dimethyl-1-benzylpiperidine (4,4-DMBP), 4,4-dimethyl-1-benzyl-2,3,4,5-tetrahydro-pyridinium (4,4-DMBP-Im⁺), 4,4-dimethyl-1-benzyl-3-piperidone (4,4-DMBP-3-one) 4,4-dimethyl-1-benzyl-2-piperidone (4,4-DMBP-2-one), 4,4-dimethyl-1-benzyl-2-cyanopiperidine (4,4-DMBP-2-CN), 4,4-dimethyl-1-benzylpiperidine N-oxide (4,4-DMBP-N-oxide), 2-methyl-1-benzylpiperidine (2-MBP), 2-methyl-1-benzyl-2,3,4,5-tetrahydropyridinium (2-MBP-Im⁺), 2-methyl-1-benzyl-2-piperidone (2-MBP-2-one), 2-methyl-1-benzyl-2-cyanopiperidine (2-MBP-2-CN), 2-methyl-1-benzyl-6-cyanopiperidine (2-MBP-6-CN), 2-methyl-1-benzylpiperidine N-oxide (2-MBP-N-oxide), α,α-dimethyl-1-
benzylpiperidine (α,α-DMBP), α,α-dimethyl-1-benzyl-2,3,4,5-tetra-hydropyridinium (α,α-DMBP-Im*), α,α-dimethyl-1-benzyl-2-cyanopiperidine (α,α-DMBP-2-CN), α,α-dimethyl-1-benzyl-4-piperidone (α,α-DMBP-4-one), α,α-dimethyl-1-benzyl-2,3-dihydro-4(1H)pyridinone (α,α-DMBP-2-en-4-one), and 1,1'-di(1-methyl-1-phenylethyl)-5-(2'-piperidiny1)-1,2,3,4-tetrahydropyridine (α,α-DMBP-THA dimer) were prepared and characterized as described (Babu, Nadkarni, Venkataraman, and Sayre, submitted to J. Org. Chem.).

1-Benzyl-4-piperidinol (1-BP-4-OH), 1-benzyl-3-piperidone (1-BP-3-one), and 1-benzyl-4-piperidone (1-BP-4-one) were obtained from Aldrich Chemical Co. All other chemicals and enzymes were AR grade as supplied commercially. All solvents used were commercially available HPLC grade.

I.4.2 PREPARATION OF MICROSONES AND STANDARD INCUBATION ASSAY

The livers of New Zealand white male rabbits and Sprague-Dawley white male rats were used for the preparation of microsomes. Phenobarbital-induced rabbits were given 0.1% phenobarbital (pH 7.0) in their drinking water for seven days, and, after overnight fasting, they were sacrificed by lethal injection of pentobarbital. Control rabbit microsomes were made from both live animals and from livers obtained from Pel-Freeze Biologicals which were stored at -70 °C until used for metabolic studies.

Livers were removed from the animals and immediately placed in ice cold
homogenizing buffer consisting of 0.1 M Tris base, 0.15 M KCl, and 1.0 mM EDTA with the pH being adjusted to 7.4 with acetic acid. During all of the following procedures, all samples and solutions were kept on ice at all times to minimize loss of enzymatic activity. Livers were patted dry, weighed, and placed in fresh ice cold homogenizing buffer at four times the weight in volume (1g=1mL). After mincing with scissors, the livers were homogenized with a Polytron Homogenizer and centrifuged in a Sorvall RC5-B centrifuge at 4 °C using a GSA rotor at 13,000 × g for 20 minutes. The supernatants (S-9) were filtered through four layers of cheesecloth, pooled, and centrifuged in a Sorvall Ultracentrifuge OTD-75B at 4 °C using a DuPont 50.38 rotor at 100,000 × g for one hour. The resulting pellets were resuspended with a teflon pestle homogenizer in a wash buffer consisting of 0.1 M sodium pyrophosphate, and 1.0 mM EDTA, adjusted to pH 7.4 with acetic acid, at two times the original weight of the livers. The samples were then centrifuged a second time at 100,000 × g at 4 °C for one hour. The resulting microsomal pellets were resuspended with a teflon pestle homogenizer in a storage buffer containing 10.0 mM Tris acetate, 1.0 mM EDTA, and 20% glycerol (v/v), adjusted to pH 7.4 with acetic acid, at one half the original liver weight. The microsomes were stored at -70 °C and thawed on ice when needed for metabolic studies. Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.
For incubations, the microsomes were diluted with an assay buffer containing 40.0 μmol tricine buffer, 1.0 μmol NADP⁺, 5.0 μmol glucose-6-phosphate, and 6.0 μmol MgCl₂ per 1.0 mL of water at pH 7.4 (from now on referred to as the standard 1 mL assay). The final protein concentration was 0.05 - 3.0 mg/mL, depending upon the amine that was being analyzed.

I.4.3 SUBSTRATE BINDING TO CYTOCHROME P-450

The binding of amines to cytochrome P-450 was determined by difference spectrum analysis as described by Estabrook et. al. (1972). Spectra were obtained using a Kontron Uvikon 860 Spectrophotometer at 37 °C with the standard 1 mL assay. The diluted sample was divided into two cuvettes and the baseline was recorded. The desired amine was titrated into the sample cuvette using 1 microliter aliquots, recording the spectrum from 350 nm to 500 nm after each addition. Aliquots were added until the magnitude of the spectral change at 385 nm relative to that at 425 nm was saturated.

I.4.4 ASSAYS FOR METABOLIC RATE ANALYSIS

Rate data were determined at 37 °C using the standard 1 mL assay with 2.0 units of glucose-6-phosphate dehydrogenase when present. Substrate concentrations varied depending upon solubility, and KCN concentrations were 0.05 - 1.0 mM for cyanide trapping experiments. Reactions were initiated by the addition of 0.5 to 3.0
mg of protein, terminated by addition of 1.0 mL of 95% ethanol, and the precipitated protein was removed by centrifugation in a 10 °C Damen IEC 5000 centrifuge at 2010 × g for 10 minutes (this centrifugation procedure was used for removing precipitated protein from all incubations unless otherwise stated). The supernatant was analyzed by HPLC at 254 nm with comparison to authentic standards. Initial rates were computed under conditions where substrate disappearance and product formation were linear with respect to time and protein concentration.

1.4.5 LARGE SCALE 1-BENZYLPIPERIDINE (1-BP) METABOLISM STUDY

Large scale incubations of 5-10 times the standard 1 mL assay were carried out to analyze the formation of minor products from the microsomal metabolism of 1-BP. Incubations (5.0 mL) were terminated by the addition of 5.0 mL ice-cold methanol, and after removal of precipitated protein, 10.0 mL of saturated K₂CO₃ was added before extracting twice with 40 mL of CH₂Cl₂. The extracts were combined and dried over anhydrous K₂CO₃. The solvent was then removed using a rotary evaporator and the residue was reconstituted in 0.5 mL of methanol for HPLC analysis.

Due to HPLC peak interference with the direct determination of 1-benzyl-3-piperidone (1-BP-3-one), the latter was quantified through conversion to its 2,4-DNP adduct and through NaBH₄ reduction to 1-benzyl-3-piperidinol (1-BP-3-OH) (with measurement of the increase in peak area). The NaBH₄ reduction was performed as
follows: At the end of 30 minutes, a 10.0 mL incubation was divided into two equal portions, and 5.0 mL of ice-cold methanol were added to each. Authentic 1-BP-3-one (50 nmol) was added to one of the halves as a control and, after removal of precipitated protein, the same basification, extraction, and evaporation procedure as above was performed on both samples. The residue obtained was reconstituted in 0.5 mL of methanol with 0.3 mL being used for direct HPLC analysis and the remaining 0.2 mL were added to 10.0 mg of NaBH₄ dissolved in 2.0 mL of methanol and stirred at room temperature for 30 minutes. Water (5.0 mL) was then added, and the samples were extracted twice with 10.0 mL of CH₂Cl₂. The organic layer was evaporated and reconstituted in 0.2 mL of methanol for HPLC analysis.

The 2,4-DNP procedure was also performed with a 10.0 mL incubation followed by quenching of two 5.0 mL portions with 5.0 mL of ice-cold 95% ethanol. Again authentic 1-BP-3-one (50 nmol) was added to one half and the precipitated protein was removed by centrifugation. The supernatants were added to 0.218 mL (20 µmol) of 2,4-DNP reagent (2.0 g 2,4-DNP, 15.0 mL concentrated H₂SO₄, 85.0 mL water, and 10.0 mL THF) and the pH was adjusted to 3.0 with saturated K₂CO₃. After letting the samples sit overnight, they were extracted twice with 20 mL of ethyl ether. The extracts were combined, the solvent was evaporated, and the residue was reconstituted in 2.0 mL of methanol for HPLC analysis.

The detection of 1-benzyl-4-piperidone (1-BP-4-one) also employed the above procedure described for the detection of 1-BP-3-one. 1-benzyl-2,3-dihydro-4(H)-
pyridinone (1-BP-2-en-4-one) was monitored by HPLC analysis at its selective $\lambda_{\text{max}}$ of 324 nm.

I.4.6 BENZPHETAMINE N-DEMETHYLASE ASSAY

The inhibition of cytochrome P-450-dependent benzphetamine N-demethylation due to a given amine was monitored by a colorimetric aliquot method using a Varian Cary 210 spectrophotometer according to the method of Nash (1953). This involved dilution of 0.05 mL of the standard 1 mL incubation assay, containing 3.0-6.0 mg of protein, to 1.0 mL with tricine buffer containing cofactors (same as the standard 1 mL assay) and 1.0 mM benzphetamine. Reactions were terminated by the addition of 0.3 mL of ice-cold 30% TCA, and the precipitated protein was removed by centrifugation. The supernatant (1.0 mL) was added to 0.5 mL of the Nash reagent and the absorbance was read at 412 nm after heating for 1 hour at 37 °C. The Nash reagent consisted of 0.45g NH$_4$OAc, 0.006 mL pentanedione, and 0.009 mL glacial acetic acid per 1.0 mL of water.

