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METABOLIC, BEHAVIORAL AND NEUROCHEMICAL STUDIES OF 3,4-METHYLENEDIOXY ANALOGUES OF AMPHETAMINE

by

KATHLEEN M. HEGADOREN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled METABOLIC, BEHAVIORAL AND NEUROCHEMICAL STUDIES OF 3,4-METHYLENEDIOXY ANALOGUES OF AMPHETAMINE by KATHLEEN M. HEGADOREN in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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DEDICATION

This thesis is dedicated to my husband Dale and our sons, Neil and Lee, who encouraged me to follow my own dreams.

And to my parents Neil and Ida McKerracher for their belief in me.

ABSTRACT

Metabolic, uptake inhibition, behavioral and neurochemical studies of three 3,4-methylenedioxy analogues of amphetamine (AMPH), 3,4-methylenedioxy-amphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxy-N-ethylamphetamine (MDE) were conducted using male Sprague-Dawley rats. A novel indirect gas chromatographic assay was developed for the simultaneous separation and quantitation of the enantiomers of MDMA and its N-demethylated metabolite, MDA. MDMA and MDA were detectable in brain and liver tissues up to 8 h after acute administration of *rac*-MDMA. Enantioselectivity favoring R-(-)-MDMA and S-(+)-MDA was demonstrated. The gas chromatographic assay was modified to examine the levels of MDE and MDA in brain tissue. In contrast to the results obtained after administration of *rac*-MDMA, whole brain levels of MDE were substantially lower, less MDA was detected and enantioselectivity was reversed [R-(-) > S-(+)]. In an *in vitro* metabolic system, employing cytochrome P450 (CYP) isozymes, *rac*-MDE was shown to be a better substrate than *rac*-MDMA. However, neither CYP2D6 nor CYP3A4 was capable of catalyzing the N-dealkylation of MDMA and MDE to the extent expected from the *ex vivo* experiments. Both *rac*-MDMA and *rac*-MDE were capable of inhibiting the uptake of tritiated neurotransmitter into rat brain prisms (potency: 5-HT > NE > DA). The S-(+)-isomers of both MDMA and MDE were more potent than their antipodes. MDA, MDMA and MDE were compared with a psychomotor stimulant and an hallucinogen, using a computer assisted methodology that examines the

frequencies and durations of discrete behaviors and the sequencing of the various behaviors. This analysis successfully classified MDMA and MDA, and differentiated them from a stimulant and a hallucinogen. MDE could be grouped with MDMA and MDA in terms of its neurochemical effects. Continued research into the metabolic fate and the biological effects of these drugs are critical in light of their continued recreational use, despite the potential for serious toxic effects.

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The above-mentioned individuals along with my fellow graduate students formed a community rich with experience, rich with opportunities to learn and grow and rich with sharing and caring. I have been most fortunate, indeed.

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TABLE LEGENDS

ABBREVIATIONS

ADH	aldehyde dehydrogenase
ADR	aldehyde reductase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPH	amphetamine
S-(+)-AMPH	S-(+)-amphetamine
AMI	amitriptyline
ANOVA	analysis of variance
BP	blood pressure
COMT	catechol-O-methyltransferase
CNS	central nervous system
CSF	cerebrospinal fluid
PCA	*p*-chloroamphetamine
pCPEA	*p*-chlorophenylethylamine
IC_{50}	concentration producing 50% inhibition of uptake
CuZnSOD	copper-zinc superoxide dismutase
CYP	cytochrome P450
°C	degrees Celsius
DMI	desmethylimipramine
DHA	3,4-dihydroxyamphetamine
DHMA	3,4-dihydroxymethamphetamine
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoylbenzo-(F) quinoxaline
DOPAC	3,4-dihydroxyphenylacetic acid
L-DOPA	L-3,4-dihydroxyphenylalanine
5,6-DHT	5,6-dihydroxytryptamine
DOM	2,5-dimethoxy-4-methylamphetamine

DIC	disseminating intravascular coagulopathy
DPM	dissociations per minute
DA	dopamine
DR	dorsal raphé
DEA	US Drug Enforcement Administration
DD	drug discrimination
EC	electrochemical
EEG	electroencephalography
EI	electron impact
t½	elimination half-life
FEN	fenfluramine
FLU	fluoxetine
GC	gas-liquid chromatography
HFPC	heptafluoro-L-prolyl chloride
HP	Hewlett Packard
HPLC	high performance liquid chromatography
HVA	homovanillic acid
h	hour(s)
8-OH-DPAT	8-hydroxy-2-(di-*n*-propylamino) tetralin
6-OH-DA	6-hydroxydopamine
5-HIAA	5-hydroxyindole-3-acetic acid
HMA	4-hydroxy-3-methoxyamphetamine
HMMA	4-hydroxy-3-methoxymethamphetamine
5-HT	5-hydroxytryptamine, serotonin
5-HTP	5-hydroxytryptophan
IMI	imipramine
I.S.	internal standard

ip	intraperitoneal
iv	intravenous
kg	kilogram
kPa	kiloPascals
LD_{50}	lethal dose to 50% of test subjects
LLC	liquid liquid chromatography
LSD	lysergic acid diethylamide
MS	mass spectrometry
MR	median raphé
MI	metabolic intermediate
MAMPH	methamphetamine
PMA	*p*-methoxyamphetamine
MHPG	3-methoxy,4-hydroxyphenylglycol
NMDA	*N*-methyl-D-aspartate
MBDB	*N*-methyl-1-(1,3-benzodioxy-5-yl)-2-butamine
MDA	3,4-methylenedioxyamphetamine
MDE	3,4-methylenedioxy-*N*-ethylamphetamine
MDMA	3,4-methylenedioxymethylamphetamine
MDP	methylenedioxyphenyl
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
AMPT	α-methyl-*p*-tyramine
µg	microgram
µl	microliter
µM	micromolar
µmole	micromole
millisec	milliseconds
MAO	monoamine oxidase

MEGX	monoethylglycinexylidide
ng	nanogram
nM	nanomolar
NT	neurotransmitter
NADPH	nicotinamide adenosine monophosphate
NPD	nitrogen phosphorus detector
NE	norepinephrine
OCS	octadecylsilane
%	percent
p.o.	per os, orally
PI	phosphotidyl inositol
rac-	racemic
REM	rapid eye movement
SCOT	support-coated open tubular
sc	subcutaneous
TFPC	trifluoroacetyl-L-prolyl chloride
THA	2,4,5-trihydroxyamphetamine
THMA	2,4,5-trihydroxymethamphetamine
Trp	L-tryptophan
TRH	tryptophan hydroxylase
TH	tyrosine hydroxylase
VMA	vanillylmandelic acid
V_{max}	maximum velocity, rate of reaction
WCOT	wall-coated open tubular

1 *INTRODUCTION*

The work described in this thesis involved metabolic, behavioral and neurochemical studies of the methylenedioxy analogues of amphetamine (AMPH). This multidisciplinary approach focused on three analogues: 3,4-methylenedioxy-amphetamine (MDA); 3,4-methylenedioxymethamphetamine (MDMA); and 3,4-methylenedioxy-N-ethylamphetamine (MDE). These drugs are used recreationally and are known on the street as "Love", "Ecstasy" or "Adam" and "Eve", respectively. Interest in these drugs has centered around three main themes. The first theme is whether these drugs represent a novel class of drugs, distinct from psychomotor stimulants and hallucinogens. These drugs have been termed entactogens to highlight their ability to facilitate positive self reflection and to increase empathy and communication with others. Results from behavioral studies with MDMA and MDA are equivocal. Neurochemical studies in the CNS have demonstrated that MDMA and MDA primarily affect the 5-hydroxytryptamine (5-HT, serotonin) system, producing decreased levels of 5-HT and 5-hydroxyindole-3-acetic acid (5-HIAA), decreased numbers of 5-HT uptake sites and a decrease in the activity of tryptophan hydroxylase (TPH). Although less information is available about MDE, it has been shown to produce similar behavioral and acute neurochemical effects, but is less potent than the other two analogues. The mechanism(s) involved in this decreased potency has not been established. The second theme is related to possible enantiomeric differences in their interactions with biological systems. MDA, MDMA and MDE each contain a chiral center which means that they exist as pairs

of nonsuperimposible enantiomers. While enantiomers have identical physical properties with the exception of the direction they distort plane-polarized light, they can exhibit very different properties in a chiral environment such as exists in biological systems. Enantioselective differences have been demonstrated in terms of their behavioral effects in animals, various neurochemical and pharmacological effects and their metabolism. The third theme is related to whether these drugs are specific 5-HT neurotoxins. Their ability to produce long-lasting deficits in 5-HT parameters and morphological changes to fine 5-HT fibres have been well replicated. However, the mechanisms involved remain unknown. Various researchers investigating the mechanisms have suggested that intraneuronal reactive oxygen-based radicals could be responsible for the observed cytotoxic effects. This has led to studies examining the relationship between the neurotoxicity and dopamine (DA), whose metabolism by monoamine oxidase (MAO) within 5-HT neurons could result in the formation of free radicals and peroxide. Other studies have focused on the role of active metabolites, in particular MDA which can be metabolized to yield α-methyl-DA, another possible source of free radical formation. The roles of 5-HT_2 receptors and excitatory amino acids and the relationship between neurotoxicity and hyperthermia have also been investigated.

These three themes are interwoven in my choices of experimental methodologies used to achieve the objectives I set out for my thesis work. One of my overall objectives included investigating possible mechanisms responsible for the decreased potency of MDE compared with MDMA. If indeed MDA, as an active

metabolite, is involved in the neurotoxic potential of these drugs, and examination of the degree of *in vivo* stereoselective metabolic N-dealkylation in brain tissue observed after administration of either racemic (*rac*)-MDMA or *rac*-MDE could provide relevant information. Gas chromatographic methodologies were developed for the separation and the quantitation of the enantiomers of MDMA or MDE and their N-dealkylated metabolite, MDA in brain tissue. Separate timed studies after acute administration of *rac*-MDMA or *rac*-MDE were conducted and the assay procedure was used to separate and quantify the enantiomers of both the parent and the N-dealkylated metabolite.

The results of the *in vivo* metabolic studies were unexpected and raised questions about whether the observed differences were related to specific metabolic enzymes involved in N-dealkylation. N-Dealkylation is a common metabolic pathway for many xenobiotics, yet much remains unknown about the mechanisms involved in the chemical reaction and the impact that the length of the N-alkyl chain has on the enzyme(s) that are capable of catalyzing the reaction. Recently, purified human cytochrome P450 (CYP) isozymes have become commercially available. Two of these (CYP2D6 and CYP3A4) were used in an *in vitro* incubation system to examine their ability to N-dealkylate MDMA and MDE. Known substrates of these isozymes were used for comparison.

Differences in the abilities to inhibit the uptake of neurotransmitter amines could be another source of potency difference between MDE and MDMA. Uptake inhibition studies in prisms from brain regions were conducted with radioisotope-labelled DA, 5-HT and norepinephrine (NE) using the racemate and the individual enantiomers of MDE and MDMA.

There were two interrelated objectives for the behavioural studies. The first was to determine if a computer-assisted methodology originally developed to examine the effects of chronic stimulants on behavior in rats could be used to distinguish the putative entactogens and the hallucinogen, *para*-methoxyamphetamine (PMA) from saline-treated animals and the second to see if MDMA, MDA and MDE as a group could be distinguished from S-(+)-AMPH and PMA. The method generated three data sets: photobeam cell interruption counts as a measure of locomotor activity, the frequencies and durations of 30 different behaviors and the temporal organization of behavior as measured by the transitions between individual behaviors expressed as a ratio of observed transitions to expected transitions.

Regional levels of DA, NE and 5-HT and the acid metabolites 5-HIAA, homovanillic acid (HVA) and 3,4-dihyroxyphenylacetic acid (DOPAC) were examined in the rats after termination of the behavioral study. Results of these neurochemical studies provided further discriminative data. In addition, information was gleaned about the relative potencies of five different AMPH analogues at equimolar doses to maintain changes in levels of biogenic amines and their metabolites in brain even after many of the acute behavioural effects were diminished or ended.

Whole brain levels of the five drugs used in the behavioral study were determined. An examination of the amount of drug remaining 3 h after drug administration helped to emphasize that such factors as pharmacokinetics and metabolism are important considerations when attempting to characterize the behavioral and neurochemical effects of specific drugs.

2 *LITERATURE REVIEW*

2.1 Overview

The written history of mankind is replete with references to the nonmedicinal use of drugs. From the early Greek writings about the drink from fermented grapes to present day concerns regarding the economic and social costs to society, much has been recorded about the use and misuse of certain types of drugs. Often, what has been written or researched has been tempered by the emotional overtones arising from a society's view on nonmedicinal drug use in general. Thus, words used to describe these types of compounds in North America and the judgements against those persons who use them have changed over time, as our society's views have shifted from open tolerance in certain periods of history to legalized prohibition during other periods to the more recent tacit acceptance of the use of specific drugs while making it illegal to even possess certain others. The emotions surrounding the entire issue of drug use and misuse have led to more legalistic and moralistic, rather than scientific, views of the topic. As society's opinions regarding certain types of drugs grew more negative, terms such as addictive and drugs of abuse became broadly applied to drugs used for their ability to alter mood, perception or state of consciousness. However, the emphasis was on the physical dependence that these drugs could create in users. As more became known about the physiological basis of dependence, it was found that some of the "abused" compounds did not produce classical physical or physiological dependence but were still powerful in their ability to maintain the desire for repeated use. A more

general definition of abusable or recreational drugs, put forth by Lecesse (1991), is "compounds, legal or illegal, that people consume voluntarily because they desire some of the psychological, and sometimes physical, consequences of ingestion". It is this more general definition that will be used in this thesis.

While use of alcohol and tobacco continues to far outstrip any other nonmedicinal drug use in terms of availability, popularity and the negative consequences of chronic use, many other types of compounds offering a variety of behavioral effects have been identified from their plant sources or have been synthesized. Attempts to classify the different types of recreational drugs have been made, using pharmacological effect, chemical structure, source of compound, primary behavioral effect, legal status or similarity of effect to well known examples within a class as the basis for differentiation. One of the first such classification systems, based on both pharmacological and behavioral effects, was put forth by Lewin in 1924, in which he described five subcategorizations: excitantia (included caffeine, tobacco, cocaine and AMPH), inebriantia (best known example was ethanol), hynotica (included barbiturates, benzodiazepines and anticholinergics), euphorica (included opium and its derivatives) and phantastica (such as peyote, marijuana and Amanita mushrooms) [Shulgin, 1978]. Excitantia and phantastica have more recently become known as psychomotor stimulants and hallucinogens, respectively.

As more and more compounds have been identified, subdivisions within classes have been made, often on the basis of chemical structure. Hallucinogens,

for example, have been subdivided into phenylisopropylamines, indolealkylamines and tricyclic carbolines (Shulgin, 1978; Glennon *et al.*, 1986) [Figure 1]. The rapid expansion of the numbers of recreationally used compounds also resulted in a growing recognition that some compounds did not fit well into the known classes. The methylenedioxy analogues of AMPH are believed to be such compounds. This thesis focuses on the three best known recreationally used methylenedioxy analogues of AMPH, namely 3,4-methylenedioxyamphetamine (MDA, "Love"), 3,4-methylenedioxymethamphetamine (MDMA, "Ecstasy", "XTC", "ADAM") and 3,4-methylenedioxy-N-ethylamphetamine (MDE, "Eve") [Figure 2], and described in this thesis are experiments that reveal their neurochemical and behavioral effects in rats and their metabolism in rats and humans. Both *in vitro* and *ex vivo* procedures that discern the unique characteristics of these controversial street drugs are described.

2.2 HISTORY

MDA was the original methylenedioxy analogue of AMPH, first synthesized by German chemists, Mannich and Jacobsohn, in 1910 (Thiessen and Cook, 1973; Shulgin, 1978). Its pharmacological properties were first studied in animals in 1939 and included marked sympathomimetic effects, central nervous system (CNS) stimulatory activity and convulsions at high doses (Gunn *et al.*, 1939). MDA has been patented in turn as an antitussive (1958), an ataractic (1960) and an appetite suppressant (1961) [Shulgin, 1978]. The CNS effects were reported by Alles (1959), who described personally experienced sensory intensities, such as increased auditory

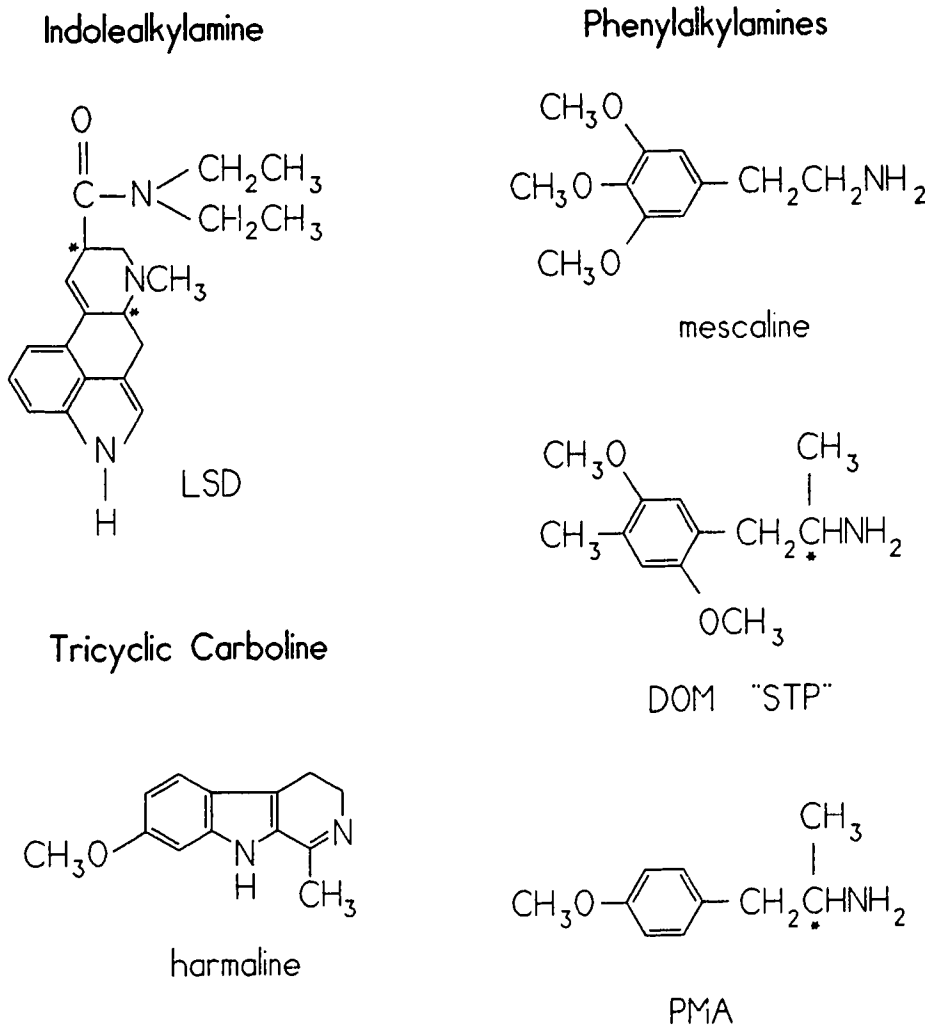
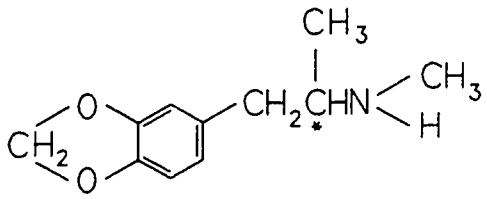
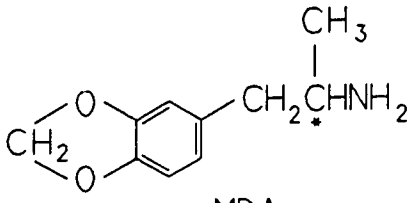


Figure 1: **Examples of three different structural classes of hallucinogens.**

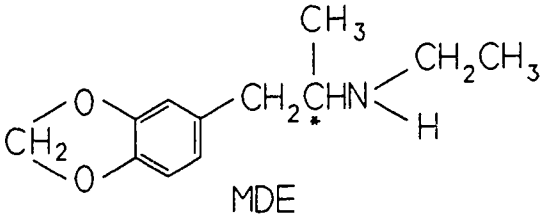
*** denotes chiral centre.**



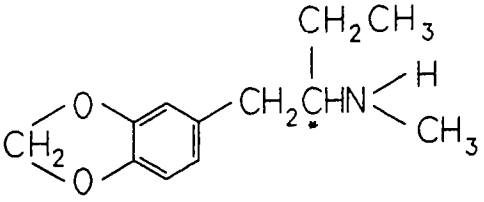
MDMA



MDA



MDE



N-methyl-1-(1.3-benzodiol-5-yl)-2-butanamine (MBDB)

Figure 2: **Structures of 3,4-methylenedioxy analogues of amphetamine.**

*** denotes chiral centre.**

acuity and tactile awareness, and depersonalization. Naranjo *et al.* (1967) recommended the use of MDA in psychotherapy for its ability to enhance emotions and empathy without producing sensory disruption or hallucinations. Its current popularity as a recreational drug and its name, the "Love" drug, arises from its emotion-enhancing qualities (Thiessen and Cook, 1973).

The first public recording of the preparation and properties of MDMA was a German patent filed in 1912 and issued in 1914 (Shulgin, 1978). A large toxicological study of mescaline and a number of ring-substituted analogues including MDMA, using five different laboratory animal species, was conducted for the U.S. Army in 1953-1954. The results were declassified in 1969 and published in 1973. The first report of the pharmacological action of MDMA in humans appeared in 1978 (Shulgin and Nichols, 1978), but the drug had been used in clinical practice prior to that as an adjunct to psychotherapy for its ability to encourage openness of emotional expression and to facilitate interpersonal communication and intimacy (Shulgin, 1990). Greer and Tolbert (1986) described the positive value of MDMA in therapy sessions with 29 patients. Grinspoon and Bakalar (1986) also supported the use of MDMA in clinical practice, classifying MDMA as a psycholytic agent or "mind loosening" agent, acting as a catalyst in the process of psychotherapy. They reported patients' claims of lasting improvement in their capacity for communication with others, in their capacity for insight and in their increased self-esteem up to two years after MDMA-assisted psychotherapy.

Unfortunately for the clinicians who believed in the efficacy of MDMA in psychotherapy, MDMA became increasingly popular as a recreational drug in the late 1970s and early 1980s (Shulgin, 1990). Articles appeared in counter-culture magazines. MDMA was actively promoted as a legally available euphoriant in Texas, with blatantly open sales in numerous bars and nightclubs (Beck, 1990). Controversy over its use, its abuse potential and its potential for toxicity spilled over into the U.S. media in 1985, with major magazines and newspapers espousing opinions, varying from supporting its clinical use to condemning it as a public safety hazard. In response, the U.S. Drug Enforcement Administration (DEA) invoked the emergency scheduling powers granted by the U.S. Comprehensive Crime Control Act of 1984 and placed MDMA on Schedule 1 of the Controlled Substances Act on July 1, 1985 (Beck, 1990). Schedule 1 controlled substances is a category of drugs considered to have a high abuse liability with no accepted medicinal use, and possession of them is illegal (Beardsley *et al.*, 1986). The Comprehensive Crime Act was enacted as an attempt to counteract the sudden advent of so-called designer drugs. Originally, designer drugs were synthetic opiate derivatives designed specifically to circumvent the prevailing laws. However, the term was soon used for all types of recreational drugs that had been structurally modified to evade legal restrictions (Lodge, 1991). MDA, MDMA and MDE were listed in Schedule H of Canada's Food and Drugs Act and Regulations in 1987. Despite its placement on the U.S. Schedule 1 list, the recreational use of MDMA continued. In 1987, out of 369 college undergraduates interviewed at a major American

university, 39% of the students admitted to having used MDMA at least once during the preceding year (Peroutka, 1990).

While MDA and MDMA are commonly referred to as "designer amphetamines" because of their popular use and AMPH-like structure, MDE can be defined as a "designer amphetamine ", both in terms of its use and its history. MDE became popular in the U.S. only after the placement of MDMA on Schedule 1 and enjoyed a brief expansion in use, until the "designer drug" legislation of 1986 which outlawed the sale of analogues of controlled substances (Beck, 1990). Although all three of these drugs have been Class A controlled substances in Britain since 1971, MDE and MDMA have attracted renewed popularity as illegal "rave" drugs in British nightclubs. The drugs are used in conjunction with rave dancing, prolonged episodes of hard and fast dancing within a large group (Henry, 1992; Tehan *et al.,* 1993). There is little information regarding the prevalence of current recreational use of MDE in North America and only anecdotal case reports from the U.K.

2.3 CHIRALITY

As indicated in Figure 2, MDMA, MDA and MDE all contain a chiral center. Thus, each exists as a set of two enantiomers. To understand the significance of this, some knowledge of stereoisomerism is required.

The foundations of stereochemistry were laid in the 1800s by J. Biot who first described optical activity as the ability of a substance to rotate the plane of

polarized light and by L. Pasteur who proposed that optical activity of organic solutions was determined by molecular asymmetry, which produces non-superimposable mirror-image structures (Drayer, 1988). Studies at the time with tartaric acid led to the recognition that the asymmetry and thus optical activity arose as a result of a carbon atom having four different constituents attached to it. It is now known that other atoms, such as nitrogen, sulphur and phosphorus are capable of acting as centers of asymmetry, also termed chiral centers, giving rise to optical stereoisomers. The word chiral derives from the Greek word "cheiros" meaning handedness. If the molecule and its mirror image are nonsuperimposable, the relationship between the two molecules is enantiomeric and the two optical stereoisomers are enantiomers. Because each member of a pair of enantiomeric molecules differs from the other only in spatial arrangement of the constituents attached to the chiral center, their physical properties (melting point, boiling point, refractive index, solubility) are identical.

2.3.1 *Nomenclature*

A brief explanation of the various nomenclature systems is necessary. Enantiomers differ in their optical activity, with one rotating plane polarized light to the right [(+) or dextrorotatory] and the other to the left [(-) or levorotatory]. The enantiomers are thus designated as (+) and (-) or prefixed by *dextro-* and *levo-* or more simply *d-* and *l-*. However, enantiomers may also be described according to their absolute configuration, referring to the order of the arrangement of the

constituents about the chiral center. Unlike optical activity which can be influenced by the solvent, temperature or light wavelength used, absolute configuration can only be modified by breaking and reforming chemical bonds. Absolute configuration is expressed using the Cahn-Ingold-Prelog method (Cahn *et al.*, 1966), prefixing compounds with R (rectus) or S (sinister) to indicate the location of the constituents about the chiral center. With this method, the substituents about the chiral center are first ranked according to their atomic number, from largest to smallest. The substituents are then oriented such that the smallest substitiuent is directed away from the viewer. The absolute configuration is then determined by whether the ordered substituents from highest rank to lowest rank follows a clockwise, designated as R, or a counterclockwise, designated as S, path. Not all optically active compounds are designated R or S; for historical reasons the absolute configuration of amino acids and carbohydrates are still referred to as D- and L-, using gylceraldehyde as the standard (Horn, 1984). The absolute configuration is not related to the optical activity in any way, and thus experimental procedures are required in addition to a polarimeter to determine absolute configuration of (+)- and (-)-isomers. It has been determined for AMPH that the optical activity of the S-enantiomer is (+) and that of the R-enantiomer (-). Because the rank order of the chiral center substituents is the same in AMPH and its methylenedioxy analogues, the same S-(+) and R-(-) relationship exists in these analogues. In this thesis, the optically active enantiomers of MDMA, MDA and MDE will be expressed as S-(+) or R-(-) and the optically inactive racemic mixture of 50% of each of the individual

enantiomers will be prefixed by *rac-*. Where no prefix is indicated in the literature references used in this thesis, it will be assumed the racemate is inferred.

2.3.2 *Implications of Chirality*

As stated earlier, enantiomers have identical physical properties except for their responses to polarized light. However, differences can arise when enantiomeric compounds are introduced into a chiral environment, such as exists in biological systems. Differences in pharmacological and behavioral effects, pharmacokinetic properties, plasma protein binding, pharmacological efficacy, toxicity and biotransformation can occur (Eichelbaum, 1988; Coutts and Baker, 1989; Jamali *et al.*, 1989). While most chiral drugs from natural sources exist as only one of the possible enantiomers, synthetically produced drugs that contain a chiral center are usually produced as racemic mixture (Drayer, 1986; Caldwell, 1992). The stereoisomeric composition of therapeutic drug substances is rapidly becoming a key issue in the development, approval and clinical use of pharmaceuticals (Campbell, 1990; Testa and Trager, 1990). Potentially clinically useful racemic mixtures are no longer likely to be approved for preclinical trials without supportive research data on the individual enantiomers and on any possible interactions between the two (Cayen, 1993).

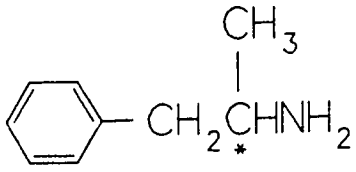
2.3.3 *Chirality and Recreational Drugs*

There are numerous examples of chiral recreational drugs (Figures 1-3).

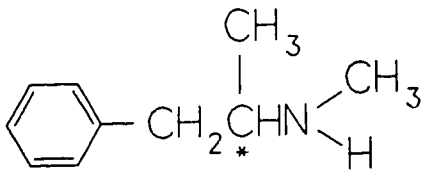
Many, such as the methylenedioxy analogues, retain chirality arising from the AMPH backbone. Other well known examples include methamphetamine (MAMPH), 2,5-dimethoxy-4-methylamphetamine (DOM) and mescaline (3,4,5-trimethoxyphenyethylamine). Natural cocaine (Figure 3) which contains 4 chiral centers is exclusively the (-) enantiomer. The absolute configuration is (1R, 2S, 3S, 5S)-(-)-cocaine (Gatley, 1991). The classical hallucinogen lysergic acid diethylamide (LSD) is also chiral with the hallucinogenic properties residing in the (5R,8R)-(+)-isomer. The (-)-isomer of LSD is more than 100 times less active as an hallucinogen than the (+)-isomer (Downing, 1964). Although both S-(+)- and R-(-)-AMPH have biological activity, the stimulatory effects reside primarily in the S-(+)-isomer (Moore, 1978).

2.4 Synthesis of MDA, MDMA and MDE

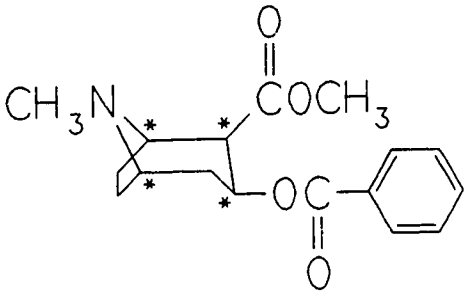
Although MDA, MDMA and MDE are referred as AMPH analogues, they are not synthesized from AMPH. Rather, they are synthesized from a number of methylenedioxyphenyl (MDP) compounds, including safrole, isosafrole, piperonal (heliotropin) and piperonylacetone [1-(3,4-methylenedioxyphenyl)-2-propanone] (Figure 4). These principal sources are interconvertible in that isosafrole is derived from safrole and piperonylacetone can be formed from any of the other three compounds (Shulgin, 1978, 1990; Clark *et al.*, 1994). When MDA was first being investigated as a psychoactive compound, there was some experimental evidence from liver homogenate studies suggesting that safrole was metabolically converted to MDA (Shulgin *et al.*, 1967; Braun and Kalhben, 1973).



amphetamine

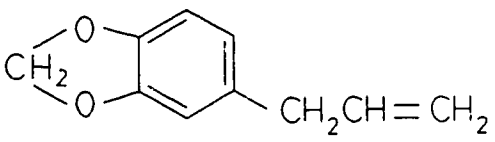


methamphetamine

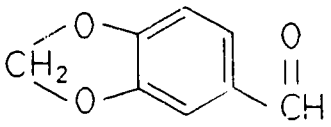


cocaine

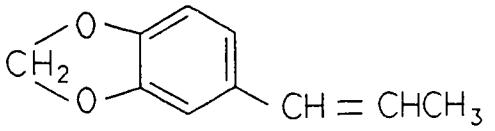
Figure 3: Structures of some common psychomotor stimulants. *Denotes chiral centre.



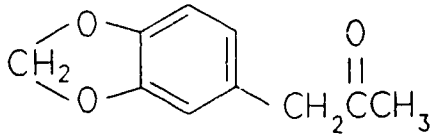
safrole



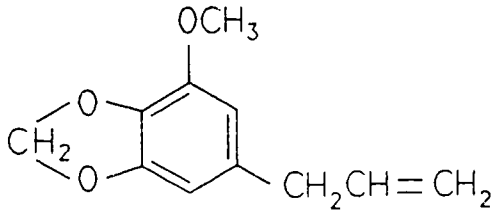
piperonal



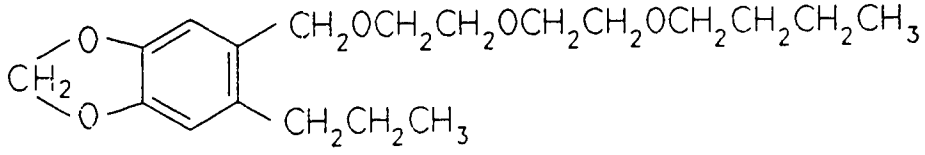
isosafrole



piperonyl acetone



myristicin



piperonyl butoxide

Figure 4: **Structures of some methylenedioxyphenyl compounds.**

The original method described for the preparation of MDA used safrole as the starting material. Safrole is a constituent of several essential oils and is used in manufacturing heliotropin in perfumery and as a food additive (Shulgin, 1978; Dahl, 1982). The synthesis of MDA or MDMA involved the addition of hydrobromic acid to safrole, followed by a reaction with methylamine (Shulgin, 1990; Clarke *et al.*, 1994) [Figure 5]. If piperonal was used as the starting material, a Darzan's reaction with ethyl-α-bromopropionate was followed by hydrolysis and methylamination (Figure 5). If the above starting materials were first converted to piperonylacetone, the synthetic reaction consisted of reductive methylamination, using sodium cyanoborohydride or amalgamated aluminum (Figure 6). The cyanoborohydride method has been used in the preparation of ^{3}H-MDMA (Shulgin, 1990). Another synthetic method involved reacting isosafrole with a mixture of hydrogen peroxide and formic acid to form a piperonylacetone intermediate. The intermediate was reacted with formamide or ammonium formate and then hydrochloric acid or with methylamine to form MDA (Frank, 1983; Clarke *et al.*, 1994) [Figure 6]. MDA can serve as the starting material for the preparation of MDMA by the addition of a one-carbon methyl group to the amine nitrogen *via* formate followed by reduction with lithium aluminium hydride (Figure 7) [Shulgin, 1990]. Rienas *et al.* (1993) looked at by-products and contamination products produced by three different MDE synthesis. Although the concentrations of the various product combinations varied strongly with the conditions of the synthetic reactions, the authors concluded that it was possible to determine the starting materials and the reaction path by the product profile.

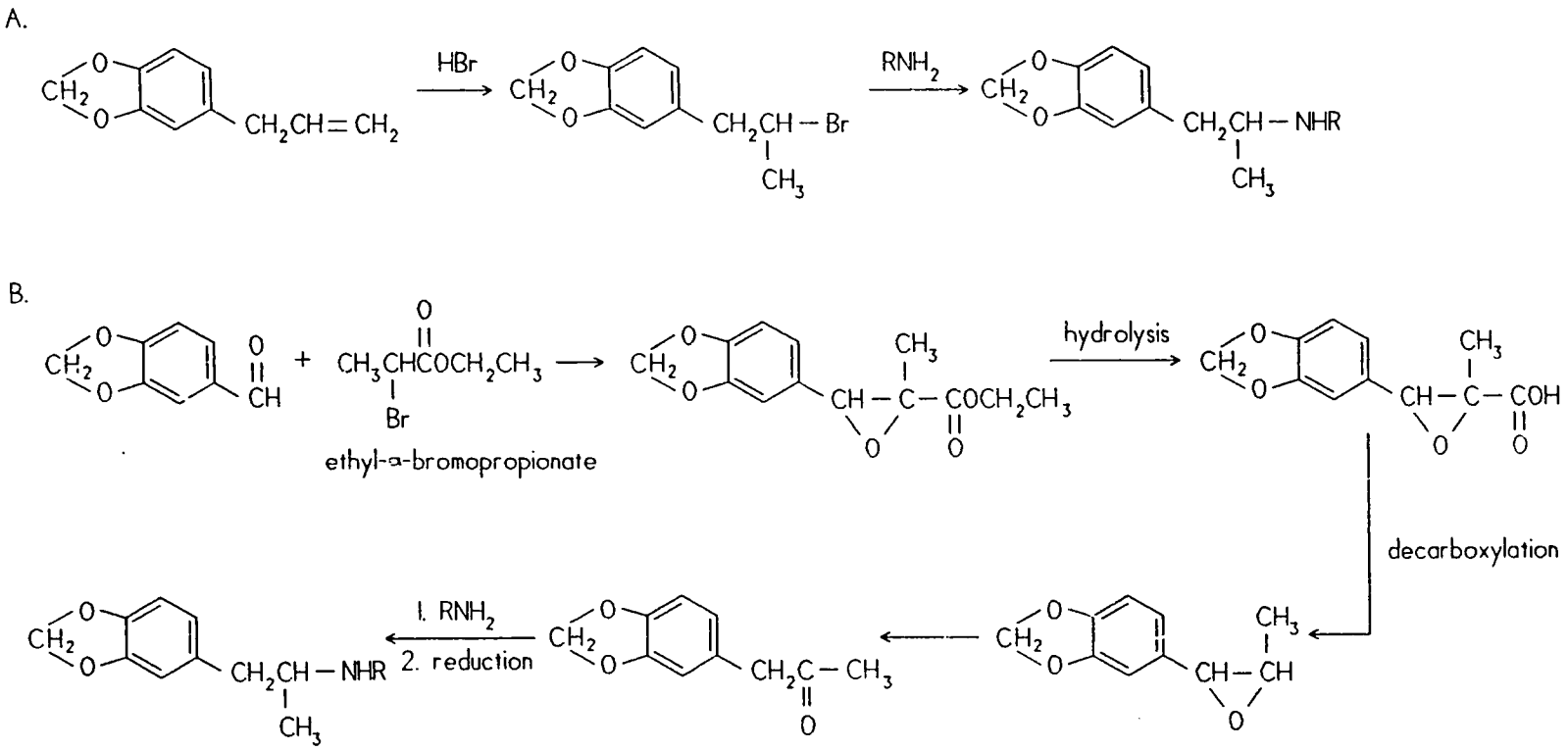


Figure 5: Chemical reactions involved in the synthesis of MDA or MDMA from (A) safrole or (B) piperonal. (R = H : MDA; R = CH_3 : MDMA).

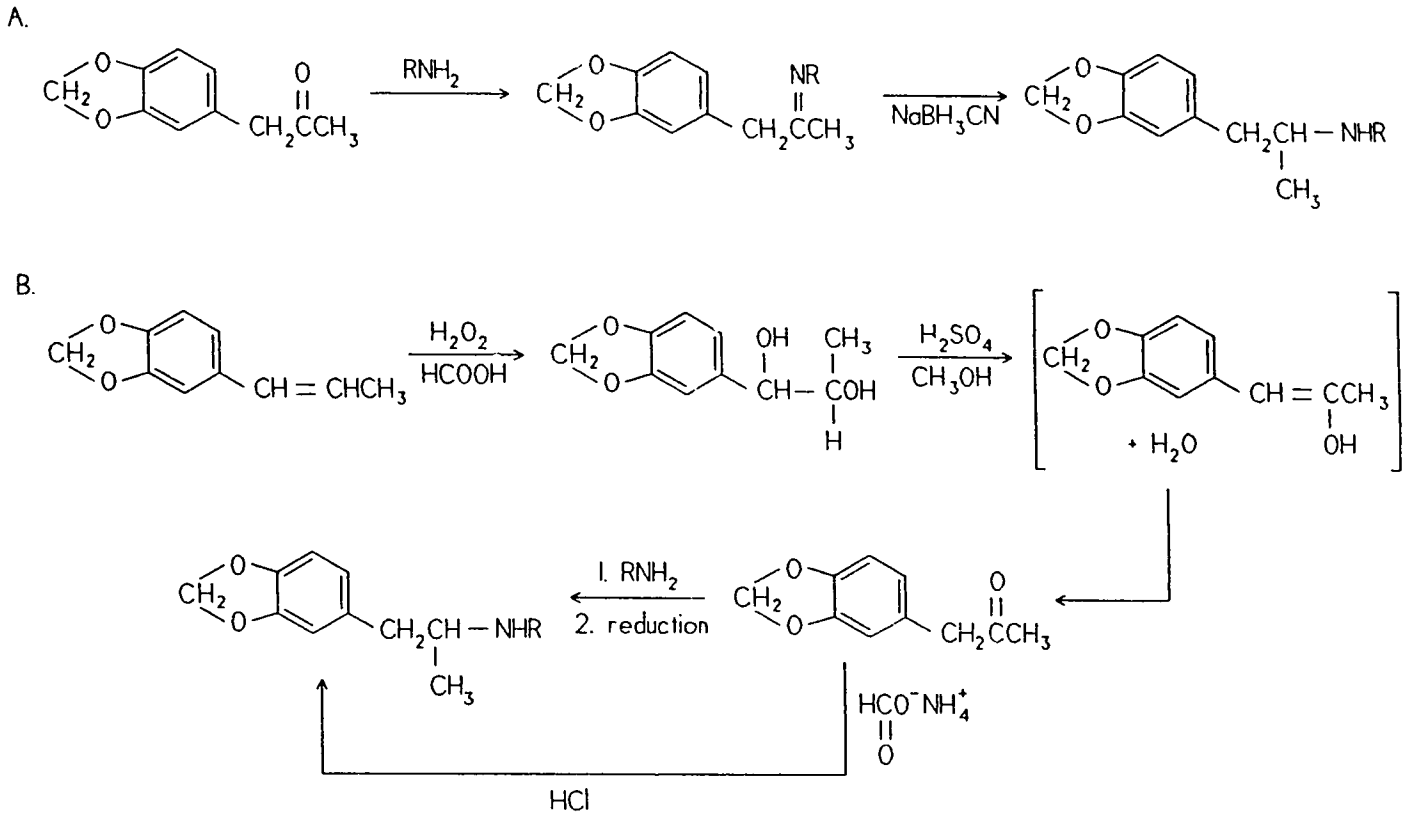


Figure 6: Chemical reactions involved in the synthesis of MDA or MDMA from (A) piperonylacetone or (B) isosafrole. (R = H : MDA; R = CH_3 : MDMA)

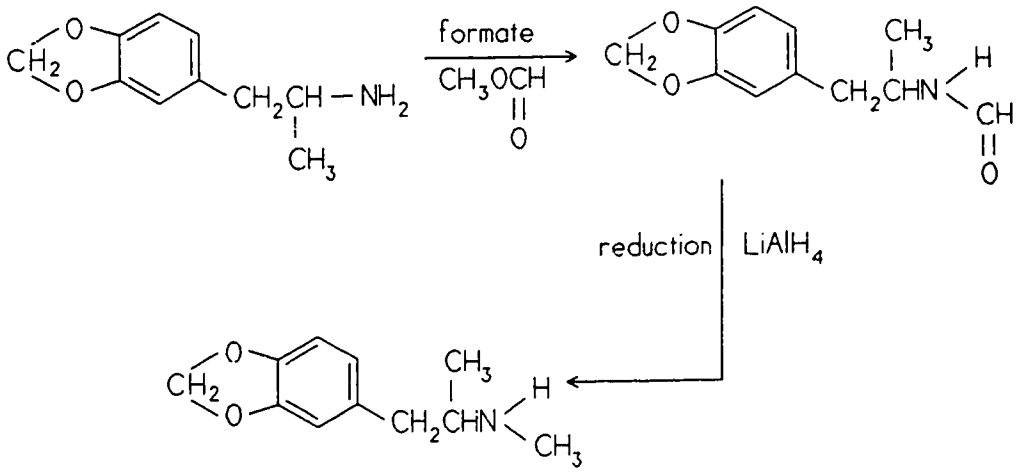


Figure 7: Chemical reactions involved in the synthesis of MDMA from MDA.

Frank (1983) reviewed reports on clandestine laboratories seized by the DEA between 1978 and 1981. Although the most common drug being made during this period was MAMPH (378 out of 751), 16 illegal laboratories were found to be specifically making MDA. It was reported that approximately 10,000 doses of MDMA were being distributed on a monthly basis by a single laboratory in California in 1976, and that number increased to 30,000 by 1984 and 500,000 by mid-1985 (Kirsch, 1986). The availability of one key precursor, piperonylacetone, was curtailed by the DEA under the U.S. Chemical Diversion and Trafficking Act in 1989 (Clark *et al.*, 1994).

2.5 **Behavioral Effects**

2.5.1 *In Humans*

MDA, MDMA and MDE have all been reported to produce very similar central and peripheral effects in humans. Any differences arise from potency, times of onset and durations of action. The central effects are described as an easily controlled altered state of consciousness, with heightened sense of well being, increased tactile sensations, increased perception of an inwardly focussed experience and a strong desire to be with and converse with people, without significant perceptual distortion or hallucinations (Alles, 1959; Mack, 1982; Nichols, 1986; Shulgin, 1990; Peroutka, 1990). Although MDA has been known to produce hallucinations at higher than typical doses, this effect appears to be either abolished by N-alkylation, or diminished to visual sensations rather than well formed

hallucinations (Mack, 1982; Nichols *et al.*, 1986; Peroutka, 1987).

MDA is more potent than MDMA and MDE, with a typical dose range of 60 to 120 mg, while single doses of 100 to 200 mg (1-4 mg/kg) of MDMA and MDE are common (Braun *et al.*, 1980; Mack, 1982; Peroutka, 1990). The onset of effect ranges from 30 to 60 min with MDA to within 30 min with MDMA and MDE. Duration of action is longer for MDA (about 8 h) than MDMA (about 6 h) and MDE (about 3-4 h) (Shulgin, 1978; Shulgin and Nichols, 1978; Nichols *et al.*, 1986; Boja and Schechter, 1987).

MDE is not illegal in Germany and has recently been used in randomized double-blind placebo-controlled cross-over design experiments with eight male volunteers (Gouzalis *et al.*, 1992; Gouzalis *et al.*, 1993b; Hermle *et al.*, 1993). Sleep electroencephalography (EEG) was used to demonstrate that the effects on sleep variables of 140 mg of MDE were AMPH-like, with decreased total sleep time and decreased rapid eye movement (REM) sleep. The same subjects were used to examine the psychological effects. The major effects from ratings scales were decreased anxiety, increased drive with pronounced partly euphoric and partly depressed mood and an overall increase in general responsiveness to emotions without any accompanying anxiety. Neuroendocrine tests revealed increased serum cortisol and prolactin and a trend towards blunting of growth hormone levels.

The peripheral effects of MDA, MDMA and MDE are largely sympathomimetic in nature, likely mediated by interaction of these drugs with α-adrenergic nerve endings, resulting in the release of NE. These include tachycardia, elevated

blood pressure (BP), mydriasis, tremor, palpitations and diaphoresis. Other common effects include increased salivation, bruxism (grinding of teeth) and trismus (tight jaw muscles) (Jackson and Reed, 1970; Shulgin and Nichols, 1978; Peroutka, 1987; McCann and Ricuarte, 1992). These two latter effects on facial muscles might be related to the central release of 5-HT. The facial motor nuclei (cranial nerve VII) are innervated by 5-HT fibers. Microiontophoretic application of 5-HT produces a slow, depolarizing action accompanied by a modest increase in membrane resistance, which facilitates the excitatory effects of iontophorectically applied glutamate. The overall increased excitability of these motor neurons can persist for several hours after only a single application (Aghajanian *et al.*, 1990).

The most common reported aftereffects of MDA, MDMA and MDE are drowsiness, muscle aches and generalized fatigue, depression lasting 1 to 2 days, difficulty in concentrating, paranoia and short-lived anxiety and irritability (Jackson and Reed, 1970; Peroutka, 1990; Winstock, 1991; McCann and Ricuarte, 1992). The aftereffects increase with successive doses. In general, it was reported that the aftereffects limit the frequency of use and tend to encourage variety in the choices of recreational drugs ingested (Peroutka, 1990).

The common use of multiple recreational drugs in combination with the questionable content and purity of drugs sold on the street has made it difficult to study long-term effects of the methylenedioxy analogues of AMPH. However, there have been some studies with chronic MDMA users. McCann *et al.* (1994) examined 58 subjects with prior histories of recreational drug use, 30 of whom were also

MDMA users while the other 28 served as controls. Lower scores were obtained from MDMA users in psychometric tests examining impulsivity and aggression, behaviors thought to be related to 5-HT systems. There was no difference between MDMA users and controls in the prolactin response to L-tryptophan (Trp). However, Price *et al.* (1989) reported a blunting of the prolactin response to Trp in 9 chronic MDMA users compared to drug-free healthy volunteers. Prolactin has been shown to be secreted in response to 5-HT agonists and in depressed patients there was a blunting of the prolactin response to Trp (Murphy *et al.*, 1986).

