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FINAL REPORT

NASA GRANT NGL 15-003-117

"Studies in Neuroendocrine Pharmacology"

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Section on Pharmacology
Medical Sciences Program
Indiana University School of Medicine
Bloomington, IN 47401

Principal Investigator: Roger P. Maickel, Ph.D.

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1.0 INTRODUCTION

The major theme in this project was to conduct as broad a series of studies as possible in areas relevant to the manned space effort utilizing the expertise and facilities available within the Section on Pharmacology of the Medical Sciences Program in conjunction with informational input from various NASA sources. Several aspects can be singled out for specific impetus, such as studies of the effects of amphetamines (which were accessible during various NASA space missions); examination of brain biogenic amines systems and stress phenomena (extremely relevant to the stresses of space flight); and the effects of commonly used therapeutic agents on a basic physiological drive such as deprivation-induced fluid consumption. In addition, many of these projects generated spin-off projects in related areas that yielded valuable data with significant relevance to aspects of the space program or to mankind.

2.0 EFFECTS OF DRUGS ON DEPRIVATION-INDUCED FLUID CONSUMPTION

A number of previous publications from this laboratory had utilized a standard test system for examining the effects of drugs on fluid consumption of deprived animals. In this system, rats are deprived of fluid for 23 hours of each day, then given access to an unlimited (but measureable) quantity during the remaining 60 minutes. Temporal drinking patterns and volume consumed are recorded; drugs are administered 15 minutes prior to the start of a test session. This test system was used to test a number of sedative-hypnotic drugs and tricyclic antidepressants, as well as to examine tolerance and taste phenomena.

2.1 Effects of Various Depressant Drugs on Deprivation-Induced Water Consumption.

The volume of water consumed in the standardized test system was significantly increased by barbiturates (amobarbital, hexobarbital, methohexital, phenobarbital), sedative-hypnotics (glutethimide, methaqualone, methyprylon), and anxiolytic agents (chlordiazepoxide, diazepam, meprobamate), but not by the antipsychotic agent, promazine. This latter finding confirms earlier studies with promazine and other phenothiazine derivatives. The results with the depressant drugs suggests that all of these agents (including the anxiolytics) are capable of causing physical dependence in man, since the only other drugs showing a similar dipsogenic action in rats are the opiate narcotics. (A-2, B-3)

2.2 Taste Phenomena Influences on Stimulation of Deprivation-Induced Fluid Consumption of Rats.

The dipsogenic action of barbital and chlordiazepoxide were found to be selectively influenced by the taste of the consummatory fluid presented to deprived rats. In the case of barbital, the potency of the drug (as a consummatory stimulant) is reduced when a more pleasant-tasting fluid (aqueous saccharin) is substituted for distilled water, while no alteration of efficacy is seen when the consummatory fluid is changed from water to an unpleasant-tasting solution (aqueous tartaric acid). Promazine, although its effect is to reduce, rather than increase, fluid consumption by deprived rats, has a similar effect in that its potency is reduced when the consummatory fluid is saccharin solution. In contrast, the potency of chlordiazepoxide as a dipsogenic agent was markedly reduced

when the taste of the consummatory fluid was altered to either more pleasant or less pleasant. (B-11)

2.3 Lack of Tolerance Development to the Dipsogenic Actions of Barbital.

Previous studies had confirmed the dipsogenic actions of barbital when given in acute dosage to rats in the test system utilized in this laboratory. Daily dosage of rats with barbital for 14 days produced a chronic dipsogenic state with severe overhydration. No tolerance could be seen to this effect; on withdrawal of drug dosage, the water intake of the rats (when computed on a body weight basis) fell to below normal levels. In contrast, when the daily intake of fluid was restricted during the period of barbital dosage, overhydration did not occur and drinking during the withdrawal phase was increased. These results suggest that the release of ADH by barbiturates could be a manifestation of an interaction involving the renin-angiotensin system. (B-8)

2.4 Interactions of Tricyclic Antidepressant Drugs with Deprivation-Induced Fluid Consumption by Rats.

A series of tricyclic antidepressant drugs, representing both secondary and tertiary side-chain amines, as well as four different ring systems, were examined for their effects on deprivation-induced fluid consumption in the standardized test system. All of the compounds reduced fluid consumption; the potency ranking was in the order: desipramine > amitriptyline > doxepin > nortriptyline > imipramine > protriptyline. Pretreatment of rats with α-methyltyrosine (in a dosage regimen that lowered brain norepinephrine by 50% with no effect on brain serotonin) reduced fluid

consumption; in such animals, a dose of protriptyline had its usual effect. Pretreatment of rats with p-chlorphenylalanine had a time-dependent effect on both brain amines and fluid consumption. At 1 day, brain serotonin was 40% of normal brain norepinephrine was lowered by 35%, and fluid consumption was reduced. At 8 days after dosage, brain serotonin was 50% of normal, but brain norepinephrine and fluid consumption had returned to normal. By 15 days after dosage, brain amines and fluid consumption had all returned to normal. Administration of protriptyline had no effect on fluid consumption on day 1, but had its usual magnitude of effect on days 8 and 15. (B-19)

2.5 Pharmacological and Toxicological Effects of Alkali and Alakline Earth Metal Ions as Measured by Deprivation-Induced Fluid Consumption.

Two series of ions were selected: alkali metals (Li^+, Na^+, K^+, Cs^+) and alkaline earth metals (Mg^{++}, Ca^{++}, Sr^{++}, Ba^{++}). The anionic component was kept constant as the Cl^- ion, and all dosages were by i.p. injection as solutions in distilled water. Dose-response data were collected over ranges of 0.02 - 0.06 m moles/kg (Ba^{++}) to 1 - 10 m moles/kg (Na^+, K^+) depending on the toxicity of each ion. Dose related stimulation of deprivation-induced fluid consumption was evoked by Na^+, K^+, Ca^{++}, and Sr^{++}, while the other ions, except for Ba^{++}, had variable actions. Ba^{++} showed only a depressant action on deprivation-induced fluid consumption and was the most severely toxic. Differential effects of the ions on fluid consumption and overt behavior were clearly observable. (A-18)

3.0 STUDIES ON BRAIN BIOGENIC AMINES.

There is little doubt that many drugs can influence mammalian behavior by virtue of interaction(s) with biogenic amine neurotransmitter systems in the brain. Thus, alteration of these neurotransmitter systems by administration of compounds with specific actions prior to administration of a test drug may cause the expected action of that test drug to be perturbed. In addition, selective alteration of brain biogenic amine systems by specific agents may yield relevant information regarding the systems themselves or the agents that influence them.

3.1 Differential Effects of Monoamine Oxidase Inhibitors.

Daily dosage of rats for 20 days with non-toxic amounts of various monoamine oxidase inhibitors had clearly differential actions on levels of brain biogenic amines and spontaneous motor activity. Open field activity was initially depressed by isocarboxazid, but was elevated by the 6th day of treatment and remained up throughout the dosage period. Pargyline caused increased activity on the 6th and 9th dosage days and Su-11,739 increased activity on the 6th dosage day; both of these compounds had no other actions. Tranyleypromine consistently decreased activity over the entire dosage course. Brain levels of norepinephrine and serotonin (on day 20) were elevated by all compounds. The effects on norepinephrine were: Su-11,739 > pargyline, tranyleypromine > isocarboxazid; the effects on serotonin were: tranyleypromine > Su-11,739 > pargyline > isocarboxazid. Su-11,739 and tranyleypromine decreased brain levels of 5-hydroxyindole-3-acetic acid, while isocarboxazid and tranyleypromine were without effect. (B-10)

3.2 On the Role of Brain Biogenic Amines in the Control of Pituitary-Adrenocortical Activity.

Pretreatment of rats with a monoamine oxidase inhibitor such as pargyline prevents the metabolic destruction of serotonin and norepinephrine released from brain stores. Administration of a subsequent dose of reserpine will thus markedly elevate the ratio of free:bound amines in the brain. Under these conditions, the ACTH hypersecretion usually evoked by reserpine does not occur, suggesting that the lowering of brain amine levels by reserpine is the mechanism of action for that drug's effect. The elevated levels of brain serotonin and norepinephrine produced by the inhibition of monoamine oxidase did not cause ACTH hypersecretion themselves, nor did they prevent the ACTH hypersecretion caused by exposure to cold or administration of sedative doses of chlorpromazine. (B-16)

3.3 Comparative Effects of 5,6-Dihydroxytryptamine and Its Benzo[b]-thiophene Analogue on Biogenic Amines in the Rat.

Administration of a single i.p. dose of either compound to rats caused a significant reduction in levels of norepinephrine in heart and spleen. In contrast, only 5,6-dihydroxytryptamine reduced spleen levels of serotonin, and neither compound had any effect on brain norepinephrine or serotonin. When administered directly into the lateral ventricle of the rat brain, both compounds reduced the levels of norepinephrine for a period of less than 24 hours. The 5,6-dihydroxytryptamine caused a prolonged lowering of brain levels of serotonin and 5-hydroxyindole-3-acetic acid; in contrast, its benzo[b]thiophene analogue was completely without effect on brain serotonin levels. (A-16, A-24, B-20, C-3)

3.4 Structure-Activity Relationships in Analogues of 5,6-Dihydroxytryptamine.

Studies of a variety of compounds related to 5,6-dihydroxytryptamine, including 5-hydroxy-6-methoxytryptamine, 6-hydroxy-5-methoxytryptamine, 5,6-dimethoxytryptamine, and 3-(β-aminoethyl)-5,6-isopropylidenedioxybenzo[b]thiophene demonstrated a wide variety of behavioral and biochemical differences related to specific structural entities. For example, the key structural factor in selective neurotoxic actions toward serotonergic systems appears to be the ability to form a quinone-type structure, while the requisite component for affecting catecholaminergic systems appears to be the catechol mority. The isopropylidenedioxy compound appears to have extreme biological stability and unique toxicological actions. (A-20, A-23, D-1, D-2)

4.0 BIOCHEMICAL RESPONSES TO STRESSFUL STIMULI

When the mammalian organism is subjected to stressful stimuli, a host of mechanisms are activated, providing a diversity of response phenomena. Many measurable changes occur in biochemical parameters, some of which are directly relevant to the stress imposed, while others are second order or incidental. In addition, responses may differ depending upon the type of stress and whether it is of an acute, repetitive, or chronic nature. Exploration of a number of parameters in response to different stress situations could lead to a descriptive characterization of the stress process in animals and indicate appropriate parameters to measure in man.

4.1 Development of a Model Stress System for Repetitive Daily Stress.

Using mice as the test system for three differentially unique stressors (cold exposure, forced immobilization, drug-induced stimulation), the effects of single and repetitive stress application were measured on a variety of biochemical parameters: plasma corticosterone, glucose, and free fatty acids, and brain norepinephrine, serotonin, and 5-hydroxyindole-3-acetic acid. In a single exposure, all three stressors elevated plasma corticosterone; amphetamine elevated plasma glucose, while cold exposure and immobilization produced a hypoglycemia; plasma fatty acids were elevated by cold exposure, depressed by immobilization, and unaffected by amphetamine. When the acute stress was repeated daily for two weeks, adaptation was seen in plasma corticosterone in the repeatedly immobilized mice, while amphetamine and cold exposure continued to evoke elevated levels; plasma glucose levels were depressed in amphetamine-treated and immobilized mice but normal in mice exposed to cold; and plasma fatty acid levels were normal in all animals. When animals were subjected to an acute stress after two weeks of repetitive stress, the degree of responding was reduced for all combinations; the effect was greatest for repetitive cold exposure and least for repetitive amphetamine dosage.