The determination of irreversible inhibition of cytochrome P-450-dependent benzphetamine N-demethylation was done by quenching the standard 1 mL incubation mixture with 25.0 mL of ice-cold 10.0 mM tris acetate buffer containing 1.0 mM EDTA and 20% glycerol at pH 7.4. The mixture was centrifuged in a Sorvall Ultracentrifuge OTD-75B at 4 °C using a DuPont 50.38 rotor at 100,000 × g for one hour. The resulting pellet was resuspended in 0.8 mL of the tris acetate buffer.
(protein determined by Lowry assay), of which 0.2 mL was diluted to 1.0 mL with tricine buffer containing cofactors and 1.0 mM benzphetamine for determining benzphetamine N-demethylase activity.

**I.4.7 CYTOCHROME C REDUCTASE ASSAY**

Incubation mixtures (1.2 mL) consisting of 0.99 mL of a cytochrome c solution (see below) and 0.0, 0.5, 1.0, and 2.0 mM KCN were used for monitoring cytochrome c reductase activity. The mixtures were incubated at 37 °C for 3 minutes, at which point, 2 units of glucose-6-phosphate dehydrogenase (0.01 mL) were added to initiate the reactions. The activities were monitored spectrophotometrically on a Cary 210 spectrophotometer at 550 nm. The cytochrome c solution was made up of 0.3 M phosphate buffer (pH 7.0), containing; 1.0 μmol NADP⁺, 5.0 μmol glucose-6-phosphate, 6.0 μmol MgCl₂, 10.8 nmol of cytochrome c, and 0.02 - 0.1 mg of protein.

**I.4.8 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY**

HPLC analyses were performed using a Shimadzu HPLC System composed of: model SCL-6A system controller, model SPD-6A UV spectrophotometric detector, two model LC-6A pumps, model C-R3A chromatopac integrator, Varian MCH-10 30 cm × 4 mm micropak columns, and a Rheodyne 7125 manual injector.

Various isocratic and gradient solvent conditions, flow rates, and pH levels
were employed depending upon the substrate and which products were being analyzed. A summary of these conditions, along with the retention times of all the compounds studied, is listed in Table I.6
Table I.6  Conditions for HPLC analysis of cyclic tertiary amine metabolism\(^a\)

<table>
<thead>
<tr>
<th>Compound</th>
<th>% MeOH</th>
<th>pH</th>
<th>Flow Rate (ml/min)</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCP</td>
<td>70</td>
<td>5.0</td>
<td>1.0</td>
<td>6.3</td>
</tr>
<tr>
<td>PCP-Im(^+)</td>
<td></td>
<td></td>
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<td>9.0</td>
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<td>PCP-2-CN</td>
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<tr>
<td>PCP-THA dimer</td>
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<td></td>
<td></td>
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<tr>
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<tr>
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<td>1.0</td>
<td>5.6</td>
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<td>10.4</td>
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<td>6.0</td>
<td>1.5</td>
<td>8.7(^c)</td>
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<tr>
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<td></td>
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<td>7.0</td>
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<td></td>
<td></td>
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</tr>
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<td></td>
<td></td>
<td>11.6(^c)</td>
</tr>
<tr>
<td>(\alpha,\alpha)-DMBP-2-en-4-one</td>
<td></td>
<td></td>
<td></td>
<td>7.4(^c)</td>
</tr>
<tr>
<td>2-MBP</td>
<td>70</td>
<td>7.0</td>
<td>1.0</td>
<td>18.8(^c)</td>
</tr>
<tr>
<td>2-MBP-Im(^+)</td>
<td></td>
<td></td>
<td></td>
<td>16.3(^c)</td>
</tr>
<tr>
<td>2-MBP-Im(^+)</td>
<td></td>
<td></td>
<td></td>
<td>8.8</td>
</tr>
</tbody>
</table>

\(^a\)reverse phase C\(_{18}\) column; 0.1 M sodium acetate with 1 % (v/v) triethylamine aqueous buffer; pH adjusted with acetic acid
\(^b\)0 - 10 min. (50 % MeOH), 10 - 25 min. (50 - 90 % MeOH), 25 - 30 min. (90 - 50 % MeOH)
\(^c\)two 30 cm x 4 mm reverse phase C\(_{18}\) columns in series
\(^d\)0.05 M sodium phosphate; mixture of mono- and dibasic to appropriate pH
PART II. NEUROTOXIC ACTIVATION OF 
$\beta,\beta^\prime$-IMINODIPROPIONITRILE (IDPN).
CHAPTER II.1 INTRODUCTION
II.1.1 BACKGROUND

Originally tested in the 1950's on account of its structural relationship to a naturally occurring substance, β-aminopropionitrile (BAPN), which induces a connective tissue disorder called osteolathyroism, β,β'-iminodipropionitrile (IDPN) was found not to be lathyrogenic, but instead produced a permanent neurotoxic condition in rodents (Azima and Grad, 1956; Rudberg, 1957). This condition was characterized by behavioral excitation and involuntary motor activity and originally termed the "waltzing syndrome". Glucose reductones of secondary amines and a large class of tertiary amine mustards had also been observed to produce this condition (Chou and Hartman, 1964). Now referred to as the ECC syndrome (excitation, circling, and choreiform head and neck movements), this condition has been proposed as an experimental model for dyskinetic abnormalities such as Tourette syndrome and Huntington’s Disease, and is believed to involve the extrapyramidal dopamine and GABA systems (Diamond et al., 1982; Gianutos and Sudzak, 1985).

In the 1960's, morphological studies were done on IDPN which showed that intoxication resulted in production of masses of neurofilaments (NF) in proximal axons (Chou and Hartman, 1965), later found to result from an impairment of slow axonal transport (Griffin et al., 1978). Demyelination was found to occur in the vicinity of the axonal enlargements, along with reduced conduction velocity, a general impairment of nerve function, and minor nerve degeneration. IDPN has thus served as an important experimental model for the development of naturally occurring
proximal axonopathies, such as those observed in amyotrophic lateral sclerosis.

II.1.2 IDPN TOXICITY

The relationship between the behavioral (ECC) and morphological effects of IDPN are unclear and very little effort has been made to identify the molecular basis of IDPN toxicity. The metabolic release of cyanide, usually associated with the toxicity of aliphatic nitriles, does not appear to be involved in the toxicity of IDPN, since its biological effects and known metabolic nature are inconsistent with this interpretation (Williams et al., 1970). The development of NF-containing axonal enlargements are believed to be a result of direct covalent modification of NF and/or related protein. The findings that systemic IDPN administration results in a dissociation of NF from microtubules (MT) (Papazomenos et al., 1981), and that local administration of IDPN directly to nerve fibers induces rapid NF-MT segregation at the injection site, support the concept of direct modification (Griffin et al., 1983). The local effect of IDPN on axonal segments also rules out any cell-body-mediated mechanism such as inhibited protein synthesis, which would affect the whole axon independent of the injection site. IDPN is a potential source of electrophilic acrylonitrile, which could be the agent responsible for covalent modification. However, the formation of acrylonitrile from IDPN, through an elimination reaction, was found not to occur under physiological conditions, making this species an unlikely candidate (Jacobson et al., 1987). Also, acrylonitrile is a
known toxic agent which produces a distinct neurotoxic condition.

All these factors suggested that the toxicity of IDPN is a consequence of one or more metabolites, leading us to consider hypothetical toxic activation pathways (Scheme II.1) consistent with known patterns of amine metabolism and the observed urinary IDPN metabolites (shown in boxes). It is important to note the implications of the proposed metabolic activation on the relationship between the behavioral and morphological effects of IDPN. Since both neurotoxic conditions can be produced independently by mutually exclusive classes of chemical compounds and through different IDPN dosage schedules (Clark et al., 1980), they are probably not directly related. However, it is still possible that the same IDPN metabolite (or toxic activation pathway) may be involved for both effects.

![Scheme II.1 Hypothetical metabolism of IDPN.](image)

Cyanoacetic acid, β-alanine, and β-aminopropionitrile (BAPN) have all been identified as urinary metabolites of IDPN (Williams et al., 1970). Cyanoacetic acid is also the major urinary metabolite of the lathyrogen BAPN (Page and Benditt, 1967), arising via oxidative deamination to cyanoacetaldehyde with subsequent oxidation. There are three different known enzyme systems which could be involved in BAPN deamination. These consist of cytochrome P-450-mediated α-hydroxylation, mitochondrial flavin-dependent monoamine oxidase (MAO)-mediated dehydrogenation, and copper amine oxidase-mediated transamination. Similarly to BAPN, IDPN metabolism should result in oxidative N-dealkylation to produce cyanoacetaldehyde, which would then be oxidized to cyanoacetic acid, and BAPN, which would hydrolyze to β-alanine (Scheme II.1). However the N-dealkylation of IDPN could not be due to copper amine oxidase, since this enzyme system only acts on primary amines.

BAPN-induced lathyrysm was originally believed to be due to the reactive electrophile cyanoacetaldehyde. However, it is now known to be a result of a direct inactivation of lysyl oxidase by BAPN (Tang et al., 1983). Nonetheless, cyanoacetaldehyde is a potential candidate for the neurotoxic effects of IDPN owing to its ability to react with protein-based nucleophiles (Jacobson et al., 1987). Alternatively, small-molecule conjugates of cyanoacetaldehyde which preserve the latter’s electrophilic reactivity (e.g. dehydro-IDPN, see below) must also be considered.
P-450-mediated $\alpha$-C-hydroxylation of IDPN or MAO-mediated dehydrogenation to the imine (Scheme II.1) would both ultimately result in formation of a resonance-stabilized cyanoenamine designated as dehydro-IDPN. Independent synthesis of the carbinolamine (from BAPN + NCCH$_2$CHO) and the imine (from 1,2-elimination of N-functional IDPN derivatives) confirmed this assumption. Microsomal flavin monooxygenase (FMO) is another enzyme system which can oxidize secondary amines. N-Hydroxylation of IDPN by FMO to HOIDPN, followed by conjugation (e.g., sulfation), could result in 1,2-elimination to imine followed by tautomerization to dehydro-IDPN (Scheme II.1). Precedent for the latter possibility was obtained through the synthesis of HOIDPN (by reaction of H$_2$NOH with an excess of acrylonitrile in methanol), conjugation of which with $p$-nitrobenzenesulfonyl chloride resulted in a spontaneous elimination-tautomerization to dehydro-IDPN (Jacobson et al., 1987).

