Pharmacokinetics, Metabolism, and Pharmaceutics of Drugs of Abuse

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Introduction

Rao S. Rapaka, Nora Chiang, and Billy R. Martin

Drugs of abuse represent a wide array of chemical classes. Therefore, it is not surprising that the consequences of drug abuse are highly dependent upon the chemical in question and vary considerably across drug classes. During the past decade, understanding of the actions of these drugs at the molecular and cellular levels has increased dramatically, thereby providing insight into developing new strategies for the treatment of drug dependence. However, the interaction of a drug with a particular biochemical system is only one factor to be considered when evaluating the pathological impact of substance abuse. The route of administration, the pharmacokinetics of the drug, conversion to toxic metabolites, and interindividual responsive-ness are just a few factors. The objective of this monograph is to evaluate the current status of knowledge of metabolism, pharmacokinetics, and pharmacodynamics of drugs of abuse. The overall goal is to identify innovative approaches for the management and treatment of the adverse consequence produced by drugs of abuse.

One of the most important challenges of drug abuse research has been providing an explanation for why all individuals do not respond or react in a similar fashion to a drug. Metabolism plays a critical role in the pharmacological and toxicological consequences of drug exposure. It is well known that the rate of formation of active, inactive, or toxic metabo-lites varies among individuals. The past decade has been marked by the characterization of substrate specificity for a large number of P-450 enzymes and the development of biochemical tools for quantitation of these enzyme levels. These advances have allowed determination of the role of metabolic enzymes in susceptibility to drug dependence, metabolic tolerance, and variability in the toxic consequence of exposure.

The future holds great promise for utilizing a "fingerprint" of P-450 enzymes as genetic markers for predisposition to either enhanced or diminished drug dependence. It is also becoming increasingly clear that brain metabolism has a more prominent role than previously thought. Notable examples include opioid, amphetamine, nicotine, and cocaine metabolism by specific isozymes to varying degrees in different organs. Both synthetic and degradative enzymes for neurotransmitters and endogenous ligands, including the putative endogenous cannabinoid ligand anandamide, deserve attention. Future

efforts should be directed toward characterization of additional isozymes responsible for metabolism of drugs of abuse, understanding regulation of gene expressions resulting in polymorphism, understanding induction of enzymes, development of selective substrates and inhibitors, and development of kinetic models, to name a few. Selective enzyme inhibitors hold promise for ameliorating the toxicity produced by metabolism of some drugs and diminishing drug use of other agents which are converted to potent compounds. The development of animal models with selective enzymatic profiles could serve to assess risk and aid in development of potential therapeutic agents for treatment of drug abuse.

While there has always been concern that metabolic transformation of agents to reactive adducts could result in neurotoxicity, 1-methyl-4-phenyl-1,2,6-tetrahydropyridine (MPTP) provided dramatic evidence. There is direct evidence, as well as considerable indirect evidence, for formation of reactive adducts for neurotransmitters, including dopamine and serotonin, and for exogenous amines such as methamphetamine, phencyclidine, and nicotine. It is now well established that certain dose regimens of methamphetamines produce neurodegeneration of dopaminergic and serotonergic neurons in several species, an effect which could also occur in humans. Several lines of evidence suggest that methamphetamine itself is not directly responsible for this neuronal damage, but rather that it produces oxygen radicals which in turn oxidize the neurotransmitters serotonin and dopamine to toxic metabolites that destroy their respective neurons. At present, the mechanism by which methamphetamine produces its neuronal toxicity has not been fully explained and is worthy of pursuit. If aberrant metabolites of dopamine and serotonin are found to be responsible for methamphetamine-induced toxicity, it is essential that these agents be identified and the mechanisms for their formation and action at the neuron be elucidated.

There is speculation that long-term exposure to cyclic tertiary amines such as cocaine, phencyclidine, and phenothiazines may result in biochemical lesions through the formation of reactive metabolites. This premise is supported by the induction of a parkinsonian state by MPTP. Although the initial product of microsomal oxidation is an electrophilic endocyclic iminium intermediate which is thought to be the reactive species primarily responsible for neurotoxicity, there is now evidence that the iminium is in equilibrium with the endocyclic enamine and that the latter is transformed to reactive species. The presence of iminium- detoxifying enzymes in cytosolic and microsomal fractions suggests that rapid inactivation of the iminium

species averts toxicity by minimizing formation of the iminium. If the premise is correct, future efforts should be devoted to designing drugs which have a higher affinity for the iminium-detoxifying enzymes. Characterizing these enzymes and identifying the reactive species of both iminium-enamine equilibrium products will provide a better understanding of the mechanisms involved in the neurotoxicity of cyclic amines and the development of strategies for minimizing their occurrence. The localization of these enzymes and establishment of substrate specificity would be of considerable interest.

Metabolism also plays a critical role in the pharmacology of cocaine. The rapid hydrolysis of cocaine via two different pathways leads to its rapid inactivation/detoxification. This rapid metabolism has been a major determinant in the methods and modes of cocaine abuse. Identification and characterization of these hydrolytic enzymes would be useful in that selective induction of these enzymes offers a potential treatment strategy for dealing with cocaine overdose. It is conceivable that long-term elevation of the enzyme or enzymatic activity could be used in conjunction with maintenance therapy for cocaine addicts. Hydrolases or esterases are also responsible for the transesterfication of cocaine. The pharmacological effect of cocaine is prolonged and enhanced when cocaine is used in conjunction with ethanol. A carboxylesterase catalyzes an ethyl transeterification of cocaine to cocaethylene, which is biologically active. In addition, ethanol inhibits cocaine metabolism. The increased levels of cocaine and cocaethylene may contribute to the prolonged and enhanced effects of cocaine. Characterization of the responsible enzymes would be important for developing substrate specificity and other means for attenuating this process. An alternative approach to detoxification involves biocatalysts. Theoretically, these agents would be devoid of pharmacological properties themselves, a feature that is not always present in therapeutic compounds. In principle, the biocatalysts should work in an exponential fashion which would enable them to detoxify large quantities of the drug. While biocatalysts have tremendous potential, there are many unanswered questions. In addition, development of appropriate models for evaluating biocatalysts is essential.

It seems highly improbable that either in vitro model systems or computer simulations will be introduced in the near future that would give researchers the confidence to administer drugs to humans without prior testing in other animal species. Most likely animal models will continue to be used as predictors for pharmacokinetics and toxicity of drugs in humans. Improvements in allometric models, or the correlation of pharmacokinetics among different species, is to be encouraged. Also, hair has been identified

as an excellent repository for detection of abused drugs. While there is relatively little controversy regarding hair analysis for the purpose of establishing the occurrence of drug use, the reliability of monitoring the incidence of prior use is far less certain. It remains to be determined whether a pharmacokinetic model can be developed which has a high degree of predictability for temporal incorporation of drugs in hair.

The primary focus of the adverse consequences of drugs of abuse has been directed toward the central nervous system (CNS), with justification. These drugs are abused because of their actions on the brain, and they serve as important probes for exploring brain function as it relates to cognition, pain perception, pleasure, and so forth. Most of the adverse consequences of drug exposure emanate from the CNS, either through impairment during intoxication or long-term behavioral changes. Increasingly, attention is being directed to the adverse effects of drugs of abuse on the entire organism rather than merely the brain. The National Toxicology Program examines the pathological consequences of long-term exposure to any drug. An important aspect of this program is the attempt to establish the relevance of the animal results with human exposure.

The acquired immunodeficiency syndrome (AIDS) epidemic has served to increase awareness that the adverse consequences of drug abuse extend beyond the acute and chronic effects produced by these drugs on the brain. The immune system has received considerable attention because both opioid and cannabinoid receptors have been identified in spleen cells. Additionally, specific effects of several drug classes on immune function are now well documented. These studies not only provide important insights into the potential harm that drugs of abuse inflict on a compromised immune system; they provide opportunities to elucidate the mechanism of action these drugs.

Lastly, the mode by which individuals abuse drugs has become an increasingly important factor. When the primary mode of cocaine abuse switched from nasal insufflation to inhalation, a transformation in the abuse of cocaine occurred. The ease by which cocaine free base could be volatilized and inhaled, coupled with savvy marketing strategies of drug pushers, have made a lasting impact on the manner in which drugs are abused. Although methamphetamine has been smoked for many years in some cultures, the recent rise in heroin inhalation underscores this point. The question arises as to whether drugs are more addictive when inhaled. While this questions remains to be answered, it is clear that inhalation and smoking represent highly efficient means of abusing some drugs. There is now concern as to whether the pattern and frequency of use of other drugs might change as dramatically as that of cocaine if the route of

administration changes to volatilized and inhaled. Furthermore, establishing the pharmacokinetics of drugs after inhalation should serve to determine whether sensitivity is enhanced via inhalation.

There is a need for new strategies in providing direct evidence for the formation of reactive intermediates and the identification of adducts. Since the formation of adducts may be without adverse consequence, it is important to establish models for assessing the outcome, particularly with regard to qualitative and quantitative analysis of adduct formation, measurement of neuronal impairment, and pathological relevance. Current treatment approaches for dealing with life-threatening conditions arising from acute drug intoxication stress amelioration of symptomatic consequences. New therapies in the future may well include highly selective enzyme inhibitors, alterations in the activity of metabolic enzymes, use of biocatalysts, and so forth. Additionally, establishing pharmacogenetic polymorphism may well explain individual sensitivity and vulnerability to CNS-acting drugs. Adding new research tools is necessary for meaningful advances in the management of drug abuse treatment.

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The Potential Role of the Cytochrome P-450 2D6 Pharmacogenetic Polymorphism in Drug Abuse

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INTRODUCTION AND BACKGROUND

The Cytochrome CYP2D6 Genetic Polymorphism

Cytochromes P-450 (P450s) are enzymes involved in the oxidative metabolism of a wide array of endogenous and exogenous molecules including steroids, plant metabolites, prostaglandins, biogenic amines, drugs, and chemical carcinogens. This broad spectrum of reactions is due to multiple P-450 isozymes with differing but overlapping substrate specificities. The mammalian P-450 superfamily consists of at least 12 families and over 400 individual genes (Nelson et al. 1993). Genetic variants of P-450 have been discovered because of atypical clinical responses in individuals who were subsequently shown to have a reduced ability to metabolize a drug. The most widely studied P-450 polymorphism was first revealed with the antihypertensive drug debrisoquin (Mahgoub et al. 1977). This drug is 4-hydroxylated by a P-450 enzyme called cytochrome P-450 2D6 (abbreviated CYP2D6) that is encoded by the CYP2D6 gene.

At least 30 drugs, many of them derived from plant alkaloids, have subsequently been shown to be oxidized by CYP2D6, including tricyclic antidepressants, some neuroleptics, beta blockers, and antiarrhythmic agents such as perhexiline, flecainide, and encainide. Several drugs of abuse including codeine (Chen et al. 1988), hydrocodone (Otton et al. 199 *a*), oxycodone (Otton et al., unpublished observation), dextromethorphan (Schmid et al. 1985), and p-methoxyamphetamine (PMA) (Kitchen et al. 1979) are known to be metabolized by this enzyme. CYP2D6-mediated metabolism of these drugs is known to be a major source of pharmaco-kinetic variation and variation in drug effect.

Functional and Molecular Characterization of CYP2D6

The activity of CYP2D6 is measured using a subclinical dose of debrisoquin or another probe drug such as dextromethorphan. Using dextromethorphan, the amounts of unchanged drug and metabolite excreted in urine over zero to 8 hours are measured and expressed as an O-demethylation ratio (ODMR) (i.e., the ratio of dextromethorphan/dextrorphan or (dextromethorphan + 3-methoxymorphinan)/(dextrorphan + 3- hydroy- morphinan)). The frequency distribution of the logarithm of these ratios is bimodal (figure 1), with 7 to 10 percent of the population comprising the upper mode (called poor metabolizers [PMs]). The frequency shows considerable interethnic variation (Kalow 1991); for example, the frequency among African Americans is about 2 percent and among Chinese less than 1 percent. The remainder of the population, who perform this reaction to varying degrees, are called extensive metabolizers (EMs). ODMR values are constant over time and are not influenced by gender, smoking, and classical P-450 inducers such as phenobarbital and rifampicin (Eichelbaum et al. 1986; Vincent-Viry et al. 1991).

In addition to functional assays for CYP2D6, polymerase chain reaction (PCR) amplification assays have been developed for the detection of specific mutations in the CYP2D6 gene (table 1). Additional genotyping

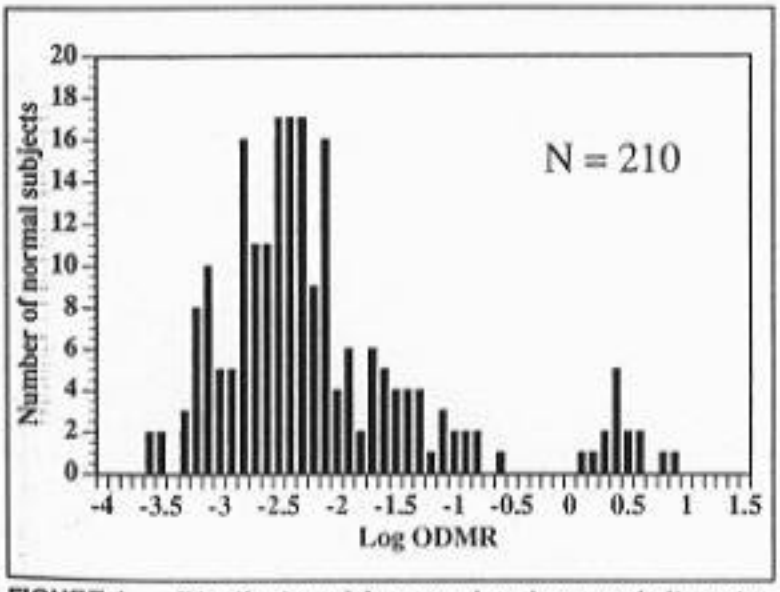

FIGURE 1. *Distribution of dextromethorphan metabolic ratio in normal subjects.*

assays use restriction fragment length polymorphisms (e.g., Xbal haplotypes) or allele-specific endonuclease digestion of PCR products to genotype the CYP2D6 locus. These assays can correctly detect > 95 percent of all mutant PM alleles found in caucasians (Broly et al. 1991). The CYP2D6 gene is part of a highly homologous gene cluster on chromosome 22 that includes CYP2D7 and CYP2D8P (Kimura et al. 1989). CYP2D8P contains several gene-disrupting insertions, deletions, and termination codons, indicating that this is a pseudogene. CYP2D7 is normal except for the presence of an insertion in exon 1 which disrupts the reading frame. The transcript for CYP2D7 has not been isolated, and it is unclear what, if any, the metabolic profile for this gene might be. The genotyping assays using PCR technology have an initial amplification that is specific for CYP2D6 introns in order to exclude the very similar CYP2D7 and CYP2D8P genes.

TABLE 1. *Frequencies of EMs and PMs among caucasians.*

Phenotype	Genotype	Frequency(%)
EM - rapid	wt/wt	54
EM - intermediate	wt/A, B, or D	39
PM - slow	A, B, or D/A, B, or D	7

Multiple CYP2D6 alleles that contain point mutations or codon deletions have been isolated (table 2). These mutant alleles, in addition to the CYP2D6 gene deletion (CYP2D6D), result in absent or impaired enzyme function (Broly and Meyer 1993; Gaedigk et al. 1991; Hanioka et al. 1990; Johansson et al. 1993, 1994; Kagimoto et al. 1990; Meyer 1994; Tyndale et al. 1991*a*). If present in the homozygous state, defective alleles result in a PM phenotype. Heterozygous combinations of mutant and wild type (wt) alleles tend to result in decreased CYP2D6 activity, although no functional means are available to fully distinguish between these heterozygous people and those who are homozygous wt/wt. In addition, CYP2D6 gene duplications that result in ultra-rapid enzyme function have recently been identified ($CYP2L_{2\&12}$) (table 2; Johansson et al. 1993).

TABLE 2. *Summary of CYP2D6 alleles, Xbal haplotypes, and their phenotypic consequences.*

Allele	Metabolic activity	Xbal haplotype (kb)
CYP2D6-wt	normal	29
CYP2D6-L	normal	29
(CYP2D6-L)2	ultra-rapid	42
(CYP2D6-L)12	ultra-rapid	175
CYP2D6-C	slightly decreased	29
CYP2D6-J	slightly decreased	29, 44
CYP2D6-W	slightly decreased	29, 44
CYP2D6-Ch1	slightly decreased	29, 44
CYP2D6-A	absent	29
CYP2D6-B	absent	29, 44, 9 + 16
CYP2D6-D	absent	11.5, 13
CYP2D6-E	absent	29
CYP2D6-F	absent	29
CYP2D6-G	absent	29

KEY: kb = kilobase.

While the majority of alleles in caucasians have now been isolated, the frequency of the CYP2D6 alleles listed in table 2 varies dramatically between ethnic groups. In addition, it is likely that many other alleles remain unidentified in other ethnic groups. Based on an understanding of the variants found in caucasians, one might predict that the unidentified variants might be unable to form a functional enzyme and have altered substrate specificity, selectivity, or turnover of the enzyme. The clinical importance of such variants is generally unknown; however, it is feasible that some ethnoracial differences in drug abuse patterns may be explainable by such differences.

The Rat Models of the Polymorphism

Rats have five CYP2D genes, CYP2D1 through CYP2D5, although CYP2D1 is the predominant enzyme expressed in rat liver. In the female Dark Agouti (DA) rat, the CYP2D1 gene is not expressed (Matsunaga et al. 1989) and these animals exhibit impaired oxidation of human CYP2D6 substrates (e.g., debrisoquin (Al-Dabbagh et al. 1981), dextromethorphan (Zysset et al. 1988)) compared to Sprague-Dawley (SD) rats. However, unlike the human deficiency, the deficiency in the CYP2D1 is due to a regulatory difference, not a defective gene for the enzyme. Nevertheless, the DA female rat may

be used as an animal counterpart of the human PM, and the SD female rat as the EM counter-part. Although females of these two rat strains are useful animal models, the rat CYP2D1 is not functionally identical to human CYP2D6. For example, quinidine is a very potent and long-lasting inhibitor of the activity of CYP2D6, both in vitro and in vivo, and its diastereoisomer quinine is 200 times less potent (Otton et al. 1984). However, quinine is the more potent inhibitor of rat CYP2D1 (Kobayashi et al. 1989).

CYP2D Enzyme in Brain

Catalytic, pharmacological, immunological, and molecular criteria have been used to identify cytochrome CYP2D in mammalian brain (Fonne-Pfister et al. 1987; Niznik et al. 1990; Tyndale et al. 1991*b*). The initial observation of CYP2D in dog brain was made during screening of central and peripheral tissues with tritiated GBR-12935, which labels the dopamine transporter protein and the so-called piperazine acceptor site or mazindol-insensitive site in brain tissue (Niznik et al. 1990). High concentrations of the piperazine acceptor site were found in liver microsomes. The similarity between amphetamine derivatives that inhibited both GBR-12935 striatal binding and hepatic CYP2D6 activity prompted further studies that demonstrated correlations between the inhibitor profile at the piperazine acceptor site purified from dog striata and the inhibition constant (K_i) for human hepatic CYP2D6 ($r = 0.85$). Immunoprecipitation and Western blotting experiments confirmed the presence of CYP2D in dog striata.

Subsequent studies focused on the catalytic activity of CYP2D in canine striata (Tyndale et al. 1991*b*). One of the classic CYP2D6 substrates, sparteine, was used as the marker for CYP2D activity in dog striata. High ($r \geq 0.95$) correlations were observed between inhibition of sparteine oxidation (K_i values) in canine striata and in human hepatic microsomes, and in human CYP2D6 expressed in HepG2 cells ($r = 0.93$). (-)-Cocaine was found to have particularly high inhibitory potency ($K_i = 74$ nanomolars (nM) for canine striatal CYP2D), and a high degree of overlap was found between compounds binding to the dopamine transporter and striatal CYP2D. The distribution of CYP2D in dissected regions of dog brain demonstrated a fortyfold range in activity, with the highest level being found in supraorbital cortex and parietal cortex, and the lowest in the cerebellum.

An uneven distribution of CYP2D activity was also observed in preliminary studies of dissected monkey *(C. Aethiops)* brain (Tyndale et al., unpublished data). Subcellular preparations of the nucleus accumbens, amygdala, and parietal cortex oxidized sparteine at rates of 300 to 600 picomoles per milligram of protein per hour (pmol/mg protein/hour). (By contrast, the rate in monkey hepatic microsomes was 62,000 pmol/mg protein/hour). The rate in striatum, hippocampus, and temporal and olfactory cortex was approximately 75 pmol/mg protein/hour. The spinal cord, frontal cortex, midbrain, medulla, and cerebellum had negligible activity. CYP2D activity in monkey brain displayed the stereoselective inhibition by quinidine and quinine that is characteristic of monkey liver CYP2D and human liver CYP2D6. Regional variation in the distribution of CYP2D messenger ribonucleic acid (mRNA) in rat and human brain has also been observed (Tyndale et al., unpublished observations). There is molecular evidence for CYP2D6 mRNA in human caudate (Tyndale et al. 1991*b*). The polymerase chain reaction was used to amplify a complementary deoxyribonucleic acid (cDNA) fragment (513 base pains) from a human caudate lambda-gt11 library. One hundred percent nucleotide identity to the CYP2D6 mRNA was found.

Relevance of CYP2D6 to Drug Abuse

A number of drugs of abuse are known substrates (e.g., codeine, hydrocodone, p-methoxyamphetamine, amphetamine) or inhibitors (e.g., (-)-cocaine, pentazocine) of CYP2D6. For some of these drugs, the pharmacokinetic differences due to the polymorphism will be so profound that they are likely to exceed pharmacodynamic sources of variation in response. For other drugs (e.g., hydrocodone to hydromorphone, codeine to morphine, oxycodone to oxymorphone), CYP2D6 may not contribute importantly to the overall clearance of the drug, but may catalyze the formation of highly active metabolites.

The consequences of absent or inhibited CYP2D6 for any particular drug depend on the relative activity of the parent drug and its various metabolites. In some cases, the pharmacology of the metabolite is qualitatively similar to the parent drug (e.g., hydrocodone to hydromorphone, codeine to morphine); in other cases, the pharmacology is different (e.g., dextro-methorphan to dextrorphan); more usually, it is not properly understood (e.g., PMA to 4-hydroxyamphetamine). In addition, CYP2D6 occurs within the central nervous system (CNS) (Fonne-Pfister et al. 1987; Tyndale et al. 1991*b*). Its role in the brain is unknown, but potential formation of active drug metabolites at their site of action makes the presence

of CYP2D6 here of great functional significance. A computer simulation study of interregional differences in CNS drug metabolism has provided a model that could account, in part, for large intersubject variability in the pharmacodynamic effects of psychoactive drugs (Britto and Wedlund 1992).

The authors hypothesize the following:

- The genetically determined activity of CYP2D6 that results in EMs (90 percent) and PMs (10 percent) of some drugs of abuse is both an important risk factor and protective factor in drug abuse and toxicity from drugs of abuse.

- Certain inhibitors of CYP2D6 or the PM state itself result in unexpected toxicity from drugs of abuse (e.g., PMA) or may have utility in the treatment of drug dependence (e.g., preventing activation of a pro-drug such as codeine or oxycodone).

- CYP2D6 may play an important neuroregulatory role in the brain, and may modulate brain functions important in drug-reinforced behavior or neurotoxicity.

CYP2D6-deficient individuals (PMs) should have a much decreased probability of abusing a drug converted to an active metabolite capable of maintaining drug-taking behavior (e.g., codeine, oxycodone, hydrocodone, dextromethorphan). In EMs, the probability is increased and is propor-tionate to the individual's genotype (homozygous versus heterozygous) and absolute CYP2D6 activity. Conversely, PMs should experience greater risk of abuse and of toxicity to a drug that is inactivated by CYP2D6 (e.g., PMA, methamphetamine); EMs should have a lesser risk.

Amphetamines and Phenethylamines. Deficiency in the p-hydroxylation of amphetamine was one of the observations that led to the discovery of the CYP2D6 polymorphism (Dring et al. 1970; Smith 1986). A single oral administration of the radiolabelled enantiomers of amphetamine to three volunteers with subsequent analysis of urine indicated that about 5 percent of (+)-amphetamine was converted to p-hydroxyamphetamine in two subjects but to a much less extent in the third subject, who was later found to have CYP2D6 deficiency (Smith 1986). The main excretion product was unchanged amphetamine (although the extent of excretion is known to be pH dependent), and the major metabolites were products of side

chain deamination (i.e., benzoic and hippuric acids). Total recovery of the radiolabelled dose in urine after 4 days was around 90 percent (Dring et al. 1970).

The hallucinogen PMA is O-demethylated by CYP2D6 to form 4-hydroxyamphetamine (Kitchen et al. 1979). A PM was observed to excrete 4.4 percent of a 5 milligram (mg) oral dose of PMA as 4-hydroxyamphetamine, compared with 50 to 65 percent excretion of this metabolite (free and conjugated) in EMs. Shortly after it made its appearance in Ontario in the early 1970s, PMA was associated with a number of deaths (Sellers et al. 1979). A cardinal feature of these deaths was cardiovascular excitation and hyperthermia; it had been noted earlier to produce marked and sustained elevation in blood pressure in some people.

Urinary excretion data of human subjects indicate that 4-hydroxylation of methamphetamine is much more extensive than that of amphetamine; the metabolic ratio (total hydroxymethamphetamine/methamphetamine) in urine averaged about 15 with individual variations of approximately fiftyfold (Shimosato 1988), suggesting considerable importance of CYP2D6 polymorphism in the fate of methamphetamine. On the other hand, there seems to be no information on the further metabolism of p-hydroxymethamphetamine as is available for p-hydroxyamphetamine.

p-Hydroxyamphetamine has been used as a drug (paredrine) in ophthalmology. Like amphetamine, it is known to release norepinephrine from postgan-glionic sympathetic nerve endings; unlike amphetamine, its use is associated with few if any CNS effects, presumably because its relative water solubility slows its passage through the blood-brain barrier (Burde and Thompson 1991). As with amphetamine, the releasing action of p-hydroxyamphetamine is thought to be a consequence of its inhibition of the reuptake mechanism and of monoamine oxidase activity, leading to a postsynaptic accumulation of not only dopamine but also of 5-hydroxy-tryptamine (Arai et al. 1990; Cho et al. 1975).

p-Hydroxyamphetamine is metabolized to pharmacologically active secondary products. Its biotransformation by dopamine-beta-hydroxylase leads to p-hydroxynorephedrine, a false neurotransmitter (Coutts and Baker 1989; Dougan et al. 1986; Smith 1986) with similar biochemical activities as p-hydroxyamphetamine (Arai et al. 1990). p-Hydroxy amphetamine is also metabolized by a neuronal

P450 to alpha-methyl-dopamine and further to alpha-methyl-noradrenaline, a CNS-active hypotensive agent that also may function as a false transmitter (Hoffman et al. 1979). Dougan and colleagues (1986) described the stereoselective neuronal accumulation of hydroxyamphetamine and hydroxynorephedrine in rat striatum and found them to have half-lives of 1.5 and 2.5 days, respectively, at this location.

d-Amphetamine hydroxylation has been observed in whole brain and in striatal preparations in both microsomal and mitochondrial membranes (Liccione and Maines 1989); manganese pretreatment increased amphetamine hydroxylation in both membranes. After administration of amphetamine, p-hydroxyamphetamine (also called alpha-methyl-p-tyramine), p-hydroxynorephedrine (also called alpha-methyl-p-octopamine), alpha-methyl-dopamine, and alpha-methyl-noradrenaline have been found in brain (studies mostly done in rat brain). Formation, or lack of formation, of these metabolites may be catalyzed by CYP2D, with the p-hydroxylation of amphetamine as the first step.

Suzuki and colleagues (1986, 1987) have tested the effects of altering methamphetamine p-hydroxylation in rats; it appeared that inhibition enhanced the methamphetamine-induced stereotyped behavior. One might conjecture that an equivalent effect could occur in humans with inborn CYP2D6 deficiency if a toxic dose of methamphetamine were applied. Unfortunately, studies of animals do not necessarily help; p-hydroxyamphetamine is the main metabolite of amphetamine in rats but not in human liver (see above), and most studies on brain metabolism have not been conducted in human tissue.

Matsuda and colleagues (1989) have shown that centrally acting amphet-amine metabolites may have different effects on different parts of the brain when applied locally or systemically. Thus the locus of formation of a metabolite within the brain could be critical, and metabolite formation in the liver might be of limited relevance.

Dextromethorphan. Dextromethorphan has the opposite steric configuration to codeine and morphine, is devoid of analgesic effects, but is as potent an antitussive as codeine. It is the most commonly used antitussive worldwide. Systematic studies concerning its abuse liability are few. In the earliest report (Isbell and Fraser 1953), no abuse liability was found with oral doses up to 100 mg. However, there are many case reports of dextromethorphan abuse and endemic use principally among young people (McElwee and Veltri 1990).

Monkeys and rats can be trained (with difficulty) to self-administer dextromethorphan, but do so more easily for its active metabolite dextrorphan. They also recognize dextrophan in particular as a discriminative stimuli, and generalize from and to phencyclidine (PCP) (Holtzman 1980). Interest in dextrorphan continues because of its similarity to PCP in mechanism of action and behavioral pharmacology, the potential role of N-methyl-D-aspartate (NMDA) antagonists in neuroprotection, and the evolving CNS pharmacology of dextromethorphan and dextrorphan (Musacchio et al. 1988; Tortella et al. 1989). The elucidation of the pharmacogenetic control of dextromethorphan kinetics, metabolism, and effects as a representative of the class could be important (Schadel et al. 1995).

In human volunteers the kinetics of dextromethorphan in EM and PM subjects are very different (Schadel et al. 1995). Hence, in a clinical practice the pharmacologic effects and consequences should be very different in the EM and PM phenotypes.

Prescription Opiates. Historically, methadone has evolved as the only widely accepted and used pharmacologial adjunct for the treatment of opiate abuse. Because methadone has strong reinforcing properties and produces physical dependence, it has generally been reserved for the management of heroin abuse and dependence. From a public health perspective, the misuse and abuse of opiate-containing prescription medications is a poorly understood and largely unaddressed treatment issue. Prescription analgesics including opioids (e.g., codeine, oxycodone, and hydrocodone) alone and in combination are extensively used worldwide and are always within the major classes of drugs prescribed in all countries. While most users have a legitimate need for these medications, there is also substantial evidence of misuse, abuse, and dependence since the frequency of use appears to exceed the frequency of acute and chronic pain in the population.

Canada has the dubious distinction of having the world's largest per capita consumption of precursor opiate-containing compounds (e.g., codeine, oxycodone, and hydrocodone) (Korcok 1979). A drug utilization review of opiate use in Canada from 1978 to 1989 shows continued increase in the use of prescribed codeine combination products. The defined daily dose (DDD)/1000 inhabitants/day for prescribed codeine-acetaminophen products has increased from 3.3 DDD/1000 inhabitants/day in 1982 to 8.1 DDD/1000 inhabitants/day in 1989. Oxycodone-acetaminophen containing products have also been increasing from 0.04 DDD/1000 inhabitants/day in 1978 to 0.21

DDD/1000 inhabitants/day in 1989 (Seto, unpublished data). Intercontinental Medical Statistics Canada reports that acetaminophen with codeine was the most frequently dispensed prescription product in Canada from 1989 to 1992 and accounted for 56 percent of all new prescriptions in 1992 (Theirriault 1992). The situation is similar in the United States, with codeine being the second most frequently dispensed chemical entity according to a 1987 drug utilization review (Tomita et al. 1988). Acetaminophen with codeine ranked as the fifth and fourth leading prescription product dispensed in 1985 and 1986 respectively (Baum et al. 1987). In 1992 and 1993, as determined by chart review and index drug report by patients, 423 patients have presented to the Clinical Research and Treatment Institute of the Addiction Research Foundation with codeine (60 percent), oxycodone (35 percent), or hydrocodone (5 percent) as the primary problem substance (authors' unpublished data). This data likely underestimate the extent of the problem because reseachers have only recently been systematically focusing on these drugs. The clinical impression is that at least half of all polydrug users have lifetime prescription opiate drug abuse or dependence.

In Vitro Oxidation of Drugs of Abuse by CYP2D6

Identification of Substrates/Inhibitors of Human Hepatic CYP2D6.

The authors have been conducting studies to identify drugs of abuse that interact with CYP2D6 enzyme in human liver microsomes. The results provide drug candidates for further studies aimed at determining whether they are CYP2D6 substrates (e.g., PMA, 3, 4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA)) or inhibitors of CYP2D6 (e.g., fluoxetine, (-)-cocaine). In table 3, competitive inhibitors of CYP2D6-catalyzed formation dextrorphan from dextrometh-orphan are identified with their K_i value. The lower the K_i value, the more potent the inhibitor (higher affinity for CYP2D6).

These in vitro results identify compounds that interact with hepatic CYP2D6, and compounds from this list become the candidates for the authors' investigations of the biological importance of this enzyme activity.

TABLE 3. *A selected list of drugs tested for an interaction with human hepatic CYP2D6.*

	K_i (M)
Phenethylamines	
(+)-Amphetamine[1]	28
(-)-Amphetamine	no interaction
(+)-Methamphetamine[1]	25
4-Methoxyamphetamine (PMA)[1]	18
3,4-Methylenedioxyamphetamine (MDA)[1]	3
Opioids	
Hydrocodone[1]	55
Oxycodone[1]	55
Morphine	150
Nalorphine	35
Methadone	3
Stimulants	
(-)-Cocaine[2]	0.74
Miscellaneous	
Nicotine	no interaction
Ketamine	no interaction
Caffeine	no interaction

KEY: 1 = identified by further studies in vitro as a CYP2D6 substrate; 2 = identified by further studies in vitro not to be a CYP2D6 substrate.

Findings that have stemmed from this screening include the following:

1. The establishment that CYP2D6 converts hydrocodone to its active metabolite hydromorphone in vivo. This drug was subsequently selected as the prototypical pro-drug opiate used to study the role of CYP2D6 activity variants in opiate plasma kinetics and in responses related to abuse (Otton et al. 1993*a*).
2. A possible clinical consequence of the potent (K_i = 0.18 micromolar (M)) inhibition of CYP2D6 by fluoxetine and its major metabolite norfluoxetine. The authors described a case report of a profound increase in the dose of oxycodone required for analgesia after the initiation of fluoxetine therapy. This led

to the observation that oxycodone is converted to its active metabolite oxymorphone via CYP2D6, and that therapeutic doses of fluoxetine inhibit CYP2D6 activity in vivo (Otton et al. 1993*b*). The kinetic profile of fluoxetine and its normetabolite favor its use as a long-acting CYP2D6 inhibitor; hence the drug might be used to inhibit the activation of codeine, hydrocodone, or oxycodone (also called producing a phenocopy because the urine metabolites look the same as in PMs) in abusers and thereby reduce the reinforcing properties of the medication.

3. In vitro inhibition of CYP2D6 activity by methadone (K_i = 3 M) predicted drug interactions in vivo. This finding was confirmed in 42 abusers of oral opiates undergoing treatment with methadone (Wu et al. 1993*a*).

4. The O-demethylation of the hallucinogen PMA to 4-hydroxyamphetamine is catalyzed in vitro by CYP2D6 (Wu et al. 1994). This confirms earlier data from three subjects (one a PM) that this reaction was catalyzed by CYP2D6 (Kitchen et al. 1979). Methamphetamine's major metabolite, 4-hydroxyamphetamine, is formed via CYP2D6 (as determined by incubations of methamphetamine with microsomes prepared from the yeast transformed with an expression plasmid containing full-length human CYP2D6 cDNA (a gift of Dr. S.W. Ellis and Dr. M.S. Lennard, Sheffield, U.K.).

5. Because of (-)-cocaine's extremely high affinity for hepatic CYP2D6 (K_i = 0.07 M), this drug is not metabolized by CYP2D6. The authors incubated (-)-cocaine with cloned human CYP2D6 enzyme expressed in yeast. Using a gas chromatography/mass spectrometry assay, no detectable ecgonine, ecgonine methyl ester, ecgonidine, ecgonidine methyl ester, norecgonidine methyl ester, norecgonine methyl ester, benzoylecgonine, o-m-p-hydroxycocaine, or norcocaine was formed during the incubations (Otton et al., unpublished observations).

In Vitro Comparison of Monkey Hepatic CYP2D6-Like Activity With Human CYP2D6. The monkey would be a valuable model for assessing the role of CYP2D6 enzyme activity in drug reinforcement and response. Catalytic, immunologic, and electrophoretic investigations indicated that the enzymes were indistinguishable (Otton et al. 1992). However, the conclusion after more extensive examination of the inhibitor specificity of the two enzymes was that

they were functionally homologous, but not identical (Wu et al. 1993*b*). These studies also predicted that in monkeys an established CYP2D inhibitor, budipine, will more readily produce PM phenocopies than quinidine.

Dextromethorphan Disposition in Rats and Monkeys

Kinetics of Dextromethorphan and Metabolites in Rat Plasma and Brain. Because of dextrorphan's higher affinity for NMDA/PCP sites than dextromethorphan, the abuse characteristics of dextromethorphan are likely due to its CYP2D1-mediated conversion to dextrorphan. Time course studies of this and other metabolites after an intraperitoneal (IP) dose of dextromethorphan indicate that conjugated dextrorphan is the predominant metabolite in plasma, and that only free dextrorphan is present in the brain (Wu et al. 1995). Brain dextrorphan levels were correlated with free dextrorphan in plasma ($r = 0.84$), but not with conjugated plasma metabolite. Similar studies of the disposition of dextrometh-orphan and metabolites following different routes of administration suggest that each will be associated with a different pharmacology (Wu et al. 1995).

FUTURE RESEARCH GOALS AND DIRECTION

Areas which would be important for future research include the following:

1. Identify and characterize more drugs of abuse, particularly phenethylamines and related compounds, that are substrates or inhibitors of the genetically polymorphic human drug metabolizing enzyme cytochrome P-450 2D6 (CYP2D6).

2. Define the localization, catalytic specificity, and regulation of CYP2D forms in rat, monkey, and human brain.

3. Using animal models, determine the importance of CYP2D deficiency or high catalytic activity to the toxicity and behavioral consequences of amphetamines, as models of active drugs of abuse with metabolites of different pharmacologic activity.
4. Determine and compare the clinical consequences of CYP2D6 genotype to the metabolism, kinetics, pharmacologic effects, and abuse liability of methamphetamine, d-amphetamine, and dextromethorphan.

CONCLUSIONS

The authors expect these studies will identify some drugs of abuse for which the EM state, the PM state, or CYP2D6 inhibitors can modify the risk of abuse; explain why some drugs of abuse are so attractive or so toxic to some individuals; explain why some patterns of drug abuse are endemic and rarely epidemic (e.g., PMA, dextromethorphan, smoked methamphetamine, phenethylamine designer drugs); identify the location of CYP2D in the brain and determine its role and importance for variation in drug response to drugs of abuse; justify the incorporation of CYP2D6 screening as part of regulatory or scheduling requirements; and establish animal models for the study of cytochrome P-450s as risk and protective factors in drug abuse.

This work can also result in needed new therapeutic strategies for the treatment of drug abuse. Such issues are particularly important for management of dependence on prescription opiates, which is the third largest drug dependence problem in North America (after nicotine and alcohol) and the least studied and understood.

With respect to treatment, depending on the particular biotransformation pattern and the activity of the metabolites, several therapeutic approaches are evident. For example, EMs (93 percent of the caucasian population) could receive an inhibitor as part of a therapeutic regimen. Such inhibition will alter the kinetics, toxicity, drug-reinforcing properties, and physical dependence liability of some drugs of abuse (e.g., hydrocodone, oxycodone, codeine) and make the drug less pharmacologically attractive.

From a broader scientific perspective, the authors' studies of the drug- metabolizing activity of the brain will contribute to psychopharmacological approaches to the treatment of mental disorders and to researchers' understanding of brain function involving endogenous neurotransmitters, exogenous drugs, neurosteroids, and neuroactive steroids.

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Human Liver Cocaine Carboxylesterases

William F. Bosron, Robert A. Dean, Monica R. Brzezinski, and Evgenia V. Pindel

The stimulant effects of cocaine are relatively short lived, as evidenced by the increase in heart rate that peaks at about 60 minutes after cocaine administration and declines thereafter (Farre et al. 1993). This duration of effect is largely due to the rapid hydrolysis of cocaine to two major deesterified metabolites that appear in serum and urine, benzoylecgonine and ecgonine methyl ester (figure 1). Neither of these deesterified metabolites is active as a stimulant when administered peripherally, even at high doses (Spealman et al. 1989; Misra et al. 1975). The rapid distribution half-life of cocaine from an intravenous (IV) dose is about 10 minutes and the elimination half-life of cocaine is 50 to 80 minutes (Jeffcoat et al. 1989). Hence, the duration of the stimulant action of cocaine is limited by its rate of hydrolysis to inactive metabolites.

The time course for toxic effects of cocaine also appears to be dependent on drug metabolism. For example, almost one-third of the deaths related to cocaine overdose occur about 2 to 5 hours after drug administration (Wang and Carpentier 1994), a time period equal to about two half-lives of cocaine in serum. Sudden cardiac death is one of the acute toxic effects of cocaine overdose, but the mechanism is not fully understood. Direct effects of cocaine on the electrophysiological properties of isolated heart muscle preparations have been shown in vitro (Wang and Carpentier 1994). Cocaine and some of its metabolites have also been shown to be potent vasoconstrictors. Hence, decreased blood flow and hypoxia of cerebral arterioles may be important contributors to cerebral infarction, hemorrhage, and developmental abnormalities, especially in newborn infants exposed to cocaine in utero (Kutrh et al. 1993; Covert et al. 1994). Cocaine-induced hepatotoxicity has been reported in both humans and rodents and the extent of hepatic necrosis appears to be dependent, in part, on the content of microsomal cocaine-metabolizing enzymes (Roth et al. 1992). Hence, the duration and magnitude of the desired stimulant effects, as well as the undesired toxic effects of cocaine use, appear to depend on the content and activity of enzymes responsible for cocaine metabolism.

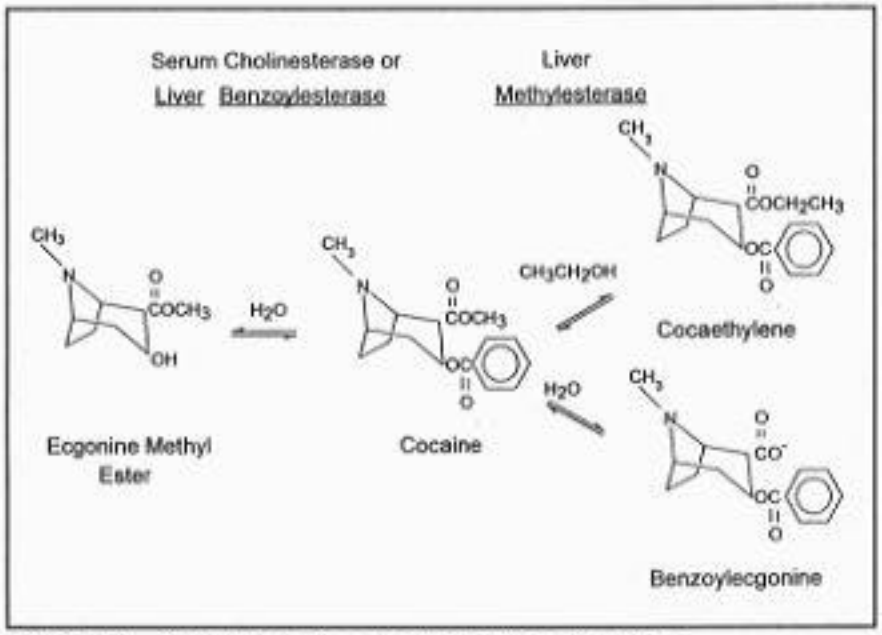

FIGURE 1. *Structures of cocaine metabolites formed by carboxylesterases.*

The hydrolysis of the benzoyl ester group of cocaine to ecgonine methyl ester and benzoate (figure 1) is catalyzed by serum cholinesterase (Stewart et al. 1977), also called pseudocholinesterase or butyrylcholines-terase, and a human liver carboxylesterase (Dean et al. 1991). The hydro-lysis of the methyl ester group of cocaine to benzoylecgonine and methanol (figure 1) occurs spontaneously in aqueous solutions at neutral to alkaline pH. However, the half-life of cocaine at pH 7.2 and 40% is about 5 hours (Garrett and Seyda 1983), which is substantially greater than the half-life of cocaine in vivo, about 1 hour (Jeffcoat et al. 1989). A carboxylesterase was recently identified in human liver that catalyzes the hydrolysis of this methyl ester of cocaine to form benzoylecgonine and methanol (Dean et al. 1991). In the presence of ethanol, the enzyme also catalyzes the ethyl transesterification of cocaine to form cocaethyl-ene plus methanol (Dean et al. 1991).

CHARACTERIZATION OF A HUMAN LIVER COCAINE BENZOYL ESTER HYDROLASE

An enzyme that catalyzes the hydrolysis of cocaine to ecgonine methyl ester and benzoate was purified from human autopsy liver by ion-exchange and affinity chromatography and gel filtration. The enzyme was purified approximately 8,700-fold and about 150 micrograms (g) of enzyme were obtained from 70 grams (g) of human liver. The enzyme catalyzed the hydrolysis of cocaine to ecgonine methyl ester and benzoate and the hydrolysis of methylumbelliferylacetate to methylumbelliferone. The Michaelis constant (K_M) for cocaine was 0.7 millimolars (mM) and the turnover number (k_{cat}) was 7.6 min^{-1}. The enzyme was inhibited by phenylmethylsulfonyl fluoride, diazinon, and eserine. The enzyme is a monomer of approximately 60 kilodalton (kDa) subunit mass. It has an isoelectric point (pI) of approximately 4.9 and is a glycoprotein.

The physiological levels of cocaine can reach a maximum of about 0.3 micromolar (M) in serum immediately after cocaine administration (Jeffcoat et al. 1989). These levels are much less than the K_M of the liver benzoyl esterase (0.7 mM). Hence, the enzyme will obey first-order kinetics, where activity equals k_{cat}/K_M, called the catalytic efficiency, times the cocaine concentration. The catalytic efficiency of the liver benzoyl esterase, 11 min^{-1} mM^{-1}, is similar to human serum cholinesterase, 7 min^{-1} mM^{-1}, another enzyme that catalyzes the hydrolysis of the benzoyl group of cocaine. The content and kinetic properties of serum cholinesterase and liver benzoyl esterase need to be evaluated to determine which enzyme has the greater capacity for hydrolysis of cocaine in human.

The catalytic efficiency of the liver cocaine benzoyl esterase and serum cholinesterase is about 50 times that of a catalytic antibody prepared to an analog of the putative cocaine benzoyl esterase transition-state structure, 0.22 min^{-1} mM^{-1} (Landry et al. 1993). The objective for preparing the catalytic antibody was to provide a reagent that could bind and enhance the clearance of cocaine (Morell 1993). It was proposed that such a reagent could be used to blunt the desire to use cocaine. However, it is not clear whether a cocaine benzoyl esterase that is more efficient than the native serum or liver enzymes can be created by catalytic antibody technology. Perhaps the clearance of cocaine might be increased by inducing the native microsomal benzoyl esterases present in liver.

CHARACTERIZATION OF A HUMAN LIVER COCAINE METHYL ESTER HYDROLASE

An enzyme that catalyzes the hydrolysis of the methyl ester of cocaine (figure 1) was purified to homogeneity by ionexchange and hydrophobic interaction chromatography and gel filtration (Brzezinski et al. 1994). Approximately 3 milligrams (mg) of enzyme were purified from 70 g of human liver obtained at autopsy. The purified carboxylesterase catalyzes the hydrolysis of cocaine to benzoylecgonine and methanol and the hydrolysis of methylumbelliferyl acetate to methylumbelliferone and acetate. The enzyme is a trimer of approximately 59 kDa subunits. It has a pI value of 5.8 and it is a glycoprotein. Fourteen different tryptic and *S. aureus* V8 peptides were purified by high performance liquid chromatography (HPLC) and their amino acid sequences were determined. After searching the amino acid and translated DNA sequences deposited in GenBank, two identical matches were found that correspond to nonspecific carboxylesterase cDNAs from human liver and lung (Long et al. 1991; Riddles et al. 1991; Munger et al. 1991).

In addition to the hydrolysis of cocaine, the purified human liver cocaine methyl ester hydrolase also catalyzed the ethyl transesterification of cocaine with ethanol to form cocaethylene and methanol as shown in figure 1 (Dean et al. 1991; Brzezinski et al. 1994). Both the hydrolytic and the ethyl transesterification reactions increased as the two activities were analyzed in protein fractions obtained during the enzyme purifi-cation by column chromatography. This suggests that the separate activities are catalyzed by the same enzyme. The K_M values for cocaine and ethanol of the purified enzyme at pH 7.3 were 116 M and 43 mM, respectively. The carboxylesterase also catalyzes the formation of ethyloleate from oleic acid and ethanol (Tsujita and Okuda 1992; Brzezinski et al. 1994). Other hydrolases or ester transferases have been reported to catalyze similar substrate "ethylation" reactions. For example, an isozyme of glutathione-S-transferase will also catalyze the fatty acid ethyl-ester synthase reaction, leading to the formation of ethyloleate from oleic acid and ethanol (Bora et al. 1989). Also, phospholipase D catalyzes the transphosphatidylation of phosphatidylcholine with ethanol to form phosphatidylethanol (Kobayashi and Kanfer 1987). The active site requirements and kinetics of the hydrolases or transferases that catalyze these ethylation reactions are not well understood. The elucidation of mechanisms and active site structures for enzyme-catalyzed

ethylation reactions should contribute to the basic understanding of alcohol-drug interactions.

The coabuse of cocaine and alcohol is all too common (Grant and Harford 1990), and is thought to result from the enhanced euphoria and sense of well-being experienced when cocaine is taken with alcohol (Farre et al. 1993). Unfortunately, coabuse of alcohol and cocaine results in an increased health risk (Farre et al. 1993). The formation of cocaethylene under defined experimental conditions in rats after administration of alcohol and cocaine and the cellular and organ toxicity of cocaethylene are discussed by Dean and colleagues elsewhere in this volume.

STRUCTURE-REACTIVITY RELATIONSHIPS FOR COCAINE CARBOXYLESTERASES

The human liver cocaine methyl and benzoyl carboxylesterases catalyze the hydrolysis of the acetyl group of 4-methylumbelliferyl acetate with specific activities of 6.8 and 140 mol/(min ß mg protein), respectively. With cocaine as substrate, however, the cocaine methyl and benzoyl estereases exhibit lower specific activities, but show absolute specificity for the methyl ester group and the benzoyl ester group, respectively. A high specificity for binding of cocaine derivatives to proteins or catalytic specificity for metabolic reactions is frequently observed. For example, Gatley reported that the benzoyl group of benzoylecgonine was not hydrolyzed by serum cholinesterase (Gatley 1991). Additionally, Gatley observed that serum cholinesterase exhibited about a 2,300-fold higher relative activity with the unnatural (+) isomer of cocaine than the natural (-) isomer (Gatley 1991).

The determination of structure-reactivity relationships of cocaine-metabolizing enzymes and binding proteins represents an important direction for research in cocaine metabolism. Binding site structures could be determined directly by protein X-ray crystallography and computerized modeling of cocaine binding. Such structure-reactivity studies will provide valuable information regarding potential drug or metabolite interactions with these important cocaine metabolizing enzymes and binding proteins.

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Effects of Ethanol on Cocaine Metabolism and Disposition in the Rat

Robert A. Dean, William F. Bosron, Frederick M. Zachman, Jing Zhang, and Monica R. Brzezinski

COABUSE OF COCAINE AND ALCOHOL

Abuse of cocaine in combination with other drugs is a widespread practice (Washton and Gold 1987) and the coabuse of alcohol is particularly common, with 99 percent of cocaine addicts reporting excessive use of ethanol in one study (Newcombe and Bentler 1987). Simultaneous ingestion of ethanol also is common, as reported by 77 percent of cocaine users (Grant and Harford 1990). Thirty percent of cocaine users ingest alcohol during almost every episode of cocaine use (Jones 1987).

Why these two drugs are so frequently coadministered is not clearly understood. Potentiation of cocaine-related euphoria by alcohol ingestion may be the basis for this behavior and is consistent with increased plasma ethanol concentrations observed when cocaine is administered after alcohol ingestion (Farré et al. 1993; Perez-Reyes and Jeffcoat 1992). Alcohol ingestion also is reported to diminish undesirable side effects, such as cocaine-induced migraine-like headaches (Weiss et al. 1988). Additionally, coadministration may be influenced by the settings in which cocaine and alcohol are abused. Regardless of the motive for combined use of cocaine and ethanol, this practice is of clinical concern, as it increases the risk of cocaine-related morbidity (Adams et al. 1987; Kreek 1987; Kreek and Stimmel 1984) and mortality (Kreek 1987; Rose et al. 1990). Epidemiological data indicate that simultaneous alcohol ingestion may increase the risk of cocaine-related sudden death by eighteenfold (Rose et al. 1990).

COCAINE METHYL ESTERASE AND ETHYL TRANSFERASE

The rapid clearance of cocaine is in large part mediated by hydrolysis of the methyl ester group. This reaction produces benzoylecgonine (figure 1), a metabolite devoid of cocaine-like psychomotor activity

(Misra et al. 1975; Spealman et al. 1989). While hydrolysis of cocaine to benzoylecgonine occurs spontaneously (Stewart et al. 1979), the slow rate of this reaction at physiological pH (Taylor et al. 1976) does not account for the relatively large percentage of cocaine recovered from urine as benzoylecgonine (Cook et al. 1985). A nonspecific carboxylesterase with cocaine methyl esterase activity was identified in human liver (Dean et al. 1991) and was subsequently purified and characterized (Bosron, this volume; Brzezinski et al. 1994). Under in vitro conditions using purified human liver carboxylesterase, ethanol was found to inhibit cocaine methyl esterase activity, decreasing hydrolysis to benzoylecgonine (Brzezinski et al. 1994; Dean et al. 1991). In the presence of ethanol, this same carboxylesterase catalyzed the ethyl transesterification of cocaine to cocaethylene (benzoylecgonine ethyl ester) (figure 1) (Bosron, this volume; Brzezinski et al. 1994; Dean et al. 1991).

EFFECTS OF ETHANOL ON THE DISTRIBUTION OF COCAINE AND METABOLITES

Cocaine metabolism and disposition following acute ethanol administration were studied in the rat to determine if the in vitro effects of ethanol on cocaine methyl esterase and ethyl transferase activities had significance in vivo (Zachman et al. 1993). The rat was used as it possesses both ethyl transferase and methyl esterase activities, is frequently employed for behavioral and toxicity studies of cocaine, and the size provides sufficient tissue for analytical work. This study was designed to address three questions. First, do significant concentrations of cocaethylene form and accumulate in tissues with controlled coadministrations of cocaine and alcohol? Second, does ethanol administration significantly diminish the hydrolysis of cocaine to benzoylecgonine and methanol, as occurs in vitro when cocaine and ethanol are coincubated with purified human liver carboxylesterase (Brzezinski et al. 1994; Dean et al. 1991)? Third, does ethanol inhibition of cocaine methyl ester hydrolysis increase the N-oxidative metabolism of cocaine, as noted when rodents are pretreated with nonspecific esterase inhibitors (Thompson et al. 1979)?

Male Wistar rats were pretreated with 2.5 grams (g) ethanol per kilogram (kg) bodyweight in water or an equal volume of water via an indwelling intragastric catheter. Sixty minutes after pretreatment, 10 milligrams (mg)/kg cocaine was infused through an indwelling intravenous (IV)

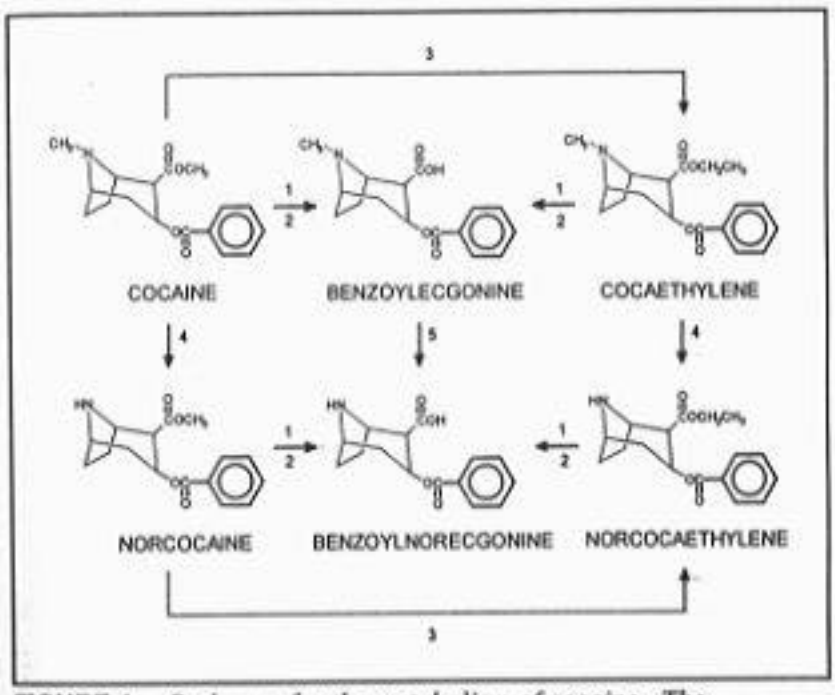

FIGURE 1. *Pathways for the metabolism of cocaine. The metabolism of cocaine and its metabolites involves at least three different classes of reactions: hyhrolysis, transesterification, and N-demethylation. The rapid enzymatic hydrolysis of cocaine and cocaethylene to benzoylecgonine (Dean et al. 1991) and norcocaine and norcocaethylene to benzoylnorecgonine (Dean, unpublished observations) are catalyzed by a carboxylesterase present in human liver (1). Slow, spontaneous hydrolysis of the methyl or ethyl esters of these compounds also occurs (2). Ethanol inhibits the methyl esterase-catalyzed hydrolytic reactions (1) and simultaneously causes the same carboxylesterase to catalyze the ethyl transesterification of cocaine to cocaethylene (Dean et al. 1991) and norcocaine to norcocaethylene (Dean, unpublished observations) (3). N-demethylation of cocaethylene to norcocaethylene occurs in isolated rat liver hepatocytes (Dean, unpublished observations) and rat and human liver microsomes (Dean, unpublished observations)(4).*

catheter. Animals were sacrificed 2.5 to 60 minutes after cocaine administration and serum and solid tissues were harvested for measurement of cocaine, cocaethylene, benzoylecgonine, norcocaine, norcocaethylene, and benzoylnorecgonine.

ALCOHOL-DEPENDENT FORMATION OF COCAETHYLENE

Cocaethylene was detected in all tissues from alcohol-pretreated animals, as previously observed following intraperitoneal (IP) coadministration of cocaine and alcohol (Dean et al. 1992). Based on area under the concentration curves (AUCs) from 2.5 to 60 minutes, the amount of cocaethylene in liver and lung was 14 and 11 percent of the measured cocaine, respectively. Peak cocaethylene concentrations in liver, lung, and kidney were noted within 2.5 minutes after cocaine administration, the earliest time point for specimen collection. These observations answered the first question, demonstrating cocaethylene formation sufficient to contribute to the combined effects of cocaine and ethanol. Cocaethylene AUCs in heart, brain, spleen, and serum were less than 2 to 4 percent of the cocaine AUCs, and peak cocaethylene concentrations in these tissues occurred approximately 10 minutes after cocaine administration.

The relatively high AUCs and rapid peak for cocaethylene in liver, lung, and kidney suggest that cocaethylene formation occurs predominantly in these tissues with subsequent distribution to other tissues. This conclusion was supported by direct measurement of cocaine ethyl transferase activity in tissue homogenate supernatants. Ethyl transferase activity, determined by measuring cocaethylene formation in the presence of saturating levels of cocaine and ethanol, was confirmed in rat liver, lung, heart, and kidney. When normalized for protein content in tissue homogenate supernatants, the relative activity in these tissues decreased in the order listed.

The relative distribution of rat cocaine ethyl transferase activity was consistent with the distribution of immunoreactive protein as determined by gel electrophoresis and Western blot analysis using rabbit anti-human cocaine ethyl transferase antibody. No ethyl transferase activity was identified in brain, spleen, or serum. When rat cocaine ethyl transferase activity was normalized for whole organ volume, liver exhibited activity that was 33-fold greater than that in lung and in excess of 600-fold greater than that in kidney and heart. Formation and accumulation of cocaethylene in liver is of potential concern; this ethylated metabolite undergoes N-demethylation to norcocaethylene and produces cocaine-like cytotoxicity in cultured rat hepatocytes (Boelsterli et al. 1993; Dean et al. 1992) and the intact mouse (Roberts et al. 1992).

Formation and accumulation of cocaethylene is thought to alter the subjective effects and enhance the toxicity of cocaine when used in combination with beverage alcohol. Support for this hypothesis is based on the following evidence. First, cocaethylene has been identified in urine, blood, brain, and liver obtained at autopsy from individuals succumbing after combined exposure to cocaine and ethanol (Hearn et al. 1991*a*; Hime et al. 1991; Jatlow et al. 1991; Rafla and Epstein 1979; Smith 1984). This metabolite was also detected in the blood of emergency room patients seeking treatment after combined use of cocaine and ethanol (Jatlow et al. 1991). Similarly, ethanol-dependent accumulation of cocaethylene in serum and plasma was noted following controlled coadministration of cocaine and ethanol, when the cocaine was known to be free of contaminants, including cocaethylene (de la Torre et al. 1991; Farré et al. 1993; McCance-Katz et al. 1991; Perez-Reyes and Jeffcoat 1992; Perez-Reyes 1994). Second, when administered under controlled conditions, cocaethylene produced similar but milder and more pleasurable subjective effects than cocaine (Peres-Reyes 1993). Comparable affinity of cocaethylene and cocaine for the dopamine transporter in human striatal membranes may explain similarities in the subjective effects of these two drugs (Hearn et al. 1991*a*), while lower cocaethylene affinity for serotonin uptake sites and the norepinephrine transporter might explain differing subjective effects. Third, controlled administration of cocaethylene in humans produced tachycardia comparable to that induced by cocaine (Perez-Reyes 1993). However, cocaethylene is a more potent blocker of cardiac sodium channels in guinea pig ventricular myocytes (Xu et al. 1994) and of muscarinic receptor-stimulated phosphoinositide metabolism in the rat (Tan and Costa 1994), and has a greater negative ionotropic effect in isolated ventricular myocytes from ferret (Qiu and Morgan 1993). If true in humans, cocaethylene may increase the risk of conduction and contractile disturbances in the heart. Finally, the median lethal dose for cocaethylene in the mouse was significantly less than that for cocaine (Hearn et al. 1991*b*; Katz et al. 1992).

ALCOHOL INHIBITION OF BENZOYLECGONINE FORMATION

Ethanol pretreatment of rats dramatically decreased the AUCs for benzoylecgonine in all tissues and serum. This in vivo effect is consistent with in vitro ethanol inhibition of carboxylesterase-catalyzed cocaine hydrolysis to benzoylecgonine and methanol. Although ethanol decreased AUCs for benzoylecgonine in all tissues, the time to peak benzoyl-ecgonine concentrations was variable. In liver, peak concentrations occurred 2.5 minutes after cocaine administration, suggesting high hepatic methyl esterase activity. By contrast, benzoylecgonine levels in serum continued to rise over the entire 60 minutes of the experiment and likely reflect redistribution of this polar metabolite. The distribution of cocaine methyl esterase activity in rat tissues was determined by directly measuring the enzymatic hydrolysis (total minus spontaneous) of cocaine to benzoylecgonine in homogenate supernatants. Methyl esterase activity was identified in kidney, liver, lung, heart, and brain. When normalized for tissue protein, the relative amount of cocaine methyl esterase activity decreased in the order listed. When normalized for whole organ volume, hepatic cocaine methyl esterase activity was 15-fold to 100-fold greater than the activity in other tissues. No such activity was detected in spleen or serum. The presence of cocaine methyl esterase activity and absence of ethyl transferase activity in brain, plus poor correlation between these two activities in other tissues, suggest that more than one enzyme catalyzes these two reactions in the rat. Although a single carboxylesterase is known to catalyze both reactions in humans (Brzezinski et al. 1994; Dean et al. 1991), it is not known if other human enzymes possess cocaine methyl esterase or ethyl transferase activities.

Although lacking psychomotor activity, benzoylecgonine recently was shown to be a more potent vasoconstrictor of cerebral arteries in cat and sheep than cocaine (Covert et al. 1994; Madden and Powers 1990; Schreiber et al. 1994). Similarly, benzoylecgonine increased mean blood pressure with no effect on heart rate or QRS duration in the anesthetized rat (Erzouki et al. 1993). By contrast, cocaine decreased mean blood pressure and heart rate and increased the QRS duration. These different cardiovascular effects of cocaine and benzoylecgonine appear to reflect differing activity within the autonomic nervous system. Unlike cocaine, which produces vasoconstriction by blocking catecholamine reuptake, benzoylecgonine-induced vasoconstriction appears to be mediated predominantly by stimulation of $_1$-adrenergic receptors (Schreiber et al. 1994). Although cause and effect have yet to be established, a temporal relationship exists between recurrent coronary artery

vasoconstriction and increasing blood benzoylecgonine concentrations in humans (Brogan et al. 1992). Similarly, increasing benzoylecgonine concentrations might contribute to the migraine-like vasospastic headaches observed in cocaine users (Satel and Gawin 1989). If so, alcohol ingestion might diminish vasoconstriction of cerebral, coronary, and possibly other vascular beds by inhibiting cocaine methyl esterase activity and benzoylecgonine production. This effect might well contribute to combined use of cocaine and ethanol.

ALCOHOL EFFECT ON N-OXIDATIVE METABOLISM OF COCAINE

In humans, N-demethylation of cocaine to norcocaine is catalyzed by a cytochrome P-450 enzyme either directly or following oxidation of cocaine to cocaine N-oxide by a flavin-adenine dinucleotide (FAD)-containing mono-oxygenase (figure 1, 4) (Kloss et al. 1983). The N-demethylation of benzoylecgonine to benzoylnorecgonine was demonstrated in the rat following administration of radiolabeled benzoylecgonine (figure 1, 5)(Misra et al. 1975).

In animals pretreated with ethanol, metabolism of cocaine to norcocaine and benzoylnorecgonine increased, as reflected by higher tissue AUCs, as compared with those receiving water. Ethanol pretreatment also resulted in measurable levels of norcocaethylene in liver and lung. These observations are consistent with the increased hepatotoxicity (presumably due to enhanced N-oxidative metabolism) observed when mice were exposed to cocaine or cocaethylene and the esterase inhibitor diazinon (Roberts et al. 1992; Thompson et al. 1979). This shift toward N-oxidative metabolism provides a mechanism to explain potentiation of cocaine hepatotoxicity by ethanol (Jover et al. 1991). Detection of norcocaethylene in ethanol- pretreated rats is consistent with norcocaethylene detected in the hair of heavy cocaine users, suggesting common pathways including hydrolysis, transesterification, and N-demethylation (figure 1) (Cone et al. 1991).

ALCOHOL EFFECTS ON COCAINE DISPOSITION

Ethanol pretreatment had variable effects on AUC curves for cocaine from tissue to tissue. Ethanol increased the AUC for cocaine in liver and decreased the AUC for cocaine in serum and the heart. Ethanol pretreatment had little or no effect on the AUCs for cocaine in brain, lung, kidney, and spleen. While lower tissue benzoylecgonine AUCs

support the notion that alcohol inhibits cocaine methyl esterase activity, variable effects of ethanol on cocaine AUCs suggest that the effects of ethanol are not fully explained by inhibition of methyl esterase activity and simultaneous initiation of ethyl transferase activity. The increased AUC for liver and decreased AUC for serum might be explained by increased hepatic extraction of cocaine when coadministered with ethanol. It is not clear how this might occur, but ethanol-mediated vasodilation could increase hepatic blood flow (Orrego et al. 1988; Shaw et al. 1977), which is typically limited by the vasoconstricting effects of cocaine (Garhart et al. 1989). Alternatively, oxidative metabolism of ethanol might decrease the intracellular pH and enhance trapping of cocaine in hepatocytes.

CONCLUSION

It is not known why coabuse of cocaine and ethanol is so common. Similarly, the specific mechanisms by which ethanol potentiates cocaine-related morbidity and mortality are not clearly defined. The studies reviewed in this chapter suggest that multiple effects of ethanol on cocaine metabolism may be contributory. In particular, ethanol inhibition of cocaine methyl esterase may slow normally rapid inactivation of cocaine to benzoylecgonine while simultaneously initiating ethyl transesterification to cocaethylene and increasing N-demethylation to norcocaine. Although acute ethanol exposure did not uniformly increase serum and tissue cocaine AUCs, the collective increase in the levels of the psychoactive compounds cocaine, cocaethylene, norcocaine, and norcocaethylene may enhance or prolong cocaine-related euphoria and toxicity. Additionally, unique and/or relative differences in the activities of these metabolites may contribute to the effects of ethanol on the pharmacokinetics and pharmacodynamics of cocaine. More extensive characterization of the enzymes responsible for cocaine metabolism and the development of pharmacokinetic and pharmacodynamic models for cocaine will facilitate efforts to understand the interactions of this drug with alcohol.