Acute amphetamine was without effect on brain biogenic amines, although repetitive amphetamine for two weeks caused lowered levels of brain norepinephrine. Acute cold exposure or immobilization elevated brain levels of serotonin and 5-hydroxyindole-3-acetic acid; on repetitive exposure, significant decreases were seen in both compounds. No effects were seen on brain norepinephrine with either cold stress or immobilization

after single or repetitive exposure. Repetitive cold stress enhanced the increased serotonin and 5-hydroxyindole-3-acetic acid caused by all three acute stresses and reduced the effects of acute amphetamine on brain norepinephrine; repetitive amphetamine enhanced the effects of a subsequent cold exposure on brain norepinephrine; repetitive immobilization was virtually without effect on subsequent acute stresses. (B-7, D-5)

4.2 Stress Effects on Conversion of Glucose to CO_2 in Mice.

Administration of ^{14}C-labeled glucose to mice under various acute stresses leads to differential excretion of $^{14}CO_2$. Cold stress increases the rate of conversion of C-1 or C-U, decreases the rate of conversion of C-2, and has no effect on C-6. Immobilization stress has no effect on the rate of conversion of C-1 glucose to CO_2, but causes a decreased rate for C-2, C-6, and C-U. Amphetamine stress increases the rate of conversion for C-U glucose to CO_2, but results in a decreased rate of conversion for C-1, C-2, and C-6. (D-6)

5.0 BIOCHEMICAL AND BEHAVIORAL PHARMACOLOGY OF AMPHETAMINES

Amphetamine and a variety of analogues and congeners are extremely interesting and cogent to this overall research program for several reasons. First of all, supplies of amphetamine, alone, or in combination with another agent, were available to the astronauts on space flights for use as antifatigue and antimotion sickness agents. Secondly, the amphetamines represent a class of pharmacological agents with intriguing actions on a variety of body systems that involve biogenic amines such as dopamine and norepinephrine. Thirdly, the known behavioral and

pharmacological effects of the amphetamines suggest that these drugs interact with a number of basic drive systems including arousal, hunger, and sleep. Finally, the structure-activity relationships of the amphetamines are most amenable to a variety of manipulations and investigative studies.

5.1 Differential Effects of αMT on Anorectic and Stimulatory Actions of Amphetamines.

Pretreatment of rats with a dosage regimen of αMT (α-methyltyrosine) that reduced brain levels of norepinephrine by 40-60% had a slight anorectic effect, but no effect on continuous avoidance responding. Such pretreatment had no effect on the anorectic action of a single dose (2 mg/kg) of d-amphetamine or methamphetamine, but markedly reduced the increased continuous avoidance responding usually evoked by those compounds. In contrast, similar pretreatment with αMT had no effect on the actions of benzphetamine in either test system. (B-15)

5.2 Interactions of Caffeine with Various Amphetamines on Rat Food Consumption and Avoidance Responding.

Pretreatment of rats with caffeine potentiated the actions of the p-chloro- and p-methyl-analogues of amphetamine and benzphetamine as depressants of deprivation-induced food consumption, although no such effects were seen with the unsubstituted compounds. Similar caffeine pretreatment completely antagonized the stimulant effect of d-amphetamine and its p-chloro analogue on continuous avoidance responding. In contrast, pretreatment with caffeine potentiated the ability of the p-methyl analogues of amphetamine and benzphetamine to decrease avoidance responding,

and converted the normally modest stimulatory action of benzphetamine on avoidance responding to that of a depressant action. (B-18)

5.3 Drug Interactions on Body Temperature Maintenance in the Mouse.

Exposure of mice to an environmental temperature of 4° C leads to a loss of body temperature and death in 5-6 hours. Intraperitoneal injection of water or solutions of d-amphetamine exacerbates this effect, while caffeine has a modest antagonistic action. The combination of d-amphetamine plus caffeine is synergistic; a rapid fall in body temperature causes death to occur in less than 3 hours of cold exposure. (C-5)

5.4 Differential Effects of d- and l-Amphetamine on Spontaneous Motor Activity in Mice.

The effects of single doses of d- and l-amphetamine on motor activity in mice differed in both quantitative and qualitative aspects. At low doses (0.5 mg/kg, i.p.) and at high doses (8.0 mg/kg, i.p.) both isomers were stimulants of spontaneous motor activity, differing only in potency. However, at intermediate doses (2.0 - 4.0 mg/kg, i.p.) the l-isomer caused a significant depression of spontaneous motor activity, while the d-isomer was stimulatory. (A-14, B-9)

5.5 Influence of Drugs Altering Brain Biogenic Amines on the Effects of Amphetamine Isomers on Locomotor Activity.

In mice, doses of d-amphetamine of 1.0 or 4.0 mg/kg, i.p. both produced a markedly increased level of spontaneous motor activity. Pretreatment of the animals with αMT reduced the duration of the stimulatory effect while pretreatment with pargyline reduced the magnitude of the amphetamine effect. Pretreatment with p-chlorophenylalanine reduced the stimulatory effect of

the lower dose of d-amphetamine but increased the action of the higher dose. Pretreatment of the mice with reserpine amplified the stimulatory effects of d-amphetamine as compared to animals given only reserpine.

Administration of a single dose of 1.0 mg/kg of l-amphetamine had only a transient depressant action on spontaneous motor activity in mice, while a larger dose (4.0 mg/kg, i.p.) caused a marked depression for 30 minutes. These effects were not significantly changed by the αMT pretreatment but were altered by pretreatment with p-chlorophenylalanine, pargyline, or reserpine. The results suggest actions of the amphetamine enantiomers occur through different biogenic amine systems. (A-14, C-6)

5.6 Differential Pharmacology of Amphetamine Enantiomers.

In rats, a single i.p. dose of 2.0 mg/kg of d-amphetamine induces a significant increase in navel spontaneous motor activity, while a similar dose of l-amphetamine induces a decrease in that activity. Chronic pretreatment with αMT blocks the stimulatory effect of the d-isomer blocks the stimulatory effect of the d-isomer but prolongs the depression induced by the l-isomer. Chronic pretreatment with pargyline induces a depression of spontaneous motor activity that is qualitatively, but not quantitatively, reversed by d-amphetamine; the pargyline pretreatment has no effect on the depressant action of a subsequent dose of l-amphetamine. Pretreatment with p-chlorophenylalanine has no major influences on the stimulatory actions of d-amphetamine, but alters the magnitude and duration of the depressant actions of l-amphetamine. The results suggest that d-amphetamine influences only catecholaminergic systems while l-amphetamine also influences serotonergic systems. (A-22, D-4)

6.0 BIOCHEMICAL STUDIES OF ANALOGUES TO BIOLOGICALLY ACTIVE INDOLE COMPOUNDS

The continuing interest of this laboratory in biologically active indole compounds and the key roles of tryptophan as an essential amino acid in mammalian nutrition, a key component in active protein molecules, and a precursor of the biogenic amines serotonin and melatonin, led to a number of studies of compounds structurally similar to tryptophan. In particular, this area of research was particularly fertile ground for studies of molecular structure relationships to biological activity, using the principles of selective molecular modifications.

6.1 Intestinal Transport of Tryptophan and Its Analogues.

A comparative study of the intestinal transport of DL-tryptophan and its 1-methylindole and benzo[b]thiophene analogues was performed *in vitro*, using the everted intestinal sac preparation of the rat and hamster. Both tryptophan and its benzo[b]thiophene analogue were actively transported by this preparation, while 1-methyltryptophan was not. The active transport of tryptophan was competitively inhibited by the benzo[b]-thiophene analogue, but not by 1-methyltryptophan, suggesting little or no interactions of the latter with the carrier process. The transport of tryptophan and its benzo[b]thiophene analogue was depressed by high concentrations of either compound (> 10 mM); all three amino acids produced subtle alterations in the barrier properties of the sacs as evidenced by increased diffusion rates observed for tetraethylammonium bromide. (A-8, B-14)

6.2 Bioisosteric Inhibition of DOPA Decarboxylase.

Using an *in vitro* test system of purified hog kidney aromatic-L-amino acid decarboxylase, the benzo[b]thiophene analogue of 5-hydroxytryptophan was found to be an effective inhibitor of DOPA decarboxylation, with a potency almost twice that of α-methyl-DOPA. When administered to mice prior to a dose of labeled L-DOPA, the compound led to significantly elevated brain levels of DOPA, dopamine, and DOPA metabolites, suggesting that sufficient inhibition of peripheral decarboxylation had been achieved to effectively increase the amount of DOPA that entered the brain. (A-4, A-17, C-1)

6.3 The Metabolic Fate of the Benzo[b]thiophene Analogue of Tryptophan in the Rat.

In mammals, the amino acid tryptophan is metabolically degraded via a number of pathways, including decarboxylation, transamination, and ring cleavage via tryptophan pyrrolase. However, when the benzo[b]thiophene analogue of tryptophan was administered to rats, no evidence was seen for decarboxylation or ring opening. Instead, > 90% of the excretion products could be identified as unchanged compound and the benzo[b]thiophene analogues of indole-3-lactic acid, indole-3-pyruvic acid, indole-3-acetic acid and indole-3-acetylglycine. (A-19, C-2)

7.0 PHARMACOLOGICAL STUDIES OF ANALOGUES TO BIOLOGICALLY ACTIVE INDOLE COMPOUNDS.

Following the aspects of biologically active indole analogues discussed in 6.0 (above) studies have also been carried out of the comparative

structure-activity relationships of analoques of tryptamine in various in vivo and in vitro systems containing responsive tryptamine receptors. The results obtained are compatible with a structural specificity for the unsubstituted indolic nitrogen.

7.1 Pressor Effects of Tryptamine Analogues.

Methylation of tryptamine in the 1-position had little effect on the potency of the drug as a pressor agent in the intact anesthetized rat, while the benzo[b]thiophene analogue of tryptamine had a markedly decreased pressor activity. Pretreatment of the animals with the α-sympatholytic agent, phenoxybenzamine, reduced the pressor effects of all three compounds. In contrast, pretreatment of the animals with reserpine reduced the pressor effects of tryptamine and its benzo[b]thiophene analogue, but increased the pressor effects of 1-methyltryptamine. (A-9, B-17).

7.2 Contractile Responses to Tryptamine Analogues in Isolated Smooth Muscle.

Tryptamine and its benzo[b]thiophene and 1-methylindole analogues had a lower affinity for receptors in rat stomach fundus than did serotonin; the interaction between methysergide and serotonin in this system was competitive, while the interactions between methysergide and tryptamine and its analogues were noncompetitive. In contrast, the affinity of tryptamine and 1-methyltryptamine for receptors in rat aortic strips was greater than the affinity for serotonin; all compounds showed noncompetitive interactions with methysergide in this preparation. (A-21, C-4)

8.0 STUDIES IN CHEMICAL PHARMACOLOGY - DRUG DISPOSITION AND DRUG METABOLISM.

In the course of carrying out a coordinated program of studies in biochemical pharmacology, a number of occasions arise when studies of the

physiological disposition and/or metabolic fate of chemical compounds is relevant to other aspects of the pharmacology of such substances.