There have been numerous case reports of diverse psychiatric symptomatology resulting from chronic use of MDMA or after only 2 or 3 doses taken over several months (McGuire and Fahy, 1991; Winstock, 1991; McCann and Ricuarte, 1992; Pallanti and Mazzi, 1992; McGuire *et al.*, 1994; Series *et al.*, 1994). The psychiatric symptoms included paranoid psychosis lasting more than a month, persistent anxiety and depression and panic disorder. Some patients had either a family history or a personal history of psychiatric symptomatology, suggestive of increased vulnerability. However, other patients had had no previous history, yet developed psychiatric disorders, sometimes requiring ongoing treatment for extended periods. Gouzoulis *et al.* (1993a) reported on a case of toxic psychosis in a volunteer after ingestion of MDE.

Only a few human studies exist related to the individual enantiomers of the methylenedioxy analogues of AMPH. Two reports using R-(-)-MDA led to the conclusion that it was this isomer that was largely responsible for the psychoactive

properties of MDA and that the less potent S-(+)-isomer had significant stimulant-like effects (Shulgin, 1978).

The unique subjective effects of MDA, MDMA and MDE ingestion has led to the suggestion that MDA and MDMA represent a novel class of drugs, labelled entactogens, distinct from psychomotor stimulants and hallucinogens (Nichols *et al.*, 1986; Nichols and Oberlender, 1990). The term entactogen is derived from the Greek roots "en" for within or inside and "gen" meaning to produce or originate and the Latin root "tactus" for touch. Hence, the connotation of entactogen is that of producing a "touching within" (Nichols *et al.*, 1986), in reference to the drugs' ability to promote inward reflection and positive self-assessment. Their prototypic entactogen was the α-ethyl derivative of MDMA, N-methyl-1-(1,3-benzodiol-5-yl)-2-butanamine (MBDB) [Figure 2]. From previous work on structure activity relationships which had showed α-ethyl substitution of DOM eliminated hallucinogenic activity, MBDB was proposed as an entactogen devoid of hallucinogenic properties and which supposedly had a less complex array of behavioral effects in comparison with MDA. Subjects given MBDB supported its entactogenic properties, reporting a pleasant state of introspection, enhanced communication and a pronounced sense of empathy, similar to MDMA except the onset of action was slower and more gentle with less euphoria (Lin *et al.*, 1993). Hermle *et al.*. (1993) supported the hypothesis that MDE also belonged to the novel entactogenic class.

Because MDA, MDMA and MDE are Schedule 1 drugs, researchers in this

field have turned to animal models to further elucidate the behavioral differences among the so-called entactogens, psychomotor stimulants and hallucinogens and the physiological mechanisms underlying the observed behaviors. Attempts to develop suitable and valid animal models and behavioral paradigms with which to study recreationally used drugs have met with many interpretive and methodological problems, especially when comparing the effects among the different classes of compounds.

2.5.2 *Animal Models*

Much has been written about the use of models in psychiatry and psychopharmacology. Carlton (1978) described three general categories of models: clinical models derived form clinical observation, equivalence models which implicitly assume an equivalence between the laboratory and clinical phenomena, and correlational models based on empirical generalizations about a set of correlations, one from the laboratory and one from a clinical setting. Because apparent relevance is not a necessary requirement of correlational models, it is this type of model that is most commonly used in behavioral studies with animals. McGuire *et al.* (1983) defined animal models as hypothetical constructs (patterns, exemplars, ideas, representatons) of events occurring within or to animals that may be used to increase our understanding of psychiatric disorders in humans. They described five different types of animal models that are correlational in nature: (a) homologous models that assume similarities exist because of shared phylogenetic

histories, (b) analogous models that assume that two or more species share the same functions, as a result of parallel evolution, (c) survey models which use data from a wide range of species in order to detect general tendencies, such as seen in toxicological studies, (d) outcome models described as independent-dependent variable models in which little may be known about the intervening physiological mechanisms causing specific outcomes and (e) mathemathical models which propose general relationships across a wide variety of species. Many of the behavioral studies employing animals to investigate drug effects are of the analogous type, where similarity is based on functional equivalency.

Irrespective of the type of animal model chosen, criteria are needed to address the central issues of reliability and validity. However, what criteria are necessary to achieve a reliable and valid model depends on the use for which the model was developed (McKinney and Bunney, 1969; Segal and Schuckit, 1983; Robinson and Becker, 1986; Greenshaw *et al.*, 1988; Martin-Iverson, 1991; Glennon, 1992). Greenshaw *et al.* (1988) set out three kinds of validity and the criteria required. Predictive validity is an index of the accuracy of an animal model in relation to the identification of a particular class of drug. This type of validity is required in tests for screening potential therapeutic agents and also for classifying novel compounds. Face validity, referring to obvious similarities between the model and clinical settings, is the most difficult to achieve. Construct validity examines the degree to which the behavior in the model is similar or homologous to the behavior in humans. Drugs whose primary behavioral effects are subjective represent a

serious challenge in developing animal models that demonstrate construct validity.

Green and Backus (1990) proposed an experimental approach that used a simple behavioral change as a marker of 5-HT function, making the behavior a type of bioassay. Monitoring the change and the way that psychoactive compounds alter the response allows predictions to be made as to what alterations are occurring in the function of the transmitter following drug treatment. From this approach, certain responses and behaviors have become typically related to activity at specific 5-HT receptors: e.g., locomotor-related responses and hypothermia being related to 5-HT_{1A} receptors and head twitch being related to 5-HT_2 receptors (Glennon, 1990; Green and Backus, 1990; Leonard, 1994). This approach has been used extensively in behavioral research related to hallucinogens.

2.5.3 *In Animals*

Behavioral studies conducted with MDA, MDMA and MDE have focussed on two broad but interrelated themes: (a) assessment of their abuse potential and (b) comparison of their effects with either psychomotor stimulants or hallucinogens. Common behavioral paradigms to assess a drug's abuse potential are self-administration and intracranial self-stimulation. The self-administration model assumes that drugs are self-administered because they act as positive reinforcers, increasing the probability of continued drug-taking behavior, indicative of high abuse potential. Using a cocaine substitution procedure where stable cocaine self-injections were maintained prior to substitution with MDMA, both Beardsley *et al.*

(1986) and Lamb and Griffith (1987) demonstrated non-human primates would self-administer MDMA, albeit to a lesser extent than was seen with cocaine. Days of high numbers of MDMA injections were interspersed with days of low numbers, a pattern of non-continuous use similar to that reported by college students (Peroutka, 1990). MDA was also shown to support self-administration (Markert and Roberts, 1991). These results were used to support the placement of MDMA and MDA on the Schedule 1 list (Jacobson, 1987).

In intracranial self-stimulation studies, electrodes are commonly implanted in the medial forebrain bundle, an area believed involved in reward processes and in maintaining drug taking behaviors. Drugs are assessed as to their ability to lower the threshold of electrical stimulation required to maintain self-stimulation (Takigawa *et al.*, 1993). Hubner *et al.* (1988) showed that MDMA, but not LSD, lowered the reward threshold in a dose-dependent manner, up to a dose of 2 mg/kg, with behavioral disruption seen at higher doses.

Comparisons of the effects of MDA, MDMA and MDE with those of psychomotor stimulants and hallucinogens have relied heavily on operant conditioning based methodologies. The central concept of operant learning is that if a response is followed by positive (or reinforcing) consequences, there will be an increase in the probability that this response will occur again under similar circumstances. The most common operant conditioning paradigms used to study the methylenedioxy analogues have been drug discrimination (DD), conditioned taste aversion and conditioned place preference.

The DD paradigm has been used extensively to compare the methylenedioxy analogues with psychomotor stimulants and hallucinogens. In this paradigm, animals are trained to press one lever in a two-lever operant situation when administered Drug A and to press the opposite lever when administered Drug B, which can be saline, different doses of Drug A or a different drug (Glennon *et al.*, 1983). Stimulus generalization to a challenge drug is evidence of similarity of effect to the training drug. DD studies can be used to qualitatively and quantitatively compare a series of agents to a particular training drug and to classify the effects of novel compounds within known classes. However, DD studies are limited in their ability to identify genuinely new classes of compounds (Nichols *et al.*, 1986).

The results from DD studies in regard to MDA, MDMA and MDE are equivocal, due to differences in the choices of training drug, the species involved, the schedule of reinforcement, the training regime and the apparent stereoselectivity of certain behavioral effects, particularly with MDA. Behavioral disruption limits the range of doses that can be employed, which can affect the degree of substitution achievable (Broadbent *et al.*, 1992). Partial generalization is difficult to interpret in terms of comparisons among classes.

S-(+)-AMPH has been used as the training drug in DD studies to identify stimulant activity of the methylenedioxy analogues. Oberlender and Nichols (1988) and Broadbent *et al.* (1992) showed that *rac*-MDMA did not substitute for S-(+)-AMPH. Shannon (1980) showed that MDA also did not substitute for S-(+)-AMPH. However, Glennon and Young (1984a,b,c) showed substitution with *rac*-MDA, S-(+)-

MDA and *rac*-MDMA (order of potency: S-(+)-MDA > *rac*-MDMA > *rac*-MDA). Evans and Johanson (1986) used S-(+)-AMPH as the training drug in pigeons and also showed both *rac*-MDA and *rac*-MDMA were capable of substituting for AMPH. In rhesus monkeys, *rac*-MDMA produced stimulus generalization, while *rac*-MDA produced only partial generalization (Kamien *et al.*, 1986).

Both LSD and DOM have been used as training drugs to identify hallucinogenic activity. Nichols *et al.* (1986) used LSD as the training drug to support their proposed novel class. They examined MBDB, MDA and MDMA (the racemates and their individual enantiomers) and found generalization occurred with *rac*-MDA and R-(-)-MDA only. Glennon *et al.* (1983,1986) evaluated a large series of AMPH analogues, including MDMA and MDA (the racemates and the enantiomers), for their ability to substitute for DOM in DOM-trained rats. *Rac*-MDA and R-(-)-MDA were again shown to be capable of substituting for an hallucinogen. S-(+)-MDA was again shown to substitute for AMPH in AMPH-trained animals. These results have led researchers to suggest that the isomers of MDA produce qualitatively different behavioral effects, with the AMPH-like effects residing in the S-(+)-isomer and the R-(-)-isomer responsible for the hallucinogenic activity (Shannon, 1980; Glennon *et al.*, 1983; Ricuarte *et al.*, 1985; Broadbent *et al.*, 1992).

Rac-MDMA itself has been employed as the training drug to evaluate similarities with other putative entactogens and among drugs of different classes. There was complete generalization seen with MDE (Schechter, 1987; Glennon and Mesenheimer, 1989), with each of its individual enantiomers [S-(+)-isomer > R-(-)-

isomer] (Schechter, 1987), with norfenfluramine (Schechter, 1989) and with *rac*-MDA (Oberlender and Nichols, 1988). Interestingly, no stimulus generalization was seen with S-(+)-AMPH, S-(+)-MAMPH, *para*-methoxyamphetamine (PMA) or the 5-HT_{1A} agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH DPAT) [Schechter, 1989; Glennon and Higgs, 1992]. Conversely, rats trained to discriminate either enantiomer of MDA from saline show cross substitution between MDA enantiomers and MDMA enantiomers (Broadbent *et al.*, 1992). Baker *et al.* (1995) studied the discriminative stimulus effects of S-(+)- and R-(-)-MDMA (training doses: 1.25 and 3.5 mg/kg, respectively) in rats. The isomers of MDMA and MDA substituted completely for both training drugs. No substitution was seen with AMPH, cocaine or DOM. Partial degeneralization was seen with LSD in only R-(-)-MDMA trained animals. FEN substituted partially for S-(+)-MDMA and completely for R-(-)-MDMA, while *p*-chloroamphetamine (PCA) substituted completely for both MDMA isomers. Rats trained to discriminate *rac*-MDE showed complete substitution with *rac*-MDMA (Boja and Schechter, 1987). Glennon *et al.* (1992) attempted to relate the MDMA stimulus in MDMA-trained rats to the various 5-HT receptor subtypes and DA receptors and found that the 5-HT_{1A} antagonist NAN-190, the 5-HT_3 antagonist pirenpirone, the nonselective DA antagonist haloperidol and the 5-HT_2 antagonists zacopride and LY 278584, all were capable of attenuating the MDMA stimulus. They concluded that both 5-HT and DA receptors are involved in the stimulus produced by MDMA and that the 5-HT mechanisms are complex and likely involve multiple receptor subtypes and multiple receptor populations. Gold *et al.* (1989)

suggested that the mesolimbic DA system was involved in the psychostimulant action of MDMA.

MDMA produced AMPH-like effects in conditioned rotational activity (Kulmala *et al.*, 1987) and conditioned taste aversion (Lin *et al.*, 1993, 1994). However, the taste aversion produced by MDMA and AMPH differed in that the aversion produced by MDMA was unaffected by DA antagonism, suggestive of mechanistic differences. Both MDA and MDMA (the racemates and the individual enantiomers) dose-dependently disrupted behavior in mice trained to bar press for food reinforcement [S-(+)-isomers more potent than their respective R-(-)-isomers] (Rosecrans and Glennon, 1987). Pirenpirone attenuated the behavioral disruption produced by R-(-)-MDA, but to a lesser extent than was seen with DOM. Bilsky *et al.* (1990) showed that doses of 2 and 6.3 mg/kg MDMA produced positive conditioned place preference, supporting abuse potential.

The effects of stimulants on spontaneous behavior in rats have been well described (Moore, 1978; Segal and Schuckit, 1983; Robbins *et al.*, 1990; Martin-Iverson, 1991; Brodkin and Nash, 1995). At low doses, S-(+)-AMPH produced a behavioral activation in rats characterized by an increase in locomotion, accompanied by relatively continuous sniffing. As the dose was increased, the locomotion during the early phase of the response was interrupted by brief periods of stereotyped behaviors consisting of focussed sniffing and/or repetitive movements of head and limbs (Segal and Schuckit, 1983).

Martin-Iverson (1991) developed a computerized methodology to collect and

analyze behavioral data from freely moving rats in order to characterize behavioral effects of chronic S-(+)-AMPH treatments. The measured behaviors included those described as "hallucination-like" (Jacobs *et al.*, 1976; Ellison, 1991). It may be important to examine not only frequency and duration of discrete behaviors, but also include the sequences of behavior because DA systems which are known to be affected by S-(+)-AMPH are involved in the temporal coordination and selection of contextually appropriate behaviors (Norton, 1968, 1973; Evenden and Robbins, 1983). Thus, S-(+)-AMPH affects not only the performance of individual behaviors, but also the organization of behavioral patterns. Hallucinogens might also disrupt the organization of behavioral patterns, with sensory misperceptions playing a role in the animal's behavioral response, possibly *via* alterations in 5-HT neurotransmission (Jacobs, 1984).

It has been more difficult to examine the effects of hallucinogens on spontaneous behaviors in animals. Hallucinations, defined as sensory experiences not based on reality, are not necessarily accompanied by consistently observable behaviors (Glennon, 1992). Jacobs *et al.* (1976) proposed the term "hallucinatory behaviors" to describe any behavior that is distinctively induced by a known hallucinogen. Such behaviors, including limb flicks and "wet dog shakes", occur at such low frequency that statistical analyses often fail to show significance. In recent reviews of the various animal models used in research into hallucinogens (disruption of conditioned avoidance responding, head twitch, hyperthermia in rabbits, "serotonin syndrome", DD studies and startle response), Glennon (1990,

1992) concluded that there is no one model that alone can consistently predict hallucinogenic properties.

The effects of MDMA on spontaneous behavior in freely moving rats have been examined, using photobeam interruption counts as a measure of locomotor activity and a 4-point scale to assess "serotonin syndrome" behaviors (forepaw treading, head weaving, low body posture, piloerection and salivation) [Spanos and Yamamoto, 1989]. MDMA produced increases in both intensity and duration of the serotonin syndrome behaviors, while locomotor activity only increased in intensity. Braun *et al.* (1980) compared MDA, MDMA and MDE [20 mg/kg administered orally (po)] in their effects on motor activity in mice and found all three produced significant motor activation, with MDE and MDMA more potent than MDA.

Other researchers have looked more closely at the microstucture (the patterns created by the frequency and durations of discrete behaviors) of spontaneous behavior after MDMA administration. Using a Behavioral Pattern Monitor which graphically reconstructs the spatial characteristics of locomotor activity, Callaway *et al.* (1990, 1992) showed that S-(+)-MDMA produced an AMPH-like increase in locomotion in rats and a decrease in investigative behaviors (hole-poking and rearing). Pretreatment with fluoxetine (FLU), a 5-HT uptake inhibitor, inhibited the increased locomotion produced by S-(+)-MDMA but had no effect on locomotor hyperactivity produced by S-(+)-AMPH. Investigative behaviors were unaffected by FLU. The role of 5-HT_1 and 5-HT_2 receptors in S-(+)-MDMA-induced hyperactivity and decreased exploratory behaviors were examined by pretreating

rats with either pindolol or ritanserin. Both pretreatments antagonized the hyperactivity produced by S-(+)-MDMA but had mixed effects or no effect on specific exploratory behaviors (Callaway *et al.*, 1992). Paulus and Geyer (1992) also used the Behavioral Pattern Monitor to compare *rac*-MDA, S-(+)-MDA, *rac*-MDMA, S-(+)- and R-(-)-MDMA, *rac*-MBDB and *rac*-MDE with a psychomotor stimulant and an hallucinogen. Three dose-dependent patterns emerged: (a) no significant change on the amount, but drastic changes in the structure of the motor activity, seen with *rac*-MDA, S-(+)-MDA and R-(-)-MDMA; (b) similar changes in structure of motor activity as seen in (a), accompanied by an increase in amount of activity, seen with *rac*-MBDB, *rac*-MDMA and high dose S-(+)-MDMA; and (c) no change in structure of the motor activity but an increase in amount of activity, seen with lower doses of *rac*-MDMA and *rac*-MDE. The stimulant and the hallucinogen produced differing patterns from each other that were also distinct from the patterns seen with the methylenedioxy compounds.

The peripheral sympathomimetic effects of MDMA and MDA have been studied and have been shown to be similar to S-(+)-AMPH. Gunn *et al.* (1939) demonstrated that MDA produced sympathomimetic effects on an isolated cat heart, but was unable to elicit similar effects on a rat heart. Fitzgerald and Reid (1994) showed that MDMA produced a concentration-dependent positive chronotropic response in isolated rat atria and induced vasoconstriction in isolated rabbit ear arteries. The sympathomimetic effects of MDMA could be blocked by desmethylimipramine (DMI). In cerebral blood flow and glucose utilization studies,

MDMA has been shown to cause marked hyperperfusion in rat neocortex without any change in glucose use, indicative that MDMA has the potential to disrupt normal cardiovascular control (Kelly *et al.*, 1994).

2.6 **Neurochemical Effects**

2.6.1 *In Humans*

Multi-drug use in combination with the questionable content and purity of drugs sold on the street has made it difficult to study neurochemical effects of the methylenedioxy analogues of AMPH. As indicated earlier, McCann *et al.* (1994) examined 58 subjects with prior histories of recreational drug use, 30 of which were also MDMA users while the other 28 served as controls. After a two week abstinence from drug use, it was found that MDMA users had lower levels of the major metabolite of 5-HT, 5-HIAA, in cerebrospinal fluid (CSF) and that the differences were greater in female users than in males. There were no differences between controls and MDMA users in the levels of homovanillic acid (HVA) and 3-methoxy,4-hydroxyphenylglycol (MHPG), metabolites of DA and NE, respectively. Ricuarte *et al.* (1990) also found lower levels of 5-HIAA in the CSF of 33 MDMA users. However, Peroutka *et al.* (1988) did not find any differences in the levels of 5-HIAA between MDMA users and controls. Differences in the subjects with differing degrees of MDMA exposure and differing tastes in other recreational drugs could account for the variability in results. Levels of 5-HIAA have been shown to be affected by diet, activity (Davis, 1989) and psychiatric disorders, including

depression (Price *et al.*, 1990), so it is difficult to interpret the decreases in levels of 5-HIAA as a single abnormality. Interviews with subjects yielded no reports of disturbances in such 5-HT-related behaviors as sleep, mood or appetite (Ricuarte *et al.*, 1990).

2.6.2 *In Animals*

The first indication that the methylenedioxy analogues of AMPH could produce long lasting neurochemical changes arose from a report that MDA caused decreases in striatal 5-HT uptake (as measured by uptake studies in crude hippocampal synaptosomal preparations) and in 5-HT and 5-HIAA content in rats examined two weeks after multiple doses of 5, 10, 20 or 40 mg/kg, without affecting the levels of DA and NE (Ricuarte *et al.*, 1985). Since then, there have been numerous studies examining at the immediate and long-term neurochemical effects and the interactions among various neurotransmitter (NT) systems that occur after administration of MDA, MDMA or MDE in rats. Most of the studies have focussed on MDMA and have demonstrated that dose (single or multiple dosing regimes), frequency of administration, species and the timing of analyses after drug administration are all critically important variables.

Stone *et al.* (1987c) summarized the acute neurochemical effects of a single dose of MDMA [10 mg/kg, subcutaneous (sc)] and observed : (a) a decline of activity of tryptophan hydroxylase (TPH), the rate-limiting enzyme in the synthesis of 5-HT; (b) a rapid decrease in the levels of 5-HT; (c) a slower rate of decline of the

levels of 5-HIAA than was seen with 5-HT; (d) increased levels of DA; and (e) opposing effects on the levels of DA metabolites, with slow increases seen in the levels of HVA and a more rapid decrease in the levels of 3,4-dihydroxyphenylacetic acid (DOPAC). Each of these acute effects have been investigated as to the time course, any dose-dependency, the degree of recovery and possible mechanisms involved in the observed changes.

The rapid decrease in TPH activity is the first effect seen following administration of MDMA (Schmidt and Taylor, 1987; Stone *et al.*, 1987c). Three hours after a single dose of MDMA [10 mg/kg. *via* intraperitoneal (ip) injection], TPH activity was decreased to 22% of control, with no further decreases seen after increasing the dose to 15 or 20 mg/kg. Only the 20 mg/kg dose was capable of producing a decrease that persisted for 7 days after administration. Multiple doses of 10 mg/kg also produces long term decreases in TPH, but not tyrosine hydroxylase (TH), activity (Gibb *et al.*, 1987; Johnson *et al.*, 1989a). Coadministration of FLU or DA depletion with 6-hydroxydopamine (6-OH-DA) or pretreatment with reserpine or α-methyl-*p*-tyramine (AMPT) have all been shown to attenuate or block the longer term persistent decreases in TPH activity, but not the acute decline (Schmidt and Taylor, 1987; Stone *et al.*, 1988). The direct addition of MDMA to brain homogenates *in vitro* at concentrations up to 100 μM had no effect on TPH activity suggesting the decrease in TPH activity seen *in vivo* was an indirect effect, related perhaps to increased release of 5-HT (Schmidt and Taylor, 1987). At lower doses, S-(+)-MDA is more potent than R-(-)-MDA at decreasing

TPH activity in the hippocampus, while S-(+)-MDMA and R-(-)-MDMA produced similar decreases in TPH activity. However, when the dose was raised to 5 or 10 mg/kg, the stereoselectivity favoring the S-(+)-MDA was lost and S-(+)-MDMA becomes more potent than R-(-)-MDMA (Johnson *et al.*, 1988). Stone *et al.* (1989) examined some of the determinants of recovery of TPH activity after administration of a single dose of either the racemate or the individual enantiomers of MDMA (10 mg/kg). The enzyme could be reconstituted by prolonged anaerobic incubation with 5 mM dithiothreitol and 50 μM Fe^{2+}, evidence that the oxidation of a sulfhydryl site within the enzyme could be the mechanism of reversible inactivation. However, if multiple doses of *rac*-MDMA or either enantiomer were used, no reconstitution was seen, suggestive of irreversible inhibition with recovery dependent on enzyme synthesis.

The decreases in the levels of 5-HT and 5-HIAA have also been shown to be both dose- and frequency-dependent. Gibb *et al.* (1987) compared MDA and MDMA both as single doses of 10mg/kg sc and as multiple doses of 10 mg/kg every 6 h for 5 doses for their effects on 5-HT and 5-HIAA in rat striata, hippocampi and frontal cortex 3 h after administration of the single dose and 18 h after the last of the multiple injections. At 3 h, MDA and MDMA produced similar decreases in the levels of 5-HT and 5-HIAA in all regions. In the striatum, both drugs increased the levels of DA but differed in their effects on DA metabolites, with MDMA producing an increase in levels of HVA and MDA producing a decrease in levels of DOPAC. Eighteen hours after multiple doses, the levels of 5-HT and 5-HIAA remained

decreased to 15-30% of control (MDA more potent than MDMA). However, DA and DOPAC concentrations had returned to normal, while levels of HVA remained elevated in the animals that had received MDA. Even single doses of 10, 20 or 30 mg/kg MDMA were sufficient to decrease levels of 5-HT and 5-HIAA in animals examined 1 week after drug treatment (Schmidt *et al.*, 1987, 1990; Johnson *et al.*, 1993). Moker *et al.* (1987) used *rac*-MDMA (40 mg/kg every 2 days for 4 doses) and demonstrated that the decline in 5-HT and 5-HIAA was still apparent 16 days later. However, Battaglia *et al.* (1987) examined various brain regions 2 weeks after a drug regime of MDMA or MDA (20 mg/kg sc twice daily for 4 days) and found that while levels of 5-HIAA were markedly decreased in all regions after either drug, less consistent decreases were seen in the regional levels of 5-HT.

Schmidt *et al.* (1987) and Johnson *et al.* (1988) found similar dose-dependent enantioselective effects of MDA and MDMA enantiomers on 5-HT, 5-HIAA and DA similar to those reported for TPH activity. With MDA, only low doses revealed enantioselectivity, favoring the S-(+)-isomer. With MDMA, enantioselectivity favoring the S-(+)-isomer was seen only with higher doses. Regioselectivity was also demonstrated. S-(+)-MDA was more potent than S-(+)-MDMA at lowering levels of 5-HT and 5-HIAA in the frontal cortex and the striatum but was equipotent in the hippocampus, where the most profound reductions were seen. R-(-)-MDMA had the least effect on all neurochemical parameters examined.

Schmidt (1987b) compared MDA, MDMA and MDE (10 or 20 mg/kg and examined either 3 h or 7 days post injection) in relation to the time-and dose-

dependency of their effects on cortical 5-HT concentrations. All three drugs decreased the levels of 5-HT to 20% or less of control at 3 h, with only MDE showing a dose-dependent difference between 10 and 20 mg/kg. After 7 days, significant decreases in 5-HT content were seen with MDA and MDMA, but not with MDE. Ricuarte *et al.* (1987) reported that MDE was approximately one fourth as potent as MDMA in producing 5-HT depletion 2 weeks after multiple doses of drug.

Species other than the rat have been examined for the dose- and time-dependency of effects of the methylenedioxy analogues of AMPH on 5-HT parameters. Mice have been shown to be less sensitive than rats to the effects of MDMA on 5-HT parameters and TPH activity, requiring multiple high doses to elicit significant decreases (Stone *et al.*, 1987a). Ricuarte *et al.* (1988) showed that squirrel monkeys were more sensitive than rats. Even with a single oral dose of MDMA that was only 2-3 times higher than the typical human dose of 1.7 to 2.7 mg/kg, there were significant decreases in 5-HT parameters. Multiple oral doses of MDMA were less effective at lowering levels of 5-HT than multiple sc injections. The capacity for recovery of 5-HT deficits produced by MDMA in squirrel monkeys was also examined. MDMA (10 mg/kg sc twice daily for 4 days) produced profound decreases in the levels of 5-HT and 5-HIAA in CSF, striatum and cortex and in the number of 5-HT uptake sites in cortex labelled with ^{3}H-paroxetine, another 5-HT uptake inhibitor similar to FLU, 2 weeks after drug treatment (Insel *et al.*,1989). There was partial recovery in animals by 10 weeks and 8 months, but when examined at 18 months, the recovery had not been maintained, and decreases

similar to what had been seen in animals at 2 weeks after drug treatment were observed (Ricuarte *et al.*, 1992). Typical human doses of 1.25 or 2.5 mg/kg MDMA were administered po twice daily for 4 days to rhesus monkeys (Ali *et al.*, 1991). The lower dose had no effect, but the 2.5 mg/kg dose was sufficient to produce decreases in hippocampal levels of 5-HT and 5-HIAA when examined 4 weeks after drug administration. Higher doses produced decreases in the hippocampus and other cortical and midbrain regions. Levels of 5-HT and 5-HIAA in the hypothalamus and brainstem were unaffected at any dose of MDMA. Despite the significant deficits in 5-HT, there were no alterations in any of the 18 home cage behaviors monitored in the monkeys at any dose.

Researchers have examined the mechanisms underlying the MDMA-induced long term deficits in 5-HT parameters in rats by combining MDMA treatment regimes with a variety of pharmacological agents. A number of agents with very different pharmacological properties have been shown to affect the long term effects of MDMA. 5-HT uptake blockers prevent the decreases in the levels of 5-HT and 5-HIAA that remain 7 days after administration of a single 20 mg/kg dose of MDMA. Selective 5-HT_2 antagonists, namely ketanserin, MDL 28,133A, MDL 100,907 or MDL 11,939, also blocked the 5-HT deficits that remained 7 days after a single dose of MDMA (Nash *et al.*, 1990; Schmidt *et al.*, 1991b; Schmidt *et al.*, 1992a,b). The protective effect afforded by MDL 28,133A was lost by co-administration of L-dihydroxyphenylalanine (L-DOPA), suggestive that 5-HT_2 antagonists interact at the level of DA synthesis. The 5-HT precursors. Trp or 5-hydroxytryptophan (5-HTP),

attenuated the MDMA-induced decreases in 5-HT and 5-HIAA (Sprague *et al.*, 1994). L-DOPA coadministered with MDMA potentiated the decreases seen in the levels of 5-HT in the striatum, the cortex and even in the hippocampus, where only a few DA inputs are known (Schmidt *et al.*, 1991a). Corticosterone potentiated the MDMA-induced decline in both TPH activity and 5-HT levels in the hippocampus only, where a high number of corticosterone receptors are found. Conversely, adrenalectomy attenuated hippocampal decreases in 5-HT content (Johnson *et al.*, 1989b). The noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, MK 801, also attenuated the ability of MDMA to decrease 5-HT concentrations, but had no effect on the MDMA-induced decrease in TPH activity (Johnson *et al.*, 1989a; Farrel *et al.*, 1992). Ohta *et al.* (1994) used *in vivo* microdialysis to demonstrate that the stimulation of presynaptic glutamatergic receptors on DA, NE and 5-HT terminals in the rat striatum by glutamate agonists evoked simultaneous release of striatal DA, NE and 5-HT in a dose-dependent manner. Thus, it is difficult to determine if the attenuation of the ability of MDMA to decrease 5-HT concentrations seen with MK 801 resulted from a direct interaction with MDMA or if it was an indirect effect through decreasing glutamate-mediated release of 5-HT. Taken together, these results emphasize the complex interactions between central 5-HT systems and those of DA, glutamate and neurohormones.

There remain many questions about the role that DA plays in the neurochemical effects of the methylenedioxy analogues of AMPH, especially in relation to the longer term effects on the levels of 5-HT and 5-HIAA. The findings

that MDA and MDMA produce dose-dependent transient increases in the levels of DA in rats are well documented. Pretreatment or coadministration with agents that block the release of DA, that deplete DA stores or that block DA synthesis all have been shown to attenuate or block the long-term deficits in 5-HT parameters induced by MDMA and MDA (Gibb *et al.*, 1987; Mokler *et al.*, 1987; Stone *et al.*, 1988; Matthews *et al.*, 1989; Schmidt *et al.*, 1991a; Ali *et al.*, 1993; Huang and Nichols, 1993). MDE which is the weakest of the three methylenedioxy analogues in terms of its capacity to produce long term deficits in 5-HT parameters is also the weakest in its effects on DA systems (Johnson *et al.*, 1987; Schmidt *et al.*, 1987b; Stone *et al.*, 1987b; Series and Molliver, 1994).

The exact mechanisms involved and the multiple interactions between DA a 5-HT systems have been difficult to delineate and to assign relative importance in terms of the long-term neurochemical effects. Intact functioning DA and 5-HT sytems appear to be fundamentally important to the development to long-term 5-HT system deficits, but the contribution of individual systems and an indication of which are "upstream" or "downstream" of the primary effect(s) of the methylenedioxy analogues of AMPH remain unclear.

AMPH and MAMPH have been shown to affect neuropeptide systems that involve neurotensin and tachykinins, *via* release of DA (Merchant *et al.*, 1987). MDA, MDMA and MDE were examined for similar effects and were found to produce increases in neurotensin-like immunoreactivity and in dynorphin A in the substantia nigra, nucleus accumbens and the striatum but not in the hypothalamus

after either single or multiple doses (Merchant *et al.*, 1987; Johnson *et al.*, 1993). Johnson *et al.* (1988) showed that the S-(+)-isomers of MDA and MDMA were more potent than their R-(-)-isomers at increasing nigral levels of neurotensin, considered to be a result of increased potency at releasing DA. Repeated administration of MDMA down regulates preprocholecystokinin mRNA expression in neurons of the rat substantia nigra (Wotherspoon *et al.*, 1994).

To further address the mechanisms involved in the neurochemical effects produced by the methylenedioxy analogues of AMPH, pharmacological methodologies, including uptake and release studies using radiolabelled biogenic amines, *in vivo* microdialysis and voltammetry, receptor binding studies and electrophysiological studies, have been employed to examine the direct effects of these drugs in the CNS.

2.7 **Pharmacological Effects**

2.7.1 *Release of Biogenic Amines*

The mechanisms by which biogenic amines are released from nerve terminals under physiological and various experimental conditions are not fully understood. However, it is generally agreed that one mechanism involves the coupling of the depolarization of nerve terminals to Ca^{2+}-dependent exocytotic release of NT (Cooper and Meyer,1984; Smith and Augustine, 1988; Augustine *et al.*, 1994). Another mechanism elicited under certain experimental conditions by various endogenous and exogenous agents has been shown to be carrier-

mediated, whereby the transporter which is responsible for removing NT from the extraneuronal spaces is driven to operate in the opposing "inside to outside" direction, releasing cytoplasmic NT. This latter carrier-dependent mechanism was originally demonstrated in the pharmacological effects of *p*-tyramine on the release of ^{3}H-NE and of S-(+)-AMPH on the release of ^{3}H-DA and ^{3}H-5-HT (Raiteri *et al.*, 1977, 1979). Carrier-mediated release has been shown to be Na^{+}-dependent, with intracellular K^{+} ions and extracellular Cl^{-} also involved in the process (Raiteri *et al.*, 1979; Sitges *et al.*, 1994).

One of the earlier *in vitro* methods developed to examine carrier-mediated release involved incubating brain tissue preparations with a radiolabelled biogenic amine (Baker *et al.*, 1980). Aliquots of the tissue preparation would then be applied to filters inserted in superfusion chambers. Using a peristaltic pump and fresh incubation medium with or without differing ionic concentrations of drug added, the filters would be superfused and the superfusate from each chamber collected in discrete fractions. The amount of radioactivity in each fraction would be determined, and an increase of radioactivity over control values would indicate a release of labelled NT from the tissue preparation. The use of the *in vitro* superfusion method to examine the releasing abilities of specific agents has advantages over *in vivo* methods which measure the amount of NT in the extracellular fluid. The superfusion method is simpler to carry out and stimulation of release can more easily be examined separately from inhibition of reuptake. Both mechanisms would cause an increase in the amount of NT in the extracellular

space *in vivo,* which could lead to interpretative difficulties (Baker and Dyck, 1985).

Using the above method, Nichols *et al.* (1982) evaluated the enantiomers of MDA and MDMA for their effects on the release of ^{3}H-5-HT from rat whole brain synaptosomes. No significant differences in enantiomeric potency was observed at the 10 μM concentration. However, at 1 μM, S-(+)-MDMA was more effective in inducing release than the R-(-)-isomer. Johnson *et al.* (1986) expanded the release studies by looking at the release of ^{3}H-5-HT from superfused rat hippocampal slices and ^{3}H-DA from caudate nuclei slices induced by the enantiomers of MDA, MDMA and MBDB. All the test compounds demonstrated similar nonenantioselective efficacies of ^{3}H-5-HT release in the μM range. In contrast, there were large differences among the drugs in their abilities to cause release of ^{3}H-DA. Neither racemic MBDB nor its enantiomers caused release of ^{3}H-DA, while both MDA and MDMA were found to produce significant release. *Rac*-MDA was more potent than *rac*-MDMA at 10 μM. Both S-(+)-MDA and S-(+)-MDMA were substantially more potent than their R-(-)-isomers. In contrast, when R-(-)-MDA was compared to R-(-)-MDMA or S-(+)-MDA compared to S-(+)-MDMA, no significant differences in ^{3}H-DA efflux was seen. Schmidt *et al.* (1987) also used superfusion experiments to show that S-(+)-MDA and S-(+)-MDMA were equipotent at inducing efflux of ^{3}H-5-HT and ^{3}H-DA. Other researchers, using other *in vitro* release procedures, have found enantiomeric differences in the ability of MDA and MDMA to induce release of ^{3}H-5-HT (Azmitia *et al.*, 1990; McKenna *et al.*, 1991). Striatal slices prepared from rats [illegible] after a single dose of 10 or 20 mg/kg MDA, MDMA or MDE were used to

demonstrate that all three drugs were more potent as 5-HT-releasing agents than as DA-releasing agents. An increased potency of MDA over MDMA and MDE was revealed only at higher concentrations of radiolabelled ligand (Schmidt, 1987b). In tissue from untreated animals, MDE was shown to be similar to MDA and MDMA in efficacy at releasing ^{3}H-5-HT but much weaker at releasing ^{3}H-DA (McKenna *et al.*, 1991; Schmidt, 1994).

Fitzgerald and Reid (1991) demonstrated that *rac*-MDMA (10μM) enhanced not only the resting outflow of ^{3}H-DA and ^{3}H-5-HT from rat superfused brain slices, but also that of ^{3}H-NE. This enhancement was blocked by neuronal uptake blockers of DA (cocaine), 5-HT (FLU) and NE (DMI). MDMA also increased the electrically stimulated outflow of ^{3}H-NE and ^{3}H-5-HT, but not that of ^{3}H-DA. When Ca^{2+} was excluded to minimize the exocytotic release of NT, MDMA-induced release from slices incubated with ^{3}H-DA was unaltered, but slices incubated with ^{3}H-NE and ^{3}H-5-HT showed enhanced response to MDMA (Fitzgerald and Reid, 1993). The doses of MDMA required for one-half maximal release (EC_{50}) of ^{3}H-5-HT and ^{3}H-NE were equivalent at about 2 μM, over 30 μM was required for the release of ^{3}H-DA.

Confirmation of the ability of the methylenedioxy analogues to release biogenic amines has come from *in vivo* voltammetry and *in vivo* microdialysis studies. Using *in vivo* voltammetry, Yamamoto and Spanos (1988) demonstrated that MDMA induced the release of DA in a regioselective, time- and dose-dependent manner. They administered MDMA (2.5, 5, or 10 mg/kg ip) and looked at DA release in the caudate nucleus and the nucleus accumbens over a 3 h period.

The magnitude of increase in DA release was dose-dependent and similar in both brain regions with low doses of MDMA but not with the highest dose. The highest dose increased release of DA to a maximum of 372% of control in the caudate and 189% in the nucleus accumbens. The time course of drug effect also differed between the two regions, with a faster onset of effect seen in the caudate. Gazzara *et al.* (1989) obtained the opposite effects using the same methodology in the striatum, with dose-dependent decreases in DA concentrations after 0.1 and 1.0 mg/kg MDMA. There were no further decreases seen with 10 mg/kg MDMA, but the time course was shortened. These authors attributed the differences to the differences in dose range and to their animals being anaesthetized, while others had used awake animals. Nash (1990) obtained the same results as did Yamamoto and Spanos using *in vivo* microdialysis in awake, freely moving animals: the systemic administration of MDMA (10 or 20 mg/kg ip) produced a dose-dependent increase in extracellular concentrations of DA accompanied by decreases in the levels of DOPAC. Ketanserin itself did not affect the levels of DA or DOPAC in the dialysate samples, but when administered 1 h prior to MDMA significantly attenuated the MDMA-induced increase in the concentration of DA without affecting the decreases in the levels of DOPAC. Using striatal *in vivo* microdialysis, Schmidt *et al.* (1992a,b) demonstrated that MDMA (20 mg/kg) not only increased the extracellular concentration of DA but also induced an acute rise in the rate of DA synthesis, as measured by the concentration of L-DOPA, 1 h after administration. Blockade of 5-HT_2 receptors with MDL 100,907 blocked both the MDMA-induced

rise in DA efflux and DA synthesis, but had no effect on basal levels of synthesis and release. The 5-HT_2 antagonist had little effect if only a 10 mg/kg dose of MDMA was used. Haloperidol and reserpine were also shown to stimulate DA synthesis but were unaffected by MDL 100,907, suggestive that haloperidol and reserpine stimulated DA synthesis *via* different mechanisms of action than those involved with MDMA-induced enhancement of DA synthesis. Direct intrastriatal, but not intranigral, infusion of MDL 100,907 produced concentration-dependent inhibition of MDMA-induced release of DA without any effect on basal release (Schmidt *et al.*, 1994). These results support the proposal by Leyson and Pauwels (1990) that 5-HT_2 receptors are seldom activated under physiological conditions, but, during states of high 5-HT activity or conditions of increased DA efflux, act in a permissive role in the activation of DA systems. The proposed mechanism by which 5-HT_2 antagonists inhibit MDMA-induced release is based on the concept that carrier-dependent release of DA induced by MDMA is dependent upon continuous availabity of a pool of newly synthesized NT. By interfering with the stimulation of synthesis, 5-HT_2 antagonists eliminate that pool available for release. White *et al.* (1994) demonstrated that MDMA depressed glutamate-evoked neuronal firing in the nucleus accumbens and suggested that the inhibition was mediated by MDMA-mediated release of DA and 5-HT.

Dissociated fetal 5-HT neurons in microculture have been used to examine both Ca^{2+}-independent and -dependent release mechanisms involved in the effects of MDMA (Azmitia *et al.*, 1990; Gu and Azmitia, 1993). The development of the

uptake capacity of ^{3}H-5-HT was half-maximally inhibited in 4-day cultures by a single application at the time of plating by either enantiomer of MDMA [S-(+)>R-(-)]. Both forms of release were shown to be involved in the loss of ^{3}H-5-HT uptake capacity, with the direct MDMA-induced Ca^{2+}-independent, FLU-sensitive release being the first step. The D_1 receptor agonist SKF 38393 and the α_2 agonist BH-T 920, both of which have been shown to block 5-HT presynaptic K^+-induced depolarization-mediated release, attenuated the MDMA-induced inhibition of the development of uptake capacity. Blockade of 5-HT_2 receptors with ketanserin attenuated the inhibitory effects of MDMA. Caffeine, which stimulates release of Ca^{2+} from intracellular stores, potentiated, while nimodipine, a calcium channel blocker, attenuated the inhibitory effects of MDMA on uptake capacity in the fetal cells. Fenfluramine (FEN), another AMPH analogue that has been shown to be a potent releaser of 5-HT, also releases 5-HT by both exocytotic and carrier-mediated mechanisms, depending on the dose of FEN used (Bonnano *et al.*, 1994) and the presence of its N-dealkylated metabolite, norfenfluramine, which also has 5-HT releasing properties (de Parada *et al.*, 1995).

2.7.2 *Uptake Studies with Radiolabelled Ligands*

The 5-HT transporter has been shown to be heavily involved in the actions of the methylenedioxy analogues of AMPH (Rudnick and Wall, 1992). The neuronal membrane 5-HT transporter, which is the major mechanism whereby the actions of synaptic 5-HT are terminated, and the vesicular membrane 5-HT transporter both

contain binding sites for 5-HT uptake. Thus, radiolabelled studies using various agonists and antagonists have been conducted examining both the cell membrane 5-HT transporter and the vesicular membrane 5-HT transporter. Other studies have examined the effects of drugs on uptake by determining their ability to inhibit the uptake of radiolabelled biogenic amines in tissue preparations similar to those used in superfusion release experiments.

^{3}H-Paroxetine has been used extensively to examine the effects of the methylenedioxy analogues on the 5-HT uptake site. Both MDMA and MDA induce similar dose-dependent effects on ^{3}H-paroxetine binding: a decrease in density of uptake sites with little effect on affinity (Battaglia *et al.*, 1987; Desouza and Kuyatt, 1987; Sprague *et al.*, 1994). Pretreatment with Trp or 5-HTP blocked the reduction in ^{3}H-paroxetine binding (Sprague *et al.*, 1994). Nash *et al.* (1991) administered single or multiple 20 mg/kg doses of MDMA to rats and examined the density of ^{3}H-paroxetine labelled binding sites in frontal cortex and platelets 24 h and 7 days post drug treatment. The single dose of MDMA decreased the number of binding sites in the frontal cortex but not in platelets at both time periods. Multiple doses decreased the binding sites in both the frontal cortex and in platelets at 24 h, but there was full recovery in platelets by 7 days after administration. De Souza and Kuyatt (1987), using autoradiography and ^{3}H-paroxetine, confirmed the selective loss of 5-HT uptake sites 2 weeks after administration of MDA (20 mg/kg sc twice daily for 4 days). The brain regions most affected by MDMA treatment were the cortex, the striatum and the hippocampus. Within the midbrain structures, regions

containing 5-HT projections, like the substantia nigra, were more affected than those regions containing 5-HT cell bodies. No changes in the density of NE and DA uptake sites were seen.

Schuldiner *et al.* (1993) examined the interaction of MDMA with the plasma membrane 5-HT transporter from platelets and the chromaffin granule vesicular amine transporter and found that MDMA was a substrate for the platelet plasma membrane transporter, therefore inhibiting both 5-HT transport and imipramine (IMI) binding. In chromaffin vesicles, ^{3}H-reserpine binding measurements were used to show that MDMA inhibited 5-HT transport by binding to the substrate site of the vesicular transporter and by dissipating the pH gradient that drives amine uptake into vesicles. MDA was also shown to inhibit ^{3}H-reserpine binding similarly to MDMA. Hashimoto *et al.* (1992a,b) conducted radiolabelled binding studies with ^{3}H-nitroquipazine, another radiolabelled selective ligand for the 5-HT uptake site, to show that coadministration of a number of benzylpiperazine derivatives attenuated the decreases in the levels of 5-HT and 5-HIAA produced by MDMA through some yet unknown mechanism that did not include the 5-HT transporter.

Published articles on the inhibition of uptake of radiolabelled biogenic amines by the methylenedioxy analogues of AMPH are limited. Steele *et al.* (1987) compared MDA, MDMA and MBDB to AMPH and DOM with regard to their ability to inhibit rat synaptosomal uptake of ^{3}H-DA, ^{3}H-NE and ^{3}H-5-HT. The individual enantiomers of each of the drugs were used to examine chiral differences in potency between enantiomers and to examine whether an R-(-)- or an S-(+)-

configuration conferred any generalized affinities at uptake sites. Th allucinogen, DOM, did not inhibit uptake of any of the radiolabelled ligands, a marked difference in pharmacological action compared to MDA and MDMA. S-(+)-AMPH was clearly the most potent inhibitor of striatal ^{3}H-DA and hypothalamic ^{3}H-NE uptake, but a weak inhibitor of hippocampal ^{3}H-5-HT uptake. Only S-(+)-MDA and S-(+)-MDMA inhibited striatal ^{3}H-DA uptake (MDA > MDMA). S-(+)-MDA and S-(+)-MDMA were equipotent at inhibiting hypothalamic ^{3}H-NE uptake and were both more potent than their respective R-(-)-isomers. MBDB was also strong inhibitor of ^{3}H-NE uptake [R-(-)- weaker than S-(+)-]. The S-(+)-isomers of MDA, MDMA and MBDB were all equipotent at inhibiting ^{3}H-5-HT and about 3-4 fold more potent than their antipodes. Uptake of ^{3}H-5-HT in synaptosomal preparations from animals treated 1 week prior with MDA, MDMA or MDE (20 mg/kg) was decreased after MDA and MDMA, likely reflecting a decrease in density of uptake sites. Uptake was unaltered by MDE treatment.