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Individual Differences in Nicotine Kinetics and Metabolism in Humans

Neal L. Benowitz and Peyton Jacob III

INTRODUCTION

Cigarette smoking remains the major preventable cause of premature disability and death in developed countries (Peto et al. 1992). Cigarette smoking is maintained by addiction to nicotine. Nicotine addiction develops in most people before the age of 20 (Department of Health and Human Services 1994). Many youth experiment with cigarettes, but only about 25 percent of high school seniors become addicted smokers (Escobedo et al. 1993). Thus, there appears to be individual variability in susceptibility to nicotine addiction.

In support of the idea of individual variability to nicotine addiction are twin studies showing genetic linkages for never smoking, for quitting (i.e., former smoker status), and even for being a light versus a heavy smoker (Carmelli et al. 1992). The basis for individual differences in susceptibility to addiction is unknown. Possible factors include differences in pharmacokinetics and metabolism of nicotine, pharmaco-dynamic differences, and factors related to personality, including affective disorders and, of course, environmental influences (Benowitz 1992). Of note is an apparent shared inheritance in susceptibility to nicotine addiction and alcohol abuse (Swan et al. 1990).

NICOTINE METABOLISM AND SMOKING BEHAVIOR

This chapter considers individual differences in the pharmacology of nicotine. While there is evidence of genetic difference in pharmacologic response to nicotine in rodents (Marks et al. 1991), there has been very little research into individual differences in pharmacodynamics in humans. Individual differences in pharmacokinetics and metabolism have been much better documented, and are the major focus of this discussion.

Individual differences in nicotine kinetics and metabolism could affect smoking behavior in two ways. First, an individual's rate of nicotine metabolism could affect how much a person smokes. Smokers tend to

adjust their smoking to maintain particular levels of nicotine in the body. A person who metabolizes nicotine quickly may need to smoke more to achieve a particular level of nicotine than does a person who metabolizes nicotine more slowly. The phenomenon of regulation has been demon-strated experimentally in a study in which the rate of nicotine elimination was increased by acidification of the urine (Benowitz and Jacob 1985). In conditions of urinary acidification, smokers consumed 18 percent more nicotine per day from cigarettes, compensating by about 50 percent for the excess loss of nicotine by increased renal clearance.

A second mechanism by which individual differences in metabolism could affect nicotine addiction is through the pattern of metabolites generated. Some nicotine metabolites may be pharmacologically active. These include nicotine iminium ion, -nicotyrine, cotinine, and nornicotine. Nicotine iminium ion is an intermediate in the metabolism of nicotine to cotinine (figure 1). Nicotine iminium ion can covalently bind to macro-molecules (Shigenaga et al. 1988) and may thereby produce tissue injury and/or promote carcinogenesis. -nicotyrine is a minor metabolite of nicotine that has been shown to inhibit nicotine metabolism in vitro (Shigenaga et al. 1989). Cotinine is the major proximate metabolite of nicotine. Cotinine is inactive toward nicotinic cholinergic receptors, but does appear to affect a number of enzyme systems, including those involved in steroid synthesis (Benowitz 1994). Cotinine may also have central nervous system (CNS) activity, reportedly modifying nicotine withdrawal symptoms in abstinent smokers (Keenan et al. 1994). The site or mechanism of CNS action of cotinine is unknown, but if there is CNS activity, cotinine, which is present at 15 times the concentration of nicotine, could contribute significantly to nicotine addiction. Nornicotine is a minor metabolite of nicotine as well as a component of tobacco itself. It is as potent in pharma- cologic activity and toxicity as nicotine (Risner et al. 1988). Thus, considering the activity of various metabolites, individual differences in the amount of various metabolites generated could influence differential susceptibility to nicotine addiction and/or toxic effects of tobacco use.

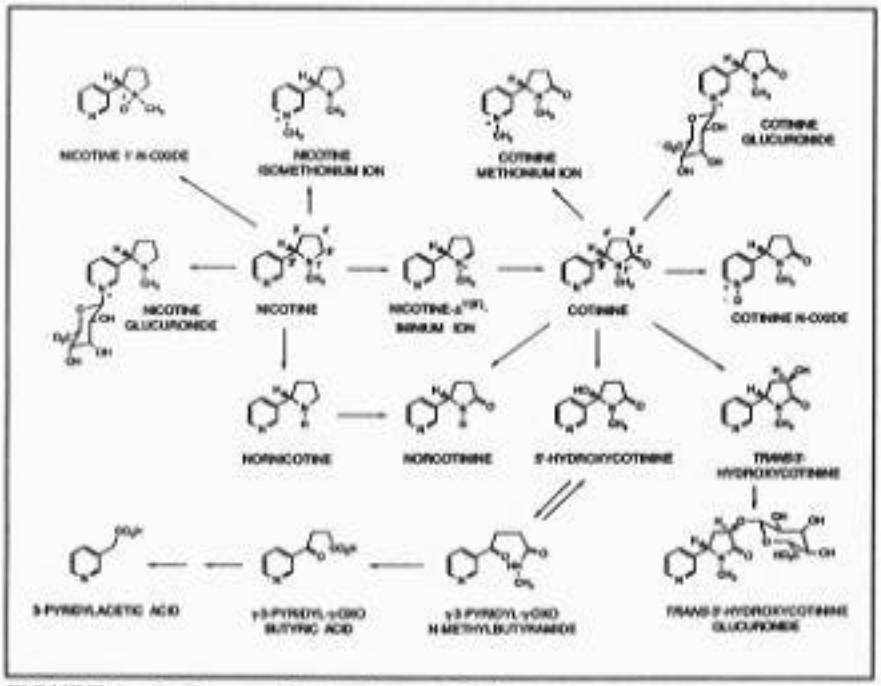

FIGURE 1. *Pathways of nicotine metabolism.*

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INDIVIDUAL VARIABILITY IN NICOTINE METABOLISM

Nicotine is metabolized via cytochrome P450 (CYP 450) to nicotine iminium ion, and then by aldehyde oxidase to cotinine (figure 1). Cotinine is the major proximate metabolite of nicotine, with 70 to 80 percent of nicotine metabolized to cotinine in most smokers (Benowitz and Jacob 1994). Cotinine is, in turn, metabolized to trans-3'-hydroxy-cotine, which is the most abundant nicotine metabolite in the urine of most smokers. Nicotine-N'-oxide is formed via a flavoprotein enzyme and is a minor metabolite, averaging about 4 percent of the systemic nicotine dose (Benowitz et al. 1994). Nicotine, cotinine, and trans-3'-hydroxycotinine are also conjugated (Benowitz et al. 1994; Byrd et al. 1992). Nicotine and cotinine form quaternary N-glucuronides, whereas trans-3'-hydroxycotinine forms an O-glucuronide (figure 1). Figure 2 shows the average pattern of nicotine metabolism and urinary recovery, based on a study in individuals receiving nicotine at steady state via transdermal nicotine patches. While this figure illustrates the average pattern, there is individual variability as shown in figure 3. Thus, for

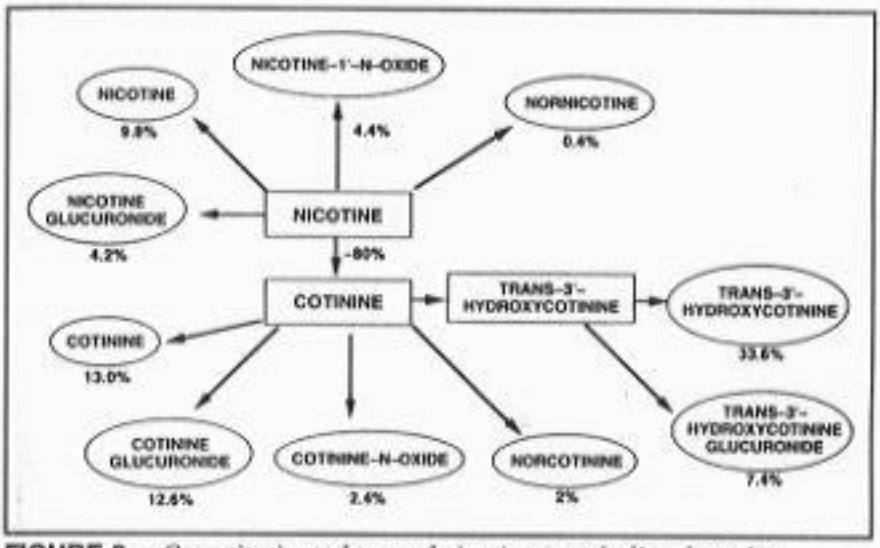

FIGURE 2. *Quantitative scheme of nicotine metabolism based on average excretion of metabolites as percent of systemic dose during transdermal nicotine application. Circled compounds indicate excretion in urine and associated numbers indicate associated percent of systemic dose of nicotine.*

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most individuals, trans-3'-hydroxycotinine is the most abundant metabolite in the urine, but cotinine is more abundant in others.

There is considerable variability in the extent of conjugation. Of note, the extent of conjugation of nicotine and cotinine within subjects is highly correlated, whereas there is no relationship between nicotine or cotinine conjugation and the extent of conjugation of trans-3'-hydroxy-cotinine. These data suggest that nicotine and cotinine are conjugated by the same enzyme, while trans-3'-hydroxycotinine is conjugated by a different enzyme.

These data show that there are considerable individual differences in the metabolism of nicotine. If metabolites contribute to nicotine addiction, individual variability in pattern of metabolism could explain some of the individual variability in susceptibility to nicotine addiction.

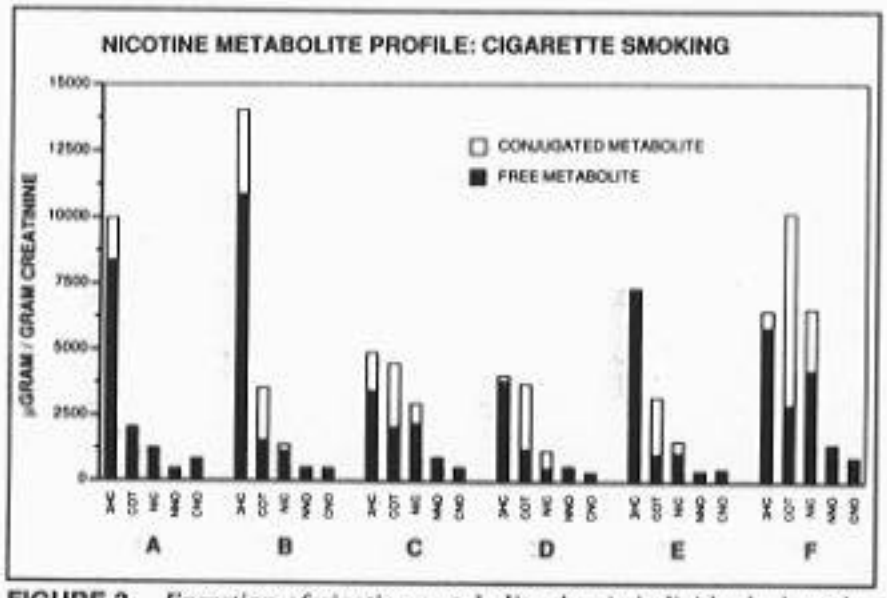

FIGURE 3. *Excretion of nicotine metabolites by six individuals, based on 24-hour urine collection during cigarette smoking.*

KEY: NIC = nicotine; COT = cotinine; 3HC = trans-3'-hydroxycotinine; NNO = nicotine-1'-N-oxide; CNO = cotinine-N-oxide.

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INDIVIDUAL VARIATION IN NICOTINE AND COTININE KINETICS

As expected by analogy to other weak bases that are extensively metabolized, there is considerable individual variability in the clearance of nicotine. Early studies on nicotine kinetics were performed by infusing nicotine in smokers who were abstinent from tobacco (Benowitz et al. 1982; Rosenberg et al. 1980). However, it is most relevant to investigate the disposition kinetics of a drug in the chemical environment where the drug is normally used. Using labeled compounds, one can study the metabolism and kinetics of nicotine in smokers while they are smoking. To do so, deuterium-labeled analogs of nicotine (3',3'-dideuteronicotine, nicotine-d_2) and cotinine (2,4,5,6-tetra-deuterocotinine, cotinine-d_4), both with the natural (S)-configurations, have been synthesized. Concentrations of natural and labeled nicotine and cotinine, as well as their metabolites, are measured by gas chromatography/mass spectrometry (GC/MS). Comparing the pharmacokinetics of labeled and natural compounds, the absence of an isotope effect was demonstrated, validating their use

in studies of nicotine and cotinine metabolic disposition (Benowitz and Jacob 1994; Jacob et al. 1991).

NICOTINE DISPOSITION IN SMOKERS AND NONSMOKERS

Cigarette smoke contains a variety of chemicals, including polycyclic aromatic hydrocarbons, that may affect the metabolism of various other drugs. For example, smokers are well known to have increased metabolic activity of liver CYP 1A2, which results in the accelerated metabolism of caffeine, theophylline, and other drugs (Dawson and Vestal 1982). Earlier research had suggested that smokers metabolize nicotine more rapidly than nonsmokers (Kyerematen et al. 1982, 1990). If true, this might be a significant factor in the natural history of tobacco addiction as a mechanism of metabolic tolerance. That is, the longer a person smoked, the faster nicotine would be metabolized; therefore, one would have to smoke more to maintain a desired nicotine level in the body.

The stable isotope technique described above was used to compare nicotine kinetics in smokers and nonsmokers (Benowitz and Jacob 1993). Labeled (S)-(-)-nicotine was infused intravenously for 30 minutes, and blood and urine samples were collected for 96 hours. Smokers and nonsmokers received the same low dose of nicotine (0.5 micrograms per kilogram per minute (g/kg/min)), and on another day the smokers also received a higher dose of nicotine (2.0 g/kg/min) that resulted in plasma nicotine concentrations similar to those they achieve with smoking. Nonsmokers are unable to tolerate this dose due to toxicity.

As shown in figure 4, nicotine levels were similar in smokers and nonsmokers. Pharmacokinetic analysis revealed that clearance was slightly but significantly greater in nonsmokers than smokers, while the steady-state volume of distribution and half-lives were similar among groups (figure 5). These findings indicate that smokers do not metabolize nicotine more rapidly than nonsmokers. In fact, the reverse appears to be true; cigarette smoking appears to inhibit the metabolism of nicotine. In any case, metabolic tolerance does not appear to be a factor in the natural history of tobacco addiction.

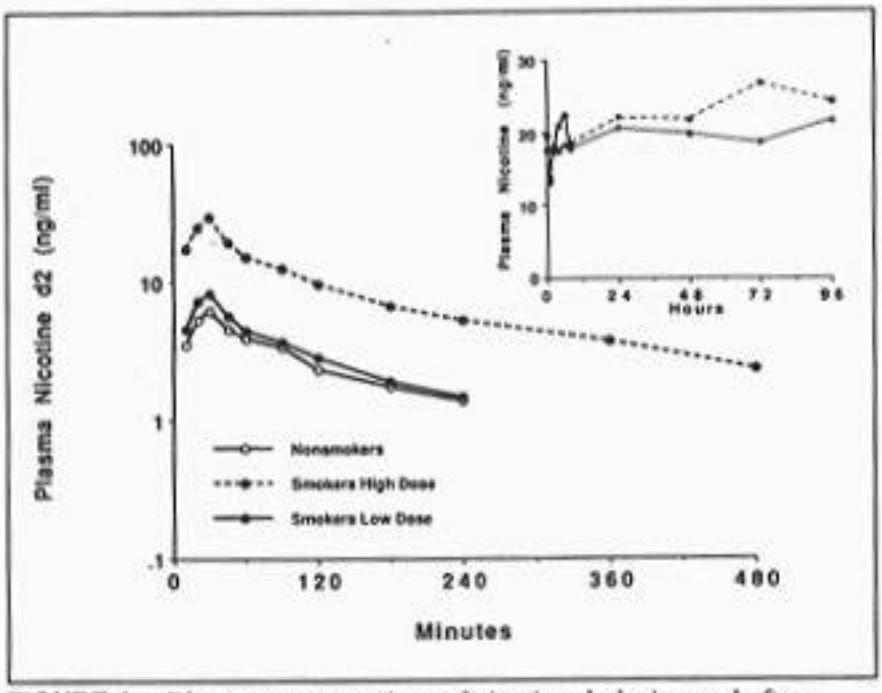

FIGURE 4. *Plasma concentrations of nicotine-d_2 during and after intravenous infusion of 0.5 µg/kg/min for 30 minutes in nonsmokers and smokers (low dose) and 2.0-µg/kg/min for 30 minutes in smokers (high dose). Inset shows plasma concentrations of natural nicotine derived from cigarette smoking during the course of the study. Data represent the mean of 11 subjects.*

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INDIVIDUAL DIFFERENCES IN THE METABOLISM OF NICOTINE TO COTININE

By simultaneously infusing labeled nicotine-d_2 and cotinine-d_4, and by measuring levels of cotinine-d_2 generated from nicotine-d_2, the fractional conversion of nicotine to cotinine can be determined (Benowitz and Jacob 1994). An example of data generated by such a study is shown in figures 6a and 6b. Using this approach in 20 smokers, it was determined that on average 72 percent of nicotine is converted to cotinine (range 55 to 92 percent) (figure 7). No differences in the clearances of nicotine or cotinine or the percentage of nicotine conversion to cotinine were seen

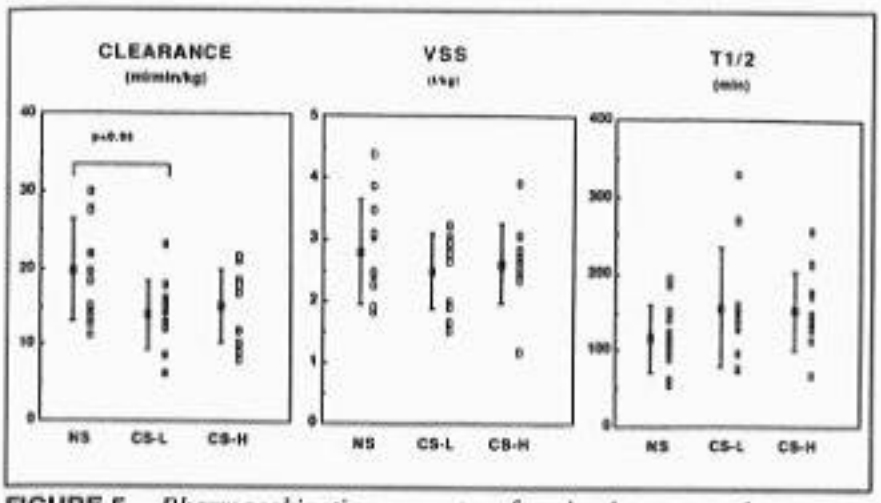

FIGURE 5. *Pharmacokinetic parameters for nicotine comparing nonsmokers, smokers receiving low dose intravenous nicotine, and smokers receiving high dose intravenous nicotine. Mean ± SD indicated next to each data set.*

KEY: Vss = steady state volume of distribution; t½ = half-life; NS = nonsmokers; CS-L = smokers receiving low dose nicotine; CS-H = smokers receiving high dose nicotine.

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when comparing data from men and women. As expected, clearances did vary among individuals, with coefficients of variation of 25 percent and 27 percent for clearances of nicotine and cotinine, respectively. The extent of individual variability and the percentage of nicotine conversion to cotinine was less, with a coefficient of variation of 12 percent.

Data on the fractional conversion of nicotine to cotinine and the clearance of cotinine for an individual can be used to compute a factor (K) that converts the steady-state plasma cotinine concentration to the intake of nicotine from smoking per day. The equation is:

$$D_{nic}\ (mg/24h) = K \times (\text{plasma COT})\ (ng/mL)$$

On average, K = 0.08 with a range of 0.47 to 0.102. The K factor, along with plasma cotinine levels, can be used to estimate daily intake of nicotine from active or passive smoking.

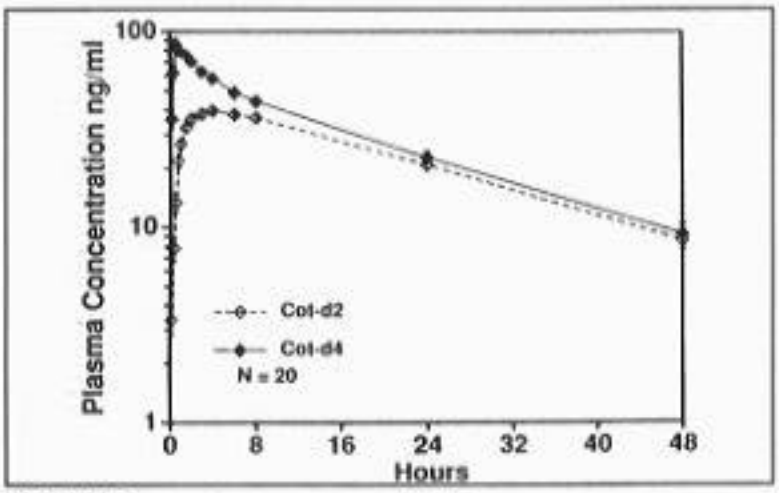

FIGURE 6a.

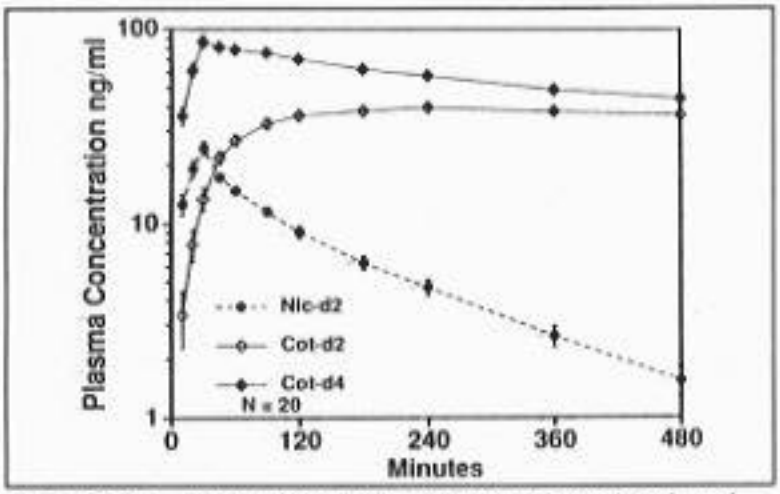

FIGURE 6b. *Mean plasma concentration of nicotine-d_2 and cotinine-d_4 during and after intravenous infusion of a 50:50 mixture of nicotine-d_2 and cotinine-d_4 (2 µg base/kg/min of each for 30 minutes, beginning at time zero). Figure 6a shows values up to 48 hours. Figure 6b shows values up to 480 minutes. Curves represent average values for 20 subjects.*

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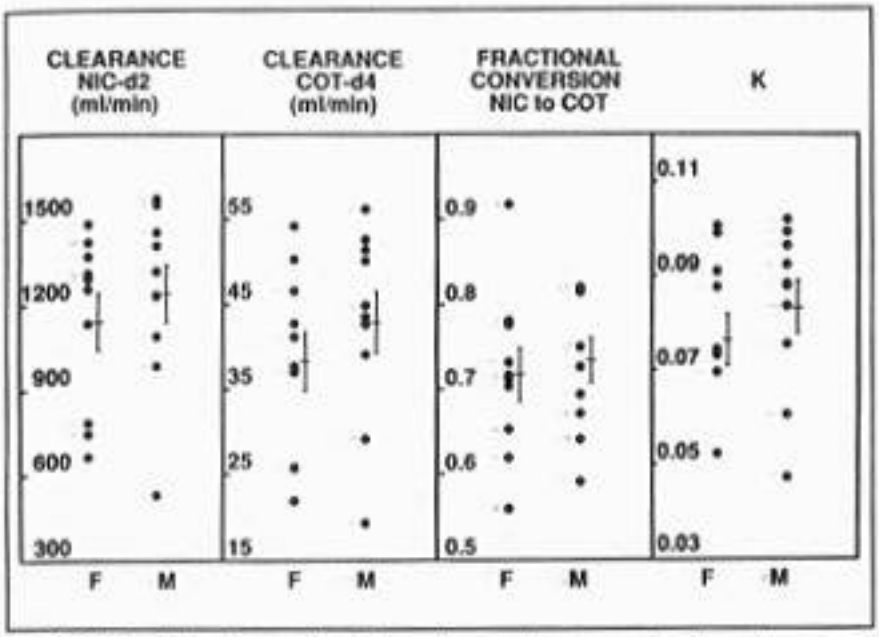

FIGURE 7. *Nicotine and cotinine plasma clearance, fractional conversion of nicotine to cotinine (F), and the factor that converts plasma cotinine concentration to daily intake of nicotine (K). Data are shown for individual subjects by gender. Bars indicate mean ± 95 percent confidence intervals.*

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Of note in the above study was the finding that the clearance of nicotine and the fractional conversion of nicotine to cotinine were significantly correlated (r = 0.59). This correlation suggests that cotinine is the most rapid or rate-limiting pathway for nicotine metabolism. Thus, people who metabolize nicotine via pathways other than those to cotinine are likely to have slower elimination of nicotine in general.

DEFICIENT C-OXIDATION OF NICOTINE

While most people metabolize nicotine extensively into cotinine, a few individuals have been identified who generate very little cotinine. One such person, a 57-year-old woman, was identified in a smoking cessation trial. The subject was found to have unexpectedly low plasma concentrations of cotinine, but normal concentrations of

nicotine (Benowitz et al. 1995*b*), both while smoking and while using nicotine patches. This individual was studied using a dual infusion of labeled nicotine and cotinine. As seen in figures 8a and 8b, little cotinine was generated from nicotine. The subject was found to convert only 9 percent of nicotine to cotinine, in contrast to the average 72 percent seen in the study described previously (Benowitz and Jacob 1994). This individual's clearance of nicotine was unusually low (6.5 mL/min/kg versus 17.2 mL/ min/kg in 20 controls), the half-life was abnormally long (348 versus 138 min), and the formation clearance of cotinine for this individual was exceedingly low (0.4 mL/min/kg) versus that seen in controls (12.1 mL/min/kg). The clearance and half-life of cotinine were, however, normal in this subject.

Thus, an individual with markedly deficient C-oxidation of nicotine has been identified. The liver enzymes responsible for C-oxidation of nicotine have not been fully characterized. In vitro studies suggested a role for CYP 2A6, 2D6, 2E1, and/or 2C9 (Cashman et al. 1992; Flammang et al. 1992; McCracken et al. 1992). Cholerton and colleagues (1994) reported five subjects with unusually high nicotine/cotinine ratios in the urine after oral nicotine who were genotypically homozygous for 2D6 mutations. They suggested that 2D6 is an important enzyme for nicotine metabolism. However, the subject described above was a normal metabolizer of dextromethorphan, and therefore a phenotypically normal metabolizer via CYP 2D6. Studies are ongoing to identify which enzymatic defects are responsible for deficient C-oxidation of nicotine.

The biological significance of deficient C-oxidation of nicotine is unclear, but could be considerable. Slow metabolizers of nicotine such as this subject might be expected to smoke fewer cigarettes and may be at less risk for smoking-related diseases linked to the consumption of cigarette smoke. On the other hand, the long half-life of nicotine may mean that nicotine levels persist at higher levels when the smoker is not smoking, and could lead to more severe physical dependence. In addition, if cotinine has significant biological activity that contributes to the pharmacologic effects of nicotine, people who do not generate cotinine will experience a different profile of pharmacologic effects from nicotine.

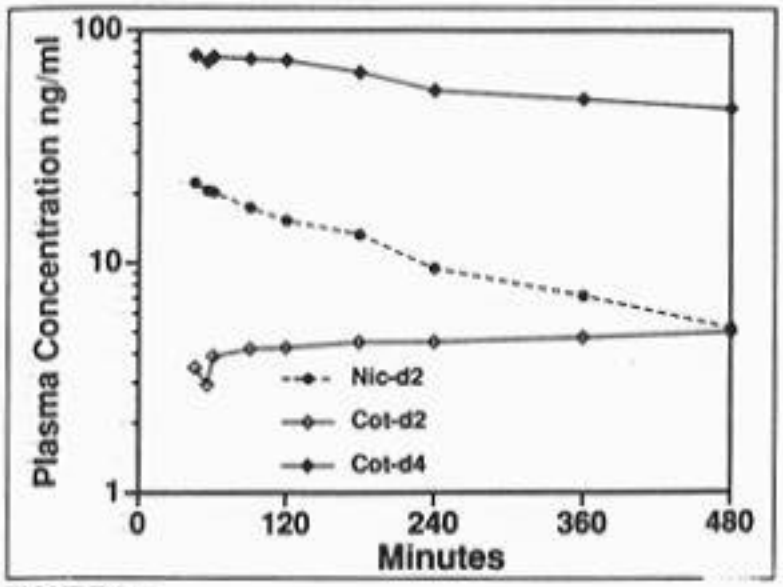

FIGURE 8a.

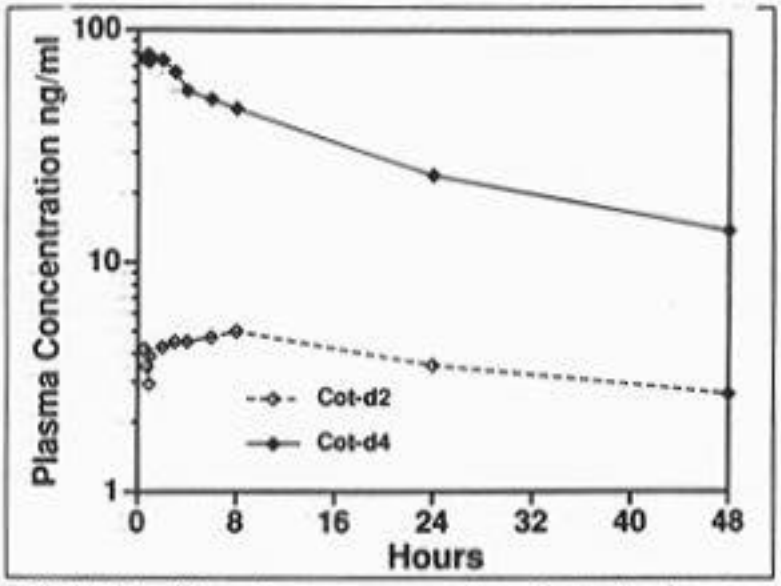

FIGURE 8b. *Mean plasma concentrations of nicotine-d_2, cotinine-d_2, and cotinine-d_4 during and after intravenous infusion of a 50:50 mixture of nicotine-d_2 and cotinine-d_4, as described in figure 6. Figure 8a shows values up to 480 minutes. Figure 8b shows values up to 48 hours.*

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ETHNIC DIFFERENCES IN NICOTINE AND COTININE METABOLISM

Ethnic differences in nicotine metabolism have been hypothesized to contribute to differences in health effects and/or susceptibility to addiction in blacks versus whites (Henningfield et al. 1990). The intriguing observation has been made that cotinine levels per cigarette smoked were significantly higher in blacks versus whites (Wagenknecht et al. 1990). In contrast, plasma levels of thiocyanate, a marker of exposure to cigarette smoke in general, were similar. There is also evidence that blacks have higher rates of lung cancer for any given level of cigarette smoking compared with whites (Satariona and Swanson 1988). Ethnic differences in the metabolism of nicotine or cotinine could help explain these observations.

To examine this issue, dual-labeled nicotine and cotinine infusions were administered to 40 black and 39 white smokers matched for age, gender, and self-reported cigarette consumption (Benowitz et al. 1995*a*). The clearance of nicotine and percentage of nicotine conversion to cotinine were similar for blacks and whites. However, the clearance of cotinine was significantly slower (0.56 versus 0.69 mL/min/kg) and the half-life of cotinine slightly longer (1,064 versus 950 min) in blacks versus whites. These data clarify at least in part the observation of higher cotinine levels when normalized for cigarette consumption in blacks. The implications of differences in cotinine metabolism regarding susceptibility to nicotine addiction or health consequences of smoking are still unclear.

SUMMARY AND CONCLUSION

Individual differences in susceptibility to nicotine addiction, the likelihood of successful smoking cessation, and the development of adverse health effects of smoking are well recognized. The basis for these individual differences is as yet unknown. This chapter examines individual differences in the metabolism and kinetics of nicotine as a possible factor.

Rare individuals appear to be deficient metabolizers of nicotine. Individual differences are described both in the pattern and rates of nicotine metabolism. Ethnic differences in cotinine metabolism have also been observed. However, the enzymes responsible for nicotine metabolism and their genetic regulation have not been fully characterized. Understanding the basis for individual differences in nicotine kinetics and metabolism, and linking these differences to

pharmacodynamic studies, may provide important clues for the prevention and treatment of nicotine and possibly other drug addictions.

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Inhibitors of Anandamide Breakdown

Dale G. Deutsch and Alexandros Makriyannis

INTRODUCTION

^{9}-Tetrahydrocannabinol (THC), the psychoactive marijuana plant- derived cannabinoid, and numerous synthetic derivatives have been shown to bind to a specific brain receptor, cannabinoid receptor 1 (CB1) (Howlett et al. 1990; Matsuda et al. 1990; Herkenham et al. 1990; Mailleux and Vanderhagen 1992). Arachidonoyl ethanolamide (anandamide), homo--linolenyl ethanolamide, and docosatetraenyl ethanolamide are naturally occurring brain constituents that bind to CB1 and as a class are called the anandamides (Mechoulam et al. 1994; Devane et al. 1992; Hanus et al. 1993; Felder et al. 1993; Devane 1994). Anandamide behaves as a cannabimimetic compound in vitro, stimulating receptor-mediated signal transduction that leads to the inhibition of forskolin-stimulated adenylate cyclase (Vogel et al. 1993; Childers et al. 1993)[1]. In a neuroblastoma cell line, anandamide causes partial inhibition of N-type calcium currents via a pertussis toxin-sensitive guanosine triphosphate binding protein (G-protein) pathway, independently of cyclic adenosine monophosphate (cAMP) metabolism (Mackie et al. 1993). Using a series of behavioral tests to evaluate cannabinoid analogs, anandamide has been shown to be a cannabinoid receptor agonist exhibiting pharmacological activity in mice parallel to that of other psychotropic cannabinoids (Fride and Mechoulam, 1993; Crawley et al. 1993; Smith et al. 1994; Abadji et al. 1994).

Soon after the discovery of anandamide, enzymatic activities responsible for its degradation (an amidase, also called an amidohydrolase) and synthesis (synthase) were described (DiMarzo et al. 1994; Desarnaud et al. 1995; Ueda et al. 1995; Deutsch and Chin 1993; Kruszka and Gross 1994; Devane and Axelrod 1994). Phenylmethylsulfonyl fluoride (PMSF) was discovered to be a potent inhibitor of the enzymatic breakdown of arachidonoylethanolamide (Deutsch and Chin 1993). A series of anandamide analogs was synthesized (trifluoromethyl ketone, -keto ester, and -keto amide derivatives) and tested in vitro and in intact cells as amidase inhibitors (Koutek et al. 1994). The trifluoromethyl ketones (e.g., arachidonyltrifluoromethyl ketone) were found to be potent inhibitors in the low micromolar (M) range. Most recently, a potent irreversible inhibitor of anandamide hydrolysis (AM 374) has

been synthesized and found to be effective in the low nanomolar (nM) range.

ANANDAMIDE SYNTHESIS

An enzymatic activity has been identified thatcatalyzes the synthesis of arachidonoylethanolamide (Deutsch and Chin 1993) as shown below:

Arachidonic Acid + Ethanolamine _ Arachidonoyl Ethanolamide + H_2O

This catalytic activity for the biosynthesis of anandamide requires ethanolamine and arachidonic acid and is readily detected in incubations of rat brain homogenates. When [^{3}H]-arachidonic acid was employed as the label, addition of exogenous ethanolamine was necessary to observe anandamide synthesis, indicating that ethanolamine is limiting in the brain homogenate. It was found that anandamide synthesis increased with increasing amounts of ethanolamine so that 35 percent of the [^{3}H]-arachidonate counts were incorporated into anandamide with 7 millimolar (mM) unlabeled ethanolamine (Deutsch and Chin 1993). (Subsequently, one of the authors (DGD) found that synthesis may be observed with as little as 0.1 mM ethanolamine.) When labeled ethanolamine was employed, the apparent increased rate of anandamide synthesis, in the presence of PMSF, was an artifact caused by the reaction of PMSF with ethanolamine to form the corresponding ethanolamide, which had the same mobility on the thin layer chromatography (TLC) plate as anandamide (W. Devane, personal communication, July 1994). Interestingly, Devane and Axelrod (1994) observed that PMSF was a potent inhibitor of the synthase. Recently, it was postulated that the amidase and synthase are the same enzyme. This is based upon the observation that the amidase and synthase activities cochromatograph during purification and that they exhibit the same behavior towards inhibitors, in their pH dependence, and in their heat inactivation profiles (Ueda et al. 1995). Synthesis of anandamide has also been demonstrated in subcellular fractions. The highest activity occurred in the synaptic vesicles, myelin, and microsomal and synaptosomal membranes (Devane and Axelrod 1994). The reaction was selective for arachidonic acid as the aliphatic constituent and ethanolamine as the polar moiety. Furthermore, it was clearly demonstrated that this reaction occurs through an adenosine triphosphate (ATP)- and CoA-independent process (Kruszka and

Gross 1994). It is interesting to note that Bachur and Udenfriend (1966) described a rat liver microsomal system utilizing aliphatic fatty acids, but not arachidonate, and several amines as substrates for the synthesis of fatty acid amides.

Burstein and Hunter (1995) observed that THC stimulated the biosynthesis of anandamide in neuroblastoma cells employing either ethanolamine or arachidonic acid as the label. Anandamide biosynthesis has also been shown to occur in primary cultures of rat brain neurons labelled with ^{3}[H]-ethanolamine when stimulated with ionomycin, a Ca^{++} ionophore (Di Marzo et al. 1994). These authors proposed an alternate model for the biosynthesis of anandamide in which N-arachidonoyl phosphatidyl ethanolamine is cleaved by a phospholipase D activity to yield phosphatidic acid and ararchidonoylethanolamide. This model is based upon extensive studies undertaken by Schmid and collaborators (1990), who have shown that fatty acid ethanolamide formation results from the N-acylation of phosphatidyl ethanolamine by a transacylase to form N-acyl phosphatidylethanolamine. Possibly resulting from postmortem changes, this compound is subsequently hydrolyzed to the fatty acid ethanolamide and the corresponding phosphatide by a phosphodiesterase, phospholipase D.

ANANDAMIDE AMIDASE

The reaction for the degradation of anandamide to arachidonic acid and ethanolamine is shown below.

Arachidonoyl Ethanolamide + H_2O _ Arachidonic Acid + Ethanolamine

The distribution of this amidase activity was characterized in tissues from the rat using a TLC assay with [arachidonoyl 5,6,8,9,11,12,14,15- ^{3}H]-ethanolamide as the substrate (Deutsch and Chin 1993). This enzymatic reaction was expressed in homogenates from brain, liver, kidney, and lung (figure 1). Under these conditions, the main degradation product detected was arachidonate. Barely detectable activity was present in homogenates of rat heart and skeletal muscle (Deutsch and Chin 1993; Desarnaud et al. 1995). In addition to the main arachidonic acid degradation product, lung and liver produced an unknown second catabolite that may be an oxidized form of anandamide. Recently, it has been shown that anandamide may be hydroxylated by the cytochrome P450s (Bornheim et al. 1993) and this may account, in part, for the additional metabolite. When the ethanolamides of a series of fatty

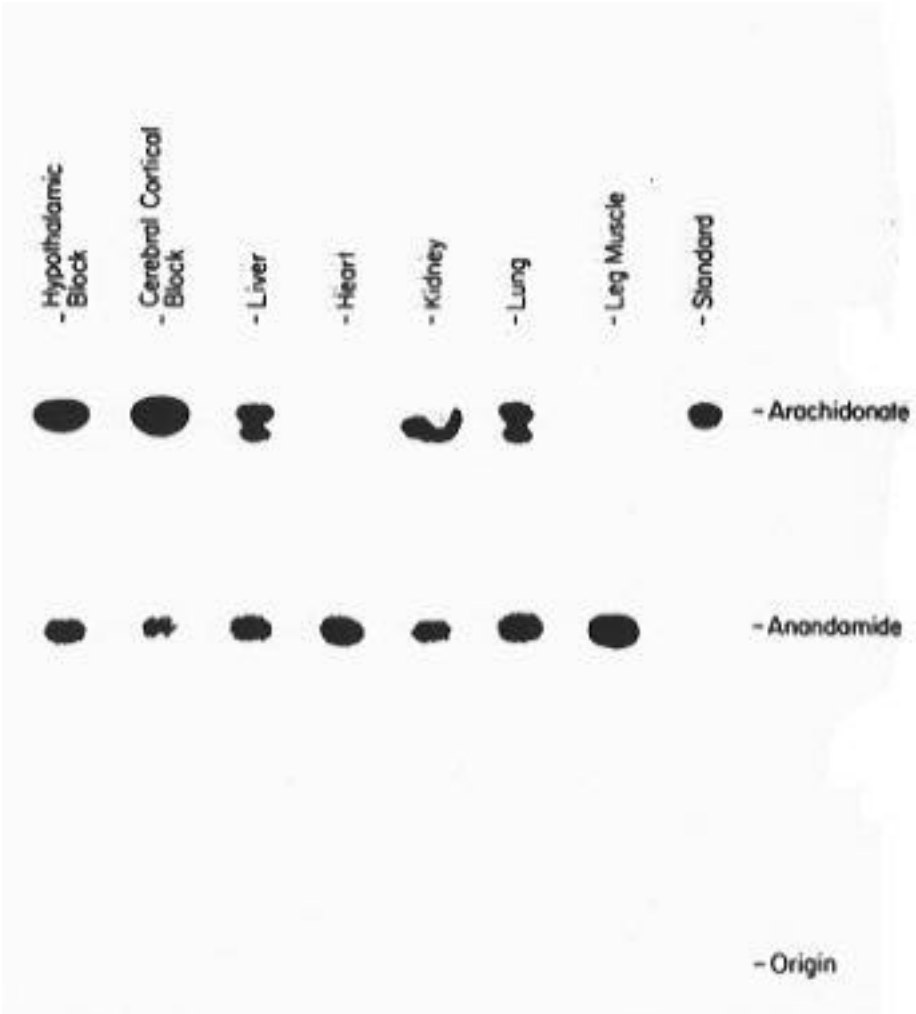

FIGURE 1. *Degradation of anandamide in homogenates of rat tissue. Incubations were at 37° for 45 min with 10 mg of tissue homogenate protein and 0.4 microcuries (μCi) [^{3}H]-anandamide in PBS.*

acids was tested as substrates for the amidase, the highest substrate specificity was for arachidonoyl ethanolamide (Desarnaud et al. 1995; Ueda et al. 1995).

CELL CULTURE EXPERIMENTS AND ANANDAMIDE AMIDASE

When ^{3}[H]- anandamide ([arachidonoyl 5,6,8,9,11,12,14,15- ^{3}H]-ethanolamide) was incubated with neuroblastoma (N18TG2) or glioma (C6) cells, there was a time-dependent decrease of its levels in the media (Deutsch and Chin 1993). Anandamide was taken up by the cells immediately, but it did not accumulate since it was converted to arachidonate and other lipids containing arachidonate that migrate on TLC near the phospholipids, triglycerides, and cholesterol esters (figure 2). After 1 hour of incubation, only about 1 percent of the total radioactivity detected in all the cell fractions was from anandamide. Arachidonylethanolamide amidase was not expressed in all cell lines. Activity was found in neuroblastoma, glioma, and nonsmall-cell lung carcinoma cells, but not in HeLa cells, larynx epidermoid carcinoma (Hep2), and hepatocellular carcinoma (HepG2) cells (data not shown). The uptake and degradation of anandamide was recently confirmed in primary cultures of rat brain (Di Marzo et al. 1994).

SUBCELLULAR FRACTIONATION AND BRAIN LOCALIZATION

When the neuroblastoma and glioma cells were fractionated into membrane and soluble fractions, the enzymatic activity for the degradation of anandamide resided mainly in the mitochondrial and microsomal membrane fractions (figure 3). Within the central nervous system, the distribution of the amidase activity correlated well with the distribution of the cannabinoid receptor. The highest activity was found in the globus pallidus, hippocampus, substantia nigra, cerebral cortex, and cerebellum, and the lowest activity was found in the brain stem and medulla where cannabinoid receptors are sparse (Desarnaud et al. 1995; Hillard et al. 1995).

A RAPID ASSAY FOR ANANDAMIDE AMIDASE

The assays (see above) for arachidonoyl ethanolamide hydrolysis (anandamide amidase) employed ^{3}H-anandamide as the substrate

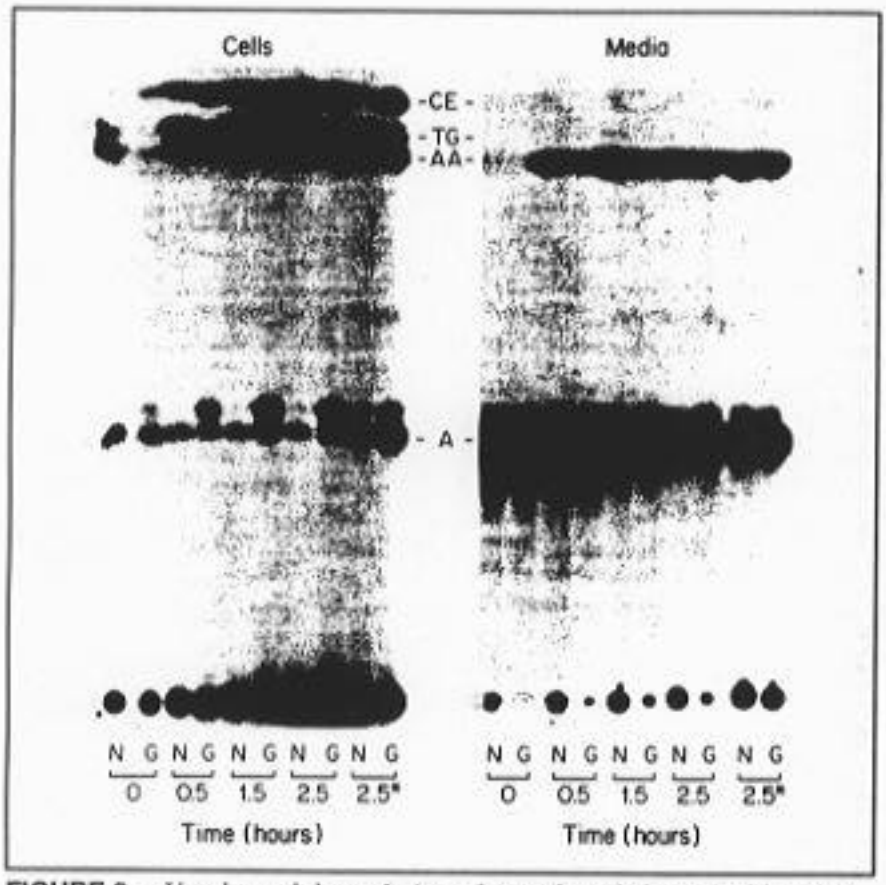

FIGURE 2. *Uptake and degradation of anandamide by neuroblastoma (N) and glioma (G) cells as a function of time. [^{3}H]-Anandamide (12 μCi) was added to the cell culture media. The radioactive products from the media and cells were analyzed by TLC and autoradiography to detect tritiated anandamide (A) and its putative metabolites: arachidonic acid (AA), triglyceride (TG), and cholesterol ester (CE). Phospholipids remained at the origin. In one set of experiments, 10^{-3} M emetine (*) was included in a 2.5-hr incubation.*

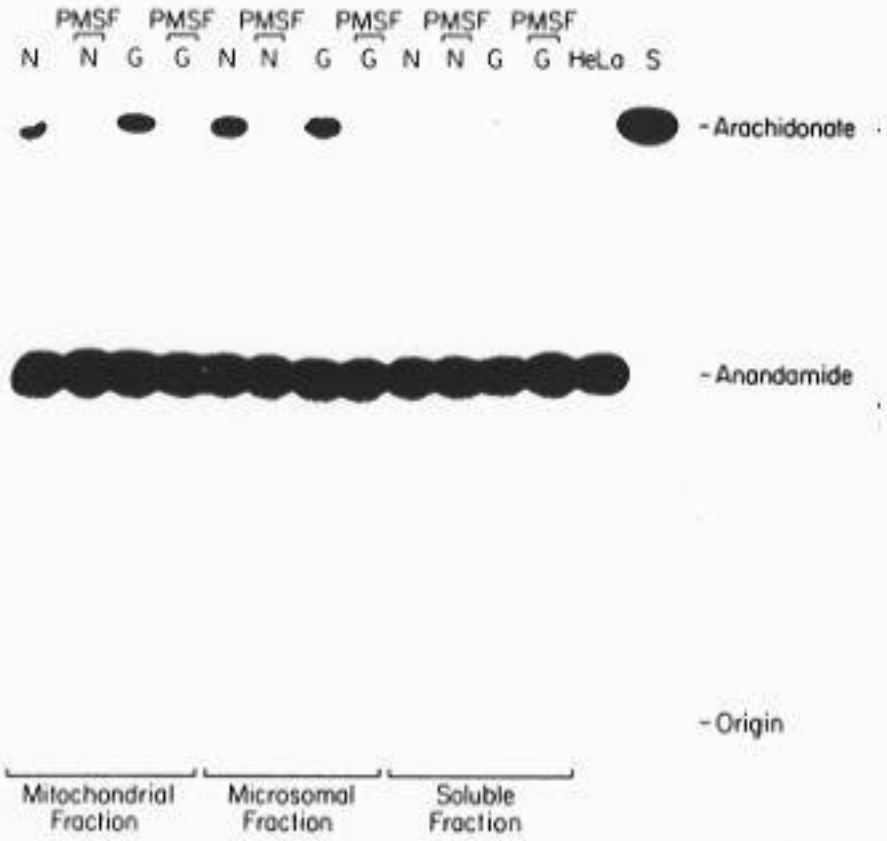

FIGURE 3. *Amidase activity in subcellular fractions of neuroblastoma (N) and glioma (G) cells. Incubations of whole fractions were for 45 min with 0.4 μCi [^{3}H]-anandamide in PBS at 37°. Where indicated, the fractions were incubated with 1.5 mM PMSF. The HeLa cells were assayed as a crude homogenate. The last sample on the plate is a [^{3}H]-arachidonic acid standard (0.16 μCi).*

(Deutsch and Chin 1993; Koutek et al. 1994). Using this assay, a TLC step is required to separate labeled arachidonic acid product from anandamide substrate. A new assay employing arachidonoyl ethanolamide labeled in the ethanolamine portion of anandamide (arachidonoyl ethanolamide-[1,2-^{14}C]) has recently been developed which obviates the need for the TLC step (Omeir et al. 1995). After incubation of the enzyme with this substrate, the reaction mixture is stopped by the addition of organic solvent. The radiolabeled anandamide substrate partitions into the organic phase while the product (ethanolamine- [1,2-^{14}C]) conveniently partitions into the aqueous phase which is subsequently measured by liquid scintillation counting. Employing this assay, it was found that the reaction proceeded linearly for at least 30 minutes. Anan-damide amidase exhibits maximal activity between pH 8 and pH 9 with a steep decline in activity at pH values below pH 6 and above pH 10. Arachidonoyl ethanolamide-[1,2-^{14}C] was found to be a good substrate to assay the amount of anandamide amidase from 10 to approximately 100 micrograms (g) of protein in brain homogenate (figure 4). Under the conditions of this assay, less than 10 percent of the substrate is hydrolyzed. Defatted serum albumin is included in the assays to bind the fatty acid product, thus preventing product inhibition. The results of a representative experiment for the initial velocity of anandamide degradation versus anandamide substrate concentration, when plotted as a rectangular hyperbole of the steady-state Michaelis-Menten equation, yields a Michaelis constant (K_m) of 30 Å 7 M and a maximal velocity (V_{max}) of 198 Å 13 nanomoles (nmoles) ethanolamine formed per hour per mg protein homogenate. The K_m and V_{max} values calculated from the saturating hyperbola must be considered approximate in view of the fact that the interfacial enzyme reaction occurs in an impure preparation, whose substrate and product have the potential to form micelles, which in turn may affect the enzyme activity. Recently Hilliard and colleagues (1995), Desarnaud and colleagues (1995), and Ueda and colleagues (1995) reported values of K_m and V_{max} for the hydrolysis of anandamine as follow: K_m 3.4, 12.7, and 60M and V_{max} 0.132, 0.337, and 28.8 moles/hr/mg protein, respectively.

The enzyme showed some specificity when two chiral methanandamide stereoisomers (Abadji et al. 1994) were tested as competitors of anandamide (figure 5). At low concentration the (R)-methanandamide stereoisomer did not affect the enzyme activity, while the (S)-meth-anandamide isomer interacted weakly. At higher concentrations this effect disappeared, with both compounds giving some inhibition of

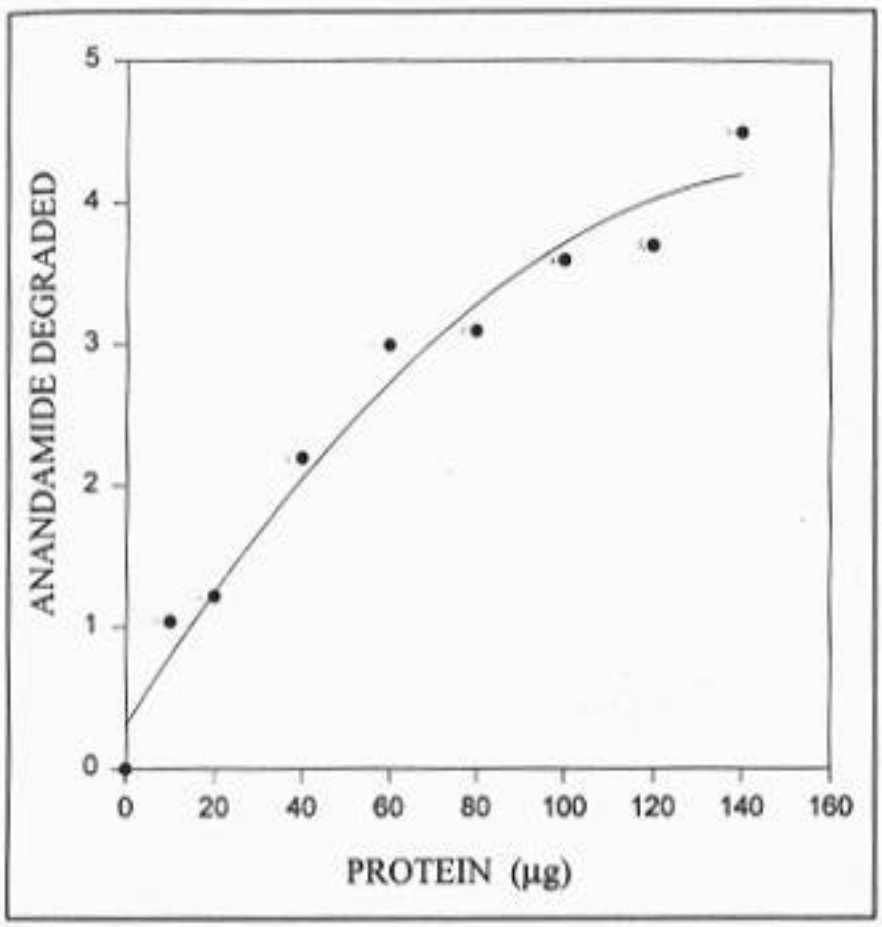

FIGURE 4. *Assay of anandamide amidase activity in crude protein brain homogentate. The amount of brain protein indicated above was incubated for 0.5 hr in a 0.2 milliliter (mL) incubation volume. Anandamide degraded is in units of nmoles/hr.*

the anandamide-amidase reaction. These results may be interpreted in terms of one stereoisomer (at low concentrations) being more effective at the substrate binding site on the enzyme.

Interestingly, the enzymatic activities found for anandamide amidase may be related to those reported in the literature for the hydrolysis of other fatty acid amides. Bachur and Udenfriend (1966) and Schmid and colleagues (1990) described a rat liver microsomal enzyme that hydrolyzed fatty acid ethanolamides. Natarajan and colleagues (1984) described activity in a dog brain microsomal fraction that hydrolyzed

palmitoylethanolamide with an apparent K_m of 53 M and a V_{max} of 667 nmole/h/mg.

PUTATIVE TRANSITION-STATE INHIBITORS OF ANANDAMIDE AMIDASE

As shown in figure 3, PMSF was discovered to be a potent inhibitor of the enzymatic breakdown of arachidonoylethanolamide in neuroblastoma and glioma cellular fractions (Deutsch and Chin 1993). It was also found that 1.5 mM PMSF (which was originally added to prevent proteolytic degradation of the enzymes in the subcellular fractions) completely abolished the amidase activity in rat brain and other tissue homogenates. This is consistent with the observation that inclusion of PMSF in receptor binding assays increased the apparent potency of anandamide and cogeners susceptible to the amidase (Abadji et al. 1994; Childers et al. 1994). The anandamide amidase activity was not inhibited by aprotinin, benzamidine, leupeptin, chymostatin, or pepstatin, suggesting that it is distinct from some of these common proteases that are susceptible to the tested inhibitors (Deutsch and Chin 1993). The amidohydrolase did not hydrolyze substrates for plasmin, aminopeptidase, elastase, or chymotrypsin (Ueda et al. 1995). The detailed mechanism by which PMSF inhibited this activity remains to be elucidated, although PMSF is known to inhibit serine proteases, some thiol proteases, and nonprotease enzymes such as erythrocyte acetylcholinesterease. Based on the behavior of anandamide with receptor preparations in the presence of PMSF, Childers and colleagues (1994) postulated that the enzyme mechanism may involve an active-site serine hydroxyl.

To further explore the question of the mechanism of anandamide amidase inhibition, analogs of anandamide were synthesized (Koutek et al. 1994). These anandamide analogs (figure 6) represent three classes of putative transition-state inhibitors: trifluoromethyl ketone (4), -keto ester (3), and -keto amide derivatives (2). The general strategy of this study was based upon the hypothesis that polarized carbonyls, such as those in trifluoromethyl ketones and -keto carboxylate derivatives, may form stabilized hydrates or enzyme adducts that mimic the tetrahedral

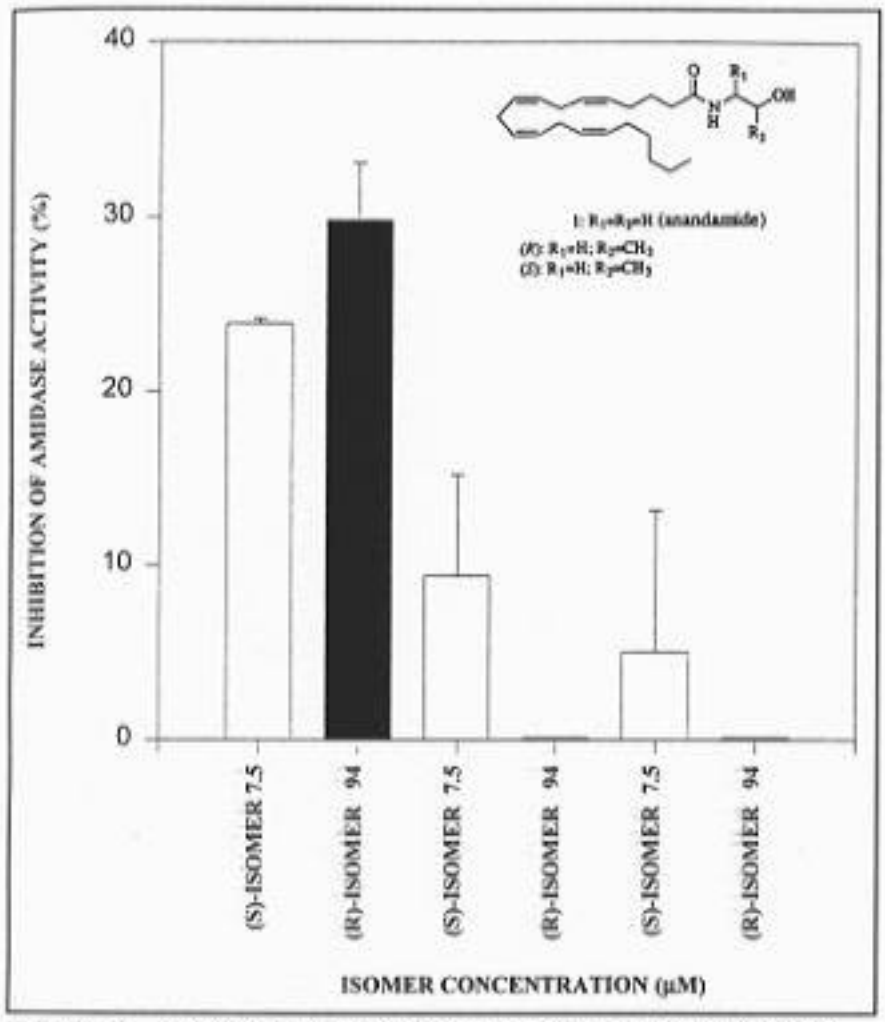

FIGURE 5. *Two chiral congeners (R and S methanandamide isomers) were tested as competitive inhibitors of anandamide amidase at 94, 7.5, and 5 μM. The percentage of inhibition was calculated by [(rate of control-rate of experimental)x100]/ rate of control. The anandamide concentration was 27.7 μM.*

intermediates formed during the reaction between the nucleophilic residue (e.g., an active-site serine hydroxyl in the hydrolytic enzyme) and the carbonyl group of anandamide (figure 7). They were tested for inhibition of anandamide hydrolysis in vitro, in cell culture, and as ligands for CB1 (Koutek et al. 1994). When tested for their ability to inhibit the hydrolysis of anandamide in vitro, the most effective classes of compounds were the trifluoromethyl ketones (4a-e) and -keto esters (3a-c). The trifluoromethyl ketones and -keto esters showed nearly 100 percent inhibition of anandamide hydrolysis in vitro in the presence of 7.5 M inhibitor and 27.7 M anandamide. Arachidonyl trifluoromethyl ketone (4a) and ethyl 2-oxostearate (3b) were the most active members of these groups, yielding nearly 100 percent inhibition of the enzyme. The inhibition of anandamide amidase by arachidonyl trifluoromethyl ketone was reversible with increasing concentrations of anandamide. Arachidonyl trifluoromethyl ketone is also a potent inhibitor of the synthase (Ueda et al. 1995). The least potent inhibitors were the -keto amides (2a-c) and the saturated analogs of anandamide (1b-d). When incubated with neuroblastoma (N18TG2) cells, anandamide is taken up by the cells and rapidly hydrolyzed to arachidonate, which is then incorporated into other lipids containing arachidonate (figure 8). However, in the presence of arachidonyl trifluoromethyl ketone (4a), there is an approximately fivefold increase of anandamide levels at 7.8 M arachidonyl trifluoromethyl ketone (figure 8). The amount of anandamide in the experimental cells increases to a twelvefold maximum, relative to the control cells, at approximately 12 M arachidonyl trifluoromethyl ketone (figure 8, inset). The mechanism apparently involves inhibition of the amidase rather than increased uptake of anandamide, since preloading the cells with labeled anandamide and then treating with 4a also resulted in a dramatic increase in anandamide levels in the cells.

The series of fatty acid derivatives was each tested, at 10 M, for their ability to displace [^{3}H]CP-55940 ([1, 2 (*R*), 5] - (-) - (1,1-dimethyl- heptyl) -2 - [5-hydroxypropylcyclohexyl] - phenol)) binding to the THC receptor in rat brain membranes (CB1). Arachidonoyl trifluoromethyl ketone was the only synthetic compound in this series of fatty acid derivatives to significantly displace [^{3}H]CP-55940 binding to CB1 with an inhibition constant (K_i) of 0.65 M. This represents approximately a fortyfold lower affinity from that of arachidonoyl ethanolamide.

Arachidonyl trifluoromethyl ketone was reported to be a slow tight-binding inhibitor of a novel 85 kilodalton-(kDa) cytosolic human

phospholipase A_2 (Street et al. 1993; Trimble et al. 1993). Some -keto acid derivatives have been shown to act as inhibitors of serine and cysteine proteinases (Ocain et al. 1992; Peet et al. 1990), and effective inhibition of cathepsin B and papain by peptidyl -keto esters, -keto amides, -diketones, and -keto acids has also been demonstrated (Hu and Abeles 1990). The disparity between the activity of ethyl 2-oxo-stearate 3b in vitro and in cell culture may be due to its susceptibility to enzymatic degradation in cell culture. It is not known if arachidonyl trifluoromethyl ketone is metabolized in the neuroblastoma cells employed in this study, but when incubated at 10 M with monocytic cells in culture for 10 min, 10 percent is converted to the corresponding alcohol (Riendeau et al. 1994).

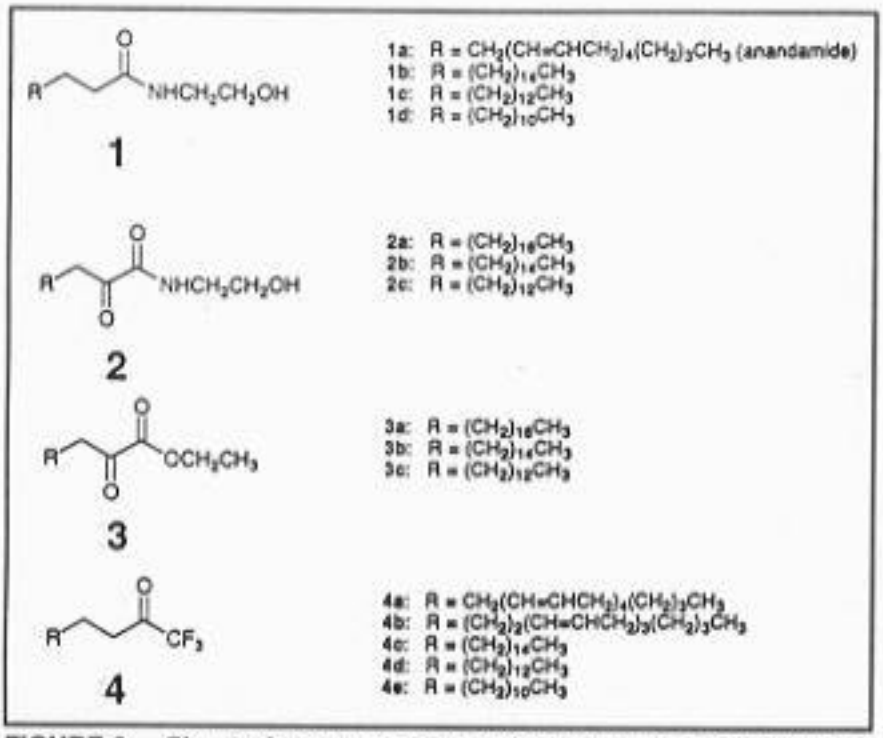

FIGURE 6. *Chemical structures of synthetic compounds. Four classes of compounds were synthesized: fatty acyl ethanolamides (1), α-keto ethanolamides (2), α-keto ethyl esters (3), and trifluoromethyl ketones (4) as described.*

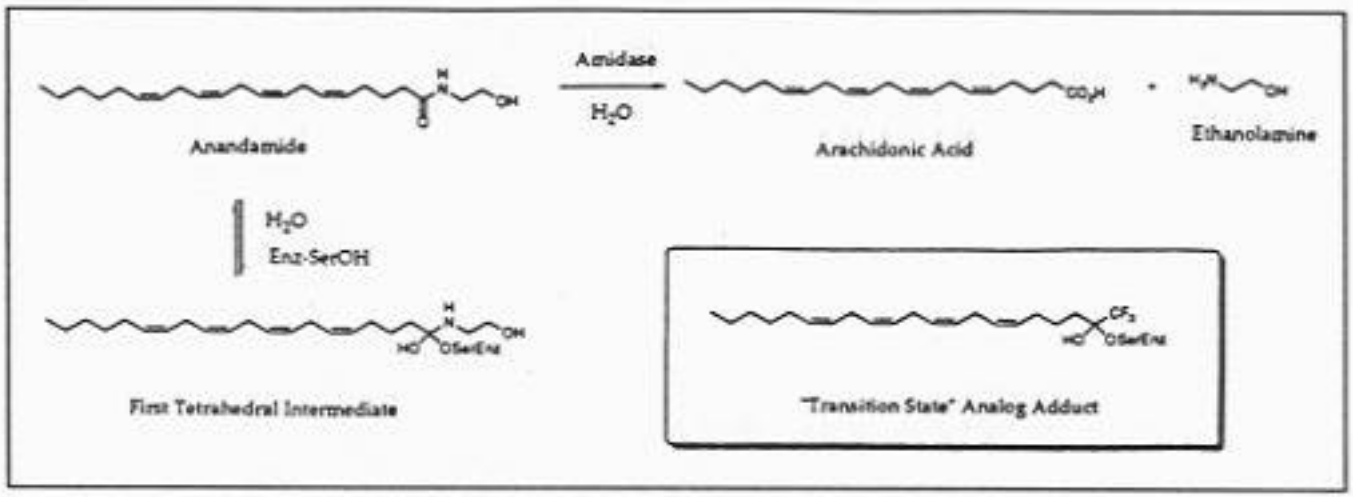

FIGURE 7. *Postulated mechanism of amidase inhibition by polarized carbonyls. Enzymatic hydrolysis of arachidonyl ethanolamide (anandamide, 1a) is postulated to proceed by a tetrahedral intermediate which can be mimicked by the adduct formed from trifluoromethyl ketone and an active-site serine residue (boxed).*

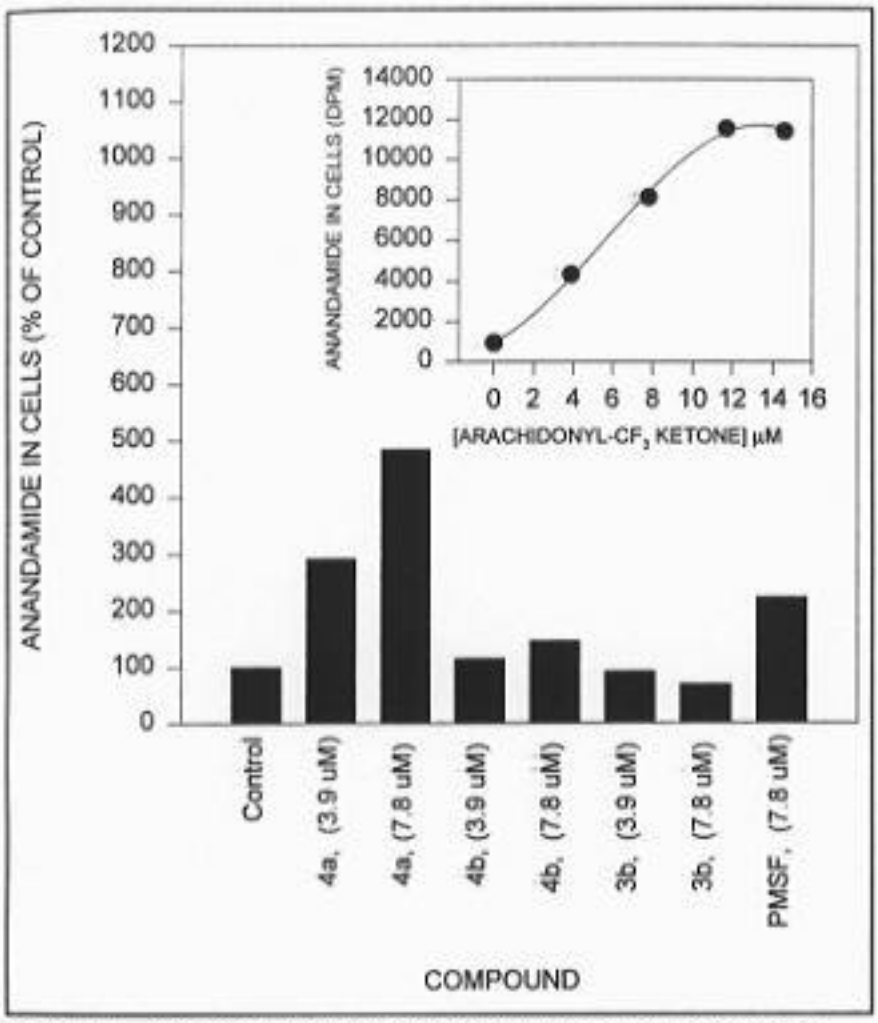

FIGURE 8. *The effect of amidase inhibitors on anandamide levels in neuroblastoma cells (N18TG2). The amount of [^{3}H]-anandamide was determined in the control and experimental cultures containing 1 x 10^6 cells, after separation by TLC and analysis of the silica gel sample scraped from the plate, by liquid scintillation counting. One hundred percent anandamide in the control cells corresponds to 1.3 percent of the total radioactivity detected in all the fractions analyzed. The inset shows the effect of increasing arachidonyl trifluoromethyl ketone concentration upon [^{3}H]-anandamide levels. Each incubation contained 4 x 10^6 cells.*

A POTENT IRREVERSIBLE INHIBITOR OF ANANDAMIDE AMIDASE

Very recently the authors developed a novel anandamide amidase inhibitor, AM374, whose potency in vitro and in neuroblastoma cells significantly exceeds that of other compounds developed to date as well as PMSF. In intact neuroblastoma cells, AM374 was found to dramatically increase the level of undegraded anandamide 55-fold at 10 nM. Interestingly, its affinity for the CB1 receptor was approximately tenfold weaker than anandamide (Deutsch and Makriyannis, unpublished data).