8.1 Inability of Rat Brain Homogenate to Oxidize Amphetamine.

Incubation of rat brain homogenate with labeled amphetamine failed to produce any decrease in the amount of amphetamine or any significant metabolites. The conversion of neotetrazolium chloride to diformazan by the tissue extract occurred with or without the presence of amphetamine. It was concluded that the literature report on metabolism of amphetamine by brain homogenate in vitro was erroneous. (B-6)

8.2 Metabolism of 3-(2-Dimethylaminoethyl)benzo[b]thiophene In Vitro and In Vivo in the Rat.

The metabolism of 3-(2-dimethylaminoethyl)benzo[b]thiophene has been studied in vivo and in vitro in rats. The results indicate the major pathways of metabolism to be 6-hydroxylation, N-dealkylation, and oxidation by monoamine oxidase. No evidence for sulfoxidation was found. Metabolites were identified by combinations of mass spectrometry, nuclear magnetic resonance spectroscopy, thin-layer chromatography, and liquid scintillation counting. (A-7, B-4)

8.3 Comparative Physiological Disposition of Melatonin and Its Benzo-[b]thiophene Analogue in the Rat.

Melatonin and its benzo[b]thiophene analog were labeled by acetylation of the corresponding 5-methoxyarylethylamines with tritiated acetic anhydride. The benzo[b]thiophene analogue had a much higher lipid solubility as measured by partition between neutral aqueous solution and organic solvents. When administered to rats, both compounds disappeared

from plasma and tissues by first-order decay. The dispositions were similar, with the higher lipid solubility of the benzo[b]thiophene analogue resulting in higher tissue:plasma ratios, especially in adipose tissue, kidney, and liver, and a longer half-life in plasma and tissues. (A-11, B-12)

8.4 Physiological Disposition of Atropine in the Rat.

The physiological disposition of atropine was studied in rats, using tritium-labeled drug and a specific assay method. At doses of 1.25 to 10 mg/kg, i.p., the greatest localization was seen in kidney and liver, with tissue:plasma ratios of > 10:1. Tissue half-lives over the period 0.5 - 4 hours ranged from 40-46 minutes in plasma to 97-106 minutes in adipose tissue. (A-10, B-5)

9.0 STUDIES IN TOXICOLOGY

Another facet of an overall project in biochemical pharmacology involves the need for testing various compounds of interest for their toxicological profiles or examination of specific toxicological phenomena. In some cases, such studies yield significant data that has relevance in a much broader scope than merely descriptive characterization of a compound.

9.1 Pharmacological and Toxicological Studies on 1,4-Butanediol.

1,4-Butanediol is a widely used agent in the manufacture of a number of polymeric materials. The LD_{50} in rats was found to be 1328 mg/kg, i.p. The compound had a significant sedative effect at dosages > 300 mg/kg. Effects of the drug on spontaneous motor activity were biphasic; doses of 50-200 mg/kg significantly reduced spontaneous motor activity, while doses > 300 mg/kg caused loss of the righting reflex. No increase in liver

triglycerides was seen even after 14 days of dosage at 1000 mg/kg daily. (A-15, B-13)

9.2 Hepatotoxic Effects of Iproniazid.

Iproniazid represents a loss of monoamine oxidase inhibitors that are derivatives of hydrazine and thus have toxic biochemical effects by virtue of their ability to chemically react with carboxyl groups in the mammalian organism. Studies in mice chronically dosed with iproniazid indicated that significant strain differences exist with regard to the ability of the drug to influence liver triglycerides of animals on normal or pyridoxine-deficient diets. The effects of acute iproniazid dosage in Swiss-Webster mice on a control diet were manifested by hypoglycemia; in animals on a pyridoxine-deficient diet, the drug induced both hyperglycemia and and hyperlipemia. In addition, the administration of tryptophan or its benzo[b]thiophene or 1-methylindole analogues to mice pretreated with iproniazid led to a marked hepatotoxicity. (A-6, A-12, A-13, D-3)

10.0 CHEMICAL METHODOLOGY

In any project of this type, a variety of situations arise in which analytical or synthetic chemical methodology is required as a supportive effort. Several of these efforts resulted in significant productivity independent of, but related to, projects described elsewhere.

10.1 Mass Spectra of Carcinogenic 4-Hydroxylaminoquinoline N-oxides.

The mass spectra of carcinogenic 4-hydroxylaminoquinoline N-oxide and some methyl substituted derivatives have been determined. Fragmentation of the parent compound proceeds from M to M-2, M-16 and M-17. The M and M-2 ions are particularly valuable. (B-1)

10.2 Isotopic Procedures in Biomedical Analyses.

The use of radioisotopes in analytical chemistry is widespread, although the applications to biomedical problems are somewhat limited. Of particular interest is the potential use of radiolabeled derivatizing agents to permit the quantitative estimation of submicrogram quantities of organic compounds in biological materials. Several examples may be cited: (1) the use of ^{3}H-acetic anhydride to determine primary and secondary amines, and steroids; (2) ^{14}C-methyl iodide to determine tertiary amines; and (3) $^{63}Ni^{+3}$ to determine 3-ethoxy-2-ketobutyraldehydedithiosemicarbazone. In addition, enzymatic procedures utilizing transferase enzymes and ^{14}C-S-adenosylmethionine have been developed for the determination of catecholamines. The potential applications of these approaches, using labeled derivatization reagents of classical semi-micro organic functional group analysis and/or purified transferase enzymes with labeled cofactors are an almost virgin field of research with tremendous potential. (A-1)

10.3 Physicochemical Properties of Benzo[b]thiophene and 1-Methylindole Analogues of Indolic Compounds of Biological Interest.

Within a series of structures, i.e., indole, benzo[b]thiophene, 1-methylindole, lipid solubility was determined by the side chain, ranking in the order: $-CH_2CH_2OH$ > $-CH_2CH_2N(CH_3)_2$ > $-CH_2CH_2NH_2$ > $-CH_2COOH$, $-CH_2CH(NH_2)COOH$. Within a given series of constant side chains, i.e., $-CH_2CH_2NH_2$, $-CH_2CH_2N(CH_3)_2$, $-CH_2CH(NH_2(COOH$, or $-CH_2COOH$, the lipid solubility ranked in the order: benzo[b]thiophene > 1-methylindole > indole. The influence of molecular alteration on pK_a values of the compounds are correlated

with lipid solubility. The results are significant in relation to the biological activities and physiological disposition of the various compounds. (A-5)

10.4 Site-Specific Deuteration or Tritiation of Benzo[b]thiophene and 1-Methylindole Analogues of Biologically Active Indole Compounds.

The site-specific deuteration and tritiation of 1-methylindole and benzo[b]thiophene analogues of biologically active indole derivatives has been achieved by the use of the facile metalation of these heterocycles with n-butyllithium and subsequent reaction with $^{2}H_2O$ or $^{3}H_2O$. Metallation occurs at the 2-position and site and extent of deuterium incorporation was determined by NMR. The use of similar procedures with $^{3}H_2O$ led to ready incorporation of tritium into these heterocycles. (A-3, B-2)

11.0 BIBLIOGRAPHY

Research supported by this grant has yielded a variety of published materials. These have been categorized as follows:

I. Abstracts of papers presented at scientific meetings (listed in Section 11.1).

II. Published papers that have appeared or are in press in the open scientific literature (listed in Section 11.2).

III. Manuscripts that have been completed and have been submitted for publication (listed in Section 11.3).

IV. Manuscripts that are in process and have not yet been submitted for publication (listed in Section 11.4).

A complete set of items in categories II and III is included as an Appendix to this report.

11.1 Abstracts

A-1 Maickel, R.P.: Isotopic procedures in biomedical analyses. Eastern Analytical Symposium, Atlantic City, NJ, November 1-3, 1972.

A-2 Maloney, G.J. and Maickel, R.P.: Effects of depressant drugs on deprivation-induced fluid consumption in rats. Society for Neuroscience, Second Annual Meeting, Houston, TX, November 8-11, 1972.

A-3 Bosin, T.R. and Rogers, R.B.: Chemical pharmacology of benzo[b]-thiophene derivatives. VIII. Facile ^{3}H labeling of compounds of pharmacological interest. The Pharmacologist 15:244, 1973.

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11.4 Papers in Preparation.

D-1 Donelson, A.C. and Maickel, R.P.: Behavioral pharmacology of 5,6-dihydroxytryptamine analogues.

D-2 Donelson, A.C. and Maickel, R.P.: Pharmacology and toxicology of 3-(β-aminoethyl)-5,6-isopropylidenedioxybenzo[b]thiophene.

D-3 Maickel, R.P., Bowman, D.R., Fedynskyj, N., Snodgrass, W.R. and Ryan, M.P.: Strain differences in effects of iproniazid on mouse liver triglycerides.

D-4 Maickel, R.P., Light, K.E., and Zabik, J.E.: Differential behavioral pharmacology of amphetamine enantiomers in the rat.

D-5 Quirce, C.M. and and Maickel, R.P.: Some biochemical correlates of stress.

D-6 Quirce, C.M. and Maickel, R.P.: Oxidative conversion of glucose to CO_2 in the mouse *in vivo*: effects of stress.

12.0 APPENDIX II

PAPERS PUBLISHED OR IN PRESS

VOL.6, No. 3
NOVEMBER 1973

Research Communications in
Chemical Pathology and Pharmacology

MASS SPECTRA OF CARCINOGENIC 4-HYDROXYLAMINOQUINOLINE N-OXIDES

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Received Sept. 10, 1973

ABSTRACT

The mass spectra of carcinogenic 4-hydroxylaminoquinoline N-oxide and some methyl substituted derivatives have been determined. The diagnostic value of these fragments may be a facile means to study the metabolism and mechanism of carcinogenesis of these compounds.

INTRODUCTION

The known chemical carcinogens are composed of a multitude of structural types, including 4-nitroquinoline N-oxides. This class of chemical carcinogens, first prepared by Ochiai and co-workers (1943), was demonstrated to be carcinogenic by Nakahara et. al., (1957). Following this demonstration of carcinogenicity, a number of papers have appeared describing the physical, chemical, and spectral characteristics of 4-nitroquinoline N-oxide and its derivatives; this data and the mechanism of carcinogenicity has been summarized in a recent monograph (Endo et. al., 1971).

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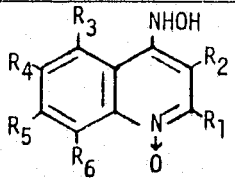
Sugimura et. al. (1966) demonstrated that mammalian cells metabolically transform 4-nitroquinoline N-oxide to 4-hydroxylaminoquinoline N-oxide, a more potent carcinogenic moiety considered to be the proximate agent. In an earlier paper (Bosin and Maickel, 1972) we reported on the mass spectral fragmentation patterns produced by carcinogenic methyl substituted 4-nitroquinoline N-oxides. As a continuation of that problem, we now report the mass spectral fragmentation patterns produced by methyl substituted 4-hydroxylaminoquinoline N-oxides.

MATERIAL AND METHODS

The structures of the compounds studied in this report are presented in Table 1.