In contrast to platelets, ^{3}H-MDMA and ^{3}H-MDA uptake into nerve terminals has been shown to be very low, suggestive that MDMA and MDA are not taken up into terminals by transporters (Schmidt *et al.*, 1987; Wang *et al.*, 1987; Zaczek *et al.*, 1989). Although Gehlert *et al.* (1985) reported high affinity specific binding to ^{3}H-MDMA to rat brain membranes, subsequent studies by Wang *et al.* (1987) suggested that these apparent binding sites represented ^{3}H-MDMA associated with glass fiber filter paper. Zaczek *et al.* (1989) reexamined the possibility that both ^{3}H-MDA and ^{3}H-MDMA associated with rat brain membranes. Saturation profiles of

^{3}H-MDA and ^{3}H-MDMA in rat synaptosomes supported the existence of multiple binding sites. These authors demonstrated that the binding of either ^{3}H-MDA and ^{3}H-MDMA fits two-site models, indicating both high and low affinity sites (for ^{3}H-MDA, K_D = 887 nM and 45 μM; for ^{3}H-MDMA, K_D = 2.9 μM and 128 μM). The rapid diffusion of MDMA and MDA across synaptosomal membranes has been attributed to their lipophilicity rather than uptake through the 5-HT transporter (Schmidt *et al.*, 1987: Schmidt, 1994). However, Zaczek *et al.* (1989) suggested that an active transport process other than that of the 5-HT transporter could be involved.

2.7.3 *Radiolabelled Ligand Receptor Binding Studies*

The receptors for 5-HT constitute a large heterogeneous superfamily of receptors. Peroutka and Synder (1979) first postulated the existence of at least two classes of 5-HT receptors in rat brain and labelled them 5-HT_1 and 5-HT_2 receptors on the basis of their affinity for radiolabelled ligands, with 5-HT_1 sites having nanomolar (nM) affinity for ^{3}H-5-HT and 5-HT_2 sites showing high affinity for ^{3}H-spiperone while ^{3}H-LSD possessed equal affinity at both receptors. Since then, there have been five additional classes of 5-HT receptors characterized and numerous subtypes identified within some of the classes (Hoyer *et al.*, 1994) (Figure 8). Further studies have led to the classification of 5-HT receptors based not only on specific ligand affinities, but also on their interface between the internal and external environments of the cell wherein the receptor resides and on the functional

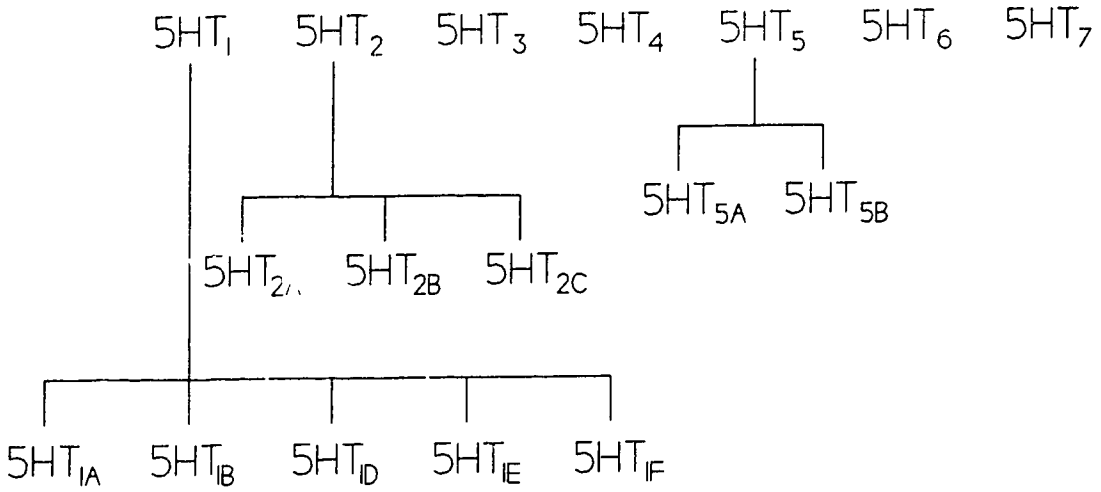


Figure 8: Proposed classification system for 5-hydroxytryptamine receptors. Adapted from Hoyer *et al.* (1994).

consequences of receptor activation. 5-HT_1 and 5-HT_2 receptors are both G protein (guanine nucleotide binding protein) coupled receptors, linked to adenylate cyclase activity or phosphotidyl inositol (PI) turnover respectively (Peroutka *et al.*, 1990). The exception is the 5-HT_{1C} receptor, which like other 5-HT_1 receptors displays high affinity for 5-HT, but is linked to PI turnover. This has led to a recent change in nomenclature, with 5-HT_{1C} receptors being renamed 5-HT_{2C} receptors and the original 5-HT_2 receptor referred to as 5-HT_{2A}, in keeping with their functional effects (Humphrey *et al.*, 1993; Hoyer *et al.*, 1994; Nash *et al.*, 1994). 5-HT_4, 5-HT_6 and 5-HT_7 receptors are also linked to adenylate cyclase activity and genes for 5-HT_5 receptors have been identified in mice and rats (Humphrey *et al.*, 1993; Hoyer *et al.*, 1994). 5-HT_3 receptors are ligand-gated cation channels (Peters *et al.*, 1992; Unwin, 1993).

Other NT receptor families, including DA, NE, muscarinic acetylcholine and histamine, display within-family heterogeneity, albeit to a lesser degree than is seen with the 5-HT receptor family. In terms of DA receptors, there have been several receptor subfamilies identified (De Keyser, 1993; O'Dowd, 1993). However, only D_1 and D_2 receptors have been examined for effects of the methylenedioxy analogues of AMPH. NE receptors, which include various subtypes of α- and β-adrenoceptors, are classified according to their affinities to specific agonists and their distribution (May and Minneman, 1986; Bylund *et al.*, 1994).

Battaglia *et al.* (1988a) examined the *in vitro* binding profile of MDMA at various brain recognition sites and assigned the following rank order of affinities: 5-

HT uptake sites > α_2-adrenoceptors = 5-HT_2 receptors = M-1 muscarinic receptors = H-1 histamine receptors > NE uptake sites = M-2 muscarinic receptors = α_1-adrenoceptors = β-adrenoceptors ≥ DA uptake sites = 5-HT_1 receptors >> D_2 receptors > D_1 receptors. These researchers also compared MDA and MDE with MDMA in terms of relative ranking of affinities at a number of receptors. The affinities of MDA were comparable (less than two fold difference) to those of MDMA at 5-HT uptake sites, 5-HT_2 receptors, α_2-adrenoceptors and muscarinic receptors. The relative affinity of MDE at the 5-HT uptake site was more than double that of MDMA and MDA but MDE was much weaker at 5-HT_2 receptors and α_2-adrenoceptors. It is interesting to note that despite its reported affinity for the 5-HT uptake site, MDE is the least potent of the methylenedioxy analogues of AMPH in terms of behavioral and neurochemical effects. The affinity for the 5-HT uptake site would appear not to be related to overall behavioral or neurochemical effects, even though interaction with the 5-HT transporter is one of the main pharmacological effects of these drugs. Post-receptor efficcy in signal transduction may be an important consideration.

The *in vitro* affinities of a number of hallucinogenic AMPH analogues at 5-HT_2 receptors are significantly correlated with both the behavioral potencies in DD studies and in their human hallucinogenic potencies (Glennon and Young, 1984a, 1984b). A comparison of the relative affinities of MDMA and MDA at postsynaptic 5-HT_2 receptors with those of AMPH-related hallucinogens suggest that MDMA and MDA would only be weak hallucinogens, but it is feasible that some of the mood

altering effects may be mediated through agonist activity at 5-HT_2 receptors (Battaglia *et al.*, 1988). MDMA showed relatively high affinity for α_2-adrenoceptors, which could contribute to the increases in both diastolic and systolic pressure in humans, and for M-1 muscarinic receptors, which could mediate the salivation seen both in humans and animals after MDMA ingestion (Shulgin, 1986; Peroutka, 1987; Spanos and Yamamoto, 1989; Gordon and Fagelson, 1994).

Scheffel *et al.* (1992) administered single and multiple doses of *rac*-MDMA to rats and then examined binding to 5-HT_2 and 5-HT_{1C} receptors, using *N*-1-methyl-2-I^{125}-LSD. A single dose of 20 mg/kg or multiple lower doses produced down-regulation of 5-HT_2 receptors, but no regionally specific choroid 5-HT_{1C} receptor down-regulation. No persistent changes were in evidence 21 days post-MDMA treatment. Down regulation of 5-HT_2 receptors is a response common to several chronic antidepressant treatments (Baker and Greenshaw, 1989; Stahl, 1994). The significance of this down regulation is not clear, especially if these receptors are not active under normal physiological conditions, as Leyson and Pauwels (1990) suggest. Yau *et al.* (1994) examined the effects of MDMA on receptor gene expression in the hippocampus of rats two weeks after multiple doses (20 mg/kg twice daily for four days). These authors found messenger RNA expression was reduced for both glucocorticoid and mineralocorticoid receptors, but was increased for 5-HT_{1C} receptors.

The enantioselectivity of MDA and MDMA inhibition of *in vitro* binding on various receptors has been examined. All four enantiomers were [illegible] es more

potent inhibitors at 5-HT_2 and 5-HT_1 receptors than at D_2 receptors. At 5-HT_2 receptors labelled with ^{3}H-ketanserin, 5-HT_1 receptors labelled with ^{3}H-5-HT and at D_2 receptors labelled with ^{3}H-*N*-methylspiperone, the R-(-)-isomers of both MDA and MDMA were more potent than the S-(+)-isomers. Only at D_2 receptors was there a difference in potency between R-(-)-MDA and R-(-)-MDMA, favoring R-(-)-MDMA (Lyons *et al.*, 1986). These results support the behavioral data showing that R-(-)-MDA has more potent psychoactive effects, but are in contrast to data discussed earlier showing that in behavioral studies S-(+)-MDMA is the more active enantiomer and that both the S-(+)-isomers of MDA and MDMA are more potent at releasing DA and at lowering the levels of 5-HT and 5-HIAA. These data illustrate that the enantioselectivity of the methylenedioxy analogues of AMPH is very much a function of the process or effect under study.

Nash *et al.* (1994) examined the direct effects of the enantiomers of either MDA or MDMA on ^{3}H-inositol monophosphate accumulation as an indicator of PI turnover in cells expressing either 5-HT_2 or 5-HT_{1C} receptors and demonstrated that MDA and MDMA had low affinity but moderate intrinsic efficacy at these receptor subtypes. In keeping with the behavioral studies, in cells expressing 5-HT_2 receptors, the R-(-)-isomers of both MDA and MDMA displayed higher affinities than their respective S-(+)-isomers. In cells expressing the 5-HT_{1C} receptors, the enantiomers of MDA were equipotent, while S-(+)-MDMA was more potent than its antipode. At both types of receptors, 5-HT displayed at least 100 times greater affinity than any of the individual enantiomers of MDA and MDMA. The rank

ordering of the maximal rate (V_{max}) of stimulation of PI turnover at 5-HT_2 receptors was 5-HT = R-(-)- and S-(+)-MDA >> R-(-)-MDMA >>S-(+)-MDMA and at 5-HT_{1C} sites was 5-HT > R-(-)- = S-(+)-MDA >> R-(-)-MDMA > S-(+)-MDMA. Schmidt and Taylor (1987) estimated that the maximum brain concentration of MDMA would be approximately 90 µM after a dose of 20 mg/kg. The peak hippocampal concentration of MDA was reported to be 165 µM (36 µg/g of tissue), observed 45 min after systemic administration of MDA 20 mg/kg sc (Zaczek *et al.*, 1989). The K_i values for R-(-)-MDA and R-(-)-MDMA at 5-HT_2 sites labelled with ^{3}H-ketanserin were calculated as 3.4 mM and 3.3 mM respectively, and for S-(+)-MDA and S-(+)-MDMA, the values were 13.0 mM and 15.8 mM (Lyons *et al.*, 1986). Thus, the primary pharmacological effects of MDA and MDMA on 5-HT_2 receptors are likely mediated indirectly *via* 5-HT release and inhibition of uptake. However, direct actions on 5-HT_2 receptors may be involved in the long-term effects observed after high or multiple doses of MDMA and MDA.

2.7.4 *Effects on Monoamine Oxidase Activity*

Monoamine oxidase (MAO) is one of the major mammalian enzymes of importance in the metabolic degradation of the biogenic amines. MAO catalyzes the reaction which converts the biogenic amines to their corresponding aldehydes. The aldehyde intermediates are rapidly further metabolized by reduction or oxidation to a glycol or acid (Tipton, 1973; Tipton and Mantle, 1977) [Figure 9]. Further metabolic O-methylation can occur. MAO in the CNS is membrane bound

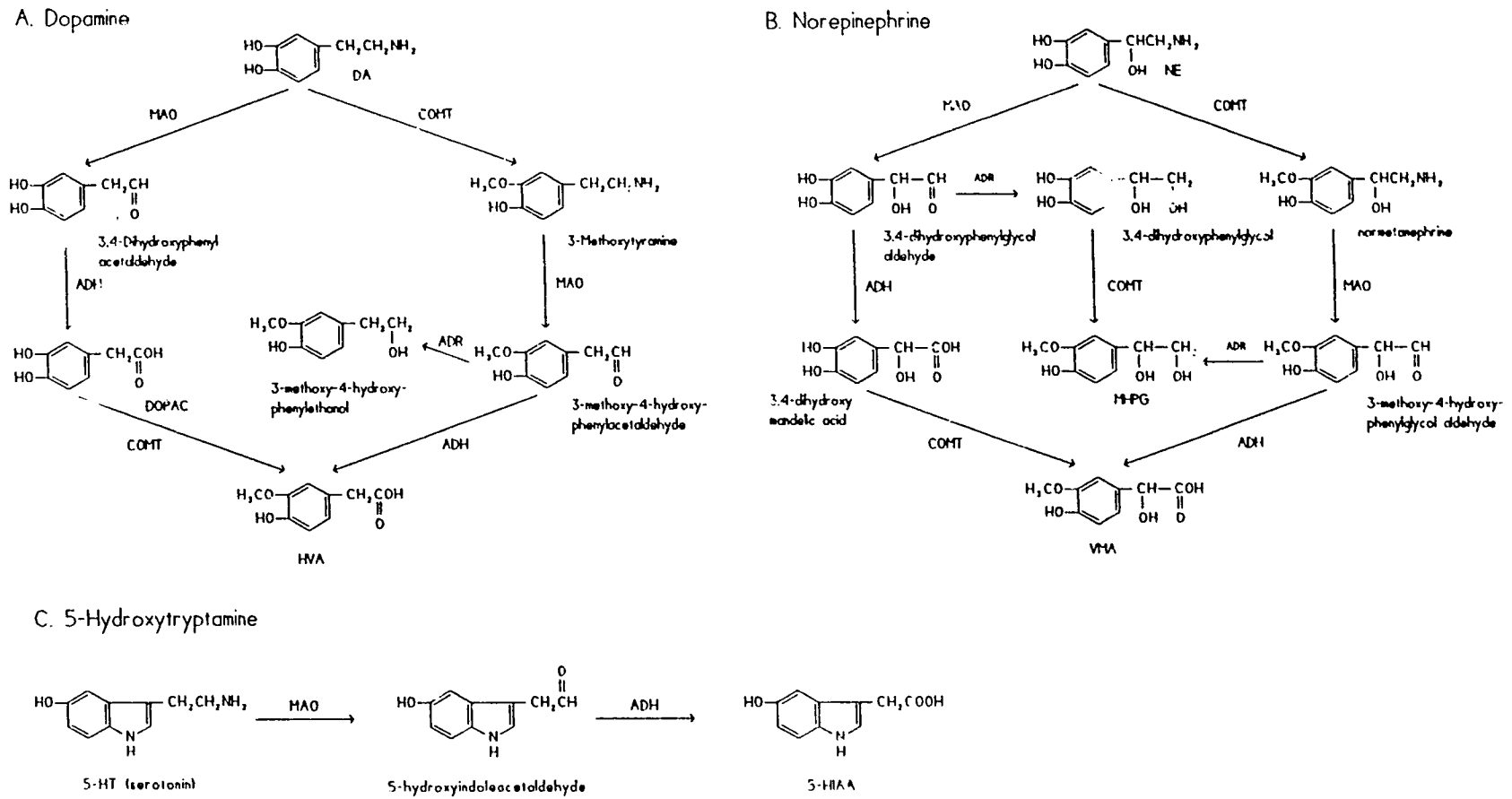


Figure 9: Monoamine oxidase-catalyzed metabolism of (A) dopamine, (B) norepinephrine and (C) 5-hydroxytryptamine. (ADH=aldehyde dehydrogenase; ADR=aldehyde reductase; COMT=catechol-O-methyltransferase; VMA=vanillylmandelic acid). Adapted from Cooper, Bloom and Roth (1991), p. 245 and 341.

protein, localized largely in the outer membrane of mitochondria in the terminal fields of neurons (Schnaitman *et al.*, 1967; Greenawalt and Schnaitman, 1970). Although MAO is generally considered to be an intraneuronal enzyme, it occurs in abundance extraneuronally, including in glial cells (Levitt *et al.*, 1982; Kalaria *et al.*, 1988; Jossan *et al.*, 1989; Grimsby *et* al., 1990). There are at least two different forms of MAO present in human and rat brain, designated type-A and type-B based on substrate specificity and sensitivity to inhibition by selective inhibitors (Tipton and Mantle, 1977; Fowler and Tipton, 1984; Bach *et al.*, 1988; for review see Berry *et al.*, 1994). The type-A enzyme (MAO-A) displays substrate preference for 5-HT and NE, while MAO-B displays preference for β-phenylethylamine and benzylamine. DA appears to be a good substrate for both types of MAO (Green *et al.*, 1977). Non-selective MAO inhibitors (e.g. phenelzine and tranylcypromine) have been developed and are marketed as antidepressants (Pare, 1985). The selective MAO-A inhibitor, moclobemide is now available commercially as an anti-depressant (Waldmeier *et al.*, 1994), and the selective MAO-B inhibitor, L-deprenyl has been shown to be efficacious in Parkinson's disease, a disease characterized by a progressive degeneration of DA neurons (Allain *et al.*, 1993).

Several different substituted AMPH analogues have been shown to be reversible selective MAO-A inhibitors. These include PMA, MAMPH, PCA, FEN and MDMA (Green and Hait, 1980; Susuki *et al.*, 1980; Gu and Azmitia, 1993; Leonardi and Azmitia, 1994). Leonardi and Azmitia (1994) compared the relative abilities of *rac*-MDMA, FEN and FLU to inhibit MAO-A and MAO-B activity in rat brai

homogenates. The relative potencies for MAO-A were MDMA > FLU > FEN and for MAO-B, FLU > MDMA > FEN. There were no significant differences found between R-(-)- and S-(+)-MDMA. The K_i value for *rac*-MDMA at MAO-A sites was calculated to be 22 μM and the IC_{50} for MAO-B was about 8-fold higher than was seen for MAO-A. The calculated IC_{50} value for MAO-B (370 μM) is more than twice the low-affinity K_D value for ^{3}H-MDMA obtained from binding studies (Zaczek *et al.*, 1989).

It is possible that selective MAO-A inhibition plays a role in the pharmacological and neurochemical effects of MDMA. While the inhibition of uptake produced by MDMA would limit the access of intraneuronal MAO to its NT substrates after their release, the MDMA-induced increase in the 5-HT released from vesicular stores into the cytoplasm would be less vulnerable to enzymatic degradation, thus allowing more carrier-mediated release into the extracellular space. This protection would preferentially affect 5-HT and NE, while DA would still be vulnerable to metabolism by MAO. The time course of the changes in DA parameters, compared to 5-HT parameters, after administration of MDMA could also be influenced by the selective MAO-A inhibition.

2.7.5 *Electrophysiological Studies*

The effects of MDMA on cell firing have been examined in both nigrastriatal DA neurons and dorsal raphé (DR) 5-HT neurons. Kelland *et al.* (1989) used standard extracellular single unit recording of rat nigrastriatal DA neurons to show

that MDMA inhibited the firing rate in a dose-dependent manner. Antagonism of the MDMA-induced slowing of the nigrastriatal neurons by 5-HT_2 receptor antagonists was attenuated by administration of L-DOPA, further supporting the proposal that 5-HT_2 antagonists act at the level of DA synthesis (Schmidt *et al.*, 1992b). Sprouse *et al.* (1989) combined extracellular single unit recording and microdialysis of an *in vitro* midbrain slice preparation and demonstrated that both enantiomers of MDMA produced dose-dependent reversible inhibition of 5-HT cell firing [S-(+)- > R-(-)-]. Pretreatment with FLU blocked the inhibitory effects, suggestive that MDMA acts indirectly to inhibit dorsal raphé (DR) cells, likely *via* increasing 5-HT concentrations, thereby activating somatodendritic 5-HT_{1A} receptors which cause hyperpolarization of the cell and a decrease in cell firing. Bradberry (1994) combined *in vitro* microdialysis and parallel *in vivo* extracellular recordings of DR neurons to add further support that S-(+)-MDMA inhibits 5-HT cell firing by releasing 5-HT which then acts upon somatodendritic receptors. The effects of S-(+)-MDMA were inhibited by FLU and augmented by Trp.

2.8 Metabolism of the Methylenedioxy Analogues of AMPH

2.8.1 *General Principles Regarding Metabolism*

The metabolism of exogenous compounds or xenobiotics is a process of detoxification, whereby the biological activity of a compound is terminated by the chemical modification of the molecule such that the polarity of the molecule is increased, allowing for ease of elimination (Creasey, 1979; Curry, 1980; Trager,

1980; Estabrook, 1984; Wolf *et al.*, 1987). The chemical modifications are generally catalyzed by metabolic enzymes. Because of its relative richness in enzymes and large mass, the liver is clearly the dominant organ in drug metabolism. However, many other tissues, including the brain, possess measurable metabolizing activity. Regardless of where metabolism takes place, a single drug may undergo several biotransformation steps, any of which may profoundly influence its biological activity. Not all metabolic processes result in less toxic or biologically inactive metabolites or intermediates. In some cases, it is a specific metabolite that is responsible for a drug's main biological effect, while with other drugs, a metabolite is the source of unwanted side effects.

For the majority of xenobiotics, metabolism involves 2 step processes, designated Phase 1 and Phase 2 metabolism (for review, see Gibson and Skett, 1986). Phase 1 metabolism is characterized by oxidation/reduction and hydrolytic reactions. Phase 2 metabolism involves enzyme-mediated conjugation, a chemical reaction combining the reactive group arising from Phase I metabolism with endogenous molecules such as glucuronic acid, sulphates, glycine and acetate. Conjugates are water soluble products which can be excreted in bile or urine.

Oxidation, a prominent route of Phase I biotransformation, involves insertion of an oxygen atom into the substrate. The process is catalyzed by an enzyme system called a monooxygenase or mixed function oxidase system (Kaufman, 1977). The overall reaction requires both atoms of molecular oxygen: one atom is introduced into the drug substrate while the other is reduced to water (Figure 10).

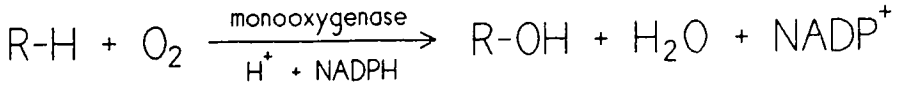


Figure 10: Overall metabolic oxidation reaction catalyzed by a monooxygenase sy[illegible].

Reduced nicotinamide-adenine dinucleotide phosphate (NADPH) is the source of reducing equivalents or electrons (Hollenberg, 1992). The most extensively studied mixed function oxidase system is the cytochrome P450 (CYP) system made up of NADPH CYP reductase, the CYP 450 hemeprotein and a lipid component, phosphotidylcholine (Lu and West, 1980). The bonding arrangement of the heme iron of CYP to the four pyrrole nitrogen atoms of protoporphyrin IX and the axial ligands produce different geometric configurations and spin states, giving rise to different UV/visible characteristics of the absorbance spectrum of CYP. Most CYP exists in a low spin configuration with a maximum absorbance wavelength of around 418 nanometers (nm).

The CYP hemeprotein is both the substrate and oxygen-binding locus of the monooxygenase reaction. The core features of the CYP catalytic cycle is the ability of the heme iron to undergo cyclic oxidation/reduction in conjuction with substrate binding and oxygen activation. The binding of substrate to the oxidized form of CYP produces changes in the absorbance spectrum of CYP. These changes are the result of the ability of various substrates to perturb the spin equilibrium of CYP and serve as the basis for classifying CYP substrates (Sligar *et al.*, 1980). Early experiments in the late 1960s categorized substrate binding to CYP into three types, namely Type I, Type II and modified Type II or reverse Type I. Binding of a Type I substrate resulted in a high spin configuration of the heme iron and the change from low to high configuration resulted in a characteristic spectral change (absorption maximum at 385-390 nm and minimum at 420 nm). Type II substrates

produced absorption peaks at 425-435 nm and troughs at 390-405 nm. Modified Type II substrates exhibited absorption peaks at 420 nm and troughs at 388-390 nm (Gibson and Skett, 1986).

The CYP hemeprotein component has been shown to consist of a superfamily of different isozymes with some differing and some overlapping substrate specificities (Nebert *et al.*, 1991; Guengerich, 1992; Wrighton and Stevens, 1992). Some isozymes display species specificity (Lu and West, 1980). Genetic polymorphism has been demonstrated with several CYP isozymes (Eichelbaum and Gross, 1990; Meyer, 1990; Meyer *et al.*, 1990; Coutts, 1994). Molecular biological techniques have allowed the classification of CYP isozymes into different gene families based on amino acid sequence (Nebert *et al.*, 1991). Each distinct gene family contains less than 40% amino acid sequence homology with other gene families and is differentiated by Arabic numbers. Within some gene families, there are subfamilies, designated by capital letters and showing at least 60% homology between members. Even within subfamilies, heterogeneity exists, with individual isozymes distinguished by another Arabic number. Thus, CYP2D6 is the sixth isozyme of the cytochrome p450 isozyme family 2, subfamily D.

The five most extensively studied CYP isozymes are CYP1A1, CYP1A2, CYP2D6, CYP3A4 and members of the CYP2C subfamily that catalyze the hydroxylation of S-mephenytoin (Coutts *et al.*, 1994). A knowledge of which CYP isozymes are involved in the metabolism of a specific drug is important as competition between substrates for the active site on the enzyme is often the basis

for drug-drug interactions. The end result of drug-drug interactions can be positive, with an increase in therapeutic effect from higher tissue levels of drug, or negative, with a decrease in therapeutic efficacy and an increase in side effects or drug toxicity. With this type of knowledge predictions of clinical efficacy could be more accurately made and potential toxicological consequences avoided. Other metabolic mechanisms involved in drug-drug interactions are induction of enzymes and their reversible inhibition.

As in the latter, chirality can play a significant role in the metabolism of drugs. Because the configuration of molecules interacting with the active sites of metabolic enzymes is important for charge attraction and three-dimensional fit, there is often a difference in fit between R-(-)- and S-(+)-isomers. This can result in enantioselective metabolism favoring the isomer whose fit allows for maximal interaction. Studies of structure-activity relationships involving enantiomers and structural analogues have allowed researchers to gain valuable insights and make predictions about the active sites on many protein structures.

Not only can the potencies of the biological activity and pharmacological effects differ between the enantiomers of the parent chiral compound, differences can also exist for any biologically active metabolites. Thus, there can be complex interactions among enantiomers of the parent compound and any chiral metabolic products within the biological organism, which in turn can affect the pharmacological actions and the metabolism of each of the individual enantiomers. Drug-drug interactions can be as significant between enantiomers as between chemically

different compounds (Coutts and Baker, 1989; Jamali *et al.*, 1989).

2.8.2 *Metabolism of the Methylenedioxyphenyl Moiety*

The oxidative metabolism of MDP compounds other than the methylenedioxy analogues of AMPH has been extensively studied. Interest in MDP compounds began with the observation that the duration of action and toxicity of many drugs in mammals and of many insecticide chemicals in insects were greatly increased with joint treatment with certain compounds containing the MDP group (Casida *et al.*, 1966). The most widely studied MDP compounds included safrole, isosafrole, piperonal (all used in the synthesis of MDA and MDMA) and piperonyl butoxide (Hodgson and Philpot, 1974; Testa and Jenner, 1981; Dahl, 1982). The ability of MDP compounds to act as insecticide synergists and drug potentiators resides in their ability to inhibit the CYP-dependent monooxygenases that play a critical role in Phase 1 metabolism of insecticides and other xenobiotics, including the MDP compounds themselves. The inhibition results in decreased levels of CYP associated with the formation of a characteristic isocyanide-like spectral complex with dual maximum absorbance wavelengths of about 455 and 427 nm. This spectrum is considered to result from the formation of a MDP-ferro-CYP complex in the presence of NADPH (Franklin, 1971; Hodgson and Philpot, 1974; Wilkinson *et al.*, 1983). Dahl and Hodgson (1978, 1979) postulated that the formation of the double spectral complexes arose from bidentate binding involving interactions with both Type I substrate binding sites and the heme moiety of CYP (Figure 11).

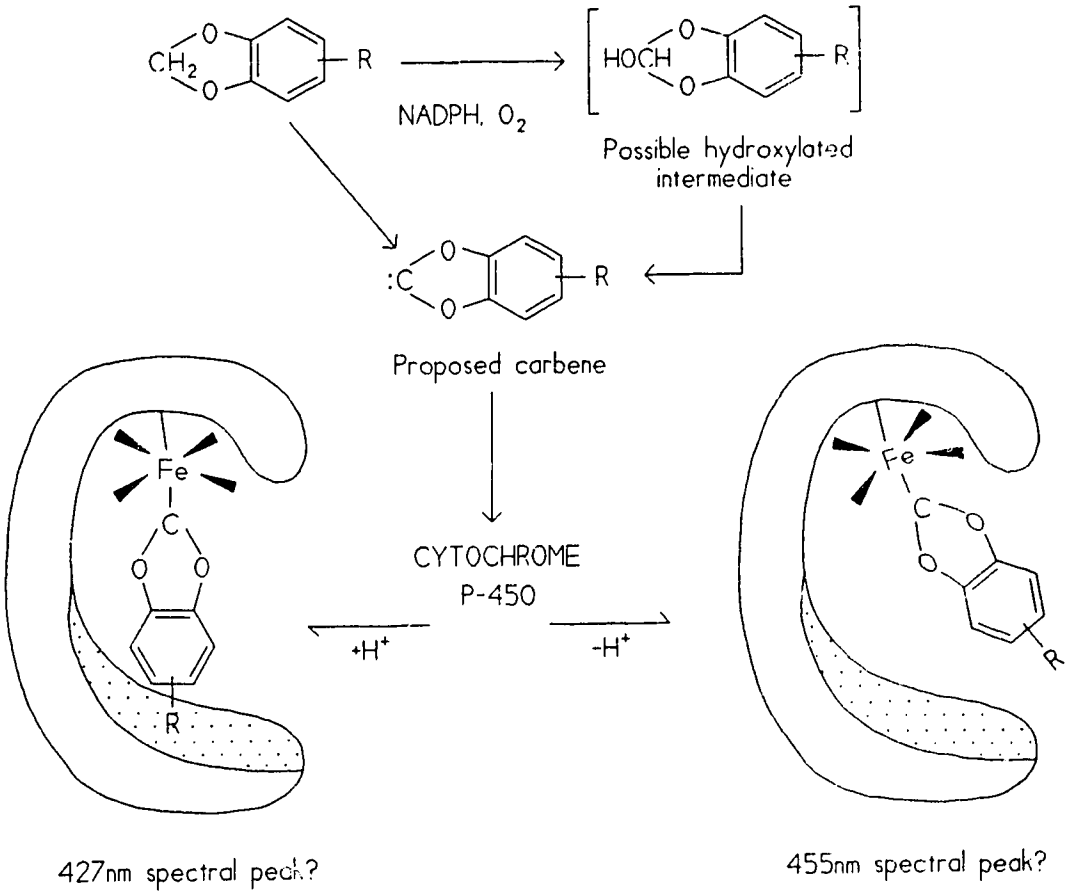


Figure 11: Dahl-Hodgson model of the methylenedioxyphenyl-cytochrome P-450 complex. Adapted from Wilkinson *et al.* (1993) pp. 46.

Franklin (1971) demonstrated the participation of O_2 in the formation of the complex between piperonyl butoxide and hepatic microsomes, indicative that an oxidative metabolic intermediate (MI) formed from piperonyl butoxide was responsible for the observed complex. MI complex formation can result in suicide inactivation of the cytochrome, not through covalent binding of the reactive intermediate to heme, but through an unusually strong ligand binding of the MI directly to the iron of reduced P450 (Johsson and Lindeke, 1992). Once formed, the MI complex was shown to be stable enough to survive oxidation and further reduction of microsomal CYP. Increased lipophilicity of MDP compounds correlated with increased metabolite-enzyme complex formation (Dahl and Brezinski, 1985). Maximum inhibition by piperonyl butoxide was about 50%, suggesting the presence of at least two forms of CYP, of which only one interacts with piperonyl butoxide to form the complex (Hodgson and Philpot, 1974). Delaforge *et al.* (1990) examined the binding of safrole to CYP P450 and P-448 and found that safrole formed a stable safrole carbene ligand complex with iron of ferro-CYP P450 and produced a concomitant loss of mixed function oxidase activity. In the presence of some type I substrates, the complex dissociated, releasing the safrole intermediate and restoring the catalytic activity. After an initial inhibitory effect on enzyme activity, MDP compounds have been shown to induce a form of CYP that is different from that induced by phenobarbital or pregnenolone carbonitrile (Bridges and Fennell, 1980; Delaforge *et al.*, 1990).

Incubation of rat microsomes with MDMA and MDA in the presence of

NADPH generated a spectrally observed inhibitory complex with CYP, similar to other MDP compounds. The complex was shown to inhibit product formation from MDA and MDMA as well as other CYP-dependent reactions (Brady *et al.*, 1986).

Other metabolic reactions involving the MDP moiety of the methylenedioxy analogues of AMPH have been identified. Summarizations of the proposed metabolic pathways for MDA and MDMA are shown in Figures 12 and 13. Foreman and Mandlik (1974) injected rats with 5 mg/kg ip of ^{3}H-S-(+)-MDA or ^{3}H-R-(-)-MDA. The elimination half-life ($t_{1/2}$) was calculated to be 48 min for both enantiomers. More than 7 metabolites were produced from both isomers; for S-(+)-MDA the major metabolite was 4-hydroxy-3-methoxyamphetamine (HMA), representing 19% of the total urine level ^{3}H as free and 25% as the glucuronide conjugate. The respective values of R-(-)-HMA were 2% and 19%. HMA was presumed by these authors to derive from the oxidation of MDA to 3,4-dihydroxyamphetamine (α-methyl-DA, DHA) and subsequent 3-O-methylation. The proposed mechanism is shown in Figure 14. Rat liver microsomes were shown to catalyze the conversion of MDA to DHA by a NADPH-dependent CYP hydroxylase (Marquart and DiStefano, 1974). The relative constancy of the sum of MDA and DHA suggested that under those conditions, the formation of DHA was the sole initial metabolic path. Fukuto *et al.* (1991) compared the demethylenation of MDA and MDMA in purified rabbit liver microsomes. With MDA, the major products were DHA, piperonylacetone and 3,4-methylenedioxy-*N*-hydroxyamphetamine. When MDMA was substituted in the rabbit liver preparation, MDA and 3,4-dihydroxymethamphetamine (DHMA) were

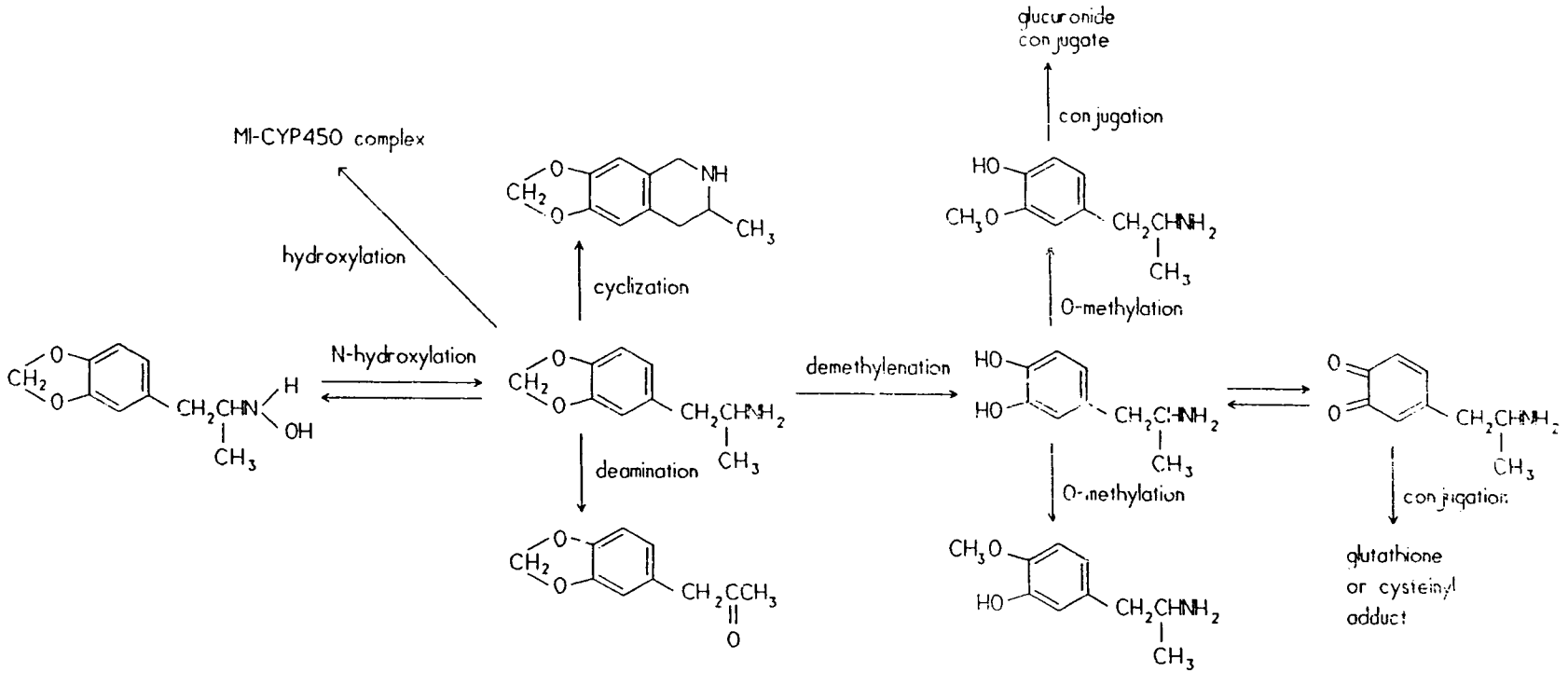


Figure 12: Proposed metabolic pathways for MDA.

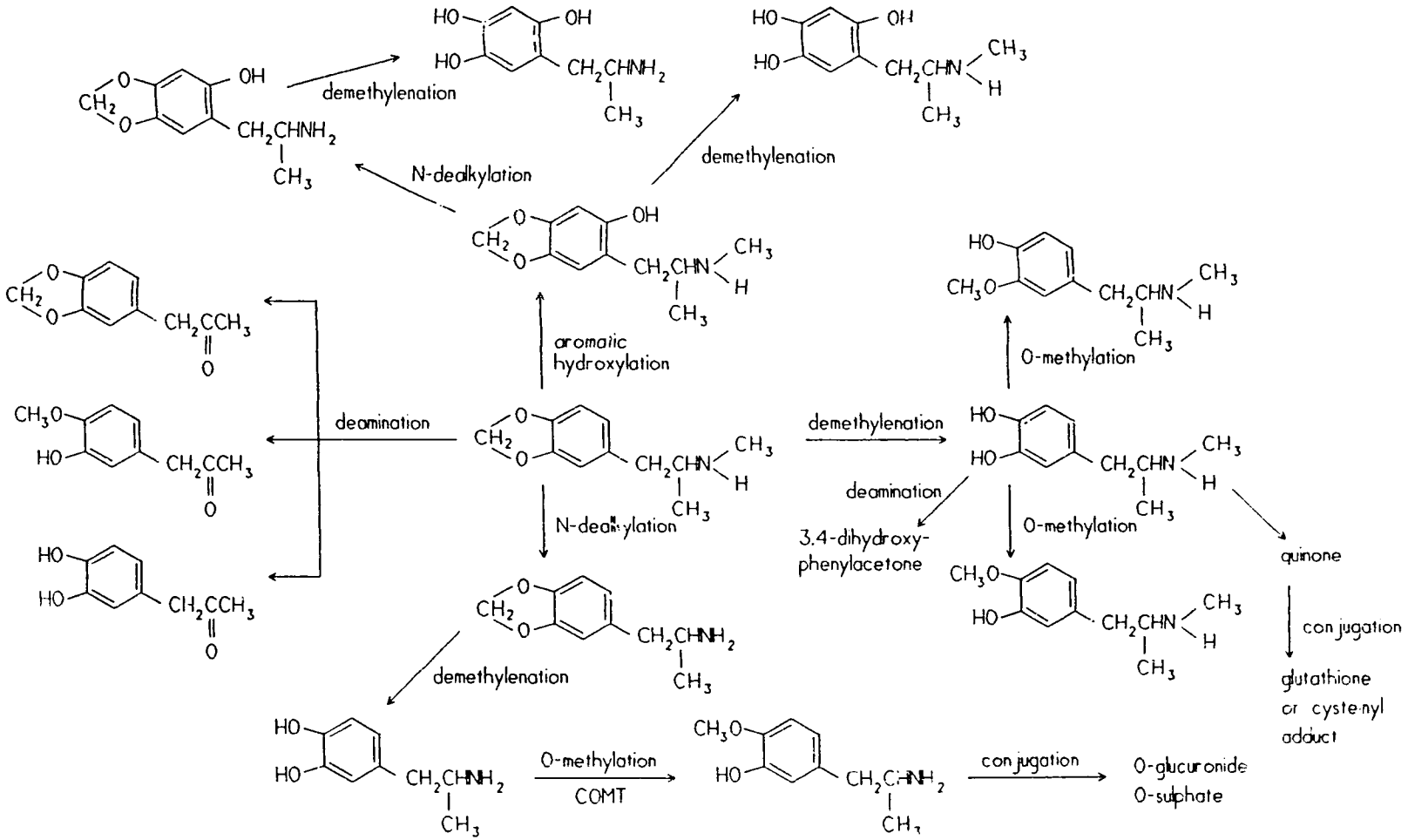


Figure 13: Proposed metabolic pathways for MDMA.

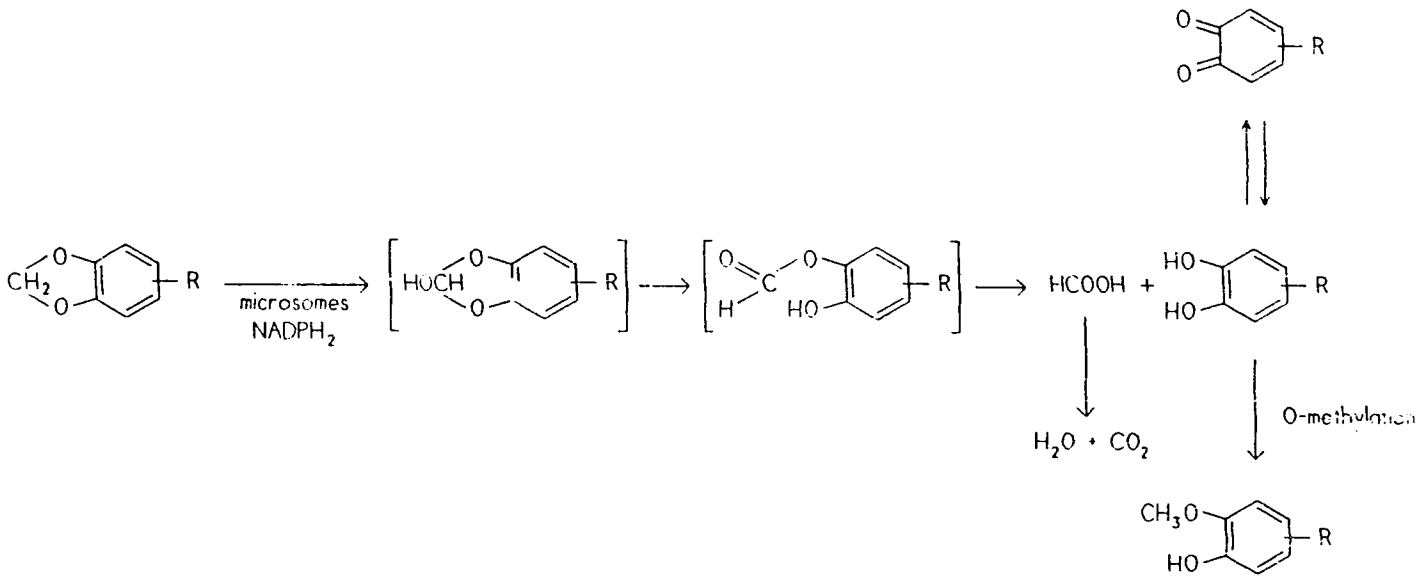


Figure 14: Proposed mechanism involved in the metabolism of the methylenedioxy constitutent of methylenedioxyphenyl compounds. Adapted from Fukuto *et al.* (1991).

identified. Hiramatsu *et al.* (1990) demonstrated that DHMA was the major metabolite of MDMA formed *in vitro* in rat liver microsomes. The reaction was CYP-dependent and displayed enantioselectivity, favoring S-(+)-MDMA. The demethylated catechol derivative, DHA, was not detected when MDMA was incubated, but was identified after incubation of MDA. DHMA was rapidly metabolized to a compound capable of forming a glutathione adduct (a conjugation reaction with glutathione, catalyzed by glutathione-*S*-transferase,usually indicative of an electrophilic center). DHA has also been shown to undergo conjugation to form glutathione and cysteinyl adducts (Patel *et al.*, 1991). The oxidation of DHMA was shown to require NADPH but not the presence of CYP450.

Yousif *et al.* (1990) examined the metabolism of MDMA after a single dose of 40 mg/kg sc in rats. In 24 h urine samples, MDA and 4-hydroxy-3-methoxy-methamphetamine (HMMA) were identified as the major urinary metabolites. Other urinary metabolites identified were HMA, DHA and 3-hydroxy-4-methoxymeth-amphetamine. MDA was also identified in brain tissues 4 h after MDMA (40 mg/kg sc). HMA, HMMA and DHMA have also been identified as metabolites of MDMA in rat brain (Lim and Foltz, 1988). These metabolites are polar and, therefore unlikely to cross the blood-brain barrier, indicating that some metabolic demethylenation can occur in the brain. The levels of HMMA and HMA were much lower than those of MDA, but this could reflect more rapid clearance rather than the relative ease of metabolic reaction (Lim *et al.*, 1993). Ketone metabolites of MDMA derived from deamination have also been identified in rats, including 4-hydroxy-3-

methoxyphenylacetone, piperonylacetone and 3,4-dihydroxyphenylacetone (Lim and Foltz, 1988).

Lim *et al.* (1992) compared mouse and rat urinary excretion of MDMA after administration of 10 mg/kg. The major urinary metabolite in both species was HMA, excreted mainly as the O-glucuronide and/or O-sulphate conjugate. These results differ from the previously discussed *in vitro* study with MDMA and rat liver microsomes which showed that DHMA was the major metabolic product (Hiramatsu *et al.*, 1990). More of the parent compound was excreted by the mouse (72%) than by the rat (35%), which may be an explanation of the observed relative resistance of mice to the development of MDMA-induced long-term deficits in 5-HT parameters (Stone *et al.*, 1987c). Urinary excretion as a % of dose was calculated for MDMA, MDA, HMMA amd HMA in 24 h urine samples after administration of individual enantiomers of MDMA (10 mg/kg) to rats and mice (Lim *et al.*, 1993). In rats the 24 h urines contained more of the R-(-)-enantiomers of the secondary amines (MDMA and HMMA), while the S-(+)-isomers were the predominant stereoisomers of the primary amines (MDA and HMA). In the 24 h urines of mice the same enantioselective pattern was obseved, with the exception that mice excreted comparable amounts of R-(-)- and S-(+)-MDMA.