Ideally, a selective amidase inhibitor should antagonize the enzyme at concentrations that fail to appreciably bind to cannabinoid receptors. Furthermore, unlike PMSF, an inhibitor should not be toxic to the cells. Many of the synthetic compounds in this study fulfill these criteria; they do not bind significantly to CB1 at concentrations that inhibit amidase activity by greater than 90 percent in cell-free preparations and appear to have low toxicity toward the cells. The role that these inhibitors play in different tissues such as spleen, where a peripheral receptor (CB2) exists (Munro et al. 1993), or as inhibitors of the cytosolic phospholipase A_2 in brain and N18TG2 cells, remains to be elucidated. Furthermore, the most successful inhibitors will be subjected to in vivo testing. The development of inhibitors that block the breakdown of anandamide may be of value in any of the therapeutic applications in which THC (Mechoulam 1986) or anandamide (Crawley et al. 1993; Fride and Mechoulam 1993; Smith et al. 1994) has been shown to be potentially useful including analgesia, mood elevation, nausea, appetite, sedation, locomotion, glaucoma, and immune function.

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Metabolic Bioactivation Reactions Potentially Related to Drug Toxicities

Neal Castagnoli, Jr. and Kay P. Castagnoli

The majority of metabolic transformations that xenobiotics undergo in mammalian systems leads to more polar and, in general, less pharmaco-logically and toxicologically potent products. This generalization has led to the proposal that evolutionary factors influencing the emergence of the principal enzymes responsible for the biotransformations of xenobiotics have a link to the improved survival potential of those individuals equipped with enzyme systems capable of converting otherwise toxic substances produced by plant- and animal-based food sources to nontoxic metabolites (Jakoby and Ziegler 1990).

The types of biotransformation reactions that many xenobiotics undergo have been organized into two principal classes: Phase I transformations that, for the most part, are oxidative in nature and generally introduce a hydroxy group into the substrate molecule; and phase II transformations that convert the newly introduced hydroxy group to polar conjugates such as glucuronides and sulfate esters (Caldwell 1986; Guengerich and Ziegler 1990). In the case of carboxylic acids, polar amido esters derived from various amino acids are formed (Killenberg and Webster 1980). Since many toxic natural products are lipophilic organic molecules, the metabolic conversion of these substances in land-bound animals to polar conjugates makes teleological sense in that polar compounds partition with difficulty across cell membranes but are readily filtered through the nephron and thus are more readily eliminated from the body through renal excretion. Depending on the structures of the molecules, however, the same metabolic events, on occasion, can generate chemically reactive and toxic metabolites (Mulder et al. 1986; Parke 1987; Korzekwa and Jones 1993; Guengerich 1994; Gonzalez and Gelboin 1994). Consequently, an important part of drug development focuses on the characterization of the metabolic profile of candidate drugs in an attempt to avoid structural features that may lead to the formation of toxic metabolites.

This chapter is concerned with the metabolic fate of cyclic tertiary amines and, in particular, a consideration of potential bioactivation

pathways that may lead to toxic metabolites. Cyclic tertiary amines form an important class of compounds in the area of drug abuse since a variety of psychoto-mimetic agents such as cocaine (figure 1, structure 1), lysergic acid diethylamide (LSD 25) (structure 2) and phencyclidine (structure 3), as well a large group of centrally acting compounds such as the neuroleptic phenothiazine chlorpromazine (structure 4), the central nervous system (CNS) stimulant mazindol (structure 5), the narcotic analgetic fentanyl (structure 6), and the antidepressant imipramine (structure 7) either have been used to treat CNS disorders and/or have the potential for abuse. These types of compounds undergo extensive oxidative metabolic transformations.

The discussion that follows focuses on selected examples of metabolic transformations that may be linked to the formation of potentially neurotoxic metabolites. Although the number of well-characterized examples of such biotransformations is relatively few, it may be reasonable to speculate that the neurological disorders associated with long-term exposure to substances of abuse and some behavior-modifying medications may involve biochemical lesions mediated by chemically reactive metabolites. Thus, it may be important when attempting to assess the possible significance of metabolic bioactivation processes to appreciate that the chemical instability of reactive metabolites which can make the identification and characterization of their biological properties difficult.

The most important metabolic transformation that cyclic amines undergo is -carbon oxidation which generates the corresponding iminium products (Koymans et al. 1993). This reaction is catalyzed by members of the cytochrome P450 superfamily of hemoproteins (Nelson et al. 1993). As discussed below, recent studies have documented that the outer mitochondrial membrane bound flavoproteins monoamine oxidase A and B (MAO-A and MAO-B) are efficient catalysts for the -carbon oxidation of a specific class of cyclic tertiary amines, namely 4-substituted 1-methyl-1, 2, 3, 6-tetrahydropyridines (Kalgutkar et al. 1995). The most generally accepted catalytic pathway (figure 2) for this reaction assumes an initial single electron transfer (SET) step from the amine substrate (structure 8) nitrogen lone pair to generate an aminyl radical cation (structure 9) which, following loss of an -proton, is converted to a highly reactive carbon-centered radical (structure 10). A second single- electron oxidation of the carbon-centered radical gives, in the case of the cytochrome P450-catalyzed reaction, the carbinolamine (structure 11),

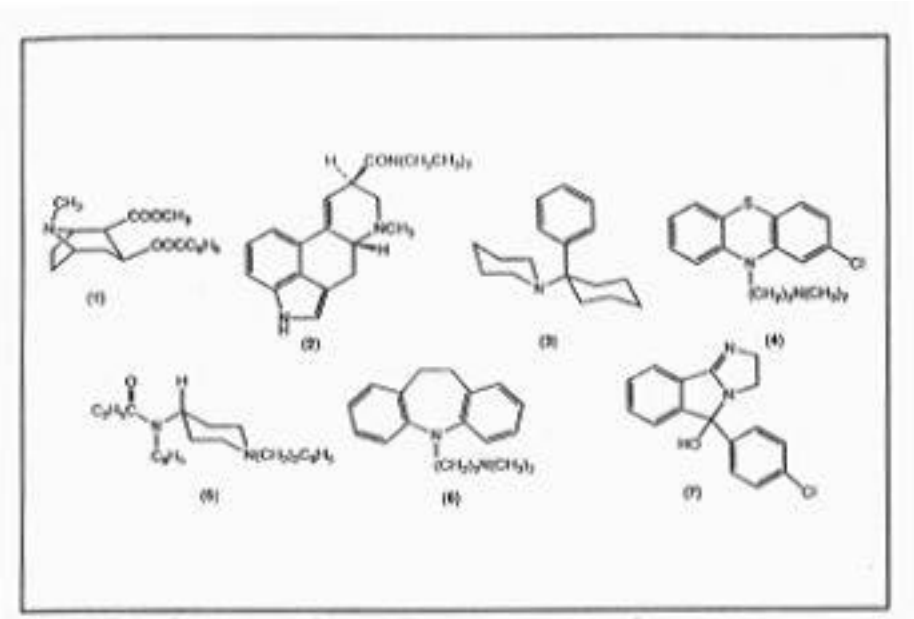

FIGURE 1. *Structures of centrally acting compounds.*

which is in equilibrium with the iminium ion product (structure 12). In the MAO-catalyzed reaction, intermediate structure 10 is converted directly to structure 12. As shown in figure 2, the electron acceptor for the cytochrome P450-catalyzed reaction is the perferryl oxo species ($Fe^{V}O$), while the electron acceptor for the MAO-catalyzed reaction is the oxidized flavin moiety (FAD).

Although the experimental evidence supporting the SET mechanism for the cytochrome P450-catalyzed (Miwa et al. 1983; Hanzlik et al. 1984; Guengerich and Macdonald 1984; Macdonald et al. 1989) and the MAO-catalyzed (Silverman 1992) reactions is extensive, it has been challenged by results from several laboratories. These include results obtained with deuterium kinetic isotope effects (Peterson et al. 1987; Peterson and Castagnoli 1988; Dinnocenzo et al. 1993; Walker and Edmondson 1994), model chemical reactions (Kim et al. 1993, 1995), and the unexpected substrate properties (Kuttab et al. 1994) of certain tertiary cyclopropyl- amine derivatives, that according to the SET pathway, would be expected to act as enzyme inactivators only (Silverman 1984). These results have led some investigators to propose a direct hydrogen atom abstraction pathway (structure 8 –> structure 12) that bypasses the aminyl radical cation intermediate (structure 9), for both the cytochrome P450-catalyzed (Dinnocenzo et al. 1993) and the MAO-catalyzed pathways (Walker and Edmondson 1994; Ottoboni et al. 1989), and a polar (2-electron)

pathway for MAO catalysis that proceeds via an amine-FAD adduct (Kim et al. 1993, 1995). The metabolic processes in figures 2 to 9 are described below.

As illustrated in figure 2, these -carbon oxidations lead to the formation of iminium ion products which undergo spontaneous hydrolysis to the corresponding aldehyde (structure 13) and secondary amine (structure 14),
the net outcome being N-dealkylation. Cyclic tertiary amines (figure 3, structure 15) also undergo oxidative N-dealkylation, via hydrolysis of the enzyme generated exocyclic iminium intermediate (figure 3, structure 16), to yield an aldehyde (structure 13) and the cyclic secondary amine (figure 3, structure 17). The corresponding oxidation of a ring -carbon atom generates the cyclic iminium intermediate (figure 3, structure 18). Unlike the acyclic regioisomer structure 16, hydrolysis of structure 18 to the aminoaldehyde structure 19 is reversible, giving rise to the possible further metabolic processing of structure 18. These intermediary metabolites are often oxidized to the biologically less active lactams (structure 20) in a reaction that is catalyzed by the liver cytosolic enzyme aldehyde oxidase (Bielawski et al. 1987). If special structural features are present in the substrate molecule or if the cyclic iminium metabolite is generated in extrahepatic tissues lacking aldehyde oxidase, these reactive

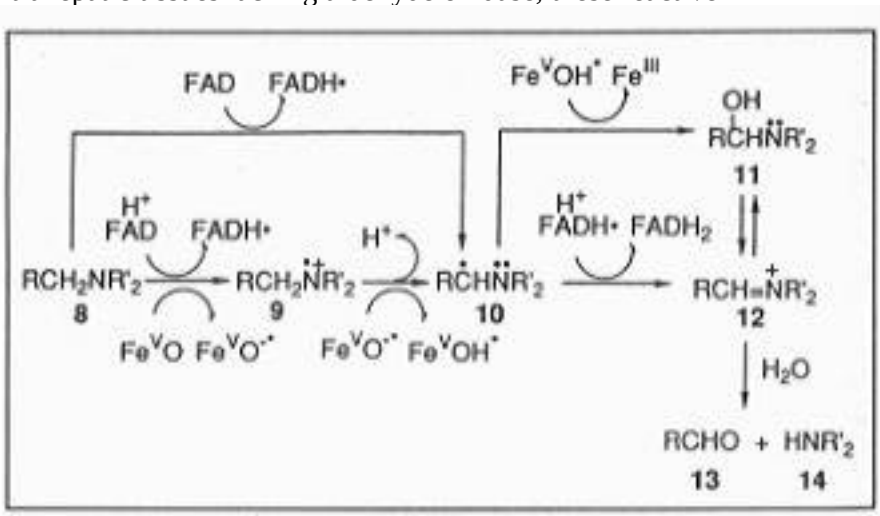

FIGURE 2. *Proposed pathways for the MAO-catalyzed and cytochrome P450-catalyzed oxidations of tertiary amines.*

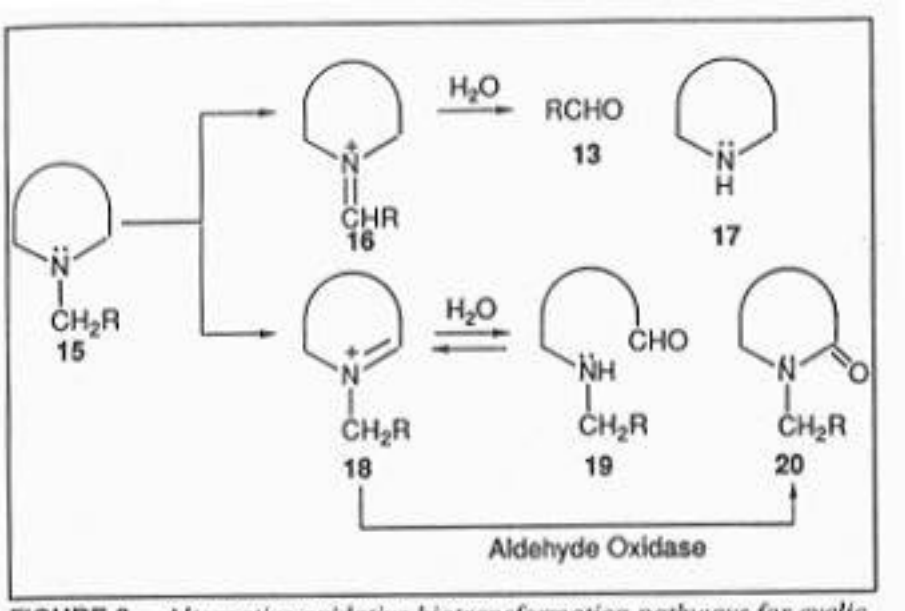

FIGURE 3. *Alternative oxidative biotransformation pathways for cyclic tertiary amines.*

intermediates may undergo alternative chemical transformations that can produce toxic products.

An example of the metabolic conversion of a 5-membered azaheterocylic system to toxic metabolites is summarized in figure 4 with the hepatotoxic and carcinogenic pyrrolizidine alkaloids (Mattocks 1986). Initial cyto-chrome P450-catalyzed, -carbon oxidation of the bicyclic parent tertiary amine (structure 21) generates the iminium intermediate (structure 22) that, upon loss of a proton, forms the pyrrolic derivative (structure 23). However, compound 23 is unstable because of the presence of the leaving groups in the side chains. Departure of the $RCOO^-$ group generates the highly reactive electrophilic intermediate (structure 24) that is attacked by a nucleophilic group present on biomacromolecules to generate an adduct (structure 25). The pyrrolizidine alkaloids are *bis*alkylating agents because of the presence of the second strategically positioned leaving group ($R.COO^-$) with the resulting formation of a crosslinked biopolymer (structure 26).

The dramatic toxicity of the pyrrolizidine alkaloids and related highly toxic compounds has led investigators to focus their attention on the

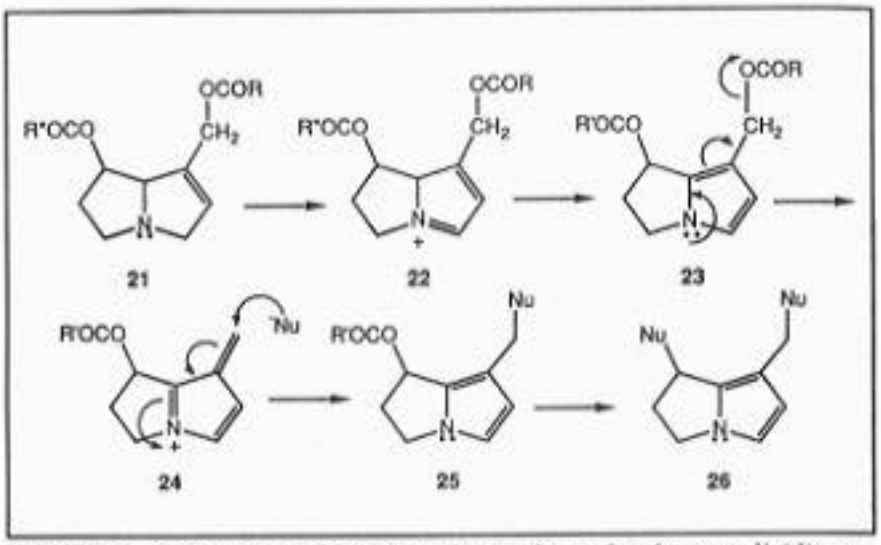

FIGURE 4. *The proposed bioactivation pathway for the pyrrolizidine alkaloids.*

underlying biochemical mechanisms. More subtle toxicities, however, may be associated with compounds that undergo similar oxidative conversions. The authors have been interested in the potential metabolic bioactivation of the tobacco alkaloid (S)-nicotine (figure 5, structure 27), a compound that is metabolically bioactivated to reactive intermediates that form covalent bonds to biomacromolecules (Shigenaga et al. 1988). The principal oxidative pathway for this compound also proceeds via cytochrome P450-catalyzed oxidation to form the corresponding iminium metabolite (structure 28). When generated in the presence of liver aldehyde oxidase, structure 28 is rapidly converted to the nontoxic lactam (S)-cotinine (structure 29). In the absence of aldehyde oxidase, however, the iminium ion (structure 28), presumably via the corres-ponding free enamine base (structure 30), can be oxidized to the pyrrolic metabolite -nicotyrine (structure 31) in a reaction that is catalyzed by MAO-B (Shigenaga 1989). -nicotyrine is an electron-rich heterocyclic aromatic system that undergoes rapid cytochrome P450-catalyzed conver- sion to the pyrrolinones (structures 35 and 36), which in turn autoxidize to the 5-hydroxypyrrolinone (structure 38) (Shigenaga et al. 1989).

The proposed reaction pathway leading to these products is depicted in figure 5. The electron-rich pyrrole ring system is oxidized to the reactive arene oxide (structure 32) which rearranges to the zwitterionic species

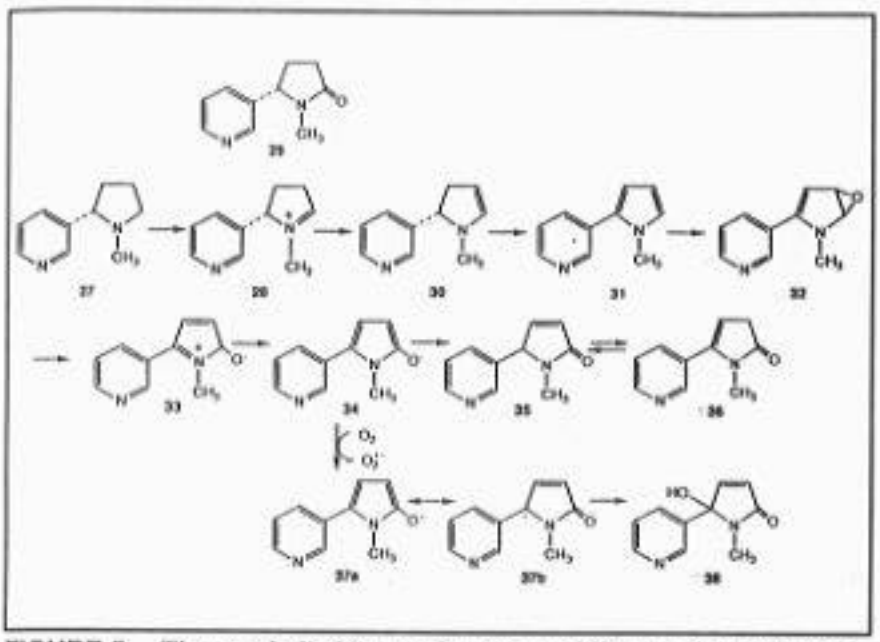

FIGURE 5. *The metabolic biotransformation of (S)-nicotine leading to potentially toxic metabolites.*

(structure 33). Proton loss generates the anion structure 34 which leads to an equilibrium mixture of pyrrolinones (structures 35 and 36). Alternatively, the anion (structure 34) undergoes autoxidation, leading to formation of superoxide radical anion ($O_2^{\cdot -}$) and the resonance stabilized radical, structure 37a <—> 37b, which eventually is converted to the final product (structure 38). The possible toxicological significance of this metabolic pathway remains to be documented. Since human exposure to tobacco products occurs over the course of many years, even low-level exposures to reactive intermediates (such as structure 37) could have a cumulative effect that may contribute to the degenerative processes linked to tobacco use. In this regard, the efficient conversion of (S)-nicotine to the corresponding iminium metabolite (Shigenaga et al. 1988) by lung cytochrome P450 may be particularly significant since the levels of aldehyde oxidase in this tissue are likely to be very low or absent (Huff and Chaykin 1967; Beedham 1985).

A major impetus for considering the bioactivation of cyclic tertiary amines is derived from studies on the parkinsonian-inducing nigrostriatal neurotoxin 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) structure 39. Extensive metabolic,

biochemical, and toxicological investigations have documented that the neurodegenerative properties of MPTP are mediated by a mitochondrial neurotoxin that is formed according to the reaction sequence shown in figure 6 (Sayre 1989; Maret et al. 1990; Kopin 1992; Tipton et al. 1993; Tipton and Singer 1993; Singer et al. 1993). The substrate amine (structure 39) undergoes C-6 (allylic) ring -carbon oxidation to form the cyclic iminium metabolite, the 1-methyl-4-phenyl-1,2-dihydropyridinium species ($MPDP^+$) (structure 40). Structure 40, although stable as its solid perchlorate salt (Chiba et al. 1985), is too unstable to isolate from incubation mixtures (Weissman et al. 1985). Since structure 40 is an excellent substrate for aldehyde oxidase, it is rapidly converted in whole liver homogenates to the corresponding lactam structure 41 (Wu et al. 1988). In the absence of the aldehyde oxidase, however, structure 40 (< 50 millimolars (mM)) in pH 7.4 buffer undergoes slow autoxidation to yield the 1-methyl-4-phenyl-pyridinium product MPP^+ depicted in structure 42 (Wu et al. 1988). At higher con- centrations, this dihydropyridinium metabolite also may participate in two alternative reactions (figure 7). The first is a bimolecular dispropor-tionation reaction in which the free base structure 43 derived from structure 40 functions as a hydride donor, while structure 40 serves as a hydride acceptor. The net result is the formation of stoichiometric amounts of MPTP and MPP^+ (Wu et al. 1988). A second and more complex reaction sequence involves the net consumption of three moles of the dihydropyridinium metabolite (structure 40) that eventually yields the isoquinoline system (structure 44), a mole of MPP^+, and a mole of methyl- amine (figure 7) (Leung et al. 1989). The extent to which these or similar reactions involving endogenous reactants occur in vivo is not known.

In vitro metabolic studies with rodent and human liver microsomal prepara- tions have established that MPTP undergoes both oxidative N-demethylation and C-6 (allylic) oxidation in reactions that are -nicotinamide adenine dinucleotide phosphate (NADPH) dependent and therefore likely to be cytochrome P-450 catalyzed (Weissman et al. 1985; Ottoboni et al. 1990). Although the latter transformation can lead to the toxic pyridinium metabolite MPP^+, the cytochrome P450-catalyzed pathway is unlikely to contribute significantly to the neurotoxicity of MPTP. As mentioned above, liver aldehyde oxidase diverts the inter-mediate dihydropyridinium metabolite away from pyridinium ion formation by catalyzing the conversion of structure 40 to the nontoxic lactim structure 41. Further-more, even if formed in the periphery, the polar pyridinium metabolite would have limited access to the central nervous system (CNS). The low

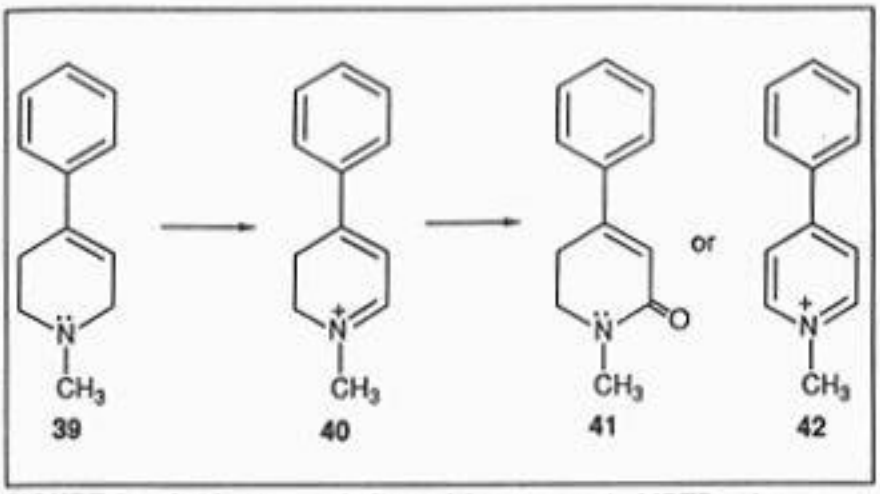

FIGURE 6. *Oxidative metabolism of the neurotoxin MPTP to its dihydropyridinium metabolite MPDP⁺ and subsequent detoxification by conversion to a dihydropyridone or oxidative toxication to the ultimate toxic species MPP⁺.*

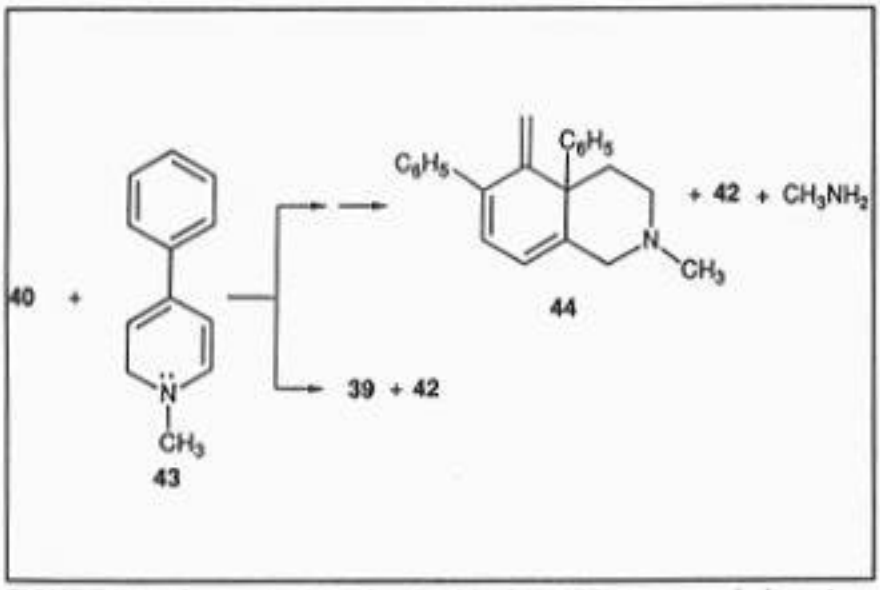

FIGURE 7. *Alternative fates for the dihydropyridinium metabolite of MPTP.*

concentrations of the P450s in the brain (Warner et al. 1993) preclude the in situ formation of toxic levels of MPP^+ within the CNS by this pathway.

Continued interest in the possible metabolic activation of MPTP led to brain tissue homogenate studies that initially established bioactivation in these tissues by MAO (Chiba et al. 1984). Later studies demonstrated the unexpected and excellent (k_{cat}/K_M = 1400 $min^{-1}mM^{-1}$ at 37%C) MAO-B substrate properties of MPTP (Kuttab et al. 1994). Subsequent studies employing a monkey model of MPTP-induced parkinsonism established the role of MAO-B in the mediation of the nigrostriatal toxicity of MPTP. The critical experiment showed that the selective MAO-B inhibitor (R)-deprenyl (structure 45, figure 8) completely protects against MPTP's toxicity in this model (Langston et al. 1984; Heikkila et al. 1984). MPTP also is a substrate for MAO-A (k_{cat}/K_M = 47 $min^{-1}mM^{-1}$ at 30%C) (Singer et al. 1986). However, since pretreatment with the MAO-A selective inactivator clorgyline (structure 46) does not protect against its neurotoxicity, this form of the enzyme does not appear to contribute to MPTP's neuro-degenerative properties.

The selective toxicity of MPTP is remarkable, particularly since there is no evidence for the presence of MAO-B in the susceptible dopaminergic nigrostriatal neurons (which do, however, contain MAO-A) (Moll et al. 1990). This apparent dilemma has been resolved by the demonstration that MPP^+ is a substrate for the dopamine transporter (Javitch et al. 1985). Once localized intraneuronally, MPP^+ is concentrated further within the inner mitochondrial membrane (Youngster et al. 1989*a*; Davey et al. 1992), where it inhibits electron transport (Nicklas et al. 1985, 1987) leading to adenosine triphosphate (ATP) depletion (Di Monte et al. 1986) and cell death.

The ability of MPTP to cause a lesion that parallels in many ways the characteristic lesion of idiopathic Parkinson's disease has stimulated efforts to identify possible environmental and endogenous compounds that may possess MPTP-type properties (Ikeda et al. 1992). Intracerebral microdialysis studies that estimate irreversible neuronal degeneration have provided evidence that a variety of pyridinium and related quaternized azaheteroaromatic systems are toxic to dopaminergic neurons (Rollema et al. 1990, 1994). Nevertheless, relatively few compounds meet all of the characteristics required for

an MPTP-type neurotoxin; these characteristics include the in situ MAO-B-catalyzed biotransformation in the brain to a pyridinium

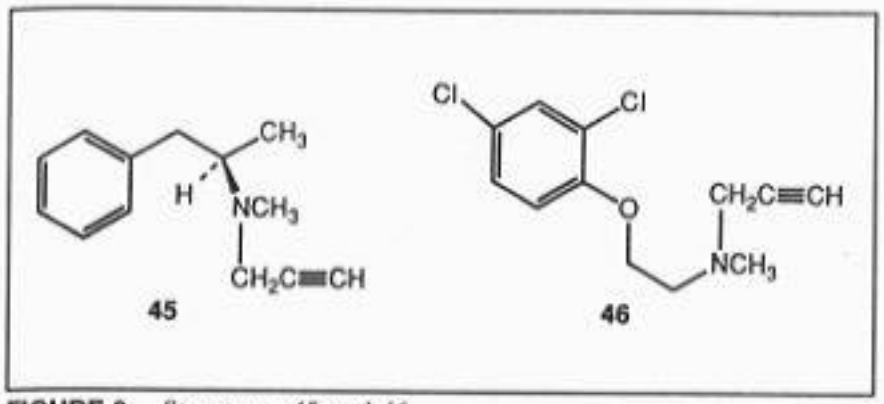

FIGURE 8. *Structures 45 and 46.*

metabolite. The metabolite is actively transported into the nigrostriatal nerve terminals and then into the inner mitochondrial membrane, where it must inhibit electron transport. Extensive studies have documented that only 1-methyl-1,2,3,6- tetrahydropyridine derivatives bearing selected substituents at C-4 are good substrates for MAO-B (Maret et al. 1990; Sablin et al. 1994; Youngster et al. 1989*b*; Kalgutkar et al. 1994). Furthermore, various types of tetrahydropyridine derivatives that are good substrates for MAO-B do not display MPTP-type activity when tested in vivo since, for various structural reasons, the intermediate dihydropyridinium metabolites are not converted to the corresponding pyridinium species (Naiman et al. 1990; Dalvie et al. 1992).

The MAO-catalyzed bioactivation of MPTP to $MPDP^+$, leading to the neurotoxic MPP^+ pyridinium metabolite, is dependent on the allylamine unit present in the tetrahydropyridine moiety. A similar reaction sequence, however, may occur with piperidine derivatives lacking the double bond but at the same oxidation state as the tetrahydropyridine. One compound of particular interest that fits this description is haloperidol (figure 9, structure 47), a potent neuroleptic agent that, like other members of this pharmacological class, causes severe extrapyramidal side effects including parkinsonism and tardive dyskinesias (Tarsy and Baldessarini 1986). This 4-piperidinol derivative resembles MPTP in that it bears an aryl group at C-4 of the piperidinol. Dehydration of HP structure 47, a reaction that is reported to occur in microsomal incubations (Fang and Gorrod 1991), gives the corresponding 1,2,3,6-tetrahydropyridine derivative HPTP, structure 50. HP 47 and HPTP 50 are not substrates for MAO-B, but

evidence obtained with the aid of mass spectral techniques and a sensitive high-performance liquid chromatography (HPLC) fluorescence assay have documented the conversion of HP (in humans) and both HP and HPTP (in rodents) to the pyridinium product HPP^+ (structure 52) (Subramanyam et al. 1990, 1991*a*, 1991*b;* Igarashi and Castagnoli 1992; Van der Schyf et al. 1994). The proposed metabolic sequence for the oxidation of HPTP to HPP^+ (figure 9) proceeds via the dihydropyridinium intermediate structure 51 followed by autoxidation of structure 51 to structure 52 (Subramanyam et al. 1991*b*). The oxidation of HP is thought to proceed via initial formation of the iminium inter-mediate structure 48 which, via the aminoenol structure 49, is converted to the dihydropyridinium species structure 51. These ring -carbon oxidations parallel the pathway outlined in figure 3 for cyclic tertiary amines in general. Since oxidative N-dealkylation (analogous to the sequence 8 –> 12 –> 13 + 14, figure 2) is a major metabolic pathway for HP (Forssman and Larsson 1977), it is not surprising to observe ring - carbon oxidation as a competing pathway.

The metabolic pathways leading to the production of these urinary pyridinium metabolites are likely to be mediated by one or more forms of liver cytochrome P450. In vitro metabolic studies with rodent (Igarashi et al., unpublished results) and human (Usuki et al., submitted) microsomal preparations have demonstrated the NADPH-dependent oxidation of both HP and HPTP to HPP^+. Ongoing studies in the authors' laboratory have shown that HPP^+ and related pyridinium metabolites are present in brain tissues obtained from C57 black mice that had been treated with HPTP (Van der Schyf et al. 1994). Additionally, results obtained from intra-cerebral microdialysis, mitochondrial respiration, and rat embryonic mesencephalic cell culture studies suggest that HPP^+ possesses MPP^+ type neurotoxic properties (Rollema et al. 1992, 1994; Bloomquist et al. 1994).

The critical question concerns the neurotoxic potential of HP in the human. Since the development of drug induced tardive dyskinesias often requires months or even years of drug exposure (Gerlach and Casey 1988; Casey 1991), the demonstration of toxin-induced lesions in experimental animals may be difficult. Furthermore, in view of the dramatic species selectivity of MPTP (Singer et al. 1987; Giovanni et al. 1994*a*, 1994*b*), the absence of a detectable anatomical lesion in HP- or HPTP-treated rodents may not provide a definitive answer to the question

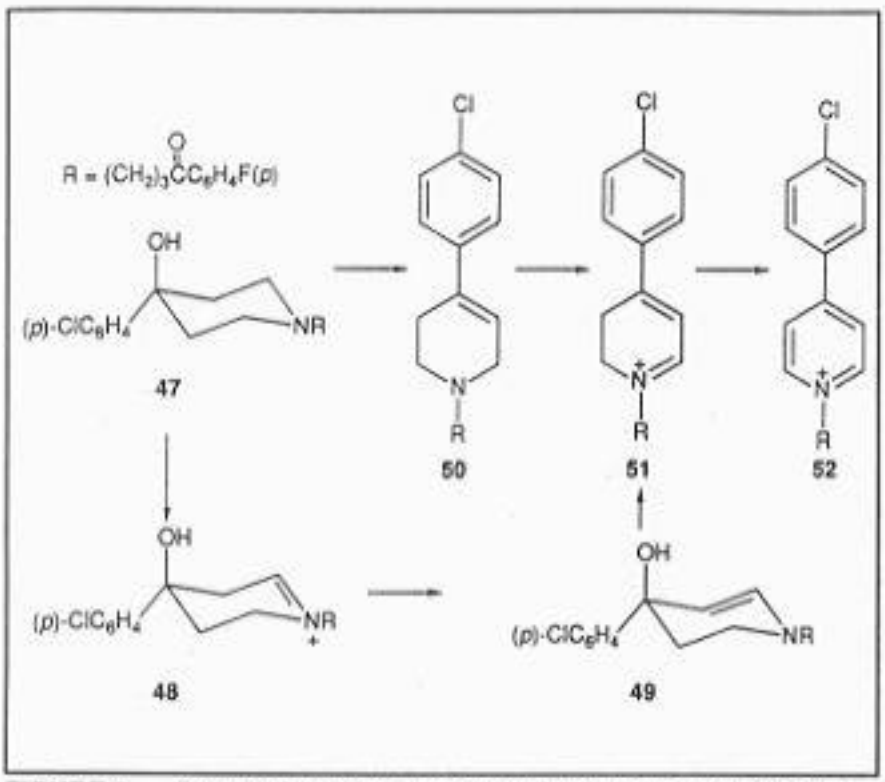

FIGURE 9. *Proposed metabolic pathways leading to the pyridinium metabolite HPP⁺.*

of the neurotoxic potential of HP. It is hoped that this issue will be resolved by an ongoing study in baboon (Van der Schyf et al., unpublished observations).

The potential neurotoxicity of pyridinium and related quaternary azaheterocyclic species and the possible formation of such metabolites from six-membered azaheterocyclic systems are of interest because of the many drugs, including many CNS-acting agents, that contain such structural features. Reports on the metabolism of compounds such as LSD (structure 3) morphine (structure 4), phencyclidine (structure 5), and fentanyl (structure 6) have not provided evidence for pyridinium ion formation. However, the proposed metabolites are quaternary cations and might not be readily detected without appropriate analytical tools. Investigations of the possible bioactivation of cyclic tertiary amines to neurotoxic quaternary cationic azaheterocyclic metabolites could provide valuable information that might lead to a better understanding of the

drug-related CNS disorders associated with long-term exposure to certain abused substances.

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The Role of Iminium-Enamine Species in the Toxication and Detoxication of Cyclic Tertiary Amines

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INTRODUCTION AND BACKGROUND

Aliphatic cyclic tertiary amines constitute a major class of naturally occurring and synthetic drugs directed at central biogenic amine receptors. Microsomal metabolism of these amines is known to be associated with low levels of covalent binding and/or suicide inactivation of the pertinent metabolizing P-450 isozymes; two of the more notorious examples are phencyclidine (1-(1-phenylcyclohexyl)piperidine) (PCP) (Hoag et al. 1984) and nicotine (Shigenaga et al. 1988).

Covalent binding was initially believed to result from alkylation of protein-based nucleophiles by the respective endocyclic iminium metabolite arising from cytochrome P-450 mediated two-electron oxidation (Hoag et al. 1984; Nguyen et al. 1979; Overton et al. 1985; Shigenaga et al. 1988; Ward et al. 1982). These iminium species are generated in equilibrium with a carbinolamine, an endocyclic enamine, and a ring-opened aminocarbonyl compound (figure 1). In contrast to *acyclic* tertiary amines, where the iminium intermediates are readily hydrolyzed via carbinolamines to secondary amines and aldehydes (or ketones) (figure 2), iminium hydrolysis for *cyclic* tertiary amines remains reversible, ensuring the persistence of all equilibrium species in figure 1. This explains why covalent binding is seen only for cyclic tertiary amines.

Support for the notion that the iminium electrophiles could be the covalent binding species is found in their ready formation of stable cyanide adducts and the fact that the presence of cyanide during metabolism of the parent amines protected against covalent binding (Hoag et al. 1984; Nguyen et al. 1979; Shigenaga et al. 1988; Ward et al. 1982). However, cyanide is a special nucleophile (it forms a C-C bond) that may not be representative of most physiologic

nucleophiles. Also, although nicotine-$^{1.(5.)}$-iminium forms a p-methylthiophenol adduct (Brandange and Lindblom 1979*a*), the iminium does not bind to nucleophilic polyamino acids except polycysteine, and this only in the presence of O_2 (Obach and Van Vunakis 1988). Unpublished studies from the authors' laboratory indicate that simple cyclic iminium species form stable covalent adducts with typical bionucleophiles only under special conditions, and no evidence has been obtained for the persistent binding of iminium species directly under physiologic conditions.

The protection against covalent binding by cyanide trapping of the initial iminium metabolite could be equally well explained if a species derived from metabolism beyond the iminium stage were the responsible culprit. In recent years, increasing evidence has arisen that most covalent binding does in fact arise from some type of *advanced* metabolite (Hoag et al. 1984, 1987; Osawa and Coon 1989; Sayre et al. 1991). For example, recent studies on the independently synthesized PCP iminium demonstrate little (Hoag et al. 1987) or no (Osawa and Coon 1989) P-450 inactivation in the absence of NADPH (the reduced form of nicotinamide-adenine dinucleotide phosphate). If the iminium species itself is not directly responsible for covalent binding, then it becomes possible to explain why glutathione (GSH) and related thiols can protect against metabolism-dependent covalent binding even though their iminium adducts only form reversibly (Hoag et al. 1984; Obach and Van Vunakis 1988; Ward et al. 1982), viz, GSH is evidently trapping the advanced reactive metabolite(s).

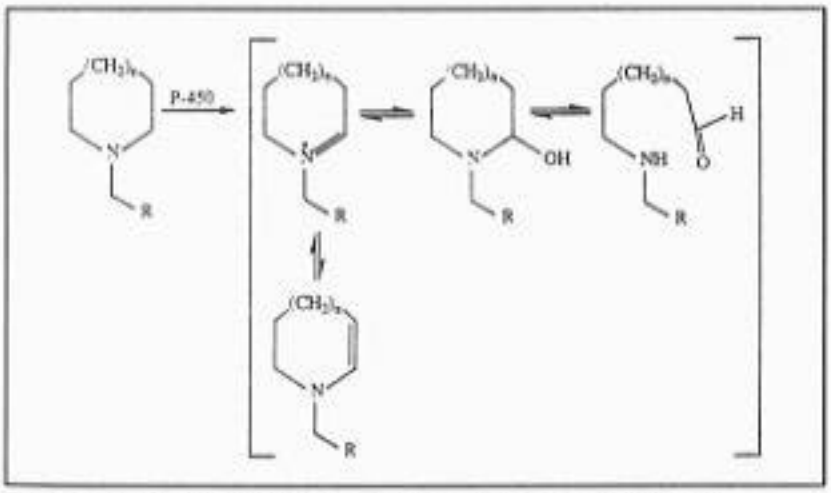


FIGURE 1. *Endocyclic N-dealkylation remains a reversible equation.*

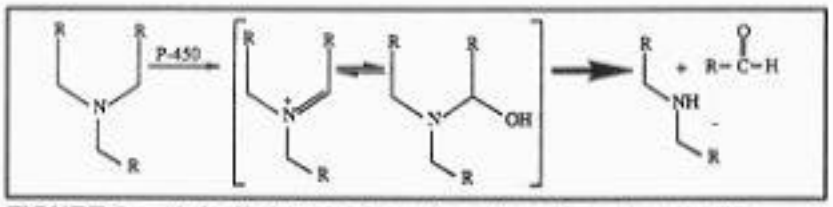


FIGURE 2. *N-dealkylation of acyclic amines is an irreversible process.*

-Aryl-substituted amines such as PCP and nicotine possess pK_as about 1 unit lower than normal tertiary amines, and enamines have pK_as about 1 unit lower than the corresponding amines (Cook 1988). Thus, cyclic enamines corresponding to PCP and nicotine, for example, should have pK_as of about 8.5, suggesting that the enamines could reach a concen- tration of nearly 10 percent that of the iminium at physiologic pH. Although higher pH also favors the carbinolamine (Brandange and Lindblom 1979*a*), the enamine would be favored over the more hydrophilic carbinolamine in a biological compartment of low dielectric constant such as the cytochrome P-450 active site. Such biological compartment will not only favor enamine relative to carbinolamine, but will also decrease the effective iminium pK_a on account of favoring charge minimization. In fact, the enamine appears to be the form of the iminium that elutes under reverse phase chromatographic conditions (Hallstrom et al. 1983; Herber et al. 1991; Mattammal et al. 1987).

The electron-rich enamines should be excellent substrates for processing by enzymes involved in oxidative metabolism, and could be giving rise to second-generation metabolites with reactive properties. A potentially important role of endocyclic enamines in xenobiotic metabolism has not been widely appreciated. It is proposed here that the enamine, rather than the ring-opened aminoaldehyde, is the iminium-derived species giving rise to metabolism-dependent P-450 inactivation. This hypothesis suggests that there should be a correlation between the degree of inactivation and the tendency of various heterocyclic ring sizes to exist in ring-closed versus ring-opened form. Such correlation is supported by a study on analogs of PCP, which revealed a rank order of $6 > 5 >> 7$ in ring size for loss of both benzphetamine demethylase activity and heme CO binding (Brady et al. 1987). As expected, N,N-diethylphenylcyclohexylamine, the acyclic version of the 5-membered ring PCP analog, exhibited no significant loss of heme.

Detoxication at the endocyclic iminium stage of metabolism is traditionally considered to involve iminium conversion to lactams by the action of the cytosolic molybdoenzyme aldehyde oxidase (AO), the prototype reaction for which is the conversion of pyridinium compounds to 2- and 4-pyridones (Felsted et al. 1973). As far as xenobiotics are concerned, the best example has been the conversion of nicotine $^{1.(5.)}$- iminium to the major human metabolite cotinine (figure 3) (Brandange and Lindblom 1979*b*). Some additional examples of lactam formation have been reported in the literature (Hammer et al. 1968; Lin et al. 1993; Wall and Baker 1989). However, although the generality of this transformation has been assumed, a consistent detoxication of iminium species by this pathway has never been demonstrated. In the key case of PCP, no lactam metabolite analogous to cotinine has ever been reported.

A preliminary report by Obach and Van Vunakis (1990) claimed that cotinine could also be formed by a microsomal nicotinamide adenine dinucleotide $(NAD)^{+}$-dependent dehydrogenase (abbreviated MND). Inhibitor studies suggested that MND is not a typical aldehyde dehydrogenase. The presence of this activity in rabbit microsomes was confirmed in the authors' laboratories (Flammang 1994). The rate of cotinine production from the $^{1.(5.)}$ -iminium by this route was found to be comparable to the rate of conversion of nicotine to the $^{1.(5.)}$ -iminium (as assessed by this enzyme might play in xenobiotic metabolism in general. It is expected that the substrate-structure dependence of MND will be quite different from that of cytosolic AO.

Detoxication at the iminium/enamine/carbinolamine stage also occurs through oxidation and reduction of the ring-opened aminoaldehyde by aldehyde and alcohol dehydrogenases, respectively, which have wide subcellar distribution. Significant differences are not expected in metabolic rates for various acyclic aldehydes arising from carbinolamine dissociation; detoxication via such routes should depend mainly on the equilibrium concentration of the ring-opened as opposed to ring-closed forms, and overall may play a minor role. Overall, the iminium/enamine stage of metabolism (figure 1) is viewed by the authors as the critical determinant between toxic activation and detoxication. Inefficient conversion to lactams by cytosolic AO and/or MND appears to correlate with increasing levels of covalent binding.

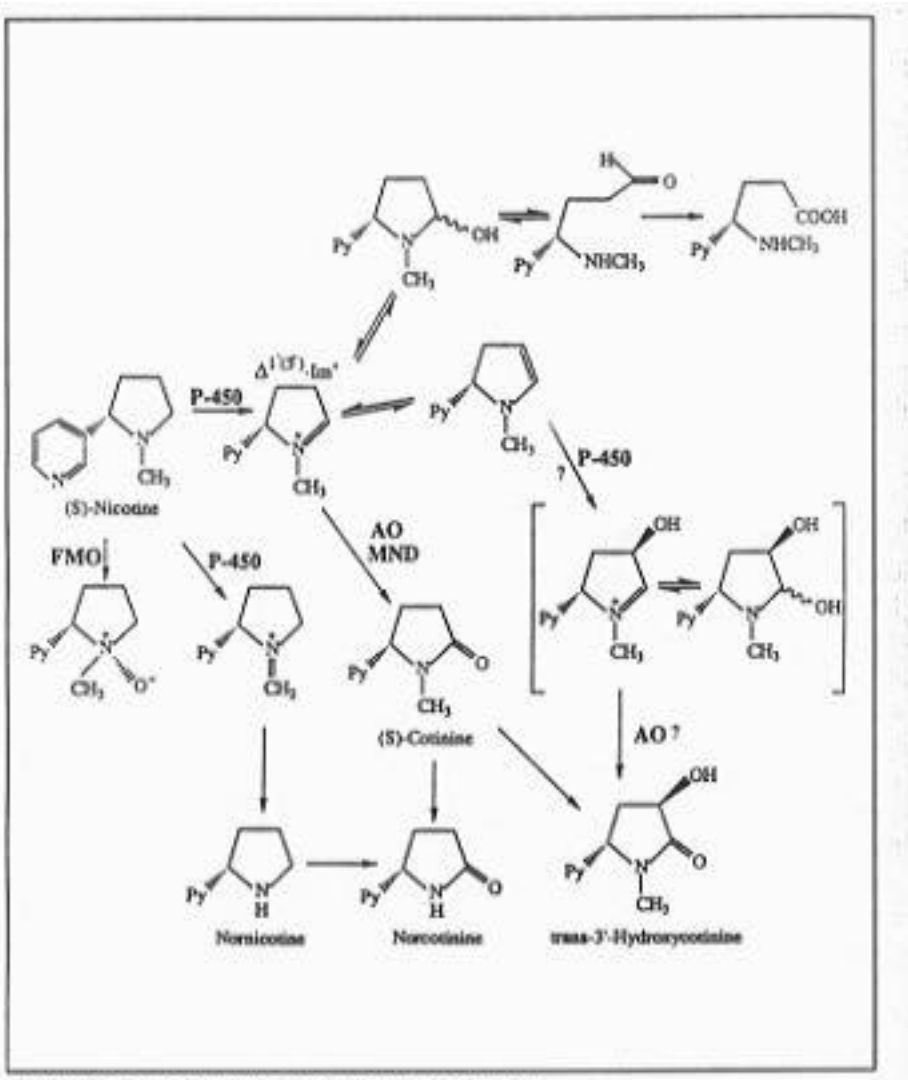


FIGURE 3. *Pathways of nicotine metabolism.*

KEY: AO = aldehyde oxidase; FMO = flavin monooxygenase; MND =microsomal NAD^+-dependent dehydrogenase.

In an effort to elucidate the nature of endocyclic iminium-derived species that give rise to metabolism-dependent covalent binding and to clarify what factors govern the balance between toxic activation and detoxication

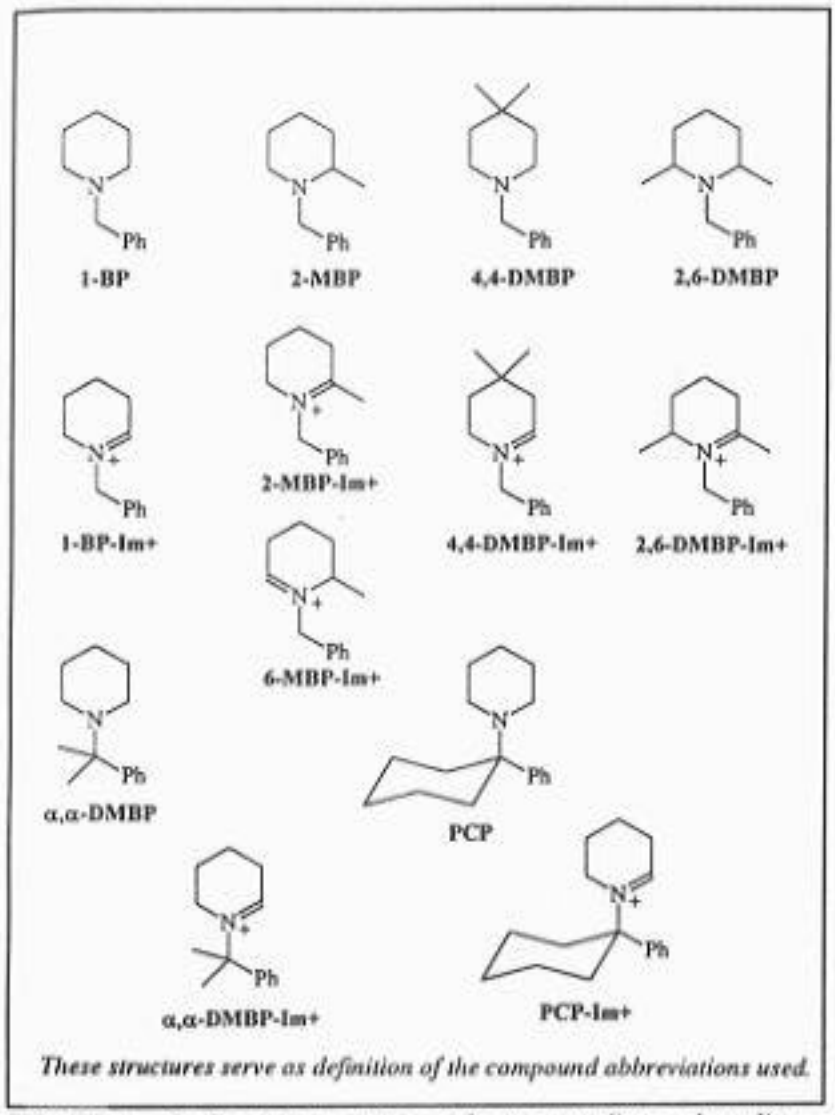


FIGURE 4. *Cyclic tertiary amines and corresponding endocyclic iminium metabolites investigated in this study.*

at the iminium stage of cyclic tertiary amine metabolism, the authors initiated a series of studies to examine the effects of systematic structural changes at the level of simple piperidine and pyrrolidine derivatives as prototypes for PCP and nicotine, respectively. These compounds permit a focus on nitrogen-directed metabolism and the role of the iminium

species, since other metabolic pathways are eliminated. For example, study of 1-benzylpiperidine (1-BP) as a structurally abbreviated form of PCP would give information about metabolic toxication/detoxication uncomplicated by the numerous cyclohexyl ring-derived PCP metabolites (Gole et al. 1988; Holsztynska and Domino 1985). The expectation that 1-BP would exhibit the endocyclic metabolism of interest and not just N-debenzylation was based on the report of metabolism-dependent covalent binding for its homolog, 1-benzylpyrrolidine (Ho and Castagnoli 1980).

Using phenobarbital-induced rabbit liver microsomes and an NADPH regenerating system, data was obtained on metabolic rates and products, cytochrome P-450 inactivation, and the effect of $CN^{å}$ trapping on these parameters (Engelhart 1994). The methods have been described in an initial report (Sayre et al. 1991), and are not duplicated here. Synthetic procedures for the amines and iminium compounds (figure 4) are described separately.

RESULTS AND DISCUSSION

Metabolic Profiles of Parent Amines

The metabolic rates and major product profiles for the tertiary amines studied are shown in table 1. The first three amines listed undergo oxidation principally at the N-C bond, leading to the endocyclic (major) and exocyclic (minor) iminium intermediates, the latter dissociating to a secondary amine (not quantified) and benzaldehyde. The preference for encyclic over exocyclic oxidation was also seen previously for 1-benzyl-pyrrolidine (Ho and Castagnoli 1980). Exocyclic N-dealkylation is blocked in the case of PCP and ,-DMBP. Cytochrome P-450 binding constants obtained for 1-BP, 2,6-DMBP, and PCP were 92, 37, and 10 micromolars (M), respectively, and appear to reflect the differences in metabolic rates and the concentrations needed to observe maximal metabolism. Although PCP was oxidized more rapidly than 1-BP and 2,6-DMBP, the fraction of metabolism resulting in formation of the PCP-Im^{+} was lower, consistent with the fact that PCP additionally gives rise to cyclohexyl ring-derived metabolites. Interestingly, 4,4-DMBP exhibited a higher rate than did 1-BP and 2,6-DMBP, but a smaller fraction of the total metabolism was accounted for by the corresponding iminium. For 1-BP, 1-BP-Im^{+} 1 was not observed directly, but the THA dimer 3 was observed instead (figure 5). Dimer 3 is the expected

TABLE 1. *Substrate activity of tertiary amines for cytochrome P-450 using microsomes from phenobarbital-induced rabbits.*

Substrate	Metabolic rate (nmol/min/mg)	Major products formed during initial metabolic phase[a]
1-BP	55 (10 mM)	C -C .dimer (50%), PhCHO (1.5%)
1-BP (1mM KCN)	52 (10 mM)	-cyano (25%), PhCHO (1.0%)
1-BP-THA dimer	40 (10 mM)	
2,6-DMBP	65 (10 mM)	iminium (71%), PhCHO (0.5%)
2,6-DMBP (1mM KCN)	59 (10 mM)	-cyano (58%), PhCHO (0.5%)
2-MBP	54 (10 mM)	iminium C -C . dimer, PhCHO[b]
4,4-DMBP	112 (10 mM)	iminium (12%), PhCHO (3.2%)
4,4-DMBP (1mM KCN)	31 (10 mM)	PhCHO (7.1%)
4,4-DMBP-Im$^+$	44 (1 mM)	
PCP	89 (1 mM)	iminium (30%)
PCP (1mM KCN)	80 (1 mM)	-cyano (11%)
PCP-Im$^+$	80 (1 mM)	
,-DMBP	37 (1 mM)	iminium (89%)
,-DMBP (0.5 mM KCN)	42 (1 mM)	iminium (37%), -cyano (50%)
,-DMBP-Im$^+$	70 (1 mM)[c]	

KEY: a = Represented as percentage of the total starting material consumed; b = yields not quantified because the authors did not distinguish between the two possible iminiums and the various possible C -C .dimers; the PhCHO was found in only trace amounts, however. c = estimated as a lower limit. PhCHO = benzaldehyde; KCN = potassium cyanide; THA = tetrahydro-anabasine. Other abbreviations are per figure 4.

outcome of iminium-enamine C -C . coupling (Leonard and Hauck 1957). Control studies on the authentic iminium perchlorate 1 established the short lifetime of this species under the incubation conditions (pH 7.4, 37%C). The endocyclic iminium could, however, be trapped as the cyano adduct 4 when metabolism was conducted in

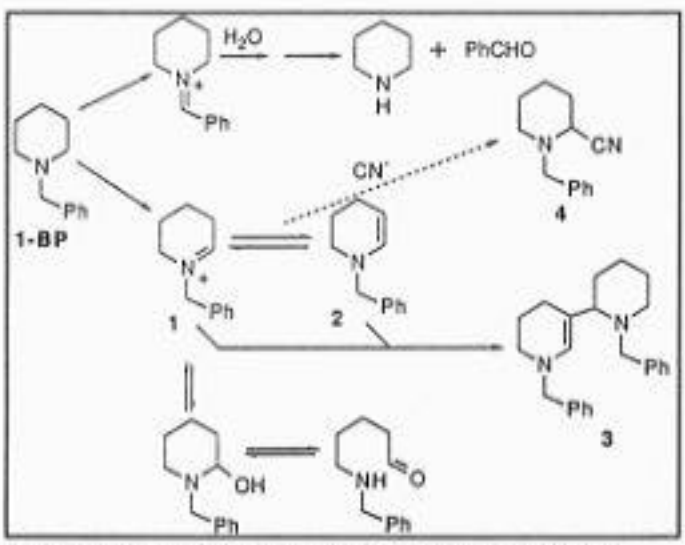


FIGURE 5. *P-450 metabolism of 1-benzylpiperidine.*

the presence of 1 mM KCN (no dimer 3 was detected in this case). For the other amines, where ring methyl substitution was expected to sterically inhibit iminium-enamine coupling (Leonard and Hauck 1957), the corresponding iminiums were observed directly. Control studies on the independently prepared iminium salts (figure 4) established their relatively long-term stability under both high performance liquid chromatography (HPLC) assay and incubation conditions, with the exception of 6-MBP-Im^+, which is analogous to 1-MBP-Im^+. On account of the instability of 6-MBP-Im^+, no attempt was made to distinguish the isomer distribution of the iminium metabolite arising from 2-MBP. As previously reported in the case of PCP and 1-benzylpyrrolidine (Hoag et al. 1984; Ho and Castagnoli 1980; Ward et al. 1982), the iminium metabolites could be trapped as the -cyano adducts in the presence of 1 mM cyanide, though this trapping was incomplete in some cases.

P-450 Inactivation

Data on the ability of various amines and metabolic intermediates to inhibit microsomal benzphetamine demthylase activity are listed in table 2. This is the standard assay for the phenobarbital-inducible P-450

TABLE 2. *Inhibition of benzphetamine N-demethylase activity.*

Substrate (1 mM)	% activity direct assay	% activity after pelleting/resuspension
1-BP, - G6PD	99[b]	99 Å 2
1-BP, + G6PD	57 Å 5	65 Å 7
1-BP, + G6PD (0.5 mM KCN)	73 Å 4	77 Å 3
2-MBP, - G6PD	99 Å 1	105 Å 3
2-MBP, + G6PD	57 Å 5	58 Å 2
2-MBP, + G6PD (0.5 mM KCN)	75 Å 9	81 Å 2
2-MBP-Im^+, - G6PD	96 Å 3	101 Å 3
2-MBP-Im^+, + G6PD	85 Å 3	89 Å 8
6-MBP-Im^+, - G6PD	81 Å 4	
6-MBP-Im^+, + G6PD	43 Å 3	
2,6-DMBP, -G6PD	98[b]	98 Å 3
2,6-DMBP, + G6PD	71 Å 3	80 Å 2
2,6-DMBP-Im^+, - G6PD	96 Å 4	108 Å 2
2,6-DMBP-Im^+, + G6PD	54 Å 2	63 Å 1
4,4-DMBP, - G6PD	95 Å 2	104 Å 3
4,4-DMBP, + G6PD	85 Å 11	93 Å 4
4,4-DMBP, + G6PD (0.5 mM KCN)	83 Å 11	86 Å 4
4,4-DMBP-Im^+, - G6PD	99[b]	102 Å 3
4,4-DMBP-Im^+, + G6PD	75 Å 6	86 Å 3
PCP, - G6PD	98[b]	104 Å 3
PCP, + G6PD	58 Å 6	57 Å 9
PCP, + G6PD (0.5 mM KCN)	78 Å 5	88 Å 4
PCP-Im^+, - G6PD	80 Å 2	78 Å 4
PCP-Im^+, + G6PD	36 Å 2	27 Å 1
,-DMBP, - G6PD	95 Å 3	104 Å 3
,-DMBP, + G6PD	43 Å 3	45 Å 5
,-DMBP, + G6PD (0.5 mM KCN)	75 Å 2	75 Å 5
,-DMBP-Im^+, - G6PD	91 Å 7	97 Å 4
,-DMBP-Im^+,+ G6PD	37 Å 4	46 Å 3

KEY: a = Incubated for 30 minutes, 37%C, pH 7.4 with microsomes from PB-induced rabbits. Values are percent of control activity Å S.E.M. of at least 3 determinations; b = Single determination; c = 0.5 mM concentration.

isoform (2B4) that is the most active in aliphatic amine metabolism. No inhibition by the parent tertiary amines was seen in the absence of metabolism (the - G6PD data). However, all parent amines exhibited a metabolism-dependent inactivation of cytochrome P-450 that was predominantly irreversible, as shown by the data obtained after pelleting/resuspension of the microsomes. The rank order of inhibitory potency was ,-DMBP > PCP ~ 2-MBP > 1-BP > 2,6-DMBP > 4,4-DMBP. The authors' interpretation of the increased level of metabolism-dependent inactivation seen for PCP and ,-DMBP relative to 1-BP is that removal of the exocyclic C-N dehydrogenation pathway (1-BP metabolism gives substantial N-debenzylation) results in an increased endocyclic metabolism, which apparently is the source of enzyme inactivation. In fact, for ,-DMBP, the level of inactivation is virtually the same as that seen for the parent amine, implying that essentially all parent amine is metabolized through endocyclic C-N dehydrogenation. The finding that ,-DMBP-Im $^{+}$and PCP-Im^{+} are equipotent inactivators confirms that the weaker inactivation of PCP relative to ,-DMBP at the parent amine stage reflects the alternate ability of PCP to be metabolized on the cyclohexyl ring.

If the iminium species is on the pathway of metabolism-dependent inactivation of P-450 by the parent amine, the independently prepared iminium should be a more potent inactivator. In an earlier study, no attempt was made to evaluate the P-450 inactivating potential of 1-BP-Im^{+} because of its rapid "dimerization" to 3 (Sayre et al. 1991). However, the iminium species corresponding to the parent amines 2,6-DMBP, 4,4-DMBP, and PCP were evaluated directly and were found to exert a greater inhibitory potency than the parent amines. The finding that most (see below) of the increased inhibition was dependent on the presence of G6PD (which again was not reversed by pelleting/resuspen-sion) indicates that the main inactivating potential arises not from the iminiums themselves but from their further metabolism.

In the case of 2-MBP, the finding that the more substituted iminium 2-MBP-Im^{+} is a weaker inactivator than the parent amine, whereas the less substituted iminium 6-MBP-Im^{+} is a more potent inactivator, indicates that endocyclic C-N metabolism on the less-substituted side is primarily responsible for inactivation by the parent amine.

The finding that PCP-Im^{+} elicits significant irreversible inactivation in the a absence of G6PD (a similar result was obtained for 6-MBP-Im^{+}) is consistent with the earlier report by Hoag and colleagues

(1987) suggesting a possible direct binding of this iminium to the enzyme. However, the G6PD-independent inactivation of the iminiums may represent residual NADPH in the microsomal preparations, because Osawa and Coon (1989) observed essentially no G6PD-independent activity loss for PCP-Im^+ using purified rabbit P-450 forms 2, 3b, and 6 in a reconstituted system.