Table 1

Structures of Compounds Subjected to Mass Spectral Studies



Substituent Position		R_1	R_2	R_3	R_4	R_5	R_6
Ring Position #		2	3	5	6	7	8
Compound Name	Compound #						
4-Hydroxylaminoquinoline N-oxide	I	H	H	H	H	H	H
2-Methyl-I	Ia	CH_3	H	H	H	H	H
5-Methyl-I	Ib	H	H	CH_3	H	H	H
6-Methyl-I	Ic	H	H	H	CH_3	H	H
7-Methyl-I	Id	H	H	H	H	CH_3	H

The precursor methyl substituted 4-nitroquinoline N-oxides were prepared as previously described (Bosin and Maickel, 1972) and were reduced to the corresponding 4-hydroxylamino derivatives by known procedures (Kawazoe and Tachibana, 1967). Melting points were in agreement with those reported (Endo et. al., 1971). Analytical purity was confirmed by thin-layer chromatography on silica gel G plates (Analtech, precoated, prescored, thickness 0.25 mm) using two solvent systems: methanol, and chloroform: methanol (1:4, V:V); visualization was accomplished by ultraviolet light (350 nm) or alkaline permanganate spray.

The mass spectra were determined on Varian MAT CH-7 and AEI MS-9 mass spectrometers at an ionizing potential of 70 ev. The direct probe method was used in all cases.

RESULTS AND DISCUSSION

Figure 1 gives the complete spectra and Table 2 selected ions of 4-hydroxylaminoquinoline N-oxide and some methyl substituted derivatives. The identification of the aromatic hydroxylamine group by mass spectrometry has been reported (Coutts and Mukherjee, 1970) and the diagnostic value of M, M-2, M-16, and M-17 emphasized. The diagnostic value of the M-16 ion, in the present case, is complicated by the possibility for loss of oxygen from both the N-oxide and the hydroxylamino groups. In the mass spectra of methyl substituted 4-hydroxylaminoquinoline N-oxides, there is no M-17 fragment, in contrast to simple aromatic hydroxylamines and methyl substituted 4-nitroquinoline N-oxides (Bosin and Maickel, 1972). In the latter case the M-17 ion was indicative of a methyl group adjacent to either the M-oxide or nitro group. Thus, for diagnostic purposes, the M and M-2 ions in combination with the select ions in Table 2 provide ready spectral characterization of these

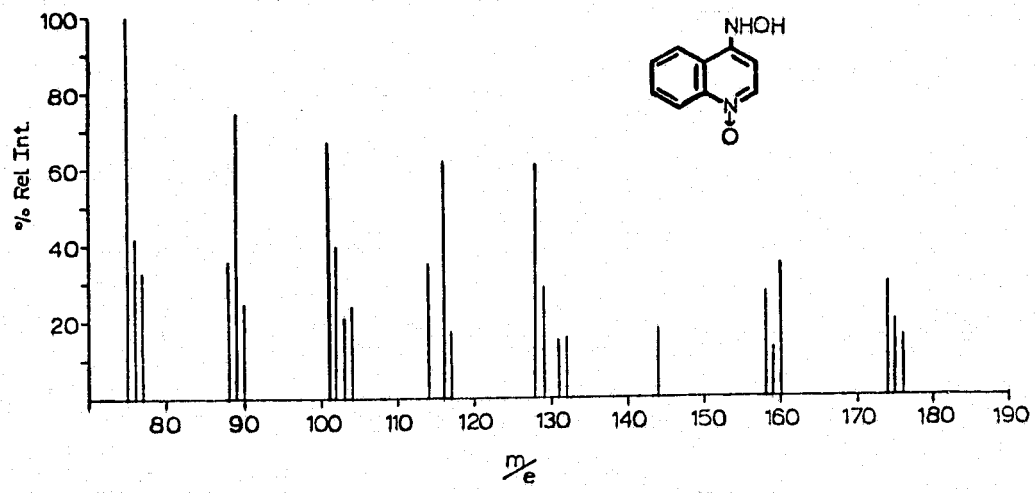
Table 2

Selected Ions of 4-Hydroxylaminoquinoline N-oxide Derivatives

COMPOUND	I	Ia	Ib	Ic	Id
Fragment	% Relative Intensity				
M	16	11	71	0	13
M-1	20	30	19	27	38
M-2	30	58	17	100	71
M-16	35	76	38	68	68
M-18	28	0	53	53	36
M-32	18	37	43	93	62
M-33	0	58	45	43	25
M-47	29	49	36	28	54
M-48	61	70	48	93	89
M-60	62	100	17	68	100
M-62	35	47	40	27	46
M-75	67	52	40	73	89
M-87	75	99	17	68	66
Base Peak, m/e	75	130	155	188	130

compounds. Figure 2 provides a proposed rationalization for the mass spectral fragmentation of Id via the M-2 pathway.

This mass spectral characterization described should be applicable to further studies of the metabolism of methyl substituted 4-hydroxylamino-quinoline N-oxides in relation to their carcinogenic effects.


NHOH
O
% Rel Int.
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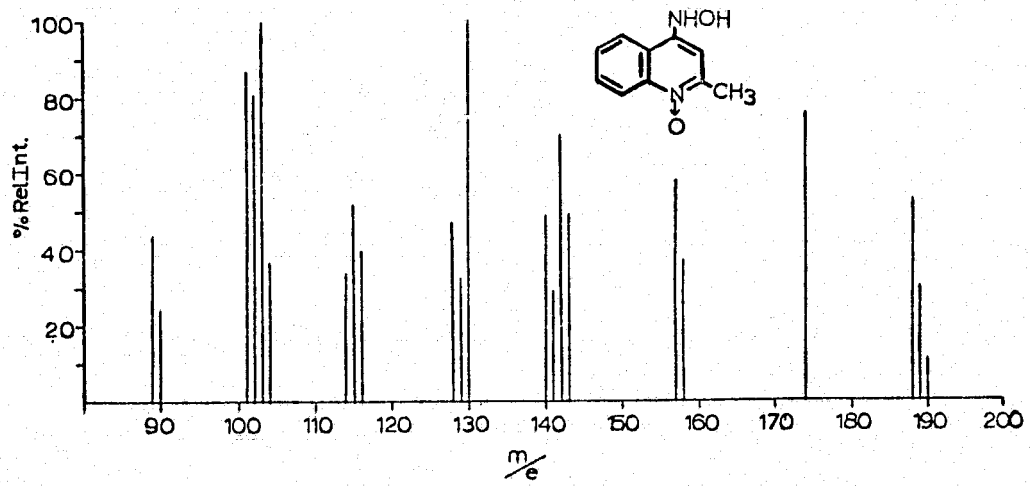
80
60
40
20
80 90 100 110 120 130 140 150 160 170 180 190
m/e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O
% Rel Int.
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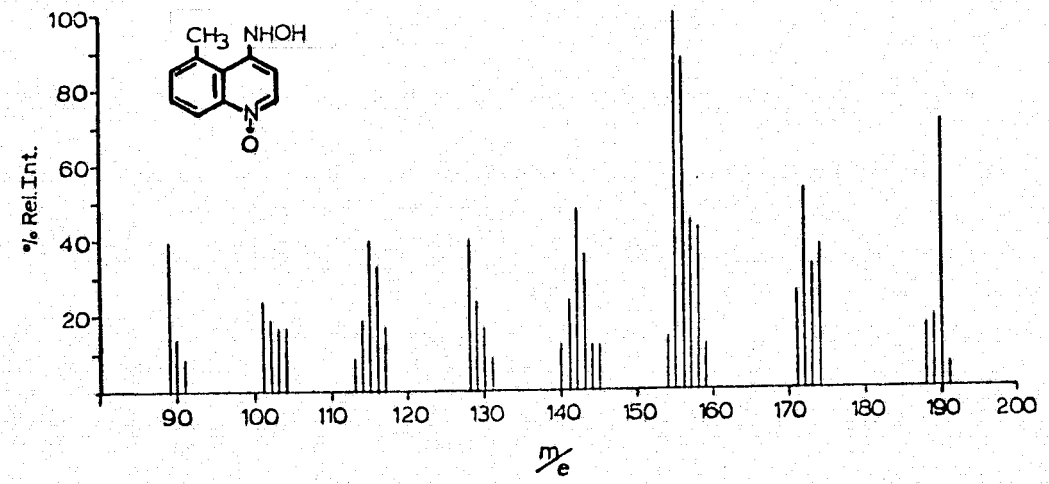
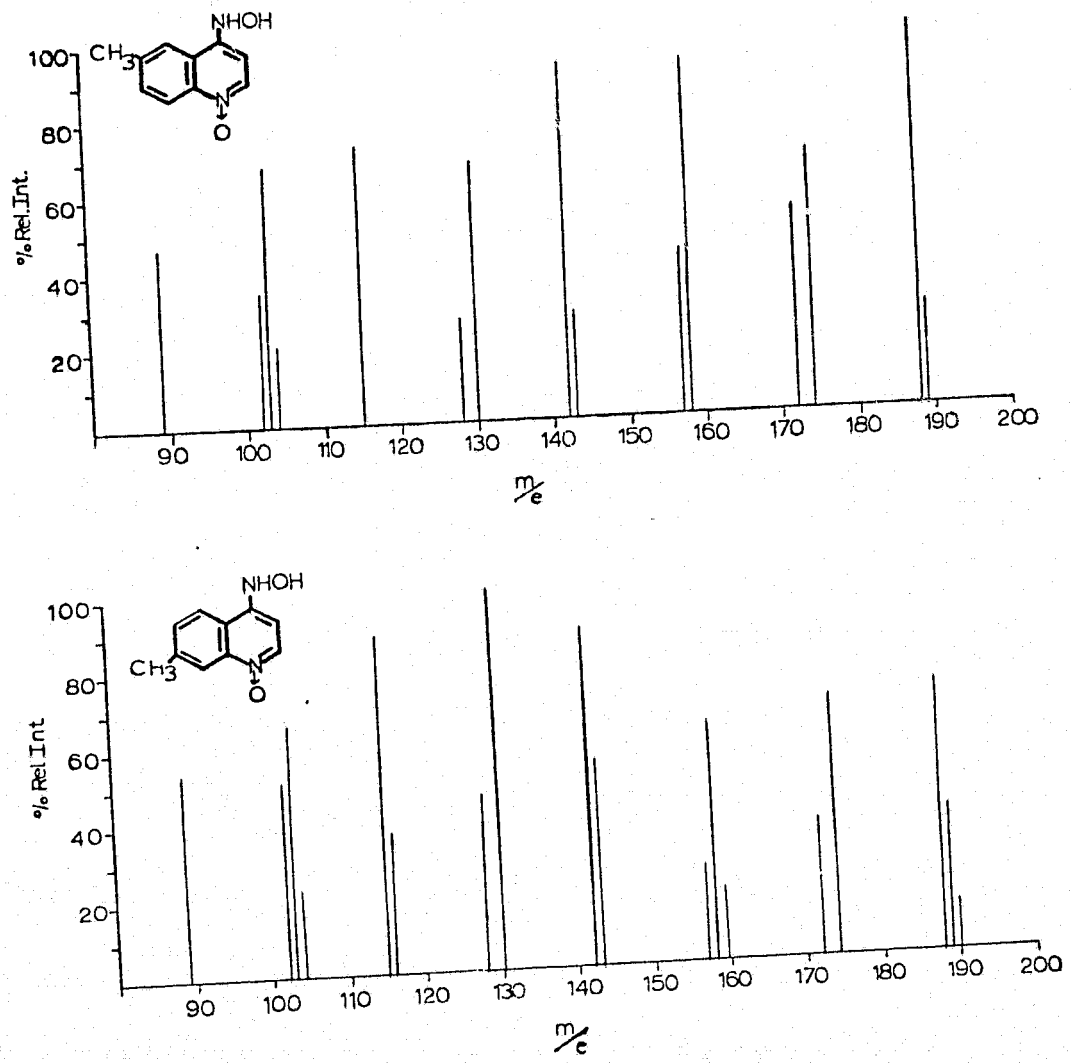
80
60
40
20
90 100 110 120 130 140 150 160 170 180 190 200
m/e