Although dehydro-IDPN is relatively stable to hydrolysis at pH 7, it undergoes a rapid transamination reaction with amines to convert the latter to the same cyanoenamines that would form from cyanoacetaldehyde directly (Jacobson et al., 1987). It is thus a good candidate for a circulating conjugate of cyanoacetaldehyde with reactive properties. Eventual hydrolysis of dehydro-IDPN through the carbinolamine is consistent with the observed urinary metabolites, thus making it a viable metabolic intermediate.

*In vivo* studies with dehydro-IDPN in rats, with administration (i.p.) of doses
up to 50% higher than the standard IDPN dose (2 g/kg), produced temporary hind-limb weakness, but no characteristic "waltzing syndrome" was observed. Also, there were seen very few axonal enlargements of the spinal cord and sciatic nerve, with the ones present being very mild. Since dehydro-IDPN is a metastable compound, i.p. administration may not be permitting its access to the required site of action, explaining the above observations. Thus, HOIDPN, which can still potentially produce dehydro-IDPN, was chosen for further studies due to its greater stability over that of dehydro-IDPN (Morandi et al., 1987).

II.1.3 ROLE OF HOIDPN

Administration of HOIDPN at 250 mg/kg, an 8-fold lower dose than the standard IDPN dosage, produced both the behavioral and morphological effects produced by intoxication with IDPN itself (Morandi et al., 1987). The much lower dose of HOIDPN needed to produce the same effects as IDPN is consistent with it being a key toxic metabolite. This finding also suggests that FMO-mediated N-oxygenation is a toxic activation pathway for IDPN. However, no conclusions can be made as to whether or not HOIDPN toxicity is due to its conversion to dehydro-IDPN, or whether an independent reaction pathway is involved.

In order to determine if HOIDPN is actually formed from IDPN under physiological conditions, an earlier collaboration with D. M. Ziegler (University of Texas) was arranged by Dr. Sayre to study the action of FMO on IDPN. Since FMO
converts secondary hydroxylamines to nitrones, the hydrolysis of which results in an oxidative N-dealkylation (Scheme II.2), this often confuses the interpretation of released aldehyde as arising from cytochrome P-450 metabolism. Thus, purified FMO was chosen over the crude microsomal system to simplify matters. IDPN was found to be a substrate for FMO with a rather high \( K_m \) of 15 mM. Nonetheless, since the usual 2 g/kg dose of IDPN corresponds to a 16 mM whole-body concentration, IDPN should be metabolized to HOIDPN by the FMO in vivo.

\[
\begin{align*}
\text{OH} & \quad \text{O}^- \\
\text{RCH}_2\text{NCH}_2\text{R} \xrightarrow{\text{FMO}} & \quad \text{RCH}_2\text{N} = \text{CHR} \quad \rightarrow \quad \text{RCH}_2\text{NOH} + \text{RCH} = \text{O}
\end{align*}
\]

Scheme II.2  FMO conversion of secondary hydroxylamines to nitrones.

The goal of the present study was to obtain evidence for the involvement of the FMO-mediated conversion of IDPN to HOIDPN in IDPN neurotoxic activation. The effects of enzyme-specific inhibitors on both the in vitro microsomal metabolism, and on the development of the neurotoxic properties of IDPN in rats in vivo, was chosen as an appropriate experimental model. By using a microsomal fraction, which contains cytochrome P-450 and FMO, the role of the mitochondrial MAO enzyme in IDPN metabolism would not be considered in these experiments. Our decision not to concentrate on the possible involvement of MAO in IDPN metabolism was based on the demonstration by Wilmarth and Froines (1991) that MAO showed only a
low-level of IDPN metabolism and that the pretreatment of rats with the potent MAO inhibitor pargyline had no effect on the development of IDPN behavioral neurotoxicity in rats.

Although the potent neurotoxicity of HOIDPN is consistent with FMO-mediated metabolism of IDPN being an activation pathway, nothing can be said about the role of cytochrome P-450. However, Llorens and Crofton (1991) have reported that CCl₄ pretreatment of rats, which drastically compromises cytochrome P-450 metabolism in the liver, enhanced the behavioral neurotoxicity of IDPN. These results suggest that cytochrome P-450-mediated metabolism represents a detoxification pathway, at least as far as the behavioral neurotoxicity of IDPN is concerned. Our comparative in vitro and in vivo inhibitor studies would test more directly the role of P-450 metabolism in IDPN toxification/detoxification, for both the behavioral (ECC syndrome) and morphological (neurofilamentous axonal enlargements) neurotoxic properties.
CHAPTER II.2  RESULTS
II.2.1 *IN VITRO* IDPN METABOLISM

II.2.1.1 TLC ANALYSIS

Before attempting any *in vivo* studies with IDPN, it was first necessary to determine if the metabolites, HOIDPN and BAPN, were formed by microsomal metabolism *in vitro*, and whether or not the formation of these two species could be altered by enzymologic manipulation. Initial studies on IDPN metabolism were performed using TLC analysis, the results being presented in Table II.1. Qualitative

Table II.1 Metabolism of IDPN by liver microsomes from PB-induced rabbits. IDPN (20 mM) was incubated at 37 °C for 60 minutes with all analyses performed qualitatively by TLC.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HOIDPN formation</th>
<th>BAPN formation</th>
<th>% FMO activity remaining(^a)</th>
<th>% P-450 activity remaining(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>yes</td>
<td>yes</td>
<td>[100]</td>
<td>[100]</td>
</tr>
<tr>
<td>(37 °C, 10 min - preincubation)</td>
<td>yes</td>
<td>yes</td>
<td>86.8</td>
<td>93.0</td>
</tr>
<tr>
<td>(50 °C, 5 min - preincubation)</td>
<td>no</td>
<td>yes</td>
<td>0</td>
<td>88.0</td>
</tr>
<tr>
<td>Triton X-100 (1 % v/v)</td>
<td>yes</td>
<td>no</td>
<td>not determined</td>
<td>16.0 ± 1.4</td>
</tr>
</tbody>
</table>

\(^a\) as determined by nicotine N-oxidase activity  
\(^b\) as determined by benzphetamine N-demethylase activity

analysis showed that both HOIDPN and BAPN were formed by incubating IDPN with rabbit liver microsomes and cofactors. The next task was to determine which metabolic pathways were responsible for the formation of these metabolites, so appropriate inhibitors could be used for *in vivo* studies.

Heat treatment is a technique that has been used to deplete microsomal fractions of FMO activity, while preserving the cytochrome P-450 activity (Poulsen
et al., 1979). Since cytochrome P-450 activity can also be heat sensitive, appropriate
controls were needed in order to determine conditions that would selectively inhibit
the FMO activity. The conversion of nicotine to nicotine N-oxide by FMO and
cytochrome P-450-dependent benzphetamine N-demethylation were used as
convenient assays. Heat treatment at 37 °C for 10 minutes did not significantly
reduce the FMO activity, however, 5 minutes at 50 °C proved to be very effective.
As was expected, this procedure inhibited HOIDPN formation while not affecting
BAPN formation. Triton X-100 is a cytochrome P-450 inhibitor (85% inhibition of
benzphetamine N-demethylase activity is seen at 1% v/v) which inhibited the
formation of BAPN and had no effect on HOIDPN formation. Thus, qualitative
evidence was obtained which showed that HOIDPN and BAPN were specific
metabolites of microsomal FMO and cytochrome P-450 pathways, respectively.

II.2.1.2 HPLC ANALYSIS

The next objective was to obtain quantitative data on the microsomal
metabolism of IDPN and the effect of specific inhibitors (methimazole for FMO and
metyrapone for cytochrome P-450). An HPLC assay was considered for such
analysis; however, it was only reliable for determining the rate of IDPN turnover by
microsomes on account of the low level of metabolites formed and the low sensitivity
of the refractive index detector that had to be employed for detection of the non-UV-
active metabolites HOIDPN and BAPN.
Data for a number of individual determinations of in vitro IDPN metabolism are listed in Table II.2. Average rates of 18.3 and 17.8 nmol/min/mg were determined for control rat liver microsomes and PB-induced rabbit liver microsomes, respectively. Although the sensitivity and peak resolution for HOIDPN were inadequate for this assay, a rate of formation was estimated to be 3.0 nmol/min/mg, which accounted for approximately 17 % of the total IDPN metabolized.