Based on the ability to trap, at least partially, the various iminium metabolites using cyanide in the incubation medium, it was expected that cyanide would inhibit the loss of cytochrome P-450 activity if the iminium species were an obligatory intermediate in the inactivation process. As indicated in table 2, partial protection in the presence of 0.05 mM (or less) KCN was observed in the case of 1-BP, 2-MBP, ,-DMBP, and, consistent with earlier findings (Hoag et al. 1984), PCP. However, the presence of higher concentrations of KCN (1 mM and above) resulted in either no apparent protection against inactivation or an actual enhancement of inactivation by the parent amines (data not shown). The latter finding is contrary to the expectation that higher [CN'] would result in more efficient trapping of the iminium species, and even at [CN'] = 1 mM, trapping of a ,-DMBP-Im^+ (table 1) and especially 2,6-DMBP-Im^+ (not shown) was incomplete. The apparent discrepancies in the cyanide protection experiments may be explained in part by the observation that the corresponding -cyano adducts (independently synthesized) are themselves rather potent inhibitors of the enzyme (data not shown). Another complicating factor is that 1 mM KCN causes a significant degree of inhibition of benzphetamine demethylase activity in its own right. Further work will be needed to arrive at a fully satisfactory explanation for the cyanide results.

Enamine-Derived Metabolites

Evidence for oxidative metabolism of the endocyclic enamines has been obtained for both 1-BP and PCP. In a previously reported (Masumoto et al. 1991) preparative scale metabolism of 1-BP, it was found that in addition to the C -C . coupled dimer 3, small amounts of 1-benzyl-3- piperidol (1-BP-3-ol), 1-benzyl-4-piperidol (1-BP-4-ol), and 1-benzyl-3-piperidone (1-BP-3-one) were obtained (figure 5). It was further found that cyanide reduced greatly the levels of 1-BP-3-one metabolite but not the 1-BP-3-ol metabolite, suggesting that 1-BP-3-one is generated principally from the initial metabolite (1-BP-Im^+) rather than from oxidation of 1-BP-3-ol (Masumoto et al. 1991; Sayre et al. 1991). Consistent with this notion is the finding of relatively large amounts of the corresponding 3-one in the

microsomal metabolism of PCP under conditions where the 4-ol is observed exclusively of any 4-one (Masumoto et al. 1989). A plausible mechanism for cytochrome P-450 metabolism of endocyclic enamines consistent with the generation of 3-piperidones is shown in figure 6.

Also shown in figure 6 is an alternate route of enamine metabolism that would lead to a 2-en-4-ol and, after subsequent oxidation, to a 2-en-4-one. Although no 2-en-4-one was detected in the microsomal metabolism of 1-BP (Sayre et al. 1991), the corresponding 2-en-4-one was found by Hoag and colleagues (1988) as a major product resulting from microsomal metabolism of PCP-Im^+. Also of interest is the report by Zhao and colleagues (1991) that exposure of PCP-Im^+ to rat brain and liver mitochondria resulted in the isolation of a 3-formyl derivative that can be rationalized in terms of formylation of PCP-enamine by a tetrahydrofolic acid formyl donor. These iminium/enamine metabolic pathways for PCP are summarized in figure 7. At this time, the information available on the fate of metabolism of the endocyclic enamines derived from 1-BP and PCP does not permit an assessment of the actual species responsible for covalent binding.

Detoxication

As mentioned above, detoxication at the enamine/iminium/carbinolamine stage occurs in large part through oxidation to a lactam by both a cystosolic AO and a newly discovered MND. Although nicotine-$^{1.(5.)}$-iminium is readily converted to cotinine by both enzymes, the lack of any reported lactam metabolite in the case of PCP suggests that the exocyclic -branching in this case was sterically interfering with oxidation at the endocylic - position. Using the rabbit liver postmicro- somal supernatant as source of AO, it was confirmed that PCP-Im^+ and ,-DMPB-Im^+ are not converted to lactam (2-one) metabolites (independently synthesized). However, additional preliminary studies indicate that even some sterically unencumbered iminium species are poor substrates for AO (see table 3). The substrate structure-activity factors which control iminium metabolism by this enzyme have never been thoroughly investigated, and deserve further research. One obvious factor may be the effect of ring size on the equilibrium between iminium, carbinolamine, and ring-opened aldehyde forms.

As far as metabolism by MND is concerned, no substrate other than nicotine $^{1.(5.)}$-iminium has been identified to date. Evidence that at

least some interaction with the enzyme can be obtained, however, is indicated by the ability of at least one iminium species to partially inhibit the conversion of nicotine [1.(5.)]-iminium to cotinine (table 3). Nonetheless, general iminium ion metabolism is certainly not the case.

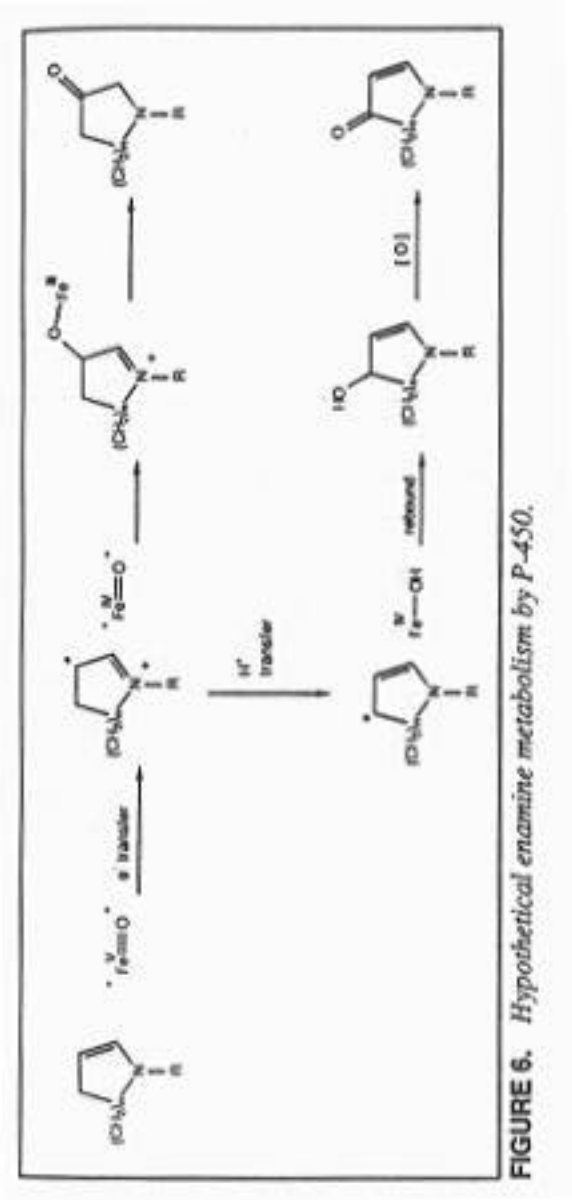


FIGURE 6. *Hypothetical enamine metabolism by P-450.*

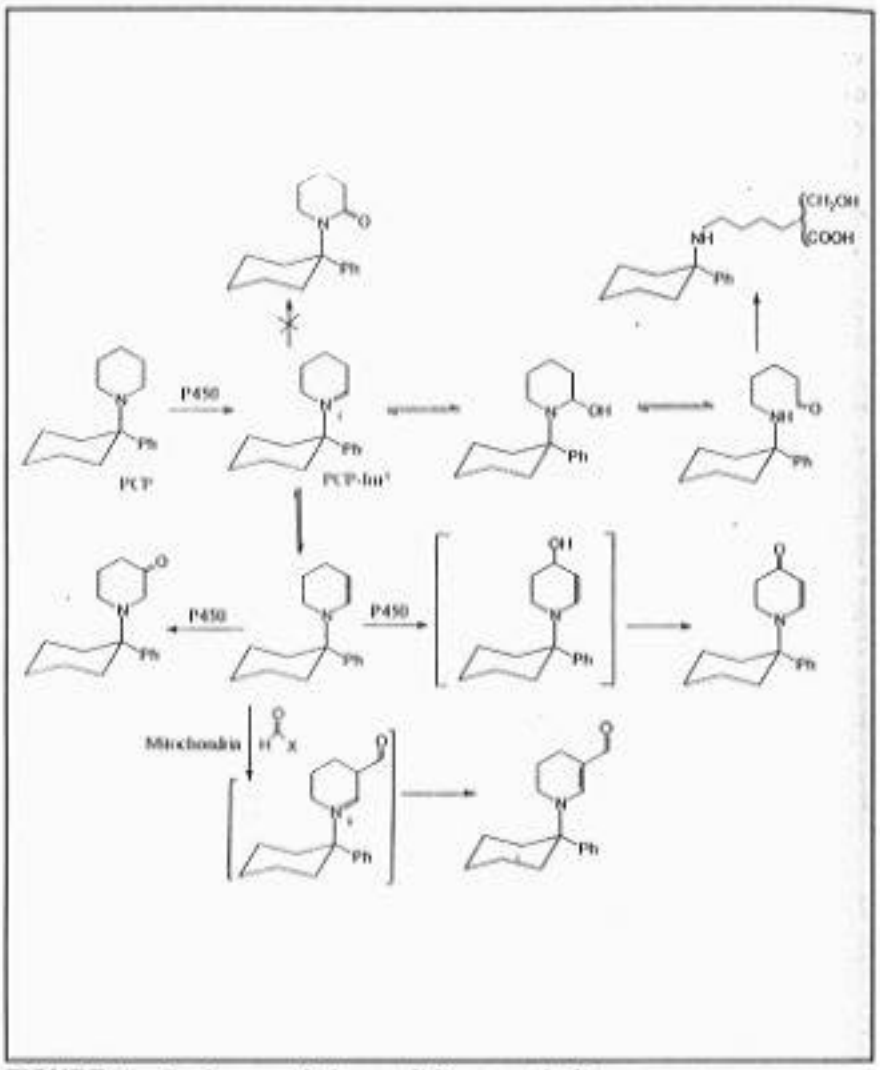
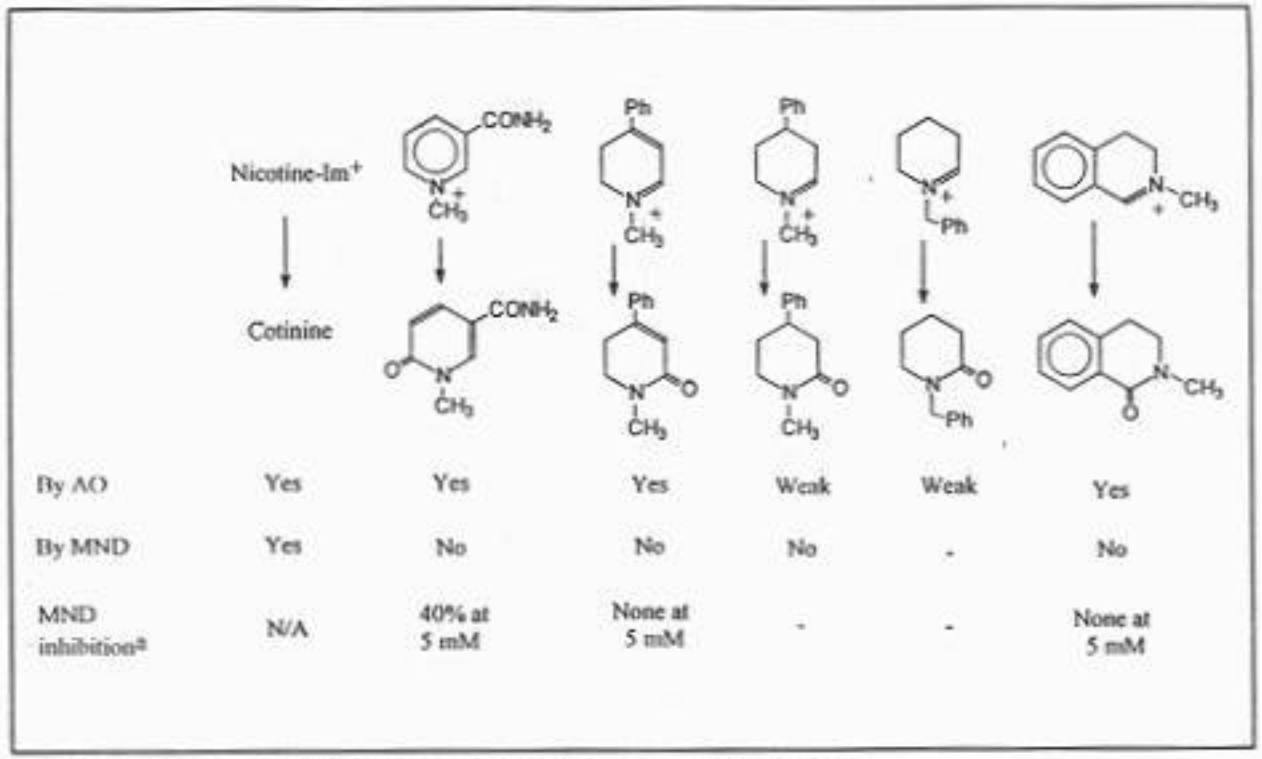


FIGURE 7. *Pathways of phencyclidine metabolism.*

In studies designed to begin characterization of MND (Flammang 1994), it was found that MND activity could be destroyed by delipidation of the microsomes (exposure to detergent), and that activity of the protein pellet could be restored by addition of dilauroyl phosphatidyl choline. Kinetic studies revealed activities associated with both a low- and high-affinity binding of NAD^+ but with a singular binding affinity of the iminium (Flammang 1994). Also, microsomes obtained from



By AO	Yes	Yes	Yes	Weak	Weak	Yes
By MND	Yes	No	No	No	-	No
MND inhibition[a]	N/A	40% at 5 mM	None at 5 mM	-	-	None at 5 mM

TABLE 3. *Conversion of iminium species to the corresponding lactams.*

KEY: a = Inhibition of conversion of nicotine-Im$^+$ to cotinine by microsomes in the presence of NAD$^+$.

phenobarbital-induced rabbits exhibited a less efficient dehydrogenase activity, in contrast to what is observed for aldehyde oxidase-mediated cotinine formation. Clearly, much more work will be needed to elucidate the nature of MND, its endogenous substrates and physiological role, and its substrate-structure profile.

CONCLUSIONS

The most telltale data in terms of mechanism of inactivation of cytochrome P-450 by the methylated 1-BP analogs is provided by the results on ,-DMBP and 4,4-DMBP. In the first case, the fact that the parent amine and iminium intermediate exhibit the same level of enzyme inactivation provides compelling evidence for a metabolic route through the endocyclic enamine. In the case of 4,4-DMBP, one might be tempted to explain the weak inactivation by the parent amine on the basis of a low level of endocyclic iminium formation. However, the independently prepared 4,4-DMBP-Im^+ is itself a weak inactivator. The decreased inactivation seen for 4,4-DMBP-Im^+ can be interpreted in terms of figure 6, wherein 4,4-dimethyl substitution would preclude functionalization at C-4 and would also sterically hinder enzymatic oxygenation at C-3. The fact that 4,4-DMBP-Im^+ still exhibits some inactivation is thus more consistent with C-3 oxygenation being the pathway leading to inactivation. This is also compatible with the decreased inactivation caused by the C-2 methyl group in 2-MBP-Im^+ and 2,6-DMBP-Im^+. Thus, although one cannot deduce at this time the nature of the reactive species generated, cytochrome P-450 oxygenation at C-3 of the enamine appears to be the most reasonable path leading to enzyme inactivation.

The significance of the covalent binding that accompanies the metabolism of nicotine and PCP is uncertain. Although there is presently no direct evidence for acute toxic effects specifically associated with such covalent binding, a role in the toxicologic effects of these or related drugs cannot be excluded at present. Any drug in clinical use has a long list of contra-indications. For any given drug, there is always a percentage of users whose individual biochemical metabolism and/or other drug use creates a situation that causes an adverse response to the new drug. The reasons for this adverse reaction are often not apparent. If drugs with associated covalent binding potential inactivate a sufficiently high percentage of a particular metabolic isozyme, then adverse drug interaction scenarios

may arise. This possibility has been recognized recently in the case of PCP (Owens et al. 1993).
A key focus of this chapter has been on the iminium/enamine stage of metabolism (figure 1) as the critical point governing the balance between toxic activation and detoxication pathways. Thus, although AO and MND efficiently intercept the $^{1.(5.)}$-iminium derived from nicotine, PCP-Im^+ is not a substrate for either enzyme. It seems quite significant that the lack of aldehyde oxidase substrate potential in this case correlates with increased levels of covalent binding of PCP relative to nicotine.

The balance between toxication and detoxication may also vary considerably between different tissues. For example, whereas microsomal rates of conversion of nicotine to the $^{1.(5.)}$-iminium ion for rabbit liver and lung are comparable on a per weight basis, nicotine is oxidized at a higher rate by lung microsomes than by liver microsomes when one corrects for the much smaller P-450 content of lung microsomal protein (McCoy, unpublished data). The higher rate of nicotine metabolism in the rabbit lung results from the enrichment in P-450 isozymes which preferentially metabolize nicotine to the $^{1.(5.)}$-iminium ion, for example, CYP2B4 and CYP4B1, which account for 90 percent of lung P-450 content (Serabjit-Singh et al. 1979) but less than 20 percent of the liver total P-450 content (Lu and West 1980). Further studies (Flammang 1994) demonstrate very low aldehyde oxidase activity in rabbit lung compared to liver, and also very low MND activity in lung compared to liver, as also reported by Obach and Van Vunakis (1990). Thus because of the high nicotine oxidation rate and the poor iminiumøcotinine activity in lung tissue, the potential exists for the intracellular concentration of nicotine $^{1.(5.)}$-iminium to be much greater in lung than in liver. Covalent binding to tissue macro-molecules (Williams et al. 1990) resulting from high intracellular concentrations of the $^{1.(5.)}$-iminium or its metabolites may thus be especially problematic in the lung.

Many naturally occurring and synthetic drugs directed at biogenic amine receptors contain a basic tertiary amine center. It is common practice in the development of new central nervous system (CNS)-active pharma-ceuticals to incorporate pyrrolidino, piperidino, or morpholino moieties (in addition to dimethylamino) in structure-activity investigations. A significant number of cyclic tertiary amine drugs have thus appeared (and continue to appear) in the marketplace despite the fact that this structural construct appears to be especially

prone to generating reactive intermediates during oxidative metabolism.

In some instances, avoiding reactive intermediate generation in the first place may be possible. If one has the choice between two pharmacologically equivalent drug analogs, only one of which has a covalent binding potential, it is clear which analog would be more desirable. The work summarized here was aimed at elucidating any patterns that might permit strategic elimination of metabolism-dependent covalent binding potential. It may be a simple matter of using 4,4-dimethylpiperidino instead of piperidino, for example, as long as the desired biological activity is not compromised. Information leading to prevention of potential toxicity should be quite welcome.

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Neurotoxicity of Amphetamines and Their Metabolites

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When amphetamine or an analog is administered in repeated high doses, neurochemical deficits in both the dopaminergic and serotonergic systems of selected areas of the brain are observed. Methamphetamine (10 to 15 milligrams per kilogram (mg/kg)), administered every 6 hours for 5 doses, decreases rat tyrosine hydroxylase (TH) activity and dopamine (DA) content in the neostriatum within 18 hours after the first dose (Koda and Gibb 1973). Interestingly, these neurochemical deficits persist in rats and nonhuman primates for extended periods of time after the drug is discontinued (Ellison et al. 1978; Bakhit et al. 1981; Woolverton et al. 1989).

Buening and Gibb (1974) demonstrated that DA plays a critical role in the neurochemical deficits observed after methamphetamine administration. The DA antagonists chlorpromazine and haloperidol, when administered concurrently with methamphetamine, completely blocked the metham-phetamine-induced alterations in neostriatal TH activity and DA content. Subsequent studies (Gibb and Kogan 1979) demonstrated that when the rate-limiting enzyme TH was inhibited by administering -methyltyrosine (MT), the neurochemical deficits normally observed with methamphet- amine were prevented. Consistent with the concept that DA is necessary for the methamphetamine-induced neurochemical deficits, the neuro-chemical deficits were again observed when DA synthesis was restored by administering L-dopa.

Not only is methamphetamine administration toxic to the dopaminergic system, but the serotonergic system in the various brain areas is also altered. Hotchkiss and Gibb (1980) reported that methamphetamine, administered as described above, decreased tryptophan hydroxylase (TPH) activity in the serotonergic nerve terminal of rat brain and spinal cord. Similarly, the content of 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindole acetic acid (5-HIAA) were also severely depressed. In contrast to the effects in the dopaminergic system, these serotonergic parameters were decreased by methamphetamine within 15 minutes after a single dose

(Bakhit and Gibb 1981) and were more pronounced than the dopaminergic deficits. Like the dopaminergic changes, these serotonergic alterations persisted for extended periods of time (Bakhit et al. 1981).

Interestingly, the serotonergic changes resulting from toxic doses of meth- amphetamine are also dependent on DA. When MT was administered concurrently with methamphetamine, the decreases in TPH activity and 5-HT and 5-HIAA content were prevented (Hotchkiss and Gibb 1980; Schmidt et al. 1985). When DA synthesis was reinstated by adding L-dopa to the dosing regimen, the neurochemical deficits were again observed. Further evidence for DA dependence was reported by Johnson and colleagues (1987), who destroyed the dopaminergic nerve terminals in the neostriatum by injecting 6-hydroxydopamine (6-OHDA) into the substantia nigra. Eleven days later the usual neurotoxic dosage regimen of metham- phetamine was administered and TPH activity in the neostriatum, cerebral cortex, and the hippocampus was determined. The usual decrease in TPH activity was observed in the cerebral cortex and hippocampus; however, in the neostriatum, which was deprived of dopaminergic input by prior administration of 6-OHDA, the deficit of TPH usually observed with methamphetamine was significantly attenuated.

The pronounced neurochemical deficits in the dopaminergic and serotonergic systems posed the question of whether methamphetamine, administered in these large, repeated doses, was a general neurotoxin affecting all neurotransmitter systems. Hotchkiss and colleagues (1979) found no effects on the cholinergic system or on the glutaminergic system as defined by an absence of any alteration of the respective synthesizing enzymes of these neurotransmitter systems.

Ricaurte and colleagues (1985) demonstrated that an analog of amphetamine, 3,4-methylenedioxyamphetamine (MDA), is neurotoxic to monoamine systems. They observed that neostriatal and hippocampal 5-HT and 5-HIAA contents were depressed after MDA administration and that hippocampal norepinephrine (NE) content was also compromised. In comparing the effects of methylenedioxymethamphetamine (MDMA) and methamphetamine, it was observed (Stone et al. 1986, 1987) that while methamphetamine decreased both TH and TPH activity, MDMA depressed only TPH activity without altering the DA-synthesizing enzyme. Although MDMA releases DA (Yamamoto and Spanos

1988), this analog is selectively neurotoxic to the serotonergic system at doses that do not cause any persisting effect on the dopaminergic system.

Further studies (Stone et al. 1988) provided extensive evidence that the neurochemical deficits induced by MDMA are DA dependent. The deficits in TPH activity and content of 5-HT and 5-HIAA normally seen in MDMA-treated rats were attenuated by concurrent administration of MT; these deficits returned when L-dopa was administered concurrently with MT and MDMA. Prior treatment with 6-OHDA (described above) selectively attenuated the serotonergic deficits in the neostriatum while no protection by 6-OHDA occurred in the hippocampus or cerebral cortex. Prior depletion of DA with reserpine attenuated the neurotoxicity of MDMA. From these experiments the authors concluded that DA and/or its metabolites play a key role not only in the neurotoxicity observed with methamphetamine, but also with its methylenedioxy analogs.

Because of the apparent role for DA in the toxicity caused by methamphetamine and its analogs, the authors reasoned that DA and/or a reactive DA metabolite may be oxidizing components of the dopaminergic and serotonergic nerve terminals to cause the persisting deficits in these two neuronal systems. To test this hypothesis, TPH activity was measured in cerebral cortex from rats treated with toxic doses of methamphetamine, MDMA, or p-chloroamphetamine (Stone et al. 1989). Three hours after receiving one of the amphetamines, the rats were killed and the supernatant fraction of the cerebral cortex containing TPH was obtained. An aliquot of the supertanant was then exposed to various reducing agents in a nitrogen atmosphere for a 24-hour period. As previously observed, enzyme activity from rats treated with the amphetamine analog was markedly impaired compared to that from untreated rats. TPH activity from rats treated with the amphetamine analog was essentially normal in those samples incubated with dithiothreitol, a reducing agent, in a nitrogen atmosphere; other reducing agents were not as effective. A time-response relationship revealed that the enzyme activity could be restored only during the first 6-hour period. After 6 hours, oxidation of the enzyme had apparently progressed to a point where it was irreversible.

METABOLITES OF AMPHETAMINE

Methamphetamine administered directly into the brain is not neurotoxic (Matsuda 1987; Molliver et al. 1986; Berger et al. 1990; Paris and

Cunningham 1990). Furthermore, when methamphetamine or its analogs are incubated with brain slices or homogenates, no impairment of TH or TPH activity is observed. This finding led to an investigation of whether an amphetamine metabolite is responsible for neurotoxicity (Matsuda et al. 1989). Ketamine-anesthetized rats received bilateral injections of either p-hydroxyamphetamine (pOHA) or p-hydroxynorephedrine (pOHN) at doses of 0.5, 5, or 50 micrograms (g) administered directly into the neostriatum. Three hours later, striatal DA and 5-HT contents were determined. The 50 g dose of pOHA or pOHN decreased the concentration of striatal DA to 27 percent and 18 percent of control, respectively. The 5-HT content was also decreased by both metabolites, but not to the same extent as DA. The DA metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) declined in parallel with DA, but 5-HIAA content was not changed by pOHA and was actually elevated after injection of pOHN. The decline in DA and its metabolites reached its nadir at 6 hours and returned to control levels by 48 hours. Surprisingly, TH activity was not altered when either pOHA or pOHN was administered intrastriatally. TPH activity was actually elevated when either of the higher doses of the two amphetamine metabolites was administered.

The effect of systemic administration of these amphetamine metabolites on the content of DA and its metabolites and on 5-HT was then examined. When pOHA was administered systemically (5, 15, or 30 mg/kg), the concentration of DA and its metabolites in the neostriatum was depressed. The effect on the neostriatal serotonergic system was not as pronounced as that on the dopaminergic system; hippocampal serotonin content was altered only at the higher dose of pOHA and hypothalamic 5-HT concentrations were decreased at both the 15 mg and 30 mg doses. The authors then determined the effect of the monoaminergic uptake inhibitors amfonelic acid and cocaine on the response to systemically administered pOHA. The decrease in DA and its metabolites was attenuated by amfonelic acid (1 mg/kg, administered 30 minutes before pOHA), but cocaine failed to alter the effects of systemic pOHA administration.

The metabolites of amphetamine, in the doses administered, decrease the concentrations of DA and its metabolites as well as 5-HT. The pronounced decrease in DA and its metabolites returned to normal within 48 hours. No decrease in either TH or TPH activity was observed concurrent with the decrease in DA. From these observations it appears that these amphetamine metabolites transiently decrease the neurotransmitter concentrations, but are not neurotoxic since the activity of the synthesizing enzymes TH and TPH are not altered.

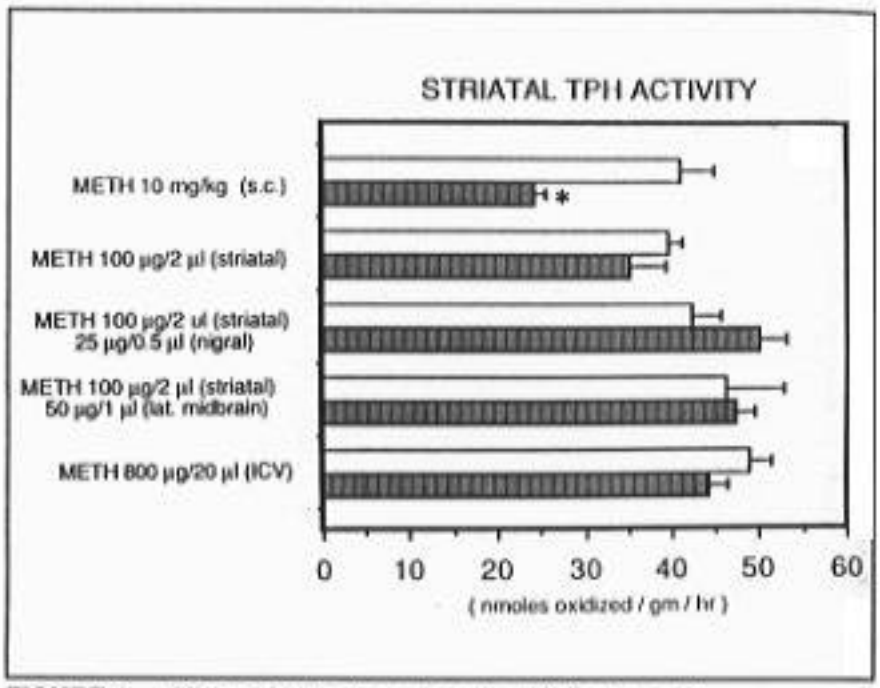

FIGURE 1. *Effects of systemic and cerebral injections of methamphetamine (METH) on neostriatal TPH activity. Rats were anesthetized with ketamine (175-225 mg/kg, IP) 30 min prior to METH treatments (both systemic and cerebral injections) and were killed 3 hr later. Regional cerebral and ICV injections were performed as described. Columns represent average striatal TPH activities (nanomoles (nmoles)/gm/hr) ± SEM (n = 3 to 8).*

KEY: □ = saline (SC)- or mannitol (cerebral injections)-treated controls; ▣ = METH-treated. * = $p < 0.05$ compared to respective controls.

The effects of cerebral injections of methamphetamine on striatal TPH activity were also investigated (figure 1). In an anesthetized rat, systemically administered methamphetamine (10 mg/kg) decreased striatal TPH activity. However, bilateral injections of methamphetamine into the neostriatum (100 g) failed to decrease striatal TPH activity 3 hours after treatment. To determine whether multiple areas of the brain are involved in methamphetamine-induced changes in striatal TPH, microinjections of methamphetamine were administered into the substantia nigra or lateral midbrain and into the striatum. Three hours after treatment, injections of methamphetamine into the striatum (100 g) and the substantia nigra (25 g) or the lateral midbrain (area of B9 serotonergic cell bodies) (50 g) did not reduce TPH activity in the neostriatum.

Similarly, in rats treated with methamphetamine (800 g intracerebroventricularly (ICV)), TPH activity was unaffected in the cerebral cortex and hypothalamus (data not shown) as well as in the neostriatum. These results suggest that direct application of methamphetamine to neural tissues has no effect on TPH activity; however, the possibility cannot be ruled out that the duration of exposure of locally injected methamphetamine, in any region, was too brief to be effective.

METABOLITES OF MDMA

As indicated above, large doses of MDMA cause profound and persisting neurochemical deficits in the serotonergic nerve terminals without any lasting effects on the dopaminergic nerve terminals. Since the parent compound is not toxic when injected directly into the brain (Molliver et al. 1986; Paris and Cunningham 1990), the possibility that one or more MDMA metabolites cause the neurotoxicity observed after administration of large doses of the drug was considered.

2,4,5-Trihydroxyamphetamine

While investigating the metabolism of MDMA, two of the authors' colleagues identified eight metabolites formed in vivo (Lim and Foltz 1991*a*, 1991*b*). The authors injected synthesized metabolites into rats to determine their possible neurotoxicity (figure 2). Rats received a single injection of either 0.25 or 0.5 micromoles (mole) of 2,4,5-trihydroxy-amphetamine (THA) ICV and were killed 7 days later (figure 3).

TPH activity was dramatically decreased in the hippocampus to 5 or 1 percent of control, respectively, with 0.25 or 0.5 moles of the metabolite. The content of 5-HT and 5-HIAA in the hippocampus was also decreased. The striatal serotonergic system was more resistant to the effects of THA. In this structure, TPH activity was decreased to 74 percent and 81 percent of control 1 week after a single injection of 0.25 or 0.5 mol of THA, while the concentration of 5-HT and 5-HIAA remained unaltered. THA administration dramatically decreased all dopaminergic parameters in the striatum.

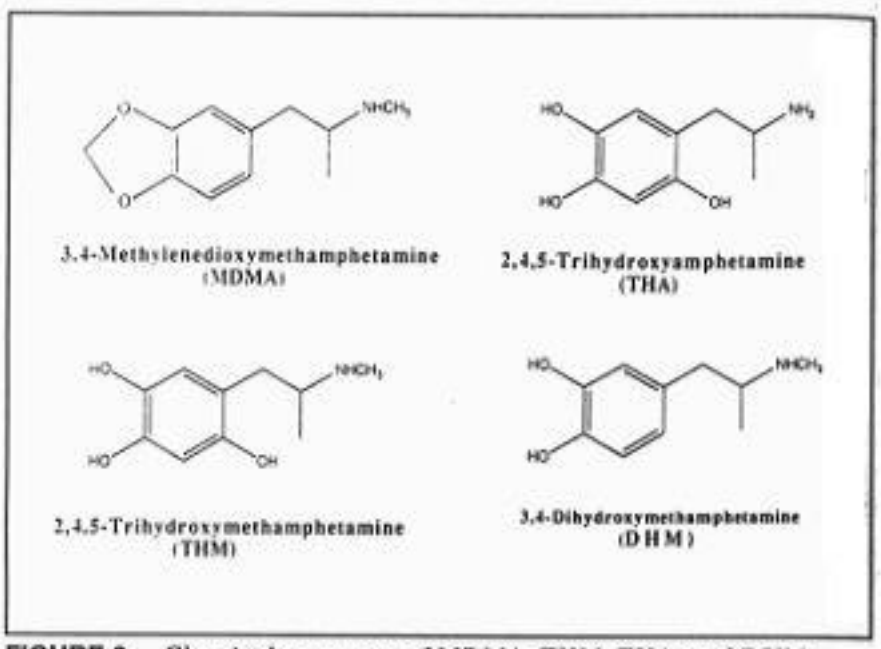

FIGURE 2. *Chemical structures of MDMA, THM, THA, and DHM.*

In response to the administration of 0.25 or 0.5 mol of THA, striatal TH was reduced to 67 and 10 percent of control, respectively (figure 4). A similar decrease in content of DA and its metabolites was observed in the striatum contralateral to the site of injection. When enzyme activity in the dopaminergic and serotonergic cell bodies from the THA-treated rats was examined, no change was observed in the medial or dorsal raphe TPH activity. However, TH activity in the substantia nigra was decreased to 59 and 20 percent of control in animals treated with 0.25 or 0.5 mol of THA, respectively. To assess the response of the noradrenergic system, hippocampal NE content was measured in the THA-treated animals. NE content was lowered to 10 and 18 percent of control after 0.25 and 0.5 mol of THA, respectively.

2,4,5-Trihydroxymethamphetamine

Corresponding experiments were conducted with another analog of MDMA, 2,4,5-trihydroxymethamphetamine (THM). A single dose of 50, 100, or 200 g was administered ICV 5 days before sacrificing the rats and TPH activity was determined in various areas of the brain (figure 5).

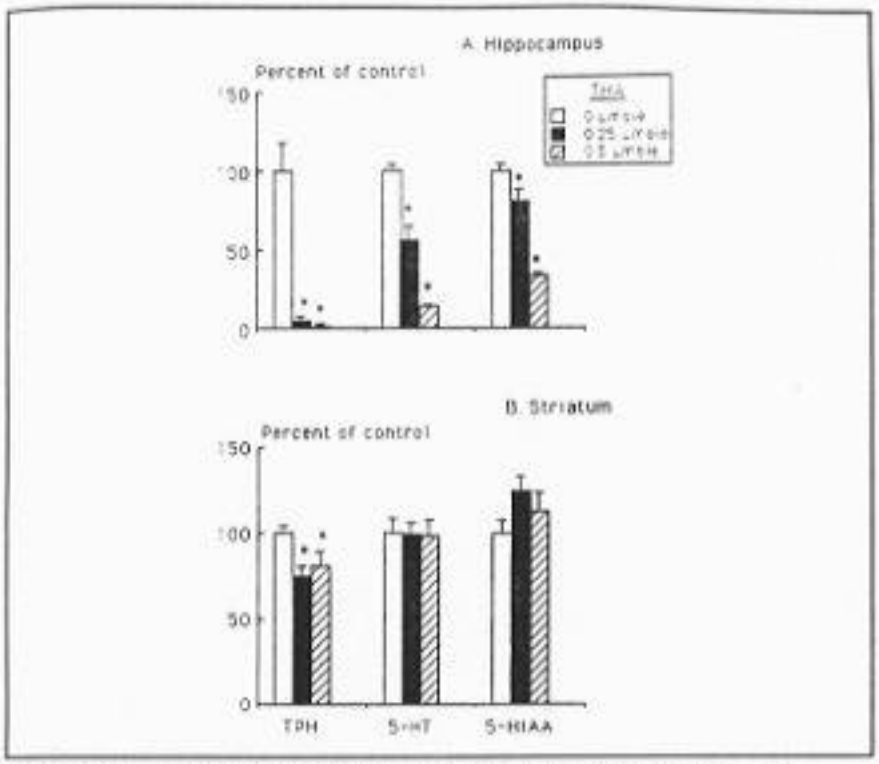

FIGURE 3. *Effect of THA on the hippocampal (A) and striatal (B) serotonergic systems. Rats received a single ICV injection of THA (0 µmol, open bar; 0.25 µmol, black bar; or 0.5 µmol, striped bar) in 20 µl of 0.1% ascorbate saline vehicle and were killed 7 days later. TPH activity was measured in the brain structure ipsilateral to the injection while 5-HT and 5-HIAA concentrations were measured in the brain structure contralateral to the injection. Means ± S.E. (n = 5-8) are expressed as a percentage of control (0 µmol). Control TPH activities, expressed in nmol of hydroxylated tryptophan per hr/g of tissue, were 49.9 ± 9.1 in the hippocampus and 120 ± 4.8 in the striatum. Control 5-HT concentrations (µg/g tissue) were 0.40 ± 0.02 in the hippocampus and 0.50 ± 0.04 in the striatum. Control 5-HIAA concentrations (µg/g tissue) were 0.30 ± 0.01 in the hippocampus and 0.40 ± 0.03 in the striatum. Statistical analysis was performed by ANOVA followed by a Fisher test.*

KEY: * = $p < 0.05$ versus control.

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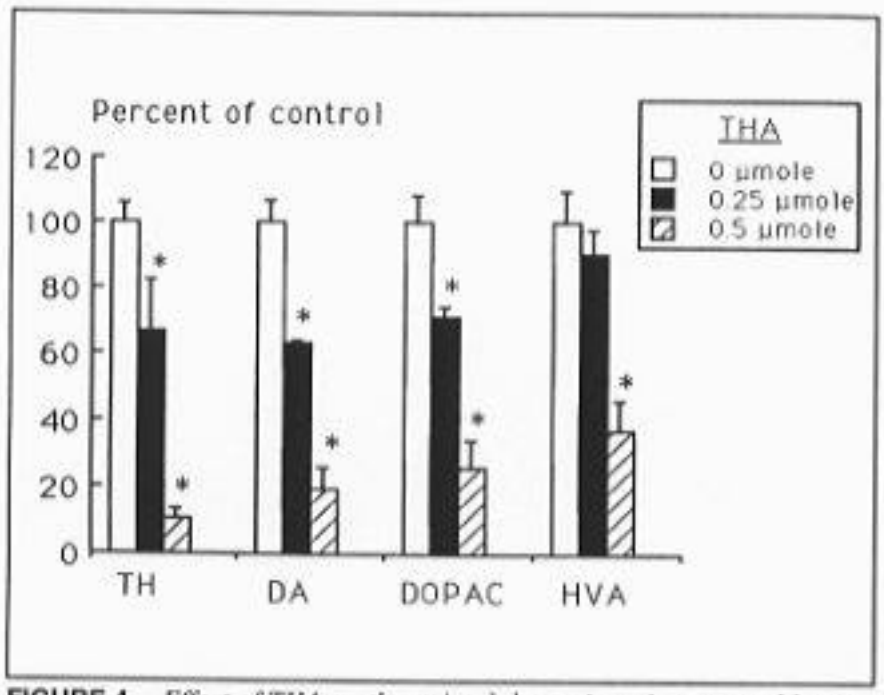

FIGURE 4. *Effect of THA on the striatal dopaminergic system. Means ± S.E. (n = 5-8) are expressed as a percentage of control (0 µmol, open bar). Control TH activity was 1.4 ± 0.1 µmol of hydroxylated tyrosine per h per g of tissue while control values were 8.0 ± 0.6 for DA, 0.60 ± 0.05 for DOPAC, and 0.50 ± 0.05 for HVA (µg/g tissue). Statistical analysis was performed by ANOVA followed by a Fisher test.*

KEY: * = $p < 0.05$ versus control.

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Of the three serotonergic terminal areas examined, TPH activity in the hippocampus was most impaired to 58 and 10 percent of control in rats treated with 50 or 200 g of THM. Enzyme activity in the frontal cortex was decreased less than that observed in the hippocampus, while the striatum was least affected. TPH activity in the cell bodies did not decrease; in fact, enzyme activity was elevated in both the medial and dorsal raphe, depending on the dose of THM. The effect of THM on 5-HT content followed a pattern similar to TPH activity in the corres-ponding brain areas, while 5-HIAA concentrations were not altered by THM in these structures.

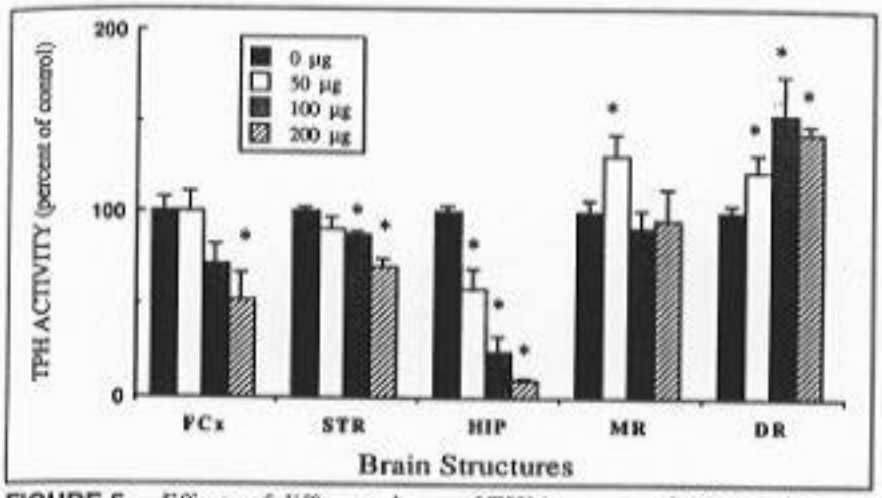

FIGURE 5. *Effects of different doses of THM on central TPH activity. The rats received one ICV injection of THM (50, 100, or 200 µg per 20 µl) or vehicle and were allowed to recover for 5 days. The results are expressed as a percentage of their respective control (means ± S.E.). The control values for TPH activity, expressed as nmol of hydroxylated tryptophan/h/g tissue, were: Fcx, 69.8 ± 5.4; STR, 109.6 ± 4.7; HIP, 143.8 ± 5.2. TPH activity in the MR and in the DR were 6 ± 0.4 and 17.3 ± 0.8 nmol of hydroxylated tryptophan/h/mg protein, respectively (n = 3-11).*

KEY: FCx = frontal cortex; STR = striatum; HIP = hippocampus; MR = median raphe; DR = dorsal raphe. * = $p < 0.05$ versus 0 µg.

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The effects of THM on the central dopaminergic system were then examined (figure 6). TH activity was markedly depressed to 76, 56, and 21 percent of control in the striatum of rats administered a single ICV injection of 50, 100, or 200 g, respectively, of THM 5 days previously. A decrease in the content of DA and its metabolites in these structures was also observed. TH activity was not altered in the substantia nigra. Another metabolite of MDMA, 3,4-dihydroxyamphetamine (DHM), was administered ICV (135 g) and the enzymatic response was examined.

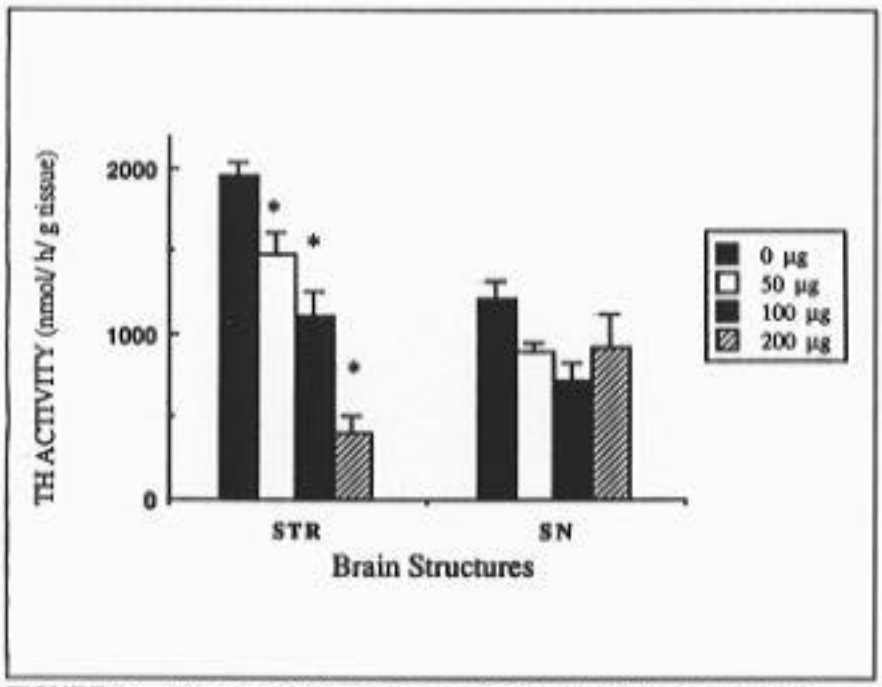

FIGURE 6. *Effects of different doses of THM on STR and substantia nigra (SN) TH activity. The animals were treated as described in figure 3. The results are expressed in nmol of hydroxylated tyrosine/h/g tissue (means ± S.E.) (n = 3-11).*

KEY: * = $p < 0.05$ versus 0 µg.

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The activity of TPH was unaltered by DHM, but TH activity was significantly elevated by this metabolite of MDMA.

SHORT-TERM EFFECTS OF MDMA METABOLITES

In the studies cited above, the authors determined the effects of MDMA metabolites 5 to 7 days after administering the drug. In other experiments (described below), rats were administered 1 mol of THA, THM, or DHM ICV and the response was determined 3 hours later. THA decreased TPH activity to 79 percent of control in the neostriatum, but in the hippocampus enzyme activity was markedly decreased to 8 percent of control. THM decreased striatal TPH activity to 87 and 54 percent of control in the striatum and hippocampus, respectively. The only effect of DHM was an elevation of enzyme activity in the striatum.

When the response of the dopaminergic system was investigated, TH activity had decreased to 75 percent of control in the striatum following THA administration, but was unaffected by THM. THA elevated neostriatal content of DA (135 percent), DOPAC (287 percent), HVA (237 percent), and 5-HT (115 percent). THM elevated DOPAC (512 percent), HVA (366 percent), and 5-HIAA (149 percent), but decreased 5-HT (74 percent) striatal content.

The authors had previously demonstrated that the decrease in enzyme activity observed in rats treated for 3 hours with MDMA was reversed by exposing the supernatant fraction containing TPH to reducing conditions (dithiothreitol in a nitrogen atmosphere) for 24 hours. Incubating hippo- campal TPH from animals treated with THM failed to prevent the decrease in enzyme activity caused by the metabolite; this finding suggests that the enzyme changes produced by the metabolite occur by a different mechanism than those caused by the parent compound.

Because THA and THM are structurally similar to 6-OHDA, it was important to ascertain whether 6-OHDA administered in a similar fashion would produce the same response. Since TPH activity from the striatum and the hippocampus was not altered by 6-OHDA after 3 hours, the DA analog is not likely to be responsible for the toxicity associated with administering THA or THM.

It is well established that 5,7-dihydroxytryptamine (DHT) is toxic to serotonergic neurons. Lim and Foltz (1991*b*) reported that THA and THM cyclize, thus forming an indole molecule that is similar in structure to DHT. The effect of DHT on hippocampal TPH activity was therefore examined (figure 7). This serotonergic neurotoxin decreased enzyme activity to 18 percent of control. When TPH was incubated under the reducing conditions described above, there was no reversal of the enzyme activity. This observation would argue against the possibility that DHT is the neurotoxin responsible for MDMA-induced neurotoxicity.

The authors then investigated the effect on hippocampal TPH activity when incubated in vitro with THA (figure 8). The hippocampus or striatum of rats was excised and prepared slices were incubated for 1 hour in buffer containing 0.001, 0.01, 0.1, 0.5, or 5.0 millimoles (mM) THA.

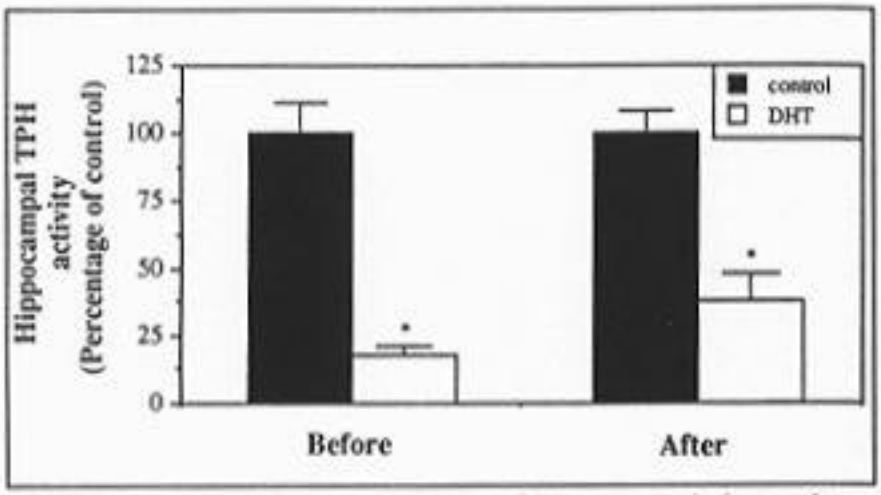

FIGURE 7. *Effect of DHT on hippocampal TPH activity before and after reducing conditions. Rats were administered 1 nmol of DHT by ICV injection and animals were sacrificed 3 hours later. Results are expressed as percentage of control. Control values, expressed as nanomoles of hydroxylated tryptophan/h/g tissue were 133.5 ± 14.5 before reducing conditions and 271.5 ± 20.4 after reducing conditions (means S.E.) (n = 5-8). TPH activity after reducing conditions was not significantly different from that before reducing conditions.*

KEY: * = $p < 0.05$

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As depicted in figure 8, TPH activity was markedly inhibited when the enzyme was incubated in vitro with THA. When the enzyme was incubated under reducing conditions, the enzyme activity was not restored to normal. As was observed in vivo, hippocampal TPH was more sensitive than striatal TPH since it was inhibited at a lower concentration of THA. The mechanism responsible for this interesting response is under investigation.

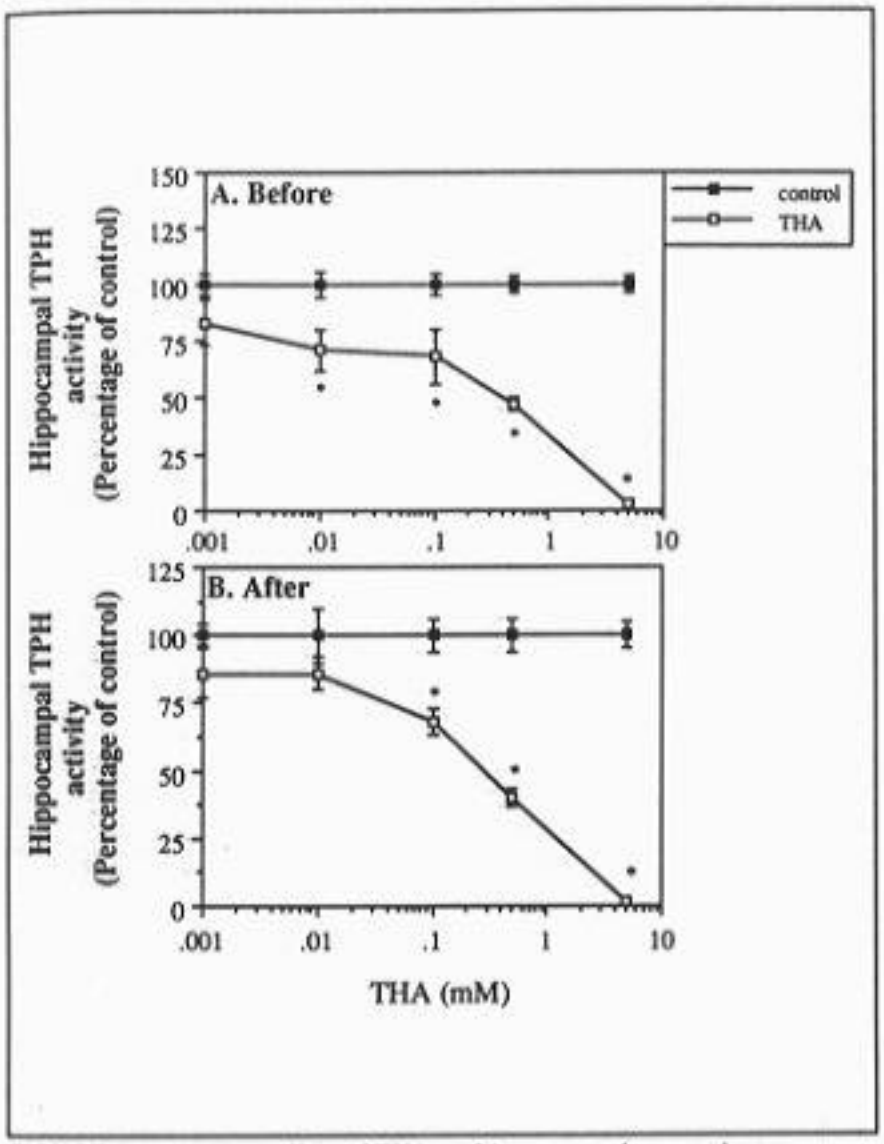

FIGURE 8. *In vitro effect of THA on hippocampal tryptophan hydroxylase activity before (A) and after (B) reducing conditions (a 20-24-h anaerobic incubation in the presence of diothiothreitol). Hippocampus was excised and incubated with 0.001, 0.01, 0.1, 0.5, or 5.0 mM THA for 1 hour under a flow of 95% O_2 and 5% CO_2. Results are expressed as percentage of control (n = 5-8).*

KEY: –■– = control; –□– = THA. * = $p < 0.05$ versus control

CONCLUSIONS

Methamphetamine, when administered in large doses, causes neurochemical deficits in both the dopaminergic and serotonergic nerve terminals of the brain that persist for extended periods of time. When the methamphetamine analog MDMA is administered, selective and persistent neurochemical deficits are observed in the serotonergic terminal regions. DA is essential for these neurochemical deficits. Reducing conditions reverse these amphetamine-induced changes, providing evidence that oxidative stress is an important component in causing the neurochemical deficits.

The role of metabolites of amphetamine or MDMA has been extensively explored. Although some of the responses to these metabolites are similar to those of the parent compounds, the neurochemical profile observed with the metabolites is distinctly different from the deficits induced by parent compounds. From these observations it appears that there are additional factors responsible for the neurochemical deficits caused by amphetamine and its congeners.

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Potential New Insights Into the Molecular Mechanisms of Methamphetamine-Induced Neurodegeneration

Monika Z. Wrona, Zhaoliang Yang, Fa Zhang, and Glenn Dryhurst

INTRODUCTION

In humans, methamphetamine evokes alertness, a decreased sense of fatigue, increased ability to concentrate or perform physical tasks, euphoria, and increased initiative and confidence (McGeer et al. 1987). Probably because of its mood-elevating and antifatigue effects, methamphetamine is self-administered by humans and a number of animal species in experimental models (Schuster 1981). Methamphet-amine is recognized as a positively reinforcing drug, and it continues to be extensively abused in many countries. However, high doses or continuous use of methamphetamine can lead to a variety of undesirable side effects. These include paranoid delusions, disordered thought, aggression, and hallucinations (Seiden et al. 1988). Neurotoxicological studies have established that certain dose regimens of methamphetamine evoke the degeneration of dopaminergic and serotonergic fibers in the brains of many animal species including the monkey (Seiden et al. 1975/1976), rats, mice, and cats (Ricaurte et al. 1980; Wagner et al. 1980; Levine et al. 1980; Seiden and Ricaurte 1987). These observations suggests that methamphetamine might also evoke similar neurodegenerative effects in the human brain.

For a variety of reasons, most studies aimed at understanding the neurodegenerative effects of methamphetamine have employed the rat. Repeated low doses or a single large dose of methamphetamine to this animal results in the degeneration of serotonergic (Ricaurte et al. 1980; Seiden et al. 1988; Bakhit and Gibb 1981; Hotchkiss and Gibb 1980*a*, 1980*b*; Commins and Seiden 1986) and dopaminergic (Seiden et al. 1988; Ricaurte et al. 1982; Wagner et al. 1980; Fibiger and McGeer 1971; Buening and Gibb 1974) nerve terminals in several areas of the brain and of a subpopulation of cell bodies in the somatosensory cortex (Commins and Seiden 1986). The latter region of the rat cortex contains no serotonergic or catecholaminergic perikarya and hence these cell bodies must be associated with another,

as yet unknown, neurotransmitter system. Based on measurements of residual levels of 5-hydroxytryptamine (5-HT) and dopamine (DA) in many brain areas following methamphet-amine administration, it appears that the most profound degeneration of serotonergic terminals occurs in the frontal cortex, hippocampus, and amygdala, whereas dopaminergic terminals are most severely degenerated in the caudate nucleus, amygdala, and nucleus accumbens (Seiden et al. 1988). Such studies have also suggested that serotonergic terminal fields are more susceptible than dopaminergic terminal fields to the neurotoxic effects of methamphetamine. Furthermore, for the various brain areas examined, it appears that depletions of DA and 5-HT are proportional. For example, if DA is not depleted then 5-HT is depleted by only a small amount; when significant dopaminergic degeneration occurs, serotonergic degeneration is even more pronounced (Seiden et al. 1988). These observations could imply that the methamphetamine-induced degeneration of dopaminergic and serotonergic terminals are not entirely independent processes, but are in some way related or connected phenomena.

The fundamental molecular mechanisms that underlie the neurodegenera- tive properties of methamphetamine are unknown, although they are probably not directly caused by the drug or its normal metabolites (Gibb et al. 1994). However, several lines of evidence suggest that oxygen radical species contribute directly or indirectly to methamphetamine-induced neuronal damage (Cadet et al. 1994). For example, pretreatment of rats with antioxidants attenuates the neurodegenerative effects of methamphetamine (DeVito and Wagner 1989). It seems to be widely accepted that oxygen radical species limit their damage in biological systems to lipids, proteins, and deoxyribonucleic acid (DNA) (Halliwell 1992). However, the two neuronal systems severely damaged as a result of methamphetamine administration utilize neurotransmitters that are very easily oxidized (i.e., 5-HT and DA). Thus, it appears unlikely that these neurotransmitters would be spared from oxygen radical-mediated oxidation reactions unless there are very sophisticated mechanisms available for their protection. Support for this view can be drawn from the observation that, following the administration of a single large dose of methamphetamine to the rat, the catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) has been detected in the caudate nucleus (Seiden and Vosmer 1984) and the serotonergic neurotoxin 5,6-dihy-droxytryptamine (5,6-DHT) has been detected in the cortex and hippocampus (Commins et al. 1987).

The only known direct chemical pathway leading to 6-OHDA and 5,6-DHT is attack of the hydroxyl radical (HO·) on DA (Slivka and Cohen 1987) and 5-HT (Wrona et al. 1995), respectively. The detection of 6-OHDA (Seiden and Vosmer 1984) and 5,6-DHT (Commins et al. 1987) in rat brain following methamphetamine administration has been reported to be somewhat sporadic and the concentrations measured extremely low. Indeed, several investigators have been unable to detect 6-OHDA in rat brain following methamphetamine administration (Rollema et al. 1986; Evans and Cohen 1989; Karoum et al. 1993). Nevertheless, a hypothesis has been advanced that under conditions of massive methamphetamine-induced release of DA and 5-HT and monoamine oxidase (MAO)-A and MAO-B inhibition (Suzuki et al. 1980), these neurotransmitters are non-enzymatically oxidized in the synaptic cleft to give 6-OHDA and 5,6-DHT, respectively (Seiden et al. 1988; Seiden and Vosmer 1984; Commins et al. 1987). Subsequent carrier-mediated uptake of 6-OHDA and 5,6-DHT into dopaminergic and serotonergic terminals, respectively, have been proposed to lead to the degeneration of these neurons. Some support for this hypothesis is provided by the fact that selective DA and 5-HT uptake inhibitors protect dopaminergic and serotonergic terminals, respectively, against metham-phetamine-induced damage (Marek et al. 1990*a*; Ricaurte et al. 1983).

On the assumption that methamphetamine in some way evokes aberrant oxidation of DA to 6-OHDA and 5-HT to 5,6-DHT, it is of interest to consider whether such reactions would occur within the cytoplasm of nerve terminals rather than in the synaptic cleft. In the dopaminergic system methamphetamine participates in an exchange-diffusion process (Raitiri et al. 1979) and displaces DA from cytoplasmic storage sites, presumably resulting in elevated levels of free or unbound neurotrans-mitter prior to its massive efflux through the uptake carrier site (Marek et al. 1990*a*; Liang and Rutledge 1982). Elevated cytoplasmic concen- trations of DA appear to expose this neurotransmitter to oxidation. To illustrate, pretreatment of rats with pargyline (which increases the cytoplasmic pool of DA) followed by methamphetamine results in increased levels of 6-OHDA in the striatum compared to those observed in animals not treated with this MAO-A and MAO-B inhibitor (Marek et al. 1990*c*). Similarly reserpine, which disrupts catecholamine storage vesicles and increases cytoplasmic levels of DA, potentiates metham-phetamine-induced damage to dopaminergic terminals (Wagner et al. 1983). Indeed, treatment of guinea pigs with reserpine alone results in a marked elevation of striatal levels of 5-*S*-cysteinyldopamine, indicative of increased oxidation of cytoplasmic DA (Fornstedt and Carlsson

1989). In contrast, the catecholamine synthesis inhibitor -methyl-*p*-tyrosine (MpT), which depletes the cytoplasmic pool of DA, attenuates methamphetamine-induced damage to dopaminergic terminals (Wagner et al. 1983) and decreases 6-OHDA formation (Axt et al. 1990). Reinstating DA synthesis by administration of L-dopa reverses the protective effects of MpT against methamphetamine-induced damage to dopaminergic terminals (Schmidt et al. 1985). However, administration of L-dopa without methamphetamine does not appear to cause any neurodegenerative effects, indicating that DA is not a neurotoxic agent (Schmidt et al. 1985).

Taken together, these lines of evidence tend to support the idea that elevated cytoplasmic concentrations of DA might favor the intraneuronal oxidation of this neurotransmitter and that such reactions are linked to the degeneration of dopaminergic terminals. The influence of manipulations of the cytoplasmic pool of 5-HT on the methamphetamine-induced oxidation of 5-HT to 5,6-DHT have not been studied. However, in view of the many apparent similarities between the neurodegenerative and other biochemical effects of methamphetamine on dopaminergic and serotonergic terminals, it might also be concluded that oxidation of 5-HT to 5,6-DHT occurs intraneuronally.

The hypothesis that 6-OHDA and 5,6-DHT are responsible for mediating the methamphetamine-induced degeneration of dopaminergic and serotonergic terminals, respectively, has been challenged on the basis of the sporadic detection and exceedingly low levels of these neurotoxins as measured in the brain (Evans and Cohen 1989). However, in the event that these neurotoxins are formed only at, or in, axon terminals, it is not inconceivable that they might transiently reach lethal localized concen-trations. Furthermore, 6-OHDA and 5,6-DHT are believed to be neuro-toxic because of their facile intraneuronal autoxidation to cytotoxic products and byproducts. Thus, the highly localized sites of formation of 6-OHDA and 5,6-DHT and their chemical instability would be expected to make the detection of these neurotoxins in the brain a significant analytical challenge.

Inhibition of DA synthesis by the tyrosine hydroxylase inhibitor MpT attenuates not only 6-OHDA formation and degeneration of striatal dopaminergic terminals, but also 5,6-DHT formation and the degeneration of serotonergic terminals and cell bodies of unknown neurotransmitter content in the somatosensory cortex (Commins and

Seiden 1986; Axt et al. 1990). These observations suggest that either DA and/or an aberrant metabolite of this neurotransmitter might play a role in the processes that result in the degeneration of both dopaminergic and serotonergic terminals and certain cell bodies in the somatosensory cortex. Selective DA uptake inhibitors such as amfolenic acid (AFA) protect striatal dopaminergic terminals against methamphetamine-induced damage (Marek et al. 1990*a*) but do not block the release of DA or 6-OHDA formation (Marek et al. 1990*b*). However, AFA does not protect striatal serotonergic terminals against methamphetamine-induced damage (Marek et al. 1990*a*).

Significantly, AFA prevents the degeneration of striatal dopaminergic terminals when administered as late as 8 hours after methamphetamine (Marek et al. 1990*a*). Selective 5-HT uptake inhibitors such as fluoxetine protect serotonergic terminals against methamphetamine-induced damage but exacerbate damage to striatal dopaminergic terminals (Ricaurte et al. 1983). Taken together, these results suggest a number of possible conclusions:

1. Methamphetamine evokes an initial increase in the cytoplasmic concentrations of free or unbound DA and 5-HT prior to their massive release;

2. Increased cytoplasmic levels of DA and 5-HT in some way permit oxidation of these neurotransmitters as evidenced by the formation of 6-OHDA, 5-*S*-cysteinyldopamine, and 5,6-DHT;

3. The toxic effects of methamphetamine toward dopaminergic and serotonergic terminals require intact and functioning neurotransmitter uptake systems;

4. DA or a toxic DA metabolite might contribute to the degeneration of both dopaminergic and serotonergic nerve terminals; and,

5. A toxic metabolite of 5-HT might contribute to the degeneration of serotonergic and dopaminergic terminals and certain cell bodies in the somatosensory cortex (there is virtually no dopaminergic input into this region of the cortex) (Lindvall and Björklund 1978).

The latter two conclusions imply that toxic aberrant metabolites of DA and 5-HT are transferred between connected dopaminergic and seroto-nergic terminals and between serotonergic terminals and

connected cell bodies in the somatosensory cortex. However, while 6-OHDA and 5,6-DHT might in principal contribute to the degeneration of dopa-minergic and serotonergic terminals, respectively, it is not likely that 6-OHDA contributes to the degeneration of serotonergic terminals or that 5,6-DHT contributes to the degeneration of dopaminergic terminals or neuronal perikarya in the somatosensory cortex. This is so because 6-OHDA (Johnsson et al. 1975) and 5,6-DHT (Baumgarten and Lachenmeyer 1972) are selective catecholaminergic and serotonergic neurotoxins, respectively, except when present in the brain in very high concentrations. Indeed, it might be argued that 6-OHDA may not be responsible for the degeneration of dopaminergic terminals because this neurotoxin cannot be detected in rat brain after methamphetamine administration at times when AFA is able to block the neurodegenerative process (Marek et al. 1990*b*).

It is possible that toxic metabolites of DA and 5-HT other than, or in addition to, 6-OHDA and 5,6-DHT might contribute to the neuronal degeneration evoked by methamphetamine. Several lines of experimental evidence provide some support for this possibility. For example, the methamphetamine-induced degeneration of dopaminergic and serotonergic terminals is significantly attenuated by N-methyl-D-aspartate (NMDA) receptor antagonists (Sonsalla et al. 1991; Farfel et al. 1992). However, there is no evidence available to suggest that 6-OHDA, 5,6-DHT, or methamphetamine are NMDA receptor agonists. Some evidence has been presented that methamphetamine evokes elevated release of glutamate in the striatum of rats (Sonsalla et al. 1991; Nash and Yamamoto 1992) that might result in enhanced activation of NMDA receptors located on dopaminergic and serotonergic terminals and resultant excitotoxic damage (Choi 1987; Lafon-Cazal et al. 1993). However, there are relatively few NMDA receptors located on these terminals in the striatum, whereas gamma aminobutyric acid (GABA), cholinergic, and substance P cell bodies in this structure have high densities of these receptors (Coyle and Schwarz 1976; Sonsalla et al. 1991). Thus, if methamphetamine evokes widespread release of glutamate from glutamatergic terminals throughout the striatum, activation of NMDA receptors and excitotoxic damage would be expected to cause serious damage to GABAergic, cholinergic, and substance P cell bodies. However, these neurons suffer no long-lasting damage following methamphetamine administration (Hotchkiss et al. 1979; Sonsalla et al. 1986).

As noted by Sonsalla and colleagues (1991), if dopaminergic and serotonergic terminal damage induced by methamphetamine is due to the direct effects of glutamate on NMDA receptors, it would clearly have to be a very discrete and highly localized action. In principle, such a localized action could be caused by aberrant metabolites of DA and/or 5-HT formed at dopaminergic or serotonergic terminals, respectively, that are either potent NMDA receptor agonists or in some way potentiate the release of glutamate from connected glutamatergic terminals. A possible clue to the mechanism that might evoke the latter process may be drawn from the observation that the methamphetamine-induced damage to dopaminergic terminals is blocked by coadministration of pharmacologic agents that elevate extraneuronal levels of GABA (Hotchkiss and Gibb 1980*a*).