CH3 NHOH
O
% Rel Int.
100
80
60
40
20
90 100 110 120 130 140 150 160 170 180 190 200
m/e


NHOH
CH3
N
O
100
80
60
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% Rel. Int.
90
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120
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140
150
160
170
180
190
200
m/e
NHOH
CH3
N
O
100
80
60
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20
% Rel Int
90
100
110
120
130
140
150
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170
180
190
200
m/e

Figure 1

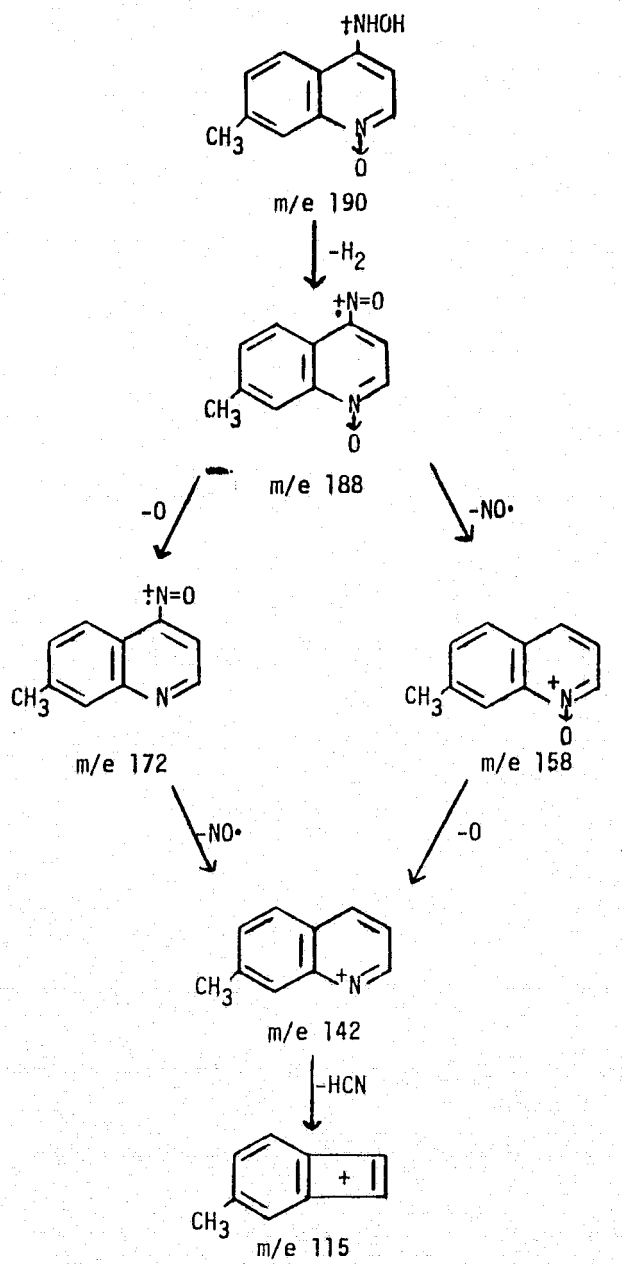
+NHOH
CH3
N
O
m/e 190
-H2
+N=O
CH3
N
O
m/e 188
-O
-NO•
+N=O
CH3
N
m/e 172
CH3
+
N
O
m/e 158
-NO•
-O
CH3
+N
m/e 142
-HCN
CH3
+
m/e 115

Figure 2

ACKNOWLEDGEMENTS

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Nakahara, W., Fukuoka, F. and Sugimura, T. (1957). Carcinogenic action of 4-nitroquinoline N-oxide. Gann 48, 129-137.

Ochiai, E., Ishikawa, M.O., and Sai, Z. (1943). Polarization of aromatic heterocyclic compounds. XXIX. Nitration of quinoline 1-oxide. Yakrigaku Zasshi 63, 280-282.

Sugimura, T., Okabe, T., and Nagao, M. (1966). Metabolism of 4-nitroquinoline 1-oxide, a carcinogen. III. Enzyme catalyzing the conversion of 4-nitroquinoline 1-oxide to 4-hydroxyaminoquinoline 1-oxide in rat liver and heptomas. Cancer Res. 26, 1717-1721.

SITE-SPECIFIC DEUTERATION OR TRITIATION OF BENZO[b]THIOPHENE AND 1-METHYLINDOLE ANALOGS OF BIOLOGICALLY ACTIVE INDOLE DERIVATIVES (1)

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Received on January 12, 1973

SUMMARY

The site-specific deuteration and tritiation of 1-methylindole and benzo[b]-thiophene analogs of biologically active indole derivatives has been achieved by use of the facile metalation of these heterocycles with n-butyllithium and subsequent reaction with $^{2}H_2O$ or $^{3}H_2O$. Metalation occurs at the 2-position and site and extent of deuterium incorporation was determined by N.M.R. The use of similar procedures with $^{3}H_2O$ led to ready incorporation of tritium into these heterocycles.

INTRODUCTION

The demand for specifically labeled organic molecules has resulted in the development of selective procedures for the incorporation of deuterium (3), tritium (4), and carbon-14 (5) into organic compounds. Specifically, radiolabeled molecules are extensively used in mechanistic and biomedical research (6); in addition, the application of stable isotopically labeled molecules to metabolic studies has recently been described (7).

As a result of our study of the chemical pharmacology of tryptophan and its isosteres, it became necessary to prepare suitably labeled

benzo[b]thiophene and 1-methylindole analogs of biologically active indole derivatives. The method which was employed utilizes the facile metalation of benzo[b]thiophene (8) and 1-methylindole (9) with n-butyllithium and subsequent reaction with ^2H_2O or ^3H_2O. Metalation has been shown (8,9) to occur at the 2-position due to the inductive effect of the heteroatom, and is therefore the active site for introduction of the deuterium or tritium atom.

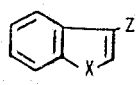
RESULTS AND DISCUSSION

A. Exchange Studies Using Deuterium

The general procedure for deuterium incorporation is as follows: The compound to be labeled was dissolved in dry, freshly distilled tetrahydrofuran (THF) and the resultant solution cooled to 0° for 1-methylindole and -78° for benzo[b]thiophene derivatives. The reaction solution was treated with n-butyllithium and allowed to stir for 30-75 minutes at the low temperature prior to reaction with ^2H_2O. The nmr spectra of 1-methylindole and benzo[b]thiophene derivatives possessed a characteristic absorption for the H-2 proton, and therefore, the decrease or loss of the H-2 absorption provided a ready means of determining the percent exchange. The compounds labeled in this study and the nmr data appear in Table I.

Table I

Compounds and Percent Exchange Based on NMR



Compound No.	Name	Substituents X	Z	NMR Data δH-2	% H-2 Exchanged
I	3-(2-Aminoethyl)benzo[b]-thiophene	S	$CH_2CH_2NH_2$	7.07	80
II	Benzo[b]thiophene-3-acetic acid	S	CH_2COOH	7.26-7.48*	70

III	3-(2-Dimethylaminoethyl)-benzo[b]thiophene	S	$CH_2CH_2N(CH_3)_2$	7.10	100
IV	1-Methyl-3-(2-dimethylamino-ethyl)indole	$N-CH_3$	$CH_2CH_2N(CH_3)_2$	6.71	75

§ These δH-2 values are those for the parent unexchanged compound.
* This proton is part of a multiplet.

The exchange labeling of the sulfur analog of tryptamine (I) was complicated by its extreme sensitivity to air. Preparation of the free base from its hydrochloride salt and subsequent handling under an inert atmosphere were required.

The metalation of the sulfur analog of indole-3-acetic acid (II) with n-butyllithium resulted in 70% exchange of the H-2 proton and 35% exchange of the methylene protons. Increasing the molar ratio of n-butyllithium and/or the reaction time failed to significantly alter the extent of methylene proton exchange.

In order to facilitate the exchange of the H-2 proton in the 1-methyl-indole derivative (IV) it was necessary to elevate the reaction temperature to 0° and extend the reaction time to 75 minutes; further elevation of the reaction temperature or increase of the reaction time led to degradation of the compound.

B. Exchange Studies Using Tritium

The use of ^3H_2O as the quenching agent for the metalated heterocycles allows for the facile preparation of specifically labeled compounds. The specific activities of the compounds so labeled appear in Table II. The chemical purity of each compound was demonstrated by thin-layer chromatography using several different solvent systems and the radiochemical purity was verified by radioscanning of the thin-layer plates.

Table II

Specific Activity of Exchanged Compounds§

Compound No.	Specific Activity (μCi/mmole)
I	518
II	729
III	431
IV	176

§ Reactions were run as per the experimental using ^3H_2O (2 mCi/mmole).

Compound II was selected for a study of the effects of the volume and the specific activity of the quenching ^3H_2O on the rate of tritium incorporation. These data appear in Table III.

The study of the effect of ^3H_2O volume used to quench 3.0 mmoles of base reveals the highest rate of tritium incorporation occurred when 1.5 mmoles of ^3H_2O and the lowest when 6.0 mmoles of ^3H_2O were used to quench the reaction. The decreased tritium incorporation observed upon utilization of excess ^3H_2O may reflect a tritium isotope effect and/or the greater availability of ^1H vs. ^3H atoms.

Table III

Effects of Volume and Specific Activity of the Quenching ^3H_2O§

Volume (μl)	mmoles ^3H_2O	^3H_2O Specific Activity (mCi/mmole)	Specific Activity of Compound II (mCi/mmole)
27	1.5	2	2.15
54	3.0	2	1.76
108	6.0	2	0.91
27	1.5	10	5.18
27	1.5	20	11.10

§ Reactions were run using 1.0 mmole of compound II and 3.0 mmoles of n-butyllithium as per the experimental.

The study of the effect of the specific activity of the quenching ^3H_2O on the rate of tritium incorporation was undertaken using 1.5 mmoles of ^3H_2O. The rate of tritium incorporation was a linear function of the specific activity of the ^3H_2O. Studies are presently in progress to develop appropriate exchange procedures for heterocycles containing NH groups, i.e., indole, imidazole, thiazole, etc., and should greatly extend the versatility of this method.

EXPERIMENTAL

Melting points were measured on a Mel-Temp capillary melting point apparatus. UV data were obtained on a Unicam SP1800 Ultraviolet Spectrophotometer and concentrations of solutions used in specific activity determinations were obtained via UV. Radioactivity was measured in a Packard Tri-Carb 4322 Liquid Scintillation System, using the scintillation solution described by Maickel, et al (10). Nmr spectra were run on a Varian Associates Model HA-100 spectrometer using deuterochloroform as the solvent and tetramethylsilane as the internal standard. Aromatic proton assignments were made by analogy to compounds previously reported (11,12). One dimensional thin-layer chromatography was carried out on Analtech silica gel G precoated plates (Analtech, Inc., Newark, Delaware). The solvent systems used in thin-layer chromatography were: A. methyl acetate-2-propanol-ammonia (45:35:20); B. chloroform-methanol-acetic acid (60:35:5); and C. methanol-ammonia (98.5:1.5). All thin-layer chromatograms were developed 12 cm and visualized with one or more of the following reagents (13): Van Urk reagent, alkaline potassium permanganate, ninhydrin, and iodine vapor. The developed thin-layer plates containing radioactive material were scanned on a Packard Model 7200 Radiochromatogram Scanner. All exchange reactions were conducted with oven-dried glassware which was assembled and placed under a continuous flow of dry nitrogen gas,

prior to the introduction of any reaction component.