<table>
<thead>
<tr>
<th>incubation (ml, min)</th>
<th>IDPN rate (nmol/min/mg)</th>
<th>HOIDPN rate (nmol/min/mg)</th>
<th>microsome type</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0, 30</td>
<td>17.02 (1)</td>
<td>5.00 (1)</td>
<td>Control-rat</td>
</tr>
<tr>
<td>5.0, 30</td>
<td>19.58 (2)</td>
<td>0.86 (2)</td>
<td>Control-rat</td>
</tr>
<tr>
<td>5.0, 30</td>
<td>n.d.</td>
<td>3.16 (1)</td>
<td>Control-rat</td>
</tr>
<tr>
<td>5.0, 30</td>
<td>n.d.</td>
<td>1.01 (1)(^c)</td>
<td>Control-rat</td>
</tr>
<tr>
<td>[5.0, 30</td>
<td>18.3 ± 1.3 (n=2)</td>
<td></td>
<td>Control-rat</td>
</tr>
<tr>
<td>1.0, 60</td>
<td>37.89 (3)</td>
<td>n.d.</td>
<td>PB-rabbit</td>
</tr>
<tr>
<td>1.0, 60</td>
<td>16.22 (2)</td>
<td>n.d.</td>
<td>PB-rabbit</td>
</tr>
<tr>
<td>1.0, 60</td>
<td>12.68 (3)</td>
<td>n.d.</td>
<td>PB-rabbit</td>
</tr>
<tr>
<td>1.0, 60</td>
<td>4.28 (3)</td>
<td>n.d.</td>
<td>PB-rabbit</td>
</tr>
<tr>
<td>1.0, 60</td>
<td>17.88 (3)</td>
<td>n.d.</td>
<td>PB-rabbit</td>
</tr>
<tr>
<td>[1.0, 60</td>
<td>17.8 ± 11.1 (n=5)</td>
<td></td>
<td>PB-rabbit</td>
</tr>
</tbody>
</table>

\(^a\)numbers in parenthesis represent replicate runs in individual experimental sessions

\(^b\)not determined

\(^c\)determined by colorimetric assay

II.2.1.3 HOIDPN COLORIMETRIC ASSAY

A colorimetric assay that has been previously used for the detection of N-hydroxylamines (Day et al., 1979; Belanger et al., 1981) was found to be a much
more sensitive and accurate means of HOIDPN detection. The time course and enzyme titration plots shown in Figures II.1 and II.2 are examples of the type of data obtained using this method. The rates of HOIDPN formation, obtained from averaging data points from the linear portions of these plots, are listed in Table II.3. Rates of 1.0, 3.3, and 3.3 nmol/min/mg were determined for control rat, control rabbit, and PB-induced rabbit liver microsomes, respectively. The similarity in the

![Graph showing time dependent conversion of IDPN (20 mM) to its N-hydroxy metabolite (HOIDPN) by liver microsomes from PB-induced rabbits. The amount of HOIDPN formed was monitored by colorimetric assay. Values are an average of two determinations.](image)

FIGURE II.1 Time dependent conversion of IDPN (20 mM) to its N-hydroxy metabolite (HOIDPN) by liver microsomes from PB-induced rabbits. The amount of HOIDPN formed was monitored by colorimetric assay. Values are an average of two determinations.

rates for control and PB-induced rabbit liver microsomes was to be expected since FMO is not induced by phenobarbital. There does, however, appear to be species differences in FMO activities for IDPN metabolism between rats and rabbits.
The first enzymologic manipulations performed using the colorimetric assay for microsomal HOIDPN formation were with Triton X-100 inhibition and heat treatment. This allowed confirmation and quantification of the results obtained earlier by TLC analysis. The results were consistent with those obtained earlier; Triton X-100 did not affect HOIDPN formation, while heat treatment inhibited HOIDPN formation by 71 % (Table II.3). Surprisingly, n-octylamine, previously reported to act simultaneously as a positive effector of FMO in hog liver microsomes and an inhibitor of cytochrome P-450 (Tynes and Hodgson, 1983), did not stimulate HOIDPN formation in rabbit liver microsomes. Again, appropriate controls were run.

![Graph](image)

**FIGURE II.2** Enzyme dependent conversion of IDPN (20 mM) to its N-hydroxy metabolite (HOIDPN) by liver microsomes from PB-induced rabbits. The amount of HOIDPN formed was monitored by colorimetric assay. Values are an average of two determinations.
to monitor the FMO and cytochrome P-450 activities independently (data not shown).

<table>
<thead>
<tr>
<th>incubation (ml, min)</th>
<th>rate (nmol/min/mg)</th>
<th>treatment</th>
<th>microsome type</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0, 30</td>
<td>0.86 (3)</td>
<td></td>
<td>C-rat</td>
</tr>
<tr>
<td>5.0, 30</td>
<td>1.01 (1)</td>
<td></td>
<td>C-rat</td>
</tr>
<tr>
<td>5.0, 30</td>
<td>1.14 (1)</td>
<td></td>
<td>C-rat</td>
</tr>
<tr>
<td>[5.0, 30</td>
<td>1.00 ± 0.11 (n=3)</td>
<td>heat (50°C, 5 min)</td>
<td>C-rat</td>
</tr>
<tr>
<td>1.0, 15</td>
<td>3.29 (6)b</td>
<td></td>
<td>C-rabbit</td>
</tr>
<tr>
<td>1.0, 30</td>
<td>0.51 (4)c</td>
<td></td>
<td>PB-rabbit</td>
</tr>
<tr>
<td>1.0, 30</td>
<td>3.11 (3)</td>
<td></td>
<td>PB-rabbit</td>
</tr>
<tr>
<td>1.0, 30</td>
<td>3.72 (8)</td>
<td></td>
<td>PB-rabbit</td>
</tr>
<tr>
<td>1.0, 30</td>
<td>3.01 (8)</td>
<td></td>
<td>PB-rabbit</td>
</tr>
<tr>
<td>[1.0, 30</td>
<td>3.28 ± 0.31 (n=3)</td>
<td></td>
<td>PB-rabbit</td>
</tr>
<tr>
<td>1.0, 30</td>
<td>3.77 ± 0.38 (n=4)d</td>
<td></td>
<td>PB-rabbit</td>
</tr>
<tr>
<td>1.0, 30</td>
<td>3.46 (5)</td>
<td></td>
<td>PB-rabbit</td>
</tr>
<tr>
<td>1.0, 30</td>
<td>3.59 (5)</td>
<td>1% Triton X-100</td>
<td>PB-rabbit</td>
</tr>
<tr>
<td>1.0, 30</td>
<td>3.72 (2)</td>
<td>1.0 mM n-octylamine</td>
<td>PB-rabbit</td>
</tr>
<tr>
<td></td>
<td>3.67 (2)</td>
<td>1.0 mM n-octylamine</td>
<td>PB-rabbit</td>
</tr>
<tr>
<td></td>
<td>3.68 (2)</td>
<td>3.0 mM n-octylamine</td>
<td>PB-rabbit</td>
</tr>
<tr>
<td></td>
<td>3.87 (2)</td>
<td>5.0 mM n-octylamine</td>
<td>PB-rabbit</td>
</tr>
</tbody>
</table>

aNumbers in parenthesis represent replicate runs in individual experimental sessions
bDetermined from linear portion of enzyme titration curve
cEnzyme not saturated
dDetermined from inhibitor data with methimazole and metyrapone
We were initially unsure of observing HOIDPN directly because the N-hydroxy FMO metabolites of secondary amines are normally further metabolized faster than the parent amines (Ziegler, 1980). However, as we had no problem detecting HOIDPN, we independently studied the microsomal metabolism of synthetic HOIDPN. At a concentration of 1 mM only a slight extent of microsomal metabolism was observed: averaging 1.3 nmol/min/mg over 60 minutes. This corresponds to only 7% of the rate of IDPN metabolism, making it clear why we were able to observe HOIDPN accumulation. Also it makes it unnecessary for us to have to correct the HOIDPN formation rates for further HOIDPN metabolism. It is unclear what factors make the IDPN/HOIDPN situation different from other $R_2$NH/$R_2$NOH systems (Ziegler, 1980).

**II.2.1.4 BAPN COLORIMETRIC ASSAY**

A phthalaldehyde-based colorimetric assay, originally designed as a fluorometric assay specific for detection of primary amines (Benson *et al.*, 1975), was used for quantitating BAPN formation from IDPN. Although BAPN was easily quantitated with this assay in the absence of any inhibitors, the results obtained from experiments with inhibitors were considered only qualitatively reliable (Table II.4). All the inhibitors used, Triton X-100, heat treatment, metyrapone, and methimazole, interfered with the absorbance readings in various ways. Appropriate controls were run in an effort to make corrections for these interferences, but the corrections were
the same order of magnitude as were the observed differences.

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>rate (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>2.57 ± 0.2 (n=14)</td>
</tr>
<tr>
<td>metyrapone (0.1 mM)</td>
<td>1.62 ± 0.02 (n=2)</td>
</tr>
<tr>
<td>metyrapone (1.0 mM)</td>
<td>1.24 ± 0.02 (n=2)</td>
</tr>
<tr>
<td>metyrapone (5.0 mM)</td>
<td>0.72 ± 0.11 (n=8)</td>
</tr>
<tr>
<td>metyrapone (10.0 mM)</td>
<td>0.79 ± 0.06 (n=2)</td>
</tr>
<tr>
<td>methimazole (0.05 mM)</td>
<td>2.49 ± 0.04 (n=2)</td>
</tr>
<tr>
<td>methimazole (0.5 mM)</td>
<td>1.66 ± 0.57 (n=7)</td>
</tr>
<tr>
<td>methimazole (5.0 mM)</td>
<td>1.08 ± 0.26 (n=3)</td>
</tr>
</tbody>
</table>

As expected, metyrapone does inhibit BAPN formation; however, methimazole also demonstrates a level of inhibition approximately one-half that of metyrapone. This is probably due to the high concentration of methimazole used in these experiments, since methimazole has been shown to inhibit cytochrome P-450 at high concentrations (Lee and Neal, 1978).

II.2.1.5 BAPN FLUOROMETRIC ASSAY

Although the phthalaldehyde-based assay we used for detection of BAPN was originally designed as a fluorometric assay, we chose initially to use a colorimetric analysis for convenience. However, due to interference with the colorimetric assay by the inhibitors, fluorometric detection was employed in order to confirm our
results. This method of detection proved to be a much more sensitive technique, and only minor corrections were needed for the use of methimazole and metyrapone in the assays. Using fluorometric detection, quantitation of BAPN generation by both NADPH-dependent (NADPH was generated from NADP⁺, isocitrate, and isocitrate dehydrogenase (ICD)) and NADPH-independent pathways could be assessed individually in rabbit liver microsomes. The NADPH-dependent rate of BAPN formation was determined to be approximately 3.0 and 2.0 nmol/min/mg of microsomal protein from PB-induced and control rabbit liver microsomes, respectively. The rate of BAPN formation for the NADPH-independent pathway was about 1.5 nmol/min/mg for the PB-induced microsomes and 1.25 nmol/min/mg for the control microsomes.