This finding suggests that interactions of GABA with GABA receptors can block the neurotoxic effects of methamphetamine. However, there are no known interactions between 6-OHDA, 5,6-DHT, or methamphet-amine and GABA receptors. Interestingly, $GABA_B$ receptors are located both on nerve terminals and at postsynaptic sites in many brain regions (Bowery 1989). Activation of presynaptic $GABA_B$ receptors by GABA reduces the evoked release of biogenic amine, excitatory amino acid (including glutamate), and neuropeptide neurotransmitters by blockade of calcium channels (Bowery 1989; Lev-Tov et al. 1988). Conversely, therefore, a $GABA_B$ inverse receptor agonist would be expected to potentiate the opening of the calcium channel and evoke elevated release of neurotransmitters. Following methamphetamine administration, formation of an aberrant metabolite of DA and/or 5-HT that was a potent $GABA_B$ receptor inverse agonist at dopaminergic and/or serotonergic terminals could, in principal, account for the release of glutamate from anatomically connected glutamatergic terminals leading to very localized NMDA receptor-mediated excitotoxicity and neuronal damage.

Available evidence thus suggests that methamphetamine evokes aberrant oxidations of DA to 6-OHDA and 5-HT to 5,6-DHT, probably in the cytoplasm of dopaminergic and serotonergic terminals, respectively. While these two neurotoxins might contribute to the degeneration of the nerve terminals where they are formed, it is not likely that they are involved with neurodegenerative mechanisms mediated by NMDA or GABA receptors. The influence of MpT and selective DA uptake inhibitors on the neurodegenerative effects of methamphetamine tend to support the notion that aberrant metabolites derived from this neurotrans-mitter

(other than or in addition to 6-OHDA) not only contribute to the degeneration of dopaminergic terminals, but also play a role in the degeneration of connected serotonergic terminals. Similarly, the effects of selective 5-HT uptake inhibitors suggest that aberrant metabolites of 5-HT (other than or in addition to 5,6-DHT) might contribute to the degeneration of serotonergic terminals, connected dopaminergic terminals, and certain cell bodies in the somatosensory cortex. These putative aberrant metabolites of DA and/or 5-HT might include compounds that either activate NMDA receptors and evoke excitotoxic damage or facilitate the release of glutamate, but only in the immediate vicinity of their sites of formation at dopaminergic and/or serotonergic terminals.

In the event that aberrant metabolites of DA and 5-HT do play roles in mediating the neurodegenerative effects of methamphetamine, it becomes of key interest to know the identities of the compounds and mechanisms that might be responsible for their formation. Extensive studies (Seiden et al. 1988; Seiden and Vosmer 1984; Commins et al. 1987; Marek et al. 1990*a*, 1990*b*; Axt et al. 1990) indicate that methamphetamine evokes oxidation of DA to 6-OHDA and 5-HT to 5,6-DHT. However, the autoxidation (i.e., oxidation by molecular oxygen in the absence of enzyme catalysis) of DA (Graham 1978) and the electrochemically driven oxidation (Zhang and Dryhurst 1993) of this neurotransmitter in aqueous solution at physiological pH do not give 6-OHDA as a detectable product. Similarly, the autoxidation (Wrona et al. 1992) and enzyme-mediated (Wrona and Dryhurst 1991) and electrochemical (Wrona and Dryhurst 1990) oxidations of 5-HT at physiological pH do not give 5,6-DHT. However, HO· attack on DA does give 6-OHDA, although in much lower yields than 2-OHDA and 5-OHDA (Slivka and Cohen 1987). The latter observation, therefore, might indicate that 6-OHDA and 5,6-DHT are marker molecules for the methamphetamine-induced HO· oxidation of DA and 5-HT, respectively. Indeed, there is good—albeit indirect—evidence that methamphetamine evokes formation of oxygen radicals in the brain (DeVito and Wagner 1989; Cadet et al. 1994). However, remarkably little is known about the HO·-mediated oxidations of DA and 5-HT, the products (i.e., putative aberrant oxidative metabo-lites) that are likely to be formed in vivo, and the neurobiological properties of these products. Accordingly, the authors have recently initiated studies aimed at elucidating the oxidation chemistry of 5-HT and DA, including that mediated by HO·, and assessing the neurobiological activities of the resulting putative metabolites and their possible roles in methamphetamine-evoked neurodegenerative processes and other neurodegenerative brain disorders.

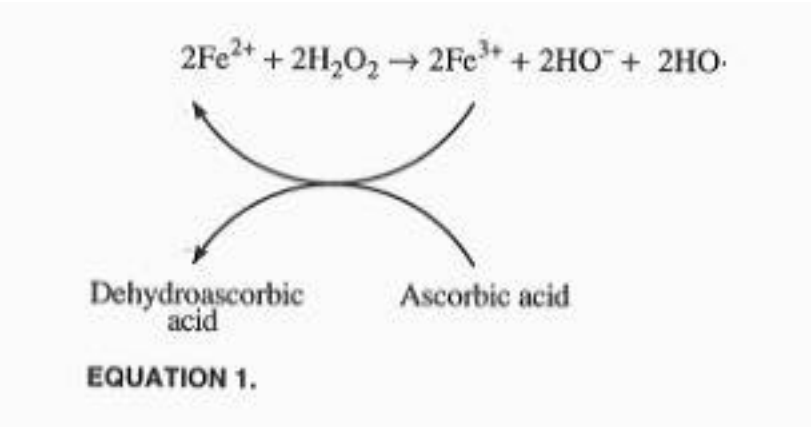
OXIDATION CHEMISTRY OF 5-HT AND DA

Location is a central question relevant to studies of the oxidation reactions of 5-HT and DA and the role of such reactions (and resulting products) in mediating methamphetamine's neurodegenerative properties. This question is important because the course of these reactions and the putative aberrant oxidative metabolites formed are likely to be strongly dependent on whether they occur in the synaptic cleft (Seiden et al. 1988; Seiden and Vosmer 1984; Commins et al. 1987; Marek et al. 1990*c*) or intraneuronally. For example, the cytoplasm of dopaminergic and serotonergic nerve terminals contain relatively high concentrations of ascorbic acid (AA) (Spector and Eells 1984), glutathione (GSH), L-cysteine (CySH) (Slivka et al. 1987), hydrogen peroxide (H_2O_2, a byproduct of many intraneuronal metabolic processes), molecular oxygen, and traces of low molecular weight Fe^{2+} species (Halliwell 1992). By contrast, extraneuronal levels of AA (Spector and Eells 1984), GSH, and CySH (Slivka et al. 1987), for example, are probably appreciably lower than cytoplasmic concentrations.

A widely used HO·-generating system consists of AA, traces of Fe^{2+}-ethylenediaminetetraacetic acid (EDTA), H_2O_2, and molecular oxygen (Udenfriend et al. 1954). In this medium, HO· is formed by decomposition of H_2O_2 by the Fenton reaction (Walling 1975) and the resulting Fe^{3+} is reduced back to Fe^{2+} by AA (equation 1). Thus, as long as AA and H_2O_2 are available, HO· is formed by this cyclic reaction. When incubated with this HO·-generating system, 5-HT is oxidized extremely rapidly (Wrona et al. 1995). The initial step in the reaction involves HO· attack on 5-HT to give the 4,5-(**1**), 2,5-(**2**) (1) and 5,6-(**3**)-dihydroxytryptamine radicals, which are then oxidized by a second molecule of HO· to give 2,5-DHT, 4,5-DHT, and 5,6-DHT in approximate yields of 60 percent, 24 percent, and 11 percent, respectively (figure 1). However, 4,5-DHT is not an isolatable product owing to its very facile oxidation by molecular oxygen to give tryptamine-4,5-dione (T-4,5-D) in a reaction that forms H_2O_2 as a byproduct (Wong and Dryhurst 1990). Furthermore, 2,5-DHT exists predominantly in solution at pH 7.4 as its 2-keto tautomer, 5-hydroxy-3-ethylamino-2-oxindole (5-HEO). The latter compound readily deprotonates to give the C(3)-centered carbanion **4** which attacks T-4,5-D to give dimer **5**. Autoxidation of the 4,5-DHT residue of **5** then yields **6**, forming H_2O_2 as a byproduct. Dimer **6** undergoes a slow intramolecular cyclization reaction to give the pyrrolo[2,3-*f*]quinoline **7**, in which the 4,5-DHT residue is autoxidized

to *o*-quinone **8**, again forming H_2O_2 as a byproduct. A relatively minor amount of T-4,5-D also dimerizes to give the 7,7.-linked dimer **9**, which is immediately autoxidized to 7,7.-D forming H_2O_2 as a byproduct. Because of its reactions with 5-HEO and its dimerization to 7,7.-D, T-4,5-D is not observed as a product of the HO·-mediated oxidation of 5-HT. 5,6-DHT is clearly formed as a result of oxidation of 5-HT by HO·, but it represents only a rather minor product of this reaction. Although 5,6-DHT can be oxidized by molecular oxygen to give *o*-quinone **10**, which subsequently polymerizes to indolic melanin, this is a relatively slow reaction in vitro (Singh and Dryhurst 1990). Furthermore, detection of this neurotoxin as a product of the HO·-mediated oxidation of 5-HT presumably reflects the reduction of *o*-quinone **10** by AA.

The rate of the reaction shown in figure 1 is dependent on the concen-tration of Fe^{2+} in the reaction medium. However, even in the absence of added Fe^{2+}, 5-HEO **6**, and 5,6-DHT are observed as products after several



EQUATION 1.

minutes, indicating that submicromolar concentrations of iron and/or other transition metal ions that always contaminate buffer constituents are sufficient to catalyze the decomposition of H_2O_2 to HO·. Similarly, in the presence of Fe^{2+} but without added H_2O_2, 5-HT is oxidized to 5-HEO, **6** (subsequently **8** forms), and 5,6-DHT. This is because autoxidation of AA to dehydroascorbic acid provides the initial source of H_2O_2 and hence HO·. When 5-HT is oxidized in the same HO·-generating system in the presence of GSH, the yields of 5,6-DHT are not significantly altered. However, yields of 5-HEO increase, those of **6** (and **8**) decrease, and a new product, 7-*S*-glutathionyltryptamine-4,5- dione (7-*S*-Glu-T-4,5-D), appears. In the presence of sufficiently high concentrations of GSH,

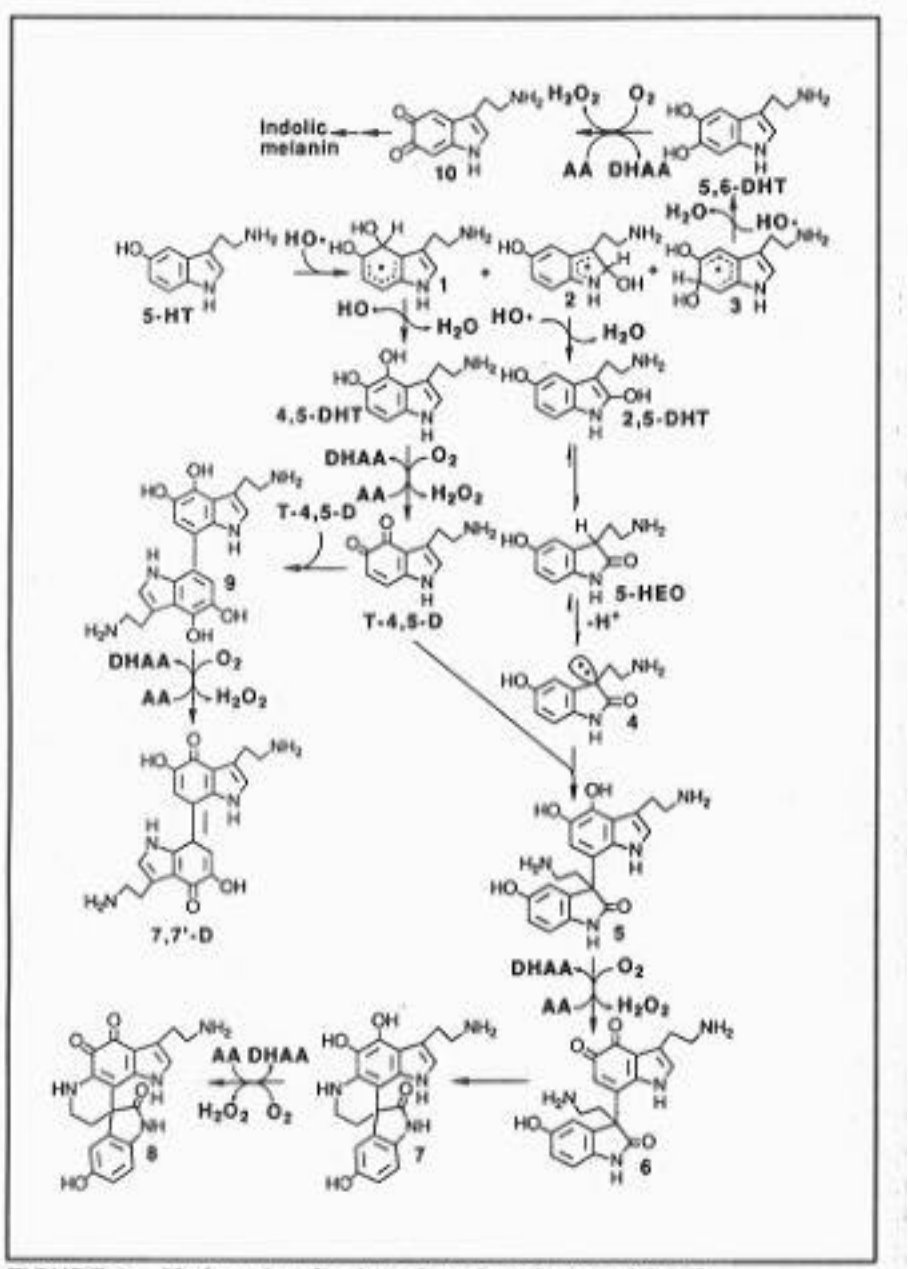


FIGURE 1. *Hydroxyl radical-mediated oxidation of 5-HT.*

formation of **6** (and **8**) is almost completely blocked and yields of 5-HEO and 7-*S*-Glu-T-4,5-D reach maximal levels. These observations are explained by the fact that GSH efficiently scavenges T-4,5-D to give

7-*S*-glutathionyl-4,5-dihydroxytryptamine (**11**, figure 2), which is then autoxidized to 7-*S*-Glu-T-4,5-D and forms H_2O_2 as a byproduct (Wong et al. 1993).

Quantitative measurements of the yields of products formed when 5-HT is oxidized with the AA/Fe^{2+}-EDTA/H_2O_2/O_2 system reveal that the initial yields of 5-HEO, 5,6-DHT, and **6** (and 7-*S*-Glu-T-4,5-D when GSH is present) are larger than expected based on the H_2O_2 (and hence HO·) concentrations employed (Wrona et al. 1995). This additional HO· probably results from autoxidations of 4,5-DHT (Wong and Dryhurst 1990), 5,6-DHT (Singh and Dryhurst 1990), **5** (and **8**) (Wrona et al. 1995), **11** (Wong et al. 1993), and redox cycling reactions of the T-4,5-D/4,5-DHT (formal potential (E%.) = -240 millivolts (mV) versus the saturated calomel reference electrode (SCE) at pH 7.4), **6**/**5** (E%. = -495 mV), **8**/**7** (E%. = -495 mV), 7-*S*-Glu-T-4,5-D/**11** (E%. = -248 mV), **10**/5,6-DHT, and 7,7./**9** (Singh et al. 1992) couples which form H_2O_2 as a byproduct.

These autoxidation-redox cycling reactions and resultant H_2O_2 and HO· formation apparently continue the oxidation of 5-HT until AA is exhausted. Although 5,6-DHT is an initial but minor product of the HO·-mediated oxidation of 5-HT, this neurotoxin almost completely disappears within 2 to 3 hours (Wrona et al. 1995) because of its autoxidation to an insoluble brown-black indolic melanin polymer (Singh and Dryhurst 1990; Singh et al. 1990). By contrast, the major reaction product, 5-HEO, is an appreciably more stable compound.

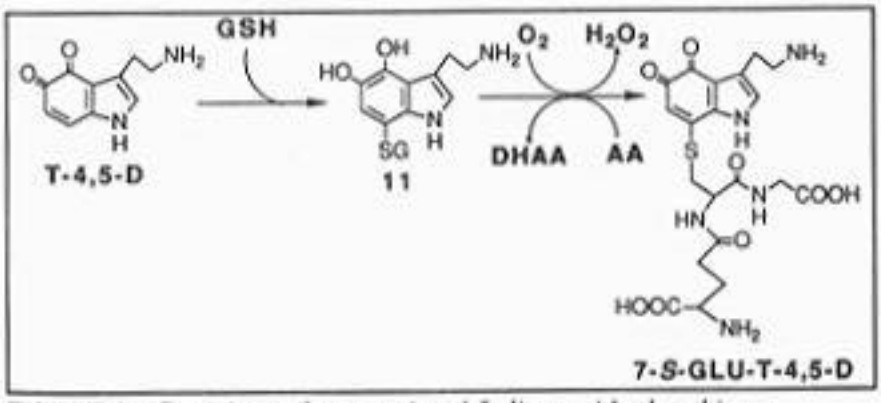


FIGURE 2. *Reactions of tryptamine-4,5-dione with glutathione.*

The oxidation of DA at physiological pH by HO·-generating systems consisting of $Fe^{2+}/H_2O_2/O_2$ or $Fe^{2+}/AA/O_2$ (H_2O_2 and hence HO· being formed as a result of AA autoxidation) has been reported to give a mixture of 2-OHDA, 5-OHDA, and 6-OHDA in relative yields of 3:2:1 (Slivka and Cohen 1987). However, the yields of all of these products were low based upon the HO· concentrations generated. The mechanism of these hydroxylation reactions is not completely clear. However, Richter and Waddell (1983) have reported that addition of HO·, generated by pulse radiolysis, to each of the available ring positions of DA is equally probable. Under the conditions employed by these investigators, which were carried out in the absence of molecular oxygen and iron salts, the intermediate radical species (**12**-**14**, figure 3) eliminate the elements of water to give semiquinones of DA. The dehydration of the 6-hydroxy- lated radical (**14**) was faster than for **12** and **13**. Slivka and Cohen (1987) have suggested that radicals **12**-**14** are oxidized by molecular oxygen or Fe^{3+} to give 2-OHDA, 5-OHDA, and 6-OHDA (figure 3). The lower yield of 6-OHDA was attributed to the more rapid water elimination reaction of **14**. In the event that methamphetamine evokes oxidation of DA by HO· in the cytoplasm of dopaminergic nerve terminals, then such reactions must occur in the presence of CySH and GSH (Slivka et al. 1987) and it is probable that these sulfhydryl compounds would influence the products formed.

Information bearing on such reactions in vivo can be drawn from reports that the 5-*S*-cysteinyl conjugates of DA and other endogenous catechols are present in human and other mammalian brains (Rosengren et al. 1985; Fornstedt et al. 1986). These conjugates are probably formed either by nucleophilic addition of CySH to the *o*-quinones formed by oxidation of these catechols or by addition of GSH to yield the 5-*S*-glutathionyl conjugates which are then hydrolyzed by peptidase enzymes (Rosengren et al. 1985).

Several lines of evidence suggest that the oxidation of DA and other catechols and formation of their 5-*S*-cysteinyl conjugates occurs in the cytoplasm of dopaminergic neurons. To illustrate, both GSH and CySH, which are clearly required to form these cysteinyl conjugates, are located in the cytoplasm of dopaminergic terminals and axons (Slivka et al. 1987). Furthermore, treatment of guinea pigs with reserpine, which disrupts DA storage vesicles and elevates cytoplasmic levels of this neurotransmitter, evokes a marked elevation of striatal levels of

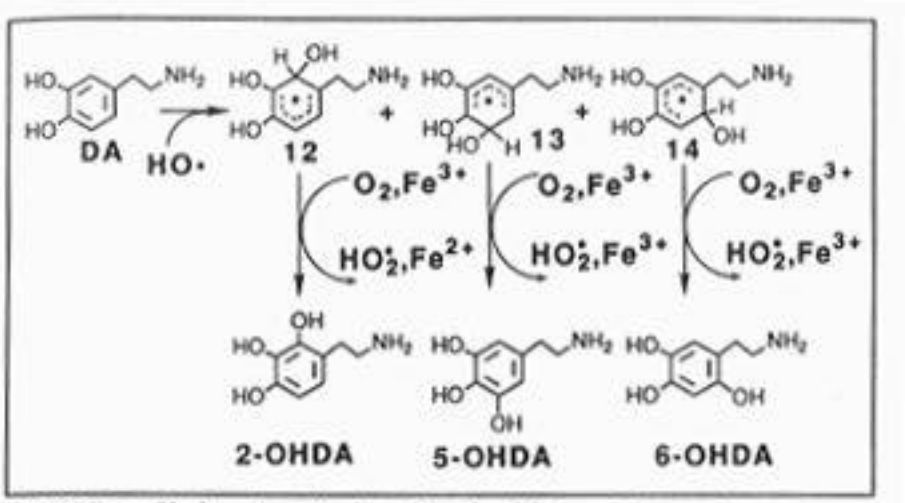


FIGURE 3. *Hydroxyl radical-mediated oxidation of dopamine.*

5-S-cysteinyldopamine (5-*S*-CyS-DA) (Fornstedt and Carlsson 1989). The mechanism of the HO·-mediated oxidation of DA in the presence of molecular oxygen and Fe^{2+}/Fe^{3+} does not appear to involve an electro- philic *o*-quinone intermediate (figure 3), the apparent precursor of 5-S-CyS-DA (Rosengren et al. 1985; Fornstedt et al. 1986, 1989). Autoxidation of DA apparently does proceed via such an intermediate, but is a relatively slow reaction at physiological pH and hence unsuitable for isolation and identification of reaction products. However, the electrochemically driven oxidation of DA is not only very facile but undoubtedly proceeds by initial formation of an *o*-quinone intermediate (Zhang and Dryhurst 1993). Accordingly, the authors have recently studied the influence of CySH on the electrochemical oxidation of DA at physiological pH (Zhang and Dryhurst 1994). This reaction results in formation of a very complex mixture of products. However, among the major products are the dihydrobenzothiazines (DHBTs) **17** to **19** and **22** (figure 4). In this reaction, DA-*o*-quinone is initially attacked by CySH to give 5-*S*-CyS-DA, which is appreciably more easily oxidized than DA to give *o*-quinone **15**. This intermediate very rapidly cyclizes to give the bicyclic *o*-quinone imine **16**. In the presence of free CySH **16** can either be reduced to DHBT **17** or nucleophilic addition of a second CySH molecule occurs to give DHBTs **18** and **19**. The latter compounds are even more easily oxidized than DA and 5-*S*-CyS-DA to *o*-quinone imines **20** and **21**, respectively, which can be either reduced by CySH or further

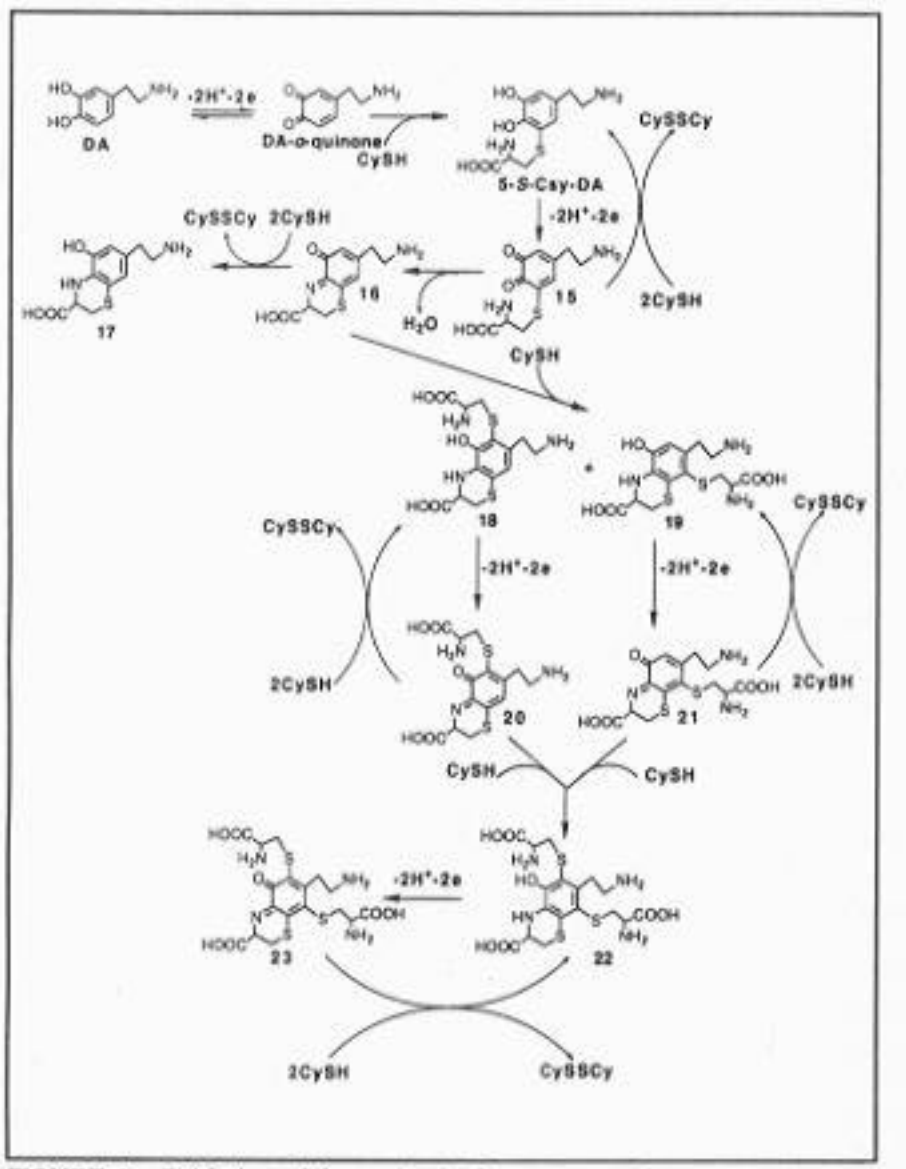


FIGURE 4. *Oxidation of dopamine in the presence of cysteine.*

attacked to give DHBT **22**. DHBTs **17** to **19** and **22** survive in the oxidation reaction simply because their oxidized forms are readily reduced by free CySH. DA-*o*-quinone is also scavenged by GSH to give a number of glutathionyl conjugates that include 5-*S*-glutathionyl- dopamine (5-*S*-Glu-DA) (Zhang and Dryhurst, in press). As suggested by Rosengren and colleagues (1985), if formed in the cytoplasm of dopaminergic terminals, 5-*S*-Glu-DA might be hydrolyzed by peptidase enzymes to 5-*S*-CyS-DA (figure 5). Under conditions when DA is oxidized, the latter conjugate must also be

oxidized because it is a significantly more easily oxidized compound (Zhang and Dryhurst 1994). In the event that this reaction is mediated by molecular oxygen, the expected byproducts would be superoxide anion radical (O_2-·) and/or H_2O_2, both precursors of HO·. The resulting products would include DHBTs **17** to **19** and **22** as conceptualized in figure 5. Again, the facile autoxidation of these DHBTs and their ability to redox cycle would be expected to potentiate additional formation of HO· precursors. The oxidation of DA in the presence of CySH in an HO·-generating system consisting of AA/Fe^{2+}/H_2O_2/O_2 also forms 5-*S*-CyS-DA in high yield; this is further oxidatively cyclized to DHBT **17** (Zhang and Dryhurst, unpublished results). Preliminary results do not indicate that 6-OHDA is a significant reaction product.

Studies in this laboratory aimed at understanding the in vitro oxygen radical-mediated oxidations of 5-HT and DA under experimental conditions that might mimic those in the cytoplasm of serotonergic and dopaminergic nerve terminals in vivo are at an early stage. Nevertheless, these investigations have established that 5,6-DHT is formed as a result of HO· attack on 5-HT. However, this neurotoxin is the most minor of the identified products of the reaction and is also a rather unstable species owing to its oxidative polymerization to indolic melanin. Furthermore, intraneuronal formation of 5,6-DHT as a result of methamphetamine administration would necessarily expose this substance to mitochondria which are known to catalyze its oxidation by molecular oxygen (Klemm et al. 1980; Singh and Dryhurst 1990). Taken together, these facts might account for the sporadic and low levels of 5,6-DHT detected in rat brain following an acute dose of methamphetamine (Commins et al. 1987).

These observations led Evans and Cohen (1989) to question the hypothesis that this neurotoxin mediates the methamphetamine-induced degeneration of serotonergic terminals. Assuming that formation of 5,6-DHT in rat brain following methamphetamine administration reflects intraneuronal oxidation of 5-HT by HO· (this is the only known chemical reaction that directly converts 5-HT and 5,6-DHT), the reaction pathways shown in figures 1 and 2 (Wrona et al. 1995) predict that 5-HEO in particular (and perhaps **6** and **8**) should be formed as major and more stable aberrant metabolites. However, intraneuronal GSH (Slivka et al. 1987) would be expected to scavenge T-4,5-D to give 7-*S*-Glu-T-4,5-D.

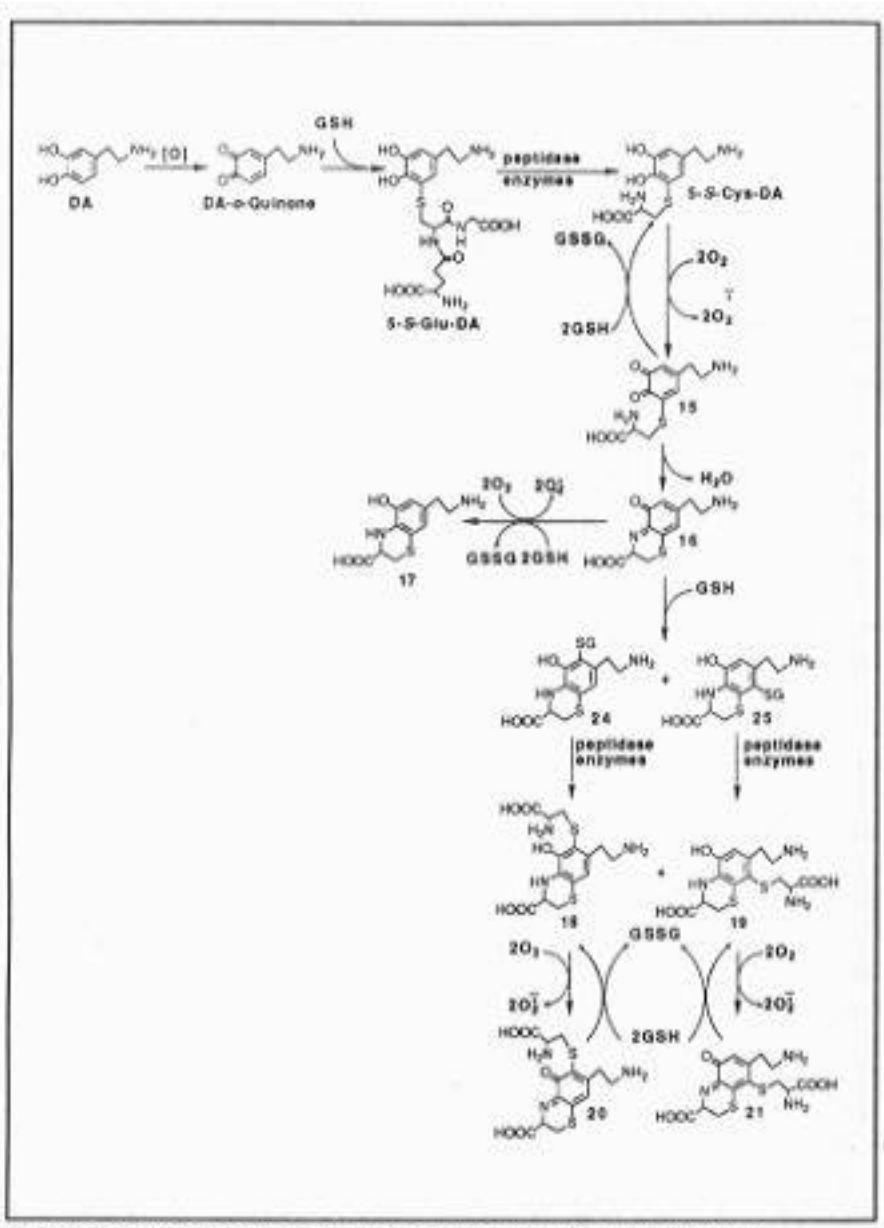


FIGURE 5. *Oxidation of dopamine in the presence of glutathione and possible in vivo effects of peptidase enzymes.*

Thus 5-HEO, **6**, **8**, or 7-*S*-Glu-T-4,5-D might represent valuable analytical marker molecules in brain tissue to provide evidence in support of the idea that methamphetamine does indeed evoke intraneuronal formation of HO·. Furthermore, the fact that the in vitro HO·-mediated oxidation of 5-HT gives additional products in higher yields than

5,6-DHT raises the possibility that one or more of these other putative aberrant oxidative metabolites might play roles in the neurodegenerative effects evoked by methamphetamine. The influence of CySH on the HO·-mediated oxidation of 5-HT remains to be studied. However, recent studies into the electrochemically driven oxidation of 5-HT in the presence of CySH suggest that many additional products are likely to be formed (Wrona et al. 1994).

In vitro studies reveal that the HO·-mediated oxidation of DA at physiological pH gives 6-OHDA (Slivka and Cohen 1987) (figure 3). Accordingly, detection of 6-OHDA in the rat striatum shortly following methamphetamine administration (Seiden and Vosmer 1984) suggests that this drug also evokes formation of HO·. Nevertheless, this conclusion must be tempered by the fact that several investigators have been unable to detect even extremely low levels of 6-OHDA in rat brain following administration of methamphetamine or other drugs that release DA into the synaptic cleft (Rollema et al. 1986; Evans and Cohen 1989; Karoum et al. 1993). However, using gas chromatography-mass spectrometry, Karoum and colleagues (1993) have observed a fluoro-propionylated derivative of an unknown compound having a retention time very similar to pentafluoropropionylated 6-OHDA in the brains of methamphetamine treated rats. In the event that methamphetamine evokes the intraneuronal oxidation of DA, it seems plausible to suggest that CySH and or GSH might intervene in the reaction as shown in figures 4 and 5 to give 5-*S*-CyS-DA and 5-*S*-Glu-DA, precursors of DHBTs such as **17** to **19** and **22**. This suggestion raises the possibility that these putative aberrant oxidative metabolites of DA, formed only from endogenous substances, might contribute to the neurodegenerative effects evoked by methamphetamine.

IN VIVO PROPERTIES OF PUTATIVE ABERRANT OXIDATIVE METABOLITES OF 5-HT AND DA

Investigations into the neurotoxicology and neuropharmacology of putative aberrant oxidative metabolites of 5-HT and DA are at a very preliminary stage. 5-HEO, the major product of the in vitro HO·-mediated oxidation of 5-HT, is not toxic (lethal) when administered into the brains of mice weighing ~ 30 grams (g) at doses as high as 100-200 micrograms (g) (Dryhurst et al., unpublished results). However, several other intermediates/products of this reaction are active in the brain. For example, T-4,5-D is lethal when injected into the brains of mice (Wong et al. 1993). Furthermore,

intracerebroventricular injections of T-4,5-D into rat brain have been claimed to evoke long-lasting decreases in 5-HT levels in the hippocampus, striatum, and cortex; reduced activity of tryptophan hydroxylase (Chen et al. 1992); and degeneration of nerve terminals (Crino et al. 1989). 7-*S*-Glu-T-4,5-D is also lethal (median lethal dose (LD_{50}) = 21 g) when administered into mouse brain, evoking extreme excitation and hyperactivity for about 30 minutes (Wong et al. 1993). During this period, 7-*S*-Glu-T-4,5-D evokes statistically significant declines of whole brain levels of norepinephrine, DA, and 5-HT and elevation of the metabolites of these neurotransmitters (Wong et al. 1993). This might suggest that 7-*S*-Glu-T-4,5-D potentiates the release and elevated turnover of the monoamines although its influence on the release of other neurotransmitters, especially glutamate, remains to be determined. 7-*S*-Glu-T-4,5-D also redox cycles in the presence of cellular antioxidants/reductants and molecular oxygen and when incubated with brain homogenates forming H_2O_2 as a byproduct (Wong et al. 1993). Dimer **6** is also lethal (LD_{50} = 19 g) when administered into mouse brain (Dryhurst et al., unpublished results). Furthermore, the E%. values of **6** and **8** (both -495 mV at pH 7.4) are such that these compounds might be expected to redox cycle under intraneuronal conditions (Yamazaki and Piette 1963) forming O_2-· and/or H_2O_2 as byproducts, that is, precursors of cytotoxic HO·.

5-*S*-CyS-DA, a putative aberrant oxidative metabolite of DA in dopaminergic terminals, is not lethal when administered into the brains of laboratory mice. DHBTs **17** (LD_{50} = 14 g), **18** (LD_{50} = 17 g), and **19** (LD_{50} = 70 g) are lethal (Zhang and Dryhurst 1994).

SUMMARY

In the event that methamphetamine evokes HO· formation within serotonergic axon terminals, the resultant oxidation of 5-HT would be expected to generate not only 5,6-DHT but also T-4,5-D, 7-*S*-Glu-T-4,5-D, **6**, **8**, and 7,7T-D (figure 1), at least three of which (T-4,5-D, 7-*S*-Glu-T- 4,5-D, and **6**) are lethal in mouse brain. Furthermore, several intermed- iates/products formed in the in vitro oxidation of 5-HT by HO· are readily autoxidized (4,5-DHT, 5,6-DHT, **5**, **7**, and **9**) or redox cycled (T-4,5-D, **6**, **8**, 7,7T-D, 7-*S*-Glu-T-4,5-D) in reactions that would be expected to yield O_2-· and/or H_2O_2 as byproducts. These byproducts, in the presence of trace levels of transition metal ion catalysts, would be readily converted into HO· (Walling 1975; Halliwell and Gutteridge 1984). Together these

putative aberrant oxidative metabolites of 5-HT and HO·-forming reactions might contribute to the degeneration of serotonergic nerve terminals. Similarly, the methamphetamine-induced intraneuronal formation of HO· in dopaminergic terminals might be expected to generate not only 6-OHDA (and 2-OHDA and 5-OHDA, figure 3) but also 5,-*S*-CyS-DA and 5-*S*-Glu-DA, precursors of DHBT **17** and other more complex dihydrobenzothiazines (figure 4).

DHBTs **17** to **19** are lethal in mouse brain, although at this time the biochemical/chemical mechanisms underlying this toxicity and specific neuronal systems affected are unknown. However, 5-*S*-CyS-DA and **17** to **19** are much more easily oxidized than DA, and the latter DHBTs appear to be capable of redox cycling reactions (Zhang and Dryhurst 1994). Thus, the HO·-mediated oxidation of DA in dopaminergic nerve terminals induced by methamphetamine might be expected to generate aberrant oxidative metabolites that (as a result of autoxidation and redox cycling reactions) potentiate formation of O_2-· and/or H_2O_2, and then HO· and neuronal damage.

A number of lines of evidence, discussed previously, suggest that aberrant metabolite(s) of DA (other than or in addition to 6-OHDA) might contribute to the methamphetamine-induced degeneration of not only dopaminergic terminals but also serotonergic terminals. Similarly, aberrant metabolite(s) of 5-HT (other than or in addition to 5,6-DHT) might be involved in the degeneration of serotonergic and dopaminergic terminals and a subpopulation of cell bodies in the somatosensory cortex. Experimental evidence indicates that some of the neurodegenerative effects evoked by methamphetamine are mediated by NMDA and GABA receptors. Thus, it will be of considerable interest to investigate the neuro- toxicity of putative aberrant oxidative metabolites of 5-HT (figures 1 and 2) and DA (figures 4 and 5) towards serotonergic, dopaminergic, and other neuronal systems and their interactions with NMDA, GABA, and other brain receptors.

A central question relates to mechanisms by which methamphetamine might evoke the intraneuronal formation of oxygen radicals that appear to play important roles in the overall neurodegenerative processes evoked by this drug (DeVito and Wagner 1989; Cadet et al. 1994). Once putative oxidative metabolites of 5-HT such as T-4,5-D, 7-*S*-Glu-T-4,5-D, 5,6-DHT, **6**, **8**, and 7,7T-D (figure 1) are formed intraneuronally, autoxidation/redox cycling reactions should, in principle, be capable of generating O_2-· and/or H_2O_2, the precursors of HO·. Similarly, intraneuronal formation of 6-OHDA, 5-*S*-CyS-DA,

and DHBTs **17** to **19** and **22** would also be expected to potentiate elevated fluxes of O_2-·, H_2O_2, and HO· as a result of the facile autoxidation/redox cycling reactions of these putative aberrant metabolites. The presence of very low concentrations of 5-*S*-CyS-DA in DA-rich regions of human and other mammalian brains suggests that autoxidation (Rosengren et al. 1985; Fornstedt et al. 1986, 1989, 1990) or perhaps some other form of DA oxidation is a normal reaction in vivo. Furthermore, available evidence suggests that it is cytoplasmic DA that is oxidized to give 5-*S*-CyS-DA (Fornstedt et al. 1989; Fornstedt and Carlsson 1989). Presumably, only free or unbound cytoplasmic DA is available for such oxidation reactions.

The ability of methamphetamine to displace DA from cytoplasmic storage sites prior to efflux through the uptake carrier site (Raitiri et al. 1979; Marek et al. 1990*a*; Liang and Rutledge 1982) would be expected to elevate the levels of this neurotransmitter available for autoxidation. Furthermore, reuptake of released DA through the uptake carrier site might continue to provide elevated cytoplasmic levels of the neurotrans-mitter for autoxidation and oxidation by HO·, generated as a result of autoxidation/redox cycling reactions of aberrant oxidative metabolites of DA. Such a sequence of events might result in the gradual increase in the levels of toxic aberrant oxidative metabolites of DA and account for the observation that administration of the uptake inhibitor AFA up to 8 hours after methamphetamine is capable of protecting dopaminergic terminals (Marek et al. 1990*b*).

There is presently no experimental information concerning the normal autoxidation of cytoplasmic 5-HT. However, 5-HT autoxidizes at about the same rate as DA at physiological pH (Creveling et al. 1975; Rotman et al. 1976). Methamphetamine displacement of 5-HT from its cytoplasmic storage sites and reuptake of the released neurotransmitter might lead to 5-HT autoxidation to aberrant metabolites; as a result of autoxidation/redox cycling reactions, these metabolites form elevated fluxes of O_2-·, H_2O_2, and HO· and thereby potentiate their own synthesis and neuronal damage.

Until recently it was believed that intraneuronal proteins known as serotonin binding proteins (SBP) were involved in the storage, protection, and/or transport of 5-HT (Tamir et al. 1976; Gershon and Tamir 1984) and catecholamines (Jimenez Del Rio et al. 1992, 1993*a*, 1993*b*; Pinxteren et al. 1993). The binding of 5-HT and DA to SBP is increased by Fe^{2+} but not Fe^{3+}. Thus, it was generally

believed that Fe^{2+} initially binds to sulfhydryl groups of SBP and that the monoamine neurotrans- mitters form reversible coordinate bonds with the trapped iron. However, more recent results (Jimenez Del Rio et al. 1993*a*) have revealed that in the presence of Fe^{2+} and molecular oxygen, SBP covalently (irreversibly) binds the oxidation products of 5-HT and DA. The latter reactions are apparently mediated by oxygen radicals formed by reaction between Fe^{2+} and molecular oxygen. These observations might imply that the physiological role for SBP is to act as a scavenger of electrophilic oxidation products of 5-HT (e.g., T-4,5-D, **10** (figure 1)) and DA (e.g., DA-*o*-quinone, figure 4), which are themselves cytotoxins or precursors of other toxic metabolites. Interestingly, reserpine inhibits the binding of 5-HT (or its oxidation products) to SBP in the presence of Fe^{2+} and molecular oxygen (Tamir et al. 1976) and hence might account, in part, for increased formation of 5-*S*-CyS-DA in the striatum of reserpinized rats (Fornstedt and Carlsson 1989). Thus, reserpine might not only evoke increased cytoplasmic levels of DA, but also impair the ability of SBP to bind electrophilic oxidation products. Similarly, elevated cytoplasmic levels of free DA and 5-HT caused by methamphetamine, followed by intraneuronal oxidation of these neurotransmitters, might result in formation of electrophilic intermediates at concentrations that exceed the scavenging capacity of SBP with resultant neurodegenerative consequences.

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Immunotoxicology of Opioids, Inhalants, and Other Drugs of Abuse

Robert V. House, Peter T. Thomas, and Hemendra N. Bhargava

INTRODUCTION

Drug abuse is a problem of increasing worldwide significance. In addition to the obvious socioeconomic problems associated with the use of so-called street drugs, the abuse of ethical pharmaceuticals may also result in serious untoward health effects depending upon a wide range of variables (Chiang and Goldfrank 1990). One possible medical complication of drug abuse is modulation of the immune system, or immunotoxicity (Pillai and Watson 1990). The immune system is a highly regulated organ system that presents a variety of potential targets for modulation by drugs. This modulation may take the form of immunosuppression, leading to an enhanced susceptibility to infection or neoplasia; conversely, it may take the form of immunostimulation, resulting in hypersensitivity (allergy) or autoimmunity (Luster and Rosenthal 1993). Closely associated with drug abuse in recent years has been the emergence of the acquired immunodeficiency syndrome (AIDS), a retroviral infection spread by sexual contact or hematogenously by the sharing of needles among intravenous (IV) drug abusers. One of the hallmarks of AIDS is a profound and irreversible suppression of immune competence, usually resulting in death of the host from opportunistic infections. Thus, the combination of drug abuse and human immunodeficiency virus (HIV) infection represents a formidable challenge to the immune system. Although the scientific literature is replete with studies describing drug-related immunosuppression, the sheer number of abused drugs precludes detailed examination using traditional methodology. Moreover, as new therapeutics are developed for the treatment of drug abuse, an efficient screening approach will be required to assess their immunomodulatory potential.

The studies described herein were performed under the aegis of National Institute on Drug Abuse (NIDA) contract 271-91-9201, entitled "Immunomodulatory Effects of Drugs of Abuse and Potential Medications." The purpose of the these studies was the evaluation of the potential for a number of drugs of abuse, opioid peptides, and established and experimental therapeutic agents to alter immune function associated with host defense. This was approached in a

twofold manner. First, test materials were evaluated in vitro using murine splenic lymphocytes from B6C3F1 (C57BL/6 *X* C3H) hybrid mice. This hybrid strain was chosen because of the large database that exists of drug and chemical effects on immunity. The panel of immune function assays was carefully chosen to represent relevant host defense mechanisms and be adaptable to drug screening. Second, standard in vivo pharmacological models of tolerance/abstinence were validated in this strain and then used for in vitro multidrug exposure studies as above.

RATIONALE AND METHODOLOGY

For the screening portion, the data were obtained following in vitro exposure of drugs to isolated splenic lymphocytes and macrophages. This experimental paradigm allows for a high degree of precision in drug concentration delivered to target cells, utilizes only small amounts of test material and a limited number of animals, targets only the cells of primary interest, and facilitates a high-output screening approach. In spite of these important advantages, this in vitro exposure system does not account for drug metabolism (and the attendant question of metabolite-associated immune effects) or potential secondary effects on other target cells or tissues. For example, a drug might exert immunotoxicity toward the bone marrow, thus affecting the precursors of all immune cells. Alternatively, a drug that targets the thymus (for example) might have selective effects on cell-mediated immunity, but only limited effects on humoral immunity. Additionally, studies have demonstrated that the metabolites of some drugs exert profound effects on immune function (Thomas et al. 1995*a*). Thus, abused drugs that do not exhibit in vitro immunomodulatory activity may still display such activity following in vivo exposure. For these reasons, drugs testing positive in the in vitro screen would require further testing following in vivo exposure to more fully evaluate the range and nature of their immunomodulatory potential.

Figure 1 illustrates the general experimental approach. Spleens harvested from naive female B6C3F1 mice served as a convenient source of lymphocytes for study. The initial consideration in the in vitro screen was the physical characteristics of the test compounds themselves. For the most part, at the in vitro concentrations tested, the compounds have

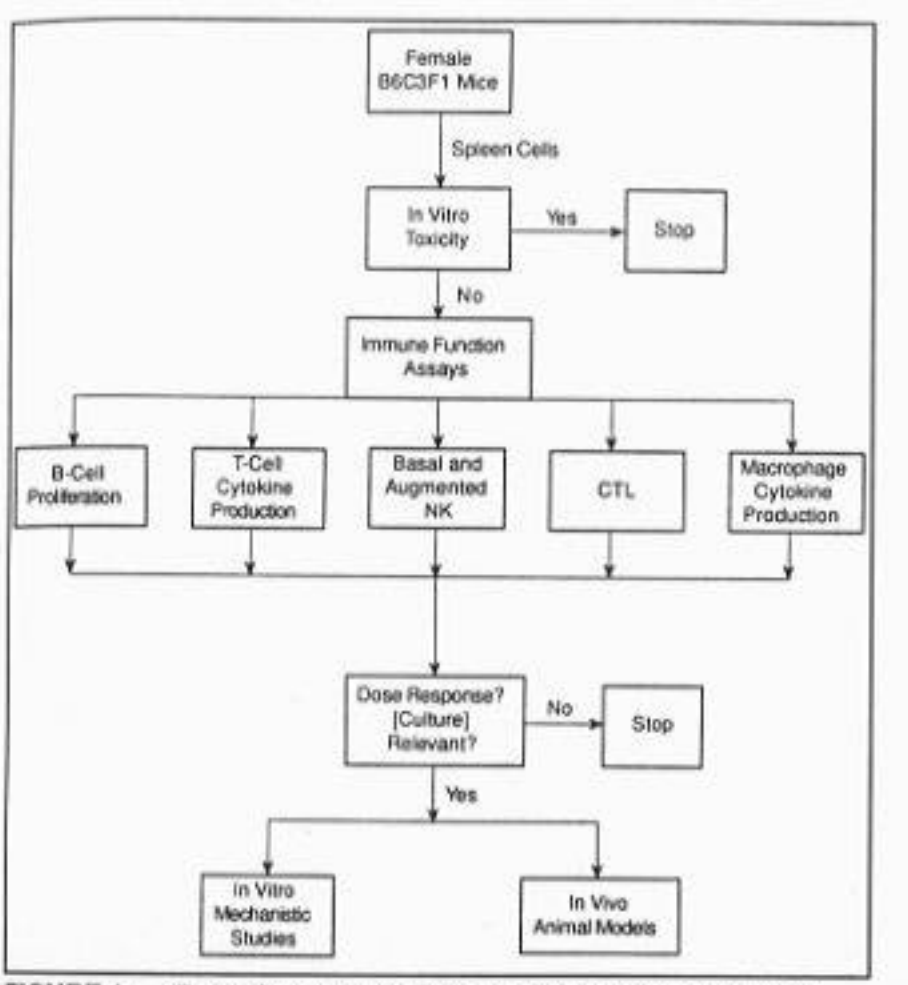

FIGURE 1. *General experimental approach for evaluating in vitro immunomodulation by drugs.*

largely been soluble in aqueous solution. However, drugs that are primarily or exclusively lipid-soluble require the use of an appropriate, nontoxic carrier material. The second consideration was the cytotoxic potential following in vitro exposure to any of these drugs. This was evaluated by exposing isolated splenocytes to the anticipated range of drug concentrations in vitro. At appropriate time points, the cells were evaluated for toxicity by vital dye exclusion. Any drug found to be cytotoxic would have been excluded from study; however, no drug yet tested has produced overt toxicity as evaluated by this approach.

Two ranges of in vitro concentrations were utilized in these studies. Most of the abused drugs (e.g., amphetamine, phencyclidine (PCP)) were examined at in vitro concentrations between 0.0001 and 100

micromolars (M). Endogenous and exogenous peptide-type compounds (e.g., [D-penicillamine^{2}, D-penicillamine^{5-}]-enkephalin (DPDPE), -endorphin) were evaluated at concentrations between 0.00001 and 10 M. These ranges were chosen to include realistic pharmacologically achievable concentrations in humans following in vivo drug exposure.

After establishment of the drug exposure paradigm, the potential of these compounds to produce immunomodulation was examined in a panel of in vitro assays. These assays were carefully selected to yield the maximal amount of information on various immune regulatory and effector mechanisms. The rationale for the use of each assay and its execution are described below.

One of the two principal arms of the immune system in vertebrates, namely humoral immunity, is mediated by soluble factors (antibodies) produced by B-cells. This form of immunity is vitally important for efficient host defense. B-cells are primarily responsible for the production of specific antibody although they also exhibit other functions, including antigen presentation and cytokine production (Brown 1992). For the purposes of this work, a rapid screening type assay of B-cell function was required. B-cell proliferation was chosen as the endpoint for two principal reasons. First, proliferation of lymphocytes is an early sequela of activation, and as such represents a relevant measurement of immune competence. Second, cellular proliferation is an easily quantitated function, whereas other assays of B-cell function (e.g., antibody production) are laborious and time consuming, precluding their efficient use in a large-scale screening study.

Isolated murine splenocytes were cultured for 3 days in the presence of various concentrations of drugs and a combination of anti-immuno-globulin M (IgM) antibody/recombinant murine interleukin-4 (IL-4) as adapted from Abbas and colleagues (1990). The activational signal is mediated via anti-Ig binding with the surface immunoglobulins present on B-cells, with IL-4 providing the proliferative signal. This system may represent a more physiologically relevant model than more routinely used mitogens such as lipopolysaccharide (LPS). Since this was a screening study requiring a high throughput of test materials, a more rapid indicator system was needed to replace the traditional thymidine incorporation method. To this end, a colorimetric assay based on mitochondrial reduction of a tetrazolium salt to a colored formazan end product (Roehm et al. 1991) was employed. This system allowed assay of a large number of samples in

a greatly reduced time, and will be used in all of the authors' future screening studies.

The action of the second principal arm of the immune system is mediated primarily by cellular mechanisms, and consequently is referred to as cell-mediated immunity. Cell-mediated immunity is determined largely by the function of T-cells. For this study, both the regulatory and the effector functions of T-cells were evaluated. The regulatory capacity of T-helper cells was evaluated by measurement of cytokine production. T-helper cells are a subset of T-cells displaying the CD3/CD4 surface antigens, and are primarily responsible for producing regulatory cytokines. T-helper cells exhibit functional differences based on their pattern of cytokine secretion; these differences are thought to function as mechanisms for controlling the immune reaction and directing its ultimate expression as either humoral- or cell-mediated immunity (Mosmann et al. 1991).

In simplistic terms, T-helper cells may be categorized as TH1, which secrete IL-2 and interferon-gamma (IFN) but not IL-4 or IL-10, and TH2, which secrete IL-4 and IL-10 but not IL-2 or IFN. Examining representa- tive cytokines from each of these groups provides mechanistic information on the potential differential effects of test materials on T-cell subset functions. In this work, the production of IL-2 and IL-4, the prototype representatives for TH1 and TH2 respectively, was evaluated. For the in vitro studies, naive murine splenocytes were cultured in the presence of various concentrations of test drugs, and were stimulated with a mono- clonal antibody directed against the T-cell antigen receptor (i.e., anti-CD3). This system polyclonally simulates the interaction of T-cells with their cognate antigen, and represents a physiologically relevant activational signal. Following culture, the supernatant fluids were collected and analyzed for IL-2 bioactivity by a modification of the method of Gillis and coworkers (1978), and for IL-4 bioactivity by a modification of the method of Hu-Li and coworkers (1989). As with the B-cell proliferation assay, a colorimetric endpoint was utilized to facilitate evaluation of a large number of samples.

The impact of drugs on effector T-cell function is determined by evaluating the in vitro induction of cytotoxic T-lymphocytes (CTLs), a population of T-cells bearing the CD3/CD8 surface antigens. These cells are capable of exhibiting cytotoxicity toward specific target cells after prior exposure to antigen, and therefore represent a central effector mechanism of cell-mediated immunity and host resistance (Berke 1989). The crucial role that CTLs are

thought to serve in host defense makes them a prime measure of cell-mediated immunity in both normal and immunocompromised states. In the course of the in vitro studies in this work, the laboratory of one author has developed a serum-free modification of the standard CTL induction method (House et al. 1994*a*). This serum- free approach results in greater reproducibility of the assay and facilitates recovery of viable cells, an important consideration when examining cells with potential immunomodulatory effects. In these studies, isolated murine splenocytes were bulk cultured for 5 days in the presence of various concentrations of drugs and mitomycin C-inactivated P815 mastocytoma cells (which also serve as the target cells). The effector cells were subsequently collected, washed, and cocultured for 4 hours with radiolabeled P815 target cells. Released radiolabel was measured in a gamma counter, and the specific lysis of the target cells was determined as a percentage of total releasable counts.

An important immune function to evaluate in the context of immuno-deficiency, and one that represents a form of nonspecific immunity/host resistance, is the function of natural killer (NK) cells. NK cells are lymphocytes distinct from either B-cells or T-cells, which contribute to immunocompetence by mediating major histocompatibility complex-independent cytotoxicity (Lotzová 1993). For the purposes of these studies, a combined measurement of both basal and augmented NK cell function was used. In this assay, murine splenocytes were exposed for 24 hours to various concentrations of drugs in the presence or absence of an optimum concentration of recombinant IL-2 (Thomas et al. 1993). The cells were then washed and cocultured for 4 hours with radiolabeled YAC-1 tumor cells (a murine NK-sensitive cell line). Tumor cell lysis was quantitated as described above for the CTL procedure.

The approach described above had a dual purpose. First, although NK cells are generally nonspecific in their cytotoxic capacity, their action is sensitive to modulation by immune mechanisms, particularly by the action of cytokines like IL-2 and interferon-gamma (Talmadge 1985). This situation would probably occur in vivo during an infection or other immune reaction. Basal NK levels are often of low activity, which would render any decrease in activity difficult to measure. Thus, measurement of enhanced (augmented) activity may reveal differential modulation of NK cell function in a physiological situation. The second reason for this 24-hour culture was the uncertainty that drug exposure would produce significant effects only during the 4-hour lytic phase of the assay. This combination assay

provided mechanistic information on NK cell functional alteration with a minimum of extra labor and materials.

Finally, the effect of drug exposure on macrophage function was evaluated. Macrophages subserve both specific and nonspecific host resistance mechanisms and, as such, are important cells in the induction and maintenance of various immune and nonimmune responses to infection. As with the T-cells, macrophages accomplish this regulatory activity largely through the action of cytokines. In these studies, the production of IL-6 and tumor necrosis factor (TNF) by macrophages exposed in vitro to drugs was implemented. IL-6 and TNF are pivotal cytokines with a multitude of effector and regulatory functions (Akira et al. 1990) and, as such, represent rational targets for determining macrophage function. For these studies, naive mice were given an intraperitoneal (IP) injection of thioglycolate to elicit peritoneal exudate cells, which were washed from the animal by peritoneal lavage following sacrifice. Peritoneal macrophages from these preparations were enriched by plastic adherence. Monolayers of enriched macrophages were exposed in vitro for 48 hours to various concentrations of drugs in the presence of an optimum stimulatory concentration of bacterial LPS. The culture supernatant fluids were harvested, and IL-6 and TNF were quantitated by specific bioassays. IL-6 was quantitated by a modification of the method of Van Snick and colleagues (1986) using the 7TD1 cell line, and TNF was quantitated by a modification of methods described by Meager and colleagues (1989) using the L929 fibroblast cell line. Both bioassays utilized a colorimetric endpoint, as described above.

Although drug action on elicited macrophages may not necessarily reflect the situation in normal animals, the elicitation procedure was necessitated by the extremely low recovery of peritoneal cells in the absence of such treatment; this low recovery would have necessitated the use of very large numbers of mice to accomplish these screening studies.

RESULTS FROM IN VITRO SCREENING STUDIES

In general, drugs selected for testing in this study may be grouped into three general categories: abused drugs previously demonstrated to suppress the immune response (e.g., heroin, cocaine); abused drugs that have not previously been associated with immune function alterations (e.g., lysergic acid diethylamide (LSD),

methylenedioxymethamphetaine (MDMA)); and drugs with therapeutic potential for treating drug abuse (e.g., ibogaine).

For the purpose of summarizing the findings of the in vitro screening assays, these drugs are further classified according to either their action or chemical description. The sections below describe the results of these in vitro assays. Representative data are presented for illustrative purposes.

Hallucinogens

In general, the hallucinogenic drugs have received scant investigation for potential immunotoxic potential. The reason for this is unclear, although the limited duration of exposure characteristic of these drugs may be an important factor. That is, although hallucinogen abuse may be a long-term habit, actual exposure is usually not chronic. This pattern of abuse may result in acute alterations of selected immune functions, but the resiliency of the immune response may compensate for any drug-induced defects.

One of the first drugs examined in this project was the dissociative hallucinogen PCP, also known as angel dust. In the absence of effects on cellular viability, PCP exposure significantly suppressed B-cell prolif-eration, cytokine production by T-cells, and the generation of specifically sensitized cytotoxic T-cells. In addition, PCP significantly suppressed IL-2-augmented NK function. By comparison, macrophage IL-6 production was not affected by any concentration of PCP examined (Thomas et al. 1993). Significantly, these alterations in immune function were observed at pharmacologically relevant concentrations. These results are in basic agreement with, and expand upon, the work of Khansari and colleagues (1984) and Dornand and colleagues (1987), who both reported immunosuppression after PCP exposure. These data suggest that chronic PCP abuse may adversely affect immune mechanisms associated with host defense.

Another hallucinogen examined in this project was LSD, a commonly abused drug in the 1960s that appears to be enjoying a renaissance. A careful review of the literature indicated that LSD had not heretofore been evaluated for immune effects, prompting its investigation in this work. It was found that in vitro exposure to LSD resulted in suppressed proliferation of B-cells, production of the cytokines IL-2, IL-4, and IL-6, and the induction of cytotoxic T-lymphocytes. In vitro exposure to LSD had differential effects on NK cell activity,

with significant enhancement of both basal and IL-2-augmented NK cell function occurring at concen-trations that may be reached upon human exposure (House et al. 1994*b*). Potential in vivo immunological effects of LSD exposure remain to be characterized.

Two additional hallucinogens examined in this project were the indole alkaloids ibogaine and harmaline. Ibogaine enjoyed a brief career as a street drug in the 1960s, and was classified by the U.S. Food and Drug Administration as a Schedule I drug in 1970 (Glick et al. 1991). Ibogaine was of special interest for study since it is the subject of two U.S. patents for the treatment of addiction (U.S. patents 4,499,096 and 4,587,243). Another indole alkaloid with hallucinogenic properties is harmaline, a -carboline compound related to ibogaine. The -carbolines are of particular interest in that, in addition to their presence in plant materials, they may be present in mammals as metabolic byproducts. Although harmaline and its congeners produce physiological reactions similar to those seen following ibogaine exposure, the two classes of compounds apparently function by different mechanisms (Deecher et al. 1992). Harmaline was evaluated along with ibogaine to determine whether any observed effects might be related to the chemical structure of these hallucinogens. With the exception of NK cell function with ibogaine, both drugs produced a biphasic effect on both natural and antigen-specific immune responses. By contrast, TH1- and TH2-derived cytokine production was unaffected by exposure to either ibogaine or harmaline. Suppression of macrophage function was noted for the different drugs, but was generally associated only with high concentrations (House et al. 1995*b*).

Stimulants

Amphetamine [(phenylisopropyl)amine] is a powerful central nervous system stimulant that also affects the cardiovascular and peripheral nervous systems, producing a decreased sense of fatigue, a mood elevation, an increase in motor activity, and often euphoria (Hoffman and Lefkowitz 1990). These properties have contributed to its continuing popularity as a recreational drug. Although amphetamine abuse represented an important problem in the United States between the 1940s and the 1960s, the Controlled Substance Act of 1970 limited the practice (Derlet and Heischober 1990). The N-methylated homolog methamphetamine is produced in numerous clandestine laboratories, and its abuse appears to be on the rise, especially in Hawaii and the U.S. West Coast (Heischober and Miller 1991). A confounding factor in methamphetamine/amphetamine

abuse is that these drugs are often self-injected intravenously. This practice poses the dual concern of an increased probability of spreading HIV infection during the process of drug abuse, as well as the potential of methamphetamine-mediated suppression of an already damaged immune system in normal or HIV-infected drug abusers (Klee 1992). Studies on the immunomodulatory effects of amphetamines (House et al. 1994*a*) indicated that in vitro exposure to amphetamine resulted in a significant suppression of IL-2, but not IL-4, production by T-cells, as well as a suppression of B-cell proliferation only at the highest amphetamine concentration examined. An interesting finding of these studies was that NK cell function was slightly suppressed by amphetamine exposure, but was enhanced by methamphetamine exposure (figure 2). The nature of this differential immunomodulatory activity is currently unknown.

Methamphetamine often serves as the parent compound for synthesis of new designer drugs (Bost 1988; Buchanan and Brown 1988). Currently, one of the more popular designer drugs is MDMA, also known as ecstasy. MDMA is the methylated derivative of the amphetamine analog methylenedioxyamphetamine (MDA), a prototype of the hallucinogenic amphetamine drugs (Bost 1988). MDMA is relatively easy to synthesize

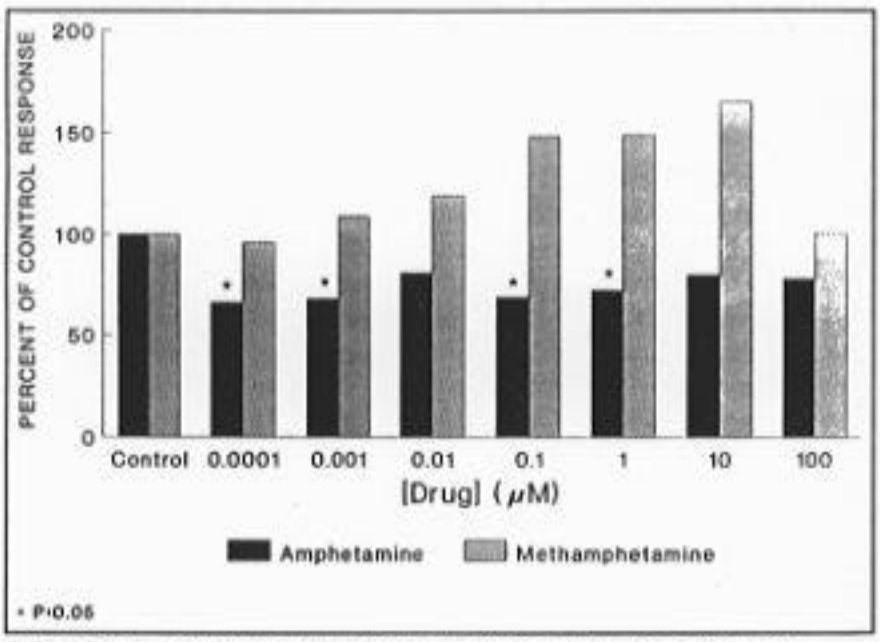

FIGURE 2. *Differential modulation of NK cell function following in vitro exposure to amphetamine or methamphetamine.*

in clandestine drug laboratories, and thus will probably remain a potential drug of abuse for the foreseeable future. Although MDMA has been used both legally and illegally for a number of years, little is known concerning its potential health effects. Studies with MDMA demonstrated that in vitro exposure resulted in a pattern of immunomodulation similar in most respects to that observed with the parent drug methamphetamine (House et al. 1995*a*). No effect was observed on B-cell proliferation at any concentration tested. In comparison, production of IL-2 was enhanced at concentrations as low as 0.0001 M and IL-4 production was unaffected. Basal and augmented NK cell function were enhanced at concentrations between 0.0001 and 1.0 M, and CTL induction was significantly suppressed at a concentration of 100 M. Finally, macrophage production of TNF was slightly suppressed at 10 and 100 M MDMA, although this inhibition was not statistically significant. The similarity in results from methamphetamine and MDMA suggests that a common mechanism of immunomodulation may be, in part, due to chemical similarities between the two drugs, although elucidation of this mechanism awaits further investigation.

It is important to remember that drug abuse is not an exclusively American problem, and increasing population mobility can only serve to increase exposure to previously unknown types of abused drugs. Therefore, the immunotoxic potential of exposure to the natural amphetamine cathinone was investigated. Cathinone is a phenylalkylamine present in the khat shrub, the leaves of which are chewed on a regular basis by residents of East Africa and the Arabian peninsula (Kalix 1992). Consumption of khat is widespread in certain cultures, but its use in countries outside its growth area has been limited. Studies with cathinone, performed in tandem with studies of the synthetic amphetamines, demonstrated that in vitro exposure to either the (S) or (R) isomers of cathinone resulted in stimulation of IL-2 production, B-cell proliferation, and CTL induction in murine cells (House et al. 1994*a*). No significant effect of cathinone was noted on NK cell function. These previously unknown findings indicate that use of this mild stimulant may significantly alter the immune response.