3-(2-Aminoethyl)benzo[b]thiophene-2-^{2}H or ^{3}H. (I). 3-(2-Aminoethyl)benzo[b]thiophene hydrochloride (0.213 g, 1.0 mmole) was dissolved in 15 ml of 1 N NaOH and extracted with 2 x 25 ml of diethyl ether. The combined ether extracts were dried over anhydrous $MgSO_4$ and the ether removed under reduced pressure. The free base was unstable in the atmosphere necessitating handling in an inert atmosphere. The free base was dissolved in 60 ml of freshly distilled (from $LiAlH_4$) THF, cooled to -78° in a CO_2/acetone bath, and treated with n-butyllithium (1.5 ml of a 2.5 M solution, 3.75 mmoles). The solution was stirred for 30 min at -78° prior to quenching with either $^{2}H_2O$ (0.1 ml, 5 mmoles) or $^{3}H_2O$ (0.1 ml, 5.0 mmoles). The quenched reaction was dried with anhydrous $MgSO_4$. The dried and filtered THF solution was treated with dry HCl gas to produce the amine hydrochloride. Recrystallization from methanol/ethyl acetate gave 0.176 g (83%) of large colorless plates, m.p. 220-221°. TLC using solvent systems A, B, and C gave R_f's 0.82, 0.59 and 0.24 respectively. Nmr of ^{2}H-exchanged I: δ 1.22-1.60 (m, 2H, NH_2), 2.97 (broad s, 4H, CH_2CH_2), 7.09 (s, 0.20H, H-2), 7.20-7.46 (m, 2H, H-5,6), 7.62-7.90 (m, 2H, H-4,7). Using $^{2}H_2O$ (99%) as the quenching agent gave 80% deuterium incorporation at the 2-position, while using $^{3}H_2O$ (2 mCi/mmole) gave a specific activity of 518 μCi/mmole.

Benzo[b]thiophene-2-^{2}H or ^{3}H-3-acetic acid. (II). Benzo[b]thiophene-3-acetic acid (0.192 g, 1.0 mmole) was dissolved in 60 ml of freshly distilled (from $LiAlH_4$) THF and the reaction vessel cooled to -78° in a CO_2/acetone bath and treated with n-butyllithium (1.28 ml of a 2.34 M solution, 3.0 mmoles). The reaction was allowed to stir for 30 min at -78° and quenched with $^{2}H_2O$ (0.108 ml, 6.0 mmoles) or $^{3}H_2O$ (0.108 ml, 6.0 mmoles). Upon removal of the THF under reduced pressure, the residue was dissolved in 20 ml of 1 N HCl and the aqueous phase extracted with 2 x 25 ml of ether.

Removal of the ether and recrystallization of the product from H_2O gave 0.155 (81%) of long colorless needles, m.p. 110-111°. TLC using solvent systems A and B gave R_f's 0.68 and 0.91 respectively. Nmr of ^2H-exchanged II: δ 3.82 (s, 1.3H, CH_2), 7.25-7.50 (m, 2.3H, H-2,5,6), 7.64-7.94 (m, 2H, H-4,7), 11.50 (broad s, 1H, OH). Using ^2H_2O (99%) as the quenching agent gave 70% deuterium incorporation at the 2-position and 35% incorporation at the methylene position. Quenching with ^3H_2O (2 mCi/mmole) gave a specific activity of 719 μCi/mmole.

3-(2-Dimethylaminoethyl)benzo[b]thiophene-2-^2H or ^3H. (III). 3-(2-Dimethylaminoethyl)benzo[b]thiophene (0.205 g, 1.0 mmole) was dissolved in 60 ml of freshly distilled (from $LiAlH_4$) THF and the reaction vessel was cooled to -78° in CO_2/acetone prior to treatment with n-butyllithium (1 ml of 2.5 M solution, 2.5 mmoles). The reaction was allowed to stir for 30 min at -78° before quenching with either ^2H_2O (0.1 ml, 5 mmoles) or ^3H_2O (0.1 ml, 5 mmoles). The THF was removed under reduced pressure, the free base was dissolved in 50 ml of diethyl ether and the ether dried over anhydrous $MgSO_4$ prior to treatment with dry HCl gas to produce the hydrochloride. Recrystallization of the hydrochloride from methanol/ethyl acetate gave 0.20 g (83%) of long colorless needles, m.p. 260-261°. TLC using solvent systems A, B, and C gave R_f's 0.92, 0.42 and 0.36 respectively. Nmr of the ^2H-exchanged III: δ 2.31 (s, 6H, $N(CH_3)_2$), 2.52-2.76 (m, 2H, CH_2N), 2.85-3.10 (m, 2H, $ArCH_2$), 7.18-7.43 (m, 2H, H-5,6), 7.63-7.86 (m, 2H, H-4,7). Using ^2H_2O (99%) as the quenching agent gave 100% deuterium incorporation at the 2-position, while using ^3H_2O (2 mCi/mmole) gave a specific activity of 431 μCi/mmole.

1-Methyl-3-(2-dimethylaminoethyl)indole-2-^2H or ^3H. (IV). 1-Methyl-3-(2-dimethylaminoethyl)indole (0.203 g, 1.0 mmole) was dissolved in 60 ml of freshly distilled (from $LiAlH_4$) THF, was cooled to 0° in an ice bath, and treated with n-butyllithium (0.8 ml of 2.5 M solution, 2.0 mmoles).

The reaction was allowed to stir for 75 min at 0° prior to quenching with either ^2H_2O (0.1 ml, 5.0 mmoles), or ^3H_2O (0.1 ml, 5.0 mmoles). The THF was removed under reduced pressure, the free base was dissolved in 50 ml of diethyl ether and the ether was dried over anhydrous $MgSO_4$ prior to conversion to the hydrochloride with dry HCl gas. Recrystallization from ethanol/diethyl ether gave 0.176 (74%) of colorless needles, m.p. 169-170°. TLC using solvent system C gave R_f 0.31. Nmr of ^2H-exchanged IV: δ 2.30 (s, 6H, $N(CH_3)_2$), 2.49-2.74 (m, 2H, CH_2N), 2.80-3.04 (m, 2H, $ArCH_2$), 3.64 (s, 3H, NCH_3), 6.80 (s, 0.25H, H-2), 6.97-7.26 (m, 3H, H-4,5,6), 7.49-7.63 (m, 1H, H-7). Using ^2H_2O (99%) as the quenching agent gave 75% deuterium incorporation at the 2-position, while using ^3H_2O (2 mCi/mmole) gave a specific activity of 176 μCi/mmole.

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EFFECTS OF VARIOUS DEPRESSANT DRUGS ON DEPRIVATION-INDUCED WATER CONSUMPTION*

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Summary—The volume of water consumed in a 60 min period by rats deprived of water for 23 hr was increased by a variety of sedative drugs such as barbiturates, glutethimide, methaqualone and methyprylon. The antipsychotic agent, promazine, decreased water consumption under similar conditions, while antianxiety agents such as chlordiazepoxide and meprobamate had a stimulatory effect similar to the barbiturates. The results may be significant in consideration of the use of liquid reinforcement in punishment attenuation studies.

The role of central mechanisms in the control of fluid intake has been the subject of considerable study, as evidenced by a number of symposia, reviews, and books (MILLER, 1965; GROSSMAN, 1967; MORGANE, 1969). Previous work from this laboratory has demonstrated that a variety of cholinergic blocking agents, cholinergic stimulants, antihistamines and amphetamines all reduce deprivation-induced fluid consumption by rats (GERALD and MAICKEL, 1969; MAICKEL, COX, KSIR, SNODGRASS and MILLER, 1970; GERALD and MAICKEL, 1972; MAICKEL and WEBB, 1972). In contrast to this diversity of pharmacological agents that reduce deprivation-induced water consumption, one specific group of drugs, the barbiturates, have been shown to stimulate fluid intake by deprived rats (SCHMIDT, 1958, 1969). In addition, several antianxiety agents have been reported to stimulate consumption of fluids that might be considered aversive in taste. For example FALK and BURNIDGE (1970) reported increased consumption of 1·5% saline solution by rats treated with chlordiazepoxide. Similarly, MARGULES and STEIN (1967) reported that oxazepam increased the consumption of milk made bitter-tasting by the addition of quinine.

The present paper reports on the actions of various sedative-hypnotic drugs, both barbiturate and nonbarbiturate, and several antianxiety agents on deprivation-induced fluid consumption by rats.

MATERIALS AND METHODS

Adult (300–350 g) male, Sprague–Dawley rats (Murphy Breeding Laboratories, Plainfield, Indiana) were used in all experiments. The animals were maintained on tap water and Purina Rat Chow *ad lib.* for at least 1 week after arrival in the laboratory. All solutions for injection were made in glass distilled water such that a dose volume of 0·1 ml per 100 g body

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weight contained the desired dose; all injections were given intraperitoneally, 15 min prior to placing the animals in the drinking cages.

The procedures used to measure deprivation-induced water consumption were basically those of Gerald and Maickel (1969). Rats were placed in cages, 7 × 7 × 14 inches, identical to the home cages of the animals. The cages were suspended in individual compartments of a sound-proofed box with uniform fluorescent lighting. Constant circulation of air by blowers maintained uniform temperature in the compartments; the noise level of blowers served as a "white noise". Each cage contained a drinking tube connected to an external 50 ml buret filled with distilled water at the start of each test run and stoppered. As the animal consumed water, the change in volume was measured visually to the nearest 0·1 ml. The front door of each compartment was equipped with an eye-piece lens to permit visual observation of the rats without disturbing their behavioral performance. For at least 1 week prior to testing drug effects, rats were deprived of water daily for 23 hr prior to testing, then placed in the "drinking cages" and allowed to drink for 1 hr. Food was available *ad lib.* in the home cages but was not available in the drinking cages.

Drug trials were started only after the animals demonstrated stable baselines (less than 5% daily variation) of water intake. The schedule for drug studies was arranged so that the rats were run daily, with drugs administered every fourth day, and placebo doses on the intervening days. Water intake was recorded at 15, 30 and 60 min of the consummatory sessions; the greatest proportion of drinking occurred in the first 15 min in all cases.

Groups of 8 rats were run in each test system; an n of <8 rats indicates that a value or values was discarded because of equipment malfunction. All data are reported as mean ± S.D. Statistical comparisons were carried out by the correlated t-test for paired scores. The day prior to each drug day was used as the pre-drug value for that drug run. Each animal served as its own control; per cent of pre-drug values are the mean of values calculated for each animal.

RESULTS

Effects of barbiturates on deprivation-induced water consumption in rats

The data in Table 1 indicate that the short-acting barbiturates, amobarbital, hexobarbital and methohexital, had no significant effect on the volume of water consumed by deprived rats, except for the highest dose of methohexital. With the longer-acting barbiturate, phenobarbital, increased water consumption was observed at all dose levels, a finding similar to that previously reported for barbital (Maickel and Maloney, 1972).