The specific CYP isozymes involved in the demethylenation of MDMA have been investigated. Tucker *et al.* (1994) used a microsomal preparation of yeast expressing the human CYP2D6 isozyme and human liver microsomes from poor and extensive metabolizers of CYP2D6 substrates. Only DHMA was detected in

the incubation mixture. No products of N-demethylation or aromatic hydroxylation were detected after 5 min incubation using microsomes from any of the human livers. At low concentrations of substrate, the demethylenation of R-(-)-MDMA proceeded more rapidly than the S-(+)-isomer. However, the efficiency of demethylenation of either isomer was very low, as indicated by the relationship between NADPH consumption and product formation. The proportion of catechol derivative formed only accounted for 5-8 % of the NADPH consumed at substrate concentrations of 10 mM or 10 μM. At higher concentrations, enantioselective substrate inhibition was noted, favoring the S-(+)-isomer, consistent with a mechanism in which the substrate formed a catalytically inactive complex. Kumagai *et al.* (1994) demonstrated that DHMA formation from MDMA in rat liver microsomes exhibited multi-enzyme kinetics. A low K_m system was shown to arise from a constitutive enzyme and a high K_m system from a mixture of inducible enzymes. Demethylenation that exhibited the low K_m was not induced by phenobarbital, 3-methylcholanthrene, pregnenolone-16α-carbonitrile or pyrazole, but was inhibited by CYP2D substrates. In contrast, demethylenation exhibiting the high K_m value was induced by phenobarbital and the activity of both untreated and phenobarbital-pretreated microsomes was suppressed by antibodies raised against CYP2B1. The stereoselectivity of R-(-)- versus S-(+)-MDMA varied with differing pretreatments, supporting the participation of different isozymes in the reaction. However, these authors also demonstrated that the experimental conditions (concentration of substrate and time of incubation) can affect the efficiency of the enzymatic reaction

and the specific isozyme that predominated. Studies with antibodies raised against specific CYP isozymes in rabbit liver and lung were conducted to investigate the oxidative metabolism of MDA. MDA demethylenation was not catalyzed or only marginally affected by CYP1A1, CYP1A2, CYP2B4, CYP2C3, CYP2D, CYP2E1, CYP3A6 or CYP4B1 (Kumagai *et al.*, 1991,1992a). MDA competitively inhibited the N-demethylation of erythromycin, a drug known to be N-demethylated by CYP3A4 (Coutts *et al.*, 1994).

Zhao and Castagnoli (1992) proposed an alternative biotransformation pathway of MDMA, involving initial C-2 oxidation of the aromatic ring, with subsequent oxidative cleavage of the methylenedioxy group to generate 2,4,5-trihydroxymethamphetamine (THMA) and the N-dealkyl metabolite, 2,4,5-trihydroxyamphetamine (THA), both of which would be structural analogues 6-OHDA. Lim and Foltz (1991) detected 2-hydroxy-4,5-MDMA and 2-hydroxy-4,5-MDA, but not the trihydroxylated metabolites, THMA and THM in rat brain after administration of MDMA. Kumagai *et al.* (1992b) also demonstrated aromatic hydroxylation of MDMA in rabbit liver microsomes, albeit to a much lower extent than demethylenation to dihydroxy metabolites. The aromatic hydroxylation was inhibited by carbon monoxide and DMI, but was relatively unaffected by phenobarbital pretreatment.

Despite the absence of confirmation of the formation of trihydroxylated metabolites in *in vivo* experiments, the short and longer term neurochemical effects of the dihydroxy- and trihydroxy- metabolites have been investigated. Acute

neurochemical changes after intracerebroventricular injection of THMA or THA included decreased hippocampal TPH activity (THA > THMA). DHA did not alter TPH activity. 5,6-Dihydroxytryptamine (5,6-DHT), but not 6-OHDA, mimicked the effect of THA on TPH activity. TH activity was also decreased by THA administration (Johnson *et al.*, 1992; Elayan *et al.*, 1993). The decreases in TPH and TH activities after intracerebroventricular administration of THA persisted for 5-7 days post-treatment (hippocampus > striatum) (Elayan *et al.*, 1992; Johnson *et al.*, 1992). Decreases in enzyme activities were accompanied by decreases in the levels of hippocampal 5-HT and NE and striatal DA. 2-Hydroxy-4,5-MDMA did not alter enzyme activity, while 2-hydroxy-4,5-MDA increased the activity of both TPH and TH. Zhao *et al.* (1992) showed that systemic, intracerebroventricular, intrastriatal or intracortical administration of 2-hydroxy-4,5-MDMA failed to lead to detectable alterations in the levels of hippocampal or cortical 5-HT or striatal DA. However, intrastriatal or intracortical administration of 2-hydroxy-4,5-MDA produced profound depletions of regional levels of both 5-HT and DA. Systemic administration of HMA or DHA produced only minor effects on the various biogenic amines and their metabolites (Yeh and Hsu, 1987). The relative lack of effect when HMA or DHA were administered systemically might have been influenced by difficulties these metabolites of MDMA would have in crossing the blood-brain barrier. Glennon and Higgs (1992) carried out substitution studies with several metabolites of MDMA in animals trained to discriminate MDMA and saline. Only partial substitution was seen with DHMA, DHA and 3-hydroxy-4-

methoxyamphetamine. Again, the decreased ability of these polar metabolites to enter the CNS might have affected the results.

2.8.3 *Metabolic N-Dealkylation*

Metabolic dealkylation reactions can occur very readily with drugs containing an alkyl group attached to an amine or ring nitrogen, leading to the formation of a primary or secondary amine product. The reaction is considered to occur in two steps, the first being hydroxylation of the α carbon to yield a carbinolamine intermediate, and the second a decomposition of the intermediate, where the alkyl group is lost as the corresponding aldehyde (Figure 15). The hydroxylation reaction has been shown to be mediated by CYP enzyme systems, although the specific isozyme(s) involved remain(s) unclear. In human and animal species, it appears that there are at least eight CYP isozymes capable of catalyzing oxidative N-dealkylation (Coutts *et al.*, 1994) The individual isozymes vary in terms of substrate specificity and relative abundance. There is also evidence that multiple isozymes can be involved in the N-dealkylation of a single drug with differing degrees of efficiency. Species and gender differences also play a role in the relative proportions of individual isozymes present and substrate specificity. In terms of human CYP isozymes, the CYP3A family is the most abundantly expressed in human liver (Gonzalez, 1992; Wrighton and Stevens, 1992). Two members of this family, CYP3A3 and CYP3A4 are known to catalyze metabolic reactions of such typical substrates as nifedipine, erythromycin, benzphetamine, lidocaine and endo-

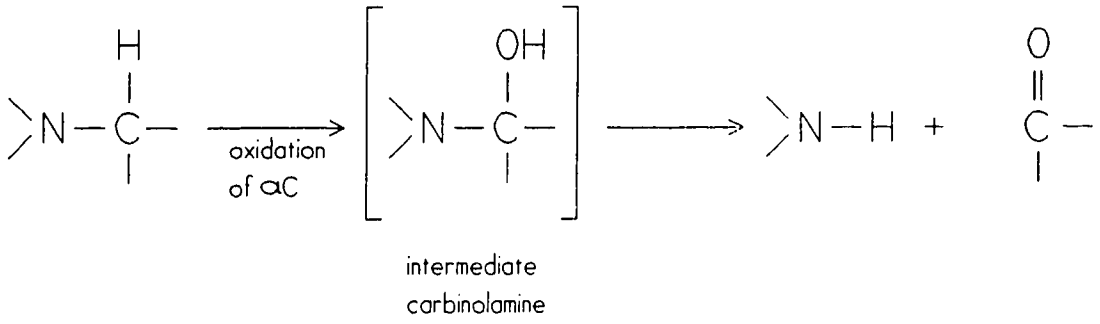


Figure 15: Proposed mechanism involved in N-dealkylation of secondary or tertiary amines. Adapted from Gibson and Skett (1986) pp. 4.

genous steriods (Gonzalez, 1992; Coutts *et al.*, 1994). These two isozymes differ by only 11 amino acids and are indistinguishable by immunohistochemical means and catalytic activity. In rats, genes for *CYP*3A1 and *CYP*3A2 have been identified (Wrighton and Stevens, 1992). CYP2C11 has been shown to play an important role in the N-demethylation of MAMPH (Baba *et al.*, 1988). However, Hiratsuka *et al.* (1995) provided evidence of stereoselective participation of other CYP isozymes in the N-demethylation of MAMPH. The authors pretreated rats with phencyclidine, a known inhibitor of CYP2B4 (Oshawa and Coon, 1989), then prepared liver microsomes to demonstrate that the *in vitro* N-demethylation of MAMPH was inhibited in a stereoselective manner [R-(-)-MAMPH more affected than S-(+)-MAMPH]. The reverse pattern was seen in the N-demethylation of MDMA, with S-(+)-MDMA being more affected than the R-(-) isomer.

The N-dealkylation of MDMA to yield MDA has been well documented in both human subjects and in animals. Vereby *et al.* (1988) studied MDMA disposition in a single subject after ingestion of 50 mg of MDMA. In plasma, MDMA levels peaked at 2 h post administration and were still detectable at 24 h. The calculated $t_{1/2}$ of MDMA in plasma was 7.6 h. Unchanged MDMA was the major urinary excretion product, accounting for 72% of the dose. MDA was detected by 1 h and the levels peaked at 4 h and were no longer detectable by 18 h. At the peak level of MDA, the ratio of MDMA/MDA was about 2.5. Russell *et al.* (1992) detected both MDMA and MDA in a 13 month old child in both serum and urine after accidental ingestion of MDMA.

Fitzgerald *et al.* (1989b) administered 40 mg/kg of *rac*-MDMA to rats and mice and examined plasma levels of the enantiomers of MDMA and MDA 4 h after administration. No stereoselective gender or species differences were found. Levels of S-(+)-MDA were greater than those of R-(-)-MDA, while the parent compound showed less enantiomeric differences. Pharmacokinetic studies with various doses of intravenous (iv) or sc *rac*-MDMA or the individual enantiomers showed that S-(+)-MDMA was cleared more rapidly than R-(-)-MDMA and that the peak concentration of S-(+)-MDA was about three times that of R-(-)-MDA (Cho *et al.*, 1990; Fitzgerald *et al.*, 1990). Cho *et al.* (1990) calculated the $t_{1/2}$ in plasma in rats as 73.8 min for S-(+)-MDMA and 100.7 min for R-(-)-MDMA, much lower than the calculated value of the racemate in humans. MDA has also been identified in rat brain and liver after administration of MDMA (Yousif *et al.*, 1990; Lim *et al.*, 1993).

The N-dealkylation of individual enantiomers of MDMA by rat liver microsomes was examined for gender differences and for effects of pretreatment with phenobarbital (a known inducer of CYP2B isozymes), 3-methycholanthrene (which induces isozymes belonging to the CYP1A and CYP2A families) and the nonselective CYP inhibitor SKF 525-A on the formation of MDA (Gollamudi *et al.*, 1989). The *in vitro* enantioselective formation of S-(+)-MDA was more pronounced in liver microsomes from females than from males. Pretreatment with phenobarbital, but not with 3-methylcholanthrene, increased the N-dealkylation of MDMA, while SKF 525-A inhibited the reaction. SKF 525-A was also shown to

attenuate the 5-HT depleting potency of MDMA. This finding is in contrast to the results of Cho *et al.* (1990) who showed that pretreatment with phenobarbital decreased MDA formation from MDMA, while SKF 525-A had little effect. Battaglia *et al.* (1990) also found that pretreatment with SKF 525-A had no effect on the 5-HT deficits produced by repeated administration of MDMA.

The degree of N-dealkylation of MDMA observed appears to vary with the tissue examined, species, gender and the experimental method chosen, leading to conflicting reports that MDA is the major metabolite of MDMA (Lim and Foltz, 1988; Vereby *et al.*, 1988; Fitzgerald *et al.*, 1989b; Gollamudi *et al.*, 1989) or that MDA is only a minor metabolite with demethylenation being the major route of biotransformation (Brady et al., 1986; Cho *et al.*, 1990; Maurer *et al.*, 1993). Irrespective of the relative abundances of the various metabolites of MDMA, it is clear that the biotransformation of MDMA results in multiple biologically active intermediates and metabolites. The complex interaction among the various compounds could profoundly influence the overall behavioral and neurochemical effects of MDMA and the time course of biological activity.

2.8.4 *Other Metabolic Paths*

MDA has been shown to undergo other metabolic reactions. Nakagawa *et al.* (1993) detected a novel metabolite of MDA, 3-methyl-6,7-methylenedioxy-1,2,3,4-tetrahydroisoquinoline, in rat brain and urine. The formation of this metabolite displayed enantioselectivity, favoring the R-(-)-isomer. In an open field

test, 3-methyl-6,7-methylenedioxy-1,2,3,4-tetrahydroisoquinoline decreased both ambulation and rearing, in contrast to MDA, which markedly increased ambulation. The formation of this metabolite is of interest for several reasons. Endogenous tetrahydroisoquinoline derivatives, assumed to be produced by the cyclization of β-phenylethylamine, have been implicated to play a role in Parkinson's disease (Tasaki *et al.*, 1991). R-(-)-1,2-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline is a specific DA neurotoxin (Takahashi *et al.*, 1994). Selective inhibition of MAO-A by catechol isoquinolinium ions has been demonstrated (Naoi *et al.*, 1995).

Although N-hydroxy-MDA has been reported as a metabolite of MDA in *in vitro* studies with rabbit liver preparations (Fukuto et al., 1991; Kumagai *et al.*, 1992a), Ravis *et al.* (1994) demonstrated the rapid conversion of N-hydroxy-MDA to MDA in rats after administration of N-hydroxy-MDA. Thus, it is difficult to assess the contribution that N-hydroxy-MDA might make in the overall biological activity of MDA and the importance of N-hydroxylation in the biotransformation of MDA.

Ensslin *et al.* (1990) examined the metabolites of MDE in human urine and identified three metabolic paths: N-dealkylation, oxidative deamination followed by oxidative reduction to yield benzoic acid derivatives and O-demethylenation (Figure 16). The metabolic paths are similar to those seen in animal studies with MDMA and MDA. However, no quantitative or stereoselectivity data were reported.

2.9 **Toxicity**

The greatest controversy surrounding the methylenedioxy analogues of

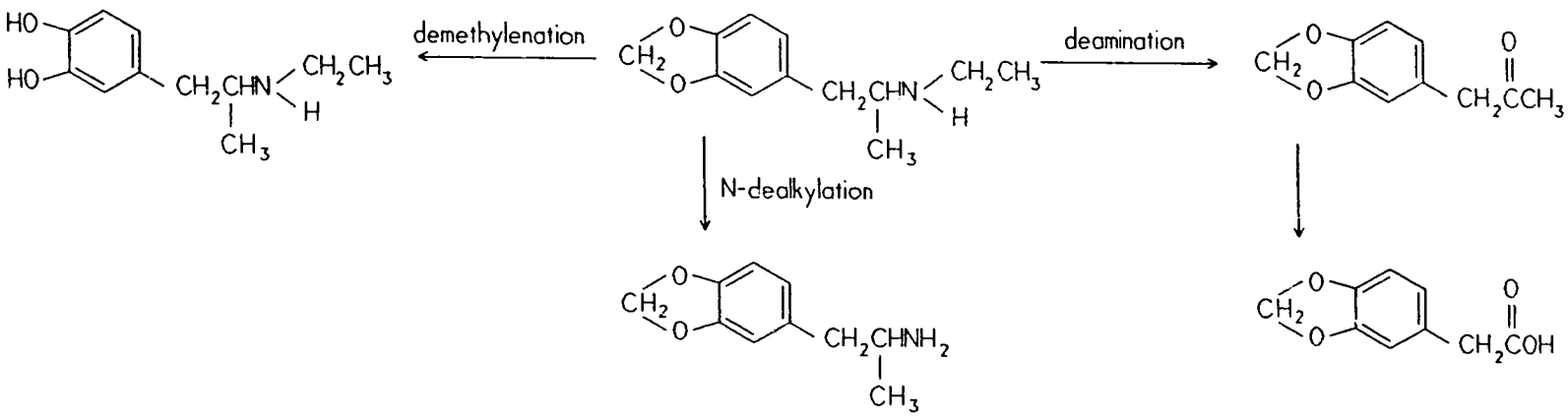


Figure 16: Proposed metabolic pathways for MDE.

AMPH is whether these compounds are neurotoxic to humans. There are numerous case reports related to generalized toxicity leading to death in humans. Lethality of these compounds has been studied in a number of animal species. Neurochemical changes and morphological changes in 5-HT neurons indicative of neurotoxicity have been well documented in animal species. However, there remains considerable disagreement whether the data from animal models are applicable to human recreational users of MDA, MDMA and MDE. Controversy also remains regarding the functional consequences of the neurotoxic changes and the mechanisms involved. A closer examination of the literature reveals some of the difficulties in assessing the neurotoxic potential of these drugs to humans.

2.9.1 *In Humans*

As indicated earlier, there have been no clinical data suggesting that the recreational use of the methylenedioxy analogues produces long term neurological sequelae. Grob *et al.* (1990) suggested that the fears of MDMA neurotoxicity may have been exaggerated as the doses used in animal models to show neuronal damage were cumulatively up to 100 times the usual human dose. These authors used FEN as an example of a clinically useful drug that has been prescribed for the last 25 years without any evidence of neurotoxicological effects, yet has been shown to be 3 times more potent than MDMA at producing long term 5-HT deficits in laboratory animals. FEN has been efficacious as an anorexiant and more recently in the treatment of infantile autism, a disorder where a significant number

of patients display hyperserotonemia, and attention deficit disorder with hyperactivity (Ritvo *et al.*, 1983; Donnelly *et al.*, 1989; Robinson *et al.*, 1988).

Despite this lack of neurological sequelae, the recreational use of methylenedioxy analogues of AMPH is not without serious risk. In addition to the risks inherent in the use of recreational drugs in general, the methylenedioxy analogues of AMPH have been implicated in the production of a number of psychiatric symptoms. As previously discussed, psychotic symptoms, generalized anxiety and depression and panic disorder have been reported. More serious and life-threatening, though, are the acute toxic effects that can occur with these drugs, taken either in high doses and/or in combination with physical exertion which exacerbates the drugs' effects.

In the early 1970s, reports of fatalities associated with the ingestion of MDA began appearing. Reed *et al.* (1972) reported *postmortem* findings of marked visceral congestion and edema and petechial hemorrhages on the surface of the heart following ingestion of MDA. Toxicology revealed levels of MDA in brain and blood of 1 mg/100 ml, suggestive of an overdose. Simpson and Rumack (1981) reported on a patient treated in a hospital emergency department after ingestion of MDA. The patient presented as unresponsive, with increased heart rate, hyperthermia, generalized rigidity, dilated pupils and hyperreflexia. By 24 h there was deepened unresponsiveness, increased pulmonary distress, decreased blood pressure and continued hyperthermia resistant to any cooling techniques used. Prior to death, there was evidence of rhabdomylitis and disseminating intravascular

coagulopathy (DIC). Autopsy revealed diffuse pulmonary and cerebral edema. These authors compared the death with nine deaths reported after ingestion of PMA, where all the patients presented with similar symptoms as was seen with MDA and also died without their conditions ever stabilizing (Cimbura, 1974).

There are many case reports of similar non-fatal clinical courses and fatalities related to MDMA and MDE (Brown and Osterlach, 1987; Campkin and Davis, 1992; Henry, 1992; Russell *et al.*, 1992; Barrett and Taylor, 1993; Tehan, 1993). Dowling (1990) in his review of *post mortem* reports from July 1985 to March 1986 found 16 cases where MDMA or MDE were the underlying cause of death or were felt to have contributed to death. The dose ingested does not appear to correlate well with the severity of symptoms and the clinical course. One fatality had a serum MDMA level of 1.26 mg/L, while another patient, whose serum level was 7.0 mg/L, received supportive treatments only and survived (Brown and Osterloh, 1987; Campkin and Davis, 1992). Barrett and Taylor (1993) reported on a patient who experienced severe rhabdomylitis and DIC, yet blood levels of MDMA were only 0.2 mg/L. Marsh *et al.* (1994) reported 2 cases of non-fatal aplastic anemia following exposure to MDMA, both of which resolved spontaneously 7-9 wks after presentation.

The recommended treatments for toxic reactions related to the ingestion of MDA, MDMA and MDE are generally supportive, with ventilation assistance, cooling measures, anticonvulsants and fluid replacements. However, there remains controversy over the use of dantrolene, a drug used in the management of malignant hyperthermia (Barrett and Taylor, 1993; Tehan, 1993). Watson et al.

(1993) reviewed 6 case histories of patients admitted after ingestion of MDA or MDMA and found that dantrolene did not affect the clinical outcome. Other pharmacological agents that have been used included naloxone, chlorpromazine, haloperidol and chlormethiazole.

It is these types of toxic reactions to the use of the methylenedioxy analogues of AMPH that have prompted various government agencies and clinicians to support the continued placement of these compounds on the Schedule 1 list of the U.S. Food and Drug Act, irrespective of whether they are also neurotoxic. In a similar fashion to Parkinson's disease, where at least 80% of DA-containing neurons are destroyed prior to the emergence of clinical symptomatology, Ricuarte *et al.* (1994) speculated that these drugs produce 5-HT neuronal death which might not become clinically evident until much later in life.

2.9.2 *In Animals*

2.9.2.1 Lethality Studies

As stated earlier, the U.S. Army contracted studies to assess the lethality of a series of AMPH analogues in the 1950s, but it was 20 years later before the results were published by Hardman *et al.* (1973). Of the compounds tested, MDA was the most potent, especially in dogs and monkeys, with a LD_{50} of 6-7 mg/kg (iv). In the rat, the LD_{50} for MDA after ip administration was 27 mg/kg, compared with 49 mg/kg of MDMA and 95 mg/kg for MBDB. Simpson and Rumack (1981) reported LD_{50} values for mice, comparing MDA, AMPH and PMA by various routes of

administration. All three compounds were equipotent when administered iv or ip, but MDA was the most potent orally administered compound. Davis *et al.* (1986) found no significant differences between the LD_{50} values of MDA and MDMA after ip doses in mice. In support of placing MDMA on the Schedule 1 list, these authors pointed out that the LD_{50} for MDMA was only 10 times the effective dose, while in comparison, for LSD the LD_{50} was 100 times the effective dose.

2.9.2.2 Neurotoxicity Studies

In the past, regulation of neurotoxins was based on the setting of allowable doses or exposures by calculating the maximum dose with no observable adverse effects and dividing by a number that took into account uncertainty factors, arising from intra- and inter-species variability between animals and humans. In general, 10 was used to allow for higher sensitivity in humans and another 10 used to allow for variability of sensitivity among humans. Thus, the maximum dose with no adverse effects was divided by 100 (Gaylor and Slikker, 1990). More recently, other schemas to assess neurotoxic risk have been suggested. Gaylor and Slikker (1990) proposed using bioassay data to estimate the risk of neurotoxic effects as a function of dose. They used MDMA as an example and found the dose required to produce an abnormal response was dependent on the endpoint or biological marker selected and the species studied. Kleven and Seiden (1992) summarized the four criteria that have been most widely used to establish neurotoxicity of AMPH analogues: (a) long lasting depletion of 5-HT or DA; (b) a decrease in high affinity uptake sites; (c)

decreased activity of synthetic enzymes; and (d) alterations in neuronal morphology. All four of these criteria have been studied in relation to the methylenedioxy analogues of AMPH and yet many questions remain.

Schmidt (1987a) was one of the first researchers to publish data on the first two criteria. He demonstrated that a single dose of MDMA (10 mg/kg) in rats produced a biphasic effect, with acute depletion of 5-HT, most prominent at 3-6 h after administration and recovery seen by 24 h, followed by another phase of 5-HT depletion seen 1 wk after drug treatment. The second phase of 5-HT depletion was accompanied by a decreased number of 5-HT uptake sites. Both enantiomers of MDMA were capable of inducing the acute depletion, with little dose dependency observed. However, only S-(+)-MDMA produced the second phase of 5-HT depletion and the reduction of number of 5-HT uptake sites seen 1 wk later. The hippocampus and the frontal cortex were the most sensitive regions to the depleting effects of MDMA (Schmidt *et al.*, 1987). Since then, many other workers in this field have replicated these findings, using differing doses and dosing schedules to highlight the importance of dose, route and frequency of administration to the neurotoxic potential of MDA, MDMA and MDE (Commins *et al.*, 1987a,b; Battaglia *et al.*, 1988b; Logan *et al.*, 1988; Ricuarte *et al.*, 1988; Kleven *et al.*, 1989; Colado and Green, 1994). The degree of recovery and the time required for recovery to occur after administration of these drugs also appear to be dependent on dose and frequency of administration. In addition, recovery depends on the specific biological marker examined, the species and the methylenedioxy analogue. For example,

Battaglia *et al.* (1988b) found full recovery of the number of cortical 5-HT uptake sites in rats by 12 months, following an initial 90% loss of the sites after MDMA (20 mg/kg twice daily for 4 days). Ricuarte *et al.* (1993) reported partial recovery of the levels of 5-HT in rats 5 months after a drug treatment regime of 20 mg/kg twice daily for 4 days, then repeated 1 week later. In contrast, monkeys examined 14 weeks after multiple doses of MDMA continued to exhibit profound loss of 5-HT uptake sites and decreased levels of 5-HT and 5-HIAA in cortex, hippocampus and striatum but not in hypothalamus and spinal cord (Insel *et al.*, 1989). MDE has been shown to produce similar acute effects as MDA and MDMA but the second phase of depletion and the loss of 5-HT uptake sites were either significantly attenuated or not apparent (Johnson *et al.*, 1987; Schmidt, 1987b; Stone *et al.*, 1987b).

The third criterion, loss of activity of synthetic enzymes, was discussed earlier. Dose-dependent long term decreases in TPH activity are seen after administration of MDA or MDMA (Gibb *et al.*, 1987; Schmidt and Taylor, 1987; Stone *et al.*, 1987b; Johnson *et al.*, 1989a).

Using a number of different experimental methodologies, all three methylenedioxy analogues of AMPH have been shown to be capable of producing morphological changes to 5-HT neurons. However, the data were obtained after using vastly larger doses than behaviorally effective doses and often even larger doses than required to cause profound neurochemical deficits. O'Hearn *et al.* (1986) used 5-HT immunocytochemistry to visualize 5-HT neurons and their axonal projections in rats 2 weeks after treatment with either MDA or MDMA (20 mg/kg sc

twice daily for 4 days). They demonstrated profound loss of 5-HT axons throughout the forebrain, especially in the neocortex, striatum and the thalamus. The loss of 5-HT axon terminals roughly paralleled the reductions in levels of 5-HT and uptake sites (Battaglia *et al.*, 1987). The loss of fine 5-HT axon terminals was selective, as there was intact staining of raphé cell bodies and of the straighter thicker axons in deep layers of the cortex and medial forebrain bundle (O'Hearn *et al.*, 1988). The spared 5-HT axons had large round varicosities and were shown to arise from the median raphé (MR) nucleus. Harvey *et al.*(1993) also used immunocytochemistry to show profound selective loss of fine 5-HT fibers after multiple high doses of MDA. Scallet *et al.* (1988) combined immunohistochemistry and degeneration-specific methods to demonstrate that MDMA produced morphological changes to 5-HT fibers.

The cell bodies of most 5-HT neurons are found within the boundaries of the raphé nuclei of the brain stem. The raphé nuclei can be divided into morphologically heterogeneous cell clusters (Tork, 1990; Axt *et al.*, 1994). The DR nuclei contain the largest number of 5-HT cells, with more than twice as many neurons as the next biggest cluster, the MR nuclei. The ascending 5-HT projections arising from these two nuclei form a dual projection system. The first system consists of numerous thin axons with small varicosities that arise from the DR nuclei and branch profusely over large areas of the forebrain. It is not believed that these fine fibers make discrete synaptic connections. However, they could function in a more neuromodulatory role, interacting with 5-HT receptors on non-5-HT neurons.

For example, stimulation of the DR nucleus produces predominantly inhibition of spontaneous activity of single neurones, serving to tonically inhibit DA neurones (Dray *et al.*, 1978). The second system ascending from the MR nuclei with thick non-varicose axons gives rise to branches with the characteristic beaded varicose axons. These large terminal areas make well defined synapses with the soma and dendrites of target cells. It remains unclear why the morphological changes induced by MDA, MDMA and MDE are selective for the fine 5-HT axons ascending from the DR nuclei. The same selectivity was seen by light and electron microscope in rat spinal cord after PCA treatment, where there was marked and selective elimination of the fine 5-HT immunoreactive fibers of the dorsal horn, but sparing of the other large axons (Ridet *et al.*, 1994). Molliver *et al.* (1990) speculated that perhaps there are distinct uptake carriers for 5-HT between the two projection systems, or the densities of uptake sites may differ such as to create differences in volume and dilution within the fibers. Intracellular mechanisms available to inactivate a potential toxin may also differ between the two types of axons. Other possibilities include differences in metabolic activity, differing receptor affinities and reported drug resistance of certain 5-HT projections which make connections with discrete targets (Axt *et al.*, 1994).

Molliver *et al.* (1990) examined the time dependency of the degenerative changes and found that 24-38 h after drug treatment, both rats and monkeys treated with MDA exhibited swollen, distorted and fragmented cortical axon terminals as well as swollen proximal axon stumps. This positive evidence for

degeneration was supported by the accumulation of 5-HT in swollen preterminal fibers, seen as intense immunoreactivity at 2 weeks after drug treatment and by subsequent disappearance of damaged fibers and the loss of fine axon terminals. These authors proposed four phases of neurotoxicity produced by such AMPH analogues as PCA, FEN, MDA and MDMA: (a) phase 1 involving transmitter release and depletion, which is immediate and reversible, (b) phase 2 involving irreversible axon degeneration seen 24-48 h after drug administration, (c) phase 3 involving regional denervation, which can persist for weeks or months and, finally, (d) phase 4 where there is a slowly progressive regeneration over a year.

The mechanisms involved in the neurotoxicity of the methylenedioxy analogues of AMPH appear complex and interrelated. It has been suggested that the mechanisms involved in the neurotoxicity of the well known neurotoxin PCA and methylenedioxy analogues of AMPH are very similar (Berger *et al.*, 1992; McKenna and Peroutka, 1991,1992). Some studies have used PCA and generalized the mechanisms to include MDMA and MDA. Functional 5-HT and DA systems appear to be basic requirements for both the acute and long term effects of these drugs, as removal of either of these systems by pharmacological or lesioning methods prevent drug-induced neurotoxicity (Sprague *et al.*, 1994). Beyond functional integrity however, the complexity of the myriad of interactions among the various direct drug effects on neuronal components and among the indirect effects from the release of 5-HT makes it very difficult to assess the relative contribution of single processes and discrete drug effects to the observed neurotoxic changes.

The proposal that either endogenous metabolites of DA or drug metabolites play a role in the neurotoxicity of the methylenedioxy analogues of AMPH has been evaluated. McKenna *et al.* (1991) provided evidence that the most potent 5-HT neurotoxins *in vivo* show the greatest potency as releasers of DA rather than 5-HT. Once released, DA can be taken up into 5-HT neurons where its metabolism by MAO-B could result in the formation of free radicals and peroxide which are potentially cytotoxic (Nash *et al.*, 1990). Sprague and Nichols (1995) showed that inhibitors of MAO-B [(-)-deprenyl and MDL 72974] prevented the loss of striatal 5-HT uptake sites when given 30 min before an acute dose of MDMA (40 mg/kg sc). However, the methylenedioxy analogues of AMPH block the reuptake of 5-HT and presumably would block DA uptake as well. If MAO-B was involved in the production of potentially neurotoxic oxidation products of DA metabolism, a selective MAO-B inhibitor should attenuate the neurotoxic effects. Benmansour and Brunswick (1994) showed that (-)-deprenyl potentiated the neurotoxicity induced by PCA. Further support for the involvement of some type of oxygen-based radicals in the neurotoxicity of MDA and MDMA comes from data showing that transgenic mice that carry the complete sequence of the human copper-zinc superoxide dismutase (CuZnSOD) gene were resistant to the lethal effects of both drugs (Cadet *et al.*, 1994). Hirata *et al.* (1995) also used transgenic mice to demonstrate that CuZnSOD-Tg mice which have high levels of CuZnSOD in the brain are protected against the neurotoxic effects of MAMPH on the striatal 5-HT system. CuZnSOD dismutates the superoxide anion (O_2^-) and thus forms the first line of defense

against oxygen-based radicals. The data from both the MDA and MDMA reports and the results with MAMPH suggest that superoxide radical formation could be a mechanism common to the three drugs. 5,6-DHT has been detected after administration of MAMPH (Commins *et al.*, 1987b). In addition, Wrona *et al.* (1995) established that 5,6-DHT was a product of the *in vitro* hydroxyl radical-mediated oxidation of 5-HT and suggested that this reaction might be involved in the neurotoxicity of MAMPH.

Demethylenation of the methylenedioxy analogues also produces a number of catechol derivatives and reactive quinones. Del Rio *et al.* (1994) demonstrated that Fe^{2+} enhances the oxidation of both 5-HT and DA in the presence of dissolved O_2 and that the oxidation products bind covalently to the cysteine residues on the 5-HT-binding protein, a cytosolic protein involved in the intraneuronal transport of 5-HT, in the sequestering of 5-HT into vesicles and in protecting 5-HT from catabolism by MAO-A. The formation of covalent bonds could represent a potential mechanism by which DA and the catechol derivatives arising from demethylenation could act as neurotoxins. With MDMA and MDE, N-dealkylation would produce MDA, another substrate for potentially neurotoxic demethylenation and an active metabolite with higher potency to produce neurochemical changes than either parent compound. Support for a metabolic intermediate or metabolite mediating at least some of the neurotoxic effects comes from the lack of neurochemical or immunohistochemical changes after direct intraraphé or intracerebral microinjections of MDMA (Battaglia *et al.*, 1990; Paris and Cunningham, 1990). A

relationship between the increased excretion of the parent compound in mice compared to rats and the relative resistance of mice to the long term 5-HT deficits would support a role of a metabolite or metabolic intermediate. However, in the single human subject, unmetabolized MDMA was also the main urinary excretion product (Vereby *et al.*, 1988).

O'Callaghan and Miller (1994) used astrogliosis, measured by glial fibrillary acidic protein, as an index of drug-induced neural damage and silver degeneration stain as direct evidence of neuronal damage to demonstrate neurotoxic changes to DA projections in the striatum and the cortex of C57BL/6J mice after administration of S-(+)-MDA and S-(+)-MDMA. The increase in astrogliosis and silver staining was blocked by the NMDA receptor antagonist MK 801, which the authors attributed to MK 801's ability to block the hyperthermia induced by S-(+)-MDA and S-(+)-MDMA. In a companion paper, Miller and O'Callaghan (1994) showed that lowering the ambient temperature from 22°C to 15°C also blocked the neurotoxic changes to the DA projections induced by S-(+)-MDA and S-(+)-MDMA. Coadministration of MK801 also prevented MDMA-induced hyperthermia if the core temperature was maintained below 38.4°C. Coadministration of CGS 19755, a competitive NMDA receptor antagonist, was also able to reduce the temperature increases produced by MDMA, but the α-amino-3-hydroxy-5-methyl-4-isoxazol propionic acid (AMPA) receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX) had no effect (Farfel and Seiden, 1995). Gordon and Fagelson (1994) supported a role for ambient temperature in the hyperthermic response to MDMA, showing that

acrylic cages exacerbated the hyperthermic response. Gordon *et al.* (1991) evaluated the effects 1 h after high dose MDMA on metabolic rate, evaporative water loss, motor activity and colonic temperature at varying ambient temperatures. MDMA produced temperature-dependent increases in metabolic rate and evaporative water loss. Motor activity was increased but did not vary with the ambient temperature. Colonic temperature was increased only when the ambient temperature was 30°C, a temperature where there was a 100% mortality rate. When the dose of MDMA was lowered to 20 mg/kg and the animals' heart rates and body core temperatures were monitored by radiotelemetry in acrylic cages at 25°C, MDMA caused rapid elevation in heart rate and core temperature. The mortality rate was greater than 60% in the animals having core temperatures of more than 42°C (control temperature was similar to human). An important observation from these studies was that the MDMA-treated rats in a hyperthermic state failed to increase blood flow to the tail, a common thermoregulatory response in the rat when body temperature is increased *via* exercise, ambient heat stress or through pharmacological manipulation. If normal heat-dissipating mechanisms are functionally impaired in an analogous manner in human subjects, it could account for the resistance of hyperthermia to supportive cooling measures in the human fatalities related to ingestion of methylenedioxy analogues of AMPH.

Simply controlling the temperature increase induced by neurotoxins does not always protect against the neurotoxic changes. MK 801 lowered the core temperature in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice

but did not prevent MPTP-induced neurotoxicity. Cocaine blocked S-(+)-MDMA-induced hyperthermia, but did not cause hypothermia itself and did not offer any protection against the neurotoxic changes induced by S-(+)-MDMA (O'Callaghan and Miller, 1994). CGS 19755 prevented MDMA-induced hyperthermia but provided only partial protection against the 5-HT depletion produced by MDMA (Farfel and Seiden, 1995). It is known that 5-HT_{1A} receptor agonists produce hypothermia (Glennon, 1990; Leonard, 1994). In contrast, the methylenedioxy analogues of AMPH which display relatively high agonist affinity for 5-HT_{1A} receptors produce the opposite effect on temperature (Battaglia *et al.*, 1990). FEN has been shown to produce selective 5-HT toxicity in a similar manner to MDMA and MDA, yet it produces hypothermia in C57BL/6J mice (O'Callaghan and Miller, 1994). Mazzola-Pomietto *et al.* (1995) demonstrated that the hyperthermia produced by the AMPH analogue 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane was mediated by stimulation of 5-HT_2 receptors. Bross and Hoffer (1995) showed that the 5-HT uptake inhibitor FLU also increased basal body temperature in human subjects. Thus, the relationship between hyperthermia and neurotoxicity induced by S-(+)-MDA and S-(+)-MDMA is complex. More information about the interaction between various NT systems and thermoregulation is required before effects of specific drugs can be well understood. This line of research is important in light of the continued recreational use of MDA, MDMA and MDE coupled with heavy physical exertion which produces life threatening hyperthermia and other severe toxic effects.

Neuroprotective agents such as chlormethiazole and dizocilipine prevented the decreases in 5-HT parameters produced by MDMA in rats, but had little effect on 5-HT neurotoxicity induced by PCA and FEN (Colado *et al.*, 1993). Chlormethiazole attenuated both MDMA- and PCA-induced hyperthermia, providing further evidence that hyperthermia is not directly related to neurotoxicity. It is interesting to note that chormethiazole can also antagonize the acute effects of MDMA. This drug was used in the successful treatment of accidental ingestion of MDMA by a 13 month old child, with dramatic cessation of convulsions and tachyarrythmias within 5-10 min after iv administration (Russell *et al.*, 1992). Other compounds known to affect 5-HT systems or to be protective have been tested: γ-butyrolactone and pentobarbital attenuated the decreases in the levels of 5-HT and 5-HIAA but ondansetron had no effect (Colado and Green, 1994). Iyler *et al.* (1994) used *in vivo* microdialysis to show that Trp pretreatment augmented PCA-induced increases in extracellular 5-HT and DA but attenuated the PCA-induced decreases in 5-HT in prefrontal cortex and striatum when examined 1 wk post drug administration. If the degree of neurotoxicity was related to the degree of release, Trp should have increased PCA-induced neurotoxicity. An alternate explanation is that Trp changes the 5-HT/DA ratio in favor of 5-HT in terms of competition for carrier mediated entry into 5-HT terminals. Less DA entering the terminal could mean lower concentrations of potentially cytotoxic oxidation products of DA catabolism.

Whitaker-Azmitia and Azmitia (1990) used a hippocampal fetal culture of 5-

HT neurons to demonstrate that S-(+)-MDMA was about 10 times more potent in producing toxicity than was the R-(-)-isomer. S-(+)-MDMA was also about 10 times as potent in causing the release of 5-HT, which these authors suggested at least partially underlies the toxicity. Support that 5-HT release is involved in the toxicity comes from data showing that the α_2 agonist BHT 920 inhibited the toxicity induced by either isomer of MDMA. Activation of alpha$_2$ adrenergic heteroreceptors on 5-HT neurons are known to cause inhibition of the release of 5-HT (Raiteri *et al.*, 1983; Blier and de Montigny, 1994). In the tissue culture model, toxicity was expressed as degeneration of 5-HT cell bodies, but there is a significant body of evidence that the methylenedioxy analogues of AMPH cause selective degeneration of 5-HT fibers, while leaving the 5-HT cell bodies intact. This difference could arise from an increased frailty of fetal cells. The direct effects of MDMA seen in tissue culture support that, while metabolic intermediates or metabolites may also be involved in the neurotoxic effects, MDMA itself is capable of producing 5-HT neuronal degeneration.

A number of researchers have examined animal behavior after administration of doses known to produce marked 5-HT depletion for evidence of behavioral sequelae. Robinson *et al.* (1993) evaluated rats at behavioral tasks of spatial navigation, skilled reaching and foraging after MDMA (10 mg/kg twice daily for 4 days). No differences between control and MDMA-treated rats were found in any of the tasks. Ricuarte *et al.* (1993) failed to find any deficits in memory in rats assessed in three variations of spatial alteration in the T maze 4 weeks after a

dosing regime of MDMA 20 mg/kg twice daily for 4 days then repeated 1 week later. St Omer *et al.* (1991) examined both the mother and rat pups after prenatal exposure to multiple doses of MDMA. Gestational duration, litter size and neonatal birth weight were not affected by MDMA. Maturational parameters, surface righting reflexes, swimming performance, forelimb grip strength and passive avoidance behaviors in the rat pups were unaffected by MDMA. While MDMA produced the characteristic decreases in the levels of 5-HT and 5-HIAA in the mothers at 27 days *post partum*, the levels of 5-HT and 5-HIAA and the density of 5-HT uptake sites in the offspring at 27 days *post natal* were the same as control animals. Bronson *et al.* (1994a) injected S-(+)-AMPH or MDMA (8,16 or 32 mg/kg egg weight) into the chorioallantois of chick eggs. Both drugs decreased motility on day 14 of embryogenesis. One day after hatching, the chicks were challenged with a high dose of the same drug they had received *in ova*. Both drugs [S-(+)-AMPH > MDMA] produced distress vocalizations, wing extension, tremor, flat body posture, loss of righting reflex and convulsant-like kicking, suggesting that chicks might be more sensitive to the behavioral effects of MDMA than rat pups. MDMA given *in ova* had no effect on body, brain or liver weight of the hatched chicks (Bronson *et al.*, 1994b).

Ricuarte *et al.* (1994) cautioned against reaching any conclusion that the depletion of 5-HT and DA by the various neurotoxins, including MDA and MDMA, was of little behavioral significance. They cite several factors that could impact on the lack of behavioral changes in response to the administration of these drugs: (a)

the severity of depletion may be insufficiently large to produce behavioral changes; (b) compensatory changes in the injured neuronal systems or in other systems may negate the impact on behavior; (c) regeneration of damaged axons and axon terminals may come into play, depending on the timing of the behavioral testing; (d) the method used to study behavior in animals may not be sensitive enough to detect subtle behavioral deficits; and (e) the development of functional consequences could be species-dependent.

Further research is critical in light of the continued recreational use of the methylenedioxy analogues of AMPH. More is known about the direct pharmacological effects of these drugs on some of the neuronal systems, but there seems a large gap in our knowledge about how the various pharmacological effects interacting with each other and among different neuronal systems come together to produce the characteristic entactogen-like responses in human users and the potential for 5-HT neurotoxicity.

2.10 **Objectives of My Research and Rationale**

A. To develop a gas chromatographic assay to simultaneously resolve the enantiomers of MDMA and MDA isolated from rat brain and liver tissues after administration of *rac*-MDMA. Any stereoselectivity observed in brain and liver tissues will be compared to that seen in blood and urine reported in the literature.

B. Using a modified gas chromatographic assay, to examine the degree of stereoselective metabolic N-dealkylation in rats after administration of *rac*-MDE compared with *rac*-MDMA. No quantitative nor stereochemical data was found in the literature regarding the levels of MDE in any tissue nor information regarding the degree of N-dealkyation *in vivo* after administration of *rac*-MDE. If indeed MDA formation is involved in the neurotoxicity observed with MDMA, perhaps the decreased ability of MDE to produce neurotoxic changes is related to differences in the degree of N-dealkylation.

C. To evaluate the ability of two human CYP 450 isozymes to catalyze metabolic reactions with known substrates and MDMA and MDE in an *in vitro* incubation system by using purified individual isozymes expressed in a human AHH-1 TK +/- cell line. These experiments will provide further information about the capacity of these isozymes to catalyze metabolic reactions involving nitrogen in a number of substrates with very different chemical structures and also about the relative ease of N-demethylation compared to N-deethylation under the same conditions.

D. To compare the abilities of the racemates and the individual enantiomers of MDE and MDMA to alter the *in vitro* uptake of radioisotope labelled DA, NE and 5-HT in rat brain tissue preparations. Although IC_{50} values for the individual enantiomers of MDMA have been reported in the literature, experiments examining the racemate and the individual enantiomers of MDMA were conducted to allow for direct comparisons with MDE.

E. Use the computer-assisted methodology developed by Martin-Iverson (1991) to study the behavioral effects of MDA, MDMA and MDE compared with a psychomotor stimulant and an hallucinogen. Two interrelated goals for the behavioral study were set out: (a) to determine if the computer-assisted methodology originally developed to examine the effects of chronic stimulants on behavior in rats could be used to distinguish the putative entactogens and an hallucinogen from saline-treated animals; and (b) to determine if MDMA, MDA and MDE as a group could be distinguished from the stimulant and the hallucinogen.

F. Utilize high performance liquid chromatography to compare the levels of the DA, NE and 5-HT and the acid metabolites DOPAC, HVA and 5-HIAA amongst the treatment groups from the behavioral study. Comparisons among the five drugs in terms of the neurochemical changes seen 3 h after acute administration would provide further discriminative data from which to classify the drugs. The use of equimolar doses of each drug will provide valuable data regarding the drugs' relative efficacies at producing lasting effects on NT amine systems.

G. Whole brain drug levels in the animals from the behavioral study will be determined using gas chromatographic procedures. Levels of the individual drugs will be compared in terms of known relative durations of action. Examination of drug levels will help to emphasize that the kinetic properties of individual drugs and metabolism are important factors that require consideration to fully characterize the behavioral and neurochemical effects of specific drugs.

3 *MATERIALS AND METHODS*

3.1 Chemicals

Chemicals	*Suppliers or Manufacturers*
acetic anhydride	Sigma (St. Louis, MO)
acetonitrile	British Drug Houses [BDH] (Toronto, ONT)
air, dry for GC-NPD	Linde, Union Carbide
S-(+)-amphetamine	SmithKline Beecham Pharma
L-ascorbic acid	Fisher Scientific (Edmonton, ALTA)
calcium chloride	Fisher Scientific
control microsomes	Gentest (Woburn, MASS)
p-chlorophenylethylamine·HCl	Sigma, HCl salt prepared by Dr. RT Coutts Faculty of Pharmacy and Pharmaceutical Sciences, Edmonton, ALTA
cytochrome P4501A1	Gentest
cytochrome P4503A3/4 with reductase	Gentest
cytochrome P4502D6	Gentest
desmethylimipramine·HCl	Sigma
dextrose	Fisher
3,4-dihydroxyphenylacetic acid	Sigma
dopamine	Sigma
[2,5,6-^{3}H]-dopamine	Amersham (Oakville, ONT)
ethyl acetate	BDH

ethylenediamine tetraacetate, disodium salt	Fisher Scientific
rac-fluoxetine·HCl	Lilly Research Laboratories (Indianapolis, IN)
D-glucose-6-phosphate	Sigma
D-glucose-6-dehydrogenase	Sigma
helium	Linde, Union Carbide
heptafluoro-L-prolyl chloride	synthesized by Drs. J. Hubbard and G. MacKay, College of Pharmacy, University of Saskatchewan, Saskatoon, Canada.
homovanillic acid	Sigma
hydrochloric acid, 37-38%	Fisher Scientific
hydrogen	Linde, Union Carbide
5-hydroxyindole-3-acetic acid	Sigma
(hydroxymethyl)aminomethane (Tris)	Fisher Scientific
5-hydroxytryptamine	Sigma
5-hydroxy[G-^{3}H] Tryptamine creatinine sulphate	Amersham
imipramine·HCl	Sigma
iprindole·HCl	Wyeth Research UK Ltd (Berkshire, UK)
isopentane	BDH
magnesium chloride	Fisher Scientific
magnesium sulfate	Fisher Scientific

rac-*p*-methoxyamphetamine	Health Protection Branch, Health Canada (Ottawa, ONT)
rac-3,4-methylenedioxyamphetamine	Health Protection Branch
R-*(-)*-3,4-methylenedioxyamphetamine	Health Protection Branch
S-*(+)*-3,4-methylenedioxyamphetamine	Health Protection Branch
rac-3,4-methylenedioxy-*N*-ethylamphetamine	Health Protection Branch
R-*(-)*-3,4-methylenedioxy-*N*-ethylamphetamine	Health Protection Branch
S-(+)-3,4-methylenedioxy-*N*-ethylamphetamine	Health Protection Branch
rac-3,4-methylenedioxymethamphetamine	Health Protection Branch
R-(-)-3,4-methylenedioxymethamphetamine	Health Protection Branch
S-(+)-3,4-methylenedioxymethamphetamine	Health Protection Branch
nialamide	Sigma
β-nicotinamide adenine dinucleotide phosphate	Sigma
nitrogen	Linde, Union Carbide
norepinephrine	Sigma
Levo-[7-^{3}H(N)]-Norepinephrine	New England Nuclear
rac-norfluoxetine	Lilly Research Laboratories

octyl sodium sulfate	Aldrich
perchloric acid, 60%	Fisher Scientific
o-phosphoric acid, 85%	Fisher Scientific
piperonyl butoxide	Aldrich
potassium chloride	Fisher Scientific
sodium borate	Fisher Scientific
sodium carbonate	Fisher Scientific
sodium chloride	Fisher Scientific
sodium phosphate, monobasic	Fisher Scientific
sodium phosphate, tribasic	Baker (Toronto, ONT)
toluene, glass distilled	BDH
N-trifluoroacetyl-L-prolyl chloride	Aldrich
L-tryptophan	Nutritional Biochemicals Corporation (Cleveland, OHIO)
p-tyramine	Sigma

3.2 **Chromatographic Analytical Instrumentation**

Partition chromatography is an analytical technique based on the separation of solutes by the use of differences in their distribution between two phases, a stationary and a mobile phase (for reviews, see Brenner and Olson, 1967; Szepesy, 1970; Johnson and Stevenson, 1978; Burtis *et al.*, 1987; Tabor, 1989) Polarity is one of the major determinants in the chromatographic process and encompasses the total interaction between the solvent and sample molecules and between the

solvent and sample molecules and the stationary phase. The polarity arises from non-covalent sources such as van der Waal's forces, dipole moments, hydrogen bonding and electrostatic interactions. Analytically useful information that can be obtained from chromatography includes testing for purity, identifying individual components of an organic mixture and quantitating the compounds of interest (Littlewood, 1970).