Opiates

Abuse of morphine and related opiates is well known to alter a number of immune response parameters, including suppression of cell-mediated, humoral-mediated, and natural (nonspecific) immunity (Bhargava 1990). Unlike the clear demonstration of morphine's effects on immune responses following in vivo exposure, in vitro studies have been limited. Although morphine's ultimate deleterious

effects on human health are the subject of greatest concern, the use of in vitro models of immune function offer the possibility of investigating, at a cellular and molecular level, the mechanisms of the immunomodulation produced by morphine, its metabolites, and related opiates.

Following in vivo exposure, morphine is eliminated in a biphasic manner. In the first phase, morphine is rapidly distributed to all tissues. In the second phase, morphine is quickly converted to its principal metabolite, morphine-3-glucuronide, and somewhat more slowly to a secondary metabolite, morphine-6-glucuronide (Karch 1993). Another metabolite, normorphine, is produced in smaller amounts than the glucuronides. Normorphine and morphine-6-glucuronide exhibit the greatest pharmacological activity (Glare and Walsh 1991).

The immunomodulatory potential of morphine sulfate and its principal metabolites normorphine, morphine-3-glucuronide, and morphine-6-glucuronide were explored. B-cell proliferation was significantly suppressed following exposure to all drugs. Production of cytokines was affected only moderately by all drugs except morphine-6-glucuronide, which produced a marked suppression at 100 M. NK cell function was unaffected by any drug except morphine-6-glucuronide, which enhanced NK cell activity at concentrations between 0.0001 and 1.0 M (figure 3). In contrast, both morphine-3-glucuronide and morphine-6-glucuronide significantly inhibited CTL induction at concentrations between 0.0001 and 100 M, whereas morphine and normorphine were inactive in this assay. These results suggest that, in general, immunomodulation produced by morphine may be at least partially independent from its pharmacological characteristics (Thomas et al. 1995*a*).

Another opiate with significant in vivo data, but only limited in vitro data, is heroin (diacetylmorphine). Heroin has been extensively associated with suppression of immunity and enhanced susceptibility to infection (Novick et al. 1991). Methadone, the treatment of choice for heroin addiction, has also been associated with immunosuppression, although limited in vitro studies have explored the nature of this immunosuppression (Novick et al. 1991). Both drugs were evaluated simultaneously in this laboratory. In general, a differential modulation of immune function was observed between these drugs. In vitro exposure to

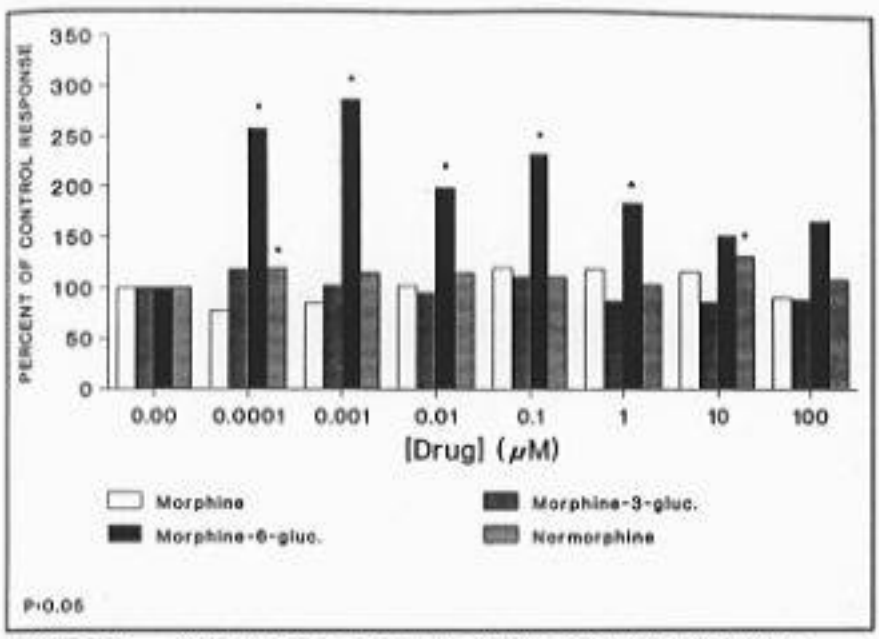

FIGURE 3. *Differential modulation of NK cell function following in vitro exposure to morphine and its metabolites.*

heroin or methadone resulted in decreased B-cell proliferation and production of IL-2 and IL-4. Cytokine production by macrophages was differentially affected, with a significant suppression of IL-6 production observed, whereas TNF production was markedly enhanced in the same cells. Induction of CTL was suppressed by exposure to heroin, whereas NK cell activity was suppressed by both drugs (Thomas et al. 1995*b*).

Finally, the potential immunotoxicity of the opioid analgesics fentanyl and meperidine was evaluated. Exposure to fentanyl and meperidine was associated with a differential suppression of IL-4 production by T-cells, as well as a more generalized suppression of cytokine production by macrophages. In addition, T-cell cytolytic activity was suppressed at high drug concentrations. B-cell proliferation and NK cell activity were also inhibited, but to a lesser degree than noted with T-cell function. Interestingly, the addition of naltrexone to the cultures did not reverse these alterations in immune function, suggesting that these changes are not mediated via opioid receptors (House et al. 1995*c*).

Mu Opioid Agonists

The mu opioid receptor agonists examined in this study have included [D-Ala^2-MePhe^4,Gly-ol^5]-enkephalin (DAMGO), metkephamide, and PL017. In general, this laboratory has shown that in vitro exposure to mu opioid receptor agonists results in immunosuppression (data not shown). More extensive studies on this group of drugs are currently in progress.

Endogenous Opioid Peptides

A number of endogenous opioid peptides have been evaluated in these studies, including met-5-enkephalin, leu-5-enkephalin, -endorphin, met- enkephalinamide, and dynorphin B. The enkephalins and endorphins are known to be secreted by cells of the immune system, and have previously been demonstrated to produce immunomodulation. This activity may be either suppressive or stimulatory, depending upon the nature of the target cells and the conditions of exposure. In the present studies, none of the peptides examined exhibited any significant effect on B-cell proliferation or production of IL-2. In comparison, production of IL-4 and IL-6 were modulated by endorphin. Although the results were statistically signifi-cant, the limited degree of bioactivity noted was of doubtful biological significance. A more consistent pattern of activity was observed with NK cell activity, which was significantly suppressed by leu-enkephalin, met-enkephalin, and met-enkephalinamide. Neither -endorphin nor dynorphin had any significant effect on NK cell activity.

Delta Opioid Receptor Agonists

Recent studies have suggested that compounds acting as delta opioid receptor agonists may exhibit immunostimulatory activity. For example, both IL-2 and IL-4 production were markedly enhanced by DPDPE at concentrations between 0.00001 and 0.1 M. Exposure to H-Tyr-D-Thr-Gly-Phe-Leu-Thr-OH (DTLET) and DPDPE was associated with a significant enhancement in NK cell activity. More extensive mechanistic studies are in progress to define the nature of this immunomodulatory activity.

Delta Opioid Receptor Antagonists—Peptidic

Representative peptides acting as delta opioid receptor antagonists, including Tyr-Tic-Phe-Phe-OH (TIPP), Tyr-D-Tic-Phe-Phe-NH_2

(d-TIPP), and ICI 174864, have been selected for testing. To date, B-cell proliferation and production of T-cell-derived cytokines following in vitro exposure to these compounds have been examined. Neither TIPP nor d-TIPP produced any consistent immunomodulation following in vitro exposure. Exposure to ICI 174864 resulted in suppression of both B-cell proliferation and cytokine production only at the 10 M concen-tration; this suppression is of doubtful biological significance due to the lack of any obvious dose relationship.

Delta Opioid Receptor Antagonists—Nonpeptidic

Several nonpeptide delta opioid receptor antagonists are currently under investigation in this project, including 7-benzylidene-7-dehydronaltrexone HCl (BNTX), naltrindole (NTI), and naltriben (NTB). In vitro exposure to BNTX or NTI results in immunosuppression at concentrations between 0.1 and 10 M, consonant with the hypothesis that delta opioid receptor agonists enhance, but delta receptor antagonists suppress, immune functional parameters. In comparison, in vitro exposure to NTB did not result in any detectable alteration in immune function.

Kappa Opioid Receptor Agonists

A number of kappa opioid receptor agonists have been examined so far, including dynorphin A (1-9), dynorphin A (1-11), dynorphin A (1-13), and dynorphin A (1-17). In general, these compounds do not exhibit a great degree of immunomodulatory activity, with the exception of B-cell proliferation. Exposure to dynorphins at concentrations between 0.1 and 10 M was associated with a significant enhancement in B-cell prolif-eration. However, due to the high (i.e., nonphysiological) concentrations, the biological relevance of these findings is uncertain.

Inhalants

A form of substance abuse of increasing interest is the recreational use of inhalants. Taken literally, this classification is useless, as it may be construed to include use of tobacco, crack cocaine, or marijuana (Sharp 1992). A better designation may be "volatile substance" abuse, a more accurate description of an important drug abuse pattern. Volatile substances that are commonly abused include glues, aerosols, anesthetics, cleaning agents, and solvents (Sharp 1992). Abuse of inhalants has been associated with a variety of toxicologies

(Tenenbein 1992). More important from the perspective of immunomodulation is the abuse of so-called room odorizers or volatile nitrites. These compounds—such as amyl nitrite, butyl nitrite, and isobutyl nitrite (IBN)—produce vasodi-lation of the cerebral vessels, resulting in euphoria (Haverkos and Dougherty 1988) and a reported enhancement in sexual function. Initially used clinically in the treatment of angina pectoris, their use appears to be prevalent in the homosexual community and may be associated with Kaposi's sarcoma (Newell et al. 1984). Use of these compounds has been demonstrated to result in decreased immune function, particularly T-cell-mediated immunity (Dax et al. 1991; Lotzová et al. 1984; Soderberg and Barnett 1991; Ratajczak et al. 1995). The association between inhalants and Kaposi's sarcoma is currently the subject of increased research interest.

EFFECT OF TOLERANCE TO AND ABSTINENCE FROM MORPHINE

In vivo exposure to morphine via implantable pellets was evaluated in female B6C3F1 mice using the model illustrated in figure 4. This system, originally developed in the rat to investigate pharmacological parameters (Bhargava and Matwyshyn 1985), was modified to accom-modate immunological assessment in the B6C3F1 mouse (Bhargava et al. 1994). The model indicated a reproducible induction of tolerance, and was subsequently used to evaluate the effect of tolerance/abstinence on immune function. Tolerance to morphine was associated with a significant suppression of host immunity. Abstinence from morphine after removal of the pellets resulted in a differential effect on the immune system, with some parameters exacerbated and other slightly ameliorated (Bhargava et al. 1994).

As a followup on these studies, additional experimental groups were included in which naltrexone pellets were coimplanted with either the morphine pellets or placebo pellets (Bhargava et al. 1995). Implantation of naltrexone pellets reversed the morphine-induced analgesic response, as was anticipated. Once this model was in place, the same exposure regimen was utilized to examine the effects on immune function. As demonstrated previously, implantation of morphine pellets produced a significant suppression in B-cell proliferation. Coimplantation of naltrexone pellets did not reverse this suppression, but rather appeared to slightly exacerbate suppression of B-cell function (figure 5). Also consistent with earlier findings, morphine exposure resulted in suppres-ion of IL-2 production by T-cells. Unlike the B-cell response, the

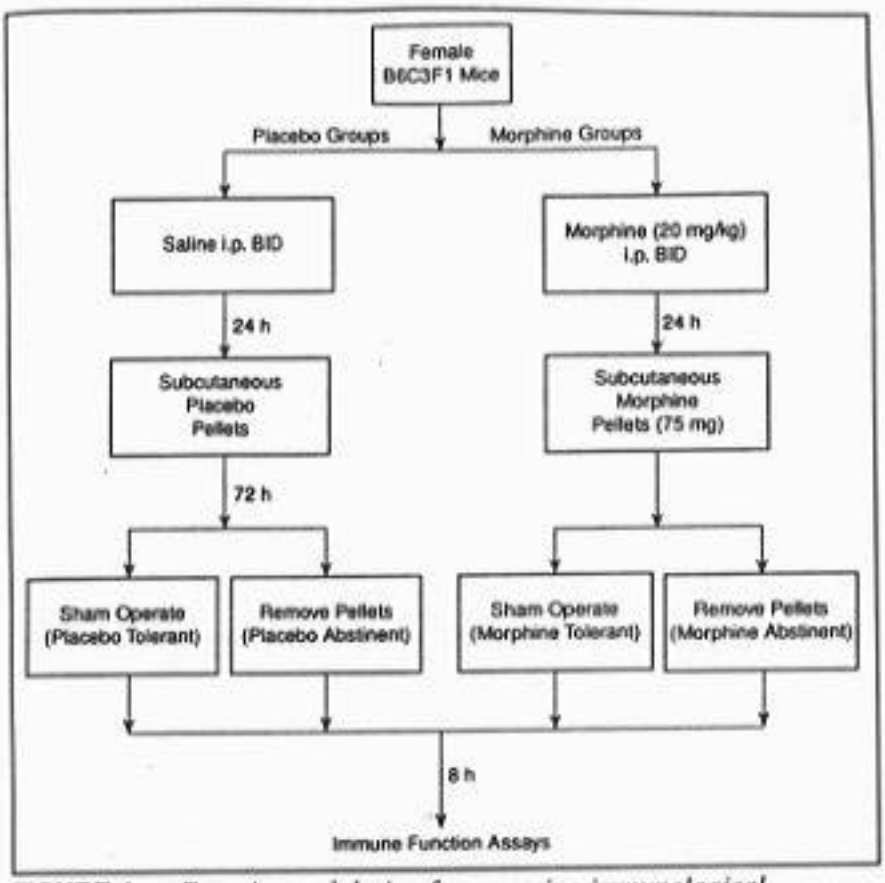

FIGURE 4. *Experimental design for assessing immunological consequences of morphine tolerance/abstinence.*

suppression of IL-2 production was completely reversed in animals coimplanted with naltrexone pellets (figure 6a). In contrast, IL-4 production was unaffected by morphine or naltrexone (figure 6b).

EFFECT OF TOLERANCE TO AND ABSTINENCE FROM L-TRANS-^{9}-TETRAHYDROCANNABIN

An experimental model of tolerance/abstinence for tetrahydrocannabinol (THC) exposure was also developed as illustrated in figure 7, and was used to evaluate the effect of THC exposure on immune function (Bhargava et al., submitted). Mice were injected subcutaneously (SC) with THC (10 milligrams per kilogram (mg/kg)) twice daily for 4 days. On day 5, analgesic and hypothermic responses to THC were determined. Multiple injections of THC resulted in the development of tolerance to both the analgesic and hypothermic effects of THC.

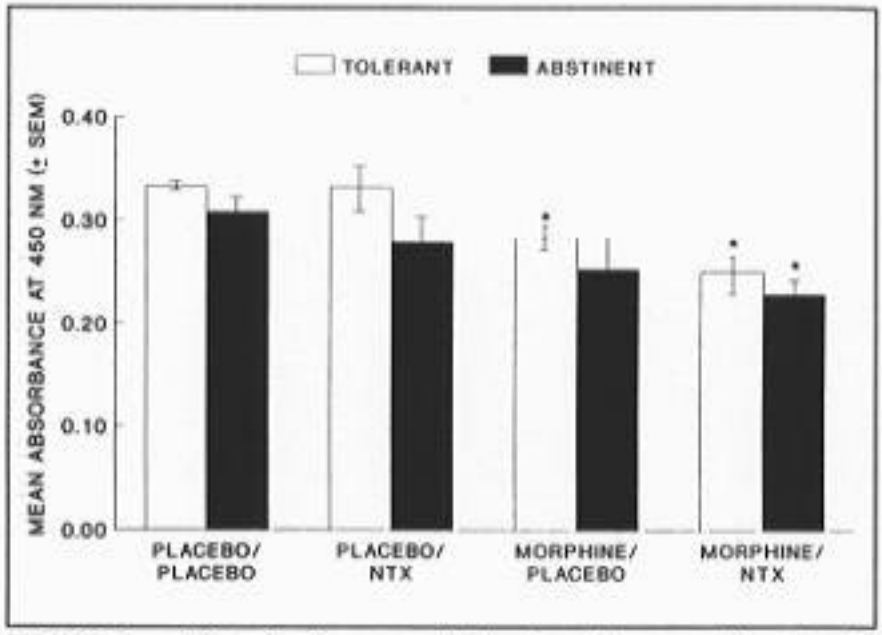

FIGURE 5. *Effect of naltrexone pellet implantation on proliferation of B-lymphocytes from placebo or morphine pellet-implanted mice.*

Immune function studies were subsequently performed on mice rendered tolerant or abstinent by this procedure. Neither tolerance nor abstinence to the 4-day THC administration had any effect on bodyweight or thymus weight and cellularity, although spleen weight and cellularity were both decreased in THC-abstinent animals. Likewise, there were no significant effects on B-cell proliferation observed in either group. Production of IL-2 by cells was suppressed in both tolerant and abstinent mice, whereas production of IL-4 was significantly suppressed only in THC-abstinent mice. Significant suppression of CTL and NK cells activity was only observed in THC-abstinent mice. These results suggest that THC-mediated modulation of the immune response may result from a differential effect on cellular populations.

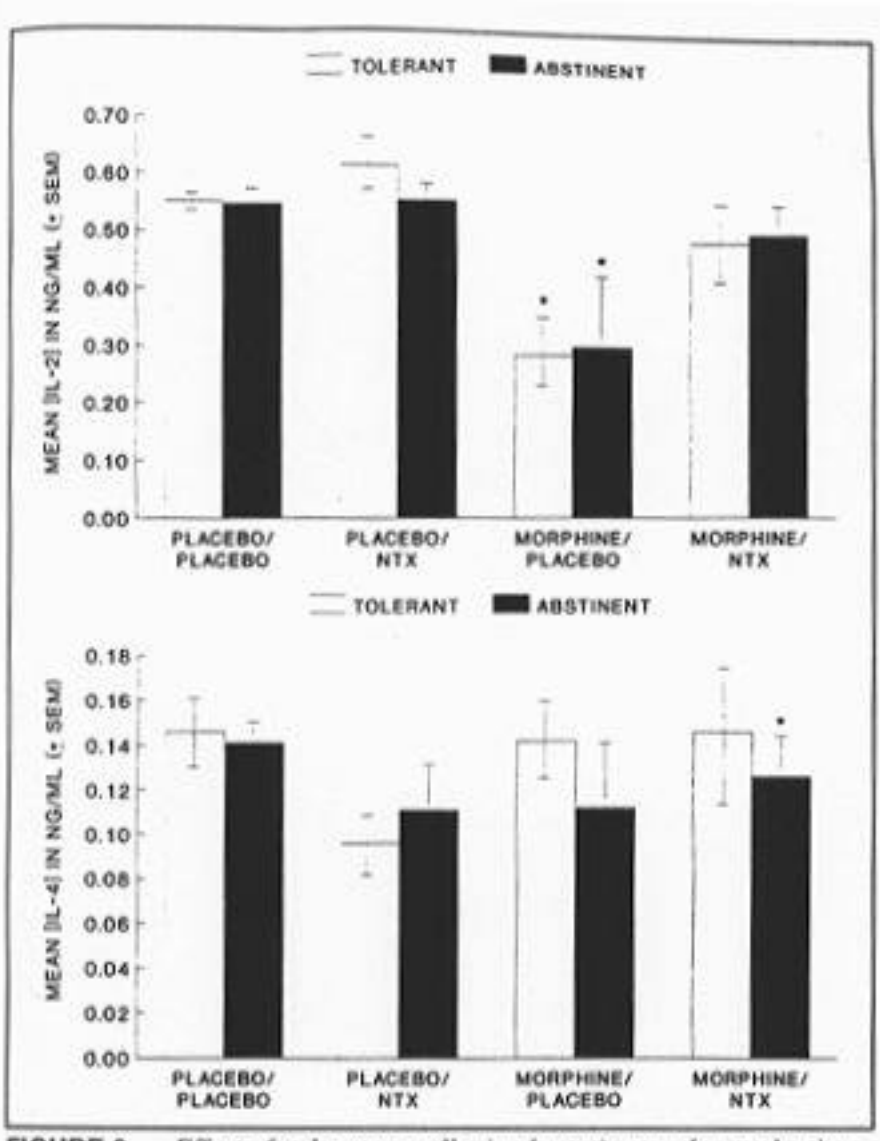

FIGURE 6. *Effect of naltrexone pellet implantation on the production of IL-2 (panel A) and IL-4 (panel B) by lymphocytes from placebo- or morphine pellet-implanted mice.*

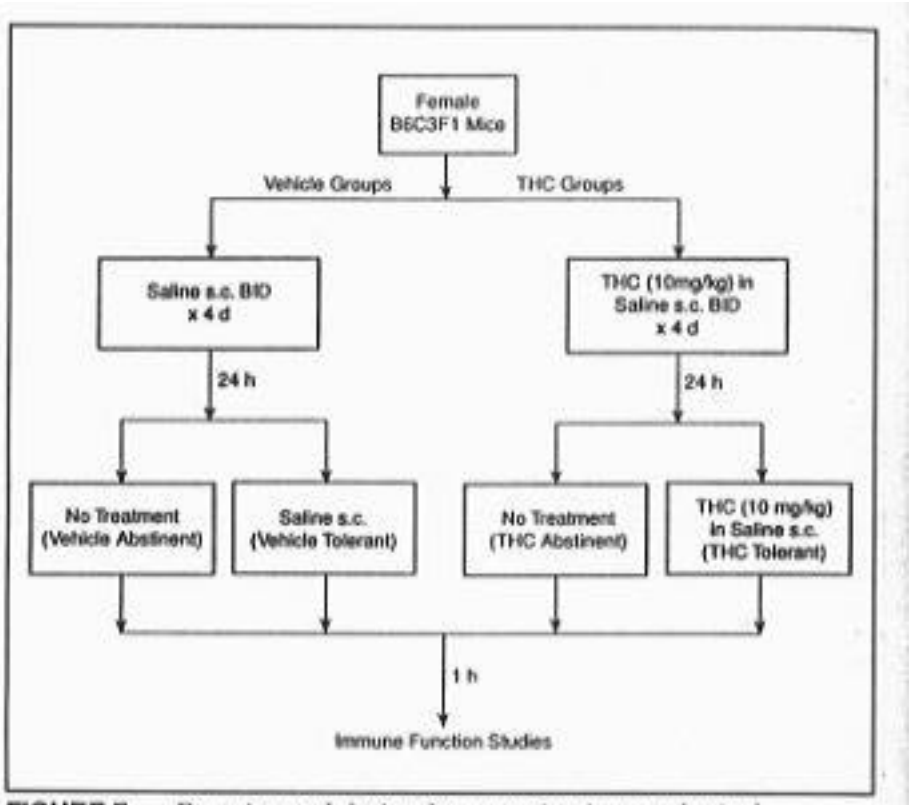

FIGURE 7. *Experimental design for assessing immunological consequences of THC tolerance/abstinence.*

RECOMMENDATIONS FOR THE FUTURE

Continuation of In Vitro Screening Program

This program has resulted in the development of a rapid and cost-effective screening protocol that has produced a substantial database of information on the in vitro and in vivo effects of abused drugs on immune function. The advantages of such a program include examination of the effects of drugs of abuse on individual cells and components of the immune system, evaluation of the immunomodulatory effects of both parent compounds and metabolites, and generation of dose-response curves for immune function at pharmacologically relevant drug concentrations.

Delta Opioid Receptor Agonist Compounds as Immunomodulators

As mentioned previously, a potentially significant observation made in these studies is that the delta opioid receptor agonist compounds

tend to exhibit distinct immunostimulatory activity at picomolar to nanomolar concentrations. In addition, both peptide and nonpeptide delta receptor antagonists tend to suppress both B-cell and T-cell function. This trend is consonant with the authors' hypothesis that, in general, delta receptor agonists and antagonists are immunostimulatory and immunosuppressive, respectively. Data obtained in these various studies will be invaluable in designing novel immunostimulant or immunorestorative drugs based on this class of compounds.

Potential of Kappa Opioid Receptor Agonists To Produce Immunomodulation

A considerable body of data has been published documenting the immunosuppressive nature of compounds active at the mu opioid receptors (e.g., heroin, morphine). As described above, the delta opioid receptor-specific compounds appear to be immunostimulatory. However, the role of the third major type of opioid receptor, the kappa receptor, in immune function has not been described in detail. Defining the role of this receptor in immunomodulation would be vital in understanding the nature of neuroimmune interactions. To date, the kappa opioid receptor agonists dynorphin A (1-9), dynorphin A (1-11), dynorphin A (1-13), dynorphin A (1-17), dynorphin B, and U-50488H have been evaluated for immunomodulatory activity in the B-cell proliferation and T-cell cytokine production assays only. This group of drugs produces only marginal effects on B-cell proliferation, cytokine production, or NK cell activity. These drugs have not yet been evaluated for effects on CTL induction or macrophage cytokine production. These assays will be necessary to more fully examine any possible immunomodulation by kappa opioid receptor specific compounds.

Development of Animal Models of Polydrug Abuse

It is well established that drug abuse increasingly involves the use of multiple agents (e.g., ethanol, cocaine, heroin, THC). As mentioned previously, the authors' laboratories have developed in vivo models for tolerance to and abstinence from morphine and ^{9}-THC in B6C3F1 mice. This particular mouse strain has been used extensively by the National Toxicology Program, resulting in a sizable database of background toxicology and immunology data. In addition, a similar model for in vivo exposure to cocaine is in the preliminary stages of development. These models reveal that in vivo exposure to these drug types results in a significant suppression of a variety of immune

effector and regulatory mechanisms; this suppression is at least partially reversible with antagonists. These models will be used for determining the immuno-modulatory effect of polydrug exposure. In these studies, mice tolerant to morphine, THC, or cocaine will also receive various other drugs in combination. These studies are expected to provide vital information on the nature of immune dysfunction following drug exposure relevant to human usage.

Abused Drugs as Cofactors in AIDS-Related Infections

Another area of interest relevant to this work is the role of drug abuse in the pathology of AIDS (Pillai et al. 1991). Two particular research areas that would generate valuable information are drug exposure in animals infected with opportunistic infections and the potential reconstitution of suppressed immunity by drug treatment. In the first instance, opportunistic infections are normally benign organisms that often lead to fulminant infections in immunocompromised hosts. Examples include tuberculosis, toxoplasmosis, and pneumocystosis. An animal model of some interest is murine acquired immunodeficiency syndrome (MAIDS), a murine analog of the human disease. There is a need for studies to evaluate the potential of drugs of abuse or potential medications to either exacerbate or ameliorate this condition.

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Inhalation Studies With Drugs of Abuse

Yun Meng, Aron H. Lichtman, D. Troy Bridgen, and Billy R. Martin

INTRODUCTION

In recent years, smoking or inhalation of drugs has become a popular route of administration among drug users. Various drugs of different classes have been abused by inhalation or smoking, including phencyclidine (PCP), cocaine, heroin, methamphetamine, and marijuana. This increased popu-larity of smoking drugs has resulted from the fast onset of drug action and fears related to contracting acquired immunodeficiency syndrome (AIDS) or other infectious diseases from intravenous (IV) injections. In particular, heroin smoking has increased dramatically; approximately 74 percent of the heroin addicts in India use this method of administration, and it is also popular in the United States (Griffiths et al. 1994). The shift toward smoking heroin has also been associated with an increased availability of illicit heroin on the streets, which has declined in price and increased in purity (Huizer 1987). Consequently, there is an increased risk of overdose.

Inhalation is a very potent route of drug administration, and is characterized by fast absorption from the nasal mucosa and the extensive lung capillaries. Inhalation results in an immediate elevation of arterial blood drug con-centration and a higher bioavailability due to avoiding drug metabolism by the liver. The fact that smoking or inhalation provides rapid delivery of drugs to the brain may result in an immediate reinforcing effect of the drug and further contribute to its abuse liability or risk of dependency. Smoking or inhalation of drugs may also lead to other adverse effects, such as pulmonary diseases or deleterious cardiovascular consequences. Moreover, the parent drugs may be degraded, leading to inhalation of toxic pyrolytic products (Benowitz 1990; Wesson and Washburn 1990). For example, there have been reports of heroin leucoencephalopathy, a life-threatening condition that has occurred in some individuals after smoking heroin (Wolters et al. 1982). This condition was not associated with IV administration and no impurities present in the drug were identified as the cause, but may have been caused by pyrolytic products of either heroin or impurities present in the samples (Wolters et al. 1982).

The rapid increase in smoking drugs of abuse raised concerns about drugs that are currently abused using other routes of administration: Can they also be

smoked? Furthermore, it is unknown whether their abuse liability, side effects, and toxicity would be increased by smoking. Thus the major goal of this research has been to develop guidelines for predicting which drugs can potentially be abused by smoking or inhalation.

To reach this goal, the first step was to establish a predictive parameter for the volatility of drugs. Underscoring the importance of volatility is the practice of smoking various drugs of abuse in combination or with other agents in an effort to enhance their volatility and thereby increase their pharmacological effects. For example, the addition of either caffeine or barbiturates has been shown to improve the volatilization of heroin (Huzier 1987).

One physiochemical parameter that might play a critical role in the volatility of a compound is vapor pressure. Accordingly, the authors hypothesized that vapor pressure could be positively correlated with both volatility and pharmacological potency after smoking. Thus, the vapor pressures of a variety of drugs were determined, and then the volatili-zation of selected compounds was studied.

A second goal of this work was to investigate the volatilization of these drugs and identify their major pyrolysis products. Analytical methods employing gas chromatography/mass spectrometry (GC/MS) and high performance liquid chromatography (HPLC) were developed to identify and quantify drugs and their pyrolytic products after volatilization.

Despite the increase in smoking and inhalation of drugs of abuse, relatively little is known about the consequences of this route of administration. Therefore, the third research goal has been to develop a reliable animal model to evaluate the pharmacology and biodisposition of drugs after inhalation. In these studies, the pharmacological effects of volatilized drugs were assessed in mice. In addition, the brain, plasma, and whole-body levels of drugs occurring after inhalation were quantified in order to obtain biodisposition information. These studies provided the strategy and the tools for prediction of drugs that can be abused by smoking or inhalation.

VAPOR PRESSURE AND VOLATILITY

In general, absorption of inhaled drug is dependent upon the physical characteristics of the drug, including particle size, lipid solubility, and volatility. Clearly, a drug's volatility would play an important role in determining its inhalation potential. Drug volatility is determined by many factors, including boiling point, melting point, and vapor pressure. Since many abused substances are less volatile than organic solvents and are smoked

at very high temperatures, effects of melting and boiling points on the volatilization would be negligible. On the other hand, vapor pressure may serve as a good indication of volatility.

$$pV = nRT$$

Based on the ideal gas law,

equation 1

where p is the partial pressure of the gas, R is the gas constant, n is the number of molecules, V is the volume of the gas phase of the compound, and T is the temperature in kelvin, the partial pressure of the gas becomes the vapor pressure (P_v) when the gas and liquid phases of the compound reach equilibrium at a particular temperature. Then, equation 1 can be expressed as

equation 2

where C = n/V and is the concentration of the compound in the gas phase and is directly proportional to the vapor pressure. This relationship suggests that the vapor pressure of a compound is positively correlated with volatility; the higher concentration of the gas, the more volatile it is. Therefore, the volatility of a drug at a certain temperature is determined by its vapor pressure and the volatilization temperature.

$$P_v = CRT$$

Due to a lack of information on vapor pressure for a variety of drugs of abuse and related compounds, the authors determined this parameter by an indirect method based on a system using gas chromatography and relative retention times. This approach is a modification those described by others (Hamilton 1980; Westcott and Bidleman 1981; Bidleman 1984). The original method is based on in the relationship between solid vapor pressure and GC column retention time (or volume retention time, V_R), and has been used in determination of vapor pressures for herbicides, pesticides, and a variety of nonpolar organic compounds. The vapor pressure (P_v) of two substances at the same temperature (as well as their latencies of vaporization, L_V) are related by

equation 3 $\ln P_1 = (L_1/L_2) \ln P_2 - C$

$\ln [(V_R)_1/(V_R)_2] = [1-(L_1/L_2)] \ln P_2 - C$

and the fact that vapor pressure has been shown to be related to column retention volumes by equation 4

The relationship between vapor pressure and column retention times can be determined from the combination of equations 3 and 4:
equation 5

Therefore, a plot of ln $[(V_R)_1/(V_R)_2]$ versus ln P_2 should yield a straight line with either a positive or negative correlation coefficient depending on the ratio of $(V_R)_1/(V_R)_2$. The value of (L_1/L_2) can be calculated from the slope and thus P_1 determined from equation 3. If substances 1 and 2 are the unknown and standard compounds, respectively, then the vapor pressure of the unknown at a given temperature can be determined. The relationship between vapor pressure and temperature can be simply described by the Clausius-Clapeyron equation:
equation 6

Therefore, the vapor pressure at any temperature can be extrapolated by the linear regression between ln P and 1/T.

Since this technique has primarily been used for estimating vapor pressures of pesticides, these chemicals were utilized to establish a working model to determine the vapor pressures of drugs of abuse. The authors' strategy was to determine the vapor pressure of drugs of abuse by employing a pesticide with known P_v values as a standard. A GC/MS was equipped with a 4-meter capillary column. The helium carrier gas was adjusted to a 1.11 milliliter per minute (mL/min) flow rate and a 78.26 mL/min split for a split ratio of 79:1. The injector port was kept at 200%C, detector port at 225%C, and the source of the MS at 200%C. The oven temperature was kept constant during any given analysis. All test compounds were dissolved in hexane or

chloroform at 0.5 to 4.0 milligrams per mL (mg/mL) in order to obtain a substantial peak from a 1 microliter (L) injection. Since eicosane and octadecane are commonly used as standard compounds in determining the vapor pressure of other pesticides, both compounds were used to standardize the GC column. Various pesticides (nonpolar organic compounds), such as naphthalene, phenanthrene, pyrene, and benzo[a]pyrene were then injected and retention times obtained for 5 to 8 temperatures at 10%C increments, ranging from 40%C to 190%C. The natural logs of the ratios of the retention times of the standard and test compounds were then plotted against the natural log of the vapor pressure of the standard at each temperature.

Both octadecane and eicosane standardization yielded vapor pressures of the pesticides very close to published values. Of these two compounds, the values obtained with eicosane exhibited a higher correlation coefficient. Thus, eicosane appeared to be the better standard for approximating the values of various drugs of abuse. However, the plot of relative retention time ratios of several of the drugs of abuse to the published vapor pressure of eicosane (equation 5) correlated poorly (table 1). This result eliminated eicosane as a standard for measuring the vapor pressures of drugs of abuse. In the search for another standard, dibutyl phthalate proved to be a good candidate. Using eicosane as a standard, the vapor pressure of dibutyl phthalate, at 25%C, was determined as 6.89×10^{-5} torr, which fell within the published vapor pressure range of 1.2×10^{-6} to 4.4×10^{-5} torr (Small et al. 1948). By the same method, the vapor pressures of dibutyl phthalate at different temperatures were then determined against eicosane. Equation 6 was then solved for dibutyl phthalate: $\ln P = A + B/T$ where $A = 25.179$, $B = -10{,}364$, P is in torr and T is in kelvins. Using dibutyl phthalate as a standard, the natural log of the relative retention time ratios plotted against the natural log of its vapor pressures at the respective temperatures yielded a high correlation for various drugs of abuse (figure 1). Thus, vapor pressures at 25%C could be approximated for drugs representing a variety of classes (table 1).

Vapor pressures of selected compounds are listed in table 1. In comparing different classes of drugs, the opioids appear to have relatively low vapor pressure, suggesting that they are less volatile than other drugs. As can also be seen in table 1, nicotine exhibited relatively high volatility, which is consistent with the fact that cigarette smoking is the most popular method of nicotine administration. Vapor pressures for methamphetamine and amphetamine appeared to be higher than that of nicotine at 25%C, but their vapor pressures could not be determined by the present method since their volatility exceeded the range of the standard at the temperatures that were

TABLE 1. *Determining the vapor pressures of drugs of abuse using dibutyl phthalate as the standard.*

Drugs (Free base)	Correlation coefficient of In $(VR)_1/(VR)_2$ to In P: Eicosane	Correlation coefficient of In $(VR)_1/(VR)_2$ to In P: Dibutyl phthalate	Vapor pressures (Torr, at 25%C)
Nicotine	A	0.871	2.61×10^{-2}
MDMA	—	0.997	4.47×10^{-3}
Caffeine	—	0.997	8.56×10^{-4}
PCP	—	0.984	1.49×10^{-4}
Secobarbital	—	0.996	5.72×10^{-5}
Pentalbarbital	—	0.948	4.16×10^{-5}
Methaqualone	0.751	0.896	1.60×10^{-5}
Cocaine	0.748	0.996	9.79×10^{-6}
Morphine	—	0.943	9.49×10^{-7}
9-THC	—	0.986	1.01×10^{-7}
Heroin	0.981	0.983	5.71×10^{-8}
Fentanyl	—	0.982	2.41×10^{-8}

KEY: A = Values were not determined.

studied. Compounds with high vapor pressures are predicted to be much more volatile and consequently more likely to be smoked than those possessing low vapor pressures.

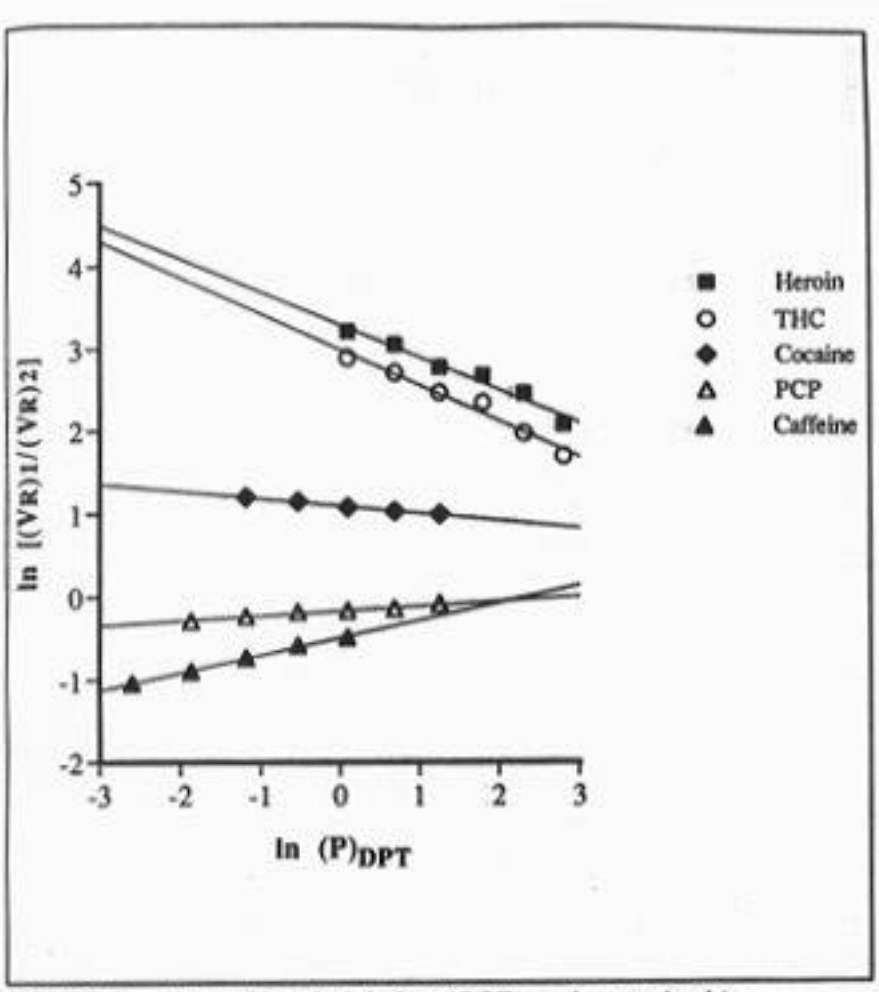

FIGURE 1. *Using dibutyl phthalate (DPT) as the standard in determining vapor pressures of drugs of abuse. Plots represent the linear correlation between the natural log of the vapor pressure of DPT and the natural log of ratios of retention times at different temperatures. Positive correlations indicate that the test compounds have higher vapor pressures than the standard, while negative correlations indicate that the test compounds have lower vapor pressures than the standard.*

INHALATION EXPOSURE OF DRUGS OF ABUSE IN THE MOUSE INHALATION MODEL: PHARMACOLOGICAL ASSESSMENT

Although drug volatility can provide fundamental information about the inhalation potential for drugs of abuse, it is only one of many factors necessary for producing a pharmacological effect. Pharmacokinetic and pharmacodynamic considerations have considerable bearing on a drug's ability to produce an effect. Presently, relatively little is known about the potency of drugs of abuse after inhalation or smoking. In order to deter-mine the relationship between volatility and pharmacological potency by the inhalation route, the authors developed an animal model to approx-imate the conditions of human inhalation. The approach involved a volatilization-inhalation drug delivery system developed over the past 10 years in this laboratory. The design of this inhalation apparatus is illustrated in figure 2.

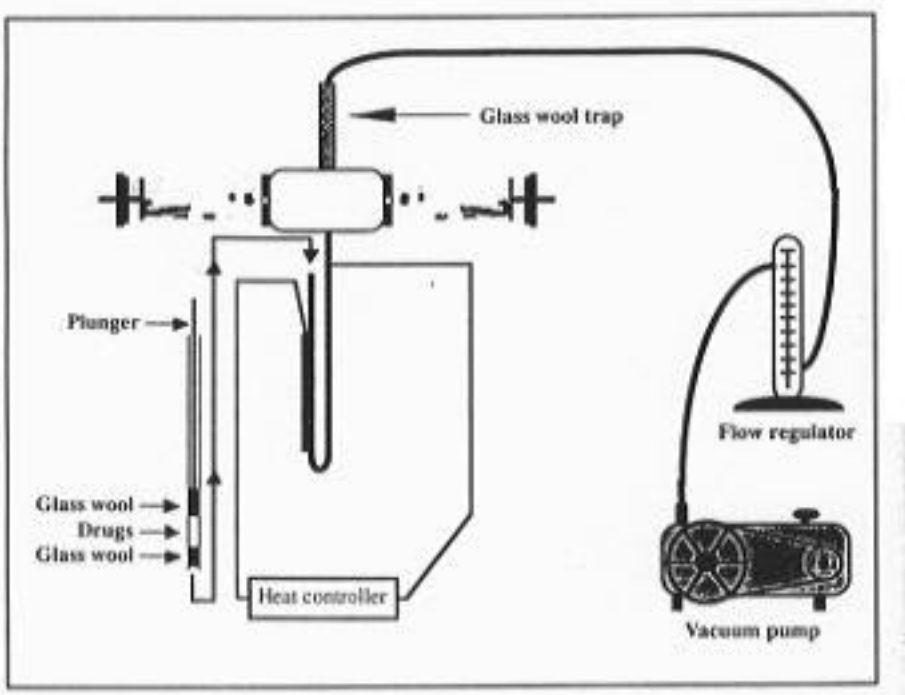

FIGURE 2. *Mouse inhalation apparatus.*

The apparatus consisted of a U-shape glass volatilization pipe preheated to a designed temperature, a nose-only exposure unit containing six mice, a glass wool trap (packed with 0.5 g of glass wool fiber) that sequestered the vaporized test compound, and a vacuum system that created negative pressure and pulled the air through the

entire apparatus. The airflow was regulated at a rate of 400 mL/min by a flow meter placed after the glass wool trap. The entire system was contained within a hood. A known amount of test compound (either in dry powder or liquid form), packed in between two pieces of glass wool in a glass plunger, was injected into the preheated pipe and volatilized. Animals were exposed to the vapor for 5 minutes, and then the appropriate pharmacological effects for each respective drug were measured.

These studies allowed the authors to optimize the volatilization conditions by using a pharmacological endpoint. Once conditions for volatilizing a drug had been established, then pharmacological potency was determined by exposing mice to different quantities of volatilized drug. This strategy represents a deviation from traditional inhalation approaches involving optimization of volatilization using analytical methods. The rationale for using a pharmacological endpoint is that failure of an agent to produce behavioral effects via inhalation renders the analytical considerations moot.

To determine the feasibility of evaluating the volatilization of drugs with different pharmacological properties, several compounds that are abused by smoking or inhalation were selected. It is well known that changing the route of administration has important effects on the development of drug dependency (Griffiths 1994); thus the authors elected to compare pharma- cological effects after inhalation and IV administration. Heroin was chosen because of the shift toward smoking among heroin users and the lack of systematic studies on the pharmacological potency, onset, and duration of action of smoked heroin. Other drugs of abuse such as PCP and meth-amphetamine are also commonly smoked (Wesson and Washburn 1990). PCP was initially abused by oral and IV administration routes associated with many adverse effects.

The discovery that smoking PCP-laced cigarettes allowed for a better titration of doses and fewer side effects propelled it to the forefront of drug abuse. Previously, the authors studied the pyrolysis of PCP in parsley cigarettes, which employed a much higher temperature than the currently used system (Freeman and Martin 1981; Lue et al. 1986, 1988; Martin and Boni 1990). Those studies demonstrated that more than 50 percent of the drug was delivered intact. The pharmacology of smoked PCP-laced cigarettes in mice and rats has also been characterized (Freeman and Martin 1982; Martin and Freeman 1983; Wessinger et al. 1985).

The incidence of inhalation or smoking of methamphetamine has risen very recently in the United States. Several investigators have examined the volatilization and pyrolysis of this compound (Cook et al. 1991, 1993; Miller and Kozel 1991). Sekine and Nahahara (1987) studied the volatilization of methamphetamine applied to tobacco cigarettes and found that about 15 percent of the drug was delivered in the main stream of the smoke and several pyrolysis products were formed. Studies of the volatilization, biodisposition, and pharmacokinetics of smoked metham-phetamine hydrocloride (Cook et al. 1991, 1993; Perez-Reyes et al. 1991) demonstrated that this compound can be easily volatilized in the temperature range of 200 to 400%C, while 90 percent of the parent drug is delivered intact. The pharmacological effects of methamphetamine administered by inhalation or IV injection appeared to be similar.

Using the rodent inhalation model depicted in figure 2, mice were assessed for locomotor activity after exposure to methamphetamine vapor, antinociception after exposure to heroin, and motor coordination after exposure to PCP. Temperatures used for volatilization of these drugs are listed in table 2. These temperatures were empirically derived

TABLE 2. *Relative potencies of drugs by inhalation exposure and IV administration.*

Drugs	Inhalation ED_{50} (mg)[A]	IV ED_{50} (mg/kg)
Heroin [B]	1.1 [B]	0.28
PCP HCl [C]	2.8 [C]	0.1
Methamphetamine-HCl [D]	3.9 [D]	0.9

KEY: A = Based upon the amount of drug added to the volatilization chamber. B = Free base volatilized at 250%C. C = Volatilized at 275%C. D = Volatilized at 200 %C. ED_{50} = Median effective dose.

based upon their pharmacological effectiveness in mice in inhalation studies. In order to measure the antinociceptive effects of heroin, the tail-flick apparatus described by Dewey and colleagues (1970) was used. Fifteen minutes after the inhalation exposure or IV administration of heroin, the mouse's tail was placed under a radiant heat lamp and the amount of time required for the animal to flick its tail from under the heat source was recorded. Baseline reaction latencies ranged from 2 to 4 seconds, and the maximal allowable reaction time was 10 seconds. The percent of maximal percent effect (percent MPE) was calculated for each animal. Volatilization of heroin free base resulted in a dose-related antinociception with maximal effects occurring in mice exposed to the vapor from 3 mg of heroin free base. Dose-response curves generated for heroin are illustrated in figure 3 and the ED_{50} values are listed in table 2. These data clearly show that heroin-induced antinociception is qualitatively similar after inhalation and IV administration. Similar to the results in the present study, smoking heroin has been reported to be as potent as IV heroin in humans (Jenkings et al. 1994). In a controlled clinical study, the pharmacokinetic and pharmacodynamic profile of smoked heroin was evaluated in human subjects. It was demonstrated that the behavioral effects of smoked heroin were as potent and rapid in onset as IV administration.

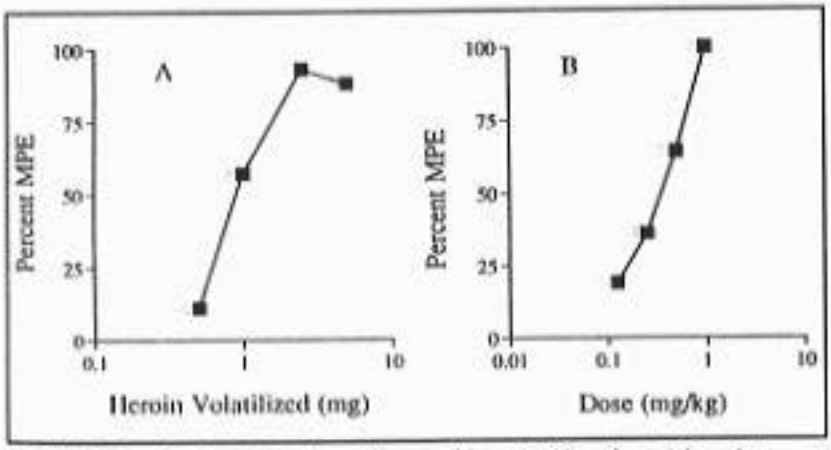

FIGURE 3. *Antinociceptive effects of heroin (free base) in mice after inhalation exposure (panel A) or IV administration of heroin HCl (panel B). All subjects were assessed in the tail-flick test 15 min after drug administration. The results are the means of at least six mice/group.*

Stimulant effects are readily quantified by measuring spontaneous activity. For these studies mice were placed in individual photocell activity cages (6.5 x 11 in) with 16 photocell beams per chamber. Individual mice were placed into one of six chambers and allowed to acclimate for 10 minutes. They were removed from the activity chambers and either injected IV with saline or drug or exposed to volatilized methamphetamine. Immediately after the injection or inhalation exposure, the mice were returned to the chambers, and interruptions of the photocell beams were recorded for the next 40 minutes using an animal activity monitor. Activity in the chamber was then expressed as the total number of beam interruptions for the total 40 minutes. Maximal possible stimulation was determined from double reciprocal plots of photocell interruptions versus dose so that the data could be expressed as percentage of maximal stimulation. Methamphetamine produced a dose-related stimulation of spontaneous activity with maximal stimulation occurring with volatilization of 25 mg of drug. This stimulation was comparable to that produced by IV administration of methamphetamine in the dose range of 0.5 to 4.0 mg/kg (figure 4). The ED_{50} of metham-phetamine by both routes of administration are summarized in table 2.

The final drug to be evaluated for pharmacological effects following inhalation was PCP hydrochloride (HCl). Motor incoordination produced by PCP HCl was evaluated by the inverted-screen test (Coughenour et al. 1977). Fifteen minutes after drug exposure, the mice were placed on a wire screen that was immediately inverted. The percentage of animals that climbed onto the top within 60 seconds was recorded. Only mice successfully completing the task in a pre-experimental test were used. Mice (in groups of six) exposed to the volatilization of PCP HCl in the range of 2 to 6 mg exhibited a dose-related inhibition of motor function as depicted in figure 5. Mice exposed to the volatilization of 1 mg of PCP displayed altered behavior to a greater degree than those exposed to 2 mg; this most likely represents an aberration. The disruption of motor function following inhalation is comparable to that produced by IV administration of PCP in the dose range of 0.03 to 1.0 mg/kg.

These inhalation studies demonstrate the feasibility of evaluating the potency of drugs with different pharmacological actions following inhalation exposure. The comparison of pharmacological effects after both inhalation and IV administration revealed very similar dose-response relationships for each of these three drugs. However, valid potency comparisons can be made between inhalation and IV administration only

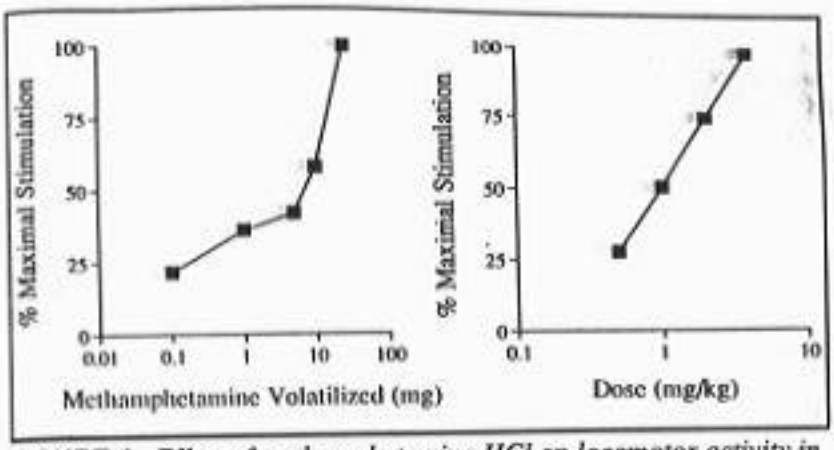

FIGURE 4. *Effect of methamphetamine HCl on locomotor activity in mice after inhalation exposure (panel A) or IV administration (panel B). Control mice produced 2,892±265 interruptions of the photocell beams whereas exposure to the vapors from 30 mg of methamphetamine produced 13,503±652. IV administration of 4.0 mg/kg of methamphetamine HCl resulted in 13,224±1420 interruptions of the photocell beams. The effects produced by the other doses of drugs are expressed as percentage of this maximal stimulation produced by methamphetamine. The results are the means of at least six mice/group.*

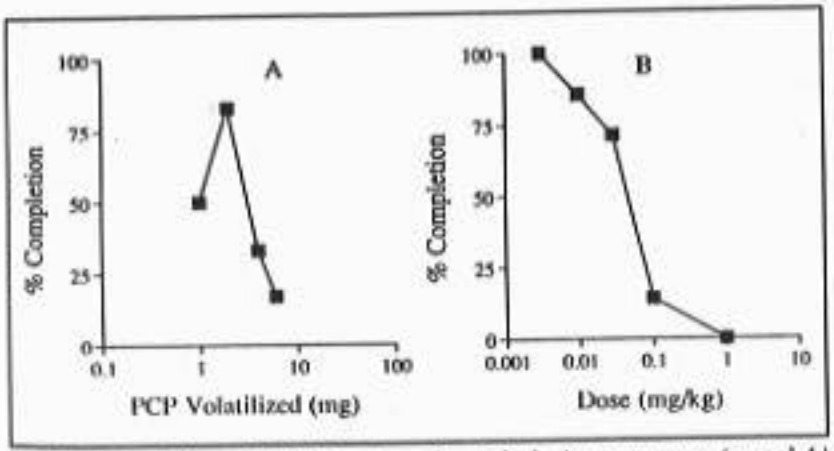

FIGURE 5. *Effect of PCP in mice after inhalation exposure (panel A) or IV administration (panel B) in the inverted-screen test. The results are the means of at least six mice/group.*

if the biological concentrations of the parent drug are known. In addition to differences in the pharmacokinetics of drugs administered by these two routes, the dose of the drug administered by inhalation must be deter- mined. In the studies described above, the potencies are expressed in terms of amount of drug added to the volatilization apparatus. In order to establish dosimetry, the animals are exposed to the volatilization of the radiolabeled drug under the same conditions used for the potency measurement. Whole-body determination of total radioactivity provides the dose of the volatilized drug.

An additional possible confound is the formation of pyrolysis products during volatilization, which could contribute pharmacological effects. The authors' strategy is to utilize volatilization conditions in such a way that minimal pyrolysis occurs, thus eliminating the possible contribution of pharmacological effects and simplifying determination of dosimetry.

To demonstrate the feasibility of this approach, the authors have chosen to determine the volatilization and dosimetry of heroin for the purpose of making valid potency comparisons between IV and inhalation exposure.

VOLATILIZATION OF HEROIN

Heroin represents a logical choice for establishing inhalation procedures. Smoking and inhalation of heroin, known as "chasing the dragon," have largely replaced opium smoking for almost a century. It has become the most popular method of heroin use in recent years due to searches for alternatives to IV injections and the drug's increased availability. The most common method of smoking heroin involves heating the drug on a piece of aluminum foil and inhaling the vapor. An often reported obser-vation is that the aluminum foil contains black residues of decomposed heroin after smoking. In pyrolysis studies, Huzier (1987) and Cook and Jeffcoat (1990) reported that heroin undergoes extensive decomposition at temperatures that are presumably required for volatilization.

Studies of the volatilization of the drugs of abuse together with the assessment of pharmacological effects in laboratory animals after inhalation exposure can provide important information for drug inhalation potential in humans. Using the same volatilization-inhalation apparatus as shown in figure 2, the volatilization of heroin was investigated. Heroin free base, 1 and 5 mg doses, was heated for 5

minutes at 250%C. The glass wool trap and the pipe were then flushed with ethanol. Concentrations of the heroin and its pyrolytic products were analyzed by GC/MS and HPLC.

Results from volatilization of heroin free base indicated high efficiency at the employed temperature. The extent of volatilization after 5 minutes of heating appeared to be independent of the drug quantity. At 250%C, heroin was found to be volatilized over 75 percent. More than 90 percent of the initial amount was recovered as unchanged heroin after volatilization. Monoacetylmorphine, the only degradation product that resulted from the volatilization of heroin, accounted for less than 5 percent of the total drug. In contrast to the authors' results, Cook (1991) found that heroin was extensively degradated to a variety of products, including 6-acetyl morphine, N,6-diacetylnormorphine, and N-acetylnormorphine, after heating in a quartz furnace tube at 250%C. On the other hand, Huizer (1987) studied the pyrolysis of both heroin free base and its hydrochloride salt by using a TAS oven (a thermomicro separation, transfer, and application procedure) or heating on a piece of aluminum foil. They found that heroin HCl required a higher volatilizing temperature than the free base to completely

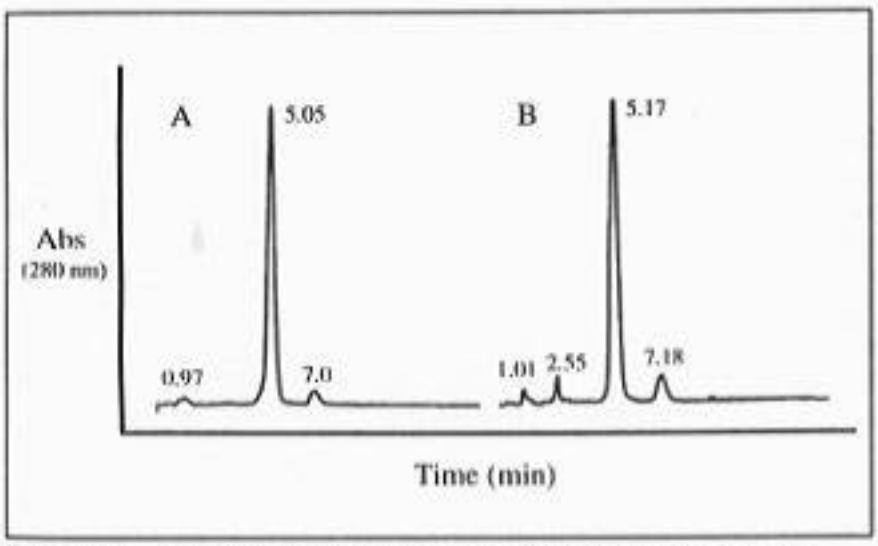

FIGURE 6. *HPLC chromatograms of standard heroin free base in ethanol solution (panel A.) and ethanol extract of glass wool from volatalization of heroin free base (panel B.). Retention times: heroin free base, 5 min; monoaceto-morphine, 2.5 min; and monoacetocodine, 7 min. Mono-acetocodeine is an impurity present in the heroin free base.*

volatilized (275 versus 225%C for the salt and free base, respectively). Heating heroin HCl resulted in the parent compound and 6-monoacetyl-morphine, along with small amounts of N,6-diacetylnormorphine and N,3,6-triacetylnormorphine; the amounts of the later two pyrolysis products increased when the temperature was increased from 275 to 325%C. Pyrolysis of heroin free base produced mainly heroin and 6-monoacetyl-morphine; as the temperature was increased from 225 to 325%C, trace amounts of morphine were detected. Analyses of the results from heating heroin on aluminum foil indicated that 17 and 62 percent of the heroin salt and free base, respectively, were recovered in the condensate. This later value is similar to the percentage of heroin recovered in the present study. Huizer's method was similar to the system described in this chapter in terms of employing a steady airflow through the system. Variations in the composition and nature of the heroin samples being smoked by users may be expected to influence volatilization efficiency. Huzier (1987) also demonstrated that the presence of caffeine and barbiturate increased the volatilization of heroin free base and the hydrochloride salt, respectively. Taken together, these results demonstrate that, in addition to volatility, temperature and airflow are important determinants of the volatilizing efficiency of a drug.

BIODISPOSITION OF HEROIN AFTER INHALATION EXPOSURE

The ED_{50} values obtained from inhalation studies (table 2) were based on the amounts of drug that were added into the pipe prior to volatilization, which precluded direct comparison of potencies to those obtained by IV administration. To evaluate the relative pharmacological potency of the inhaled drugs, the actual tissue concentrations of the drugs resulting from inhalation are required. Therefore, the biodisposition of heroin was investigated by exposing mice to volatilized [^{3}H.]-heroin.

Results of the biodisposition analysis of [^{3}H]-heroin are summarized in table 3. The whole-body concentrations of heroin equivalents that produced a 50 percent MPE for inhalation exposure and IV injection were 0.60 and 0.28 mg/kg, respectively. The concentrations of heroin equivalents in brain and plasma resulting from inhalation and IV administration were also very close. Doses of heroin that produced 100 percent MPE through inhalation exposure or IV injection showed a similar pattern of results. These data suggest that heroin is

equipotent when administered by inhalation exposure or IV injection. It is known that the acetyl groups on the heroin molecule enable it to enter the brain easily, suggesting that the equilibrium of heroin between the brain and plasma can be reached rather quickly regardless of the route of administration. Furthermore, since heroin is extensively metabolized by the liver and other tissues, the immediate metabolite of heroin, 6-monoacetylmorphine, has been reported to be both as potent and lipophilic as heroin (Way et al. 1959). Thus, the concentrations of drug equivalents found in brain in the present study undoubtedly reflect heroin and its metabolites.

It has also been demonstrated that, in humans, the time course of heroin in plasma and the appearance and disappearance of heroin metabolites after inhalation and IV administration are similar (Jenkings et al. 1994). The half-lives of heroin after smoking and IV administration were 3.3 and 3.6 minutes, respectively.

TABLE 3. *Recovery of heroin free base after volatilization.* [A]

	Heroin recovered after volatilization (mg)		
Amount (mg)	Glass wool	Pipe	Total
1.10 Å 0.01	0.77 Å 0.03	0.16 Å 0.03	0.92 Å 0.06
5.11 Å 0.03	3.36 Å 0.22	1.42 Å 0.13	4.73 Å 0.18

KEY: A = Volatilization was carried out at 250%C. Values represent means Å SE.

This rodent inhalation model has proven to be reliable in studying the pharmacological effects of a variety of opioids, stimulants, and other drugs of abuse. In addition to predicting the inhalation potential of drugs of abuse by the comparison of inhalation and IV routes of administration, actual tissue concentrations of drug can be approximated with the use of radiolabeled compounds.

CONCLUSION

Inhalation has become known to drug abusers as a rapid and potent route of administration. This route has also increased in popularity because of

TABLE 4. *Biodisposition of [H]- heroin free base.*

			Drug equivalents[A]			Drug equivalents ratio[A]	
Route	Dose	% MPE	Brain (&g/g)	Plasma (&g/ml)	Body (&g/g)	Brain/body	Brain/plasma
IV N = 6	0.28 mg/kg	50	0.04Å0.01	0.20Å0.05	0.19Å0.01	0.20Å0.02	0.15Å0.03
	1.0 mg/kg	100	0.24Å0.02	0.82Å0.14	0.82Å0.04	0.30Å0.01	0.34Å0.07
Inhalation N = 3	1.0 mg	50	0.11Å0.02	0.38Å0.08	0.06Å0.14	0.31Å0.01	0.18Å0.01
	3.0 mg	100	0.24Å0.01	0.86Å0.10	1.20Å0.25	0.29Å0.04	0.22Å0.05

KEY: A = Values represent means Å SE.

fears of contracting diseases such as AIDS from IV injection. However, smoking may increase the risk of other hazardous effects caused by pyrolytic products that are not associated with other modes of administration. Studies of the volatility and inhalation of drugs of abuse can provide important information for developing guidelines to predict their abuse potential upon inhalation.

One of the goals of this investigation was to use vapor pressure to predict drug volatility and thus the abuse potential through inhalation. Preliminary evidence from studies on the vapor pressure and pharmacology of the drugs of abuse is consistent with this notion. In table 5, the estimated vapor pressures of several drugs were compared with the temperature required to produce optimum pharmacological effects following inhalation. These results suggest that as vapor pressure increases (i.e., a more volatile drug), lower volatilization temperatures are required to produce a pharmacological effects.

Drugs in the salt form are much less volatile than their free bases and would be expected to require high temperatures for volatilization. It should be noted that vapor pressure information on the salts not currently available; however, the relative order of their vapor pressures is assumed to be the same as their free bases (table 5). In addition, the present results suggest that methamphetamine and amphetamine are more volatile than most of the other drugs tested. This is consistent with the popularity of methamphetamine smoking among drug users. Conversely, a drug that has extremely low vapor pressure would not be expected to be readily used by inhalation.

In order to study the pharmacological effects of a drug when smoked, and presumably its potential for abuse by inhalation, efforts were made to examine the relationship between a drug's volatility and its pharmacological potency upon inhalation. A rodent inhalation model was developed that enabled systematic investigation of the inhalation route of drug admini-stration. Using this model of inhalation, the pharmacological effects of a variety of drugs, including opioids, stimulants, and PCP, were evaluated in mice. It was demonstrated that inhalation produced pharmacological effects similar to those obtained from IV administration of heroin, PCP, and methamphetamine. Biodisposition studies with radiolabeled heroin enabled the authors to evaluate the relative potency of a drug following inhalation and IV injection. The tissue concentrations of heroin equivalents obtained after these two routes of administration revealed that smoking is equipotent to IV injection.

TABLE 5. *Comparison of vapor pressures and volatilizing temperatures.*

	Vapor pressures at	Volatilizing temperature (%C)	
Drug	25%C (Torr)	Free base	Salt
Methamphetamine	$> 10^{-2}$		200
Amphetamine	$> 10^{-2}$		200
PCP	8.56×10^{-4}		275
Cocaine	9.79×10^{-6}	220	
Morphine	9.49×10^{-7}	250	
Heroin	5.71×10^{-8}	250	
Fentanyl	2.41×10^{-8}	300	

Although the authors propose that vapor pressure can be used to predict whether a drug of abuse may be smoked, other physiochemical parameters, including particle size (Snyder et al. 1988) and lipid solubility (McQuay et al. 1989), also influence drug potency. The determination of these physiochemical parameters used concurrently with a reliable inhalation animal model will serve as useful tools for identifying which drugs may potentially be abused by inhalation.

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Biocatalysts in Detoxication of Drugs of Abuse

John R. Cashman

INTRODUCTION

Detoxication of drugs of abuse has generally followed a traditional approach involving pharmacological intervention in the biochemical action of the abused substance. This intervention has usually taken the form of the development of inhibitors or antagonists of biological macromolecules such as central nervous system (CNS) receptors. Some of the shortcomings of this approach include the fundamental biological activity of the antagonist, the possible occurrence of side effects or pharmacological liabilities of the antagonist itself, and the stoichiometric nature of the antagonism.

The idea that biocatalysts (e.g., enzymes, catalytic antibodies, ribozymes) could play a role in human therapy is not a new one. However, few examples of the application of biocatalysts to the detoxication of drugs of abuse have been reported. The use of biocatalysts in the detoxication of drugs of abuse has tremendous potential. First, the catalyst would likely have no intrinsic pharmacological activity. That is, the biomacro- or small-molecule catalyst would probably have minimal direct effect on CNS receptors. As such, the biocatalyst itself should have a markedly reduced potential for pharmacological side effects. Of course, numerous advances in "humanizing" biocatalysts must be undertaken before they are useful in the clinical setting, but it is likely that those advances will come to fruition in the near future based on the recent rapid progress of recombinant biological approaches to developing human therapeutics. Finally, biocatalysts work in an exponential catalytic fashion; it is possible that less material will be needed to be employed therapeutically, thus decreasing the possible side effects and increasing the therapeutic efficacy as a detoxication agent.

In principle, there are two fundamentally different ways to develop biocatalysts useful in detoxication of drugs of abuse. The first approach involves the procurement or design of a small molecule or macro-molecule that will selectively catalyze the detoxication reaction of interest. The second approach relies on selection of a catalyst from a large pool of candidates. However, several important

but difficult questions associated with such an exercise need to be addressed. For example, what is the critical rate-limiting step in the chemical detox-ication process? How will the catalyst be synthesized or otherwise obtained? Will the detoxication catalyst be of clinical relevance?

Selection of a catalyst for specific chemical detoxication reactions from a large biologically or chemically generated library also poses several important questions. How does one create a large biologically or chemically generated library of putative detoxication catalysts? How will the library be screened for catalytic detoxication activity? Are dependable methods available to amplify the catalyst to reasonable amounts for further study? Is it sufficient to simply screen existing stockpiles of manmade chemicals or natural products for detoxication catalytic activity, or is it imperative to develop fundamentally new technology so that novel approaches and novel agents are introduced for evaluation? It is likely that the time to develop new technology to address important questions of drug abuse is now; traditional approaches are limited, and have not appeared to solve the clinical problems thus far.