Effect of various sedative drugs on deprivation-induced water consumption

The data in Table 2 are those obtained with three sedative drugs, glutethimide, methaqualone, and methyprylon as well as the antipsychotic agent, promazine. With the exception of the lowest doses of glutethimide and methyprylon, a significant elevation of water consumption was seen with all three sedatives. In contrast, promazine, at all three doses tested, produced a significant decrease in deprivation-induced water consumption, a finding that is in agreement with a previous publication from this laboratory (Maickel, Gerald, Warburton and Mahju, 1968) examining several phenothiazine tranquilizers.

Table 1. Effects of barbiturates on deprivation-induced water consumption in rats

Drug	n	Dose (i.p.) (μmol/kg)	Volume consumed: Pre-drug (ml)	Drug (ml)	% of Pre-drug	P
Amobarbital	6	1·9	14·9 ± 1·6	16·0 ± 1·6	109 ± 13·0	
	8	3·8	16·2 ± 3·9	17·3 ± 4·3	108 ± 23·8	
	8	7·6	10·5 ± 3·4	10·9 ± 3·4	111 ± 37·2	
Hexobarbital	8	3·6	12·5 ± 2·1	13·1 ± 3·4	105 ± 20·9	
	7	7·2	16·1 ± 2·7	17·8 ± 3·2	109 ± 17·4	
	8	14·4	15·7 ± 4·1	16·8 ± 3·9	110 ± 15·4	
Methohexital	8	1·6	11·9 ± 3·1	14·0 ± 4·2	120 ± 30·5	
	8	3·2	13·9 ± 3·2	15·1 ± 3·9	109 ± 18·7	
	8	6·4	12·7 ± 2·9	14·9 ± 2·5	121 ± 23·2	*
Phenobarbital	8	1·95	14·7 ± 2·5	18·7 ± 2·5	129 ± 19·8	*
	8	3·9	13·5 ± 1·6	18·3 ± 3·3	137 ± 24·6	*
	8	7·8	12·9 ± 1·9	20·0 ± 3·3	158 ± 38·8	*
	8	15·6	11·2 ± 2·5	18·6 ± 1·8	172 ± 40·5	*

Animals were treated as described in Materials and Methods section; drug was administered 15 min prior to start of drinking session. Results are expressed as mean ±S.D. of the values obtained. Statistical comparisons were made by the correlated *t*-test for paired scores; significant differences from the corresponding pre-drug values ($P < 0·05$) are indicated by.*

Table 2. Effects of various sedative drugs on deprivation-induced water consumption in rats

Drug	n	Dose (i.p.) (μmol/kg)	Volume consumed: Pre-drug (ml)	Drug (ml)	% of Pre-drug	P
Glutethimide	7	4·6	15·8 ± 2·6	15·4 ± 2·2	99 ± 11·3	
	8	9·2	12·4 ± 1·5	15·0 ± 3·9	121 ± 26·3	*
	8	18·4	13·3 ± 3·4	15·6 ± 3·4	121 ± 21·4	*
Methaqualone	8	2·0	15·1 ± 2·3	17·1 ± 7·8	116 ± 20·1	*
	8	4·0	14·3 ± 2·7	18·5 ± 1·6	134 ± 23·1	*
Methyprylon	8	2·7	12·4 ± 2·1	13·2 ± 1·4	109 ± 19·7	
	8	5·4	11·8 ± 2·8	15·2 ± 3·1	142 ± 37·3	*
	7	10.8	12·4 ± 2·2	16·2 ± 3·2	133 ± 26·4	*
Promazine	7	0·78	15·1 ± 3·1	12·9 ± 3·7	85 ± 16·4	*
	8	1·56	10·2 ± 3·1	8·1 ± 2·8	47 ± 14·7	*
	8	3·1	14·4 ± 3·3	6·3 ± 3·7	40 ± 24·7	*

Animals were treated as described in Materials and Methods section; drug was administered 15 min prior to start of drinking session. Results are expressed as mean ±S.D. of the values obtained. Statistical comparisons were made by the correlated *t*-test for paired scores; significant differences from the corresponding pre-drug values ($P < 0·05$) are indicated by.*

Effect of various antianxiety drugs on deprivation-induced water consumption

In Table 3 are presented the data obtained when chlordiazepoxide, diazepam, and meprobamate were administered to deprived rats prior to the start of the drinking session. Except for the lowest dose of meprobamate, significant increases in water consumption were observed in all test situations.

Table 3. Effect of antianxiety drugs on deprivation-induced water consumption in rats

Drug	*n*	Dose (i.p.) (mol/kg)	Volume consumed Pre-drug (ml)	Drug (ml)	% of Pre-drug	*P*
Chlordiazepoxide	8	1·1	13·1 ± 2·4	16·4 ± 2·8	125 ± 15·1	*
	8	2·2	10·7 ± 2·2	15·5 ± 1·2	150 ± 29·3	*
	8	4.4	12·5 ± 2·9	18·5 ± 2·5	152 ± 31·1	*
Diazepam	8	1·8	14·3 ± 3·1	18·4 ± 3·5	133 ± 30·6	*
	8	3·6	14·2 ± 2·1	19·9 ± 3·5	136 ± 32·7	*
	7	7·2	12·5 ± 1·9	18·7 ± 3·2	151 ± 26·0	*
Meprobamate	8	9·2	12·6 ± 2·2	13·4 ± 3·4	106 ± 18·5	*
	8	18·4	13·4 ± 2·3	16·7 ± 3·5	124 ± 17·7	*
	8	36·8	10·6 ± 2·5	13·7 ± 3·0	132 ± 21·7	*

Animals were treated as described in Materials and Methods section; drug was administered 15 min prior to start of drinking session. Results are expressed as mean ± S.D. of the values obtained. Statistical comparisons were made by the correlated *t*-test for paired scores; significant differences from the corresponding pre-drug values ($P < 0·05$) are indicated by.*

A comparison of the slopes of the dose–response curves for all the drugs tested is seen in Table 4. The slopes of chlordiazepoxide, phenobarbital, methyprylon, and meprobamate are similar, as are those of diazepam and glutethimide. Promazine is dissimilar to all others in sign since it causes a reduction in fluid intake, while amobarbital, hexobarbital and methohexital did not yield dose–response curves.

Table 4. Slopes of dose response plots of drug effects on fluid consumption

Compound	Slope	≃
Amobarbital	3·4	0·64
Chlordiazepoxide	43·4	0·90
Diazepam	29·9	0·93
Glutethimide	36·5	0·87
Hexobarbital	8·3	0·94
Meprobamate	43·0	0·98
Methaqualone	28·2	0·99
Methohexital	1·7	0·08
Methyprylon	35·4	0·67
Phenobarbital	49·8	0·99
Promazine	74·7	0·93

DISCUSSION

In many reports of the effects of sedative drugs on fluid consumption, a lack of standardization of procedures, dose schedules, and thirst-inducing stimuli has made comparisons difficult. In the present study, the stimulus was constant (23 hr deprivation of fluids), the pretreatment time was constant (15 min), all drugs were studied in dose–response schedules, and all experiments were run at the same time of day to minimize the effects of circadian rhythm variables.

The results obtained are consistent with the hypothesis that sedative-hypnotics and antianxiety agents stimulate water consumption in deprived rats. The sedative action of the barbiturates cannot be responsible for this phenomena since promazine, like other phenothiazine tranquilizers (MAICKEL *et al.*, 1968) decreases fluid consumption in a similar test system. In addition, previous work from this laboratory has demonstrated that antihistamines, despite their having sedative actions in humans, also reduce fluid intake in a similar test system (GERALD and MAICKEL, 1972)

In a recent publication (MAICKEL and MALONEY, 1972) we have shown that careful attention must be paid to time- and dose-response characteristics in order to separate the sedative and dipsogenic actions of barbital. Such restriction hold true for all sedative drugs; the depressed animal may not be able to drink even though the thirst drive is present. In this regard, the use of combination test system such as actophotometers and water consumption tests (MAICKEL and MALONEY, 1972) may be a requisite for suitable interpretation of the data obtained.

The implications of the findings reported in the present manuscript towards the mode of action of antianxiety drugs merits special attention. According to the hypothesis of Geller (GELLER and LEIFTER, 1960, 1962; GELLER, HULAK and LEIFTER, 1962), the responsiveness of rats on a bar pressing schedule for fluid reinforcement is suppressed by punishment. Administration of chlordiazepoxide, meprobamate, or phenobarbital increases the rate of responding during the punished period, leading to the interpretation that these drugs have abolished the effect of punishment. In view of the present findings demonstrating the increased water intake in response to these drugs, one must pose the question of whether this dipsogenic action is sufficient to "override" the inhibition of behavior due to punishment. In view of evidence in the literature on increased food consumption elicited by sedative drugs (RANDALL, SCHALLEK, HEISE, KEITH and BAGDON, 1960; OPITZ and AKINLAJA, 1966) and the present findings on fluid consumption, the possibility of a motivational component in the explanation of consummatory behavior suppressed by punishment should receive further study.

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METABOLISM OF 3-(2-DIMETHYLAMINOETHYL)BENZO[b]THIOPHENE *IN VITRO* AND *IN VIVO* IN THE RAT

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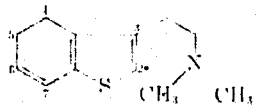
ABSTRACT

The metabolism of 3-(2-dimethylaminoethyl)benzo[b]thiophene has been studied *in vivo* and *in vitro* in rats. The results indicate the major pathways of metabolism to be 6-hydroxylation, N-dealkylation, and oxidation by monoamine oxidase. No evidence for sulfoxidation was obtained. Metabolites were identified by combinations of mass spectrometry, nuclear magnetic resonance spectroscopy, thin-layer chromatography, and liquid scintillation counting.

Benzo[b]thiophene derivatives constitute a growing class of biologically active compounds (1). Despite the large number of benzo[b]thiophene derivatives that have been synthesized, metabolic studies have been reported for only one compound, 4-benzo[b]thiophene methylcarbamate, a pesticide, in the rat (2), and in dairy goats and a lactating cow (3). Of particular interest was the identification, by infrared spectroscopic techniques, of benzo[b]thienyl-4-sulfate-1-oxide, as a urinary metabolite in the goat and cow (3).

Continuing interest in the biochemical pharmacology of benzo[b]thiophene derivatives has led this laboratory to investigate several compounds analogous to biologically active indoles (4–9). No information is available on the metabolism of 3-substituted benzo[b]thiophenes, and because many of the naturally occurring indoles of pharmacological interest are 3-substituted derivatives, 3-(2-dimethylaminoethyl)benzo[b]thiophene (I), the benzo[b]thiophene analog of N,N-dimethyltryptamine (DMT), was chosen as a model compound for the present study.



I

In view of the reported sulfoxidation of 4-benzo[b]thiophene methyl carbamate (2, 3), it was of interest to determine whether I might undergo sulfoxidation. Other metabolic pathways for I might be predicted on the basis of the structural analogy between I and DMT.[2] Metabolites of DMT result from N-dealkylation, N-oxidation, aromatic hydroxylation, and oxidative deamination (10, 11). Jaccarini and Jepson (12) reported that the positional specificity of indole hydroxylation by liver microsomal enzymes was the same regardless of substrate, species, or attempted induction. The 6-hydroxy derivative was the only hydroxylated product formed. When incubated with the 10,000*g* supernatant fraction of rat liver homogenate (no pretreatment with inducing agent), DMT was found to yield 1.5 μmol of 6-hydroxy-DMT per g of liver per hr (12).