Chromatographic methods employing columns are referred to as liquid chromatography (see above reviews). In addition, chromatographic methods are classified according to the physical state of the mobile phase, the two most common methods being gas-liquid chromatography (GC) and liquid-liquid chromatography (LLC). In LLC, most commonly high performance liquid chromatography (HPLC), the phase support is usually coated with a polar substance, with separations accomplished by using an immiscible nonpolar mobile phase, termed normal phase. An alternative to normal phase is reversed phase, where a silica phase support is modified to produce a surface layer of non-polarity and, in contrast, the mobile phase is highly polar. This results in the most polar molecules eluting first and the least polar being retained in the column longer (Johnson and Stevenson, 1978; Warsh *et al.*, 1982; Bowers, 1989). In GC, partition is also used to achieve separation. The mobile gas phase carries the sample to the column, through the column and to the detector. Although the inert mobile phase in GC does not directly interact with the sample molecules or the stationary phase, it plays an important role on the column efficiency, the length of time required for analysis and in some cases

the sensitivity of a given detector (Schwartz, 1967; Jones, 1970; Simpson, 1970). Temperature dependency is another important parameter for separation in GC (Jones, 1970; Szepesy, 1970; Karger *et al.*, 1973). Advantages of GC are: (*a*) due to the low viscosity of the moving gas phase, long columns with high separation efficiency can be used (b) speed of sample analysis; (c) only small sample size required; (d) the simplicity and reliability of operation of the analytical instrument; (e) method applicable to widely divergent sample type , due in part to the availability of a large number of derivatizing reagents and column types and advances in instrumentation technology; and (f) relatively low expense, particularly if compared with mass spectrometry (MS) (Brenner and Olson, 1967; Szepesy, 1970; Baker *et al.*, 1982; Coutts and Baker, 1982). Limitations of GC include: (a) compounds that exhibit thermal lability and/or poor volatility are not suitable; (b) retention times are characteristic but not unique to a single analyte [however GC coupled with MS can be used for confirmation of structure, then routine analysis can be conducted with GC only (Brenner and Olson, 1967; Baker *et al.*, 1982; Coutts and Baker, 1982)]. Advantages of HPLC are: (a) the ability of the solute to interact selectively with both the stationary and mobile phases provides additional parameters to improve resolution; (b) relatively polar compounds present in body fluids are readily dissolved in commonly used mobile phase solvents; (c) increased sample volume injectable can increase sensitivity; and (d) for several of the more common applications, less preparation of the analyte mixture is required (Johnson and Stevenson, 1978; Warsh *et al.*, 1982). Limitations of HPLC include: (a) technically

more complex to maintain and operate; and (b) retention times are more sensitive to changes in environmental conditions than is the case with GC (Warsh *et al.*, 1982; Bowers (1989). Both GC and HPLC were used as analytical techniques within this thesis and will be discussed separately.

3.2.1 *Gas Chromatography*

The process involved in the separation and quantification of volatile components of a mixture using GC consists of partitioning them as vapors between a stationary liquid phase and a mobile gas phase (for reviews, see Jones, 1970; Littlewood, 1970; Simpson, 1970; Walker *et al.*, 1977; Coutts and Baker, 1982; Coutts *et al.*, 1985; Poklis, 1989). The stationary phase, most commonly a high-boiling non-volatile liquid, is affixed to the column through which the vaporized mixture to be separated and the mobile phase flow. The mobile phase or carrier gas is a stream of gas(es) that carries the vaporized components through the column. Helium, nitrogen, argon and hydrogen are commonly used carrier gases. A basic GC system consists of: (a) a heated inlet to allow entry and vaporization of the mixture to be analyzed and entry of the inert carrier gas; (b) a column; (c) an oven which heats the column as programmed; (d) a detector that detects each component as it elutes from the column; and (e) an integrator recorder that integrates the electrical signal from the detector into a recording of a series of peaks versus time (Coutts and Baker, 1982; Coutts *et al.*, 1985; Burtis *et al.*, 1987) [Figure 17].

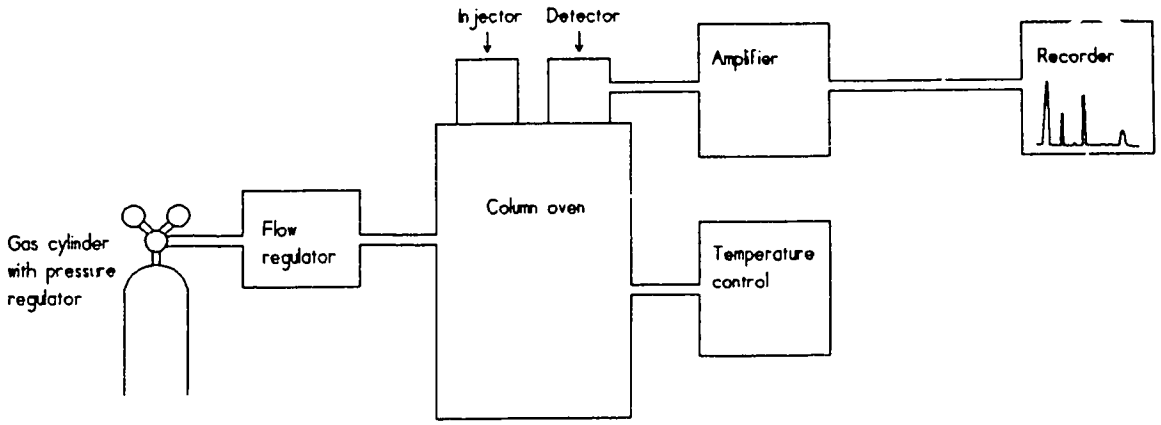


Figure 17: Schematic diagram of the components of a GC system. Adapted from Burtis *et al.* (1987).

3.2.1.1 Injection Systems

The two main types of injection systems available are the splitless and the splitter systems (Jennings, 1978; Coutts *et al.*, 1985). In the splitless system the sample is introduced and vaporized in a glass-lined cylinder that extends from the septum to the head of the column. The splitter system involves splitting the carrier gas stream in such a way that only a small proportion of the injected sample enters the column. The splitless system was used in the GC analysis described in this thesis as it offers optimal analysis of low concentrations of analytes.

3.2.1.2 Columns

GC columns are available commercially in a variety of materials, including glass, stainless steel, nickel, copper, aluminum, nylon and silica (Baker *et al.*, 1982; Coutts and Baker, 1982; Coutts *et al.*, 1985). In the experiments described in this thesis a fused silica capillary column was used. Two types of capillary columns are available, wall-coated open tubular (WCOT) and support-coated open tubular (SCOT), differing in the absence or presence of a support between the liquid phase and the inside surface of the capillary tubing. A WCOT column was used in the experiments described here as they are better suited for relatively low concentrations of analytes. There are also a wide variety of liquid phases that differ in their degree of polarity. The selection of the optimum liquid phase rests on the nature of the compounds to be separated. In general, separations are best achieved through matching the polarities of the solute and the liquid phase (Coutts *et al.*,

1985). GC columns with optically active liquid phases have become available for the direct resolution of enantiomeric compounds. These columns can offer the advantage of eliminating the requirement for derivatization with an optically and chemically pure reagent (Allenmark, 1984; Pasutto, 1992; Welch, 1994). However, chiral columns exhibit considerable batch to batch variability and are, in general, less rugged, less efficient and have lower capacities than standard achiral columns (Pasutto, 1992). The relatively high cost of chiral GC columns also limits their use, especially in method development (Pasutto, 1992).

3.2.1.3 Detectors

GC detectors sense the separated components of the sample as they elute from the column and provide a corresponding electrical signal (Coutts and Baker, 1982; Coutts *et al.*, 1985). Only one type of GC detector, the nitrogen-phosphorus detector (NPD) was used in the analytical work described in this thesis. The NPD is a type of flame ionization detector, where ions of an alkali metal (rubidium) are introduced into the mixture of eluted components in carrier gas and hydrogen and burned in air to produce enough heat to ionize the organic eluants. A pair of electrodes, charged by a polarizing voltage, collects the positive ions and generates a current proportional to the number of ions present. When a compound containing nitrogen or phosphorus is burned, the rate of release of alkali metal vapor is increased and ultimately increases the current flow which results in increased sensitivity for nitrogen- or phosphorus-containing analytes (Burtis *et al.*, 1987;

Poklis, 1989).

Mass spectrometry (MS) is a specialized chromatographic detector that provides extremely sensitive detection and highly specific identification of components of a mixture (Teranishi *et al.*, 1967; Karger *et al.*, 1973; Poklis, 1989). The generation of a spectrum by MS involves three steps: ionization, mass filtration and detection. There are three modes of ionization, the most common being electron impact (EI). In MS operating in EI mode, the sample is converted into a vapor and then bombarded with high energy electrons to produce a charged molecule or to shatter the molecule into ionic fragments (Figure 18). Both the molecular ion and positively charged molecular ion fragments are separated and detected according to their mass/charge ratio. The mass spectrum is a display of the different masses of the molecular ion and the charged fragments and their relative abundances and is unique to each of the molecules analyzed. MS in EI mode was employed to confirm the identification of the derivatives of the methylenedioxy analogues of AMPH and the internal standard used in the GC analysis of extracts from brain and liver tissues.

3.2.1.4 Derivatization

Derivatization of compounds of interest for GC analysis involves the chemical alteration of the molecules. The purposes of derivatization include: (a) increased volatility; (b) increased sensitivity; (c) reduction of the polarity of the analytes to improve chromatographic quality; (d) improved extraction efficiency from aqueous

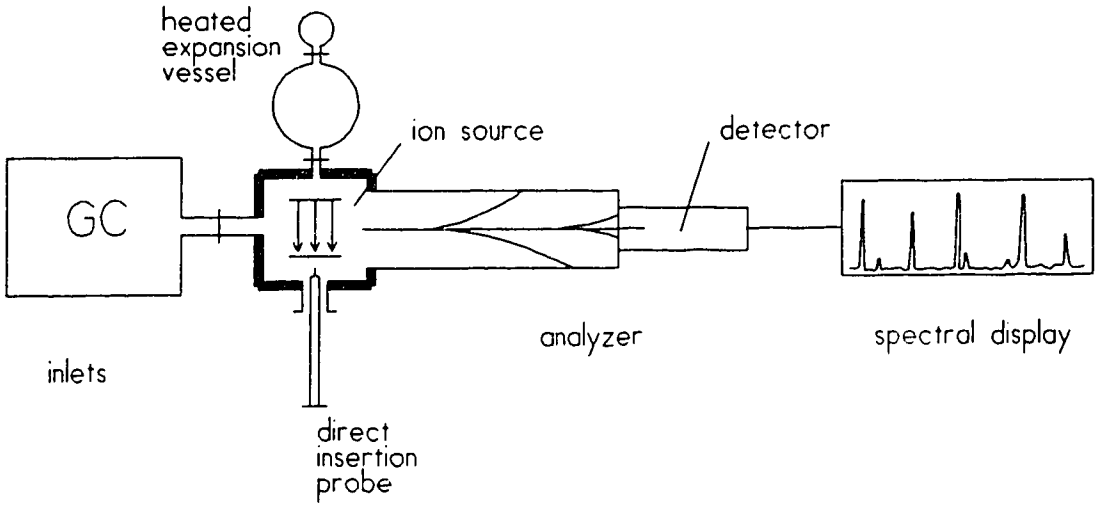


Figure 18: Schematic diagram of an electron impact mass spectrometer with three different types of inlets. Adapted from lecture materials with permission from Dr. R.T. Coutts.

solutions; and (e) introduction of a functional group that is sensitive to a particular detector (Coutts and Baker, 1982; Coutts *et al.*, 1985; Baker *et al.*, 1994). Derivatizations typically involve the replacement of the active hydrogen on polar groups, such as N-H, O-H or S-H, via acylation, alkylation, silylation or condensation (Figure 19).

Another purpose for derivatization is the production of diastereomers from mixtures of enantiomers by reaction with an optically active derivatizing reagent, allowing separation on a standard achiral column. This methodology has been termed the indirect method and has been the subject of many review articles (Dennis, 1986; Gal, 1988; Mutschler *et al.*, 1990; Pasutto, 1992; Wright and Jamali, 1993; Ahn *et al.*, 1994; Srinivas *et al.*, 1995). In general, there is agreement that the optically active derivatizing reagent should satisfy several criteria to avoid errors in quantifying the respective enantiomers in analytical samples. These criteria include exceptional optical purity, stability against isomerization during storage and use and identical reactivity with, and detector sensitivity to, both diastereomeric derivatives (see above review articles).

The indirect method was employed in the separation and quantification of the enantiomers of MDMA, MDA and MDE described in this thesis. The commercially available optically active derivatization reagent, N-trifluoracetyl-L-prolyl chloride (TFPC) was used to produce N-trifluoroacetyl derivatives of the enantiomers of the methylenedioxy analogues of AMPH. This particular reagent has been used in stereoselective assays of propanalol (Hermansson and Von Bahr, 1980; Silber and

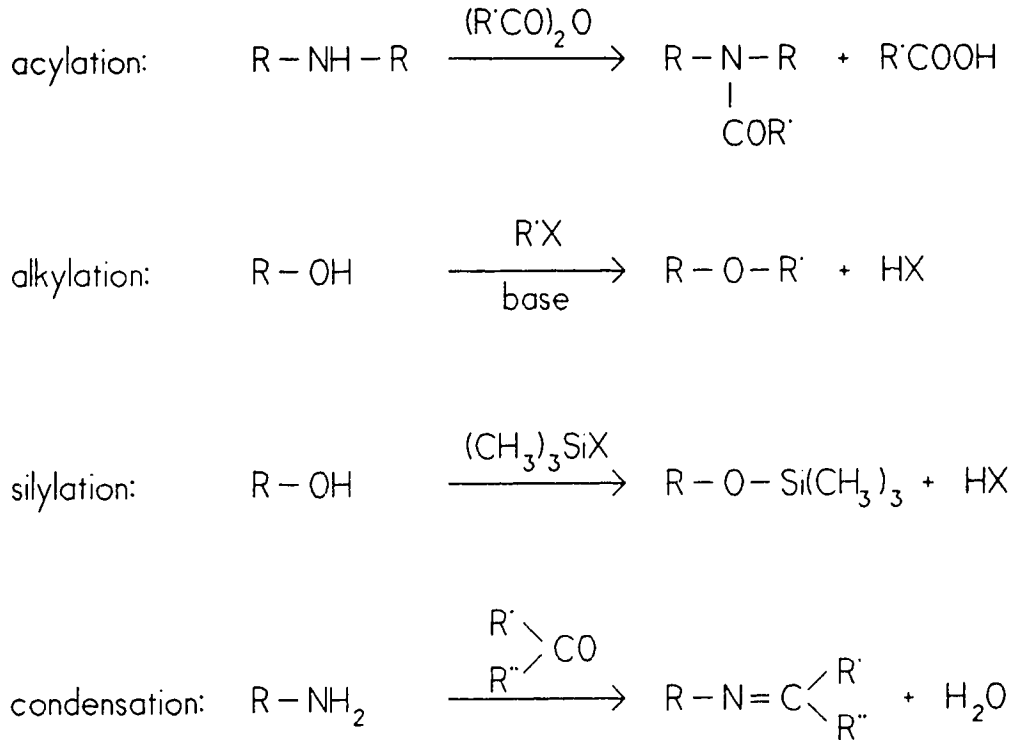


Figure 19: Some common derivatization reactions used in GC analysis. Adapted from Baker *et al.* (1982).

Riegelman, 1980), FLU and norfluoxetine (Torok-Both *et al.*, 1992), tranylcypromine (Aspeslet *et al.*, 1992) and AMPH and MAMPH (Sievert, 1994). Adams *et al.* (1982) used TFPC in the derivatization of the sterically hindered chiral amine ketamine and found the TFPC was subject to a degree of racemization. Ahn *et al.* (1994) observed that the degree of racemization of the derivatization reagent was sensitive to the reaction conditions, including temperature and duration of the evaporation. The harsher the conditions, the greater the risk of racemization. Fitzgerald *et al.* (1989a) reported that the company that manufactures TFPC was employing milder conditions in TFPC synthesis, yielding a reagent with optical purity of 97-99%.

Fitzgerald *et al.* (1989a,b, 1990) used TFPC to form diastereomers of MDMA and MDA from rat plasma and whole blood samples. Optical purity of the TFPC, found to be 96.1%, was checked by chromatographing optically pure standards of S-(+)-MDA and calculating the area of the minor peak. These authors also demonstrated that kinetic racemization was not occurring during their on-column derivatization procedure by derivatizing and chromatographing racemic MDMA and MDA, then comparing the resultant peak areas from racemic standards. No statistical differences were seen in the peak areas of the R-(-) and S-(+) peaks. After showing the linear regression analyses from optically pure, racemic and 75:25 standards produced the same slope, the three groups were compared and no statistical differences found. Thompson and Dasgupta (1994) also used TFPC in a microwave-induced rapid preparation procedure for the derivatives of MDMA.

3.2.2 *High Performance Liquid Chromatography*

HPLC is a form of partition chromatography in which a liquid mobile phase is forced at high pressure through a column coated with a thin layer of stationary phase (for reviews, see Walker *et al.*, 1977; Krstulovic, 1982; Stern, 1982; Warsh *et al.*, 1982; Kissinger, 1983; Hashimoto and Maruyama, 1983; Bowers, 1989). The differential equilibrium of the analytes between the mobile and stationary phases results in their separation. Interactions involve competition in terms of solubility. The components of a HPLC system include: (a) a solvent reservoir for the mobile phase; (b) a pump, most commonly a reciprocating piston pump, to provide the driving force for the mobile phase; (c) a precolumn packed with silica to saturate the mobile phase with silica to prevent the dissolving of the packing material in the analytical column; (d) a sample delivery system, commonly a nitrogen-driven automated sample system; (e) a guard column between the injector and the analytical column protecting the analytical column from particulate matter or any strongly retained components of the sample; (f) an analytical column, usually of stainless steel with a stationary phase on a support matrix; (g) some type of detector (absorbance, fluorescence or electrochemical); and (h) an integrator and recording device (Figure 20).

HPLC combined with electrochemical (EC) detection represents an analytical method well tailored for the simultaneous analysis of the catecholamines and 5-HT and their acid metabolites (Warsh *et al.*, 1982; Hashimoto and Maruyama, 1985). This method was employed to examine the changes in the levels of DA, NE and

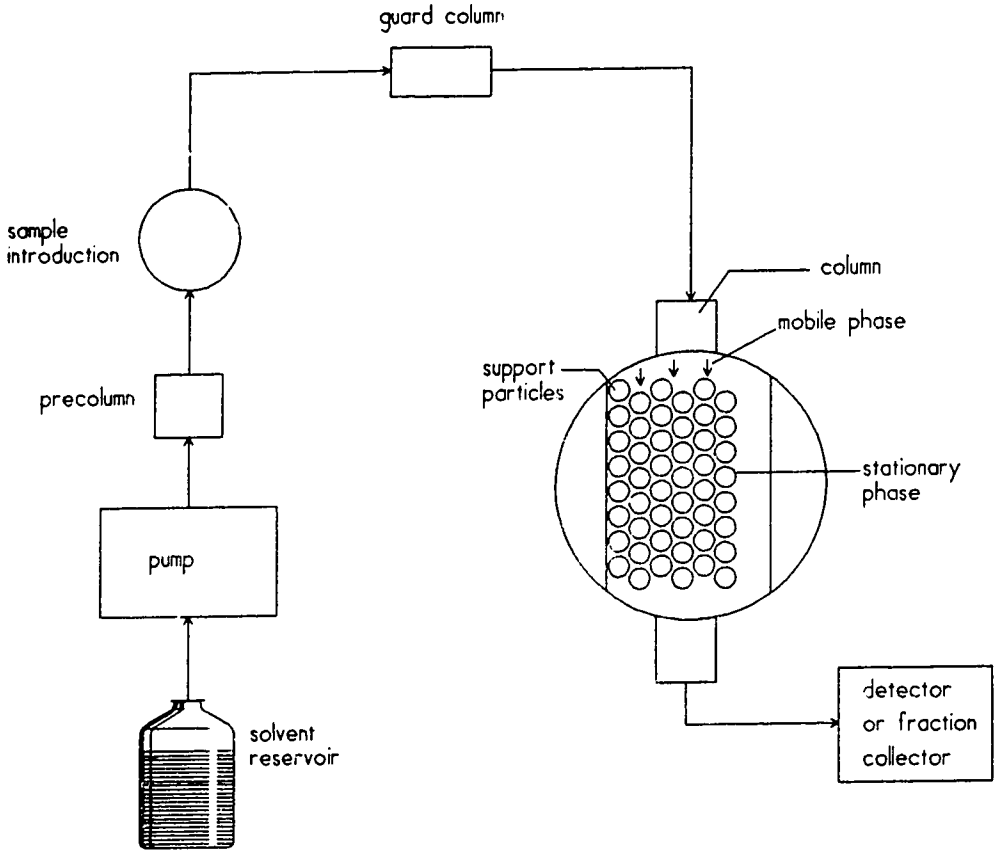


Figure 20: Schematic diagram of the components of a HPLC system. Adapted from Bower (1989).

5-HT and the acid metabolites, HVA, DOPAC and 5-HIAA, following administration of the methylenedioxy analogues of AMPH, a representative psychomotor stimulant and an hallucinogen. Further discussion regarding HPLC will focus on reversed phase HPLC-EC, the system employed in the experiments described in this thesis.

The most popular stationary phase support matrix for reversed phase HPLC is octadecylsilane (ODS, C-18) because of the ease with which polar biological molecules are separated. The stationary phase consists of organic moieties chemically bonded onto the support matrix, accomplished by reacting silanol groups with the ODS matrix, resulting in a surface containing octadecyl groups bound to the surface by siloxane bonds (Si-O-Si). Chemical bonding is advantageous because polar and ionic compounds are readily separated and the phase is not easily stripped with repeated use. However, this type of column operates most effectively at pH between 2 and 8.

The characteristics of the highly polar water-based mobile phase that allow optimal separation of the biogenic amines include (a) being buffered to a fixed pH value and maintained there to limit ionization of analytes; (b) the use of organic modifiers such as acetonitrile to decrease the surface tension between the stationary and mobile phases, thus decreasing the retention times of the analytes; and (c) ion-pairing reagents to decrease the analytes' polarity.

Principles of the EC detection method are based on EC reactions on a electrode surface and are quite different from those of other spectroscopic detection methods, in which physical phenomena, such as light absorbance and fluorescence

emission, are the basis of detection (Walker *et al.*, 1978). Because the EC method works on the principle of EC reactions on intact molecules, there is no need for derivatization before or after separation by HPLC. A typical example of an EC reaction with DA is shown in Figure 21. These oxidation-reduction (redox) reactions can be brought about electrochemically since they require only relatively simple charge (electron) transfer processes. EC methods are based on the measurement of current (either attracted or provided) associated with oxidation or reduction of electrochemically active substances on the surface of an electrode. Changes in the current, reflecting changes in the concentration of chromatographic eluates, are simply monitored, while the applied potential is kept at a fixed value. The release of the electrons (reaction current) from the oxidative reaction is amplified by an amplifier and signals are recorded on a recorder as responses to the oxidative reaction. The intensity of the response is proportional to the concentration of the electrochemically active analyte on the electrode surface. Quantitative analysis of the components of the biological mixture is possible if the parameters which influence the reaction rate and the degree of the reaction are kept constant. The parameters are the composition of the mobile phase, the temperature and pH of the reaction medium and the ionic strength of the solution.

EC detectors can be divided into two categories on the basis of cell design and electrolytic efficiencies: amperometric and coulometric. An amperometric EC detector was used in the experiments described in this thesis. Amperometric detectors have a relatively small surface area and their electrolytic efficiency is low.

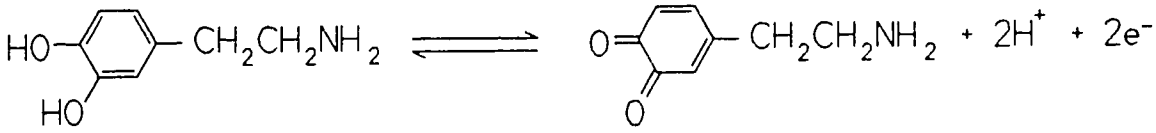


Figure 21: The proposed electrochemical reaction involving dopamine in an HPLC system equipped with electrochemical detection.

However, compensation of background current, flowing steadily through the circuit, is achievable because the background current is very low. In addition, because the surface area is small, it is possible to place the working electrode where the effluent flow is continuous and steady. As a result, these detectors are usually free from noises induced by fluctuations in liquid flow in the cell.

Although HPLC-EC has been widely employed for the analysis of the levels of biogenic amines in biological tissues or fluids, this analytical technique can also be applied to the analysis of drug levels. Michel *et al.* (1993) developed a HPLC-EC method to analyze MDMA and MDA in whole blood standards (MDE was used as the internal standard). The retention times were such that simultaneous analysis was complete within 15 min. The reported sensitivity of 1 ng/ml allowed the use of very small volumes of blood (200 µl) which would be advantageous for repeated blood sampling from animals. This method could be helpful in pharmacokinetic studies of the methylenedioxy analogues of AMPH.

3.3 **Apparatus and Animals**

3.3.1 *Tissue Homogenizer*

A combination of TRI-R S63C (TRI-R Instruments, Rockville Center, NY, USA) variable speed laboratory motor with a Teflon glass pestle and a glass grinding tube was used for homogenizing tissue samples.

3.3.2 *Centrifuges*

A Sorvall GLC-2b or Sorvall GLC-1 General Laboratory Centrifuge (Dupont Instruments, Wilmington, DE, USA) was used for low-speed, small volume centrifugations. Higher speed and/or larger volumes centrifigations were carried out in a Damon-1EC B-20 (Needham Heights, MA, USA) refrigerated high-speed centrifuge or a Beckman Model J-21B refrigerated preparative centrifuge (Palo Alto, CA, USA).

3.3.3 *Savant Evaporator*

A Savant Speed Vac SS1 (Savant Instruments, Inc., Farmington, NY, USA) was used for evaporating samples. The instrument consists of a concentrator (a rotor chamber with a heater), a chemical trap that accepts disposable cartridges, a refrigerated condensation trap and a vacuum pump. The process of drying involves combining centrifugal force and vacuum.

3.3.4 *Shaker-Mixer*

Two types of vortex-shakers were used: Ika-Vibrax VXR2 Shaker (Janke and Kundel GMBH and Co., Staufen, Germany) and a Thermolyne Maxi Mix vortex mixer (Thermolyne Corp., Dubuque, IO, USA).

3.3.5 *Glassware Cleaning*

All glassware was initially rinsed with tap water. Further cleaning was

completed using a Mieie Electronic 6715 dishwasher and biodegradable Sparkleen (Fisher Scientific Co.) For test tubes, an additional cleaning step was added. The tubes were sonicated (Ultra-sonic cleaner, Mettler Electronics) in a solution of Decon 75 concentrate (BDH Chemicals). Tubes were then cleaned in the dishwasher without Sparkleen. All glassware was then air-dried in a mechanical convection oven (Model 28, Precision Scientific Group, Chicago, IL, USA).

3.3.6 *Animals*

The male Sprague-Dawley rats (250-400 g) used for all experiments in this thesis were obtained from Bio-Science Animal Services, Ellerslie, Alberta, Canada. The animals were housed in pairs in plastic cages on cedar chip bedding in a temperature controlled room (21±1°C). They were subject to a 12 h light/dark cycle (lights on at 0730h) throughout all experiments with the exception of the behavioral experiments which were conducted in specially equipped cages and under reversed light/dark cycle (see 11.6.1 and 11.6.2 for details). Water and lab chow were provided *ad libitum*. The lab chow (Lab-Blox feed, Wayne Feed Division, Continental Grain Company, Chicago, IL, USA) was 4.0% (min) crude fat, 4.5% (max) crude fibre and 24% (min) crude protein.

Procedures involving the use of animals were approved by the University of Alberta Health Sciences Animal Welfare Committee and were conducted according to the guidelines put forth by the Canadian Council on Animal Care.

3.4 **Metabolic Studies**

3.4.1 *Development of GC assay for the enantiomers of MDMA and MDA*

Male Sprague-Dawley rats were randomly assigned to drug or vehicle treatment groups. The rats were injected ip with either physiological saline or *rac*-MDMA (10 mg/kg, free base weight) dissolved in physiological saline. Animals were killed by cervical dislocation and guillotine decapitation 1, 2, 4 or 8 h post injection. Whole brain and liver tissues were removed immediately and frozen in solid carbon dioxide-cooled isopentane. All samples were stored at -80°C until analysis. Regional dissections were carried out on half brains from rats killed 2 h post injection.

The assay is summarized in Figure 22. Brain or liver tissue samples were partially thawed, weighed and homogenized in 5 volumes of ice-cold 0.1N perchloric acid. Homogenates were centrifuged at 1200 g for 10 min at 0-4°C. Portions (0.5 or 1 ml) of the supernatants were used for analysis, and the internal standard (I.S.) [1500 ng of *p*-chlorophenylethylamine (pCPEA)] was added to all samples. Trisodium phosphate (200 µl of 10% solution) was added and the pH value checked (pH 11.5-12). Ethyl acetate (4 ml) was then added and the tubes were shaken vigorously for 10 min and centrifuged at 1000 g for 10 min. The organic phase was transferred to smaller test tubes and taken to dryness under a gentle stream of nitrogen in a heating block at 45°C. A derivatization solution, consisting of 2 µl TFPC per 100 µl toluene, was prepared and 100 µl were added to each of the tubes. The samples were vortexed and placed in a heating block at 60°C for 30

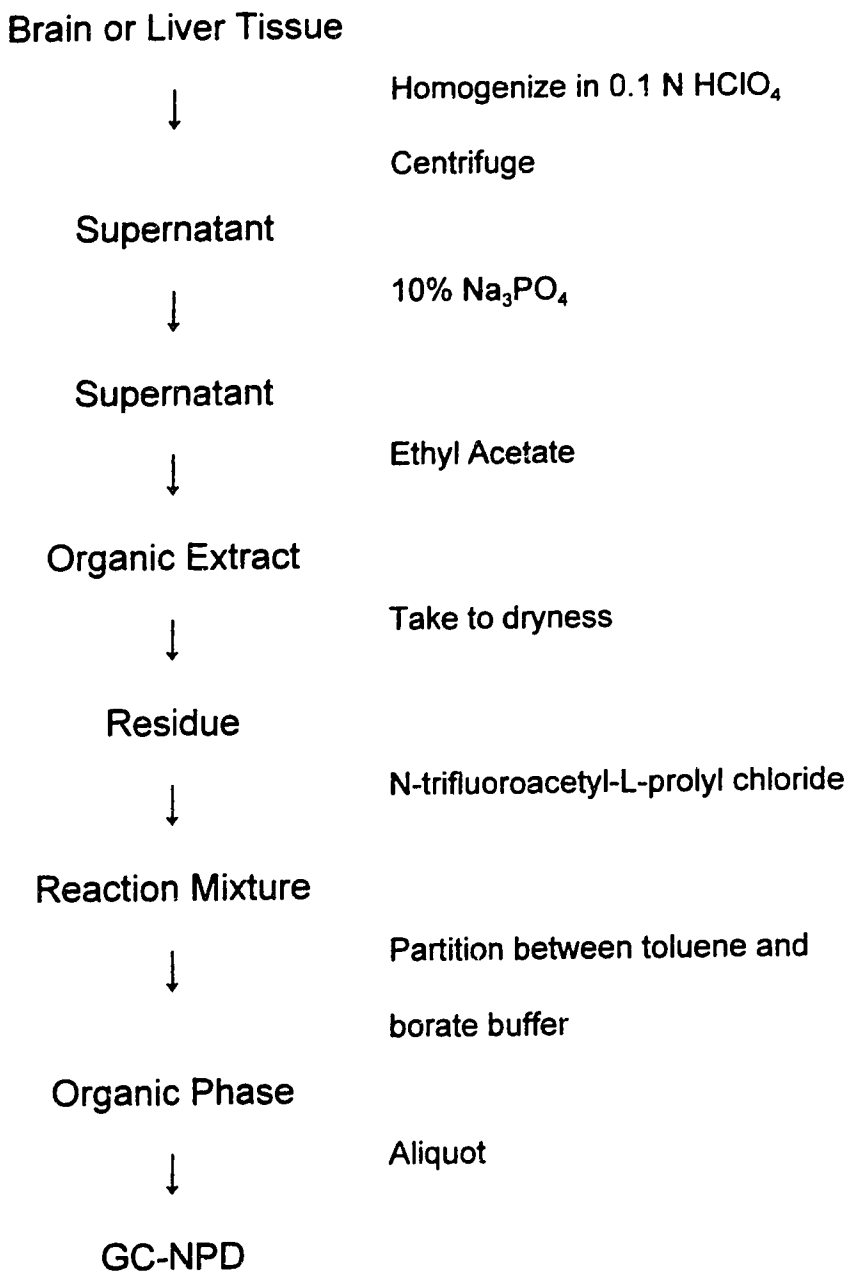


Figure 22: Flow chart demonstrating the assay procedure developed for simultaneous analyses of the enantiomers of MDMA and MDA in brain and liver tissues.

min, then removed and allowed to stand at room temperature for 10 min. A further 100 µl toluene and 1 ml saturated sodium borate solution were added. The toluene layer was retained and 1 µl portions were injected on the gas chromatograph.

A Hewlett Packard (HP) model 5890 gas chromatograph equipped with a NPD and linked to a HP 3392A integrator was used. A fused silica capillary column (25 m × 0.32 mm I.D.) coated with a 0.52 µm film thickness of 5% phenylmethyl silicone (HP Co., Palo Alto, CA, USA) was employed. The carrier gas was pure helium at a flow rate of 2 ml/min. The detector was purged with pure hydrogen at 3.5 ml/min mixed with dry air. Temperatures at the injection port and detector were 250°C and 325°C, respectively. The initial oven temperature of 105°C was programmed to increase at a rate of 15°C/min to 270°C.

A standard curve ranging from 50 ng to 750 ng for each of the 4 enantiomers was run in parallel with each assay. These curves were prepared by adding 1500 ng of I.S. (pCPEA) and varying amounts of *rac*-MDMA and *rac*-MDA to 1 ml 0.1N perchloric acid. A comparison was carried out between using 0.1N perchloric acid and brain or liver supernatant from vehicle-treated rats in preparing the standard curves. No statistical differences were found, so for economic and ethical reasons, 0.1N perchloric acid was used routinely in the preparation of the standard curves. The ratio of the peak heights of the individual derivatized enantiomers to that of the derivatized I.S. was calculated and plotted against the concentrations of the individual enantiomers. Blank samples were also included during assay development.

Confirmation of the structures of the derivatives was carried out using combined GC-MS. The mass spectrometer (HP 5985A with HP 7920 data system) was operated in the EI mode and the GC conditions were as described above. Operating conditions for the mass spectrometer were: ion source temperature, 200°C; interface temperature, 275°C; column pressure, 34.5 kiloPascals (kPa); accelerating voltage, 2200 eV; ionization voltage 70 eV; scan speed, 200 amu/sec; and dwell time, 200 msec.

Stability of the derivatives of the drugs was examined by repeated GC analysis of the same set of standards and tissue samples six times over a one week period. Intra-assay coefficients of variation (standard deviation/ mean × 100%) for each of the four enantiomers were determined by comparing the results of the same assay injected six different times. Inter-assay coefficients of variation for the four enantiomers were determined for 11 different calibration curves. Mean absolute recovery rates were calculated by comparing the recovery of 200 ng of the individual enantiomers of MDMA and MDA in brain supernatant and methanol.

In the variable time study, regression analysis was done on the differences between the R-(-) and S-(+) enantiomers of MDMA and MDA over time.

3.4.2 *Modification of GC assay for the Enantiomers of MDE and MDA*

Male Sprague-Dawley rats were given acute ip injections of *rac*-MDE (10 mg/kg, free base weight) and killed 1,2,4, or 8 h post injection. Whole brain tissues were removed and treated in the same manner as described above.

The GC analysis of the enantiomers of MDE proved to be more difficult than was the case with the enantiomers of MDMA and MDA. Several modifications of the assay developed for separating the enantiomers of MDMA and MDA described above were necessary (Figure 23). The ethyl acetate used as the organic solvent for extraction of the drugs from the aqueous supernatant in the above assay gave poor chromatographic results with interfering peaks on the GC trace and was replaced with a solvent mixture of toluene:ethyl acetate (9:1). Standard curves prepared from 0.1N perchloric acid differed from those prepared from brain supernatant of drug naive animals, so all standard curves were prepared from brain supernatant. Instead of drying the organic solvent under a gentle stream of nitrogen, a Savant Speed Vac evaporator was used.

To monitor possible racemization of TFPC, standards of the individual enantiomers of MDE and MDA were reacted with the TFPC 6 months after the bottle had been opened to examine the degree of impurity. It was found to range from 9%-11%. Interestingly, a newly opened bottle of TFPC was compared with the old bottle that had been in use for 6 months. The new bottle was found to have 22%-26% impurity and was not used in any subsequent assays. As further confirmation of the MDE and MDA values obtained with TFPC the experiment at the 1 h time period was repeated using heptafluorobutyryl-L-prolyl chloride (HFPC), another stable optically active derivatization reagent used for the separation of chiral compounds (Lim *et al.*, 1986; Srinivas *et al.*, 1987, 1995). The HFPC was obtained from Drs. J. Hubbard and G. MacKay, College of Pharmacy, University of Saskat-

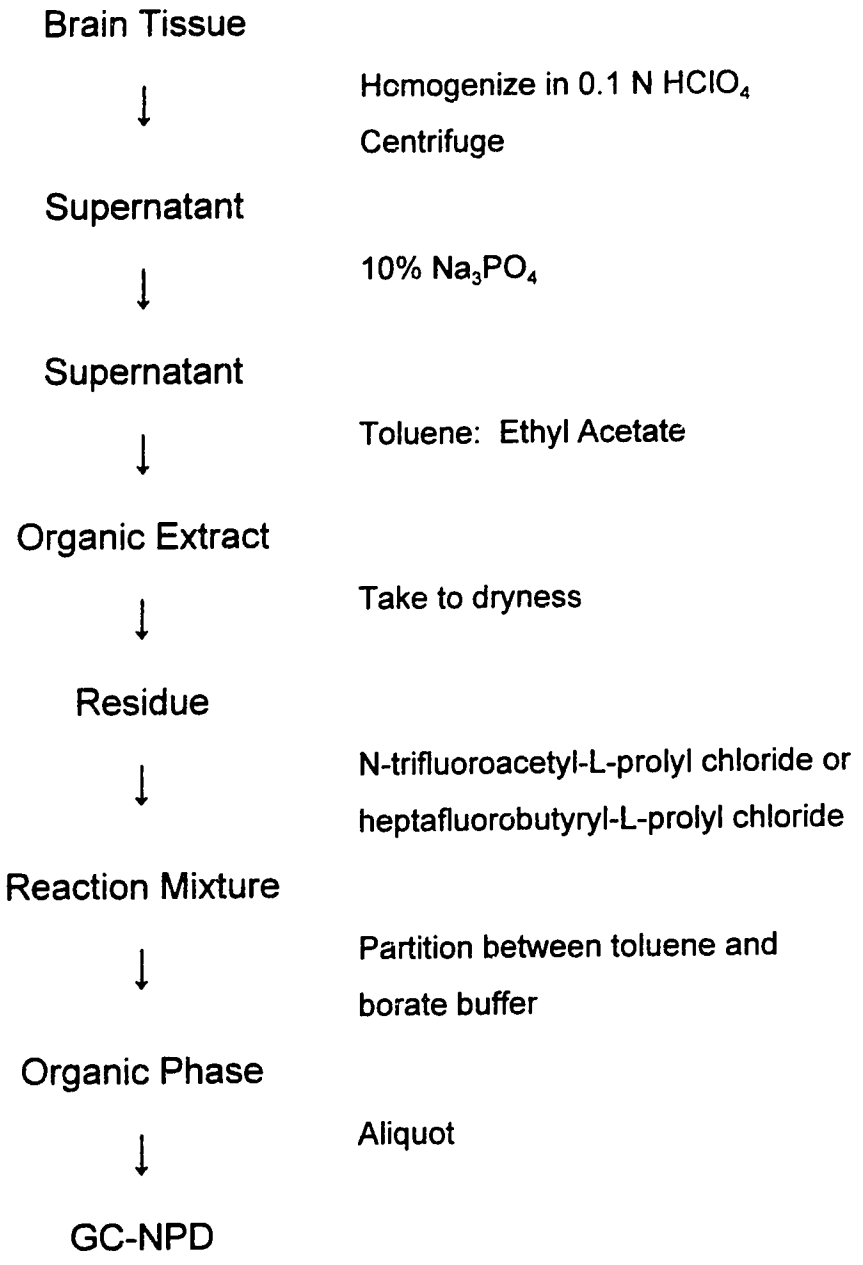


Figure 23: Flow chart demonstrating the assay procedure to simultaneously analyse the enantiomers of MDE and MDA in brain tissues.

chewan, Saskatoon, Canada and had been prepared according to the reported procedure of Lim *et al.* (1986). The extraction and reaction conditions were as described above, except that the reaction with HFPC was allowed to proceed at room temperature instead of 60°C. The GC conditions were as reported for MDMA with the exception that the oven temperature was programmed to increase at a rate of 10°C/min instead of 15°C/min to better separate a small interference peak from the peaks of interest.

Calibration curves were constructed with each assay run and were prepared by spiking drug-naive brain supernatant after homogenization and centrifugation with known quantities of *rac*-MDE and *rac*-MDA and the I.S., pCPEA. Peak height ratios of each of the four enantiomers to the I.S. were used to calculate linear regression lines and the peak height ratios from the brain extracts were compared to those of the calibration curve to calculate the quantities of the enantiomers in the brain sample. Mean absolute recovery rates were calculated using 300 ng and 25 ng of the individual enantiomers in spiked supernatant or methanol. Stabilities of the derivatives were also examined by storing derivatized samples at -80°C and reinjecting them on to the gas chromatograph 2, 6, 10 and 14 days later.

Analysis of variance (ANOVA), with enantiomer as the repeated factor and hour as the between factor, were conducted for both MDE and MDA. Multiple F tests (α=0.05) were conducted to calculate the critical difference required between each set of enantiomers to achieve statistical significance.

3.4.3 *Specific CYP Isozyme Studies*

The human microsomal isozymes used in these series of experiments were commercially prepared and included human CYP2D6 and CYP3A4, both derived from a human AHH-1 TK +/- cell line which had been transfected with complementary DNA that encoded each of the microsomal isozyme proteins (Gentest, Woburn, MA, USA). The specific human CYP2D6 product was CYP2D6-Val with reductase. This product was chosen as it was reported by the manufacturer to have a higher specific activity than their original CYP2D6-Met product in the test assay with (+)-bufuralol 1'-hydroxylase. The CYP3A4 product also contained reductase which resulted in a higher specific activity in the manufacturer's test assay with testosterone 6 β-hydroxylase. The isozyme studies were subdivided into three sets of experiments; the first used CYP2D6. CYP2D6 had been shown to catalyze hydroxylation reactions in the presence of imipramine (IMI) and to a limited degree, the N-demethylation of IMI to yield DMI (Coutts *et al.*, 1993,1994). Another tricyclic antidepressant, amitriptyline (AMI) has also been shown to be a substrate for N-demethylation by CYP2D6 (Coutts *et al.*, unpublished). Thus, IMI and AMI were compared with MDMA and MDE at doses similar to those reported by Coutts *et al.*, (1993) [40 nmoles] in terms of their ability to be N-dealkylated in the presence of CYP2D6. The next set of experiments was conducted under the same conditions, except CYP3A4 was substituted for CYP2D6.

The third set of experiments was conducted with two known substrates for

CYP3A4, nifedepine and lidocaine (Imaoka *et al.*, 1990; Gonzalez, 1992; Guengerich, 1992). Nifedepine, as one of the model substrates for CYP3A4, was compared with MDMA and MDE, using an amount of substrate (28.9 nmoles) that had been shown to produce maximum nifedepine metabolite (personal communication, Ms. M. Bach). Although nifedepine has been used extensively as a model substrate, it is not dealkylated by CYP3A4; rather it undergoes initial hydroxylation followed by dehydration to yield nitropyridine (Gonzalez, 1992; Guengerich, 1992). To demonstrate that CYP3A4 is capable of being involved in N-dealkylation reactions, lidocaine which is deethylated to monoethylglycinexylidide (MEGX) (Imaoka *et al.*, 1990) was also used as a CYP3A4 substrate and compared to MDMA and MDE at equimolar concentrations. A summary of the substrates and their proposed metabolites which were used in experiments to compare with the ability of the same isozymes under the same experimental conditions to catalyze the N-dealkylation of MDMA and MDE is seen in Figure 24.

The detailed procedure used to evaluate the ability of specific CYP isozymes to catalyze the metabolic reactions was a modification of the method developed by Coutts *et al.* (1993). The *in vitro* metabolic incubation medium (for a final volume of 1000 µl) consisted of: (a) 220 µl of a NADPH-generating system [20 µg/µl $NADP^+$ in 0.1 M potassium phosphate buffer (pH=7.4); 20 µg/µl D-glucose-6-phosphate dissolved in the same buffer; 6.7 µg/µl $MgCl_2 \cdot 6H_2O$ in distilled water; 1 unit (20 µl) of D-glucose-6-phosphate dehydrogenase (1 unit will oxidize 1.0 µmole of D-glucose-6-phosphate to 6-phospho-D-gluconate per min in presence of NADP at pH

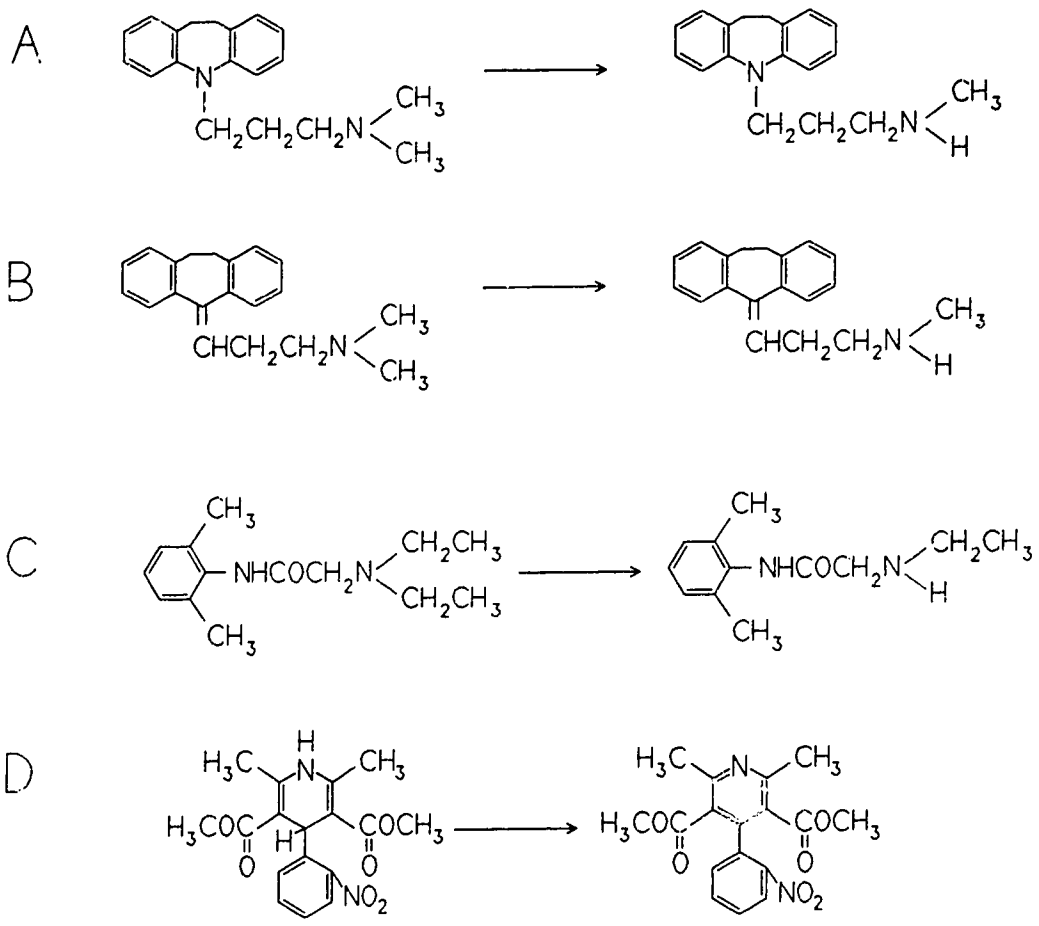


Figure 24: Proposed metabolic paths catalyzed by CYP2D6 and/or CYP3A4: (A) N-demethylation of IMI; (B) N-demethylation of AMI; (C) N-deethylation of lidocaine; (D) dehydration of nifedepine.