There are a number of secondary questions that arise from a consideration of the above-mentioned approaches to procuring detoxication catalysts or developing new detoxication strategies. Of course, one of the first such questions is: Will the catalysts obtained by design or selection possess any clinical relevance or be useful in human therapy? If so, will the clinical use be limited to acute toxic overdose situations or will the catalyst be useful only in drug cessation paradigms? Will the catalyst play any role in decreasing or reversing addiction liability of drugs of abuse? To a certain extent these latter questions are somewhat premature (like the field of catalyst design and selection itself). At present, a larger question should be: What is the fundamental technology useful in discovering catalysts or underlying principles of catalysis that might later support rational design? What can the technology reveal about the way detoxication catalysts (such as they are) naturally evolved? How should a drug of abuse detox-ication catalyst be evaluated in the clinic?

Several fundamentally distinct approaches to the procurement and evalu-ation of biocatalysts useful in the detoxication of drugs or chemicals of abuse are outlined below. The author has arbitrarily limited the presen-tation to a few select detoxication catalysts in the field of drug abuse. In some cases, other nondetoxicating biochemical routes of transformation are included as well.

The first section focuses on naturally occurring adult human biocatalysts that transform (*S*)-nicotine. (*S*)-Nicotine is discussed because it is one of the most well-studied CNS-active compounds and because it constitutes perhaps the most widely used pharmacologically active agent in humans. In the second section, discussion of the metabolism of cocaine focuses on cocaine hydrolysis as a major route of detoxication. Cocaine has been chosen because it illustrates some of the advantages and pitfalls of the use of cocaine esterolytic catalytic antibodies as a new potential therapeutic approach. Finally, a brief discussion of catalytic ribonucleic acid (RNA) (i.e., ribozymes) is presented. The use of catalytic RNAs as therapeutic agents is in its infancy but some of the relevant concepts discussed for enzymes or catalytic antibodies are also relevant to ribozymes. In addition, other principles observed in RNA catalysis have found considerable application in the use of oligonucleotides in antisense therapy, and there may be some useful extensions of antisense drug design that could be useful in the field of detoxication catalysts.

ENZYMES IN (S)-NICOTINE METABOLISM AND DETOXICATION

(*S*)-Nicotine[1] is one of the most widely used psychoactive drugs in the world. Almost 25 percent of adult Americans smoke despite convincing evidence of the health hazards of smoking (Surgeon General 1988). Nicotine causes complex CNS, behavioral, cardiovascular, and endocrine neuromuscular effects in humans (Benowitz 1988). Most notably, nicotine is the primary cause of tobacco addiction in humans (Surgeon General 1988). The molecular basis for tobacco addiction is unknown but it is possible that a biologically active metabolite of nicotine plays a role in nicotine tolerance and dependence, and individual differences in the metabolism and disposition of nicotine may explain why some humans become highly dependent on nicotine and others do not. Clearly, a full appreciation of the role of human enzymes in nicotine metabolism and detoxication could provide much insight into the interindividual variation in the biological responses to smoking and the variation in the pharmacological effects of nicotine.

The untoward biological properties of nicotine and nicotine metabolites are controversial. Extensive epidemiological studies have supported a role of smoking in several types of cancer. However, although nicotine is a prominent component of cigarette smoke, there are thousands of other chemicals present in tobacco smoke, including most classes of known chemical carcinogens (Hecht and

Hoffmann 1989). The determination of the agent(s) in tobacco smoke responsible for causing cancer has been and continues to be an extremely challenging problem. Most notable for this discussion, however, is that several nicotine-related metabolites are formed in humans in apparent detoxication processes. Study of these enzymatic processes could reveal why certain people are less susceptible to the harmful effects of nicotine. That is, certain humans may be endowed with the ability to decrease the potential untoward effects of nicotine by an abundance of detoxication processes. Careful study of the enzymes involved in nicotine detoxication could provide valuable clues for designing new catalysts useful in decreasing the pharmacological effects of nicotine. For example, nicotine-, cotinine-, and 3-hydroxy-cotinine glucuronides are all formed in apparent enzymatic detoxication processes. The apparent detoxication of nicotine by glucuronidation metabolic pathways in humans is quite variable and it is possible that an undiscovered relationship exists between glucuronidation and susceptibility to the untoward effects of nicotine ingestion. While nicotine addiction is a complex phenomenon involving many biological, behavioral, and other parameters, it is unknown what relationship, if any, exists between metabolic biotransformation of nicotine and nicotine addiction.

Even though the pharmacokinetics and metabolism of nicotine have been extensively studied (Beckett et al. 1971; Benowitz and Jacob 1994; Booth and Boyland 1970; Byrd et al. 1992; Caldwell et al. 1992; Jacob et al. 1988; Kyerematen et al. 1990; Neurath et al. 1987; Schepers et al. 1992; Scherer et al. 1988), the molecular basis for addiction to nicotine remains unclear. Following smoking or intravenous (IV) infusion, the terminal half-life of nicotine is about 2 hours (and is urine pH-dependent) (Benowitz et al. 1982). Total clearance averages 1300 milliliters per minute (mL/min) (and is highly variable between individuals) and the relatively large volume of distribution (183 liters (L)) explains the relatively long half-life in the presence of rapid clearance. Plasma protein binding of nicotine is very low (Duan et al. 1991). In humans, nicotine is rapidly and extensively metabolized. Liver monooxygenases and, to a lesser extent, lung monooxygenases have been implicated, and at present about 90 percent of ingested nicotine can be accounted for as urinary metabolites (Benowitz and Jacob 1994). The major excreted urinary metabolites of nicotine in humans are cotinine (10 to 15 percent),

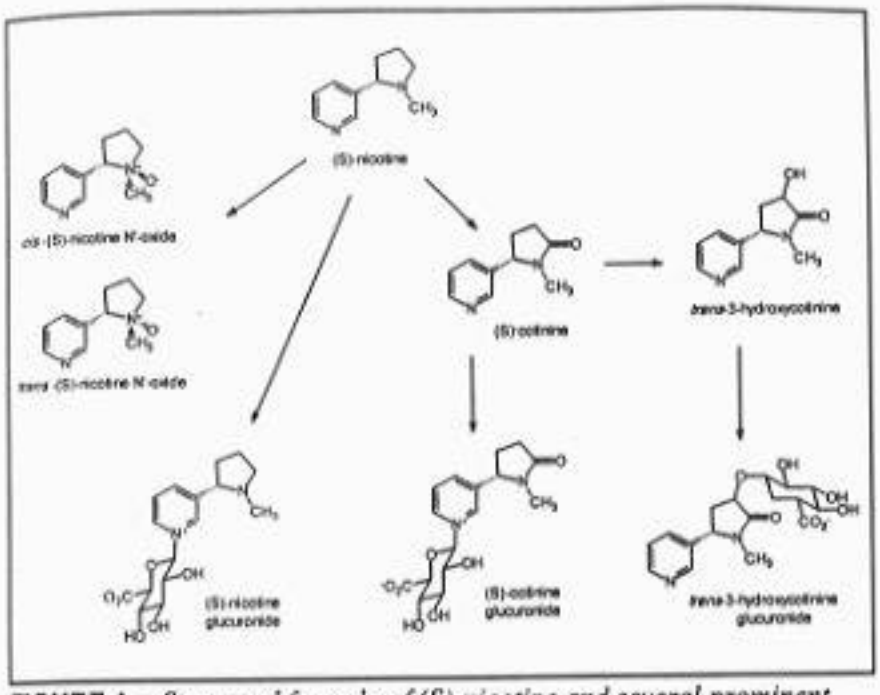

FIGURE 1. *Structural formula of (S)-nicotine and several prominent metabolites.*

nicotine N-1'-oxide (4 percent), *trans*-3-hydroxycotinine (30 to 35 percent) and cotinine-, 3-hydroxycotinine-, and nicotine-glucuronides (together, approximately 24 percent) (figure 1) (Benowitz and Jacob 1994). Cotinine and nicotine N-1'-oxide have been used as markers of nicotine exposure. Cotinine has a long half-life (16 hr) and blood levels are stable (Benowitz et al. 1983). However, it may be the glucuronide metabolites of nicotine or cotinine that are the most useful as bioindicators of nicotine exposure. Regardless, cotinine and nicotine N-1'-oxide have historically been used to verify human nicotine exposure.

In 26 smokers, mean cotinine excretion was 1.39 mg/24 hr, while nicotine N-1'-oxide was excreted at a rate of 0.56 mg/24 hr in the same smokers (Jacob et al. 1986). The disposition of nicotine N-1'-oxide in humans has been characterized (Park et al. 1993). Administration of deuterated nicotine N-1'-oxide (figure 2) to humans results in the rapid excretion of the N-1'-oxide largely unchanged in the urine. Examination of the stereochemistry of the nicotine N-1'-oxide before and after administration showed that the stereochemistry of the excreted material was essentially identical to that of the administered compound. That no

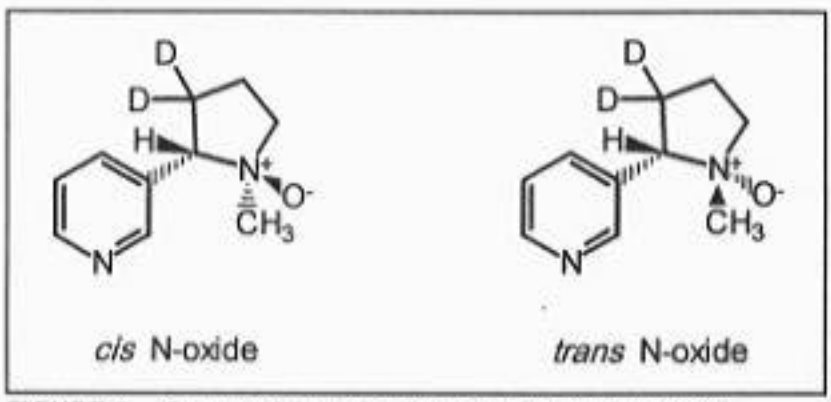

FIGURE 2. *Structural formulas for cis and trans deuterated (S)-nicotine N-1'-oxide.*

change in stereochemistry and no reduction of IV-administered N-1'-oxide to nicotine was observed in humans (Beckett et al. 1971; Park et al. 1993) suggests that N-1'-oxygenation of nicotine is a detoxication process. In animals, considerable reduction of oral or intraperitoneally (IP)-administered nicotine N-1'-oxide was observed (Dajani et al. 1975). In rabbits, less than 3 percent is reduced following IV dosing, but after oral administration 45 percent of nicotine N-1'-oxide is reduced. It is likely that gut bacteria or intestinal reductases are responsible for the reduction of nicotine N-1'-oxide after oral administration (Duan et al. 1991). In rabbits, nicotine N-1'-oxide clearance was 7.5 mL/min per kilogram (kg), had a half-life of 42.6 min, a volume of distribution of 340 mL/kg, and was not plasma protein bound (Duan et al. 1991). The idea that nicotine N-1'-oxide serves as a reservoir for nicotine (i.e., by reduction back to nicotine) and participates in maintaining nicotine levels required for addiction and nicotine-related toxicity is apparently not true in humans (Park et al. 1993). This is not to imply that nicotine N-1'-oxide does not have pharmacological activity; it causes vasoconstrictive effects on isolated rabbit-ear vessels and increases the tonus of isolated rabbit intestine, and in mice causes qualitatively similar tremor, dyspnea, and spasms that are similar (IV administration) in effects to nicotine, but the dose required is much larger (Barass et al. 1969). More important, the N-1'-oxide administered before challenge with nicotine conferred protection against a subsequent lethal dose of nicotine (Barass et al. 1969). The conclusion is that formation of nicotine N-1'-oxide constitutes a true detoxication route of disposition, but in most adult humans, this pathway represents only a small percentage of the overall metabolism of nicotine. In principle, development of catalysts that could form

nicotine N-1'-oxide may be a useful adjuvant in smoking cessation therapy.

Cytochromes P-450

Human cytochrome P-450 is a ubiquitous heme-containing enzyme that participates in the oxidation of a wide variety of chemicals, drugs, and even some endogenous materials (Guengerich and Shimada 1991). Although the cytochrome P-450 transformation of nicotine has been extensively described for animals, nicotine has not been extensively used as a probe substrate for adult human metabolism. The molecular basis for metabolism of nicotine in humans is considerably different from that observed in animals (Cashman et al. 1992). In humans, nicotine is primarily eliminated by metabolism, with total clearance varying by about fourfold among individuals. Men metabolize nicotine more rapidly than women (Benowitz and Jacob 1984). Age also influences urinary nicotine metabolite levels in humans. The pathway leading to cotinine is the major route of nicotine metabolism, and the initial metabolic step is nicotine $^{1',5'}$-iminium ion formation. In previous studies, the author and colleagues identified human liver cytochrome P-450 2A6 as the principal enzyme responsible for nicotine $^{1',5'}$-iminium ion formation (Cashman et al. 1992). This result is in contrast to the situation in animals where cytochrome P-450 2B (identical to the phenobarbital-inducible form) has most often been implicated as the putative nicotine oxidase (McCoy et al. 1989; Williams et al. 1990). In rats and rabbits, phenobarbital pretreatment increased cotinine formation in liver microsomes supplemented with aldehyde oxidase that was isolated from the animals. In the absence of aldehyde oxidase, cytochrome P-450 is responsible for the metabolism-dependent covalent binding of nicotine to tissue macromolecules, implicating nicotine $^{1',5'}$-iminium ion as an electrophilic metabolite (Williams et al. 1990; Shigenaga et al. 1988). In rabbit lung microsomes, cytochrome P-450 2 (or LM2), which is similar to the phenobarbital-inducible hepatic form, is primarily responsible for nicotine $^{1',5'}$-iminium ion formation that can be trapped by cyanide or converted to cotinine by aldehyde oxidase (Williams et al. 1990). It is important to note that the cytochrome P-450 corresponding to the rat or rabbit liver phenobarbital-inducible form (i.e., adult human liver cytochrome P-450 2B6) is highly variable and is detectable in less than 3 percent of adult human livers examined (Mimura et al. 1993).

Because there is strong evidence that P-450 2A6 is the primary enzyme forming nicotine $^{1',5'}$-iminium ion in adult human liver

microsomes, it is likely that in human tissues about 90 percent of the nicotine iminium ion formation is mediated by cytochrome P-450 2A6 (Berkman et al. 1995; Cashman et al. 1992). Interestingly, increased hepatic cytochrome P-450 2A6 levels have been observed in humans with a history of barbiturate administration (Cashman et al. 1992). Production of nicotine $^{1',5'}$-iminium ion showed some dependence on the previous drug administration history of the subject from which the hepatic microsomes were isolated (table 1). Two of the highest rates of iminium ion formation occurred in the presence of human liver microsomes from barbiturate-pretreated subjects (i.e., E and I) (table 1). Of the seven major human cytochrome P-450 enzymes examined (i.e., 1A2, 2A1, 2C8, 2C9, 2D6, 2E1, 3A5, and 3A total), the greatest linkage between immunoreactivity and nicotine iminium ion formation (i.e., at 10 micromolar (M) substrate concentration) was observed for cytochrome P-450 2A6 (r = 0.9) (Berkman et al. 1995). Neither gender, age, nor smoking history provided a direct relation between nicotine $^{1',5'}$-iminium formation and cytochrome P-450 2A6 levels. Others have suggested that cytochrome P-450 2D6 or 2B6 plays a role in adult human liver nicotine oxidase activity. However, these studies employed extremely high nonphysiological concentrations of nicotine (Flammang et al. 1992). Based on their studies, the author and colleagues anticipate that non-cytochrome P-450 2A6 enzymes contribute less than 10 percent to the oxidation of nicotine (Berkman et al. 1995).

In summary, in adult human liver, cytochrome P-450 2A6 is responsible for formation of nicotine $^{1',5'}$-iminium ion that is subsequently converted by aldehyde oxidase to cotinine. Because cotinine is extensively glucuronidated or metabolized to 3-hydroxy-cotinine (which is in turn glucuronidated), detoxication of nicotine via the cotinine glucuronidation pathway must pass through the relatively electrophilic iminium ion pathway. However, with the exception of a few studies (most notably by Castagnoli and coworkers) (Peterson and Castagnoli 1988; Peterson et al. 1987), the pharmacological or toxicological effects of nicotine $^{1',5'}$-iminium ion have not been reported. It is interesting to note that human liver cytochrome P-450 2A6 and 2E1 catalyze metabolic activation of the tobacco smoke-related nitrosoamines 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanol (NNAL), and N-nitrosonornicotine (NNN). Thus, cytochrome P-450 2A6 and 2E1 could catalyze the conversion of several tobacco smoke-related nitrosoamines to genotoxic products (Yamazaki et al. 1992).

				Metabolite formed [pmol/(min mg of protein)]				
patient code	gender	age	drug history	FMO immuno-reactivity [b]	nicotine N-1[1]-oxide	cotinine	nornicotine	
A	M	25	none	3	226	294	ND	
B	M	50	none	2	88	238	ND	
C	M	22	ethanol	2	181	187	ND	
D	M	31	MPME	1	54	568	ND	
E	M	14	pentobarb	2	180	376	ND	
F	F	50	ethanol	2	55	353	ND	
G	F	48	teldrin	5	225	299	ND	
H	F	28	none	2	34	284	ND	
I	N	43	phenobarb	3	45	750	ND	
J	F	55	none	4	133	377	ND	
K	M	23	ethanol	4	192	431	ND	
L	F	58	dopamine	4	141	208	ND	
M	M	18	ethanol	3	67	234	ND	
N	M	21	ethanol	2	127	311	ND	

KEY: a = Incubations were performed with 0.4-2.0 mg of microsomal protein, 4.3 mg of rat liver microsome supernatant (as a source of aldehyde oxidase), 0.5 mM NADPH at pH 8.4 and 37%C for 10-20 min, and products were quantified by HPLC. For cotinine, nornicotine, and 7-OH-coumarin formation, incubations were at pH 7.4. Values are the mean of 2-4 determinations 10%. b = Relative immunoquantitation; 5 = greatest, 1 = weakest, ND = not detected.

SOURCE: From Cashman et al. 1992, used with permission.

The conclusion of the study was that interindividual variations in the amount of human cytochrome 2A6 and 2E1 could contribute to the susceptibility of environmental procarcinogens including those derived from tobacco smoke. Cytochromes P-450 largely bioactivate nicotine and nicotine-related nitrosoamines to electrophilic materials. Presumably, the balance between bioactivating enzyme activity and detoxicating enzyme activity is important in determining the overall susceptibilities of humans to exposure to chemicals. Below, the properties of several additional enzymes in nicotine detoxication are described.

Aldehyde Oxidase

Aldehyde oxidase is a cytosolic, iron-containing molybdoflavoprotein that mediates the oxidation of aldehydes and nitrogen-containing heterocyclic drugs, chemicals, and endogenous substrates (Beedham 1985). The structural biology of the various forms of the enzyme are just beginning to be described, but animal and human aldehyde oxidase apparently consists of homogenous homodimers of subunit molecular size (approximately 150,000 daltons (Da)). The substrate specificity of human liver aldehyde oxidase has not been as exhaustively examined as animal liver aldehyde oxidase but some data point to a similar spectrum of substrate activity (Beedham 1985; Krenitsky et al. 1974). Thus, prototypic substrates such benzaldehyde, 6-methylpurine, and N-1-methylnicotinamide appear to be good substrates for both animal and human forms of aldehyde oxidase (Rodrigues 1994).

Electronic factors and the relative lipophilicity of the molecule probably help to determine the affinity of the substrate for the enzyme as well as turnover properties. It is likely that the fundamental instability of the enzyme has hampered progress in the characterization of human liver aldehyde oxidase. At least in animals, the specific activity of the enzyme is quite dependent on the way the tissue is procured, processed, and stored; this may lead to considerable intersample variability. Enzyme instability may at least in part explain why aldehyde oxidase activity from different species is so variable (Duley et al. 1985). However, it is likely that in addition to intrinsic differences in stability, the determination of aldehyde oxidase activity for a given substrate in various tissue preparations is dependent on the analytical methodology employed to assay the enzyme and the likelihood of the presence of different forms of the enzyme that possess distinct substrate specificity and kinetic properties (Johns 1967; Beedham 1985). For example, in the

presence of six different adult human liver preparations (i.e., the 9000 *g* supernatant or S-9 fraction), the oxidation of benzaldehyde, 6-methylpurine, and N-1-methylnicotinamide by aldehyde oxidase varied by 3.6-, 2.3-, and > 40-fold, respectively (Rodrigues 1994) (table 2).

TABLE 2. *Aldehyde oxidase activity in human liver 9000 g supernatant (S-9) fractions.*

				Activity* (nmol/ min/mg S-9 protein	
Subject code	Age (years)	Sex	BA	6-MP	NMN
FGL	26	F	11.4	17.5	1.4
GDD	25	F	14.5	14.7	<0.5
GEQ	20	M	19.5	14.1	<0.5
GFE	50	F	40.7	32.8	20.4
GC4	29	M	26.3	23.1	<0.5
FRX	58	M	22.2	15.5	1.5
Mean Å SD			22.4 Å 10.4	19.6 Å 7.2	7.78 Å 10.9
Fold variation**			3.6	2.3	>40

KEY: * = Data for each subject represent the mean of duplicate determinations. BA = benzaldehyde; 6-MP = 6-methylpurine; NMN = N-1'-methylnicotinamide. ** = Ratio of the highest/lowest activity.

SOURCE: From Rodrigues 1994, used with permission.

In agreement with what has been observed in animals, the level of aldehyde oxidase activity present in human liver is also markedly dependent on the substrate employed, the analytical method used to evaluate the substrate, and most importantly, the intrinsic activity of the preparation (Beedham et al. 1992). For N-1-methylnicotinamide oxidase activity, the rank order was cynomolgus monkey > rat > beagle dog > human liver (Rodrigues 1994). The relative levels of aldehyde oxidase activity may help determine the relative extent of lactim formation from N-heterocyclic compounds such as N-1-methylnicotinamide. Of course, formation of lactim metabolites from iminium ions can also be coordinately regulated by other enzyme activities including cytochromes P-450. Thus, formation of

iminium ions by cytochrome P-450 and conversion to lactams by aldehyde oxidase can be thought of as an important detoxication mechanism whereby N-heterocyclic compounds are converted into more polar materials that are excreted (Ohkubo and Fujimura 1982). In addition to its role as an oxidative enzyme, aldehyde oxidase has been reported to reduce a number of tertiary amine N-oxides back to the parent tertiary amine (Kitamura and Tatsumi 1984*a*, 1984*b*). Because a number of heterocyclic tertiary amines can undergo sequential 1-electron oxidation (i.e., via the iminium ion by cytochrome P-450) or 2-electron oxygenation (i.e., to the tertiary amine N-oxide by the flavin-containing monooxygenase), the participation of aldehyde oxidase activity in the disposition of tertiary amines may represent an important aspect of drug of abuse detoxication.

Flavin-Containing Monooxygenase

The mammalian flavin-containing monooxygenase (FMO) is a widely distributed membrane-associated family of enzymes that catalyze the oxygenation of nucleophilic nitrogen-, sulfur-, and phosphorous-containing xenobiotics, drugs, and endogenous substances (Ziegler 1993). Nucleophilic tertiary amines such as nicotine are readily converted to relatively stable tertiary amine N-oxides that are more polar and readily excreted unchanged (Park et al. 1993). As described above, nicotine N-1'-oxygenation is a route of detoxication for nicotine in humans. In contrast to cytochromes P-450, the mechanism of human FMO form 3 (FMO3)-catalyzed N-1'-oxygenation of nicotine involves 2-electron oxygenation to provide exclusively the trans nicotine N-1'-oxide (Cashman et al. 1992). Formation of nicotine N-1'-oxide diastereomers is highly FMO enzyme form-dependent. Thus, pig FMO1 forms approximately a 40:60 mixture of cistrans nicotine N-1'-oxides (Damani et al. 1988; Park et al. 1993). In contrast, human stereoselective formation of trans nicotine N-1'-oxide may serve as a highly sensitive stereochemical probe of adult human FMO3 activity in vitro and in vivo.

In the presence of liver microsomes, human FMO3 nicotine N-1'-oxygenase activity is not strongly dependent on the age, gender, or drug administration history of the subject from whom the liver was obtained (Cashman et al. 1992). In good agreement with in vitro studies, in vivo metabolism of nicotine in humans by three routes of administration including ad libitum smoking, intravenous infusion of nicotine-d_2, and dermal patch administration of nicotine all produced

only the trans diastereomer of nicotine N-1'-oxide in the urine (Park et al. 1993). In addition, trans nicotine N-1'-oxide is not appreciably reduced or oxidized further because infusion of nicotine-d_2 N-1'-oxide in humans with a known ratio of cis to trans N-1'- oxide diastereomers gave recovered urinary metabolite N-1'-oxides in high yield and with metabolite diastereoisomer ratios essentially identical to that of the infusate. In addition, human administration of highly purified preparations of stereochemically characterized nicotine-N-1'-oxide resulted in the material being rapidly excreted unchanged. This data supports the idea that formation of nicotine N-1'-oxide is a detoxication process.

Although a slight difference between the average amount of nicotine-N-1'- oxide formed and excreted in the urine of smokers versus the transdermal route of administration (i.e., 3.7 versus 2.7 percent of total urinary metabolites) could possibly reflect a minor contribution from pulmonary human FMO2 (Benowitz and Peyton 1994), the preferential N-1'-oxygen-ation of nicotine by hepatic FMO as opposed to lung FMO observed in animals (Williams et al. 1990) suggests that human liver FMO3 is the major contributing metabolic pathway. Formation of trans nicotine N-1'-oxide has been proposed as a selective functional marker for adult human FMO3 activity. The fact that nicotine N-1'-oxide formation possesses significantly less pharmacological activity than nicotine suggests that catalysts designed to convert nicotine to nicotine N-1'-oxide might be useful in smoking cessation treatment.

As stated above, it is likely that human FMO3-catalyzed formation of trans nicotine N-1'-oxide constitutes a detoxication process whereby the pharmacologically active nicotine alkaloid is converted to a polar, readily excreted tertiary amine N-1'-oxide. Previously, it has been suggested that the role of FMO in human metabolism is to metabolize xenobiotics from plant sources to benign materials that do not pose a pharmacological or toxicological challenge to the organism ingesting the chemical (Ziegler 1993). Because many CNS drugs commonly abused by humans contain a tertiary amine center, elaboration of catalysts to form tertiary amine N-oxides could constitute the basis for a new class of detoxication catalysts.

URIDINE DIPHOSPHOSPHATE GLUCURONOSYL TRANSFERASE

Glucuronosyl transferases mediate the transfer of uridine-5'-diphospho-- D-glucuronic acid to acceptor molecules in metabolic transformations that are generally considered detoxication reactions. While acyl glucu-ronides in some cases have been associated with potentially toxic conse-quences, in most instances, formation of glucuronide metabolites is associated with detoxication reactions. In humans, there have been numerous reports of the urinary excretion of quaternary N-linked glucu-ronides (Chaudhary et al. 1988; Lehman et al. 1983; Macrae et al. 1990). Recently, studies have confirmed that major metabolites of nicotine in humans are in fact glucuronide conjugates (Caldwell et al. 1992). Thus, humans administered nicotine by smoking or by a dermal patch route of administration excrete the glucuronide conjugates of (*S*)-nicotine, cotinine, and 3-hydroxy cotinine (i.e., on average, 30 percent, 49 percent, and 16 percent of a dose is conjugated, respectively) (Benowitz and Jacob 1994). While there is significant interindividual variability in the excretion of glucuronide conjugates, the pattern of metabolism is generally similar when nicotine is inhaled or absorbed transdermally. However, formation of nicotine and cotinine glucuronides does not exactly parallel the formation of 3-hydroxy cotinine glucuronide. Apparently, conjugation of 3-hydroxy cotinine is done by a different glucuronosyl transferase than the enzyme responsible for nicotine and cotinine glucuronidation (Benowitz and Jacob 1994). This may reflect the fact that one enzyme form is a nicotine N-glucuronosyltransferase while the other enzyme form is a nicotine O-glucuronosyltransferase.

Conjugation is a quantitatively important route of nicotine detoxication in humans. The large amount of nicotine glucuronosyl conjugates observed in human urine following nicotine administration and the significant degree of interindividual variability in the amount of glucuronide formed have confounded accurate assessment of nicotine metabolic disposition. By taking into account the glucuronidation pathway, a more complete quantification of nicotine metabolism and disposition could enhance the accuracy of bioindicators of nicotine exposure. In the gut, glucuronosyl conjugates of nicotine may become substrates for microflora - glucuro- nidase. As such, it is possible that enterohepatic recirculation of nicotine could arise via this pathway. A cycle of conjugation and hydrolysis could represent a mechanism to prolong the bioavailability of nicotine or its metabolites. While glucuronidation of nicotine or one of its metabolites represents a detoxication reaction, considering

other possible metabolic pathways makes the analysis of the contribution of conjugation to detoxication less straightforward.

GLUTATHIONE TRANSFERASE

While no published studies of the conjugation of electrophilic nicotine metabolites with glutathione have been reported, in principle, enzyme-catalyzed glutathione addition could produce metabolites that are more polar and more readily excreted. Nucleophilic addition of low molecular weight thiols to nicotine $^{1',5'}$-iminium ions has been shown to occur under pseudo first-order reaction conditions (Brandage and Lindblom 1979). In the liver where glutathione reaches millimolar concentrations, it is possible that glutathione conjugation contributes to the disposition of the nicotine iminium ion, especially in animals with low amounts of aldehyde oxidase. Presumably glutathione conjugates, if formed, could also be transformed into mercapturates that should be readily excreted. While investigations of a role for glutathione conjugates or mercapturates of nicotine have not been reported in the literature, the feasibility of trapping electrophilic iminium ion metabolites of nicotine with thiol nucleophiles has ample precedence (Brandage and Lindblom 1979). At this point it is only speculation that addition of biologically relevant sulfur-containing nucleophiles constitutes a detoxication reaction for metabolites of nicotine. Finally, because nucleophilic addition to the nicotine iminium ion is reversible, from a practical standpoint, an addition compound will probably only be formed and detected under conditions where the nucleophile is present in large excess.

Tobacco Addiction

Tobacco addiction is complex, and involves behavioral as well as pharmacologic factors. The importance of nicotine in tobacco dependence has been demonstrated in many studies (Benowitz and Jacob 1990; Surgeon General 1988). However, the neurochemical mechanisms of nicotine tolerance and dependence are not known, and the possibility exists that a reactive metabolite may be involved. Thus, identification of nicotine-derived electrophilic metabolites that covalently modify human proteins may provide insight into the fundamental mechanisms underlying tobacco addiction. This is a largely unexplored area of research. Knowledge of the bioactivation as well as detoxication steps in the metabolism of nicotine may help

identify the reasons for the interindividual variation and susceptibility to the injurious effects of this widely used chemical.

Cocaine Use and Related Toxicity in the United States

During recent years, cocaine has been most commonly ingested in the United States by smoking cocaine base (in chunk or crack form) (NIDA 1990). Smoking crack cocaine is preferred by abusers because the concentration of cocaine in venous blood peaks sooner after smoking crack (or after IV administration of cocaine) than by other routes of administration. Cocaine is generally not abused by the oral route of administration, but oral absorption is efficient and is a significant route of dosing as a cause of toxicity (Jones 1990). Thus, cocaine toxicity could be greater for children who have swallowed cocaine in mother's milk, for example.

Cocaine is hydrolyzed by esterases of the blood, liver, and other organs (Benowitz 1992). The major (inactive) metabolite of cocaine is benzoylecgonine, but ecgonine and ecgonine methyl ester are also formed (Benowitz 1992) (figure 3). Hepatic metabolism of cocaine to norcocaine (pharmacologically active) accounts for only 2 to 6 percent of the total amount eliminated (Benowitz 1992). After IV administration, cocaine has a plasma half-life of 70 mins. The transient nature of the CNS effects of cocaine is due to the relatively rapid redistribution of cocaine into and out of the brain and into other tissues. This type of rapid influx-efflux brain distribution is typical of lipophilic agents such as cocaine. In contrast to IV or smoking routes of administration, systemic effects and plasma levels may be sustained for longer periods because of continuous absorption after oral administration and selective binding to CNS monoamine reuptake transporters.

The primary effects of cocaine in humans are CNS stimulation resulting in euphoria and activation of the peripheral sympathetic nervous system with tachycardia and blood pressure elevation. Tolerance to the euphoric effects of cocaine develops quickly (Foltin and Fischman 1991), but is incomplete for the cardiovascular effects of cocaine (Ambre et al. 1988). It is possible that repeated use of cocaine to seek the high may lead to progressive cardiovascular toxicity. Medical problems arising from cocaine abuse are among the most common causes for emergency room visits in U.S. hospitals, especially in the inner cities (Brody et al. 1990). Three types of life-threatening medical toxicities reported include

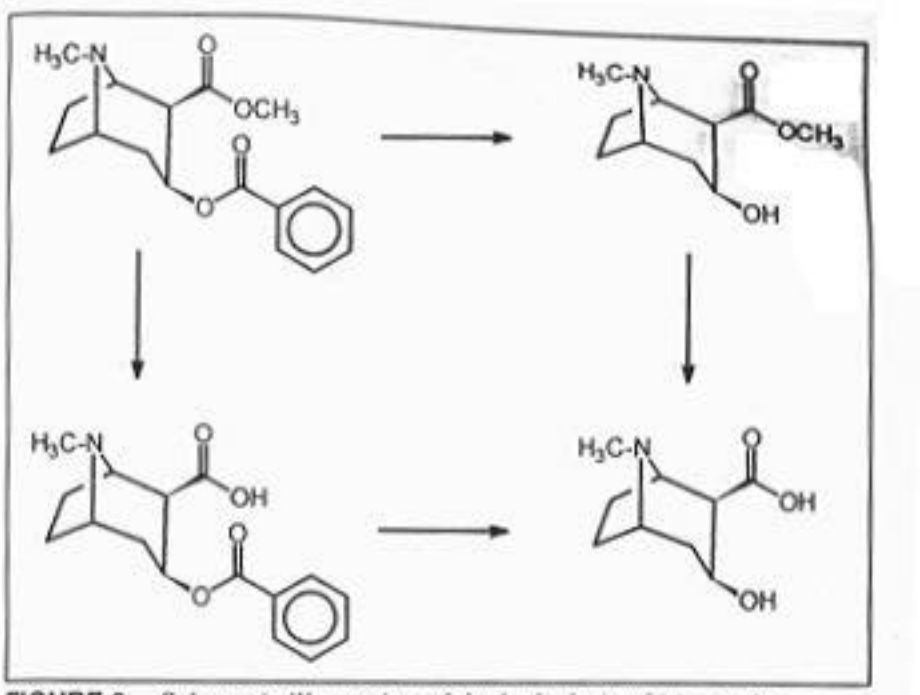

FIGURE 3. *Schematic illustration of the hydrolysis of (-)-cocaine.*

cocaine-related sudden death, cardiovascular disease, and reproductive disturbances (Benowitz 1992). Sudden death is believed to result from cardiac arrhythmias due to massive catecholamine release with or without acute myocardial ischemia. However, a review of 935 cocaine-related deaths reported in 1986 in New York City showed that the top causes of death were homicide (37.5 percent), acute narcotic exposure (12 percent), other natural causes (11.1 percent), and suicide (6.6 percent) (Litovitz et al. 1991). Another serious type of injury from cocaine use with consequences for long-term harm involves brain damage. Episodes of angina and myocardial infarction due to cocaine abuse have also become commonplace in reports from major U.S. metropolitan medical centers.

In inner-city areas of the United States, surveys of pregnant women reported rates of maternal cocaine use as high as 30 percent. Maternal cocaine use can affect reproduction by adversely affecting the pregnancy. In addition, cocaine use may directly injure the fetus and/or produce behavioral and developmental abnormalities in the neonate. Indirect toxicities of cocaine abuse may result in severe health consequences including child neglect and abuse, loss of family structure, an increased risk of AIDS, and congenitally acquired sexually transmitted diseases (Benowitz 1992). A study of data from 72 U.S. Poison Control Centers in 1990 that analyzed 1.7 million

human drug exposure cases (Litovitz et al. 1991) showed that cocaine was implicated in more major medical complications and deaths than any other chemical agent. Cocaine use among polydrug abusers, presumably occurring at increasingly higher doses via IV injection or by smoking, has become more toxic to humans. In summary, the current picture of cocaine abuse is associated with more emergency room visits and more deaths than any other illicit drug of abuse.

Completely new strategies for preventing the toxicity and abuse of cocaine are needed and novel methods may be best developed by employing modern biological and biochemical approaches (Jones 1992). It is unlikely that antagonists of neurotransmitter transporters or other pharmacological intervention (i.e., desipramine or other antidepressants) will be efficacious because the blood levels required to saturate the cocaine receptor will be too toxic in humans (Ritz et al. 1987) or have an induction period of several weeks (Fischman et al. 1990). The reasons for cocaine deaths probably stem partly from a contribution of the intrinsic toxicity and mechanism of action of cocaine and partly from the current lack of appropriate treatment for cocaine overdose. With heroin, the opiate receptor antagonist naloxone is likely to save someone brought to the emergency room with a heroin overdose. A comparable antagonist, such as the opiate antagonist naloxone, is currently not available to treat cocaine overdose. Antibodies against opiates have been reported to antagonize the reinforcing effect of low doses of heroin (Bonese et al. 1974) but because the antibodies were removed from circulation presumably by clearance processes, the therapeutic paradigm failed at high doses of heroin. Only symptomatic treatment of cocaine overdose is available today.

Powerful selective antidotes or antagonists to treat cocaine abuse are necessary to offset the increased cocaine toxicity currently observed in the United States. The use of catalytic antibodies in the creation of selective binding agents and detoxication catalysts of cocaine could represent a novel approach that may result in significant advances in the field of detoxication of drugs of abuse. An anti-cocaine catalytic antibody directed to hydrolyze the benzoyl ester could in principle catalyze the formation of ecgonine and benzoic acid, hydrolysis products of cocaine that do not possess the reinforcing or CNS stimulation properties of cocaine (Spealman et al. 1989).

Catalytic Antibodies

Antibodies are proteins that can recognize a wide array of chemicals, drugs, and other biological materials with great specificity and efficacy

(Harlow and Lane 1988). An antibody may bind to its ligand with an equilibrium dissociation constant (Kd) equal to 10^{-12} molar (M). In contrast, an enzyme typically devotes only a fraction of its overall free energy to binding phenomena and generally reserves most of its expendable free energy on catalytic events. In principle, an antibody can afford to bind a ligand with less avidity and channel the difference in free energy into catalytic activities. The fundamental similarity between antibodies and enzymes has important implications for combining the catalytic action of enzymes with the selective recognition function of antibodies.

The general approach to producing catalytic antibodies is based on the principle of reaction intermediate-enzyme transition state complementarity (Pauling 1946; Wolfenden and Kati 1991). Small molecule analogs of putative enzyme reaction transition states have been used as haptens to induce antibodies with complementary binding sites (Janda et al. 1989; Lerner et al. 1991; Pollock et al. 1986; Tramontano et al. 1986*a*, 1986*b*). As in the case of enzymes that have evolved to preferentially stabilize the transition state, antibodies induced to recognize the transition state have been shown to stabilize the putative transition state of the reaction and afford catalysis (Lerner et al. 1991; Tramontano et al. 1986*a,* 1986*b*). One of the first examples of catalytic antibody activity led to the development of hydrolytic or acyl transfer catalytic antibodies (Janda et al. 1988, 1989). Thus, simple tetrahedral carbon mimics representing the transition state for ester hydrolysis (i.e., phosphonates) were synthesized and antibodies directed against these materials (i.e., antibodies directed against small molecules covalently attached to a large carrier molecule such as a protein) possessed esterolytic activity against the corresponding ester substrate.

In principle, catalytic antibodies can be developed to facilitate selective chemical reactions that do not have biological counterparts (Benkovic et al. 1988). For example, toxins could be selectively detoxicated, novel chemical reactions could be developed, or extremely sensitive bioprobes of antibody ligands could be readily developed.

Esterolytic Catalytic Antibodies

Simple structures such as phenyl phosphonates have been synthesized and used to generate monoclonal antihapten antibodies (Tramontano et al. 1988). Antibody-producing hybridoma cells can be cloned to obtain colonies, each producing a single type of antibody

(Tramontano and Schloeder 1989). Because a large number of hybridomas can be generated, it was thought to be impractical to screen all cultures for catalytic activity. Today, with advances in rapid screening techniques, it is now practical to screen hundreds if not thousands of hybridomas for antibodies with catalytic activity. Typically, an antibody is produced in microgram quantities in cell culture and a subset of the population that recognizes the original hapten itself is chosen. One of the first catalytic antibodies studied (i.e., esterase 50D8) (Tramontano et at. 1986*a*, 1986*b*) has been well characterized. Esterase 50D8 shows a high degree of substrate specificity; the rate advantage of 6×10^6 over the background (i.e., uncatalyzed rate) and the catalytic efficiency (i.e., k_{cat} 29 sec^{-1}) both illustrate the catalytic properties of the antibodies; and the antigen or hapten inhibits the substrate (i.e., K_i=60-80 nM). However, the rate factor (i.e., k_{cat}/K_m of 10^4 M^{-1} sec^{-1}) is still several orders of magnitude less than those of a good enzyme that catalyzes an analogous reaction (i.e., values of 10^7 or 10^8 M^{-1} sec^{-1}) and the activity increases sharply above neutral pH and becomes pH-independent above pH 10. This latter observation has been found with most esterolytic catalytic antibodies, and probably reflects the fact that deacylation of the antibody by hydroxide ion is rate limiting. The design of future esterolytic catalytic antibodies must address this apparent shortcoming.

Cocaine Catalytic Antibodies

Typical antibody-catalyzed reaction rates are several hundredfold to 100,000-fold faster than the uncatalyzed reaction of the substrate. Several fundamental postulates have been proposed to explain the rate enhancements that nevertheless fall short of the enormous rate accelerations of enzymes. Is activity truly due solely to transition state stabilization by antibody-binding interactions? Can additional binding interactions be built into the combining site or into the substrate molecule itself to increase the overall rate of the reaction? Can new screening methods and immunological methods be developed to uncover novel catalysts with diverse activities? Most important, can novel esterolytic catalysts be developed based on currently available catalytic antibody technology to efficiently hydrolyze and detoxicate cocaine?

The answer is unquestionably yes. In fact, cocaine is quite immunogenic; a catalytic antibody was obtained that catalyzed the hydrolysis of cocaine, but the rate enhancement was considerably less than desirable (Landry et al. 1993). The ratio of the catalytic rate constant (k_c) to the spontaneous or water-catalyzed rate constant (k_o)

was 540 and 440 for two catalytic antibodies, respectively, which is considerably less than the rate of hydrolysis of other esters by catalytic antibodies reported in the literature. Thus, immunization of mice with a bovine serum albumin conjugate linked to a phosphonate analog of cocaine (figure 4) afforded antisera that provided monoclonal antibodies after using standard purification procedures. The primary screening for anti-cocaine antibodies was done with an enzyme-linked immunosorbent assay (ELISA) against a phospho-nate analog of cocaine (figure 4). Hybridoma cells immunopositive for anti-phosphonate analog antibodies were next screened for cocaine benzoyl ester hydrolytic activity. The selectivity of the most active anti-cocaine catalytic antibody was shown by the inhibition of the hydrolysis reaction in the presence of the phosphonate transition state analog (figure 4). The K_m values for the two most active anti-cocaine catalytic antibodies were 490 and 1020 M, with k_{cat} values of 0.11 and 0.07 min^{-1}, respectively. These values were significantly less impressive than the values obtained for butyryl cholinesterase, the principal cocaine esterase in human serum (Gatley 1991; Stewart et al. 1978), that provided K_m and k_{cat} values of 38 M and 1.2 min^{-1}, respectively. As stated by Landry and colleagues, a catalytic antibody against cocaine should ideally have a turnover number of at least 2 sec^{-1} and a K_m of approximately 30 M to deactivate cocaine before the molecule partitions into the CNS (Landry et al. 1993). Clearly, room for significant improvement in the catalytic and binding properties of antibodies against cocaine is required before such biocatalysts become part of a human therapeutic regime. In addition, advances in humanizing catalytic antibodies are also needed before artificial esterases will find clinical usefulness in decreasing the serum levels of cocaine during overdose or intoxication in humans.

Structure and Modification of Antibodies

The attachment of drugs and enzymes to antibodies is a common practice in commercial diagnostics, experimental immunoassays, and drug-delivery therapeutic approaches (Chaudhary et al. 1989; Pinchera et al. 1985). Antibody-enzyme conjugates are now being used for targeting cytotoxic pro-drugs to cancerous tissue (Chaudhary et al. 1989). In principle, antibody-enzyme conjugates could offer another possible way

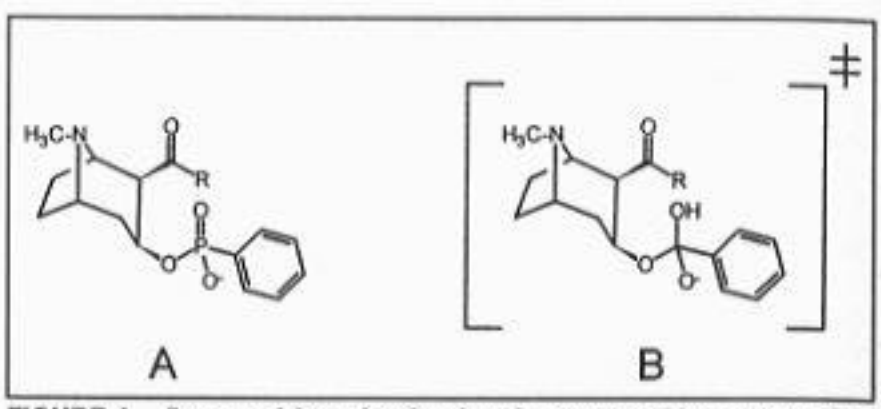

FIGURE 4. *Structural formula of a phosphonate transition state analog of cocaine benzoyl ester hydrolysis (compound A, R = OCH_3) and structural formula of the phosphonate transition state analog of cocaine used in the ELISA assay (Compound A, R = ester tether). Compound B is the structural formula of the putative transition state for benzoyl ester hydrolysis.*

to pharmacologically interdict cocaine or other drugs of abuse. For example, a biocatalyst with cocaine hydrolytic activity could be coupled to an antibody to produce a conjugate with enhanced catalytic and pharmacokinetic properties.

Coupling methods between antibodies and ligands generally rely on chemical modification to selectively introduce the enzyme onto the antibody. Antibodies offer a range of sites for chemical modification. As shown in figure 5, the combining site where antibody-hapten recognition occurs is contained in the Fab fragment. The Fab fragment can be produced by selective proteolytic digestion of the antibody. Thus, a minimal high-affinity binding protein can be obtained by papain digestion of an antibody prepared against the hapten. The variable domains of the light chains (V_L) and heavy chains (V_H) have been frequently documented as having the same antigen-binding activities as the whole antibody (Weir 1986). V_L and V_H fragments can be engineered by molecular biological means to have reduced background (i.e., nonspecific) binding sometimes associated with intact antibodies. In addition, use of the light and heavy chain fragments may be clinically advantageous in that it is possible that they are less immunogenic than the intact antibody but still possess high affinity (Condra et al. 1990). In principle, V_L and V_H fragments are also easier to construct by recombinant methods than intact antibodies because they possess fewer disulfide bonds (Better et al. 1989). However, the stoichiometric expression of V_L and V_H fragments must be linked (Pluckthun 1991). The Fab fragments are

coupled through cysteine residues at the C-terminal end of the heavy chains. Additional cysteines, which are normally involved in interchain disulfide linkages in the hinge region, are retained in a pepsin-generated $F(ab')_2$ fragment (figure 5). Selective coupling of small or large biomacromolecules to $F(ab')_2$ fragments is simple and straightforward. The potential for antibody-enzyme or antibody fragment-enzyme conjugates for use in detoxication of drugs of abuse is essentially unexplored. It is possible that combining the favorable properties of antibody-mediated localization with enzymes or other biocatalysts could provide a new class of detoxication agents.

Anti-Idiotype Antibodies

The immune system discriminates between self and nonself (i.e., foreign) antigen. The foreign antigen can stimulate an immune response, and the immune system sets in motion a steady-state memory lymphocyte which, upon reinfection with the same or closely related antigen, is able to respond with a vigorous immune response. If the foreign antigen is a complementary structure, then one might influence the immune system to recognize the mirror image of the complementary structure. Thus, immunization of an animal with a monoclonal antibody that recognizes the transition state for detoxication of a drug of abuse should elicit an immune response and produce antibodies that resemble the structure of the drug of abuse. The second antibody, the anti-idiotype antibody, thus mimics the transition state for detoxication. Injection of this second antibody into an animal could create a relatively long-term memory effect to give protection against the drug of abuse; the third antibody produced could act as a catalyst to detoxicate the drug (Landry et al. 1993). For example, passive immunization with an anti-cocaine catalytic antibody "could provide a window for appropriate psychosocial and relapse-prevention interventions" (Landry et al. 1993). Of course, researchers are a long way away from passively immunizing humans with catalytic antibodies. However, future use of antibodies in a therapeutic paradigm may provide novel approaches to promoting cessation of use of drugs of abuse as well as in the maintenance of abstinence (Rocio et al. 1995).

Catalytic Antibodies and Catalytic RNA

As discussed above, in vitro selection techniques have been used to isolate biocatalysts useful in the development of catalytic antibodies (Tawfik et al. 1990). While the cocaine catalytic antibodies reported thus

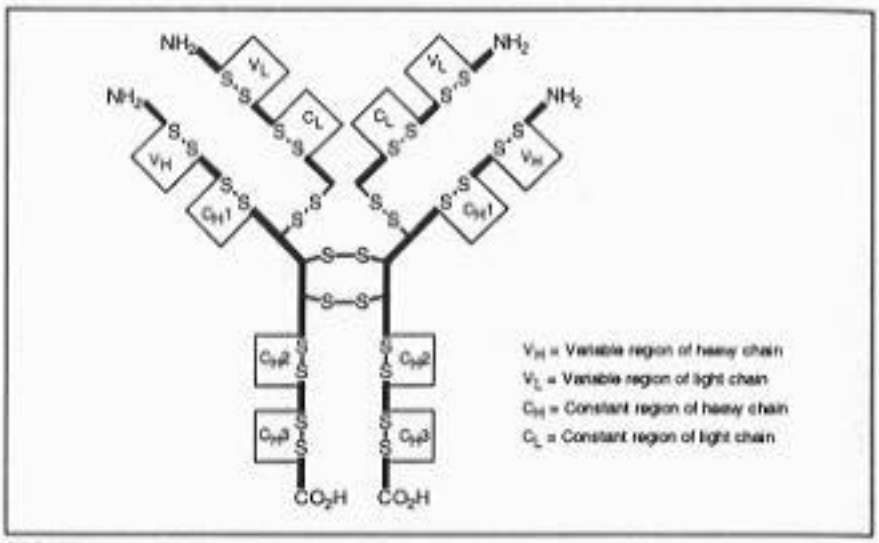

FIGURE 5. *Two-dimensional depiction of an antibody.*

SOURCE: Harlow and Lane 1988.

far do not possess impressive hydrolytic catalytic rate accelerations, the selection technology has nevertheless allowed the discovery of some fundamentals of catalysts that might later support rational design of detoxication catalysts and answer fundamental questions in catalysis (Benner 1993). Catalytic antibody technology relies on the selection of biocatalysts using the enormous diversity of the immune system. Procurement of catalytic species depends on the ability of substrates to chemically bind or transform a target molecule. The key feature of the catalytic antibody is the specificity of the reaction catalyzed. It is interesting to note that the relatively recent advances in catalytic RNA technology have revealed considerable parallels between the concepts of catalytic antibodies and catalytic RNA.

Catalytic RNA

Several structurally and mechanistically distinct classes of catalytic RNAs have been discovered (Altman 1990; Cech 1990; Pace and Smith 1990). Generally, for catalytic RNA reactions reported thus far, RNA and deoxyribonucleic acid (DNA) are substrates and transesterification or hydrolysis of phosphate esters are the reactions catalyzed (Piccirilli et al. 1992). However, binding of amino acids, organic dyes, and other small molecules to RNA suggest that catalytic RNAs may catalyze other reactions (Prudent et al. 1994). Some catalytic RNA molecules are metalloenzymes and require magnesium for their three-dimensional structure and catalytic activity. Like

catalytic antibodies, catalytic RNAs (i.e., ribozymes) catalyze very specific reactions. For example, DNA-cleaving ribozymes with relatively high catalytic efficiency that undergo relatively rapid turnover and operate in a highly specific manner have been discovered (Tsang and Joyce 1994). Future studies may show that catalytic RNA has utility as sequence-specific DNA endonucleases. The specificity stems from the first step in the ribozyme reaction which is due to specific Watson-Crick base-pairing interactions (Mueller et al. 1993). In every ribozyme thus far examined, a template region (named the internal guide sequence) (Seiwert and Stuart 1994) has been observed near the 5'-end of the molecule that forms Watson-Crick base pairs with the target RNA substrate. The 3'-hydroxy of a base at the other end of the ribozyme attacks the phosphodiester bond within the ribozyme-bound substrate complex. A transesterication reaction occurs that results in cleavage of the substrate and ligation of the 3'-portion of the substrate (Tsang and Joyce 1994). Just like in the case of enzyme- or catalytic antibody-catalyzed reactions, the rate depends upon substrate binding affinity and the intrinsic catalytic rate parameters. For example, in ester hydrolysis there is a hyperbolic dependence on the concentration of the ribozyme: at low concentration of catalyst the rate of hydrolysis is first order, while at high concentration of catalyst the reaction rate is indepen-dent of ribozyme concentration (Piccirilli et al. 1992). This type of saturation or Michaelis-Menten kinetic behavior is typical of ribozymes and is completely analogous to the enzyme-substrate complex observed for enzymes and catalytic antibodies. Like other biocatalysts, RNA-cleaving ribozymes are highly specific for RNA and catalyze hydrolysis with high catalytic efficiency, undergo rapid turnover, and operate in a highly selective manner. In addition, inhibition of ribozyme catalysis has been observed for substrate analogs; like enzymes and other biomacro-molecules, changes in ribozyme solvation and conformation have been observed when the inhibitor binds (Piccirilli et al. 1992).

Ribozymes may be developed in the future to act as aminoacyl tRNA synthetases. Ribozymes might be developed to selectively break acyl bonds to oligonucleotides or might also be engineered to selectively charge oligonucleotides with amino acids. Again, due to the specificity of Watson-Crick base-pair interactions, oligonucleotide catalysts may find use in the therapeutics of human disease. Highly selective ribozyme endonucleases may be created to perform sequence-specific cleavages of potential therapeutic value. The ratio of a toxic dose to a therapeutic dose (i.e., [toxic dose]/[therapeutic dose]) presumably will be very high for an effective ribozyme

primarily due to the high specificity of oligonucleo-tides (i.e., containing 15 to 17 nucleotides) that should have a unique sequence relative to the entire human genome (Stein and Cheng 1993). In principle, suitable oligonucleotides should be able to interfere in a sequence-specific manner with processes such as translation of messenger RNA (mRNA) into a specific receptor or other protein. If the synthesis of the target protein encoded by the mRNA is required for susceptibility to drug addiction, it may be possible to develop new therapeutic strategies based on elaboration of ribozymes or oligonucleotides targeted to inhibit or inactivate key biological processes.

SUMMARY

Currently there is a significant amount of information about the way biocatalysts from animals detoxicate and bioactivate drugs of abuse. In some cases, biotransformation data concerning drugs of abuse obtained from animal systems are analogous to the human situation, but in many cases the data are not. Clearly, significant work needs to be done with human biocatalysts to define a role in the biotransformation of drugs of abuse and to relate the work that has already been done in animals. New metabolic pathways will likely be discovered that may link drug metabolism to addiction liability or drug susceptibility in humans.

New design and selection technologies are providing the basis to allow the discovery of new biocatalysts that may be useful in the detoxication of drugs of abuse in humans. Fundamentally new approaches using biocatalysts including rationally engineered enzymes, catalytic antibodies, catalytic antibody fragments, ribozymes, oligonucleotides, and other biomacromolecules may provide basic information that may later support the rational design of biocatalysts, which may in turn provide the basis for designing detoxication catalysts for drugs of abuse.

NOTES

1. In this chapter, nicotine or nicotine metabolites shall all be assumed to have the (*S*) stereochemistry at the benzylic center.

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Antibodies as Pharmacokinetic and Metabolic Modifiers of Neurotoxicity

S. Michael Owens

INTRODUCTION

Abuse of psychoactive chemicals can result in neurotoxic effects that are difficult to treat medically. Successful therapy is often hindered by the lack of useful antagonists for many of these chemicals and by the extensive distribution of these chemicals out of the bloodstream. Although there are treatments for opiate addiction and an antagonist for opiate overdose, there are no such medical treatments for most drugs of abuse such as phencyclidine (PCP) and cocaine. Therefore, this chapter focuses on recent advances in immunotherapy which suggest this novel approach could be beneficial in the treatment of drug abuse.

PROBLEMS ASSOCIATED WITH TREATMENT OF PCP TOXICITY

PCP use by drug abusers sometimes results in behavioral toxicity that manifests as a wide variety of frightening effects that include extreme violence; self-destructive and psychotic behavior; and, at very high doses, even death (Peterson and Stillman 1978). Often the acute behavioral toxicity is not realized or even felt by the individual because of the anesthetic effects of the drug. Indeed, the lack of a pain sensation may contribute to PCP's ability to make users become violent and self-destructive. The acute toxicity of PCP is often misdiagnosed because of its similarity to schizophrenic episodes. Arylcyclohexylamines like PCP are considered to produce one of the best drug-induced models of schizophrenia (Luby et al. 1962; Itil et al. 1967). These clinical manifestations are even more frightening when misinterpreted by the public. In addition, chronic PCP use in some individuals can produce a long-lasting schizophrenic episode, which in some cases may not be reversible. These effects can be produced in humans at doses ranging from 5 milligrams (mg) to greater than 10 mg. Consequently, treatment for PCP abuse is needed and, in fact, may be most necessary to offset the behavioral toxicity that occurs at only moderate doses.

The pharmacological effects of PCP and related arylcyclohexylamines are produced through interaction with several neurotransmitter systems, ion channels, and catecholamine uptake systems. These sites include the so-called PCP receptor that is associated with the N-methyl-D-aspartate (NMDA) receptor complex (Lodge et al. 1983; Vincent et al. 1979; Zukin and Zukin 1979) and the dopamine reuptake site that may also significantly contribute to PCP abuse and psychosis (Chaudieu et al. 1989; Vignon et al. 1988).

NMDA receptor antagonists such as PCP can be protective against brain damage in neurological disorders such as stroke (Olney et al. 1989; Rothman 1984), but can also cause dose-dependent morphological damage to neurons in the cerebral cortex of rats (Olney et al. 1991). Although these effects appear to be reversible after a single dose, effects after chronic doses have not been studied and it is conceivable that chronic use of the drug could be associated with more permanent neurological damage. In addition, female rats are twice as sensitive to these effects as male rats (Olney et al. 1989). This increased potency of the drug in female rats is also found with other pharmacological effects of PCP. The mechanisms underlying these sex differences in the pharmacological effects in rats are poorly understood, but they are dose dependent and are found after single and chronic doses of the drug (Olney et al. 1989; Wessinger 1995). In addition, females are much less efficient at metabolizing PCP than males. Although sex differences of this type are not found in humans, this sexual dimorphism in the rat could be a useful model for mimicking the wide range of differences in effects and metabolism of the drug in humans.

IMMUNOTHERAPY FOR TREATMENT OF DRUG OVERDOSE

Treatment of the adverse effects of PCP is difficult for several reasons. PCP has a very high volume of distribution (6.2 liters per kilogram (L/kg) in humans) and its clearance is primarily by metabolism (Cook et al. 1982) with only a small contribution from renal excretion. Its major sites of action in the central nervous system (CNS) are far removed from the beneficial effects of most traditional treatment methods such as dialysis. In addition, there is no specific antagonist for PCP's adverse effects. These pharmacokinetic and receptor-medicated characteristics make it very difficult to develop effective treatment strategies. Some of the current methods for treatment of overdose are urine acidification, diazepam administration to control convulsions (Aronow and Done 1978;

Peterson and Stillman 1978), and simply waiting for the patient to get better. Nevertheless, Mayersohn (1985) predicts that even under ideal conditions, urine acidification would only increase systemic clearance (Cls) by about 28 percent and would only decrease the half-life (t_) by about 23 percent (t_ in humans averages about 17 hrs, but can range from 7 to 58 hrs; Cook et al. 1985). This small change in kinetic parameters is not surprising due to PCP's very high volume of distribution and low renal clearance (Mayersohn 1985). Consequently, treatments that can only reduce the amount of drug in the bloodstream will not be effective.

One approach to treating drug overdose resulting from CNS-acting, high- distribution volume drugs is the use of high-affinity, drug-specific antibodies (Colburn 1980; Owens and Mayersohn 1986; Smith et al. 1979). Currently, the only drug toxicity routinely treated by immunotherapy is the cardiac toxicity due to digitalis (Smith et al. 1982). The use of anti-drug antibodies for treating toxicity due to CNS-acting agents like PCP is currently being tested in a rat model (McClurkan et al. 1993; Valentine et al. 1994). These studies show that the anti-PCP Fab can dramatically change PCP pharmacokinetics (table 1).

Earlier pharmacokinetic studies with a tracer dose of [^{3}H]PCP (4 micrograms (g)) and very low doses of goat anti-PCP Fab (0.5 to 1 mg) in dogs suggested the therapy could be beneficial (Owens and Mayersohn 1986). However, the major limitation at the time of these earlier studies was the inability to produce large quantities of drug-specific antibody. The development of large-scale techniques for the production of monoclonal antibodies of desired specificity has made immunotherapy a more attractive possibility for treating toxicity associated with drugs of abuse. These large-scale techniques for monoclonal antibody production include ascites production and hollow fiber bioreactors. Hollow fiber bioreactor techniques for large-scale production of a high-affinity murine monoclonal antibody are capable of producing gram quantities of monoclonal antibodies on a daily basis for extended periods of time. This technique is the preferred method since it does not require the use of live animals, and the production scale can be significantly increased over other methods. These are essential features since treatment of PCP overdose in humans will probably require several grams of Fab.

TABLE 1. *Comparison of the pharmacokinetics of PCP, anti-PCP Fab, and PCP after treatment with anti-PCP Fab in Sprague-Dawley rats.*

Pharmacokinetic parameter	Anti-PCP Fab[a]	PCP[b]	PCP after treatment with anti-PCP Fab[b]
Vss*	0.55 L/kg	12.6 L/kg	0.6 L/kg
Cls	2.7 mL/min/kg	66.3 mL/min/kg	6.8 mL/min/kg
t_	7.5 hrs	3.9 hrs	4.9 hrs

KEY: * = Vss = volume of distribution at steady state.

SOURCES: a = data from McClurkan et al. 1993; b = data from Valentine et al. 1994.

From a pharmacokinetic standpoint, the potential for this new class of biologically based drugs (designer antibodies) is very exciting. Antibodies would be expected to have a totally different set of disposition parameters (volume of distribution, clearance, metabolism, and t_) from the drugs against which they are used as antagonists. Table 1 shows a comparison of the pharmacokinetic parameters for monoclonal anti-PCP Fab, PCP, and PCP after administration of anti-PCP Fab. In addition, the antigenicity can be significantly decreased or completely eliminated simply by digesting the immunoglobulin G (IgG) with papain and removing the antigenic Fc (crystalline fragment) portion by chromatographic procedures (Porter 1959; Smith et al. 1979). This yields two Fab fragments (antigen-binding fragments) each with the same affinity as the parent IgG (figure 1). The Fab fragments also have a ninefold greater apparent volume of distribution compared to intact IgG (Smith et al. 1979). Furthermore, the use of monoclonal antibodies allows greater refinement of both antibody specificity and affinity as well as the possibility for the production of large quantities of a homogeneous protein. When the therapeutic use of human monoclonal antibodies becomes more practical, the potential for protein antigenicity should be reduced even further when used in humans.

From a basic science point of view, it is very interesting to study the effects of a high-affinity antibody on the disposition of a drug such as PCP. This is because PCP has some unique characteristics in terms of its metabolism and pharmacokinetics. First, in the rat and other animal

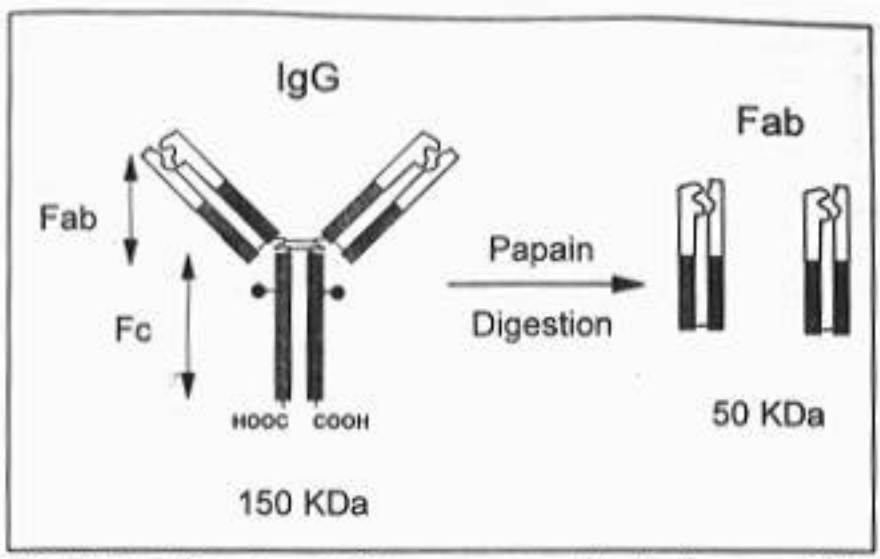

FIGURE 1. *Digestion of IgG into two antigen-binding fragments (Fab) with papain.*

species including humans, PCP has an extremely high volume of distribution (12.6 L/kg in the rat (table 1; Valentine et al. 1994); 6.2 L/kg in man (Cook et al. 1982; see Owens et al. 1987 for a review of PCP pharmacokinetics across species). In contrast, monoclonal anti-PCP Fab appears to have a much smaller volume of distribution (0.55 L/kg or about twice the value of extracellular fluid volume (table 1) (Valentine et al. 1994)). Second, the systemic clearance of PCP in the male rat is over 95 percent due to metabolism, with renal clearance composing only about 1 to 5 percent of total body clearance (Valentine et al. 1994). For comparison, the renal clearance of Fab in the rat comprises about 17 percent of total body clearance. Also, the kidney is an important site for metabolism of Fab (Arend and Silverblatt 1975; Wochner et al. 1967). Consequently, it is essential to determine how the high-affinity monoclonal Fab changes PCP effects and disposition at toxic doses of PCP (Valentine et al. 1994).

A possible drawback to the use of antibodies for the treatment of toxicity is the possibility of anti-idiotypes being produced against the monoclonal antibody binding site. These antibodies could potentially mimic drug-like structural features necessary for binding to a receptor. However, antibodies do not cross the blood-brain barrier in significant amounts and studies to date have not found this to be a problem, especially when Fab fragments are used.

With careful consideration for hapten design, anti-drug antibodies can be used in the treatment of toxicity due to other structurally related compounds. These compounds are usually Schedule I drugs because of their potential for abuse (e.g., 1-[1-(2-thienyl) cyclohexyl] piperdine (TCP) and N-ethyl-1-phencyclohexylamine (PCE). If these other drugs were to suddenly become available to drug abusers, as have a wide variety of fentanyl- and amphetamine-like derivatives, the medical community would not be prepared to treat the toxic effects of these other arylcyclohexylamines. Therefore, it would be most prudent if strategies could be developed for the treatment of a whole class of drugs rather than individual drugs. Indeed, several arylcyclohexylamines appear to have similar effects to PCP, except that they are even more potent. TCP and PCE are about 1.3 to 6 times more potent (respectively) than PCP in drug discrimination assays (Shannon 1981, 1983) and PCP receptor binding assays (see Owens et al. 1988 for a review).

After careful consideration for the structure-activity relationship of arylcyclohexylamines, monoclonal antibodies against a PCP-like hapten (5-[N-(1'-phenylcyclohexyl)amino]pentanoic acid) were generated (McClurkan et al. 1993). These antibodies bind to PCP, TCP, and PCE with a greater affinity than the binding of these ligands to the PCP receptor (Owens, unpublished observation). The development of antibody-based approaches for treating drug classes, rather than just one specific drug, is an exciting possibility and could provide a prototypic model for designing immunotherapeutic approaches for other classes of drugs of abuse.

CATALYTIC ANTIBODIES FOR TREATMENT OF DRUG ABUSE

Another experimental approach for the treatment of drug abuse is the use of catalytic antibodies. The idea behind this treatment is to create an artificial, antibody-based enzyme that can convert the parent drug to an inactive metabolite. This catalytic antibody would then be administered in medical emergencies for overdose or perhaps administered on a chronic basis to persons trying to withdraw from addiction to the drug. In the field of drug abuse, the possibility of using cocaine catalytic antibodies as a therapeutic aid to the treatment of toxicity and addiction has generated the most clinical interest.