II.2.1.6 EFFECT OF INHIBITORS ON HOIDPN AND BAPN FORMATION

Before conducting experiments on the effects of metyrapone and methimazole on IDPN metabolism, the effects of these inhibitors on known metabolic reactions were determined. As shown in Figures II.3 and II.4 (see next page), metyrapone and methimazole are very effective inhibitors of cytochrome P-450 (monitored by benzphetamine N-demethylation) and FMO (monitored by nicotine N-oxide formation), respectively. As expected, methimazole inhibited FMO-mediated conversion of IDPN to HOIDPN (Figure II.5, page 84), while metyrapone had little effect on this pathway (Figure II.6, page 84).
FIGURE II.3 Metyrapone inhibition of P-450-dependent benzphetamine N-demethylation by liver microsomes from PB-induced rabbits. Benzphetamine N-demethylase activity was monitored spectrophotometrically by the method of Nash. Values plotted are an average of four determinations. (IC$_{50}$ ~ 15 $\mu$M)

FIGURE II.4 Methimazole inhibition of the FMO-dependent conversion of nicotine to its N-oxide by liver microsomes from PB-induced rabbits. The amount of nicotine N-oxide formed was monitored by HPLC analysis. Values are an average of two determinations. (IC$_{50}$ ~ 100 $\mu$M)
FIGURE II.5 Methimazole inhibition of IDPN (20 mM) conversion to its N-hydroxy metabolite (HOIDPN) by liver microsomes from PB-induced rabbits. The amount of HOIDPN formed was monitored by colorimetric assay. Values are an average of two determinations. (IC$_{50}$ = 80 μM)

FIGURE II.6 Effect of metyrapone on IDPN (20 mM) conversion to its N-hydroxy metabolite (HOIDPN) by liver microsomes from PB-induced rabbits. The amount of HOIDPN formed was monitored by colorimetric assay. Values plotted are an average of two determinations.
As shown in Figure II.7, metyrapone effectively inhibits the NADPH-dependent component of BAPN formation from PB-induced microsomes (IC$_{50}$ ~ 50 µM), while having no effect upon the NADPH-independent pathway. Interestingly, metyrapone appears not to have any effect on BAPN formation using control microsomes (Figure II.8).

![Graph showing the inhibition of BAPN formation by metyrapone](image)

**FIGURE II.7** Metyrapone inhibition of IDPN (20 mM) conversion to BAPN by liver microsomes from PB-induced rabbits. The amount of BAPN formed was monitored by fluorometric assay. Values plotted are an average of three determinations. (IC$_{50}$ ~ 50 µM for the ICD-dependent component)
Methimazole was also found to be an inhibitor of the NADPH-dependent formation of BAPN with both PB-induced (Figure II.9) and control microsomes (Figure II.10). As expected, the estimated IC$_{50}$ of 2 mM for methimazole inhibition was much higher than that for metyrapone. Methimazole was also found to partially inhibit the NADPH-independent pathway of BAPN formation with both PB-induced and control microsomes.
FIGURE II.9 Methimazole inhibition of IDPN (20 mM) conversion to BAPN by liver microsomes from PB-induced rabbits. The amount of BAPN formed was monitored by fluorometric assay. Values are an average of three determinations. (IC$_{50}$ ~ 2 mM for the ICD-dependent component)

FIGURE II.10 Methimazole inhibition of IDPN (20 mM) conversion to BAPN by control rabbit liver microsomes. The amount of BAPN formed was monitored by fluorometric assay. Values are an average of three determinations. (IC$_{50}$ ~ 2 mM for the ICD-dependent component)
II.2.2 IN VIVO IDPN METABOLISM

II.2.2.1 BEHAVIORAL NEUROTOXICITY

The behavioral effects associated with IDPN administration (1-2g/kg) to rats were monitored daily. One very obvious effect was that the IDPN treated rats lost approximately 30-35 grams of weight over a seven day period, while the control animals (no IDPN or inhibitors) showed a gain in weight of about 50 grams. While co-administration of metyrapone had very little effect on the IDPN-induced weight loss (which averaged about 25-30 grams), methimazole, on the other hand, resulted in the rats showing only a somewhat lower weight gain than the control animals. A summary of the weight measurements obtained is presented in Figure II.11 (see next page). For the same treatment group, the behavioral effects associated with the IDPN-induced ECC syndrome were completely inhibited by co-administration of methimazole, whereas the animals co-treated with metyrapone demonstrated a daily enhancement of the ECC syndrome, almost indistinguishable from that seen for the animals treated with IDPN alone (Table II.5, see next page).
Figure II.11  Effects of IDPN (1 g/kg) and inhibitors on rat weights. Values represent the average weight of each group consisting of three animals per group. Metyrapone (90 mg/kg) and methimazole (300 mg/kg) were administered 30 minutes prior to and 24 hours after administration of IDPN.

Table II.5  Effects of specific enzyme inhibitors on the behavioral neurotoxicity of IDPN in rats. Numbers are an average of behavior ratings on three rats in each group as described in Methods.

<table>
<thead>
<tr>
<th></th>
<th>day 1</th>
<th>day 2</th>
<th>day 3</th>
<th>day 4</th>
<th>day 5</th>
<th>day 6</th>
<th>day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDPN (1g/kg)</td>
<td>-0-</td>
<td>-0-</td>
<td>1.7</td>
<td>2.7</td>
<td>3.7</td>
<td>4.7</td>
<td>5.7</td>
</tr>
<tr>
<td>IDPN (1g/kg) +</td>
<td>-0-</td>
<td>-0-</td>
<td>-0-</td>
<td>-0-</td>
<td>-0-</td>
<td>-0-</td>
<td>-0-</td>
</tr>
<tr>
<td>methimazole (300 mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IDPN (1g/kg) +</td>
<td>-0-</td>
<td>-0-</td>
<td>1.0</td>
<td>2.3</td>
<td>3.0</td>
<td>4.0</td>
<td>5.3</td>
</tr>
<tr>
<td>metyrapone (90 mg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
II.2.2.2 MORPHOLOGICAL NEUROTOXICITY

The effects of the two inhibitors, metyrapone and methimazole, on the extent of the axonal enlargements varied with the IDPN and inhibitor dosage schedules. At both 1 and 2 g/kg dosages of IDPN, metyrapone (60 and 90 mg/kg) afforded a slight amelioration of the severity of axonal enlargements (Table II.6). Methimazole (300 mg/kg), however, demonstrated complete protection at the lower dose of IDPN, and about two-thirds reduction of the enlargements at the higher dosage, while the lower dosage of methimazole, 200 mg/kg, afforded an approximately 50% reduction of the observed enlargements at the high IDPN dose schedule (Table II.6).

<table>
<thead>
<tr>
<th>inhibitor</th>
<th>IDPN (2g/kg)</th>
<th>IDPN (1g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>+ 5.0</td>
<td>+ 5.0</td>
</tr>
<tr>
<td>metyrapone (60 mg/kg)</td>
<td>+ 4.0</td>
<td>not determined</td>
</tr>
<tr>
<td>metyrapone (90 mg/kg)</td>
<td>+ 4.0</td>
<td>+ 4.3</td>
</tr>
<tr>
<td>methimazole (200 mg/kg)</td>
<td>+ 2.3</td>
<td>not determined</td>
</tr>
<tr>
<td>methimazole (300 mg/kg)</td>
<td>+ 1.2</td>
<td>-0-</td>
</tr>
</tbody>
</table>

Table II.6 Effects of IDPN and specific enzyme inhibitors on axonal enlargements in rats at the level of the lumbar spinal cord. Values represent the average of at least three determinations with -0- representing normal morphology and + 5.0 representing the maximal observed axonal enlargements. The inhibitors were administered 30 minutes prior to and 24 hours after administration of IDPN.
CHAPTER II.3 DISCUSSION
II.3.1 *IN VITRO* IDPN METABOLISM

There has been only a limited effort made to elucidate the metabolic basis of IDPN neurotoxicity. As discussed in the introduction, the fact that the two neurotoxic conditions (ECC syndrome and peripheral neuropathy characterized by proximal neurofilamentous axonal enlargements) can be produced independently by other chemical agents, indicates that there is no direct causal relationship between them. Furthermore, the fact that the ECC syndrome and peripheral neuropathy can be produced individually by manipulation of the IDPN dosage schedule, suggests some point of divergence in IDPN toxic activation.

Our lab previously showed that HOIDPN can produce both IDPN neurotoxicities at approximately an eight-fold lower dosage (Morandi *et al*., 1987). One possible explanation for this finding is that FMO-mediated formation of HOIDPN is an IDPN toxic activation pathway common to both neurotoxic conditions (i.e., the point of divergence between the two neurotoxicities must be at a subsequent metabolic stage). However, it is also possible that the higher toxicity of HOIDPN reflects merely the structure/activity characteristics of these two neurotoxicities (viz. HOIDPN is just a more potent IDPN analog for these two activities), in which case HOIDPN need not be on the IDPN metabolic activation pathway.

Indirect support for the possible involvement of FMO-mediated production of HOIDPN in IDPN toxic activation is that the other two principal enzyme systems involved in secondary amine metabolism, namely mitochondrial monoamine oxidase
(MAO) and cytochrome P-450, have been shown not to be involved in IDPN toxic activation. In the case of MAO, IDPN is only a very weak substrate (Wilmarth and Froines, 1991). In the case of cytochrome P-450, although IDPN is potentially metabolized to BAPN and cyanoacetaldehyde (see section II.1.2), this appears to be a detoxification rather than a toxic activation pathway, at least in the rat. Llorens and Crofton (1991) found that compromise of rat liver function in (the main site of cytochrome P-450 metabolism) by CCl₄ enhanced IDPN neurotoxicity in terms of weight loss and behavioral symptomology. This result suggests that cytochrome P-450 metabolism is not only not needed for expression of IDPN behavioral neurotoxicity, but that it provides a detoxication metabolic channel. However, this result does not bear on the issue of the IDPN peripheral neuropathy.