7.4 at 25°C, according to the manufacturer's specifications)]; (b) varying concentrations of substrate; and (c) buffer to make up a final volume of 850 µl. Polypropylene microfuge tubes (1.5 ml) were used for the incubation, as recommended by the manufacturers. The tubes were preincubated 5 min at 37°C in a water bath. Ice-cold isozymes were added (50 µl: protein content=500 µg), the isozyme containers were rinsed twice with 50 µl buffer and the solution gently mixed. This step was carried out in a Labconco Purifier Class II Safety Cabinet (Labconco Corporation, Kansas, Miss, USA) biohazard fumehood, as indicated by the manufacturer. In the third set of experiments for economic reasons, the final volume was reduced to 500 µl with corresponding reductions of the various additions to the incubation mixture. In the case of lidocaine, several modifications of the experimental procedure were shown to increase the reaction efficiency (personal communication, Ms. M. Bach). Phospholipids (phosphotidylserine.19.8 µg and cholic acid,100 µg for a 500 µl final volume) were added to the incubation mix, as Shigura *et al.*, (1994) had demonstrated that for the *in vitro* metabolism of FK506 (a potent immunosuppressive agent) using purified CYP3A enzymes, the addition of the phospholipids increased the efficiency of the metabolic reaction. A lower concentration of lidocaine (12.9 nmoles) was shown to produce more metabolite per mole of substrate than higher concentrations of lidocaine. The amount of CYP3A4 isozyme required was doubled over that used in the experiments with nifedepine.

The tubes were then returned to the water bath and incubated for varying

lengths of time. Following the incubation time and still under the fumehood, the tubes were quickly immersed in ice to stop the metabolic reaction. The internal standard, if used, and a basifying agent (the specific agent was dependent on the substrate) were added. At the point when the basifying agent was added, the samples were no longer considered a biohazard risk and could be removed from the fumehood and handled in the same manner as tissue supernatant.

In the samples where the substrate was either MDMA or MDE, 200 µl of 10% trisodium phosphate was added to each tube. The samples were then assayed according to the GC assay for MDMA and MDE detailed previously.

The GC assay for determining the levels of IMI and DMI was modified from the procedure described by Drebit *et al.* (1988). After the incubation period, solid sodium carbonate was added in excess and the samples acetylated with 300 µl of acetic anhydride. Further sodium carbonate was added until the bubbling stopped, signalling the acetylation reaction was complete. Toluene (5 ml) was added and the tubes were placed in a shaker-mixer for 5 min, then centrifuged for 5 min. The organic layer was retained and dried in a Savant evaporator. The analytes were reconstituted in toluene (100 µl) and 1 µl aliquots were injected on a gas chromatograph equipped with the same column and detector as used for MDMA and MDE. The GC conditions were as follows: temperatures at the injection port and the detector were 250°C and 325°C, respectively and the initial oven temperature of 105°C was programmed to increase at a rate of 15°C/min with a final temperature of 270°C. The assay for AMI and its N-demethylated metabolite,

nortripyline was similar to the assay for IMI and DMI, with the exception that the amount of acetic anhydride was reduced to 200 µl and the amount of extraction solvent was reduced to 4 ml. The GC conditions were changed to: temperatures at the injection port and the detector were 250°C and 325°C, respectively and the initial oven temperature of 105°C was programmed to increase at a rate of 25°C/min with a final temperature of 295°C.

The assay for nifedepine and its metabolite involved basifying the incubation mixture with 50 µl of 25% K_2CO_3. The extraction solvent was 2% acetone in hexane (4 ml). The two phases were shaken for 10 min in a shaker-mixer and then centrifuged for another 10 min. The organic phase was removed and dried in a warm water bath under a gentle stream of nitrogen. The analytes were reconstituted in 100 µl of toluene and 1 µl aliquots were injected on the same gas chromatograph as previously described. The GC conditions were as follows: temperatures at the injection port and the detector were 250°C and 325°C, respectively and the initial oven temperature of 105°C was programmed to increase at a rate of 12°C/min with a final temperature of 280°C.

The assay for lidocaine and MEGX involved adding solid sodium bicarbonate and acetic anhydride (250 µl) to the incubation mixture. The sodium bicarbonate was kept in excess until bubbling had ceased. Following this, 25% K_2CO_3 (200 µl) was added to increase the pH. A solution of 2% acetone in hexane (4 ml) was used as the extraction solvent. The two phases were shaken for 5 min in a shaker-mixer and then centrifuged for 5 min. The organic layer was removed and dried in a warm

water bath under a gentle stream of nitrogen. The analytes were reconstituted in 100 µl of toluene and 1 µl aliquots were injected on the same gas chromatograph as above. The GC conditions were modified in the following manner: temperatures at the injection port and the detector were 250°C and 325°C, respectively and the initial oven temperature of 105°C was programmed to increase at a rate of 10°C/min with a final temperature of 280°C.

The ratios of the peak heights of the metabolite and the parent drug were calculated to provide a qualitative comparison of the relative degree of metabolism that had occurred (n=4-6).

3.5 *In vitro* Uptake Studies With *rac*-MDE, R-(-)-MDE and S-(+)-MDE

The protocol was based on the procedure established by IL Martin *et al.* (1978) and had been previously used to examine the inhibition of uptake of radiolabelled DA, NE and 5-HT in the presence of PMA and 4-ethoxyamphetamine (Hegadoren *et al.*, 1994). Rats were killed by guillotine decapitation, their brains were removed, placed on an ice-cooled plate and striatal and hypothalamic tissues dissected out. Tissue prisms of 0.1×0.1×approximately 2 mm were obtained with a McIlwain tissue chopper (The Mickle Laboratory Engineering Company, Gomshall, Surrey, U.K.) and dispersed in cold incubation medium containing 123 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 20 mM Tris-HCl buffer, pH 7.5, 10 mM glucose, 12.5 µm nialamide (an inhibitor of MAO) and 1 mM ascorbic acid. The tissue suspension at a concentration of 1 mg/ml was then equilibrated at 37°C in

a Heto Denmark shaking water bath (Bach-Simpson Ltd., London, Ontario, Canada) for 15 min. Tritium-labelled DA, NE or 5-HT was added simultaneously with various concentrations of drug and the incubation continued for a further five min. Blank samples with no tissue and tissue samples with no drug added were included with each set of samples to obtain values for background radioactivity and for maximal uptake. The tissue was subsequently separated from the incubation medium by rapid filtration using a Millipore filtration device [Millipore (Canada) Ltd., Nepean, Ontario, Canada] and was washed twice with 5 ml of warm (37°C) incubation medium. The filter containing the tissue was then placed in a scintillation vial, and 10 ml of Ready Safe™ scintillation cocktail (Beckman Instruments Canada, Mississauga, Ontario, Canada) was added. The radioactivity was counted [in disassociations per minute (DPM)] on a Beckman LS 6000SC or a LS 7500 scintillation counter (Beckman Instruments Canada, Mississauga, Ontario, Canada).

A series of concentrations of *rac*-MDE and each of the individual enantiomers were studied, and the percentage of inhibition was plotted against drug concentrations (on a log scale) to determine the mean concentration giving 50% inhibition of uptake (IC_{50}) (n=5). Two-tailed t tests on the IC_{50} values were conducted between the racemate and each of the individual enantiomers of MDE and also between the R-(-)- and S-(+)-isomers (α=0.05).

A series of concentrations of *rac*-MDMA and each of the individual enantiomers of MDMA were also studied under the same experimental conditions to provide a basis for direct comparison between MDMA and MDE. Two-tailed t

tests on the IC_{50} values were conducted between the racemates of MDE and MDMA and between corresponding enantiomers (α=0.05).

3.6 **Behavioral and Neurochemical Studies**

The methodology developed by Martin-Iverson (1991) was applied to determine the effects of equimolar doses of racemic MDA, MDMA and MDE in contrast to S-(+)-AMPH and the hallucinogenic amphetamine analog, *rac*-PMA. The representative stimulant and hallucinogen were chosen on the basis of their similarity in core structure to the methylenedioxy analogues of AMPH. The dose of 32 μmoles/kg was chosen so that the dose used would be similar to that reported in other behavioral and neurochemical studies of the individual compounds (Shulgin *et al.*, 1969; Menon *et al.*, 1976; Shulgin, 1978; Shulgin and Nichols, 1978; Martin-Iverson *et al.*, 1991). The racemates of the methylenedioxy analogues of AMPH were used in this study to more closely resemble the situation when the drugs are used recreationally.

3.6.1 *Equipment*

Male Sprague-Dawley rats (250-400 g) were randomly assigned to drug or vehicle treatment groups (n=12). Each animal was used only once. The light/dark cycle was reversed so that the animals were in their active phase during the testing period. Animals were individually placed into one of 48 stainless steel cages (24 cm wide by 26 cm long),with one Plexiglass side and a wire-mesh floor for 10 days prior

to drug treatment to acclimatize them to the cages and the reversed light/dark cycle. In front of each cage was an infrared-sensitive CCD video camera (Canon Ci-20R) mounted to view one cage at a time. Each camera was equipped with a 12 mm auto-iris lens (Canon C 61205) and an infrared light source (IR-20W) that did not provide light in the visible spectrum. The video signal from each camera was forwarded to a video monitor (Hitachi VM-900), a time date generator (Pelco TD200DT), and an extended video cassette recorder (NEC PV-1200A), all of which were kept in a room adjacent to the testing room. The cages were also equipped with infrared photocell beams.

3.6.2 *Behavioral Procedure*

On the test day under infrared light, an equimolar dose of one of the five drugs (32 µmoles/kg dissolved in saline such that each rat received a 2ml/kg of drug solution) or saline was administered *via* ip injection. The rats were videotaped with infrared sensitive cameras using a time-sampling procedure: 2.5 min observation periods every 30 min for 1.5 h prior to and 3 h after injection, with a total post drug sampling time of 15 min. Photobeam interruption counts were collected at 30 min intervals for the 1.5 h prior to and the 3 h after drug administration.

3.6.3 *Neurochemical Procedure*

All animals were killed by guillotine decapitation immediately following their last taped observation period (at 3 h post drug). After midline sagittal splitting of the

brain, dissections were carried out on one of the halves to obtain hippocampal, hypothalamic, striatal and cortical tissues. The other half of the brain was kept for drug analysis. An equal proportion of right and left halves were used for the neurochemical and the drug level determinations. All tissues were stored at -80°C until assayed. The HPLC analyses were carried out using the method described by Baker *et al.* (1987) and were performed using a Waters (Milford, MA, USA) WISP 710B sample injector system and a model 510 pump, a Waters M460 detector and a glassy carbon electrode set at 0.88 V vs an Ag/AgCl reference electrode. The flow rate was set at 0.8 ml/min through a Phenomenex C18 column (4.6 mm × 250 mm: 5 µm particle size, CA, USA) coupled to a precolumn. The mobile phase consisted of $NaH_2PO_4 \cdot H_2O$ (55 mM), sodium octyl sulphate (0.85 mM), disodium EDTA (0.37 mM) and acetonitrile (10%). The mobile phase was filtered through a type HA filter (0.45 µm, Millipore) before being degassed and adjusted to a pH of 3 using phosphoric acid.

The concentrations of DA, NE and 5-HT and the acid metabolites of DA and 5-HT (5-HIAA, DOPAC and HVA) were determined by comparing peak areas for the regional brain samples to authentic standards processed in parallel with each assay (range 5-100 ng).

Drug levels were obtained using a modification of the GC assay described earlier. Due to an interference peak, benzylamine was substituted as the I.S. for the analysis of the levels of PMA. Drug levels were expressed as the sum of the two enantiomers, with the exception of AMPH, as only the S(+)-isomer was

administered.

3.6.4 *Data Analyses*

Videotape records were analyzed by an observer blind to the drug conditions of the animals and the purpose of the experiment. Frequency, duration and sequences of thirty different behaviors were scored with the Basic Experimental Behavioral Observation Program (BEBOP) [Table 1]. The clock in this program was linked to the time/date signal on the videotapes for timing behaviors. A key was pressed at the initiation and again at the termination of a bout of behavior, allowing determination of bout duration. Behavioral categories were not necessarily mutually exclusive. For example, locomote, sniff, head movement and snout contact with cage surface may all occur simultaneously. However, they are not likely to be initiated simultaneously. The program allowed editing of scoring, such that a tape could be rewound and replayed at normal or slow speeds until the observer was confident that the behaviors were accurately scored. To establish the level of intra-observer reliability, selected tapes were analyzed twice for the frequencies and durations of behaviours and frequencies of transitions and compared using Hoyt's reliability analysis.

Statistical analyses of the photobeam interruption counts and the frequencies and durations of behaviors were completed using ANOVA followed by multiple F-test comparisons (α=0.05). Principal components analysis was conducted on the totals of both the frequencies and the durations of the 17 behaviors that were signifi-

Table 1: Basic Experimental Behavioral Observation Program (BEBOP) coded behaviors and their operational definitions.

1. *Wet-dog shake:* abrupt shaking/shivering predominantly of the shoulders.
2. *Eating:* gnawing of food pellets.
3. *Backwards walking:* moving in reverse, using all 4 limbs with a result of altering the animal's position in the cage.
4. *Stretch:* extension of the forequarters with the forelimbs stretched out in advance of the animal.
5. *Yawn:* mouth gaping widely.
6. *Hindlimb scratch:* scratching of the body or head with one of the hindlimbs.
7. *Pause:* a momentary cessation of all active behaviours (e.g. sniffing, walking, but not of postural behaviours).
8. *Sniff:* rapid flaring and contracting of the nostrils accompanied by movements of the whiskers.
9. *Drink:* licking the water spout.
10. *Limb flick:* a rapid twitch of one of the limbs.
11. *Groom:* face, head or body stroking with the forelimbs, or licking of any part of the body.
12. *Head movements:* head weaving (lateral movements) or bobbing (vertical movements).
13. *Jump:* a leap upwards into the air such that all 4 limbs leave the cage floor at some point.
14. *Lick:* the tongue can be observed making contact with some surface other than the animal's own body.
15. *Locomote:* movement with all 4 limbs that alters the animal's position in the cage.
16. *Sleep:* eyes are closed, breathing is even and slow.
17. *Retrieve food:* reaching into the food bin with forepaws or mouth and bringing out a food pellet.
18. *Snout contact with a cage surface:* self-explanatory.
19. *Postural adjustment:* a movement of the trunk of the body that alters the posture of the animal excluding major transitions of posture (i.e. excluding changes between stand, sit, lie, locomote, rear, reverse locomote, jump). For example, a shift of the body while lying down so that a slightly different area of the body is in contact with the cage floor.
20. *Gnaw:* chewing on something in the cage other than food.
21. *Mouth movements:* jaws opening and closing in empty air (excludes gnawing, licking, eating and drinking).
22. *Can't tell:* the animal faces away from the camera, occluding the view so no adequate assessment of the behaviour can be determined.
23. *Rear:* the animal is standing with its hindlimbs extended to the floor and the forelimbs are in the air or resting on a wall.
24. *Lie:* the animal is lying down such that its legs are retracted close to the body or stretched out.
25. *Sit:* the animal is on its haunches, without supporting its body with its forelimbs.
26. *Stand:* all 4 limbs are extended and apparently supporting the weight of the animal.
27. *Twitch:* a sudden quick contraction of some part of the trunk or head.
28. *Scrabble:* pawing at the floor or wall.
29. *Clockwise rotation:* turn to the right of at least 90°.
30. *Counter-clockwise rotation:* turn to the left of at least 90°.

cantly affected by drug treatment. Seven factors were identified as accounting for 81% of the variance in the behaviors. These components were computed by summing the products of the correlation coefficients and the behavioral measures that contributed to the factor, and then subjected to ANOVA with drug as the independent factor. In two cases (factors 2 and 7), there was a lack of homogeneity of variances across groups. Therefore, the square roots of the values were used for the ANOVA.

Behavioral sequences were analyzed by the method of adjusted residuals, comparing the actual observed behavioral transitions with the random expected transitions, given the frequency of initiations of behavior (Haberman, 1973). Computer software for these analyses was developed by M.T. Martin-Iverson. Multiple correlations in terms of the ratios of the observed and expected transitions across treatment groups were determined.

Cluster analyses were carried out on the frequencies and durations of the behaviors significantly affected by drug treatment and on the ratios of the observed and expected transitions across treatment groups to further identify homogeneity among the drug groups. Agglomerative hierarchial clusterings were completed using the commonly used distance measure, the squared Euclidean distance, which is the sum of the squared differences over all the variables being considered. Squaring the differences gives more weight to greater differences. The Euclidean distances are plotted by rescaling the actual distances to numbers between 0 and 25 and are represented in the form of dendograms.

Statistical analyses on the neurochemical findings were conducted using

ANOVA followed by multiple F-test comparisons (α=0.05). 5-HT turnover was expressed as the ratio of the levels of 5-HT to 5-HIAA DA turnover was defined as the ratio of DA to the sum of the two DA metabolites, DOPAC and HVA All ANOVAs, principal components analyses and cluster analyses were carried out using commercial software (SPSSPC).

4 *RESULTS*

4.1 Metabolic Studies

4.1.1 Levels of MDMA and MDA in Rat Brain and Liver

Under the GC assay conditions described above, the enantiomers of MDMA and MDA were readily separated and quantitated. The standard curve was linear over the range of 50-750 ng, with a correlation coefficient > 0.99 obtained routinely. Mean recovery of 200 ng of MDMA was 89.2% and 88.9% for the R-(-) and S-(+) enantiomers. Recoveries of S-(+)-MDA and R-(-)-MDA under the same conditions were 95.2% and 98.0%, respectively. Typical chromatograms of derivatized brain extracts from rats treated with vehicle or *rac*-MDMA are shown in Figure 25. In Figures 26, 27 and 28 are shown the proposed mass fragmentations of the derivatives of MDMA, MDA and the I.S., *p*-CPEA, respectively. The proposed mass fragmentations of the derivatives of MDMA and MDA shown here are very similar to the mass spectra published by Fitzgerald *et al.* (1989a). Intra- and inter-assay coefficients of variation for the four enantiomers ranged from 1.2 to 10.9%, and these values are shown in Table 2.

Levels of the four enantiomers in whole brain are shown in Figure 29. Regression analysis revealed significant linear relationships [MDMA: $F(1,20)=39.4$; MDA: $F(1,20)=42.3$, $p<0.0001$)] between the R-(-)- and S-(+)-MDMA and between R-(-)- and S-(+)-MDA over time. With the exception of the 1 h time period, the levels of R-(-)-MDMA were significantly greater than those of S-(+)-MDMA. The reverse was seen with MDA, in that there was more S-(+)-MDA than R-(-)-MDA at the studied periods.

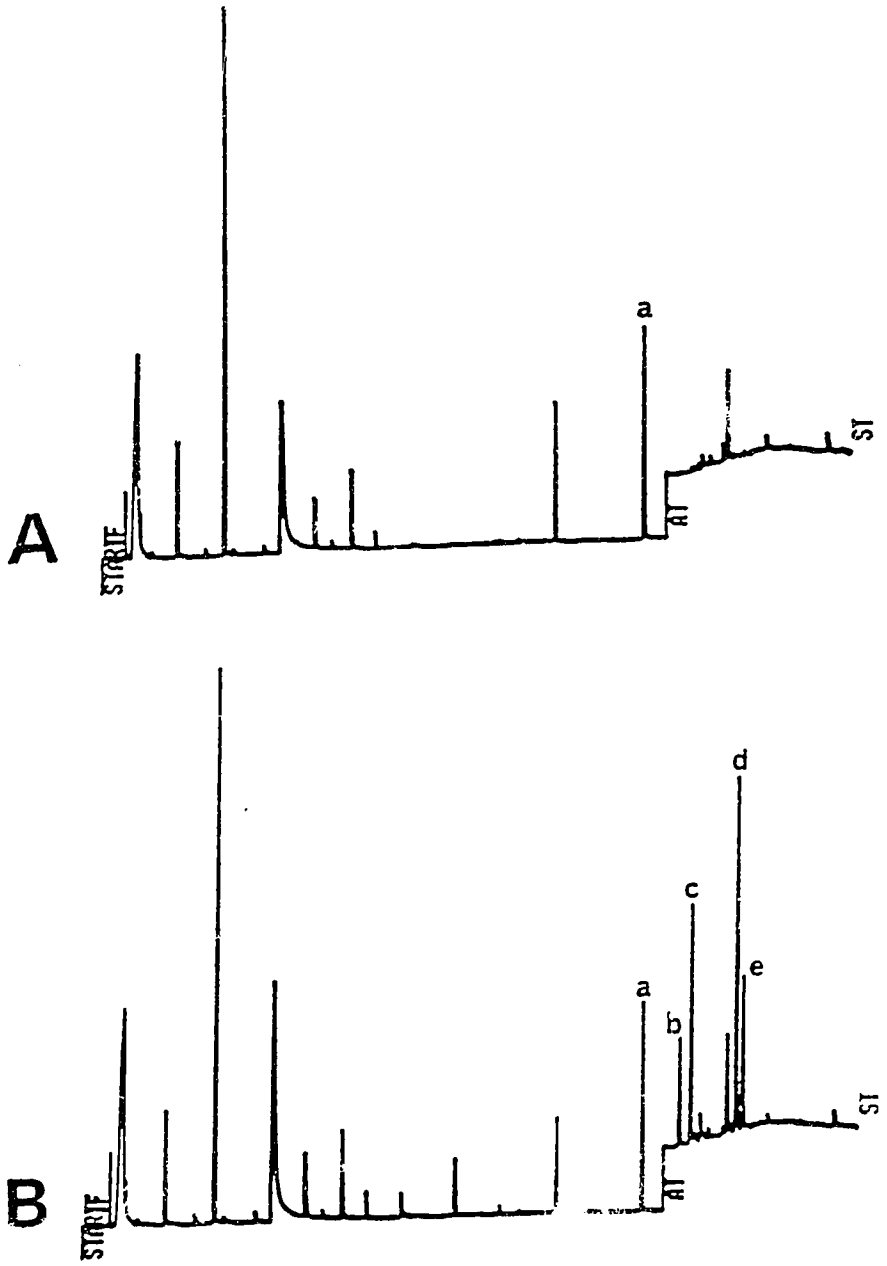


Figure 25: Typical chromatograms of derivatized brain extracts from rats treated with (A) vehicle or (B) *rac*-MDMA. Retention times (in min) of the derivatives: (a) *p*-chlorophenylethylamine 14.39; (b) R-(-)-MDA 15.41; (c) S-(+)-MDA 15.69; (d) R-(-)-MDMA 16.88; and (e) S-(+)-MDMA 16.96.

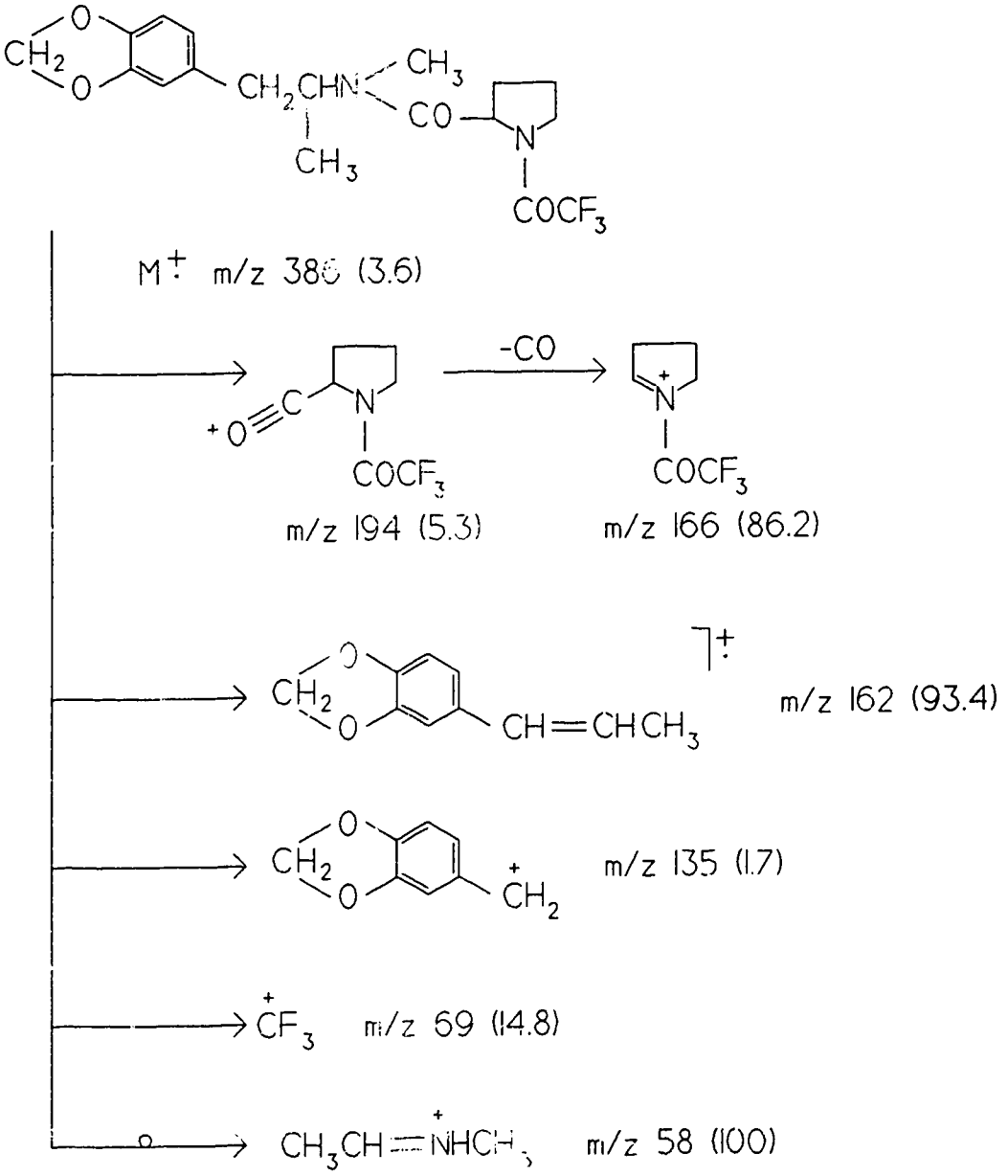


Figure 26: Proposed mass spectrometric fragmentation of the trifluoracetyl derivative of MDMA. Relative abundances (in %) are shown in parentheses.

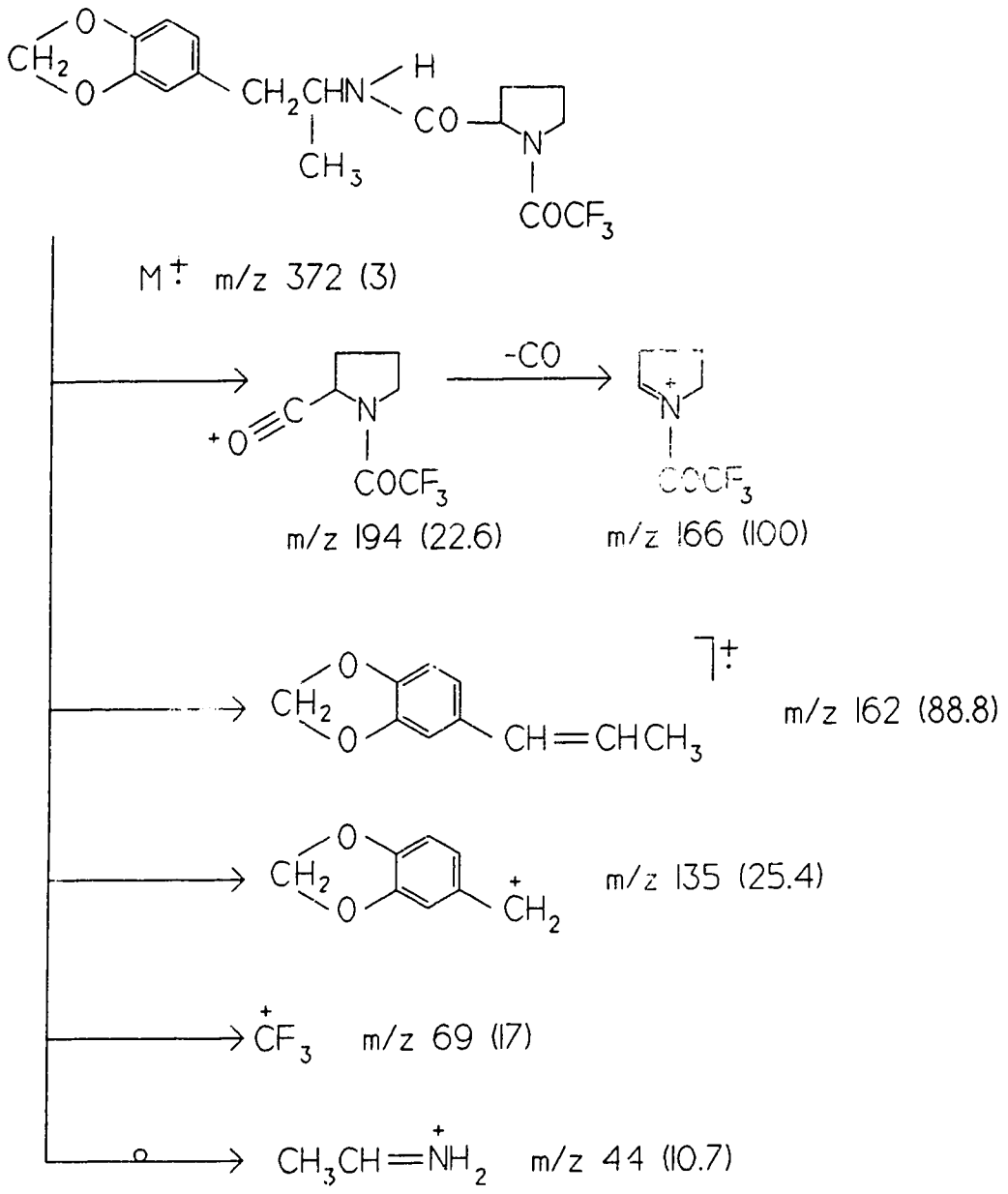


Figure 27: Proposed mass spectrometric fragmentation of the trifluoracetyl derivative of MDA. Relative abundances (in %) are shown in parentheses.

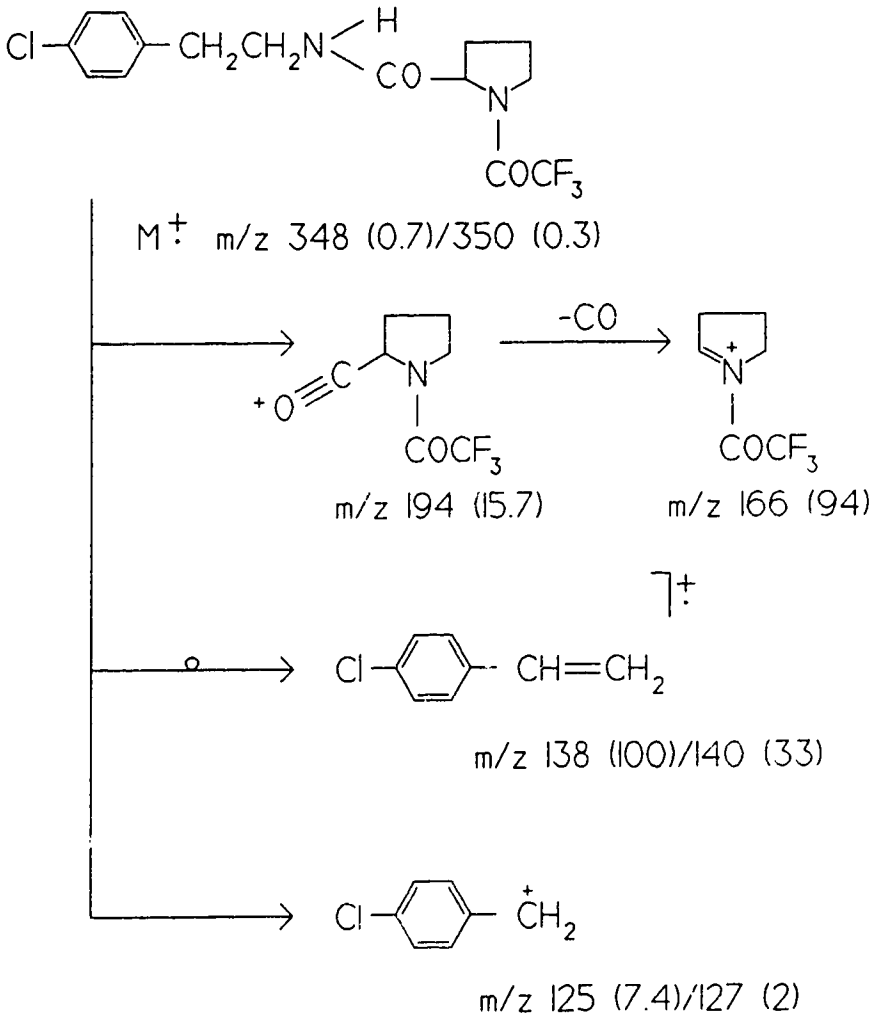


Figure 28: Proposed mass spectrometric fragmentation of the trifluoracetyl derivative of *p*-chlorophenylethylamine. Relative abundances (in %) are shown in parentheses.

(A)

Enantiomer	50 ng	100 ng	250 ng	500 ng
R-(-)-MDMA	10.9	5.9	5.0	3.1
S-(+)-MDMA	9.7	4.9	4.9	3.0
R-(-)-MDA	4.8	3.8	2.0	1.2
S-(+)-MDA	4.6	3.0	2.7	2.0

(B)

Enantiomer	50 ng	100 ng	250 ng	500 ng
R-(-)-MDMA	9.7	7.8	9.8	9.3
S-(+)-MDMA	5.6	7.8	8.8	8.9
R-(-)-MDA	5.8	6.9	3.9	6.7
S-(+)-MDA	6.2	7.6	3.7	6.0

Table 2: (A) Coefficients of variation (%) for the MDMA/MDA assay: intra-assay, n=6. (B) Coefficients of variation (%) for the MDMA/MDA assay: Inte assay, n=11.

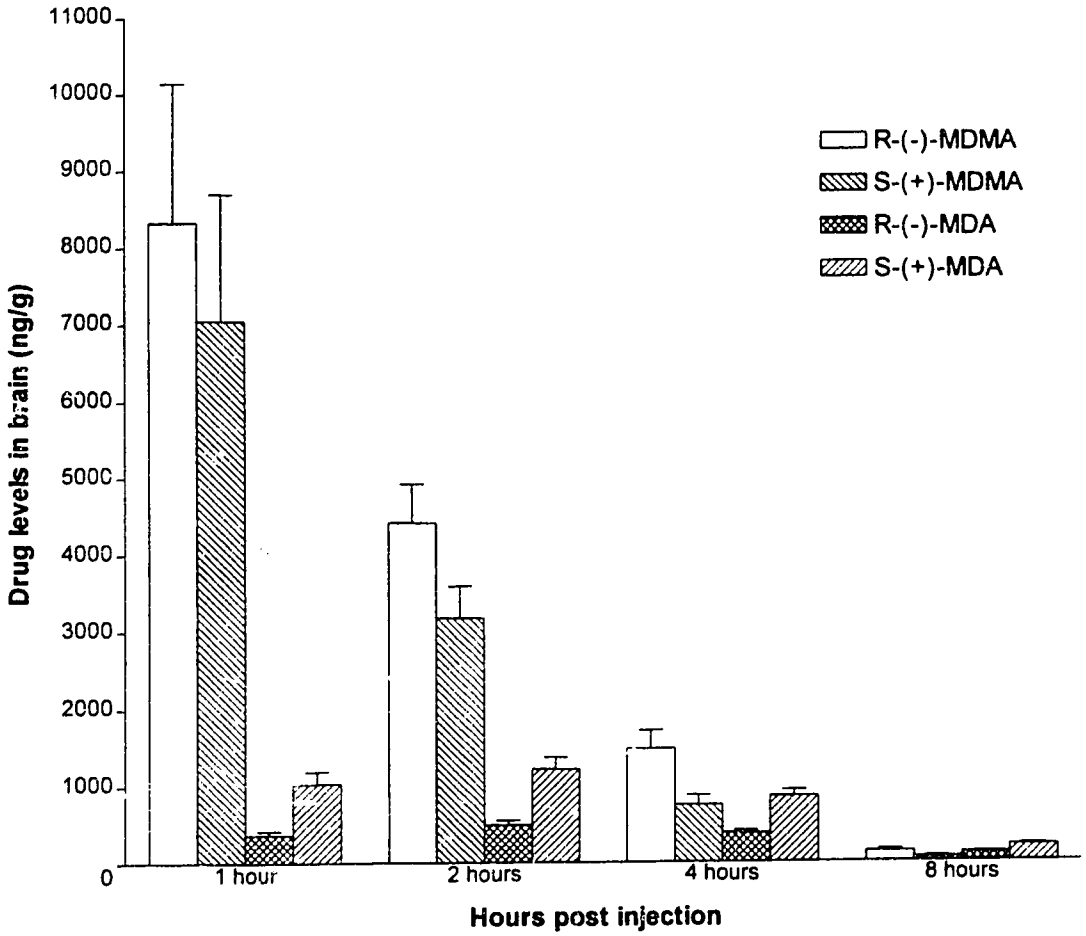


Figure 29: Whole brain levels of the enantiomers of MDMA and MDA after acute injections of *rac*-MDMA (10 mg/kg ip). Values represent means ± SEM (n=6).

The pattern of decline of the levels of S-(+)-MDMA and S-(+)-MDA compared to the pattern seen with R-(-)-MDMA and R-(-)-MDA was different. At 1 and 2 h, there was significantly more S(+)-MDMA than S-(+)-MDA. By 4 h, the difference is no longer significant and by 8 h the pattern had reversed, with significantly more S-(+)-MDA than S-(+)-MDMA. In the case of R-(-)-MDMA and R-(-)-MDA, R-(-)-MDMA remained higher than R-(-)-MDA levels until the 8 h period.

Regional analyses of drug levels 2 h post injection are shown in Table 3. The same pattern of ratios of the enantiomers was observed in the regions as in whole brain, i.e. levels of R-(-)-MDMA were higher than those of S-(+)-MDMA, while the reverse was true for the enantiomers of MDA. The highest drug levels of S-(+)-MDMA and R-(-)-MDMA were in the cortex, the striatum and the hippocampus.

Levels of S-(+)- and R-(-)-MDMA and S-(+)- and R-(-)-MDA in liver are shown in Figure 30. The overall levels of the enantiomers are much lower than was seen in whole brain tissue at the 1, 2, 4 h time periods. The highest levels of R-(-)-MDMA in liver was reached at 2 h, whereas in whole brain the highest level was seen at 1 h. The same relationships between the pairs of enantiomers were seen in liver tissues as were seen in the brain tissues, with more R-(-)-MDMA and S-(+)-MDA than their antipodes. By 8 h, there remained very little R-(-)-MDMA, no S-(+)-MDMA detectable and similar levels of S-(+)- and R-(-)-MDA to those seen in whole brain.

	R-(-)-MDMA	S-(+)-MDMA	R-(-)-MDA	S-(+)-MDA
Whole brain	4412 ± 502	3172 ± 414	489 ± 54	1210 ± 155
Cortex	5994 ± 1318	4232 ± 922	510 ± 107	1289 ± 244
Hypothalamus	3804 ± 354	3010 ± 92	1730 ± 894	2300 ± 1084
Striatum	5529 ± 1108	2584 ± 392	897 ± 50	1862 ± 440
Hippocampus	5114 ± 758	3414 ± 549	986 ± 90	1547 ± 149
Pons	2311 ± 292	1492 ± 153	277 ± 39	658 ± 79
Cerebellum	2887 ± 264	1608 ± 320	272 ± 19	663 ± 32
Rest of Brain	4470 ± 2378	3183 ± 1634	459 ± 153	1153 ± 23

Table 3: Enantiomers of MDMA and MDA in whole brain and brain regions 2 h after ip injection of *rac*-MDMA (10 mg/kg). Values (in ng/g of tissue) represent means ±SEM (n=6).

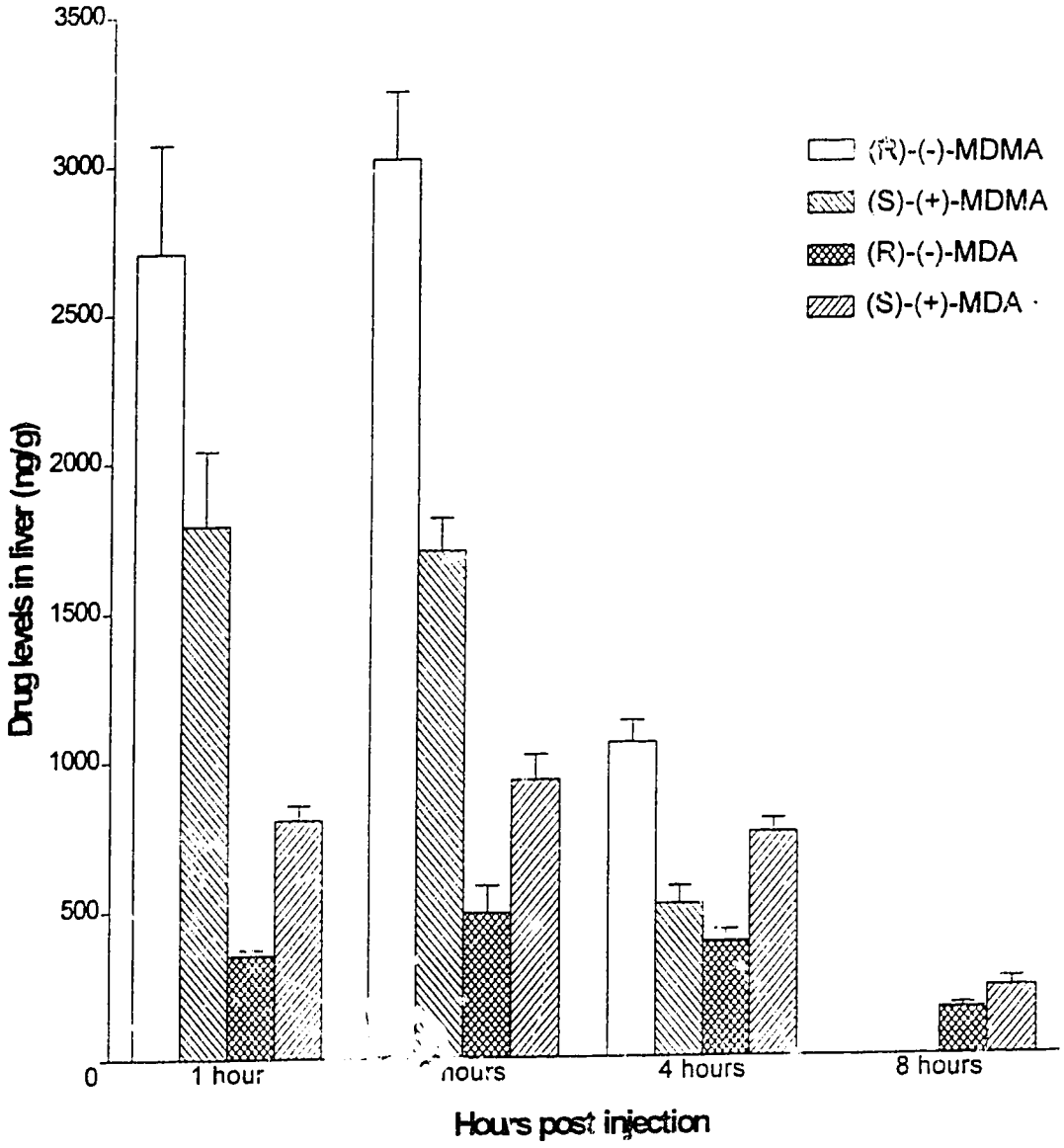


Figure 30: Hepatic levels of the enantiomers of MDMA and MDA after acute injections of *rac*-MDMA (10 mg/kg ip). Values represent means ± SEM (n=6).

4.1.2 *Levels of the Enantiomers of MDE and MDA in Rat Brain*

With the modifications as described above, the enantiomers of MDE and MDA were also readily separated and quantitated. Over a range of 25-200 ng for R-(-)- and S-(+)-MDE and 10-250 ng for R-(-)- and S-(+)-MDA, the standard curves were linear, with correlation coefficients > 0.99 obtained routinely. The degree of impurity in standards prepared from the four individual enantiomers 6 months after the derivatizing reagent bottle had been opened was found to range from 9-11%. Brain concentrations calculated from the calibration curves were adjusted to take into account the small quantities of impurity found when each of the enantiomers were assayed separately. The mean recovery rates of 300 ng and 25 ng of MDE (n=4) were R-(-)=89.5% and 83.6%; S-(+)=91.2% and 84.9%, respectively. Recoveries of R-(-)- and S-(+)-MDA were reported with the MDMA data. Typical chromatographs of derivitized brain extracts from rats treated with vehicle or *rac*-MDE are shown in Figure 31. A peak representing an endogenous unidentified substance was present immediately in front of derivitized R-(-)-MDA, but did not interfere with the determination of peak heights. The proposed mass fragmentation of the derivitive of MDE is depicted in Figure 32. Intra- and inter-assay coefficients of variation for R-(-)- and S-(+)-MDE at concentrations of 25 to 500 ng (N=6) ranged from 4.1 to 10.2% and 9.5 to 11.6%, respectively (Table 4). The derivatized samples, stored at -80°C and reinjected into the gas chromatograph after 2, 6, 10 and 14 days, provided almost identical data, indicating that the solutions of analytes remained stable over the 2 week period.

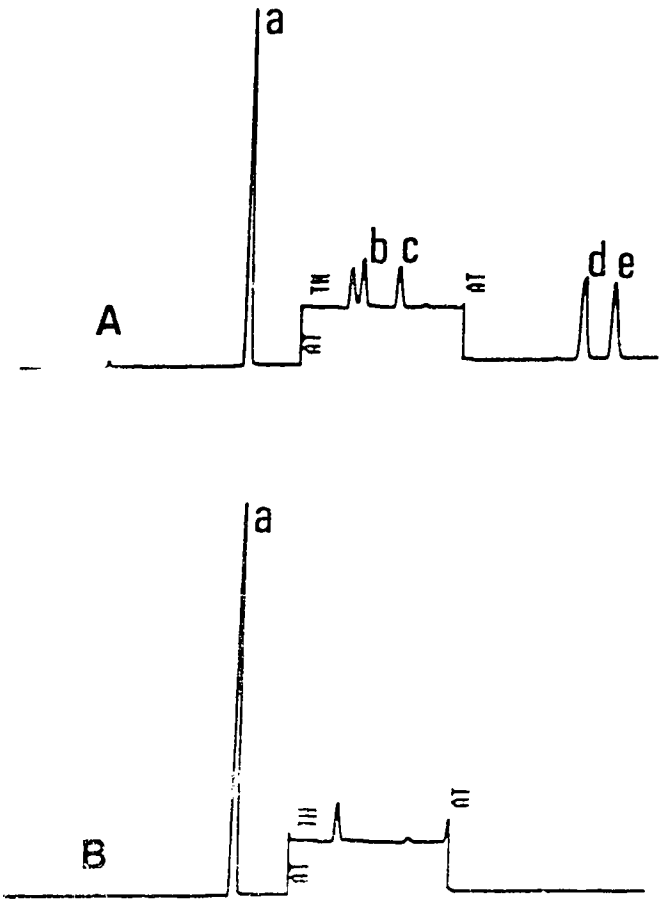


Figure 31: Typical chromatograms of derivatized brain extracts from rats treated with (A) *rac*-MDE or (B) vehicle. Retention times (in min) of the derivatives: (a)*p*-chlorophenylethylamine 18.62; (b) R-(-)-MDA 20.15; (c) S-(+)-MDA 20.58; (d) R-(-)-MDE 23.40; and (e) S-(+)-MDE 24.00.