Cocaine is a good prototypic ligand to study for this purpose because the drug is naturally metabolized by in vivo estearases, and catalytic monoclonal antibodies with esterase activity are among the few

metabolic reactions that have been successfully mimicked. Landry and colleagues (1993) have generated a monoclonal antibody that can metabolize cocaine to ecgonine methyl ester and benzoic acid. While this approach may have some theoretical advantages over the use of high-affinity anti-drug antibodies, it also has some serious and perhaps insurmountable disadvantages.

The major theoretical advantage to catalytic antibodies for therapy is that, unlike most anti-drug antibodies, they release the breakdown product (metabolite) and are ready to metabolize more drug. Since they continuously regenerate their capacity (like a natural enzyme), they could reduce the body burden of drug with perhaps lower molar ratios of antibody to drug than would be needed for an anti-cocaine antibody. It has been suggested that they could even be used for passive immunization of cocaine addicts during periods of psychological and social rehabilitation to help prevent or blunt drug effects if the patient self-administered cocaine. While these suggested uses could be important from a pharmacokinetic, pharmacodynamic, and immunological point of view, these uses may prove difficult to achieve.

The use of catalytic antibodies for acute toxicity in humans would most likely be only marginally helpful. It is difficult to conceive that this medical approach would have significant advantages over high-affinity anti-cocaine antibodies such as those currently in use for treating digoxin toxicity. It is true that catalytic antibodies could potentially regenerate their capacity after enzymatic turnover of cocaine, theoretically allowing a higher overall capacity for the antibody antagonist. However, in terms of therapeutic effect, rapid reduction of tissue concentrations may be the critical factor in saving lives.

Sudden death is a major factor in cocaine-related fatalities. The mechanism for the cardiotoxicity is not fully understood, but it appears to be poorly predicted from patient to patient. Nevertheless, a rapid immuno-therapeutic response would be essential in treating cocaine-related toxicity, and even catalytic antibodies with low micromolar (M) Michaelis-Menton constant (K_m) values may not be fast-acting enough. By comparison, high-affinity anti-drug antibodies typically have dissociation constant (K_d) values in the low nanomolar (nM) to high picomolar (pM) range. Therefore, to test the usefulness of catalytic antibodies, it will be important to conduct full dose-response curves with the cocaine catalytic antibodies and to

compare these results with the therapeutic effects of similar doses of high-affinity anti-cocaine antibodies as the control.

Currently the immunotherapy literature is not clear about the dose of antibody that would be needed to rapidly reduce the body burden, and thereby the toxicity, of drugs. Nevertheless, some studies suggest it is necessary to use approximately 0.3 to > 1.0 mole equivalent doses of anti-drug antibodies. Since the K_m values for cocaine catalytic antibodies are likely to be relatively high compared to the K_d values for anti-drug antibodies, it is especially difficult to estimate the amount of catalytic antibodies that would be necessary to be effective in various medical situations. Nevertheless, based on the expected high K_m values for cocaine metabolism, the amount of antibody needed for effective treatment will likely be significantly greater than the 0.3 to 1.0 mole equivalents of a high-affinity (low K_d value) anti-cocaine antibody.

Therefore, the greater potential capacity for enzymatic inactivation by catalytic antibodies might be more than offset by their lower effective affinities (i.e., higher K_m values). The higher K_m values would result in very slow metabolic inactivation. The metabolic capacity of endogenous enzyme systems that metabolize cocaine are very high and very efficient relative to the currently available catalytic antibodies. In addition, high- affinity anti-cocaine antibodies are easier to produce than cocaine catalytic antibodies. Since the terminal elimination t_ of cocaine is less than 2 hours (Cook et al. 1985) and the terminal elimination t_ for Fab fragments and IgG are on the order of several hours to a day or more, the normal in vivo metabolic pathways for cocaine could also serve to metabolize unbound drug and thereby regenerate these cocaine-specific antibodies. This would assume that the high-affinity anti-cocaine antibodies would not significantly cross-react with cocaine metabolites.

As another minor point, the use of catalytic antibodies with esterase activity could theoretically lead to autoimmunity against endogenous enzymes. This problem could be partially overcome if Fab fragments were used, or perhaps completely overcome if humanized antibodies or their fragments were used. Also, it is not currently known if endogenous ligands will be substrates for these catalytic enzymes; this could potentially decrease the effective rate for cocaine metabolism or lead to other medical problems.

It has also been suggested that cocaine catalytic antibodies could be used for chronic treatment (for months) of cocaine addicts to augment behavioral modification therapy while patients are withdrawing from the drug. The theory is that if high doses of cocaine catalytic antibodies were present in a recovering addict who then used cocaine, the drug would be efficiently removed or the effects would at least be blunted by the catalytic antibodies. Although this treatment would presumably prevent addicts from adding to their dependence on the drug, it could possibly lead to a dangerous clinical situation. It is difficult to imagine that enough cocaine catalytic antibody could be continuously admini-stered to blunt the effects of cocaine, since enterprising patients would simply take increasing amounts of drug until they obtained the desired effect. In addition, the presence of cocaine catalytic antibodies could actually be a problem because the patient's attempts to titrate effects would be very unpredictable. For instance, if the patient surmounted the effects of the catalytic antibody by self-administering high doses soon after immunotherapy, this dose of cocaine would be too high even a day later when the concentration of the catalytic antibodies had significantly deceased. This could lead to an unexpected overdose.

For now, the major strength in the use of catalytic antibodies is the basic science underlying generation of this unique pharmacological tool. However, it is proving extremely challenging to develop a catalytic antibody with a turnover rate that even approaches the turnover rates of natural cocaine-metabolizing enzymes such as butyrylcholinesterase. Therefore, researchers should first determine if similar approaches, which are currently more technically feasible, might serve the same purpose. For instance, butyrylcholinesterase purified from human blood or high- affinity monoclonal antibodies might be more useful. Nevertheless, basic research on cocaine catalytic antibodies should be pursued since the development of pharmacokinetic and metabolic modifiers of abused drugs is an underexplored area of medications development.

ANIMAL MODELS FOR TESTING IMMUNOTHERAPY

As discussed earlier, high-affinity anti-drug antibodies produce effects on the pharmacokinetics and pharmacodynamics of drugs in animals and humans. For new therapeutic applications, these effects need to be fully tested in animals before administration to humans. In addition, from a basic science viewpoint, it will be necessary to develop relevant pharmacokinetic and pharmacodynamic models of

the effects of immunotherapy on drug abuse. Examples of the scientific questions that need to be addressed are as follows.

The relationships between antibody dose, antibody affinity (or catalytic antibody K_m), and their effects on the drug's pharmacokinetics and pharmacodynamics are poorly understood. For instance, if the drug effect compartment is associated with the pharmacokinetic peripheral compartment, the time needed to reverse drug effects with antibodies would be predicted to be slower than if the effect compartment is associated with the pharmacokinetic central compartment. In addition, it appears that high-affinity antibodies block the metabolism and/or change the metabolic profile of drugs (Owens and Mayersohn 1986; Valentine et al. 1994). These are complex changes that need to be studied in detail, and pharmacokinetic and pharmacodynamic models of these effects need to be developed in animal models before use in humans.

One way to model the genetic polymorphism of metabolism and drug effects in humans is to use the genetic diversity in different strains and sexes of animals such as rats. Although there are not significant sex-related differences in metabolism in humans, sex-related differences in rat liver function have been identified in the last few decades (see Zaphiropoulos et al. 1989 for a review). For example, sexual dimorphism in metabolism with cytochrome P-450 (CYP) rat isozymes occurs with CYP2C13, CYP2C11, CYP2C12, CYP3A2, CYP2A2, and CYP2E1 (Zaphiropoulos et al. 1989). In particular, there are major differences in either the amounts or the expression of the CYP family of enzymes. These enzymes catalyze the oxidation of many steroids, xenobiotics, and drugs (Waxman et al. 1985). These sexual differences are most extensively characterized in rats; in general, the CYP-catalyzed reactions are more efficient in male rats than in female rats. Studying these differences in the pharmacological response resulting from sex-related differences in rat metabolism should provide a useful model for predicting differences in the pharmacological response resulting from genetic polymorphism in human metabolism.

Although these sex-related differences in metabolism have been known for a long time, there is very little data available concerning the role of gender in the metabolism and pharmacokinetics of drugs of abuse. For some drugs of abuse, sexual dimorphism could have important implications and adverse consequences. For PCP, a subject of this discussion, metabolism is the major mechanism for inactivation of pharmacological effects (Mayersohn 1985). In

normal rats, recent data implicate the constitutive CYP2D1 and CYP2C11 isozymes in the formation of PCP metabolites (Hiratsuka et al. 1995; Shelnutt et al., in press).

CONCLUSIONS

Immunotherapy for treating drug abuse appears to be a viable therapeutic approach. To make this experimental treatment a reality, some major hurdles must be overcome. These include the production and purification of very large quantities of antibody at a reasonable cost and increasing the safety of using antibodies in humans. Increased production is currently possible through the use of bioreactors for large-scale production of monoclonal antibodies. Nevertheless, the cost of the antibody is still too high for the day-to-day use in most emergency rooms and clinics. As the technology improves, the cost should be dramatically reduced. To make antibodies safer for use in humans it should be possible to engineer human monoclonal antibodies or to make very small, high-affinity binding proteins based on the knowledge gained from antibody binding site templates.

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Toxicokinetics in the National Toxicology Program

Thomas J. Goehl

BACKGROUND

The National Toxicology Program (NTP) was established by the Department of Health and Human Services (DHHS) in 1978 to coordinate and manage the department's toxicology studies. The objective of DHHS' toxicology efforts is to develop scientific information necessary to protect the health of the public from exposure to hazardous chemicals. The NTP consists of the National Institutes of Health's (NIH) National Institute of Environmental Health Sciences (NIEHS), the Centers for Disease Control and Prevention's (CDC) National Institute of Occupational Safety and Health (NIOSH), and the Food and Drug Administration's (FDA) National Center for Toxicological Research (NCTR). The NTP is administered by a director, who is also the director of NIEHS. An executive committee consisting of heads of Federal health research and regulatory agencies (Consumer Products Safety Commission (CPSC), Environmental Protection Agency (EPA), FDA, National Cancer Institute (NCI), NIEHS, NIOSH, and Occupational Safety and Health Administration (OSHA)) and a board of scientific counselors (composed of governmental, industrial, and academic scientists) provide guidance for the program (figure 1).

The NTP toxicological evaluations are initiated on chemicals that are nominated for study. These nominations can come from the private or public sector, and are sent to Chemical Nominations, National Toxicology Program, NIEHS, PO Box 12233, Maildrop A001, Research Triangle Park, NC 27709. After an extensive nomination review process, selections are made and each chemical assigned to an NTP scientist who serves as the toxicology study project director. A project team is then formed by the project director to review the literature and identify data gaps. Basic information on chemical disposition, chemistry, genetic toxicology, and health and safety are developed as needed and provided to the project director for consideration in the design of toxicology protocols. The toxicology study protocols that are developed may include a general toxicological evaluation and/or specific target organ studies (figure 2).

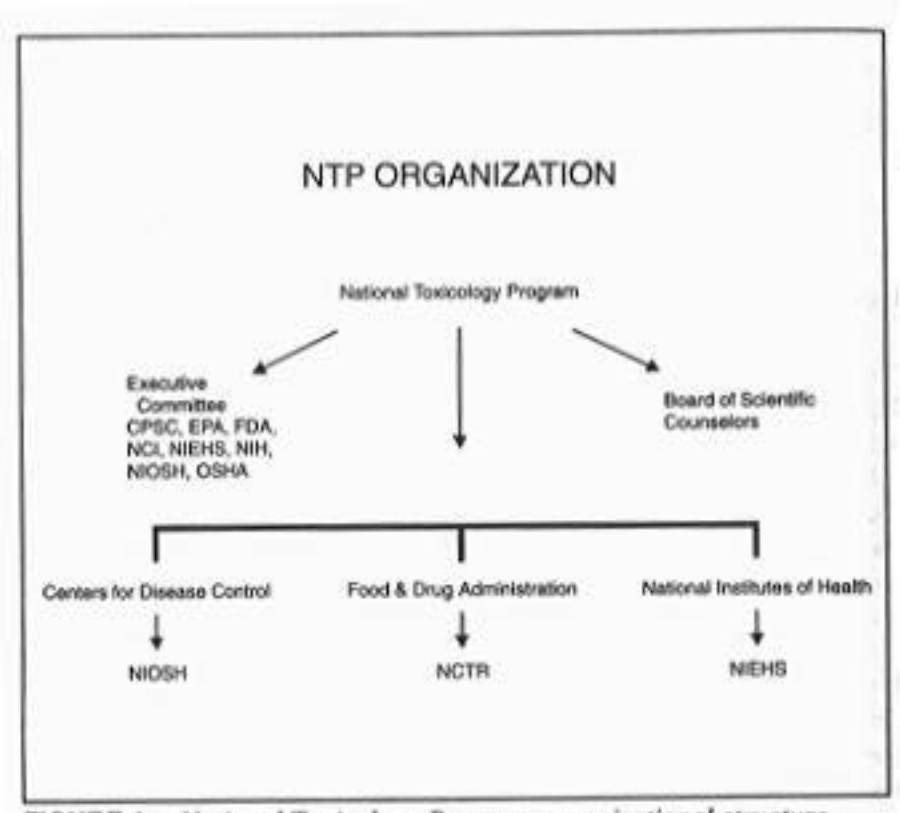

FIGURE 1. *National Toxicology Program organizational structure.*

OVERVIEW

Toxicokinetics is a term used for describing kinetic studies conducted in conjunction with toxicology evaluations (Di Carlo 1982) that deal with absorption, distribution, and elimination processes of chemicals present at concentrations that produce toxic effects. By monitoring the blood concentrations of the chemical and/or metabolites over time after administration by different routes, the test chemical's bioavailability and kinetic characteristics can be readily obtained. The data also permit the determination of the so-called linear dose range based on area under the plasma versus time curve and clearance or other related toxicokinetic parameters, as well as the prediction of possible bioaccumulation after multiple doses. Changes in kinetic parameters after multiple exposures

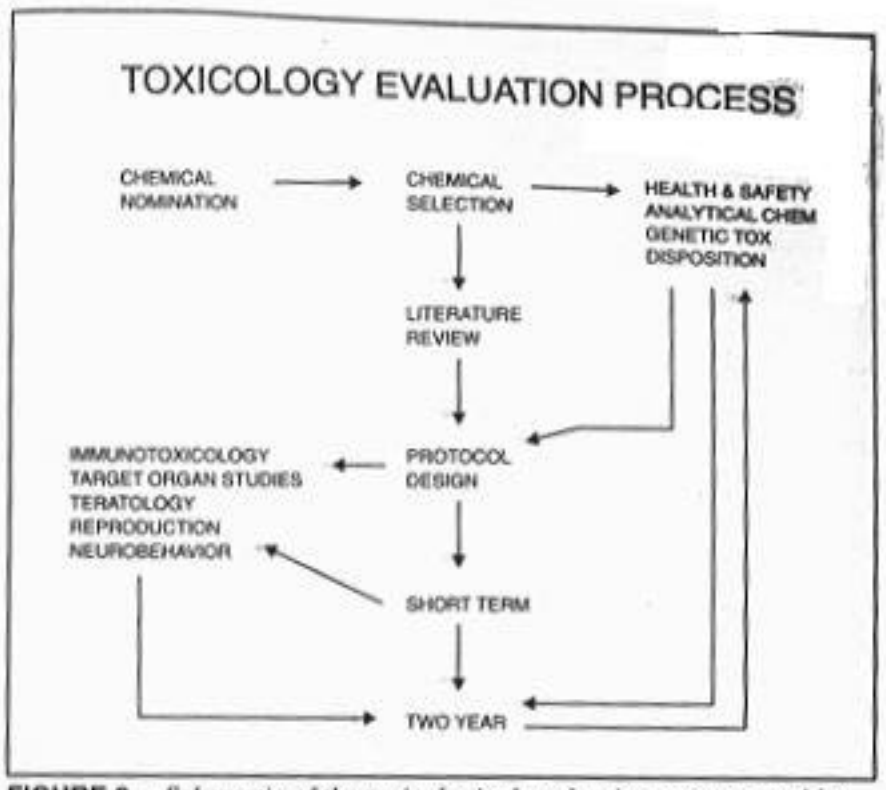

FIGURE 2. *Schematic of the toxicological evaluation process used by the National Toxicology Program.*

are indicative of perturbations in the normal way the animal handles the chemical (e.g., enzyme induction or inhibition). Techniques such as physiologically based toxicokinetic (PBTK) modeling have been used (Bischoff 1967; Ramsey and Andersen 1984; Teorell 1937) to predict the target organ and blood concentrations at various doses. Other work has centered on developing interspecies scaling factors (Mordenti 1986; Travis and White 1988).

During recent years, the importance of toxicokinetic information in risk evaluation has become more widely recognized (Yacobi et al. 1989), including by the NTP. In fact, as part of the activities prior to the conduct of an NTP toxicology study, the determination of basic toxicokinetic information is often conducted to aid in study design. During the conduct of NTP toxicology studies, evaluation of internal dose and changes in kinetic parameters are also frequently conducted to aid in the interpretation of toxicology study outcomes. Generally the internal dose is based on plasma concentrations, since the target organ for the toxicologic effect generally has not been identified as yet. However, after a PBTK model has been developed,

the target organ dose can later be estimated using the collected plasma data. Most of the NTP effort to date has been given to 2-year toxicology and carcinogenicity studies. The application of NTP's toxicokinetic studies to toxicology study design and interpretation is discussed in this chapter.

GOALS

Over the years, NTP, a worldwide leader in toxicology, has conducted chemical disposition studies. More recently, NTP and others (Jameson and Goehl 1994; World Health Organization 1986) have placed even greater emphasis on such studies, especially those that evaluate the kinetics. The scope of the NTP toxicology effort can be seen in the listing of projected NTP toxicology studies for Fiscal Year 1996 (figure 3). Many of the studies listed will include toxicokinetic evaluations. It is anticipated that the toxicokinetic data collected will ultimately improve the usefulness of the toxicology study for risk characterization.

Specifically, the goals of the toxicokinetic evaluations conducted prior to the conduct of toxicology studies are to assist in the selection of the animal species and strain, dose vehicle, dosing route, and dosages. The goals of the determination of concentrations of test chemical or metabolite concentrations in biological samples during the course of the toxicology study are to estimate the internal dose and its possible correlation to toxic effects, as well as to evaluate the effect of age and multiple exposure on the disposition kinetics (figure 4).

APPROACH

If the available toxicokinetic information does not provide enough information to aid in toxicology study design, NTP designs an upfront toxicokinetic study. The doses for the toxicokinetic study are chosen to reflect those anticipated to be used in the toxicology studies. The most common species used by the NTP are Fischer 344/N rats and B6C3F1 mice. The approach used by the NTP involves three steps: preliminary

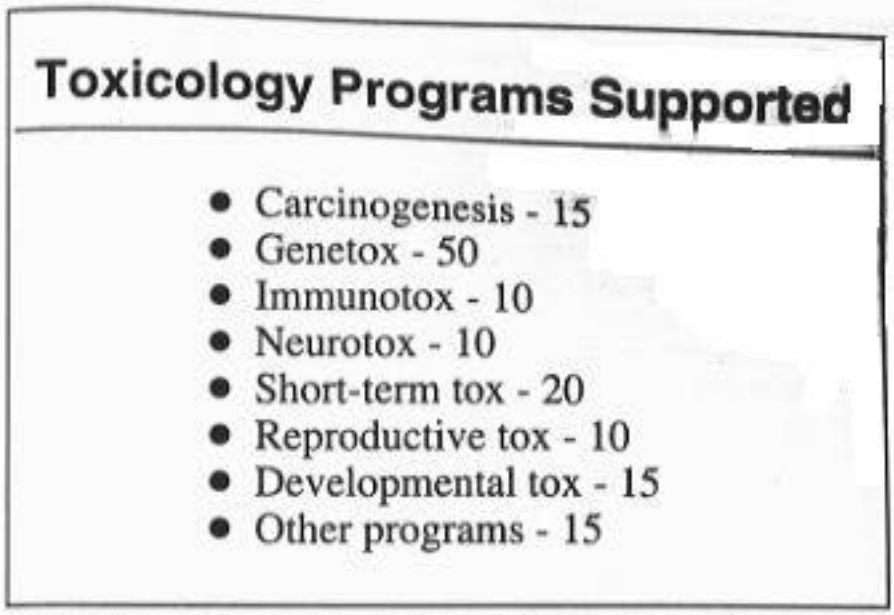

FIGURE 3. *List of the number of chemicals in each program area scheduled for study by the National Toxicology Program in FY 1996.*

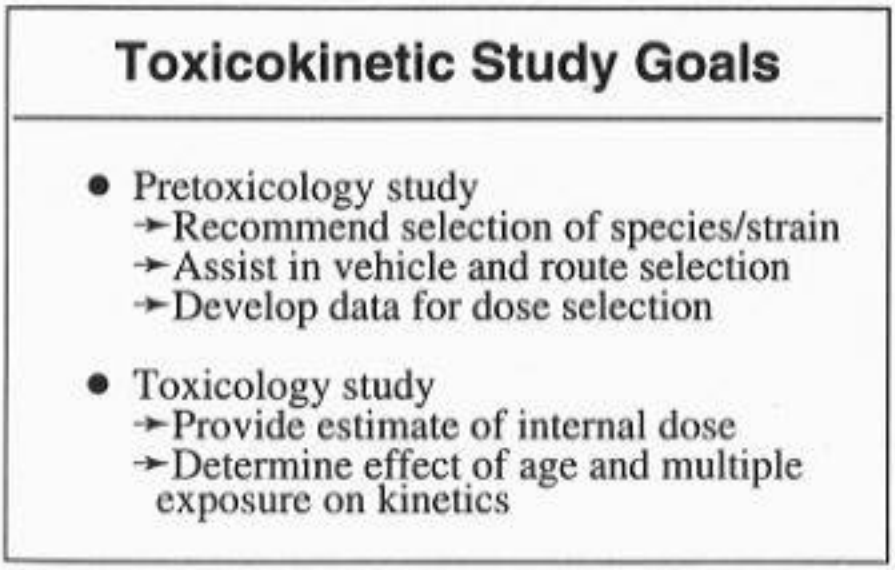

FIGURE 4. *Goals for the conduct of toxicokinetic studies prior to and during the actual toxicology study.*

studies, definitive studies, and studies conducted during the in life toxicology evaluations. The first step in producing the needed toxicokinetic data is to develop an analytical method to quantify the test chemical or metabolite in a biological sample. Most of the time blood plasma is used; all subsequent discussions will specifically refer to the analysis of the analyte in plasma. If the anticipated plasma concentrations and optimal blood sampling times are not known, it is often necessary to conduct a preliminary toxicokinetic study. This type of study involves the administration of the test chemical at the lowest and highest doses proposed for the toxicology study to a small number of animals by appropriate routes and then drawing blood samples at approximately 12 time points. The samples are analyzed using an unvalidated analytical method. Based on these results, the analytical method is further developed to accommodate the determined concentration range and then validated (see next section on analytical method validation). The stability of the analyte in the biological material is subsequently determined at 75 percent of the highest expected concentration for the period of time over which the samples are to be stored prior to analysis (figure 5).

The second step is to conduct definitive toxicokinetic studies (figure 6). In the initial single-exposure definitive study, the chemical is intravenously administered to the animals at two doses, using sufficient animals so that three data points are available at each of 10 sampling times. (The number of sampling times depends on the kinetic profile suggested by the preliminary toxicokinetic studies.) It is hoped that the intravenous (IV) single-exposure definitive study results in plasma concentrations that are directly proportional to dose and provides basic toxicokinetic parameters. The IV study is conducted regardless of the exposure route planned for the toxicology study.

In the case of toxicology studies that will use the gavage or dermal route of exposure, additional animals are dosed by gavage or dermally at three doses covering the proposed toxicology study dose range, again using sufficient animals so that three data points will be available at each sampling time. Often the doses for the toxicokinetic evaluation are the minimally toxic dose (MTD) and fractions of the MTD. This study allows for the determination of the absorption rate, dose proportionality, and, along with the results of the IV route study, the bioavailability. It is important to note that since the interpretation of the results of the toxicokinetic studies depends on the accuracy of animal dosing, the dose

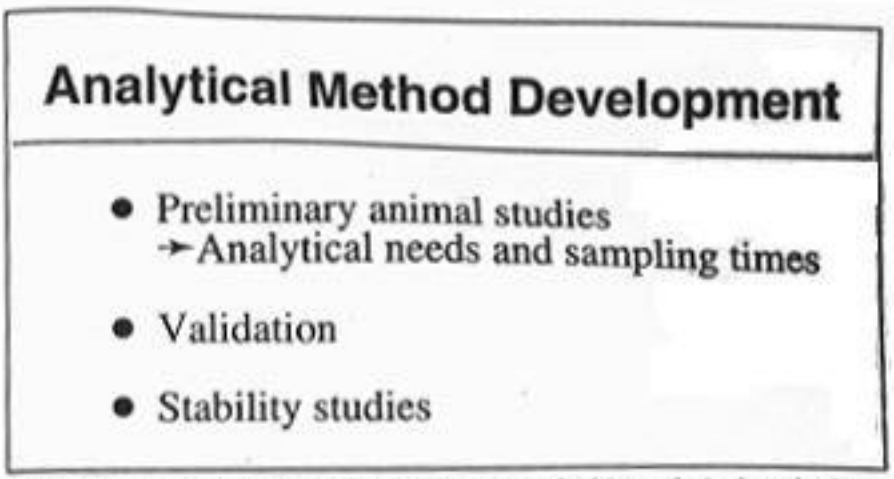

FIGURE 5. *Basic steps in the development of a bioanalytical method.*

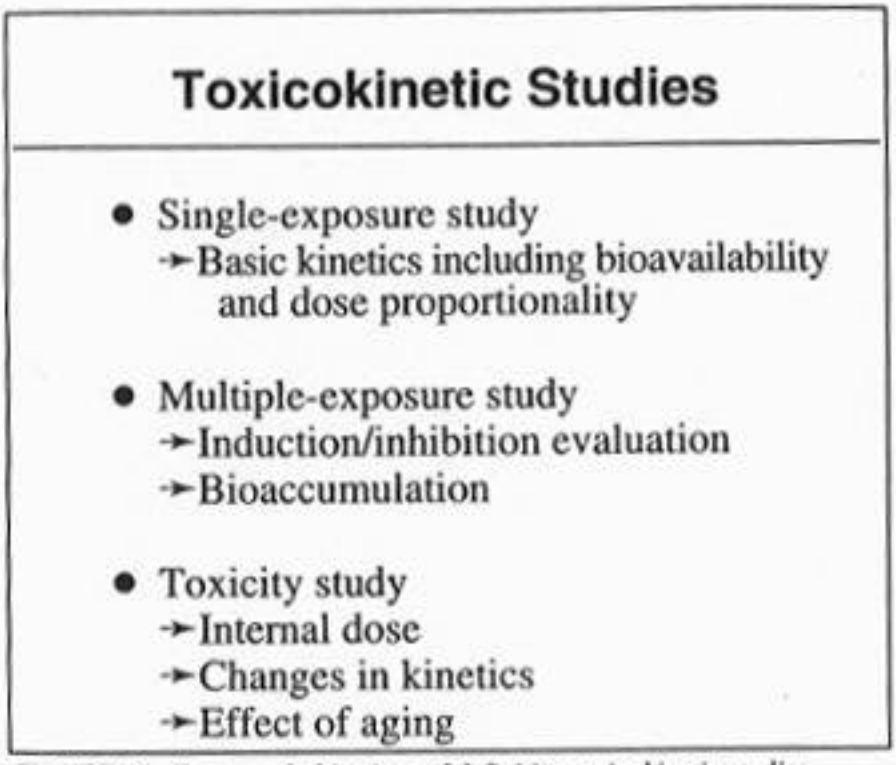

FIGURE 6. *Types and objectives of definitive toxicokinetic studies.*

formulations need to be characterized by homogeneity, stability, and accuracy of dose preparation. A number of the NTP 2-year

toxicology and carcinogenicity studies use the feed or drinking water route for test chemical exposure. How to use the kinetic data from single bolus dose studies in the design and evaluation of feed or drinking water studies is a challenge to the toxicokineticist. To help address this issue, a computer model was developed (Yuan 1993) to incorporate the animal feed or drinking habits together with the kinetic behavior of chemicals to predict the blood concentration profiles during dosed-feed or drinking water studies. The needed absorption rate and dose proportional range are determined from kinetic studies using the gavage route. The top dose for the gavage toxicokinetic study is calculated to be between one-half and one-quarter of the total daily exposure at the MTD based on normal feed or water consumption and bodyweight while the other doses are fractions of the top dose. Using the model, plasma concentrations are predicted for the planned feed or drinking water study. A feed or drinking water toxicokinetic study is then conducted and blood samples are taken over the course of a 7- to 14-day exposure period. If the determined plasma concentrations fall in the range of those achieved after gavage dosing, which themselves resulted in dose proportional concentrations, then the dosed-feed/water concentrations are assumed to be in the dose- proportional range as well.

In support of inhalation studies, blood samples are taken after a 4- to 6-hour whole-body or nose-only exposure period. The steady-state plasma concentration and the elimination rate are determined. The steady-state concentrations are plotted versus exposure concentration to make the determination of dose proportionality. A kinetic model is developed and used to predict steady-state concentrations and the time to reach steady state.

Multiple exposure studies may include obtaining information on possible enzymatic inhibition or induction effects as well as the possibility of bioaccumulation. In such studies, animals are dosed by gavage (or other appropriate route) for 14 days with one dose, usually the highest anticipated dose; sufficient animals are used so that three data points are available at each blood sampling time. Blood samples are taken at multiple time points after dosing and analyzed for test chemical or metabolite. These results are compared to the results of the single- exposure definitive study to determine possible enzymatic inhibition or induction effects and bioaccumulation of the test chemical.

The third step is to conduct toxicokinetic studies in conjunction with the actual toxicology studies to estimate internal dose as well as possible changes in the kinetics after repeated dosing and aging. The doses used in the 2-year carcinogenicity study are chosen so that, if possible, the two lower doses are in the linear kinetic range to facilitate extrapolation to lower doses and interspecies scaling while the top dose is chosen to be the MTD.

Another challenge to the toxicokineticist involves relating any observed changes in kinetics during a long-term toxicology study to age effects. In an attempt to address this issue, the kinetics of the chemical in a control group of animals are determined at 18 months of a 2-year study. Extra animals are added to each dose group in the 2-year study and used at 2 weeks and at 3, 12, and 18 months of dosing so that it is possible to follow any changes in kinetic behavior as the dosing progresses.

An appendix is included that provides examples of four current toxicokinetic study protocols for chemicals scheduled for toxicological evaluation using oral (gavage and feed), topical, and inhalation routes.

ANALYTICAL METHOD VALIDATION

Validation of biological sample analysis methods is critical to the evaluation of the final data. A description of the validation procedure utilized by the NTP is provided here. The method needs to demonstrate the appropriate specificity, precision, absolute recovery, measurement limits, and relative error. An evaluation of the blank biological sample matrix contribution to responses seen in spiked samples also needs to be determined.

In conducting the validation, spiked biological sample standards are prepared at six different concentrations, using two independently prepared stock standards of different concentrations. Spiked biological sample standards at the lowest and highest concentrations are prepared in triplicate. Single spikes are prepared at the four intermediate concentrations. Biological sample blanks collected from five animals are prepared in triplicate. The spiked standards are prepared so that every other standard comes from one stock solution while the remaining standards are prepared from the second stock solution. The instrumental response from a single analysis of each biological standard and blank is then recorded. A series of solvent

standard solutions with the same final concentrations (i.e., after any extraction, dilution, or concentration step) are also prepared. The same standard stock solutions, prepared as described above, are used for making these solutions. A reagent blank in the same solvent as the standards is also prepared. Once all standards are prepared, the analytical response is determined and calculations made to evaluate the correlation coefficient for the solvent and biological sample standard curve data as well as the precision, percent relative standard deviation (percent RSD), the percent recovery (based on a comparison of responses of the biological and solvent standards), the relative error, and the measurement limits.

The limit of detection (LOD) is defined as three times the standard deviation (SD) of the blank response expressed as concentration. The limit of quantitation (LOQ) is defined as 10 times the SD of the blank response expressed as concentration. If there is no blank value, the SD of the lowest concentration should be used in the calculations.

Often another estimate of the LOQ is used, the experimental limit of quantitation (ELOQ). This value is defined as the lowest biological sample standard concentration that has been analyzed and that has a defined relative error and a defined RSD (e.g., 15 percent relative error and 15 percent RSD).

During the analysis of the actual study samples, a standard curve using a series of spiked biological sample standards is generated with each batch of samples. The standard curve is analyzed prior to any study samples. Once analysis of study samples has been initiated, quality control samples are run at fixed intervals throughout the analytical run. An acceptable frequency depends on the method being used, but a common approach is to run a quality control sample after every 10 study samples. Quality control samples are spiked biological sample standards that are prepared at the time the actual study samples are collected and stored under the same conditions as the study samples. Quality control samples are usually prepared at two or three concentrations that cover the expected study sample concentrations.

EXAMPLES

Over the last several years, the NTP has increasingly focused on the generation of toxicokinetic data and its application in toxicology study design and interpretation. Examples are presented to illustrate how NTP has utilized the toxicokinetic data.

3'-Azido-3'-deoxythymidine

3'-Azido-3'-deoxythymidine (AZT) is the first FDA-approved drug in the United States for the treatment of acquired immunodeficiency syndrome (AIDS). To aid in the design of a planned 2-year carcinogenicity study, the bioavailability and dose proportionality of AZT in B6C3F1 mice of both sexes were evaluated (Trang 1993). The chemical was administered IV or intragastrically at doses of 15, 30, and 60 milligrams per kilogram (mg/kg). There were no differences in the kinetics of AZT between the sexes. The elimination half-life was 18 minutes and the bioavailability was 85 percent. Dose proportionality was confirmed over the doses planned in the chronic study.

Benzyl Acetate

Benzyl acetate is widely used as a flavoring agent in the food industry and as a fragrant ingredient in a variety of consumer products such as soaps and lotions. The NTP conducted two 2-year carcinogenicity studies in F344 rats and B6C3F1 mice, one using the daily gavage administration and the other using dosed-feed administration. Although the daily doses of benzyl acetate were comparable between the two studies, hepatic tumorigenicity was only observed in the gavage studies of mice. In contrast, necrotic lesions were seen in the brain in both the feed and gavage studies. Comparative toxicokinetic studies were conducted in F344 rats and B6C3F1 mice to estimate the impact of gavage (500 mg/kg in rats and 1000 mg/kg in mice using corn oil as the vehicle) versus dosed-feed administration (dosed-feed pentachlorophenol concentrations provide a daily dose of 648 mg/kg for rats and 900 mg/kg for mice) on the toxicokinetics of benzyl acetate and to provide further information for interpreting the toxicity differences observed (Yuan et al. 1995). In an in vitro study, the half-life of benzyl acetate was found to be less than a minute; therefore, it was decided to monitor internal dose based on benzyl alcohol, hippuric acid, and benzoic acid plasma concentrations. In rats and mice, the benzoic acid plasma concentrations after gavage dosing were about a hundred times the concentrations found in a study in which benzyl acetate was administered in the feed. In both studies the benzyl alcohol plasma concentrations were less than 0.1 micrograms per milliliter (g/mL) while hippuric acid concentrations were about 20 percent of the benzoic acid concentrations. In the benzyl acetate study, results of the plasma analyses showed that the method of dosing had a major impact on the internal dose of the

metabolite, benzoic acid, which may explain the route-dependent differences in toxicity.

Cadmium Oxide

Cadmium oxide has many industrial uses, including anticorrosion coating for iron, copper, and steel. The lung burden and systemic exposure in male Fischer 344 rats were evaluated during a 13-week toxicology study in which the animals were exposed to aerosolized cadmium oxide at concen-trations of 0.1, 0.25, or 1.0 mg per cubic meter (mg/m^3) for 6 hours a day (Dill et al. 1994). Concentrations of cadmium were deter-mined in the blood, kidney, and lung during the course of the toxicology study. Accumulated cadmium in the lungs was not proportional to exposure concentration. Although the concentrations of cadmium in the blood were very low, the amount in the kidneys represented a significant fraction of the lung burden. Thus there was a significant systemic exposure after inhalation of the aerosolized cadmium oxide.

p-Chloro-,,-trifluorotoluene

p-Chloro-,,-trifluorotoluene (CTFT) is a widely used chemical intermediate in the manufacture of dinitroaniline herbicides. Various routes of administration were considered. The inhalation route was excluded because of the expense involved in the conduct of inhalation studies. The chemical's volatility excluded the conduct of a dermal or dosed-feed study, while its insolubility in water excluded the conduct of a drinking water study. Therefore a gavage study was selected. Corn oil was considered as the vehicle but administration of corn oil has been related to increases in the incidences of pancreatic lesions in male F344 rats. Therefore a new approach was considered, that is, molecular encapsulation of CTFT with a-cyclodextrin (CD), which might then be soluble in water. To evaluate the acceptability of this approach a toxicokinetic study was conducted in male Fischer 344N rats (Yuan et al. 1991*a*). Animals were dosed IV at 4.7 mg/kg using a 10 percent Tween 80 aqueous solution, and intragastrically at 10, 50, and 400 mg/kg doses in either corn oil or CD-complex in water. Bioavailability was shown to be complete for both vehicles and dose proportionality was established up to at least 400 mg/kg. It was concluded that CD could be used in the planned toxicology studies of CTFT.

Codeine

Codeine is an opioid that is an effective analgesic and antitussive therapeutic agent. NTP conducted a 2-year carcinogenicity study in Fischer 344 rats of both sexes with codeine administered in the feed (400, 800, and 1,600 parts per million (ppm)). Toxicokinetic information from rats in the 2-year study was developed for comparison to human data to serve as one basis for extrapolating the results from the rodent study to humans (Yuan et al. 1992, 1994*c*). Blood samples were collected from the rats on days 7, 21, and 90 at 7:00 pm, 11:00 pm, 3:00 am, and 7:00 am. Additional collections were made at 16 and 24 months between 6:00 and 8:00 am. Plasma concentrations of codeine and its metabolite, morphine, were determined. Bioavailability of codeine using the dosed feed route increased with dose (from 10 to 25 percent). The concentrations of codeine in rats receiving 800 ppm in the feed were comparable with the mean concentration reported in humans receiving a 60 mg oral dose, while the determined concentrations of morphine conjugates in the rat are much higher than reported in humans. The presence of morphine conjugates might have a significant impact on the interpretation of the rat codeine 2-year toxicology study.

2',3'-Dideoxycytosine

2',3'-Dideoxycytosine (DDC) is a drug approved for the treatment of AIDS. Plasma concentrations of DDC were determined at the conclusion of the 180-day short-term toxicology study, and the kinetic profile of DDC was determined for B6C3F1 and NIH Swiss mice. The incidence of thymic lymphoma found in this 6-month study correlated directly to internal dose as measured by area under the plasma versus time curve, but not the administered dose. This finding was applied to both strains of mice studied (B. Collins, personal communication, September 12, 1994).

2'3'-Dideoxyinosine

2'3'-Dideoxyinosine (DDI) is a therapeutic agent used in the treatment of AIDS. DDI is known to be easily hydrolyzed in the presence of acid. To aid in the selection of a vehicle for administration of DDI in planned toxicological evaluations, the bioavailability of DDI was determined in B6C3F1 mice of both sexes after intragastric administration in buffered and unbuffered formulations (R. Handy, personal communication, January 11, 1994). The significant effect of vehicle pH on the absorption of DDI was demonstrated by comparison of plasma DDI concentrations from buffered versus unbuffered oral dose formulations. The bioavailability

was 60 percent from buffered aqueous formulations and only 10 percent from unbuffered formulations.

o-Nitroanisole

o-Nitroanisole is widely used as a dye intermediate. A toxicology study was planned with the chemical administered in feed. In preliminary work to develop a method for confirmation of the accuracy of dose formulations, it was found that the complete recovery of the chemical from feed formulations required more severe extraction conditions as the feed formulations aged. There was concern that the increased strong binding of the chemical to feed constituents might affect the bioavailability in toxicology studies. To evaluate the bioavailability of o-nitroanisole from feed formulations, the urinary concentrations of o-nitroanisole's main metabolites, free and conjugated o-nitrophenol, were determined in 7-day dosed-feed studies conducted in male F344 rats using freshly prepared and aged dosed-feed formulations (Yuan et al. 1991*b*). No differences were found in the extent of bioavailability from the fresh and aged feed.

Oxazepam

Oxazepam is a widely prescribed benzodiazepine antianxiety agent and a common metabolite of many other benzodiazepines including diazepam and chlordiazepoxide. To aid in the assessment of risks associated with human use of this drug, the comparative toxicokinetic studies were conducted in F344 rats, B6C3F1 mice, and Swiss-Webster mice of both sexes after an IV dose of 20 mg/kg and oral gavage doses of 50, 200, and 400 mg/kg (Yuan et al. 1994*b*). In addition, since the NTP 2-year toxicology study used dosed feed, the toxicokinetics of oxazepam were also investigated in a 3-week dosed-feed study in male B6C3F1 mice at 125 and 2,500 ppm. Results indicated that the elimination of oxazepam from plasma after IV injection in both rats and mice were first-order and could best be described by a two-compartment model with a terminal elimination half-life of 4 to 5 hours for rats and 5 to 7 hours for mice. At all doses studied, the females had significantly higher plasma concentrations than males. At 50 mg/kg the bioavailability of oxazepam in rats (< 50 per-cent) was lower than in Swiss-Webster mice (> 80 percent). Based on the maximum plasma concentration achieved (Cmax), dose proportionality was not observed in rats or mice after gavage dosing. In the B6C3F1 mouse dosed-feed study, plasma concentrations increased proportionally with concentration of oxazepam in feed.

The estimated relative bioavailability from dosed feed (relative to the gavage study at 50 mg/kg) was about 43 percent.

Pentachloroanisole

Pentachloroanisole (PCA) has been found to be an environmental pollutant even though it has no major industrial uses. It is postulated that PCA is derived from methylation of pentachlorophenol, a widely known environmental pollutant. Since no internal dose determinations were conducted during the NTP-sponsored 2-year toxicology studies in B6C3F1 mice and F344 Fischer rats in which some evidence for carcino-genic potential was found, a retrospective toxicokinetic study was conducted to evaluate its basic kinetics as well as its potential for bioac-cumulation (Yuan et al. 1993). It this toxicokinetic study, B6C3F1 mice and F344 Fischer rats of both sexes were administered PCA intravenously (10 mg/kg) and intragastrically (10, 20, and 40 mg/kg). Both PCA and pentachlorophenol plasma concentrations were determined. PCA was shown to be rapidly demethylated to pentachlorophenol in both species, and the resulting pentachlorophenol plasma concentrations were in the range of one hundred times higher than those found for the parent PCA. Bioavailability of PCA was only about 36 percent in rats but was about 74 percent in mice. Plasma concentrations increased with dose, but were not directly proportional. No sex differences were observed. Significant potential for bioaccumulation of pentachlorophenol from exposure to PCA was predicted.

Pentachlorophenol

Pentachlorophenol is an effective broad-spectrum biocide widely used as a wood preservative. Two-year carcinogenicity studies had been conducted in B6C3F1 mice and similar studies were planned in Fischer 344 rats. To aid in future comparison of the results of the toxicology studies in both species and to provide information for dose-response relationships, toxicokinetic evaluations were conducted. In single- and multiple-exposure studies the toxicokinetics of pentachlorophenol were studied in the Fischer 344 rat using IV and oral (gavage and dosed-feed) routes of exposure (Yuan et al. 1994*a*). The elimination kinetics of pentachlorophenol after IV administration of 5 mg/kg were independent of sex. After gavage administration, dose proportionality was established up to at least 38 mg/kg. To aid in establishing dose-response relationships, the bioavailability of pentachlorophenol administered in dosed feed was calculated and compared to that after gavage dosing. The

bioavailability of pentachlorophenol administered in dosed feed at concentrations of 302 and 1,010 ppm ranged from 30 to 52 percent, which was significantly lower than bioavailability of pentachlorophenol administered by gavage (86 to 100 percent). The time course of pentachlorophenol plasma concentrations during the dosed-feed study was simulated using a computer model based on linear theory (Yuan 1993). The simulations were comparable with the experimentally determined concentrations.

SUMMARY

Toxic responses to test chemicals are known to be dependent on the exposure route, the kinetic behavior of the chemical, and the dose used in the toxicology study. Therefore, knowledge of internal dose is indispensable for the interpretation of toxicology study results, for the facilitation of interspecies scaling, and for risk assessment. By monitoring the blood and/or tissue concentrations of test chemical and/or metabolites versus time after administration of study chemicals by different routes, the bioavailability and kinetic characteristic of test chemicals can be readily obtained. This data can define the so-called linear dose range using area under the plasma concentration versus time curve, clearance, or other related toxicokinetic parameters, and can also be used to predict the possible bioaccumulation under multiple dose regimes. Changes in kinetic parameters after multiple exposures indicate alteration in how the animal handles the chemical (e.g., that there was enzyme induction or inhibition).

A recommended approach for conducting toxicokinetic studies generally involves three steps. Step 1 is a preliminary study, which uses a minimum number of animals to estimate the range of blood/tissue concentrations, the required quantitation limit for the analytical method, and the optimal sampling times for the definitive toxicokinetic studies. Step 2 is the definitive study and generates blood and/or tissue concentration data for calculating the toxicokinetic parameters. Step 3 is the toxicokinetic study conducted in conjunction with the toxicology study to determine the internal dose and the effects of age and continuous exposure on kinetic parameters.

Examples of the application of NTP toxicokinetic evaluations were also presented in this chapter, demonstrating their use in the design and interpretation of toxicology studies.

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Appendix:
Examples of NTP Toxicokinetic Protocols

TOXICOKINETICS STUDY PROTOCOL FOR METHYLENE BLUE (AN ORAL STUDY USING INTRAGASTRIC DOSING)

Rationale

The toxicokinetic studies are designed to: determine the basic kinetics including dose proportional range and bioavailability, provide an estimate of the internal dose, and provide information about long-term exposure on the kinetics.

Preliminary Study

Analytical Method. A preliminary analytical method shall be developed for quantitation of test chemical in plasma over the target concentration range of 0.1 to 50 g/mL.

Preliminary IV Study. Groups of 12 male Fisher 344N rats and female B6C3F1 mice (13Å2 weeks old) shall be dosed intravenously with test chemical (2.5 mg/kg for rats and 2.5 mg/kg for mice). The preferred vehicle for IV formulation is Emulphor/ethanol/water (1:1:8). The volumes used for dosing by the IV route will be 2 mL/kg for rats and 4 mL/kg for mice. As much heparinized blood as possible shall be collected from the vena cava from one dosed animal at each of 12 time points (3, 5, 10, 20, 30, and 40 min; 1, 1.5, 2, 3, 4, and 6 hours). Test chemical concentrations in plasma shall be determined using the unvalidated analytical method.

Preliminary Gavage Study. Groups of 12 Fisher 344N rats and B6C3F1 mice of both sexes (13Å2 weeks old) shall be gavaged at one of two doses of test chemical (2.5 and 50 mg/kg for rats and 2.5 and 25 mg/kg for mice). The vehicle for the gavage formulation will be 0.5 percent aqueous methylcellulose. The volumes used for dosing by the gavage route will be 5 mL/kg for rats and 10 mL/kg for mice. As much heparinized blood as possible shall be collected from the vena cava from one dosed animal at each of 12 time points (5, 10, 20, 30, and 45 min; 1, 1.5, 2, 3, 4, 6, and 8 hours). Test chemical concentrations in plasma shall be determined using the unvalidated analytical method.

General Information. Confirmatory dose formulation analyses shall *not be* conducted in the preliminary IV and gavage studies, but *shall be* conducted for the dosed-feed studies. Pooled blank plasma from rats shall be used to construct the standard curves for each preliminary study if no species differences are noted in the method using blank plasma.

Method Performance Evaluation. If the preliminary study results are satisfactory and quantitation of test chemical in plasma is in a range for which quantitation is technically feasible, then the analytical method shall be validated over the range dictated by the preliminary study results. Meanwhile, a stability study of the analyte in plasma shall be conducted over a period of time covering the expected storage time of the biological samples. The concentration used in the stability study shall be the midpoint concentration of the standard curve.

Single Administration Study

Rat IV Route. Groups of 12 Fisher 344N rats of both sexes (13Å2 weeks old) shall be dosed intravenously with a test chemical IV formulation at one dose (2.5 mg/kg). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Two blood samples at different times (preferably >2 hr apart) shall be collected via alternating orbital sinuses from each dosed rat. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Mouse IV Route. Groups of 24 B6C3F1 mice of both sexes (13Å2 weeks) shall be dosed intravenously with a test chemical IV formulation at one dose (2.5 mg/kg). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Each mouse shall be bled once. Test chemical concentrations shall be determined in plasma using a validated method.

Rat Gavage Route. Groups of 12 Fisher 34N rats of both sexes (13Å2 weeks old) shall be gavaged at each of three doses of test chemical (2.5, 25, 50 mg/kg). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after gavage administration, with three animals bled at each time point. Two blood samples at different times (preferably >2 hr

apart) shall be collected via alternating orbital sinuses from each dosed rat. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Mouse Gavage Route. Groups of 24 B6C3F1 mice of both sexes (13Å2 weeks old) shall be gavaged at each of three doses of test chemical (2.5, 12.5, 25 mg/kg). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after gavage administration, with three animals bled at each time point. Each mouse shall be bled once. Test chemical concentrations shall be determined in plasma using a validated analytical method.

General Information. Confirmatory dose formulation analyses shall be conducted for the single administration studies. Pooled control plasma from rats shall be used to construct the standard curves if no species differences are noted in the blank plasma.

Two-Year Studies

Special Study Animals. Special groups of 10 rats and 10 mice of both sexes shall be added to each dose group of the 2-year study. No control rats or control mice are needed for this study. For rats, blood samples shall be collected at 3, 6, 12, and 18 months using orbital sinus bleeding, with five rats being bled at each of two time points (to be specified by NTP). Rats shall be returned to their cages after the 3-, 6-, and 12-month bleeding intervals and sacrificed after the 18-month interval. Test chemical concentrations shall be determined in plasma using a validated analytical method.

For mice, blood samples shall be collected only at 12 months with five mice being bled at each of two time points (to be specified by NTP). After sampling, mice shall be sacrificed. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Aged Rat Study. Using the sentinel rats in the 2-year study, 15 male and 15 female rats (18 months old) shall be gavaged at the middle dose. Blood samples shall be taken at five time points (to be specified by NTP), with three animals bled at each time point. Each rat shall be bled only once. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Aged Mouse Study. Using the sentinel mice in the 2-year study, 15 male and female mice (18 months old) shall be gavaged at the middle dose. Blood samples shall be taken at five time points (to be specified by NTP), with three animals bled at each time point. Each mouse shall be bled only once. Test chemical concentrations shall be determined in plasma using a validated analytical method.

TOXICOKINETICS STUDY PROTOCOL FOR 2-HYDROXY-4-METHOXYBENZOPHENONE (AN ORAL STUDY USING DOSED FEED)

Rationale

The toxicokinetic studies are designed to: determine the basic kinetics including dose proportional range and bioavailability, provide an estimate of the internal dose, and provide information about long-term exposure on the kinetics.

Preliminary Study

Analytical Method. A preliminary analytical method shall be developed for quantitation of test chemical in plasma over the target concentration range of 0.1 to 50 g/mL.

Preliminary IV Study. Groups of 10 Fisher 344N rats and B6C3F1 mice of both sexes (13Å2 weeks old) shall be dosed intravenously with test chemical (8 mg/kg for rats and 50 mg/kg for mice). The preferred vehicle for IV formulation is Emulphor/ethanol/water (1:1:8). The volumes used for dosing by the IV route will be 2 mL/kg for rats and 4 mL/kg for mice. As much heparinized blood as possible shall be collected from the vena cava from one dosed animal at each of 10 time points (3, 5, 10, 20, and 40 min; 1, 2, 3, 4, and 6 hours). Test chemical concentrations in plasma shall be determined using the unvalidated analytical method.

Preliminary Gavage Study. Groups of eight Fisher 344N rats and B6C3F1 mice of both sexes (13Å2 weeks old) shall be gavaged at one of two doses of test chemical (8 and 80 mg/kg for rats and 50 and 500 mg/kg for mice). The vehicle for the gavage formulation will be 0.5 percent aqueous methylcellulose. The volumes used for dosing by the gavage route will be 5 mL/kg for rats and 10 mL/kg for mice. As much heparinized blood as possible shall be collected from the vena cava from one dosed animal at each of 8 time points (5, 15, 30, and

45 min; 1, 2, 4, and 8 hours). Test chemical concentrations in plasma shall be determined using the unvalidated analytical method.

Preliminary Feed Study. Groups of eight Fisher 344N rats and B6C3F1 mice of both sexes (13Å2 weeks old) shall be dosed with the test chemical in the feed for up to 7 days (time to be dosed will be based on the preliminary IV study results) at one of two dosed-feed concentrations (1,000 and 10,000 ppm). As much heparinized blood as possible shall be collected from the vena cava from one dosed animal at each of seven time points (4 am, 6 am, 8 am, 10 am, 12 pm, 4 pm, and 6 pm).

General Information. Confirmatory dose formulation analyses shall *not be* conducted in the preliminary IV and gavage studies, but *shall be* conducted for the dosed feed studies. Pooled blank plasma from rats shall be used to construct the standard curves for each preliminary study if no species differences are noted in the method using blank plasma.

Method Performance Evaluation. If the preliminary study results are satisfactory and quantitation of test chemical in plasma is in a range for which quantitation is technically feasible, then the analytical method shall be validated over the range dictated by the preliminary study results (determined by NTP) per the current NTP general specifications requirements. Meanwhile, a stability study of the analyte in plasma shall be conducted over a period of time covering the expected storage time of the biological samples. The concentration used in the stability study shall be the midpoint concentration of the standard curve.

Single Administration Study

Rat IV Route. Groups of 12 Fisher 344N rats of both sexes (13Å2 weeks old) shall be dosed intravenously with a test chemical IV formulation at one dose (to be chosen based on results of the preliminary study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Two blood samples at different times (preferably > 2 hr apart) shall be collected via alternating orbital sinuses from each dosed rat. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Mouse IV Route. Groups of 24 B6C3F1 mice of both sexes (13Å2 weeks) shall be dosed intravenously with a test chemical IV formulation at one dose (to be chosen based on results of the preliminary study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Each mouse shall be bled once. Test chemical concentrations shall be determined in plasma using a validated method.

Rat Gavage Route. Groups of 12 Fisher 34N rats of both sexes (13Å2 weeks old) shall be gavaged at each of three doses of test chemical (to be chosen based on results of the preliminary study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after gavage administration, with three animals bled at each time point. Two blood samples at different times (preferably >2 hr apart) shall be collected via alternating orbital sinuses from each dosed rat. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Mouse Gavage Route. Groups of 24 B6C3F1 mice of both sexes (13Å2 weeks old) shall be gavaged at each of three doses of test chemical (to be chosen based on results of the preliminary study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after gavage administration, with three animals bled at each time point. Each mouse shall be bled once. Test chemical concentrations shall be determined in plasma using a validated analytical method.

General Information. Confirmatory dose formulation analyses shall be conducted for the single administration studies. Pooled control plasma from rats shall be used to construct the standard curves if no species differences are noted in the blank plasma.

Two-Year Studies

Special Study Animals (GLP). Special groups of 10 rats and 10 mice of both sexes shall be added to each dose group of the 2-year study. No control rats or control mice are needed for this study. For rats, blood samples shall be collected at 3, 6, 12, and 18 months using orbital sinus bleeding, with five rats being bled at each of two time points (to be specified by NTP). Rats shall be returned to their cages after the 3-, 6-, and 12-month bleeding intervals and sacrificed after

the 18-month interval. Test chemical concentrations shall be determined in plasma using a validated analytical method.

For mice, blood samples shall be collected only at 12 months, with five mice being bled at each of two time points (to be specified by NTP). After sampling, mice shall be sacrificed. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Aged Rat Study. Using the sentinel rats in the 2-year study, 15 male and 15 female rats (18 months old) shall be gavaged at the middle dose. Blood samples shall be taken at five time points (to be specified by NTP), with three animals bled at each time point. Each rat shall be bled only once. Test chemical concentrations shall be determined in plasma using a validated analytical method.

Aged Mouse Study. Using the sentinel mice in the 2-year study, 15 male and female mice (18 months old) shall be gavaged at the middle dose. Blood samples shall be taken at five time points (to be specified by NTP), with three animals bled at each time point. Each mouse shall be bled only once. Test chemical concentrations shall be determined in plasma using a validated analytical method.

TOXICOKINETICS STUDIES FOR CAMPHOR (A TOPICAL EXPOSURE)

Rationale

The camphor toxicokinetic studies using the topical exposure route are designed to determine: absolute bioavailability and basic kinetics, percent of internal dose from nontopical exposure, the extent of bioaccumulation, the internal dose, and the effects of age and long-term exposure on the kinetics.

Preliminary Study

Analytical Method Development. A preliminary analytical method shall be developed for quantitation of camphor in plasma over the target concentration range of 0.5 to 50 g/mL.

Preliminary IV Study. Groups of 10 rats and 10 mice of each sex (13Å2 weeks old purchased by the contractor) shall be dosed intravenously with camphor at 6 and 50 mg/kg for rats and mice,

respectively. Emulphor/ethanol/water (1:1:8) is to be used as the vehicle. The volumes used for dosing by the IV route will be 2 mL/kg for rats and 4 mL/kg for mice. As much heparinized blood as possible shall be collected from the vena cava from one dosed rat and mouse at each of 10 time points (5, 15, 30, and 45 min; 1, 2, 4, 6, 8, and 10 hrs). Camphor concentrations in plasma shall be determined using the unvalidated analytical method.

Preliminary Topical Exposure Study. Groups of eight rats and eight mice of each sex (13Å2 weeks old) shall be topically exposed to camphor in ethanol/water//1/1 at two dosages (25 and 400 mg/kg for rats and 200 and 1,000 mg/kg for mice). The volumes used for dosing by the topical route shall be the same as in chronic study (i.e., 0.6 mL/kg for rats and 2.0 mL/kg for mice). The concentrations of the dose formulations are to be kept constant. The site of application shall be protected to avoid grooming by the rodent but the site shall not be occluded. As much heparinized blood as possible shall be collected from the vena cava from one dosed rat and mouse at each of 10 time points (15, 30, and 45 min; 1, 2, 4, 8, 12, 16, and 24 hrs). Camphor concentrations in plasma shall be determined using the unvalidated analytical method.

General Information. Confirmatory dose formulation analyses shall *not be* conducted in the preliminary IV or topical exposure studies. Pooled control plasma from male and female rats shall be used to construct the standard curves for each preliminary study.

Method Performance Evaluation. If the preliminary study results are satisfactory and quantitation of camphor in plasma is in a range for which quantitation is technically feasible, then the analytical method shall be validated over the range dictated by the preliminary study results (determined by NTP). Meanwhile, a stability study of the analyte in plasma shall be conducted over a period of time covering the expected storage time of the biological samples. The concentration in plasma evaluated in the stability study shall be 75 percent of the highest concentration used for the standard curve.

Single Administration Study

Rat IV Route. Groups of 12 male and 12 female rats (13Å2 weeks old) shall be dosed intravenously with a camphor IV formulation at one dose (to be chosen based on results of preliminary study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with

three animals bled at each time point. Two blood samples at different times (preferably >2 hr apart) shall be collected via alternating orbital sinuses from each dosed rat. Camphor concentrations in plasma shall be determined using the validated analytical method.

Mouse IV Route. Groups of 24 male and 24 female mice (13Å2 weeks old) shall be dosed intravenously with a camphor IV formulation at one dose (to be chosen based on results of the preliminary study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Camphor concentrations in plasma shall be determined using the validated analytical method.

Rat Topical Exposure Route. Two groups of 12 male and 12 female rats (13Å2 weeks old) shall be exposed to camphor at three doses (to be chosen based on results of the preliminary study). One group will have the site of application protected to prevent grooming by the rodents while the second group will not have the site protected. Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after topical exposure, with three animals bled at each time point. Two blood samples at different times (preferably >2 hr apart) shall be collected via alternating orbital sinuses from each dosed rat. Camphor concentrations in plasma shall be determined using the validated analytical method.

Mice Topical Exposure Route. Two groups of 24 male and 24 female mice (13Å2 weeks old) shall be exposed to camphor at three dosages (to be chosen based on results of the preliminary study). One group will have the site of application protected to prevent grooming by the rodents while the second group will not have the site protected. Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after topical exposure, with three animals bled at each time point. Camphor concentrations in plasma shall be determined using the validated analytical method.

General Information. Confirmatory dose formulation analyses shall be conducted for the single administration studies. Pooled control plasma from male and female rats shall be used to construct the standard curves.

Chronic Studies

Special Study Animals (GLP). Special groups of 10 rats and 10 mice of both sexes shall be added to each core dose group of the chronic study. No control rats or control mice are needed for this study. For rats, blood samples shall be collected at 3, 6, 12, and 18 months using orbital sinus bleeding, with two rats being bled at each of five time points (to be specified by NTP). Rats shall be returned to their cages after the 3-, 6-, and 12-month bleeding intervals and sacrificed after the 18-month interval. Plasma concentrations of camphor shall be determined.

For mice, blood samples shall be collected only at 12 months, with two mice being bled at each of five time points (to be specified by NTP). After sampling, mice shall be sacrificed. Plasma concentrations of camphor shall be determined.

Aged Rat Study. Using the sentinel rats in the chronic study, 15 male and 15 female rats (18 months old) shall be dosed at the middle dose. Blood samples shall be taken at five time points (to be specified by NTP) after topical exposure, with three animals bled at each time point. Each rat shall be bled only once. Plasma concentrations of camphor shall be determined.

Aged Mouse Study. Using the sentinel mice in the chronic study, 15 male or female mice (18 months old) shall be dosed at the middle dose. Blood samples shall be taken at five time points (to be specified by NTP) after topical exposure, with three animals bled at each time point. Each mouse shall be bled only once. Plasma concentrations of camphor shall be determined.

TOXICOKINETICS STUDIES PROTOCOL FOR DECALIN/TETRALIN (AN INHALATION EXPOSURE)

Rationale

The NTP toxicokinetic studies are designed to: determine the kinetics of decalin/tetralin in blood, determine the internal dose during inhalation studies as well as any possible changes in kinetics, establish dose proportional range, provide information on the effect of age on the kinetic parameters, and determine the concentrations of known metabolites in kidneys (only for the rat decalin studies).

Preliminary Studies

Analytical Method. A preliminary analytical method shall be developed for quantitation of decalin/tetralin in blood. In the decalin studies methods are also to be developed for the determination of the concentration of known metabolites (cis-2-decalone and trans-2-decalone) in rat kidneys. This range shall be estimated from literature and previous experience.

Preliminary IV Study. Groups of 10 rats and 10 mice of each sex (13Å2 weeks old purchased by the contractor) shall be dosed intravenously with decalin/tetralin at two doses proposed by the contractor and approved by NTP. Emulphor/ethanol/water (1:1:8) solutions shall be used for IV administration. The volumes used for dosing by the IV route will be 2 mL/kg for rats and 4 mL/kg for mice. As much heparinized blood as possible shall be collected from the vena cava from one dosed rat and mouse at each of 10 time points (5, 10, 20, and 40 min; 1, 2, 4, 6, 8, and 12 hours). Blood concentrations of decalin/tetralin shall be determined using the unvalidated analytical method.

Concentrations of the two decalin ketone metabolites are to be determined in the kidneys from the 1-, 4-, 8-, and 12-hour time point in rats of both sexes.

Preliminary Inhalation Study. Groups of five rats and five mice of each sex (13Å2 weeks old purchased by the contractor) shall be exposed for 6 hours to decalin/tetralin vapor via inhalation route at each of two exposure concentrations. The exposure concentrations shall be set after the completion of the 14-day toxicology study and shall be the highest and lowest concentrations proposed for the 90-day study. Blood samples shall be taken from five animals per sex per specie immediately after shutdown of the exposure (not to exceed 10 minutes). Samples shall be analyzed to estimate the possible steady-state concentration range.

Concentrations of the two decalin ketone metabolites are to be determined in the rat kidneys of both sexes.

General Requirement. Confirmatory analyses of the dose formulation shall *not be* conducted in the preliminary study. Pooled control blood from male and female rats shall be used to construct the standard curves for *each* preliminary study.

Validating the Method. If the preliminary study results are satisfactory and quantitations of decalin/tetralin in blood are in a technically feasible range, then the analytical methods shall be validated over the range dictated by the preliminary study results (determined by NTP). A stability study of the analytes in blood shall be conducted over a period of time covering the expected storage time of the biological samples. The samples are to be analyzed as soon as possible but not to exceed 3 weeks. The concentrations used in the stability study shall be the *midpoint* concentration of the standard curve.

Single Administration Study

Rat IV Route. Groups of three jugular-cannulated rats of each sex (13Å2 weeks old purchased by the contractor) shall be dosed intravenously with a decalin/tetralin IV formulation at each of two doses (to be chosen based on results of preliminary study and the results of the 90-day inhalation toxicology study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Blood concentrations of decalin/tetralin shall be determined using the validated analytical method.

Concentrations of the two ketone metabolites of decalin are to be determined in the rat kidneys of both sexes at four time points (to be determined by NTP).

Mouse IV Route. Groups of 12 mice (13Å2 weeks old purchased by the contractor) of each sex shall be dosed intravenously with a decalin/ tetralin IV formulation at each of two doses (to be chosen based on results of the preliminary study and the 90-day inhalation toxicology study). Blood samples shall be taken at eight time points (to be determined based on the results of the preliminary study) after IV administration, with three animals bled at each time point. Each mouse shall be sampled twice via alternating orbital sinuses at different times (preferably > 2 hr apart). Blood concentrations of decalin/tetralin shall be determined using the validated analytical method.

Rat Inhalation Route (Single Exposure). Groups of 12 rats (13Å2 weeks old purchased by the contractor) of each sex shall be exposed to decalin/tetralin vapor for 6 hours at each of three exposure concentrations via inhalation route. The exposure concentrations

shall be based on the results of the 90-day toxicology study. Immediately after shutdown of the exposure, the decline of blood concentrations of decalin/tetralin in rats shall be followed at eight time points (to be determined based on the results of the preliminary study), with three animals bled at each time point. The first time point shall be as close as possible to time zero. Time zero is defined as the time of shutdown of the chamber exposure. Each rat shall be sampled twice via alternating orbital sinuses at different times (preferably > 2 hr apart). Blood concentrations of decalin/tetralin shall be determined using the validated analytical method.

Concentrations of the two ketone metabolites of decalin are to be determined in the rat kidneys of both sexes at four time points (to be determined by NTP).

Aged Rat Inhalation Route. Groups of 24 aged rats (18 months old purchased by the contractor) of each sex shall be exposed to decalin/ tetralin vapor for 6 hours using the proposed *middle* chronic exposure concentration via inhalation route. Immediately after shutdown of the exposure, the decline of blood concentrations of decalin/tetralin shall be followed at eight time points (to be determined based on the results of the preliminary study), with three animals bled at each time point. The first time point shall be as close as possible to time zero. Time zero is defined as the time of shutdown of the chamber exposure. Each rat shall be sampled only once. Blood concentrations of decalin/ tetralin shall be determined using the validated analytical method.

Mouse Inhalation Route (Single Exposure). Groups of 12 mice (13Å2 weeks old purchased by the contractor) of each sex shall be exposed to decalin/tetralin vapor for 6 hours at each of three exposure concentrations via inhalation route. The exposure concentrations shall be based on the results of the 90-day toxicology study. Immediately after shutdown of the exposure, the decline of blood concentrations of decalin/tetralin in mice shall be followed at eight time points (to be determined based on the results of the preliminary study), with three animals bled at each time point. The first time point shall be as close as possible to time zero. Time zero is defined as the time of shutdown of the chamber exposure. Each mouse shall be sampled twice via alternating orbital sinuses at different times (preferably > 2 hr apart). Blood concentrations of decalin/tetralin shall be determined using the validated analytical method.

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General Requirement. Confirmatory dose formulation analyses for the IV studies shall be conducted for the single administration studies. Pooled control blood from male and female rats shall be used to construct the standard curves.

Chronic Studies

Special Study Animals. If the single administration study is successful, then groups of nine rats of each sex shall be added to each dose group of the chronic study. No control rats are needed for this study. Blood samples shall be collected at 2 weeks and 3, 6, 12, and 18 months using orbital sinus bleeding, with three rats being bled at each of six time points (to be determined based on the single administration study). Each rat shall be sampled twice via alternating orbital sinuses at different times (preferably >2 hr apart). Rats shall be returned to their cages after being sampled and sacrificed after the 18-month bleedings. Blood concentrations of decalin/tetralin shall be determined using the validated analytical method.

Groups of nine mice of each sex shall be added to each dose group of the chronic study. No control mice are needed for this study. Blood samples shall be collected only at 12 months with three mice being bled at each of six time points (to be determined based on the single-administration study). Each mouse shall be sampled twice via alternating orbital sinuses at different times (preferably >2 hr apart). After sampling, mice shall be sacrificed. Blood concentrations of decalin/tetralin shall be determined using the validated analytical method.

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