In order to obtain direct evidence for the involvement of HOIDPN in IDPN neurotoxicity, in vitro metabolic studies were performed to confirm the enzymes involved in HOIDPN formation. Once this was accomplished, selective enzyme inhibitors could be used to probe the involvement of specific enzymatic activations in vivo.

In our initial qualitative studies (TLC analysis), we found that the selective heat inactivation (50 °C, 5 min) of the FMO but not cytochrome P-450 activity of microsomes, inhibited HOIDPN but not BAPN formation. On the other hand, the presence of 1% Triton X-100, a detergent known to disrupt microsomal cytochrome P-450 function, inhibited the formation of BAPN but not HOIDPN. A straightforward
colorimetric assay for HOIDPN formation allowed us to place these effects on a more quantitative ground: heat treatment (50 °C, 5 min) lowered the HOIDPN production by rat liver microsomes from a rate of 1.0 to 0.3 nmol/min/mg of microsomal protein; on the other hand, 1% Triton X-100 slightly raised the HOIDPN production by PB-induced rabbit liver microsomes from 3.5 to 3.6 nmol/min/mg. Overall, HOIDPN was produced more efficiently by liver microsomes from rabbit than from rat, and phenobarbital induction did not change this.

The bulk of our studies were performed with the cytochrome P-450 inhibitor metyrapone (2-methyl-1,2-di-3-pyridyl-2-propanone) and the FMO "alternate substrate" methimazole (2-mercapto-1-methylimidazole). There is currently no known inhibitor of FMO; thus, the standard approach is to utilize a fairly high concentration of the substrate methimazole to act effectively as a competitive inhibitor (Prough and Ziegler, 1977). Metyrapone and methimazole have been used together in other studies to probe the relative contribution of cytochrome P-450 and FMO in toxic activation/detoxicication of chemical agents in vivo (Chieli and Malvaldi, 1984, 1985; Mori et al., 1993).

We found that methimazole was very effective at inhibiting the conversion of IDPN to HOIDPN using liver microsomes from PB-induced rabbits. The IC₅₀ can be seen from Figure II.5 to be approximately 80 μM. This is very similar to the concentration needed (approx. 100 μM) for 50% inhibition of conversion of nicotine to its N-oxide, the standard assay for FMO activity. On the other hand, metyrapone,
up to 10 mM, showed no reduction in the conversion of IDPN to HOIDPN (Figure II.6), whereas 50% inhibition of benzphetamine demethylation (the standard cytochrome P-450 assay) was seen at a very low concentration (approx. 0.1 mM, see Figure II.3). It is clear that FMO rather than cytochrome P-450 is the enzyme system responsible for conversion of IDPN to HOIDPN.

In regard to BAPN production, although a fluorometric assay using phthalaldehyde is the method of choice when sensitivity is the crucial factor, we believed that simple colorimetric monitoring of the same assay would suffice in our case and would be more conveniently run. Using this method, we were able to determine a control rate of BAPN formation by PB-induced rabbit microsomes of 2.6 nmol/min/mg, which was reduced to 1.2 nmol/min/mg by 1.0 mM metyrapone (the apparent IC₅₀ concentration, but see below). Methimazole also displayed an inhibition of BAPN formation (Table II.4), though the effect was somewhat weaker (an IC₅₀ of 2 mM can be estimated). Since methimazole has been reported to inhibit cytochrome P-450 activity at higher concentration (Lee and Neal, 1978), and since much lower concentrations are sufficient for potent inhibition of FMO activity, our results suggest that cytochrome P-450 is the main enzyme system involved in the production of BAPN from IDPN.

Because large data corrections were required for colorimetric determination of BAPN in the presence of the inhibitors, we felt it was important to confirm our results using the standard fluorometric approach. In this case, methimazole and
metyrapone required only minor corrections to be made in the BAPN formation data. Evidently, the combined excitation/emission criterion of the fluorometric method results in less interference by other absorbing species. The fluorometric assay permitted us to obtain more reliable data on BAPN formation so that its dependence on the NADPH regenerating system could also be evaluated.

Using the fluorometric assay, the NADPH-independent rate of BAPN generation by PB-induced rabbit microsomes (1.5 nmol/min/mg) was now seen to be about one-half of the rate of BAPN formation when NADPH is available (Figure II.7). Therefore, the cytochrome P-450-dependent production of BAPN amounts only to about 1.5 nmol/min/mg, and this activity is inhibited potently by metyrapone (the IC$_{50}$ appears to be about 50 μM). Thus, the apparent high IC$_{50}$ for metyrapone inhibition of BAPN formation estimated above was an artifact of our not knowing the "background" rate of BAPN formation.

Methimazole was seen to inhibit BAPN formation as before, but some inhibition of the NADPH-independent generation of BAPN was also observed (Figure II.9). Thus, the extent of methimazole inhibition of BAPN formation that can be ascribed to cytochrome P-450 is lower, and the IC$_{50}$ for this can be estimated to be about 2 mM, as was estimated from the colorimetric study. Clearly, metyrapone is now seen to be a much more potent inhibitor of the NADPH-dependent production of BAPN than is methimazole, consistent with our proposal of cytochrome P-450 as the responsible enzyme.
As nothing is known about the involvement of various cytochrome P-450 isoymes in BAPN formation from IDPN, we wondered whether the results we obtained reflected principally the 2B4 isozyme enriched by the PB induction. Thus, we repeated the BAPN fluorometric study with control rabbit liver microsomes. The rate in the absence of inhibitors appears to be approximately 2.0 nmol/min/mg (see Figures II.8 and II.10), but a little over half of this (approx. 1.2 nmol/min/mg) appears to arise from a NADPH-independent process. Thus, the specific cytochrome P-450-dependent production of BAPN is significantly lower using control microsomes, consistent with a major contribution being made by the 2B4 isozyme. Interestingly, the virtual lack of effect of metyrapone on this process (Figure II.8) suggests either (i) that metyrapone is a weak inhibitor of whatever cytochrome P-450 isozyme is producing BAPN in control microsomes, or (ii) that the 0.8 nmol/min/mg of NADPH-dependent production of BAPN represents a different enzyme system altogether.

As far as the NADPH-independent generation of BAPN is concerned, there is currently no precedent for such a microsomal N-dealkylation activity for xenobiotics. However, there are a number of NAD⁺- or NADP⁺-dependent dehydrogenases present in liver (Dixon et al., 1979). There are also a number of O₂-dependent oxidases (generating H₂O₂) present in liver (Dixon et al., 1979). Both classes of enzymes act on secondary biogenic amines (e.g. spermidine and octopamine) and amino-acids (e.g. sarcosine). It is likely that IDPN could be a
substrate for one of these enzymes involved in normal intermediary metabolism. Further work will be needed to evaluate possible IDPN substrate activity for individual candidate enzymes.

The conclusion from our in vitro studies is that IDPN is converted at a low level rate in microsomes to HOIDPN and BAPN principally by FMO and cytochrome P-450, respectively, in a manner which could be reliably evaluated by the sensitivity to inhibition by methimazole and metyrapone. This set the stage for the use of these two inhibitors to ascertain the toxic consequences of these two metabolic pathways in vivo.

II.3.2 IN VIVO IDPN METABOLISM

As has been thoroughly documented previously, administration of IDPN to rats at a level of 1-2 g/kg results in generation of both a behavioral "ECC" neurotoxic condition, characterized by excitation, circling, and choreiform head movements (twitching), as well as a peripheral neuropathy, characterized by neurofilamentous enlargements at the first axonal nodes. The behavioral condition can be produced by lower dosages of IDPN given over several days, but production of the axonopathy requires high acute dosages of IDPN. This factor, coupled with our findings of only slow (microsomal) metabolism of IDPN, meant that any effort to intervene pharmacologically with the axonal neurotoxicity would have to recognize that high levels of IDPN would be present in the animal for a significant period of time. We
thus chose to administer the inhibitors both 30 minutes before as well as one day following the IDPN administration. The levels of drugs chosen for *in vivo* inhibition were based on the previous work of Chieli and Malvaldi (1984, 1985). The usage of a higher dose of methimazole compared to metyrapone is based on these studies (see also Mori *et al.*, 1993) and the fact that the "inhibition" of FMO achieved by methimazole is due to the latter's substrate activity; higher levels would compensate for its consumption.

It is first apparent that methimazole prevented most of the IDPN-induced weight loss incurred over 7 days, whereas metyrapone had no preventative effect. Although the significance of the IDPN-induced weight loss is not clear, most workers ascribe this to a correlate of neurotoxicity. As far as the ECC syndrome is concerned, whereas metyrapone exhibited no detectable reduction in the severity of the IDPN effect at any time over the course of the 7-day observation period, the methimazole treatment completely prevented the occurrence of the ECC syndrome.

Observing a statistically significant effect of the inhibitors on the severity of axonal enlargements was a more challenging problem. Since these enlargements are optimally produced only at the 2 g/kg dosage, we first attempted to observe an effect of the inhibitors at this dosage. Although we could easily conclude that metyrapone had no inhibiting effect, the methimazole-treated animals still exhibited about one-third to one-half the severity of axonal enlargements seen for the control IDPN cases. However, at the lower IDPN dosage (1 g/kg), although the severity of the
IDPN-induced enlargements is more subtle, we were able to observe that whereas metyrapone had no effect again, methimazole now afforded complete inhibition of the appearance of axonal enlargements.