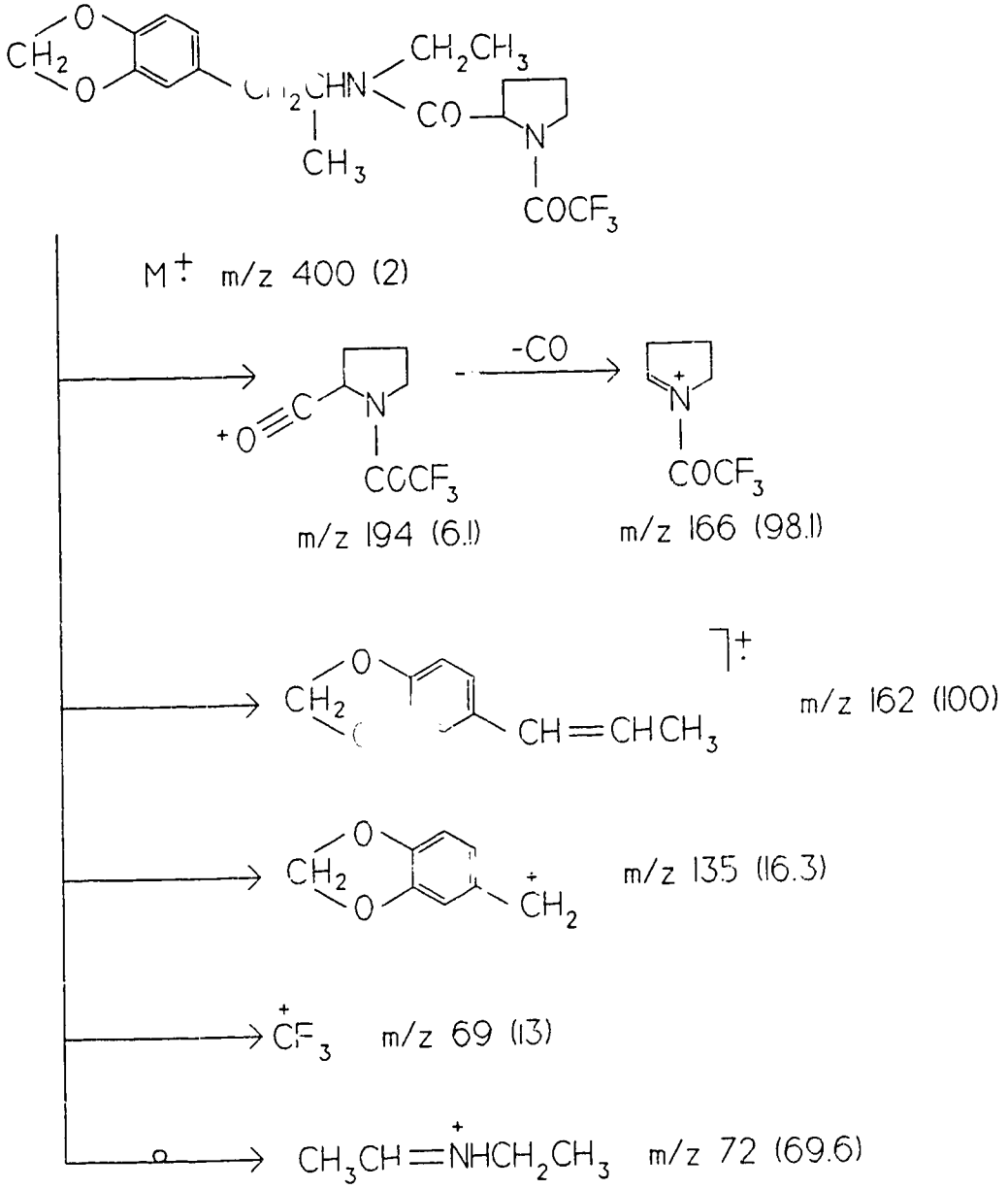


Figure 32: Proposed mass spectrometric fragmentation of the trifluoroacetyl derivative of MDE. Relative abundances (in %) are shown in parentheses.

(A)

Enantiomer	250 ng	10 ng
R-(-)-MDE	4.1	9.7
S-(+)-MDE	4.9	10.2
R-(-)-MDA	3.5	5.1
S-(+)-MDA	2.7	8.9

(B)

Enantiomer	50 ng	100 ng	250 ng	500 ng
R-(-)-MDE	9.8	8.7	11.7	9.5
S-(+)-MDE	10.2	10.1	11.7	11.6
R-(-)-MDA	5.1	4.4	3.5	3.4
S-(+)-MDA	8.9	6.2	5.5	4.9

Table 4: (A) Coefficients of variation (%) for the MDE/MDA assay: Intra-assay, n=6. (B) Coefficients of variation (%) for the MDE/MDA assay: Inter-assay, n=6.

In the confirmatory experiment at 1 h in which the analyses were done with both TFPC and HFPC, the levels of the enantiomers of MDE and MDA and the ratios to each other were very similar (R/S ratios for MDE of 0.97 and 0.98, respectively and R/S ratios for MDA of 1.26 and 1.34, respectively).

Levels of the four enantiomers in whole brain are shown in Figure 33. By 8 h, trace amounts of MDE and MDA were seen but were below the limits of detection. The levels of both MDE enantiomers at 1 h and 4 h were considerably lower than the levels of MDMA reported above after an equal dose (10 mg/kg) of *rac*-MDMA. At 2 h post injection, the levels of both MDE enantiomers were lower than those of R-(-)-MDMA but not S-(+)-MDMA. There was significant MDA, especially the S-(+)-enantiomer, seen after MDE administration than was seen with MDMA.

The ratios in brain of the levels of the R-(-)- and S-(+)-enantiomers of MDE and MDA after *rac*-MDE administration were markedly different than what had been previously reported after MDMA administration (Table 5). At 1 h, there was no difference between the levels of R-(-)- and S-(+)-MDE and small but significant differences between the levels of R-(-)- and S-(+)-MDA, with R-(-) greater than S-(+) [MDE: $F(1,18)=87.5$; MDA: $F(1,18)=17.7$; $p<0.05$]. At 2 h, there were small but significant stereoselective differences in the levels of both MDE and MDA, with more S-(+)-MDE and R-(-)-MDA than their antipodes. At 4 h, the difference between the levels of R-(-)- and S-(+)-MDA was no longer significant. Not only were the enantioselective differences much smaller in the case of MDE compared to

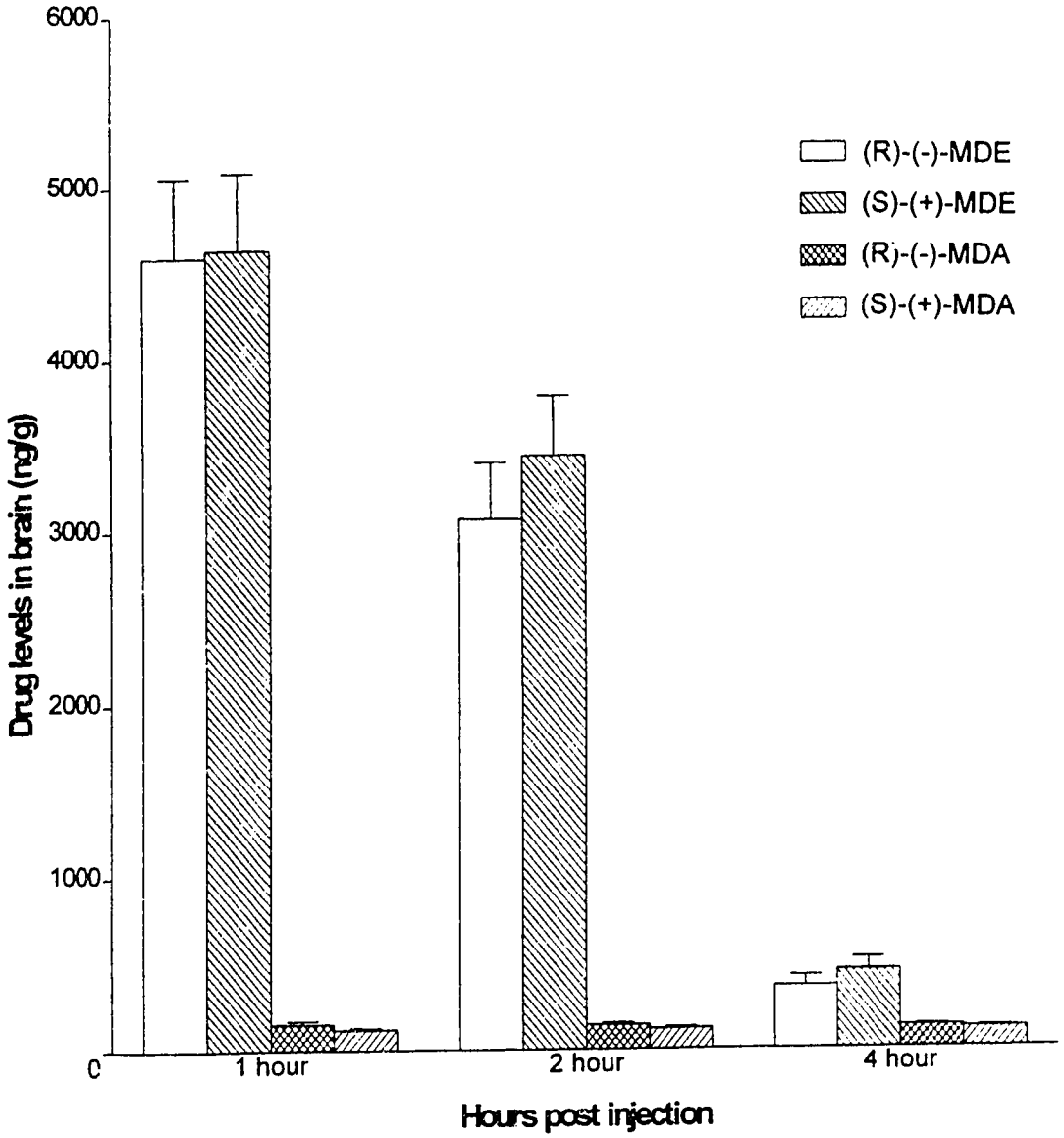


Figure 33: Whole brain levels of the enantiomers of MDE and MDA after acute injections of *rac*-MDE (10 mg/kg ip). Values represent means ± SEM (n=7).

(A)	1 Hour	2 Hour	4 Hour
(R)/(S)-MDE	1.01 ± 0.009	0.89 ± 0.005*	0.79 ± 0.013*
(R)/(S)-MDA	1.31 ± 0.076*	1.21 ± 0.086*	1.11 ± 0.074
(B)			
(R)/(S)-MDMA	1.20 ± 0.03*	1.40 ± 0.048*	2.16 ± 0.27*
(R)/(S)-MDA	0.36 ± 0.015*	0.41 ± 0.018*	0.44 ± 0.013*

Table 5: Enantiomeric ratios [R-(-)/S-(+)] of both parent drug and metabolite in rat brain after administration of either (A) *rac*-MDE or (B) *rac*-MDMA (10 mg/kg ip). Results are expressed as means ± SEM (n=6 or 7). * Indicates significant difference between levels of R- (-) and S-(+) enantiomers ($p<0.05$).

MDMA, with regard to both the parent drugs and the dealkylated metabolite formed from them, the pattern of differences was reversed.

4.1.3 *In Vitro Metabolic Studies with Specific CYP450 Isozymes*

In the first series of experiments with CYP2D6, S-(+)-MDE was shown to be the best substrate for N-dealkylation compared with the other substrates under the conditions described (Table 6). The metabolite/parent ratio for IMI was similar to the ratio seen with S-(+)-MDE and was at least twice that of the values obtained for AMI, both enantiomers of MDMA and R-(-)-MDE. The enantioselectivity reflected in the ratios was the same for both MDMA and MDE [S-(+) > R-(-)], however, MDE displayed a greater degree of enantioselectivity than did MDMA.

In the second series of experiments, CYP2D6 was shown to be superior to CYP3A4 in its ability to catalyze the N-dealkylation of IMI, both enantiomers of MDMA and S-(+)-MDE under the same experimental conditions. With AMI and R-(-)-MDE, there was little difference between the ratios obtained with CYP2D6 than those obtained with CYP3A4. In the presence of either isozyme and using AMI or *rac*-MDMA as substrates, the N-dealkylated metabolites were less than 1% of the level of the parent drug. This same low level of metabolite formation was also seen with R-(-)-MDE. A lower degree of enantioselectivity was seen between S-(+)-MDE and R-(-)-MDE when CYP3A4 was used compared to the results obtained with CYP2D6, although the S-(+) > R-(-) relationship was retained. R-(-)-MDMA was not detected.

SUBSTRATE 40 NMOLES	CYP ISOZYME 50µL/1000 µL	PHOSPHOLIPIDS	INCUBATION TIME (h)	METABOLITE/ PARENT RATIO[1]	METABOLITE/ PARENT RATIO[2]
IMI	2D6	NO	1	0.0165±0.0006	
AMI	2D6	NO	1	0.0071±0.0004	
rac-MDMA	2D6	NO	1	0.0024±0.0006	0.0064±0.0008
rac-MDE	2D6	NO	1	0.0041±0.0003	0.0222±0.0008
IMI	3A4	NO	1	0.0049±0.0006	
AMI	3A4	NO	1	0.0087±0.0006	
rac-MDMA	3A4	NO	1	ND	0.0013±0.0004
rac-MDE	3A4	NO	1	0.0043±0.0007	0.0075±0.0005

Table 6: Metabolite/parent ratios of potential substrates for *in vitro* N-dealkylation in the presence of CYP2D6 or CYP3A4. Values are means ± SEM (n=4). [1]Values of nonchiral substrates or values of the ratios of the R-(-)-isomers of the metabolite and the parent. [2]Values of the ratios of the S-(+)-isomers of the metabolite and the parent.

The studies with CYP3A4 using nifedepine and lidocaine as model substrates and comparing them with *rac*-MDMA and *rac*-MDE under identical experimental conditions are summarized in Table 7. Nifedepine was by far the superior substrate for CYP3A4, requiring less enzyme and no additions to the incubation mixture to yield substantial amounts of nitropyridine. When control enzyme (the cell line containing the expression vector without cDNA) was substituted for CYP3A4 in parallel samples, there was a small amount of nitropyridine detected (metabolite/parent ratio = 0.11±0.01, n=6). This was subtracted from the results obtained from incubation with CYP3A4. No metabolites were detected after incubation of the other substrates with control enzyme.

With increasing the amount of enzyme, lengthening the incubation time and adding phosphotidylserine and cholic acid, lidocaine deethylation was observed. The metabolite/parent ratio was at least five times those seen with IMI or AMI. No MDA was detected from incubating *rac*-MDMA with the isozymes under either of the parallel experimental conditions to that of nifedepine or lidocaine. However, small amounts of R-(-)- and S-(+)-MDA were detected when *rac*-MDE was used as the substrate. The metabolite/parent ratios obtained with *rac*-MDE under the same experimental conditions as lidocaine were larger than were seen with *rac*-MDE under the same experimental conditions as nifedepine, even though the amount of substrate was smaller.

SUBSTRATE IN NMOLES	CYP3A4 µL/µL FINAL VOL	PHOSPHOLIPIDS	INCUBATION TIME (h)	METABOLITE/ PARENT RATIO[1]	METABOLITE/ PARENT RATIO[2]
NIFEDEPINE 28.9	25/500	NO	1	8.227±0.95	
MDMA 28.9	25/500	NO	1	ND	ND
MDE 28.9	25/500	NO	1	0.0032±0.0003	0.007±0.0003
LIDOCAINE 12.9	50/500	YES	2.5	0.103±0.006	
MDMA 12.9	50/500	YES	2.5	ND	ND
MDE 12.9	50/500	YES	2.5	0.0061±0.0003	0.0185±0.0006

Table 7: Metabolite/parent ratios of potential substrates of CYP3A4 under differing *in vitro* incubation conditions. Values are means ± SEM (n=4-6). [1]Values of nonchiral substrates or values of the ratios of the R-(-)-isomers of the metabolite and the parent. [2]Values of the ratios of the S-(+)-isomers of the metabolite and the parent.

4.2 Inhibition of Uptake of Radiolabelled Biogenic Amines

The IC_{50} values for the racemate and the separate enantiomers of MDE in rat brain prisms prepared from hypothalamic tissue in the case of ^{3}H-NE and striatal tissue for ^{3}H-DA and ^{3}H-5-HT are shown in Table 8. The potency of MDE at inhibiting the uptake of the biogenic amines was 5-HT>NE>DA. S-(+)-MDE was a more potent inhibitor of the uptake of each of the biogenic amines than its antipode, especially in inhibiting the uptake of ^{3}H-5-HT, where S-(+)-MDE was 8 times more potent than R-(-)-MDE. S-(+)-MDE was more potent than *rac*-MDE at inhibiting the uptake of 5-HT but was not significantly different from *rac*-MDE in its ability to inhibit the uptake of DA and NE. Although the differences in the IC_{50} values for uptake inhibition of DA between *rac*-MDE and S-(+)-MDE were not statistically significant, there was a trend towards *rac*-MDE being more potent than S-(+)-MDE. This trend was also observed when *rac*-MDE, R-(-)-MDE and S-(+)-MDE at equimolar concentrations of 2 μM were used in the uptake procedure with ^{3}H-DA (n=5).

The comparative IC_{50} values for uptake inhibition by MDMA under the same experimental conditions are also shown in Table 8. *Rac*-MDMA and *rac*-MDE were equipotent at inhibiting the uptake of 5-HT and NE, but *rac*-MDMA was slightly more potent than *r*ac-MDE at uptake inhibition of DA. Interestingly, the trend toward the racemate being more potent than the S-(+)-isomer at inhibiting the uptake of DA seen with MDE was also seen with MDMA. The S-(+)-isomers of MDE and MDMA were compared and significant potency differences in uptake inhibition were found: (a) for DA, MDMA > MDE; (b) for 5-HT, MDE > MDA; and (c) for NE, MDMA > MDE.

(A)	*rac*-MDE	R-(-)-MDE	S-(+)-MDE
^{3}H-DA	1.67 ± 0.15	7.13 ± 1.17[a,b]	2.00 ± 0.15
^{3}H-5-HT	0.38 ± 0.04	1.25 ± 0.08[a,b]	0.16 ± 0.01[a]
^{3}H-NE	0.98 ± 0.07	2.04 ± 0.42[a,b]	0.81 ± 0.07
(B)	*rac*-MDMA	R-(-)-MDMA	S-(+)-MDMA
^{3}H-DA	1.00 ± 0.07[c]	9.67 ± 0.83[a,b,c]	1.18 ± 0.05[c]
^{3}H-5-HT	0.40 ± 0.05	2.34 ± 0.10[a,b,c]	0.40 ± 0.03[c]
^{3}H-NE	0.92 ± 0.06	1.68 ± 0.20[a,b]	0.45 ± 0.04[a,c]

Table 8: IC_{50} values (in μM) for the racemates and the individual enantiomers of (A) MDE and (B) MDMA in rat brain prisms prepared from hypothalamic tissues (in the case of ^{3}H-NE uptake) and striatal tissue (for ^{3}H-5-HT and ^{3}H-DA uptake). [a] Indicates significant difference between an individual enantiomer and the corresponding racemate. [b] Indicates significant difference between R-(-)- and S-(+)-MDE or R-(-)- and S-(+)-MDMA. [c] Indicates significant difference between the racemates of MDE and MDMA or between the R-(-) or S-(+) isomers of MDE and MDMA ($p<0.05$), n=5-9.

The R-(-) isomers also displayed significant potency differences, with MDE > MDMA at both DA and 5-HT uptake inhibition. The R-(-) isomers of MDE and MDMA were shown to be the weakest inhibitors of labelled neurotransmitter uptake, especially at inhibiting the uptake of DA. The potency differences between *rac*-MDMA and *rac*-MDE and between the R-(-) and S-(+) isomers were confirmed by conducting uptake procedures with both compounds at the same time using equimolar concentrations close to the calculated IC_{50} values obtained from the uptake experiments with each compound separately.

4.3 **Behavioral and Neurochemical Studies**

4.3.1 *Intra-Observer Reliability in Behavioral Studies*

The results of Hoyt's Reliability analysis indicated that reliability coefficients (r^2) were 0.996 for frequencies [standard error (SE) of measurements=±1.02), 0.9996 for durations (SE of measurement=±3.07) and 0.960 for sequences (SE for measurements=±1.2).

4.3.2 *Locomotor Activity*

Locomotor activity, as measured by photobeam interruption counts, is shown on Figure 33. There were no significant differences among the 6 treatment groups in the 1.5 h predrug time period [F (1,66)=1.60, p>0.15]. By 90 min after drug administration, 3 distinct patterns emerged and continued throughout the remaining time periods. As expected, S-(+)-AMPH resulted in the largest increase, which peaked at 120 min after drug, and remained above vehicle counts the entire study

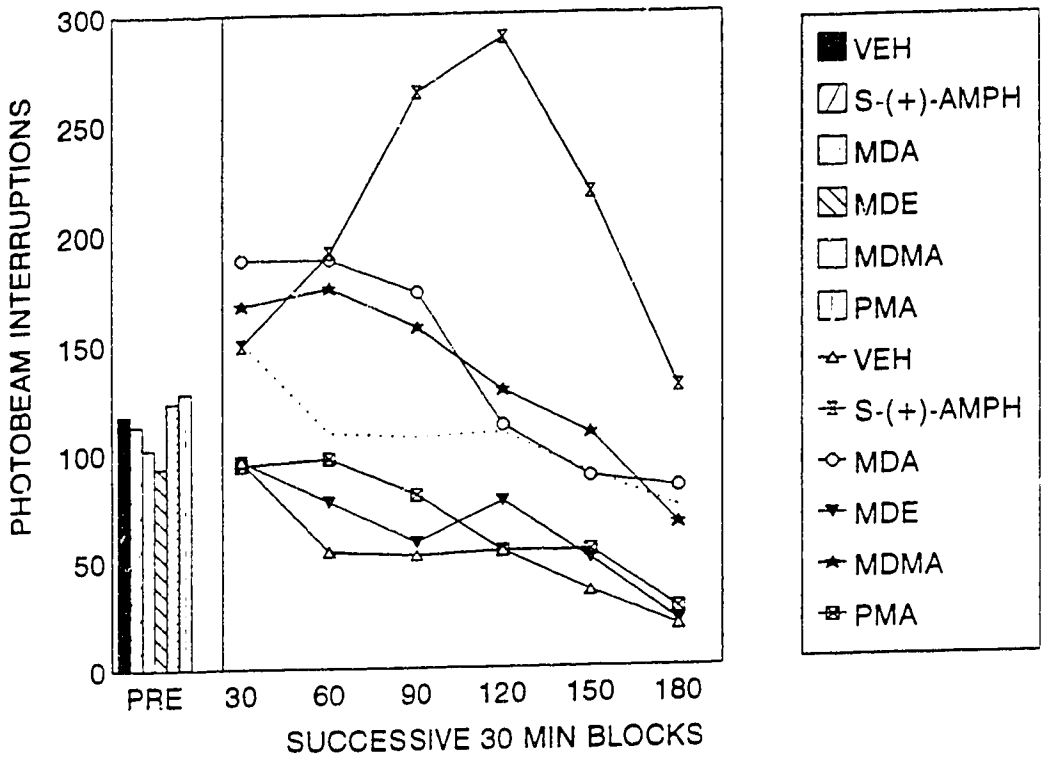


Figure 34: Photobeam interruption counts at successive 30 min blocks for the six drug treatment groups (32 μmoles/kg ip) [n=12 for each group]. Bars on left represent total counts for the 1.5 h prior to drug treatment. Dotted line represents minimum counts required to achieve statistical significance ($p<0.05$).

period. Both MDMA and MDA caused a rapid increase over the first 30 min, then fell to almost vehicle levels by 180 min. Neither PMA nor MDE produced any significant change from vehicle at any of the time periods.

4.3.3 *Principal Components*

Factor 1 is labelled "large body movements" since it included locomote, rear and clockwise rotation. No significant drug effects were seen [$F(1,66)=0.35$, $p>0.5$]. Factor 2, labelled "stimulus-induced behaviors", included jumping, retrieval of food and scratching and is shown in Figure 35. MDE was the only drug that did not significantly decrease these behaviors [$F(1,66)=10.5$, $p<0.001$]. Factor 3, labelled "olfactory exploration", included sniffing, head movements, snout contact with the cage surface and standing (Figure 36). The same pattern of drug groupings was seen as in the photobeam count data, with S-(+)-AMPH > MDMA = MDA > MDE = PMA. Factor 4 involved "yawning behaviors" (yawning, mouth movements, lying down and stretching) [Figure 37]. S-(+)-AMPH produced the greatest decrease; MDMA and MDA also caused significant decreases and MDE and PMA had no significant effects [$F(1,66)=5.7$, $p<0.001$]. Factor 5, which was limited to grooming only, was not affected by drug treatment [$F(1,66)=1.9$, $p>0.1$]. Factor 6, labelled "postural adjustment" (Figure 38), included body adjustments and clockwise rotations. Here, the difference between S-(+)-AMPH and the other compounds was not one of potency, but of the direction of drug effect: S-(+)-AMPH significantly decreased these behaviors; MDMA, MDA and MDE all resulted in increases; and PMA had no significant effect [$F(1,66)=8.7$, $p<0.001$]. Factor 7 involved sleep. All drug treatments caused a significant decrease in sleep [$F91,66)=5.72$, $p<0.001$].

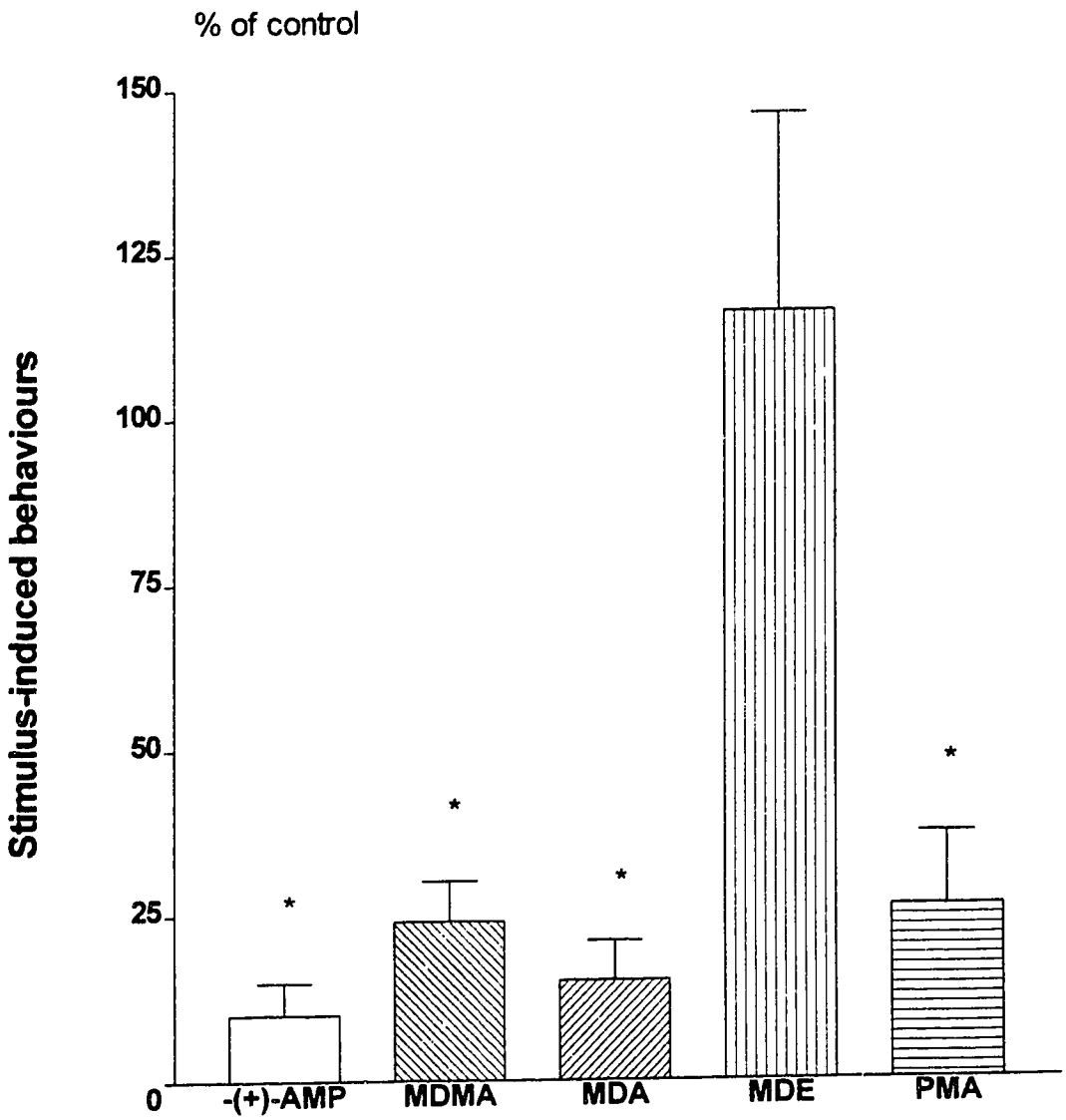


Figure 35: Factor 2 from principal component analysis of behavior following acute drug administration (32 μmoles/kg ip): stimulus-induced behaviors (including jumping, retrieval of food and scratching) expressed as mean % ± SEM of control. * indicates significant difference from control ($p<0.05$).

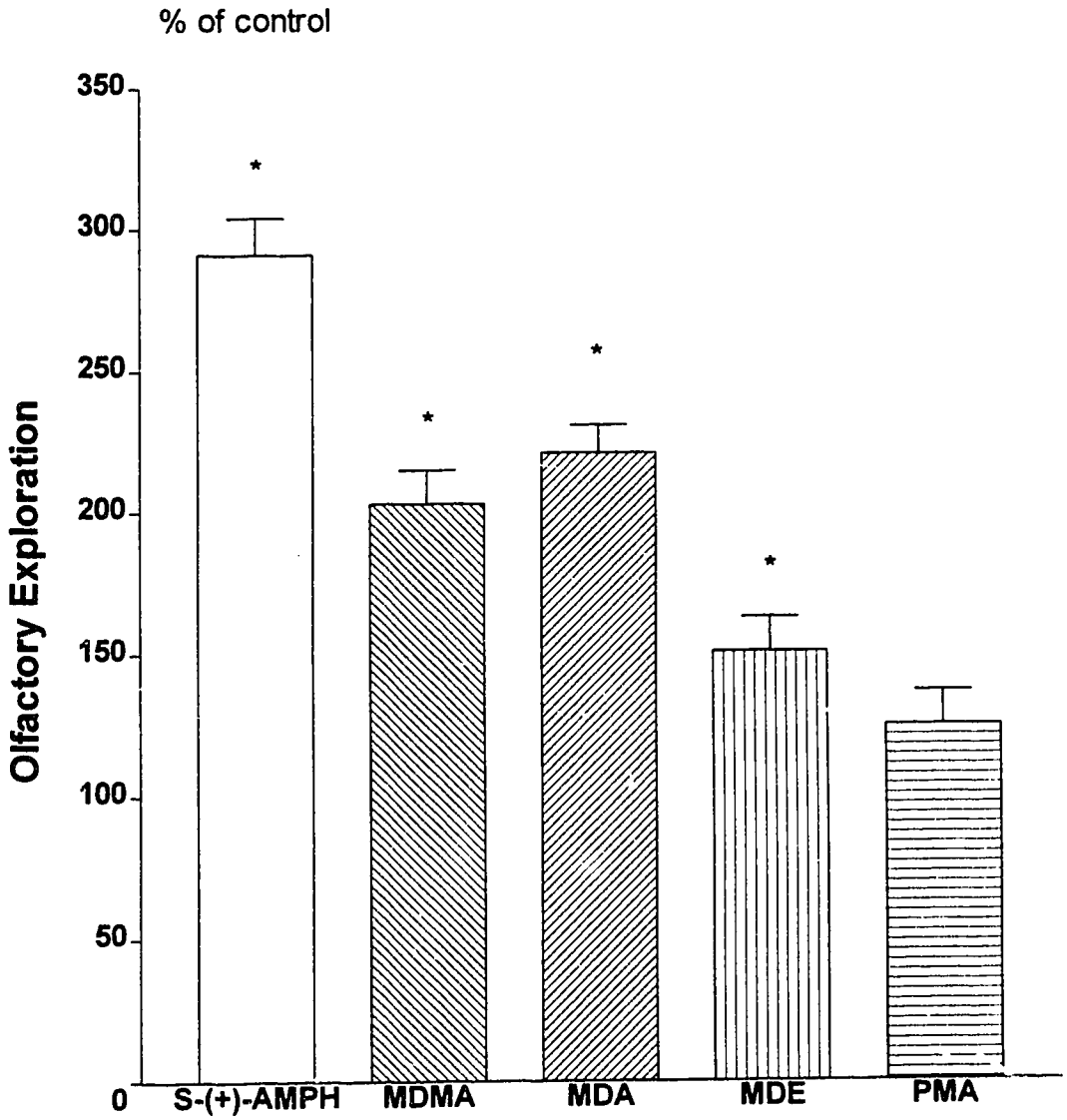


Figure 36: Factor 3 from principal component analysis of behavior following acute drug administration (32 μmoles/kg ip): olfactory exploration behaviors (including sniffing, head movements, snout contact with the cage surface and standing) expressed as mean % ± SEM of control. * indicates significant difference from control ($p<0.05$).

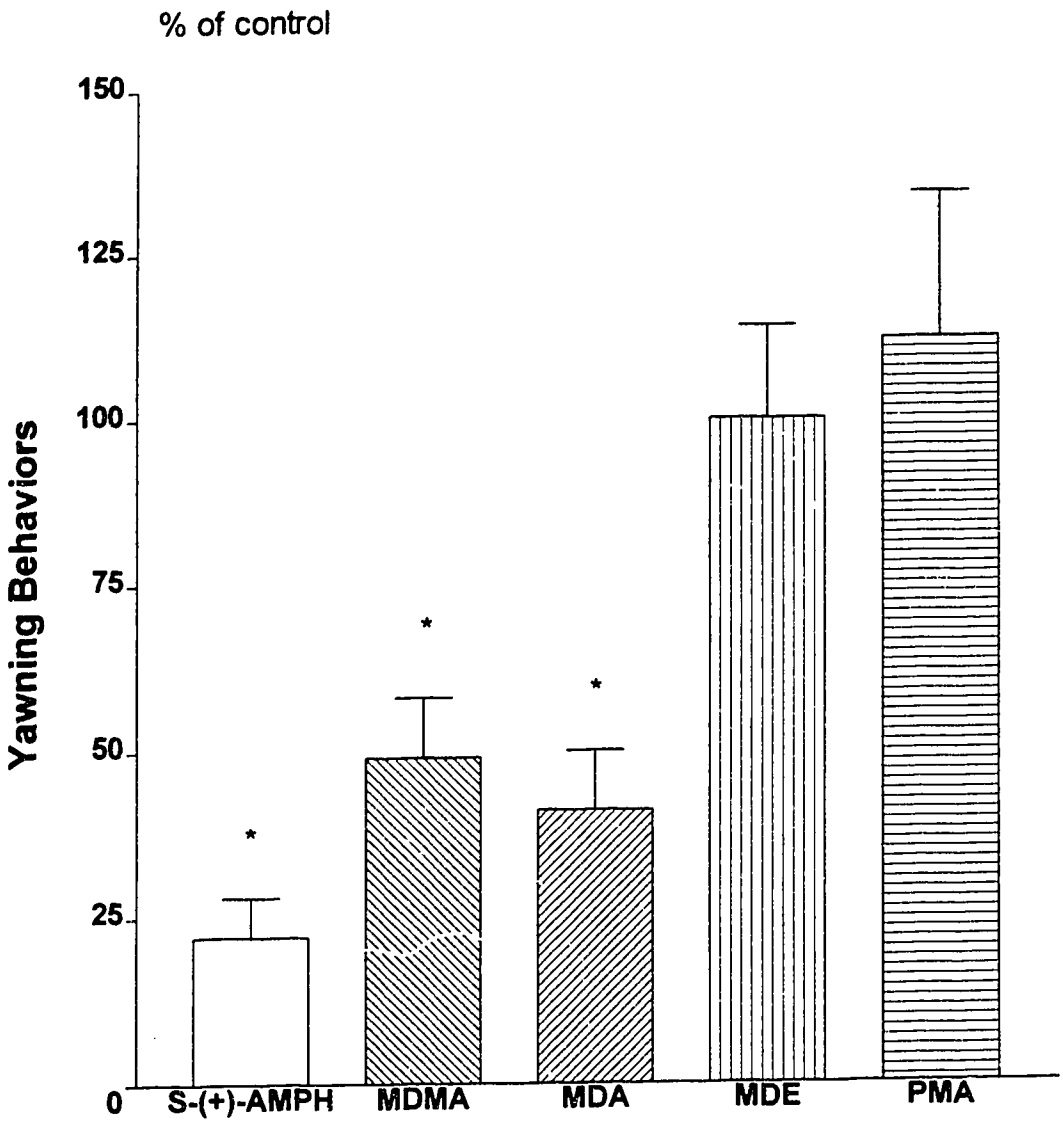


Figure 37: Factor 4 from principal component analysis of behavior following acute drug administration (32 µmoles/kg ip): yawning behaviors (including yawning, mouth movements, lying down and stretching) expressed as mean % ± SEM of control. * indicates significant difference from control ($p<0.05$).

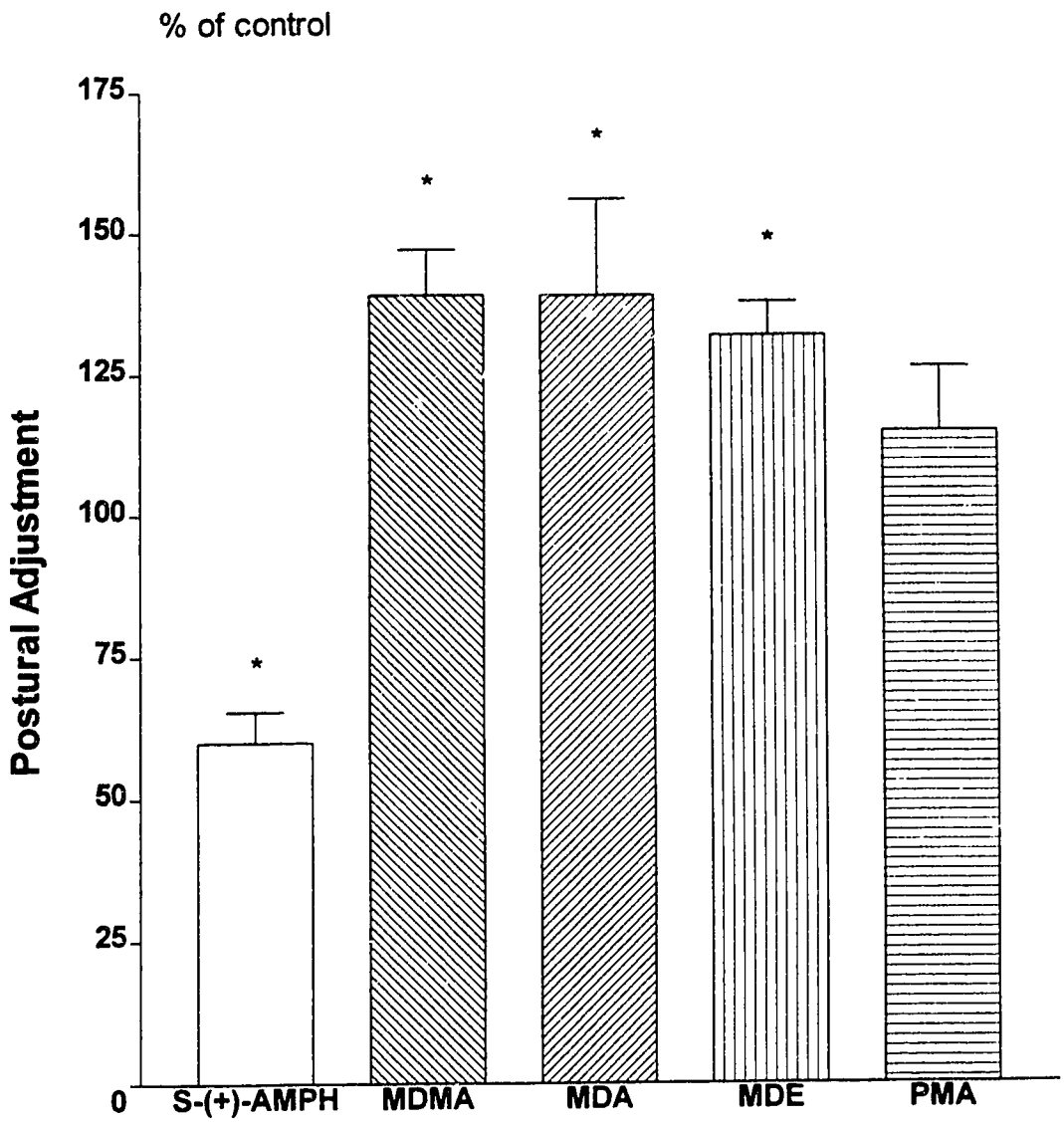


Figure 38: Factor 6 from principal component analysis of behavior following acute drug administration (32 μmoles/kg ip): postural adjustment behaviors (body adjustments and clockwise rotations) expressed as mean % ± SEM of control. * indicates significant difference from control ($p<0.05$).

Administration of S-(+)-AMPH, MDMA and MDA caused a complete loss of sleep, while treatment with MDE or PMA has a less potent effect (decreased to 38% and 32% of control, respectively).

4.3.4 *Transitional Analyses*

The significant transitions between two sets of behaviors for all treatment groups are listed in Table 9. The transition from contact to head movement was the most frequently observed transition in the vehicle-, MDMA-, MDE- and PMA-treated groups, while locomote to stand was the most frequently observed in the S-(+)-AMPH-and MDA-treated groups. The observed frequency of the transition from stand to sniff was significantly different from that expected by random in only the S-(+)-AMPH-, MDMA- and MDA-treated groups. The opposite pattern was seen in the transition from lie to body adjustments, where only vehicle, MDE and PMA produced significant differences. A summary of the multiple correlations among all the ratios of observed/expected transitions and the treatment groups is provided in Table 10. The highest degree of similarity existed between PMA and MDE. The pattern of drug grouping was consistent with the photobeam interuption counts and the principal components analyses of the frequencies and durations of the individual behaviors: S-(+)-AMPH constituted a distinct group; MDMA and MDA another; and MDE, PMA and vehicle a third group.

4.3.5 *Cluster Analyses*

The dendogram of the clustering of the treatment groups by the frequencies and durations of behaviors is shown in Figure 39. From the relative Euclidean

TRANSITION	VEH	AMPH	MDMA	MDA	MDE	PMA
Contact → Head Movement	122/48.5	99/38.6	142/66.4	147/59.5	151/70.2	139/66.5
Locomote → Stand	101/21.8	199./50.7	114/18.5	173/31.5	148/27.6	129/21.7
Stand → Contact	98/48.4	145/90.1	78/51.9	105/63.4	106/56.2	80/46.1
Sniff → Head Movement	97/32.8	NS	NS	77/30.3	107/43.1	114/46.6
Stand → Locomote	58/22.9	166/55.3	60/21.1	80/34.3	79/29.7	65/23.2
Sniff → Contact	48/27.5	73/30.1	52/35.5	54/30.6	63/35.6	52/33.1
Rear → Contact	46/12.7	35/21.3	20/12.7	32/16.2	36/10.9	32/9.2
Stand → Rear	24/14.4	NS	NS	27/16.2	20/9.7	18/8.4
Body adjust → Body adjust	23/16.7	NS	72/35.6	57/25.1	61/46.7	90/47.7
Head Movement → Mouth Movement	20/14.1	2/2.8	13/8.5	12/5.6	25/17	27/15.5
Mouth Movement → Sniff	19/7.8	17/2.1	18/4.4	14/2.5	31/8.1	24/7.3
Stretch → Yawn	18/0.3	1/0	2/0	3/0	10/0.2	11/0.2
Lie → Body adjust	15/5.9	NS	NS	NS	17/10.6	20/12.2
Contact → Locomote	NS	73/49.1	NS	44/32.5	48/30.7	33/23.8
Stand → Sniff	NS	45/29.1	47/30.9	43/28.1	NS	NS

Table 9: Frequencies of observed transitions significantly different from those expected from random, using method of adjusted residuals (ratio = observed/expected). NS = nonsignificant.

	PMA	MDE	MDA	MDMA	AMPH	VEH
VEH	92	90	30	16	3	
AMPH	11	16	64	55		
MDMA	40	41	91			
MDA	55	58				
MDE	99					

Table 10: Percent variation in common of the ratios of observed/ expected transitions across treatment groups. Results of multiple correlations (% variation = correlation value^2 x 100).

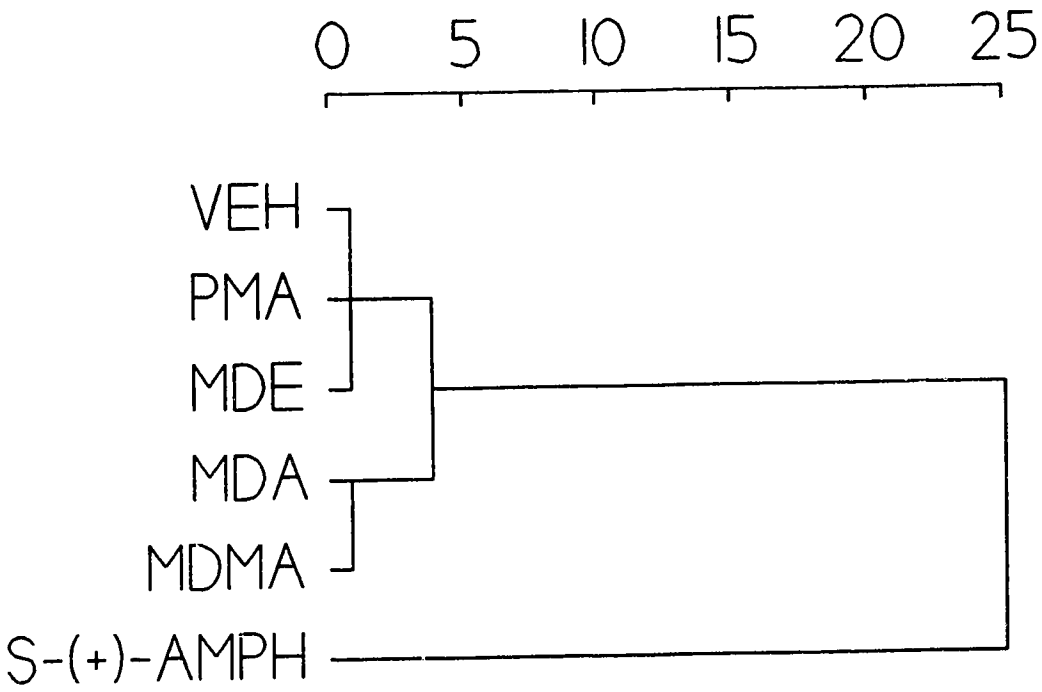


Figure 39: Dendogram displaying the clustering of the treatment groups by the frequencies and durations of behaviors significantly affected by drug treatment. The scale represents the relative distances derived from the squared Euclidean distances (the sums of the squared differences in frequencies and durations of behaviors significantly affected by drug treatment).

distances, the pattern of drug groupings is consistent with the locomotor activity data, with S-(+)-AMPH as a distinct cluster, MDMA and MDA closely related and PMA and MDE closely related but not distinguishable from vehicle. However, the dendogram of the clustering of the treatment groups by the ratios of the observed and expected transitions, as seen in Figure 40, shows that PMA and MDE were more closely related to each other and only distantly related to vehicle. S-(+)-AMPH remained a distinct cluster and MDMA and MDA remained another cluster with some degree of similarity to S-(+)-AMPH.

4.3.6 *Neurochemistry*

Regions analyzed for the levels of DA, NE and 5-HT and the acid metabolites DOPAC, HVA and 5-HIAA included the hypothalamus, the hippocampus, the striatum and the cortex. In general, the data supported a change in the pattern of drug groupings from that derived from the behavioral data, with MDE-treated animals sharing more similarity in effects with MDMA and MDA than with PMA. Data from the S-(+)-AMPH- and PMA-treated animals supported S-(+)-AMPH and PMA remaining as distinct drug groupings from each other.