II.3.3 CONCLUSIONS

The overall conclusion to be drawn from a combination of our in vitro and in vivo studies is that the metyrapone-sensitive cytochrome P-450 metabolism of IDPN (generating BAPN) is not involved in either the behavioral or peripheral neurotoxic effects of IDPN. One might even suggest a role of cytochrome P-450-mediated N-dealkylation of IDPN as a detoxification process. However, the fact that metyrapone did not enhance the severity of IDPN neurotoxicity suggests to us that the low level of metabolism to BAPN does not provide pharmacologically significant detoxification. This conclusion contrasts that arrived at recently by Denlinger et al. (1992, 1994). These workers found that deuteration α to nitrogen in IDPN reduced the production of cyanoacetic acid (from oxidation of the released cyanoacetaldehyde), consistent with a relative reduction of the cytochrome P-450 dealkylation pathway via manifestation of a primary kinetic isotope effect. The finding that α-deuteration then enhanced both behavioral and peripheral neurotoxic effects suggested a detoxicating role of cytochrome P-450-mediated dealkylation. Our results now suggest that one or more metabolic process(es) slowed by deuteration α to nitrogen other than cytochrome P-450 metabolism are probably involved in
detoxification. We will not speculate on what such processes may be.

Clearly, the ability of methimazole to prevent both IDPN neurotoxicities is consistent with a role of FMO-mediated formation of HOIDPN as a requisite step for both toxic conditions. Our finding of increased neurotoxicity by directly administered HOIDPN, relative to that seen with IDPN itself, is then consistent with the former being on the IDPN toxic activation pathway instead of representing merely a structure-activity phenomenon. It is interesting that methimazole inhibition of the ECC toxicity was so much more readily detectable than inhibition of the axonal enlargements. This may indicate an effect of methimazole that discriminates between the two sub-pathways of IDPN neurotoxic activation involved in the ECC and axonal pathologies. Alternatively, many central nervous system agents have been found to effect pharmacological inhibition of the ECC syndrome (Cadet et al., 1987 a,b; Cadet, 1988). Thus we cannot rule out the possibility that inhibition of the IDPN-induced ECC syndrome by methimazole reflects a pharmacologic effect of the drug and/or such effect combined with its effect on IDPN metabolism. Further work will be needed to clarify this possible ambiguity.

Our finding of direct evidence for a role of HOIDPN in IDPN-mediated neurotoxicity (at least the axonal pathology) now raises the question of the mechanism(s) involved. Normally, the hydroxylamine products of FMO action on secondary amines are even better substrates for further transformation to nitrones. However, in the case of HOIDPN, we could find no significant turnover by
microsomal FMO activity. Nonetheless, there is a possibility that HOIDPN is converted to the nitrone via an autoxidation process, which is common for hydroxylamines. This nitrone should tautomerize to a resonance stabilized N-hydroxy cyanoenamine (Scheme II.3), which is expected to be quite reactive toward further autoxidation and/or alkylation of biomacromolecular nucleophiles. Thus, there is at least precedent to support a proposal of a free-radical/"active oxygen" involvement in IDPN toxicity. Such a notion is interesting in that methimazole has been reported to exert antioxidant activity (Sausen et al., 1992). Thus, assuming an "active oxygen" toxicity is responsible for the IDPN-induced neuropathy, the protective effect of methimazole may be at least in part due to an antioxidant effect. Clearly, further investigation of HOIDPN is the obvious next stage of research.
II.4.1 MATERIALS

Nicotine N-oxide was synthesized previously in the laboratory by oxidation of nicotine with m-chloroperoxybenzoic acid as described by Craig and Purushothaman (1970).

N-hydroxy-β,β'-iminodipropionitrile (HOIDPN) was prepared and characterized as described by Morandi et al. (1987).

All other chemicals and enzymes were AR grade as supplied commercially. All solvents used were commercially available HPLC grade.

II.4.2 IN VITRO IDPN METABOLISM

II.4.2.1 HEAT INACTIVATION OF MICROSONAL FLAVIN MONOOXYGENASE (FMO) ACTIVITY

Assays involving the heat treatment of microsomes were done by mixing 0.7 mL of microsomes, 10-20 mg of protein, with a solution of butylated hydroxytoluene (BHT)/CH₃CN to give 200 μM BHT and 2% CH₃CN (v/v) [make 10 mM BHT in CH₃CN and add 0.014 mL to 0.7 mL microsomes] and preincubating the microsomes/BHT/CH₃CN for 3 minutes at 50 °C. The microsomes were then immediately cooled on ice for 2 minutes before adding them to the standard assay above.
P-450 activity was monitored spectrophotometrically on a Varian Cary 210 spectrophotometer by using a benzphetamine demethylase assay according to the method of Nash (1953). This consisted of adding 1.0-3.0 mg of protein, before or after heat treatment, to the standard 1 mL incubation assay (see section I.4.1) containing 1.0 mM benzphetamine. Reactions were terminated by the addition of 0.3 mL of ice-cold 30% TCA, and the precipitated protein was removed by centrifugation. The supernatant (1.0 mL) was added to 0.5 mL of the Nash reagent and the absorbance was read at 412 nm after heating for 1 hour at 37 °C. The Nash reagent consists of 0.45 g NH₄OAc, 0.006 mL 2,4-pentanedione, and 0.009 mL glacial acetic acid per 1.0 mL of water.

A control for FMO activity was done by adding 1.0-3.0 mg of protein, before or after heat treatment, to a nicotine assay. This also consisted of the standard 1 mL incubation assay containing 0.005 mL of nicotine as the substrate. Reactions were terminated by the addition of 1.0 mL ice-cold acetone, and the precipitated protein was removed by centrifugation. The supernatant (1.0 mL) was then extracted with 5.0 mL chloroform and the aqueous layer was monitored for nicotine N-oxide formation by HPLC analysis. Quantification of nicotine N-oxide was determined vs. a standard sample.
II.4.2.2 ASSAYS FOR TLC ANALYSIS

Incubations five times the scale of the standard 1 mL incubation assay were initiated by the addition of 2.5 - 5.0 mg of protein and terminated at the end of 45 minutes with 3.0 mL of ice-cold acetone. When present, Triton X-100 was used at 1 % (v/v). The samples were centrifuged to remove the precipitated protein, and the supernatant was added to 15.0 mL of acetonitrile. This mixture was placed in a 30 mL test tube and submerged in an acetone/dry ice bath. After the aqueous solution froze, the acetonitrile was decanted and evaporated to dryness (rotovap) and the remaining residue was resuspended in of chloroform for TLC analysis. Samples were run on Merck silica gel 60 TLC plates using 9:1 methanol/acetonitrile as the mobile phase solvent against standards. Visualization was by ninhydrin, sprayed on as an acetone solution: characteristic colors and Rₚ values were BAPN (blue, 0.035), IDPN (weak blue, 0.45), and HOIDPN (remains white against red background on strong heating, 0.69).

II.4.2.3 ASSAYS FOR HPLC ANALYSIS

The standard 1 mL incubation assay containing 20.0 mM IDPN and 2.0 units glucose-6-phosphate dehydrogenase when present, was incubated at 37 °C and pH 7.4 in a Dubnoff metabolic shaking incubator. Reactions were initiated by the addition of 0.5 - 1.0 mg of microsomal protein and terminated by addition of 5.0 mL ice-cold acetone. Precipitated protein was removed by centrifugation and the supernatant was
taken to dryness using a rotary evaporator in a 30 °C water bath. To the residue was added 10.0 mL of 3% MeOH/EtOAc, and the resulting precipitate was removed using a 0.45 micron filter. The filtrate was concentrated with a rotary evaporator (30 °C), and the remaining residue was dissolved in 0.2 mL of 3% MeOH/EtOAc for HPLC analysis. Larger scale incubations of 5.0 mL were performed using 5 times the amounts above except for termination with 15.0 mL acetone, addition of 25.0 mL of 3% MeOH/EtOAc, and final resuspension of the residue in 1.0 mL of 3% MeOH/EtOAc.

HPLC analyses were performed using a Shimadzu HPLC System composed of: model SCL-6A system controller, model SPD-6A UV spectrophotometric detector, Varian Refractive index detector, two model LC-6A pumps, model C-R3A chromatopac, and a Rheodyne 7125 manual injector.

Analysis of IDPN metabolic assays employed isocratic solvent conditions of 3% MeOH/EtOAc (reservoir B), two normal phase microPorasil 10 cm x 4 mm columns in tandem, a refractive index detector, and a flow rate of 0.5 mL/min.

Nicotine N-oxide analysis was done using spectrophotometric detection at 254 nm with isocratic solvent conditions of 70% MeOH (reservoir B) and 30% 0.1 M NaOAc containing 1% triethylamine, and brought to pH 7.0 with glacial acetic acid (reservoir A), a Varian MCH-10 30 cm x 4 mm micropak column, and a flow rate of 1.0 mL/min.
II.4.2.4 HOIDPN COLORIMETRIC ASSAY

Reactions were initiated by the addition of 0.5 - 1.0 mg of microsomal protein to the standard 1 mL incubation assay and terminated by addition of 1.0 mL ice-cold 10% TCA. Precipitated protein was removed by centrifugation, and the formation of HOIDPN was determined spectrophotometrically on a Varian Cary 210 spectrophotometer according to the method of Day et al. (1979). The supernatant (1.0 mL) was added to 2.5 mL of the "color reagent" and the absorbance at 595 nm was read after letting the samples sit at room temperature for 1 hour. A standard curve of nmol of HOIDPN vs. absorbance at 595 nm was constructed for quantification of HOIDPN.

The "color reagent" was prepared by adding 0.5 mL of 48 mM 2,4,6-tripyridyl-s-triazine in 0.1 M HCl (Note: Refrigerate and protect from light - good for 6 months) and 0.4 mL of 20 mM FeCl₃ in 0.2 M HCl (Note: Store at room temperature - good for 3 months) to 99.1 mL of an acetate buffer. The acetate buffer was prepared by dissolving 340.2 g of NaOAc in 200 mL of hot water, adding 5.0 M glacial acetic acid (18 mL diluted to 62.5 mL with water) and 140.5 mL MeOH to give 20% (v/v), adjusting the pH to 5.9 with HCl or NaOH, and bringing the total volume to 703.0 mL with water (acetate buffer is good for three months).

Inhibitor data was obtained using the above assay in the presence of 1% Triton X-100 (v/v), or various concentrations of methimazole and metyrapone. Assays involving the heat treatment of microsomes were done as described above for
TLC analysis.

II.4.2.5 BAPN COLORIMETRIC ASSAY

Detection of BAPN was performed spectrophotometrically on a Varian Cary 210 spectrophotometer according to a modification of the method of Benson et al. (1975). This involves quantification of the primary amine as a cyclic derivative ($\lambda_{\text{max}}$ 332 nm) formed from o-phthalaldehyde and mercaptoethanol as shown in Scheme II.3.

\[
\text{RNH}_2 + \text{Cyclic Derivative} \rightarrow \text{Product}
\]

Scheme II.4 Primary amine detection by derivitization with o-phthalaldehyde and mercaptoethanol.

Microsomal protein (0.4-1.0 mg) was added to the standard 1 mL incubation assay to initiate the reaction and 1.0 mL of ice-cold 10% TCA was added to terminate the incubation. Precipitated protein was removed by centrifugation and 1.0 mL of the supernatant was added to 2.0 mL of the "working solution". The absorbance at 332 nm and 370 nm was determined after the samples sat at room temperature for 2 hours. The specific absorbance due to BAPN was determined as the increased $A_{332}$.
relative to the change in $A_{370}$ which corrects for background absorbance changes:

$$A_{BAPN} = (A_{\text{sample} 332\text{nm}} - A_{\text{control} 332\text{nm}}) - (A_{\text{sample} 370\text{nm}} - A_{\text{control} 370\text{nm}})$$

A standard curve of nmol of BAPN vs. $A_{BAPN}$ was used for quantification. The "working solution" was prepared by dissolving 24.74 g of boric acid in 900 mL $H_2O$, adding 10.0 g of Tween 20, 800 mg of freshly recrystallized $\sigma$-phthalaldehyde in 10 mL of 95% ethanol, adding 2.0 mL of 2-mercaptoethanol, adjusting the pH to 9.7 with 10.0 M KOH, and bringing the final volume to 1.0 liter.

The effects of metyrapone and methimazole on BAPN formation were determined by using the above colorimetric assay in the presence of various concentrations of the two inhibitors. In these cases, controls with inhibitors in the absence of IDPN were needed to correct for background absorbance due to the inhibitors alone.

II.4.2.6 BAPN FLUOROMETRIC ASSAY

BAPN was also detected fluorophotometrically using a similar assay to that used for the colorimetric detection. In this assay, however, the 1.0 mL incubation assay consisted of 50.0 mM phosphate buffer containing: 15.0 $\mu$mol $MgCl_2$, 15.5 $\mu$mol trisodium isocitrate, and 1.0 $\mu$mol of NADP$^+$. When present, 0.2 units of isocitrate dehydrogenase (ICD) were used to initiate the NADPH-dependent metabolic
reactions. This particular "NADPH regenerating system" was used rather than the glucose-6-phosphate-based system in order to prevent possible interference of amine analysis arising from reaction with the glucose C-1 carbonyl.

Also, for this assay, the microsomal storage buffer was found to give a large background fluorescence due to the Tris acetate. This was replaced with an equal molar concentration of tricine buffer, which did not interfere with the detection method. BAPN detection was accomplished by using an Aminco-Bowman Spectrophotofluorometer. The excitation and emission wavelengths used were 352 and 440 nm, respectively.

II.4.2.7 METABOLISM OF HOIDPN

To study the metabolism of HOIDPN, the same colorimetric assay described above for the detection of HOIDPN was used except that 0.01 mL of the supernatant was added to 2.5 mL "color reagent", 0.495 mL 10% TCA, and 0.495 mL of H₂O.

HPLC analysis of HOIDPN metabolism was performed using the same incubation and HPLC conditions that were used for IDPN metabolism studies.
II.4.3 IN VIVO IDPN METABOLISM

II.4.3.1 COMPOUND ADMINISTRATION

Three experimental studies were performed with intraperitoneal administration to male Sprague-Dawley rats. Rats were received from Zivic Miller Laboratories, Zelienople, PA and housed three per cage in standard plastic cages. Food (Purina Formulab Diet 5008) and water were provided ad libitum. Fifteen rats (9-10 weeks old, 270-300g) were used for the first study. Two rats received one dose of IDPN (2g/kg); 3 received one dose of metyrapone (60 mg/kg) 30 minutes prior to administration of IDPN (2g/kg); 3 received one dose of methimazole (200 mg/kg) 30 minutes prior to administration of IDPN (2g/kg); 3 received doses of metyrapone (90 mg/kg) 30 minutes prior to and 24 hours after administration of IDPN (2g/kg); 3 received doses of methimazole (300 mg/kg) 30 minutes prior to and 24 hours after administration of IDPN (2g/kg); and 1 received saline only as a control.

Ten rats (6-7 weeks old, 190-220 g) were used for the second study. Three rats received one dose of IDPN (2g/kg); 3 received one dose of metyrapone (90 mg/kg) 30 minutes prior to administration of IDPN (2g/kg); 3 received one dose of methimazole (300 mg/kg) 30 minutes prior to administration of IDPN (2g/kg); and 1 received saline only as a control.

Ten rats (6-7 weeks old, 190-220 g) were used for the third study. Three rats received one dose of IDPN (1g/kg); 3 received doses of metyrapone (90 mg/kg)
30 minutes prior to and 24 hours after administration of IDPN (1g/kg); 3 received doses of methimazole (300 mg/kg) 30 minutes prior to and 24 hours after administration of IDPN (1g/kg); and 1 received saline only as a control.

II.4.3.2 PERFUSION AND DISSECTION OF SPINAL CORD

Under halothane anaesthesia, all the animals were perfused through the ascending aorta with 0.2 M cacodylate buffer, pH 7.4, followed by 3.5% (v/v) glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4, with 0.03% CaCl₂ (fixative solution). The spinal columns were excised, from the base of the skull to the near base of the tail, and immersed in the fixative solution overnight.

The spinal cord was exposed by cutting away the vertebrae using small bone cutters, being careful to keep the tissue always wet. After exposing the cord, sections, along with roots if possible, were cut out with a scalpel and placed on a wax block (3 sections approximately 3 mm in length, one each from the top, middle, and near bottom). A 0.5 mm slice was cut off of the sections using a very thin razor blade, and a very slight piece of the posterior part of the cord was cut away. The remaining piece of the cord was then cut in half and both pieces were placed in a solution of 3.5% glutaraldehyde in 0.2 M Na-cacodylate buffer containing 0.5% CaCl₂. The sections were embedded the next day by the following embedding procedure.
II.4.3.3 EMBEDDING PROCEDURE

After removing the Na-cacodylate/glutaraldehyde solution, 0.2 M Na-
cacodylate buffer (warmed to room temperature) was used to rinse the cord sections. This solution was removed and replaced with a solution of 2% osmium tetroxide/50% Na-cacodylate, diluted 1:2 with water, at room temp for 6 hours (Note: OsO₄ is toxic and volatile. Perform in the fume hood). After removal of this solution, the sections were rinsed with 30% acetone, placed in 30% acetone for 15 minutes, and then placed in 60% acetone and refrigerated overnight. The next day the sections were placed in 90% acetone for 15 minutes, followed by three repetitions of placing in 100% acetone for 15 minutes.

While performing this dehydration process, the "Spur" plastic medium was prepared. This was done by weighing out: 13.0 g 2-non-1-ylsuccinic anhydride; 5.0 g 4-vinylcyclohexene dioxide; 3.0 g DER-736 resin; and 0.4 g 2-dimethyl-
aminoethanol. Due to the high carcinogenicity of these chemicals, disposable pipets and containers were used. This solution was mixed gently and thoroughly and then covered to prevent absorption of water.

The acetone was removed from the sections and a 1:1 mixture of 100% acetone/spur was added for 1 hour. Another solution of acetone/spur (approximately 1:2) was then added, and the sections were left in this combined solution for another hour. This solution was removed and replaced with 100% spur. The vials were capped and the sections were left in the spur overnight.
The bottom of the plastic molds were cut off, and labels (marked with pencil) were put in them. One drop of fresh spur was put in the mold and a section was added making sure it laid flat in the mold. The rest of the mold was filled with spur and placed in an oven at 70 °C for 24 hours. After cooling, the mold was cut away and the samples were taken to be cut for observation. Sections were cut by the electron microscopy service of the Case Western Reserve School of Medicine, and slide mounted for observation.

II.4.3.4 DETERMINATION OF NEUROTOXIC CONDITIONS: ECC SYNDROME AND PERIPHERAL NEUROPATHY

The severity of the head movements associated with the ECC syndrome along with the weights of the rats were monitored and recorded daily. Weights were recorded in grams while the head movements were reported qualitatively by using a rating scale of 0 - 6, with zero representing normal head movements and 6 representing the severest lateral and vertical head jerks. A summary statistic was obtained by averaging the score for each treatment group on a daily basis.

By observing the slides of the embedded samples under a microscope, the extent of the axonal swellings was also rated qualitatively on a scale of 0 - 5, with zero representing normal morphology and 5 representing the maximal swelling observed. Again, a summary statistic was obtained by averaging the score for each treatment group on a daily basis.
LITERATURE CITED
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PART I. METABOLIC ACTIVATION OF CYCLIC TERTIARY AMINES


PART II. NEUROTOXIC ACTIVATION OF β,β'-IMINODIPROPIONITRILE