5-HT parameters in the cortex are shown in Figure 41. S-(+)-AMPH produced no changes. MDMA, MDA and MDE produced significant decreases in the levels of 5-HT and 5-HIAA and increases in 5-HT turnover, although the increase in turnover following MDE did not achieve statistical significance. PMA also produced decreased levels of 5-HIAA, but this was accompanied by increased

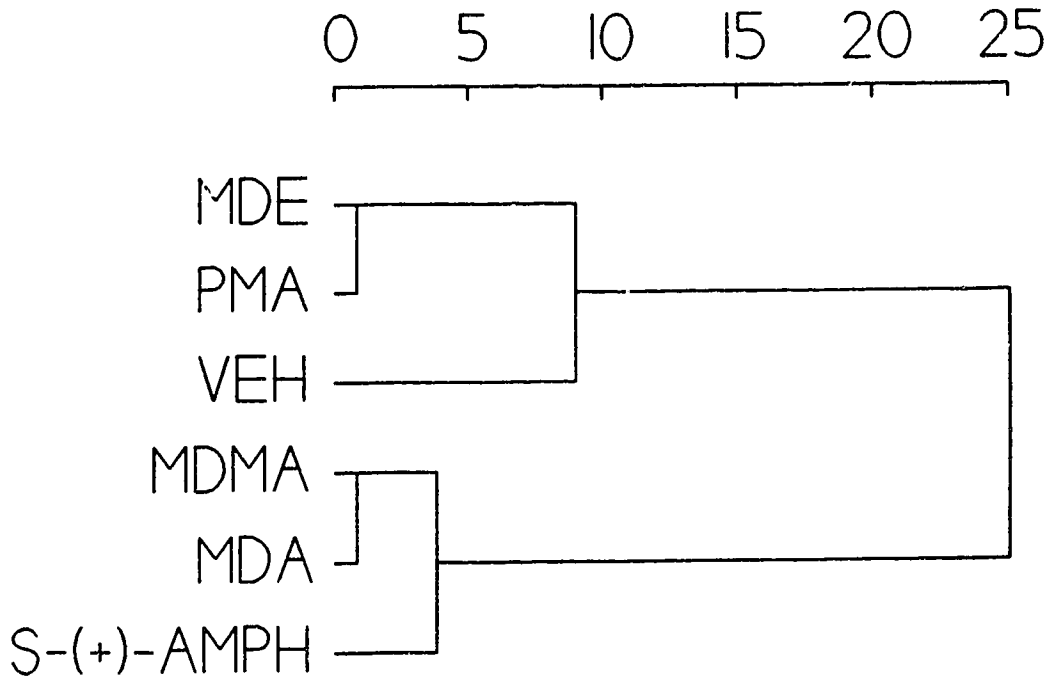


Figure 40: Dendogram displaying the clustering of the treatment groups by the ratios of the observed and expected transitions between individual behaviors. The scale represents the relative distances derived from the squared Euclidean distances (the sums of the squared differences in the ratios of the observed and expected transitions between individual behaviors).

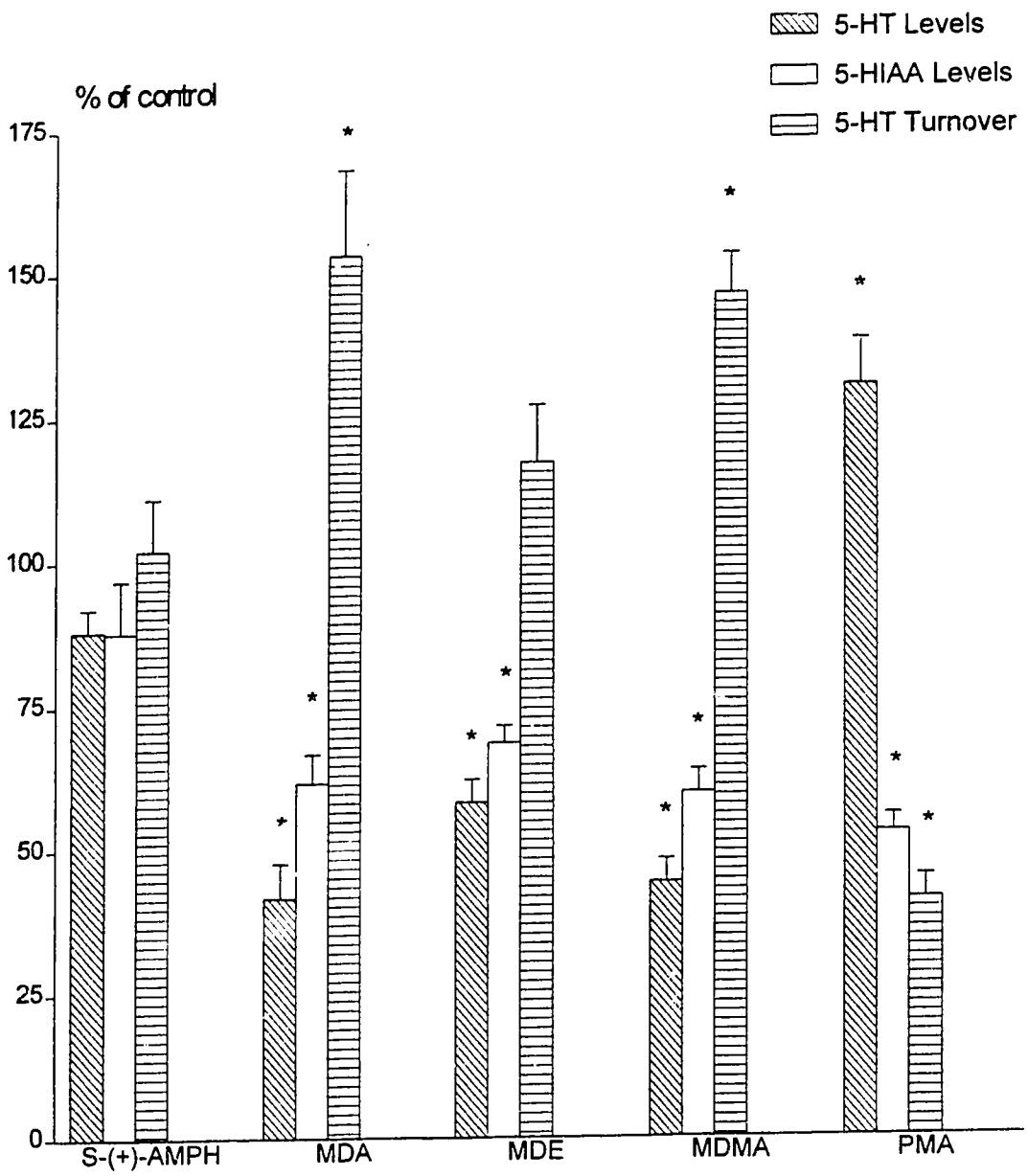


Figure 41: 5-HT parameters in rat cortex 3 h after acute injection of equimolar doses (32 µmoles/kg ip) of the drugs. Values are expressed as % of control ± SEM (n=12). Control values in ng/g tissue: 5-HT = 176.4 ± 17.4; 5-HIAA = 252.3 ± 15.1; 5-HT Turnover = 0.88 ± 0.094. * indicates significant difference from control.

5-HT and decreased 5-HT turnover. A similar pattern of 5-HT/5-HIAA changes was also seen in the striatum and the hippocampus. There were no significant drug effects on levels of NE in the hypothalamus. The changes in NE levels in the other three regions are depicted in Figure 42. MDMA and MDE had no effect. S-(+)-AMPH and MDA produced significant decreases in the levels of NE in the cortex, while in the hippocampus, decreases were only seen in the S-(+)-AMPH-treated animals. PMA caused significant decreases in all three regions.

Outlined in Figure 43 are the changes in DA parameters in the striatum. Some regional differences (both in the degree and direction of change) are seen. Overall, levels of DA were not affected by drug treatment, with the exception that animals treated with MDA showed a significant increase in the levels of DA in the hypothalamus. MDE was the only drug that failed to produce decreased levels of DOPAC and HVA and a decrease in DA turnover. Similar drug effects on DA parameters were seen in the cortex: DA turnover was decreased by all drug treatments except MDE; DOPAC levels were decreased after MDMA, MDA and PMA administration; and HVA levels were decreased by MDMA and PMA. S-(+)-AMPH increased HVA levels in the cortex and MDE increased DOPAC levels in the cortex, hypothalamus and hippocampus.

4.3.7 *Drug Levels*

The drug levels in whole brain at 3 h after injection are shown in Figure 44. There are large differences in the levels of the various drugs, with MDA > MDMA > S-(+)-AMPH > MDE > PMA.

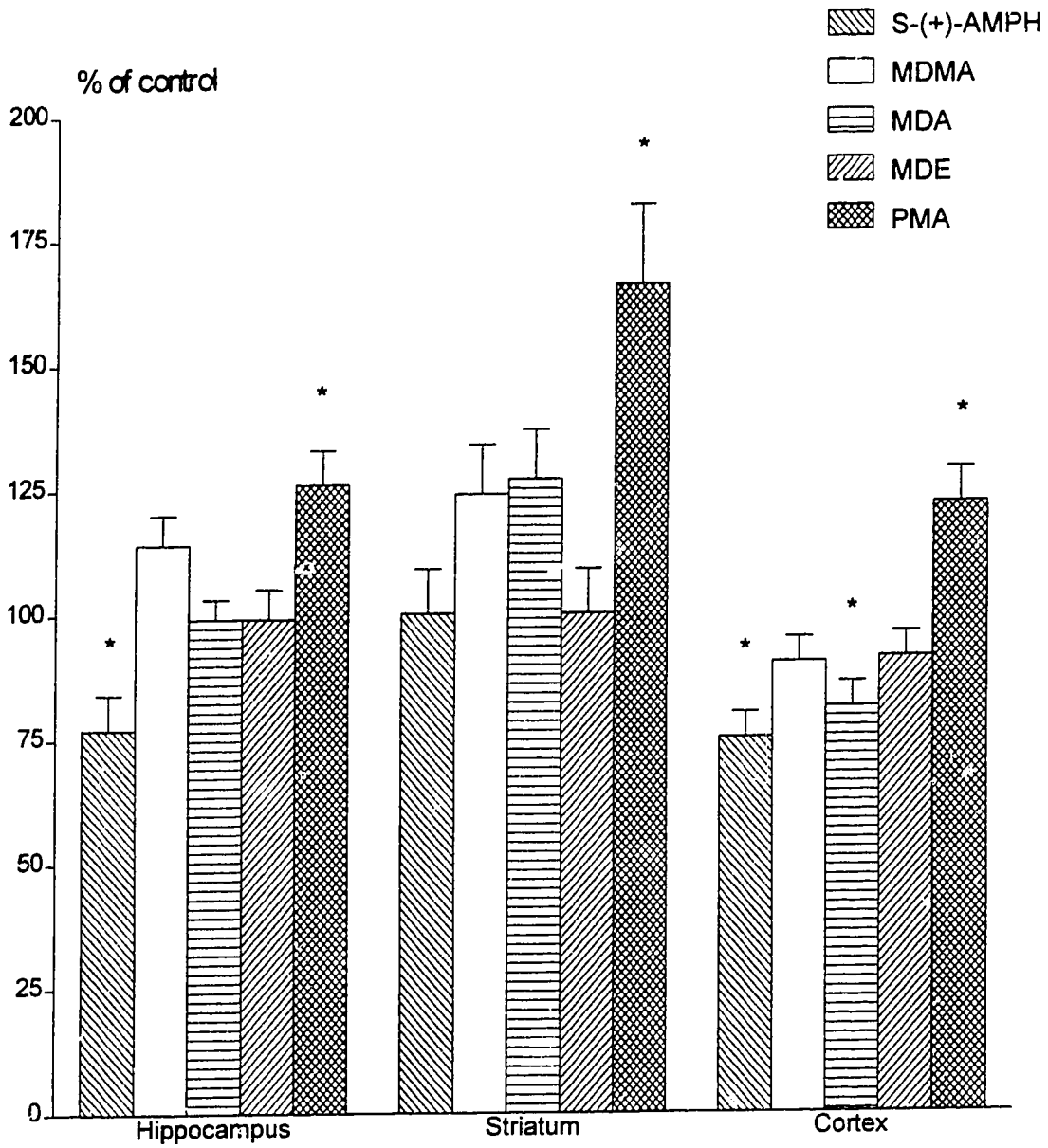


Figure 42: NE levels in three rat brain regions 3 h after acute injection of equimolar doses (32 µmoles/kg ip) of the drugs. Values are expressed as % of control ± SEM (n=12). Control values in ng/g tissue: hippocampus = 278.9 ± 15.6; striatum = 216.1 ± 23.6; cortex = 297.9 ± 13.9. * indicates significant difference from control.

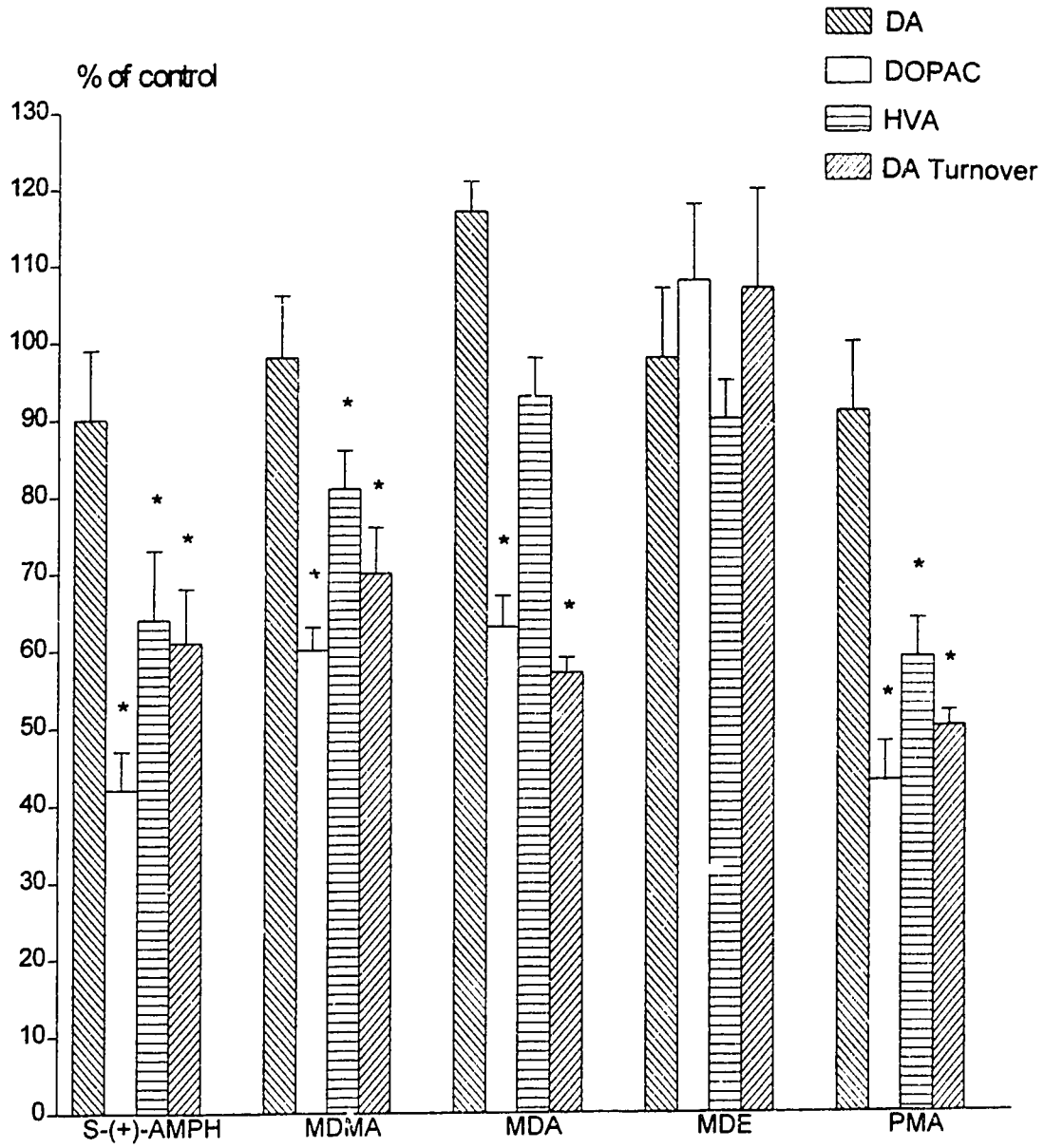


Figure 43: Striatal DA parameters in rats 3 h after acute injection of equimolar doses (32 µmoles/kg ip) of the drugs. Values are expressed as % of control ± SEM (n=12). Control values in ng/g tissue: DA = 5235.1 ± 282.5; DOPAC = 1279.3 ± 74.7; HVA = 433.4 ± 34.8; DA Turnover = 0.34 ± 0.03. * indicates significant difference from control.

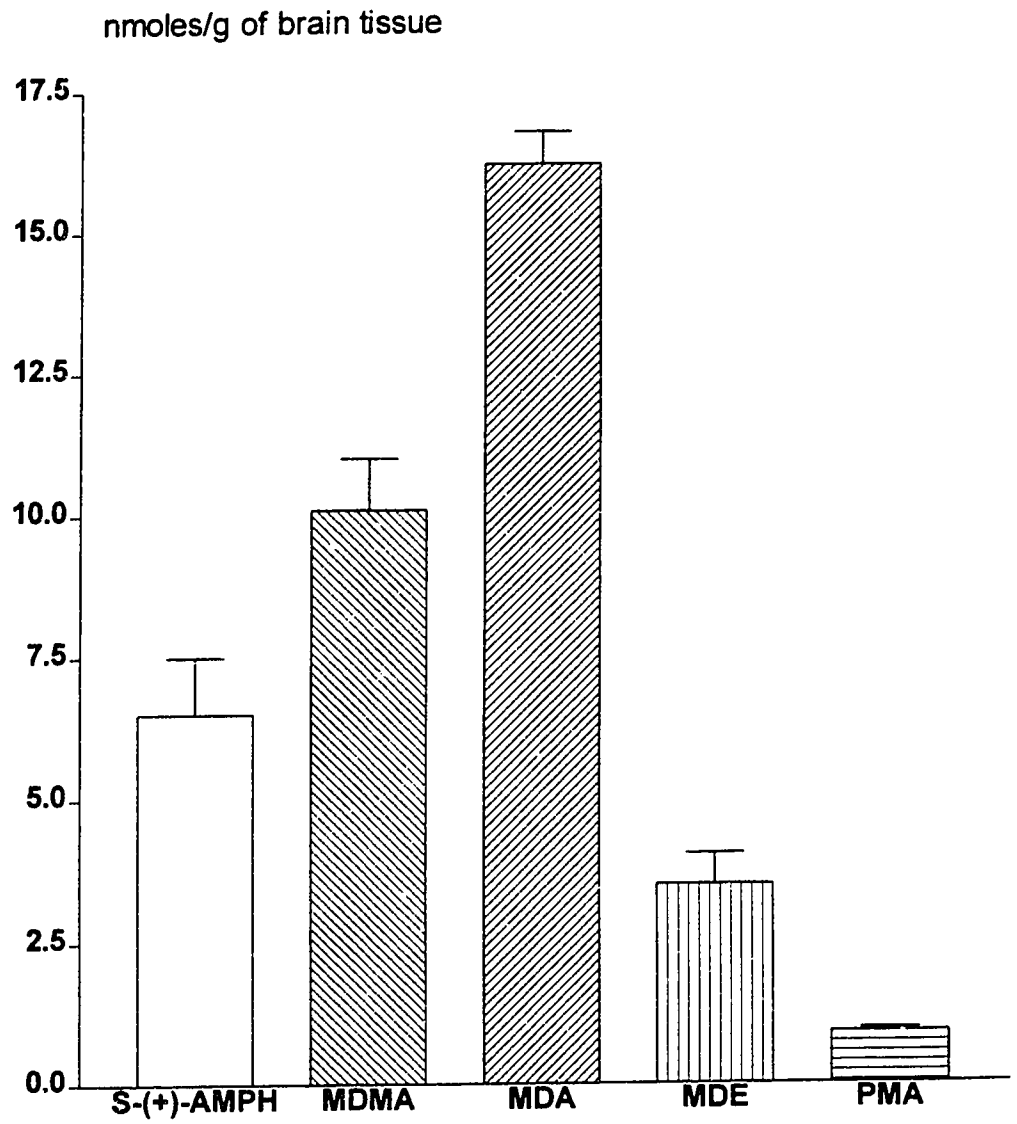


Figure 44: Drug levels in whole brain 3 h after acute administration of equimolar doses (32 µmoles/kg ip) of the drugs. Values are expressed in nmoles/g of tissue ± SEM (n=12).

5 *DISCUSSION*

5.1 Metabolic Studies

5.1.1 *Levels of MDMA and MDA in Rat Brain and Liver Tissues*

In both brain and liver tissues levels of MDMA decreased at a faster rate than was seen with MDA. Once formed as a metabolite of MDMA, levels of MDA remained fairly stable over at least the first 4 h after administration of MDMA. These data are in keeping with data from others that showed MDA had a longer duration of behavioral action and a longer half-life than MDMA in whole blood (Nichol and Oberlender, 1990; Fitzgerald *et al.*, 1990).

The pattern of decline in the levels of S-(+)-MDMA and S-(+)-MDA in brain tissues compared to the pattern of the R-(-) isomers was different. At 1 and 2 h, there was significantly more S-(+)-MDMA than S-(+)-MDA. By 4 h, the difference was no longer significant and by 8 h the pattern had reversed, with significantly more S-(+)-MDA than S-(+)-MDMA. In the case of R-(-)-MDMA and R-(-)-MDA, R-(-)-MDMA remains higher than R-(-)-MDA until the 8 h period. In the liver tissues the same patterns of decline of the S-(+) and R-(-) isomers were seen at the 1 h and 2 h periods. However, the pattern reversal [more S-(+)-MDA than S-(+)-MDMA] occurred earlier, at the 4 h time period. The enantioselectivity demonstrated here could arise from complex interactions among and/or competition for binding proteins, active transport processes, metabolic enzymes and receptor sites. Detailed pharmacokinetic and pharmacodynamic studies would be needed to discern the relative contribution of the various interactions to the observed

differences in the levels of enantiomers and the rates of decline of the levels.

The regional distribution of high levels of MDMA corresponds well with the data regarding the regions that are most affected by MDMA and MDA, namely the hippocampus, the striatum and the cortex. It is in these regions that the most profound depletions of 5-HT have been shown to occur and the morphological changes to fine 5-HT fibers are also more concentrated in these regions (O'Hearn *et al.*, 1986; Battaglia *et al.*, 1987; Gibb *et al.*, 1987; Schmidt *et al.*, 1987a; Johnson *et al.*, 1988).

5.1.2 *Levels of the enantiomers of MDE and MDA in whole brain*

These data indicate that TFPC was a suitable derivatizing reagent for the chiral separation of MDE and MDA, producing stable diastereoisomers that were readily separated and quantitated. Monitoring and the added confirmation of the results obtained using HFPC gave confidence in the results reported here.

Although there remains controversy regarding MDA's role in MDMA neurotoxicity (Molliver *et al.*, 1978, 1986), MDA could be an important intermediate preceding O-demethylenation to produce potentially neurotoxic catechol derivatives. No reports were found comparing the dihydroxy and trihydroxy metabolites of MDMA prior to N-demethylation to those following the loss of the N-methyl group in term of neurotoxic potential and potency. Various N-alkyl substitutions of AMPH and other analogues of AMPH have been carried out and in the majority of cases the length of the N-alkyl chain was negatively related to potency (Shulgin *et al.*,

1969; Shulgin, 1978). The lower ratio of MDA to parent drug levels in the case of MDE compared to that with MDMA may partially account for the decreased potency of MDE to produce long-term effects on 5-HT systems in the brain. The decreased potency of MDE relative to MDMA might also be related to the lower levels of the parent compound seen in brain tissues. The levels in brain of MDE are only approximately 50% of the levels of MDMA at 1 h and 4 h after administration. Pharmacokinetic studies are required to establish when the peak level of MDE is attained after administration of the drug.

The differences in both the degree and pattern of stereoselectivity between the parent drugs MDMA and MDE and their N-dealkylated metabolite MDA are very interesting and somewhat surprising. Perhaps because N-deethylation becomes a minor metabolic path, the levels of MDA enantiomers reflected retention of stereoselectivity, but other less stereoselective paths overwhelmed any impact N-deethylation had on the levels of MDE enantiomers. Thus, initially levels of MDE did not demonstrate any stereoselectivity. Over time as the levels of MDE enantiomers declined, small but significant differences were revealed. With both MDE and MDA, the differences between the sets of enantiomers at all time periods studied, even when significant, were small, suggesting that stereoselectivity might be influenced by the length of the N-alkyl chain. Further metabolic studies are needed to examine subsequent or alternate metabolic reactions involved in the biotransformation of MDE and any stereoselectivity of the reactions. If the O-demethylenated product of MDA as a source of free radicals is the final path leading

to neurotoxicity, the presence of the N-ethyl group on the O-demethylenated product of MDE might account for the decrease in neurotoxic potential.

As previously discussed, it had been suggested that the metabolic N-dealkylation of MDMA is catalyzed by the CYP family of enzymes (Hiramatsu *et al.*, 1990). It is not presently known if a specific CYP isozymes is involved or whether a number of different isozymes can catalyze the reaction. Until more is known about the specific isozyme(s) that catalyzes the N-demethylation of MDMA, it will not be possible to examine whether it is the same isozyme(s) involved in the N-deethylation of MDE and hampered by the length of the alkyl chain or whether it is a different isozyme with different kinetics. Other metabolic pathways and metabolites that are seen with MDMA and MDA are also likely to occur with MDE.

5.1.3 *Isozyme studies*

Under the same experimental conditions, CYP2D6 was superior to CYP3A4 in terms of its ability to catalyze the N-demethylation of IMI. The small degree of product formation *via* metabolic N-dealkylation when IMI was incubated with CYP2D6 supports the results of Brøsen *et al.* (1991) and Coutts *et al.* (1993). Both of these groups showed that while 2-hydroxylation of IMI was the major metabolic path catalyzed by CYP2D6, small amounts of DMI were consistently found.

CYP2D6 was also superior to CYP3A4 in its ability to N-deethylate the S-(+) isomer of *rac*-MDE and to a lesser extent demethylate both isomers of *rac*-MDMA. The results obtained from incubating MDE with CYP2D6 fit with previous *ex vivo*

work with iprindole and FEN (Hegadoren *et al.*, 1991). Iprindole is an antidepressant drug known to inhibit metabolic reactions thought catalyzed by CYP2D6, including the ring hydroxylation of AMPH (Freeman and Sulser, 1972; Steranka, 1982) and the N-dealkylation of FLU (Aspeslet *et al.*, 1994). When administered 1 h prior to an acute ip injection of FEN, iprindole inhibited the N-deethylation of FEN.

Under each of the various experimental conditions, *rac*-MDE proved to be a better substrate for CYP2D6 and CYP3A4 than MDMA. This was an unexpected finding, not in keeping with the results obtained in the *ex vivo* metabolic experiments, where more MDA was produced after administration of MDMA than was seen after MDE administration (see Results 4.1.1-2). The metabolic ratios in liver tissue from those experiments obtained at 1 h after administration of *rac*-MDMA (10 mg/kg ip) were 0.13 and 0.46 for the R-(-)- and S-(+)-isomers respectively. This suggests that other CYP isozymes are also involved in the metabolic N-dealkylation of MDMA and MDE. These results might also reflect species differences between rats and humans. There are significant species differences that exist in the metabolism of AMPH (Gibson and Skett, 1986) and this variability might extend to some of its analogues.

The same pattern of stereoselectivity in terms of N-dealkylated product formation [S-(+)-MDA > R-(-)-MDA] was seen with both MDE and, when detected, MDMA as was observed in whole blood after MDMA administration (Fitzgerald *et al.*, 1989a,b) and in brain and liver tissues reported earlier in this thesis. This

provides additional support to enantioselective metabolism being involved in the differences between the levels of R-(-)- and S-(+)-MDA seen *in vivo* after administration of MDMA and MDE.

When *rac*-MDE was incubated with CYP3A4, the metabolite/parent ratios were higher when a smaller amount of substrate was used. Comprehensive dose-response experiments would be needed to examine if increased amounts of MDE had an inhibitory effect on MDA formation. As indicated earlier, MDA and MDMA are capable of forming spectrally observed inhibitory complexes with CYP when incubated with rat microsomes (Brady *et al.*, 1986). The differences in enzyme concentration and/or incubation time could also account for the resultant differences in the ratios.

Nifedepine was by far the best substrate for CYP3A4 and could serve as an excellent comparative compound. Further *in vitro* kinetic studies with nifedepine and CYP3A4 could provide information about the metabolic capability and the speed at which CYP3A4 can function. Further comparative experiments between lidocaine and nifedepine could help examine the relative ease of N-dealkylation compared to other metabolic reactions.

Although the procedure involved in the *in vitro* metabolism studies was relatively simple to carry out, care must be taken with the interpretation of the preliminary and qualitative data obtained thus far. It is difficult to know the relative contributions that substrate specificity and substrate concentration might make in the overall efficiency of the metabolic reactions. The substrate concentration that

would yield optimal metabolite formation is likely different for each CYP isozyme. Thus, the K_m and V_{max} values would be required for each isozyme and substrate under investigation. However, Wrighton and Stevens (1992) suggested that at concentrations of the substrates found under physiological conditions, enzyme kinetics often favor a single form of CYP450 being the primary catalyst of the metabolism. An awareness of both optimal *in vitro* concentrations and physiologically relevant concentrations is needed.

The optimal incubation conditions also seem to vary with individual isozymes and substrates. Time course studies, keeping in mind the elimination half-life of the substrate if known, could provide optimal incubation times. Other condition parameters including enzyme concentration, specific electron-generating systems and the use of phospholipids all need to be systematically examined. The results reported here represent a preliminary qualitative examination of the capacity of two CYP isozymes known for their involvement in the biotransformation of numerous xenobiotics. The large number of purified CYP isozymes made available through molecular biological technologies will provide extensive opportunities for further understanding of this enzyme superfamily and its individual members.

While an *in vitro* metabolic system cannot duplicate the immense complexity of reactions and interactions that occur in the liver hepatocyte, it is important to pursue this line of research as it can identify specific CYP isozymes responsible for clinically used drugs. Many of the CYP isozymes exhibit genetic polymorphism and thus knowledge of drugs whose metabolism could be altered by poor metabolizers

could avoid loss of efficacy and/or increased side effects. MDMA and MDE would also be useful substrates to study structure-activity relationships in regard to metabolic enzymes responsible for N-dealkylation.

5.2 Uptake Inhibition Studies

Both MDE and MDMA were shown to be potent inhibitors of ^{3}H-NE and ^{3}H-5-HT uptake in rat brain tissues. The lower potency of *rac*-MDE and S-(+)-MDE at inhibiting DA uptake compared with MDMA supports other lines of research showing that of the three methylenedioxy analogues of AMPH, MDE was least able to affect DA systems (Johnson *et al.*, 1987; Ricuarte *et al.*, 1987; Stone *et al.*, 1987b; Series and Molliver, 1994). Although the racemates of MDMA and MDE were equipotent at NE and 5-HT uptake inhibition, comparisions between the individual enantiomers revealed significant differences. As expected, the S-(+) isomers were consistently more potent than the R-(-) isomers for both MDMA and MDE. While S-(+)-MDMA was more potent than S-(+)-MDE at DA and NE uptake inhibition, the reverse was true for uptake inhibition of 5-HT. Battaglia *et al.* (1988a) conducted *in vitro* binding studies and found that MDE also had a higher affinity for the 5-HT uptake site than MDMA and MDA. This is surprising considering the potency differences between MDMA and MDE favoring MDMA in terms of long term neurochemical effects and behavioral effects. These results do, however, support the suggestion that DA systems may play a key role in the long term neurochemical effects. Not only was *rac*-MDE weaker at inhibiting the uptake of ^{3}H-DA as shown here, it was also shown

to be weaker at releasing ^{3}H-DA (McKenna *et al.*, 1991; Schmidt, 1994). Thus, the acute effects on serotonin systems appear not to be directly related to the ability to cause long term changes in 5-HT parameters.

Interestingly, the IC_{50} values of *rac*-MDE *and rac*-MDMA for inhibiting ^{3}H-DA uptake into striatal tissues showed a trend towards being less than the IC_{50} values for the S-(+) isomers. Commonly, if there is a substantial difference in results obtained between the two individual enantiomers, then the racemic mixture will produce results that are in between the two enantiomers. These data suggest that there could be an interaction between the S-(+)- and R-(-)-isomers at the DA transporter.

The IC_{50} values obtained here for MDE and MDMA were compared with the IC_{50} values for the enantiomers of MDMA and MDA reported by Steele *et al.* (1987). The same pattern of enantioselectivity [S-(+) > R-(-)] and the same rank order of potency (5-HT > NE > DA) were seen. Direct comparisons are difficult as Steele and his colleagues used hippocampal synaptosomes for uptake studies with ^{3}H-5-HT, hypothalamic synaptosomes with ^{3}H-NE and striatal synaptosomes with ^{3}H-DA. In addition, these researchers did not include the racemates of MDMA and MDA in their uptake inhibition studies for comparision with the individual enantiomers.

5.3 **Behavioral and Neurochemical Studies**

5.3.1 *Behavioral Study*

The behavioral study was undertaken to examine the effects on spontaneous behavior in freely moving rats of drugs with a similar structure but functionally separated into the putative entactogens, a stimulant and an hallucinogen. It was hypothesized that a detailed analysis of the duration and frequency of individual behaviors, the sequences of these behaviors and the neurochemical effects of the drugs could provide a basis to differentiate among the three drug classes and vehicle treatment. Data from the photobeam interruption counts supported the division into three distinct drug groupings: the first being AMPH, the second MDMA and MDA and the third PMA, MDE and vehicle. The results support the literature in terms of similar but less potent effects of MDMA and MDA than is seen with AMPH (Thiessen and Cook, 1973; Glennon and Young, 1984a,b; Matthews *et al.*, 1989; Spanos and Yamamoto, 1989). Although Hermle *et al.* (1993) reported increased psychomotor drive in human volunteers after oral administration of MDE, no information was found in the literature regarding effects of MDE on locomotor activity in rats. Braun *et al* (1980) reported an increase in motor activity in mice after administration of MDE (20 mg/kg po). It is difficult to know whether the differences in effect on motor activity reported here were due to differences in the dose, in the species studied, in the route of administration or a combination of multiple factors. The lack of effect on locomotor activity seen with the dose of PMA used in this study supports the findings of Hitzemann *et al.* (1971) that doses of 30

mg/kg of PMA were required to increase psychomotor activity. However, Menon *et al.* (1976) showed that PMA produced increased locomotor activity at doses of 5 and 10 mg/kg. This discrepancy might arise from procedural differences. Menon and his colleagues measured locomotor activity in a social context, in that the rats were paired. In addition, their animals were in locomotor boxes for the test period only, whereas the animals used in the experiments reported here were in the test boxes for 10 days prior to the experiment. Locomotor activity measured during the animals' dark cycle in this study could be different from values obtained during the more quiesent day cycle. The timing of the study by Menon *et al.* was not reported.

Principal components analysis and cluster analysis of the frequency and duration measures of behaviors did not provide any further discriminative data than that from the photobeam interruption counts. Only the cluster analysis of transition frequencies as a ratio of expected frequencies differentiated vehicle from the hallucinogen and MDE. In this case, four groupings were found: (1) MDMA and MDA; (2) PMA and MDE; (3) AMPH; and (4) vehicle. In addition, this analysis revealed that AMPH was more closely related to MDMA and MDA than to vehicle and the hallucinogen. Thus, the cluster analysis of the behavioral transitions produced a classification scheme of the drugs closest to human subject reports. The one discrepancy was that MDE was consistently classified with the hallucinogen and not with the entactogens. As indicated earlier, dose is an important factor in determining overall behavioral effects of these compounds. The administered dose of 32 μmoles/kg represents a relatively large dose of AMPH and

PMA in rats (5.9 mg/kg and 5.3 mg/kg, respectively) but a fairly modest dose of the other drugs (MDMA 6.2 mg/kg, MDA 5.8 mg/kg and MDE 6.7 mg/kg) The possibility remains that a more accurate drug classification requires behavioral transition analysis coupled with dose-response curves. Dose-response curves for each of the five drugs would have provided information about the optimal dose to yield maximum effect. However, the generation of dose-response curves could have become an extensive undertaking as the optinal dose may have varied depending on the specific response examined, that is, whether the response was photobeam interruption counts or various discrete behaviors, maximum effects on the sequences of behaviors or maximum changes to levels of the individual neurotransmitter amines. The choice of equimolar doses for each of the five structurally similar drugs provided the opportunity to examine differences in a number of behaviors produced by aromatic or N-substitution on the basic AMPH structure under the same experimental conditions.

Further studies using the same methodology, but with continuous videotaping instead of the time-sampling procedure used here could add further information regarding behavioral changes over time, especially with the drugs with short durations of action. However, time and resource constraints limited the length of videotape per animal that could be analyzed. Randomizing the time segment that was videotaped also could have provided data across the 3 h period after drug administration. Recently, reports have been published regarding interanimal variation in response to AMPH being related to variability in baseline behavior (Sills

et al., 1993; Sills and Vaccarino, 1994). Perhaps more significant behavioral changes than reported here would have been observed if each animal was used as its own control to establish baseline behaviors and behavioral sequences.

The limitation of the stimulant and hallucinogen representatives to single examples needs to be expanded to stimulants and hallucinogens with different structures. The choices made in this experiment were guided by structural similarity: all the drugs used in the present experiment shared the same basic skeleton. Although Shulgin *et al.* (1969), on the basis of extensive clinical studies reported PMA to be hallucinogenic, DD studies in rodents and studies in chronic spinal dog have produced equivocal results (WR Martin *et al.*, 1978; Winter, 1984). I was interested in testing the hypothesis that this methodology could separate drugs according to their subjective effects in humans and thus felt PMA was an appropriate choice as a structurally similar compound. However, the analysis must be generalizable to other drug structures before confidence in this method can be firmly established.

5.3.2 *Neurochemical Effects*

The neurochemical effects of the different drugs provided further discriminative data that differentiated among the drug classes, but not consistently between the individual drugs and vehicle treatment. The NE levels in the hippocampus could be used to discriminate among AMPH, PMA and the methylenedioxy compounds, but the methylenedioxy compounds could not be

differentiated from vehicle treatment. The cortical 5-HT measures also discriminated among AMPH, PMA and the methylenedioxy compounds, but failed to differentiate between AMPH and vehicle treatment. Striatal DA measures were not helpful in discriminating among the drugs. AMPH, MDMA, MDA and PMA all caused significant decreases in the levels of DOPAC and in DA turnover.

In general, the changes seen in the levels of NTs and NT metabolites support the literature regarding the multiplicity of neurochemical effects these drugs have on neuronal systems. PMA was the only treatment that produced an increase in the levels of NE. PMA has been shown to have potent indirect sympathomimetic effects due to the release of NE from adrenergic nerve terminals (Cheng *et al.*, 1974). *In vitro* studies have shown PMA to also possess NE and 5-HT releasing effects coupled with competitive inhibition of uptake of ^{3}H-NE and ^{3}H-5-HT into striatal and corical tissues (5-HT > NE) (Hitzemann *et al.*, 1971; Tseng *et al.*, 1974, 1976; Nichols *et al.*, 1979). Although the NE releasing and uptake inhibition properties of PMA are similar to those of MDMA and MDA, perhaps the time course was different. There was a trend to increased levels of NE in the striatum after MDMA and MDA treatment, but they did not achieve statistical significance.

None of the drug treatments produced an effect on regional levels of DA. Gibb *et al.* (1990a,b) investigated the effects of MDMA and MDA (10 mg/kg) on striatal DA and found a time-dependent increase in DA. Schmidt *et al.* (1986) and Matthews *et al.* (1989) also reported increased striatal levels of DA 3 h following administration of MDMA (10 mg/kg). However, Gough *et al.* (1991) reported levels

of DA were unchanged in the caudate nucleus under the same dosing schedule. The differences in results obtained by others compared to those reported here might be due to dose and/or time dependency of the increase in the levels of DA. MDE administration did not alter any of the DA measures from those obtained in the vehicle-treated animals. This supports other lines of research that have shown that MDE had little effect on DA systems (Johnson *et al.*, 1987; Schmidt, 1987b, 1994; Stone *et al.*, 1987b; Series and Molliver, 1994). The low levels of MDE in brain tissue as seen in the *ex vivo* experiments and the *in vitro* findings that *rac*-MDE had reduced effects on DA uptake compared to *rac*-MDMA might account for the lack of effect on the DA measures. Although the other four drug treatments had no effect on striatal levels of DA, all four had inhibitory effects on levels of DA metabolites (decreases in DOPAC > decreases in HVA) and decreased DA turnover.

MDMA, MDA and MDE decreased the levels of 5-HT in all regions except the hypothalamus. Levels of 5-HIAA were also significantly decreased by the methylenedioxy analogues of AMPH. The direction and degree of change in the 5-HT parameters were similar to others who have examined regional levels of 5-HT and 5-HIAA 3 h after administration of MDMA or MDE (Schmidt *et al.*, 1986; Johnson *et al.*, 1987; Schmidt and Taylor, 1987; Stone *et al.*, 1987b).

MDMA and MDA increased 5-HT turnover in the cortex, while PMA decreased the 5-HT turnover rate. The specific processes responsible for the changes in turnover rate are difficult to discern. MDMA and MDA both have been

shown to decrease the activity of TPH, thus decreasing 5-HT synthesis (Schmidt and Taylor, 1987; Stone *et al.*, 1987b; Johnson *et al.*, 1988). These drugs are also potent 5-HT releasers and inhibitors of 5-HT uptake, producing large increases in the levels of extracellular 5-HT, followed by depletion, with persisitent lowered levels of 5-HT (Nichols *et al.*, 1982; Steele *et al.*, 1987; Stone *et al.*, 1987c; Azmitia *et al.*, 1990; Fitzgerald and Reid, 1991). MDMA has been shown to have a selective inhibitory effect on MAO-A activity, although less potent than PMA (K_i for PMA = 0.22 μM, K_i for MDMA = 22 μM) (Green and El Hait, 1980; Leonardi and Azmitia, 1994). The increased levels of 5-HT, decreased levels of 5-HIAA and the decreased 5-HT turnover seen with PMA treatment were probably related to the 100-fold greater potency of inhibition of MAO-A activity compared with MDMA, despite the similarity of effects on release and uptake of 5-HT.

Although the changes seen in the levels of the neurotransmitter amines and the acid metabolites cannot be correlated with the behavioral changes observed over the 3 h time period prior to the collection of brain tissue for HPLC analysis, the fact that there remained significant neurochemical changes after most of the behavior changes had diminished or ended highlights that these drugs are potent, centrally-acting compounds. The after-effects reported by humans are likely related to the drugs' long-lasting neurochemical effects.

5.3.3 *Drug levels in whole brain*

The large differences in the brain levels of the various drugs support the

literature regarding the relative durations of behavioral effects on humans: MDA > MDMA and AMPH > MDE and PMA (Shulgin, 1978; Shulgin and Nichols, 1978; Segal and Schuckit, 1983; Nichols *et al.*, 1986). MDA levels remained higher at 3 h than those of MDMA, supporting results from the metabolic studies reported earlier (see Results 4.1.1) showing that MDA as a metabolite of MDMA was cleared from the brain at a slower rate than was seen with the parent compound, MDMA. The levels of MDE were lower than those of MDMA, again in keeping with the metabolic studies discussed previously (see Results 4.1.2). The level of PMA was the lowest of the drugs studied, a result similar to that observed by Sherry-McKenna and Baker (personal communication) when comparing brain levels of the AMPH analogues, tranylcypromine and *para*-methoxytranylcypromine.

Separate kinetic studies with each of the five drugs using 32 μmoles/kg ip would be required to correlate drug concentrations in brain and the observed behavioral or neurochemical changes over time. However, the determination that there existed wide variation in drug levels 3 h after drug administration served to emphasize that such factors as pharmacokinetics and metabolism are important considerations when attempting to characterize the behavioral and neurochemical effects of specific drugs.

6 *CONCLUSIONS*

The studies mentioned below involved a novel, multidisciplinary approach to the investigation of the 3,4-methylenedioxyamphetamines that provided considerable new information about the behavioral, neurochemical and metabolic effects of these drugs, particularly MDE.

1. A novel indirect GC method was developed for the simultaneous separation and quantitation of the enantiomers of MDMA and its N-demethylated metabolite, MDA. The procedure was readily applicable to brain and liver tissues.
2. MDMA and MDA were detectable in brain and liver tissues up to 8 h after acute administration of MDMA. MDMA levels decreased more rapidly than levels of MDA. The enantioselectivity favoring R-(-)-MDMA and S-(+)-MDA was similar to that found in blood and urine by other researchers.
3. The highest regional levels of MDMA corresponded with the regions of the brain most affected by MDMA administration, namely the hippocampus, the cortex and the striatum.
4. The indirect GC method developed for MDMA was adaptable to the separation and quantitation of the enantiomers of MDE and its deethylated metabolite, MDA. In contrast to the results obtained after administration of MDMA, whole brain levels of MDE were substantially lower, less MDA was found and enantioselectivity, when demonstrated, was reversed [R-(-) > S-(+)]. This is the first time, to my knowledge, that a metabolic study on MDE

involving investigation of enantiomers has been conducted.

5. In an *in vitro* metabolic system, nifedepine and to a lesser degree, lidocaine were shown to be good substrates for human CYP3A4. Under the experimental conditions employing either of the CYP isozymes, *rac*-MDE was a better substrate than *rac*-MDMA. However, neither CYP2D6 nor CYP3A4 were capable of catalyzing the N-dealkylation of MDMA and MDE to the extent that would be expected from the *ex vivo* experiments.

6. There is a paucity of information available about the effects of the enantiomers of MDE on uptake of neurotransmitter amines, and I undertook such a study using MDMA as a comparative drug. Both *r*ac-MDMA and *r*ac-MDE are capable of inhibiting the uptake of tritiated neurotransmitter into rat brain tissue prisms (potency: 5-HT > NE > DA). *Rac*-MDMA and *Rac*-MDE were equipotent at inhibiting the uptake of ^{3}H-5-HT and ^{3}H-NE, while *rac*-MDMA was more potent at ^{3}H-DA uptake inhibition. The S-(+)-isomers of both MDMA and MDE were more potent than their antipodes. S-(+)-MDMA was more potent than S-(+)-MDE at inhibiting uptake of NE and DA, while the reverse was true for 5-HT.

7. The results of the behavioral study comparing the three 3,4-methylenedioxy analogues of AMPH with a psychomotor stimulant and an hallucinogen suggested that analysis of the effects of the drugs on the temporal organization of behavior in rats more closely paralleled the subjective effects reported by humans than does measurement of frequency and duration of

discrete behaviors. This analysis successfully classified MDMA and MDA, and differentiated them from a stimulant and an hallucinogen. Furthermore, the hallucinogen was successfully differentiated from vehicle treatment.

8. When overall changes in NE levels, 5-HT parameters and DA parameters were considered together, the neurochemical effects of the different drugs provided further discriminative data that differentiated among the drug classes. In contrast to the results of the behavioral data, MDE could be grouped with MDMA and MDA in terms of its neurochemical effects.

9. The large differences in the levels of the various drugs in whole brain tissue from the animals are in keeping with the relative durations of behavioral effects in humans. These differences may reflect differences in pharmacokinetics which could play an important role in the results of the behavioral analysis. The role of active metabolites must also be considered.

7 *FUTURE RESEARCH*

Certain studies from this thesis require further investigation:

1. Our results show that marked differences exist between the levels in brain of the parent drug and the N-dealkylated metabolite and the enantioselectivity after administration of *rac*-MDE compared with *rac*-MDMA. Further comparative studies between these two structurally similar compounds, including pharmacokinetic studies could aid in our understanding of the role that pharmacokinetics, metabolism and chirality play in the long term effects of these drugs on serotonin systems. The continued recreational use of these drugs, despite the potential for toxic effects, makes further research critical.

2. Further *in vitro* studies with human CYP isozymes are required to accurately interpret the results reported in this thesis. Calculation of the K_m and V_{max} values for each of the various substrates could provide an optimal concentration of each substrate for specific metabolic reactions. Further investigation to identify the optimal incubation conditions (including incubation time, enzyme concentration, requirements for additions to the incubation mixture and/or appropriate electron-generating system) is also required. Finally, expansion of the choices of CYP isozymes could be useful in ultimately elucidating which isozyme(s) are responsible for the N-dealkylation of MDMA and MDE.

3. The behavioral analysis method must be extended to other representatives of the psychomotor stimulant and hallucinogen classes. In addition, further examination of dose-response relationships could clarify whether MDE is perhaps a "hybrid", sharing properties of more than one class, or is an entactogen devoid of any stimulatory activity.

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