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[2] The abbreviations used are: DMT, N,N-dimethyltryptamine; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

We report here the identification of metabolites of I by high resolution NMR and mass spectrometry. The quantitative metabolic profile of I is presented *in vitro* and *in vivo* in the rat.

Materials and Methods

Liver samples were obtained from adult male Sprague-Dawley rats, 300-400 g (Murphy Breeding Laboratories, Plainfield, Ind.). Rats were fasted overnight before use.

The following compounds were generously supplied by Dr. E. E. Campaigne, Department of Chemistry, Indiana University, Bloomington, Ind.: 3-(2-dimethylaminoethyl)benzo[b]thiophene hydrochloride, 3-(2-methylaminoethyl)benzo[b]thiophene hydrochloride, benzo[b]thiophene-3-acetic acid, 3-(2-hydroxyethyl)benzo[b]thiophene, and 5-hydroxy-3-(2-dimethylaminoethyl)benzo[b]thiophene picrate. 2-Tritio-3-(2-dimethylaminoethyl)benzo[b]thiophene hydrochloride, 8.9 mCi/mmol, was prepared by the method of Bosin and Rogers (13). The radiochemical purity of this compound was shown by thin-layer chromatography to be greater than 99.5%.

Liver Microsomal Incubation. Preliminary studies were performed with the crude microsomal fraction of rat liver prepared and incubated by the method of Mazel (14). Optimum conditions for the microsomal 6-hydroxylation of indolealkylamines *in vitro* were established by Jaccarini and Jepson (12); these conditions were employed in all experiments performed for the purpose of quantitating metabolites. Appropriate buffers (12, 14) were prepared according to Stauff and Jaenicke (15). All experiments employed the 10,000*g* supernatant fraction of rat liver homogenate. Centrifugation was carried out at 5°C for 20 min in an IEC model B-20 refrigerated centrifuge. Incubations were carried out in 25-ml glass vials at 37°C with an Aminco-Dubnoff shaking incubator operated at 125 cpm. Reactions were terminated after 1 hr by immersing the vials in a Dry Ice-acetone bath.

Isolation of Metabolites. For preliminary TLC studies, 0.5-1.0 ml of incubation mixture, pH 7.2, was added to a glass-stoppered 15-ml centrifuge tube containing 6.0 ml of ethyl ether. Sufficient solid NaCl was added to saturate the aqueous phase. Tubes were mechanically shaken for 10 min and centrifuged for 10 min. The ether phase was then transferred to a disposable culture tube from which the ether was evaporated under a stream of N_2. The residue was taken up in methanol and subjected to TLC on Silica Gel G plates (Analtech, precoated, prescored Uniplate; 0.25-mm thickness) in one or more of the following solvent systems: A, chloroform/methanol/acetic acid (60:35:5); B, methanol/ammonia (50:0.75); C, methyl acetate/isopropanol/ammonia (45:35:20). A spray of 1% potassium permanganate in 5% aqueous sodium carbonate was employed for visualization.

To obtain a given metabolite in a quantity sufficient for spectroscopic study, 2.0 ml of incubation mixture were added to each of at least 30 60-ml glass-stoppered tubes containing 25 ml of ethyl ether and sufficient solid NaCl to saturate the aqueous phase. Amines were extracted after adjustment of the aqueous phase to pH 11 with solid Na_2CO_3. Acids were extracted after adjustment of the aqueous phase to pH 1 with 0.5 ml of 5 N HCl. After shaking and centrifugation, the pooled ether extracts were evaporated under reduced pressure. The residue was taken up as an aqueous solution at appropriate pH (pH 1 for an amine, pH 11 for an acid), transferred to a 60-ml glass-stoppered tube, diluted to a final volume of 6 ml, and washed with 2-3 10-ml portions of benzene. The aqueous phase was then adjusted to pH 7 with solid Na_2CO_3 or 0.5 N HCl as appropriate, saturated with NaCl, shaken with 30 ml of ethyl ether, and centrifuged. The ether was evaporated, and the residue was taken up in methanol. This methanolic solution was applied, by means of a 1-ml glass syringe and 30-gauge hypodermic needle, as a streak to a Silica Gel H (Brinkmann) layer (20 × 20 cm, 0.75-mm thickness), which was then developed in one of the three solvent systems described above. A band of silica gel at an appropriate R_F was scraped into a sintered glass filter funnel and washed with 0.5 N HCl if the metabolite was an amine or 0.2 N NaOH if the metabolite was an acid. The filtrate was adjusted to pH 7, saturated with NaCl, shaken with at least 2 volumes of ethyl ether, and centrifuged. The ether phase was transferred to a clean sample vial and evaporated. The residue was then examined by NMR and/or mass spectrometry. NMR spectra, 220 MHz, were obtained using a Varian HR-220 NMR spectrometer and, when necessary, were time-averaged with a Nicolet Instrument Corp. 1080 computer. Mass spectra were obtained using a Varian Mat CH7 mass spectrometer using an ion current of 70 eV.

Quantitation of Metabolites. [2-^{3}H]3-(2-Dimethylaminoethyl)benzo[b]thiophene was used. Radioactivity was measured in a Packard Tri-Carb model 2425 liquid scintillation spectrometer, with polyethylene vials and a scintillation medium consisting of 14 g of 2,5-bis-[2-(5-*tert*-butylbenzoxazolyl)]thiophene and 280 g of naphthalene in a mixture of 2100 ml of toluene and 1400 ml of ethylene glycol monomethyl ether. A counting system of 15 ml of this medium, 0.5 ml of 0.5 N HCl, and silica gel (*vide infra*) was used for these determinations. A 4-μl (Drummond Microcap) aliquot of incubation mixture or urine was applied to a Silica Gel G (Brinkmann) layer (20 × 20 cm, 0.50-mm thickness). The chromatogram was developed in either one or two dimensions, as appropriate, with one or more of the three solvent systems already described. The layer was then divided into 1 × 1-cm squares, and each square was scraped into a polyethylene counting vial. To each vial was then added 0.5 ml of 0.5 N HCl, and after thorough mixing the counting system was completed with 15 ml of scintillation medium as described above. This system gives 24% efficiency for tritium with a background of 19 cpm. Results for this procedure allow the construction of two- and three-dimensional histograms for graphic presentation of the quantitative metabolic profile. Recovery

of labeled compounds incubated in the absence of tissue or in the presence of boiled tissue was > 90% on a 60-min incubation, followed by extraction and TLC as described above.

Radiochromatogram Scanning and Thin-Layer Autoradiography. When sufficient radioactivity was present, the location of ^{3}H metabolites on thin-layer chromatograms (Silica Gel G, Brinkmann NM-Polygram, precoated plastic sheets 5 × 20 cm, 0.25-mm layer thickness) was determined using a Packard model 7200 radiochromatogram scanner. Thin-layer autoradiograms were produced by exposing chromatograms to Kodak RP Royal X-Omat medical X-ray film for 4 weeks. Films were processed in a Kodak X-Omat processor.

***In Vivo* Metabolism.** In a typical experiment, a rat was injected with ^{3}H-**I** (30 mg/kg and 125 μCi/kg ip) and placed in a metabolism cage (Maryland Plastics, New York, N.Y.) for collection of urine and feces. At 5, 24, 48, 72, and 96 hr after injection, urine was collected and stored under toluene at 5°C, and feces were collected and frozen. The animal was killed and exsanguinated, and samples of plasma, liver, kidney, and brain were collected and frozen.

The volumes of the urine and toluene phases were measured. Duplicate aliquots (0.1 ml) of each toluene phase were counted directly. Duplicate aliquots (0.1 ml) of each urine phase were digested by the perchloric acid method of Kobayashi and Maudsley (16) before counting.

The total feces collected were homogenized in 200 ml of 0.5 N HCl. Duplicate aliquots (1.0 ml) were digested with perchloric acid (16), and 0.4 ml of each digest was counted.

Tissues were homogenized in 3 volumes of 0.1 N HCl. Duplicate aliquots (0.1 ml) of each homogenate were digested with perchloric acid (16) and counted.

Urine was examined by two methods for the presence of radioactivity. Aliquots (5.0 ml) of urine at pH 1 (1.0 ml of 5 N HCl added) and pH 11 (solid Na_2CO_3 added) were saturated with solid NaCl and shaken with 30 ml of ethyl ether in a 60-ml glass-stoppered tube. After centrifugation, the ether phases were transferred to disposable culture tubes, and the ether was evaporated. The residue was taken up in methanol and chromatographed on a Silica Gel G (Brinkmann thin layer 0.25 mm). The chromatogram was then scanned for radioactivity.

A second method entailed the application of 4 μl of whole urine to a Silica Gel G (Brinkmann) layer (0.50 mm). After development in solvent system A, a histogram was produced as described earlier.

Results

Identification of the *in Vitro* Metabolites of 3-(2-Dimethylaminoethyl)benzo[b]thiophene (I). The presence of amine metabolites was demonstrated without the aid of ^{3}H-**I**. Extraction of the incubation mixture at pH 1, 7, and 11 followed by TLC examination of the organic phase revealed two amines. TLC characteristics of *in vitro* metabolites of **I** are presented in table I.

Preparative TLC in solvent system A yielded approximately 1 mg of the major amine metabolite from an extract of the incubation mixture. A time-averaged 200-MHz NMR spectrum of the metabolite was obtained, and the aromatic region is presented in fig. 1. The spectrum shows that either the 5- or the 6-proton of **I** has been replaced. If one compares this spectrum with the same region from that of the benzo[b]thiophene analog of bufotenine, 5-hydroxy-3-(2-dimethylaminoethyl)benzo[b]thiophene (17), shown in Fig. 2, a striking difference is apparent. The chemical shifts of the 2-protons are quite dissimilar, leading to the conclusion that the amine metabolite in question is a 6-substituted-3-(2-dimethylaminoethyl)benzo[b]thiophene. The possibility of 4- or 7-substitution was ruled out after a consideration of the aromatic splitting pattern. 4-Hydroxylation for example, as seen in the spectrum of the benzo[b]thiophene analog of psilocin (18), does not give rise to the well resolved multiplets that occur in a 5- or 6-substituted benzo[b]thiophene. The mass spectrum of this sample is presented in fig. 3. It exhibits a molecular ion *m/e* 221, indicating that the metabolite represents the incorporation of an oxygen atom into the parent compound. Fragments *m/e* 162 and *m/e* 91 are attributable to this metabolite, but the sample was not sufficient to yield a spectrum upon which a meaningful interpretation of mass fragmentation could be based. This evidence does, however, sup-

TABLE I

TLC characteristics of in vitro metabolites of 3-(2-dimethylaminoethyl)benzo[b]thiophene

Compound	R_f[a]		
	A	B	C
3-(2-Dimethylaminoethyl)benzo-[b]thiophene[b]	0.38	0.50	—
6-Hydroxy-3-(2-dimethylamino-ethyl)benzo[b]thiophene[b]	0.20	0.42	—
3-(2-Methylaminoethyl)benzo-[b]thiophene[b]	0.43	0.29	—
Benzo[b]thiophene-3-acetic acid[c]	0.62	0.45	0.47

[a] A, chloroform/methanol/acetic acid (60:35:5); B, methanol/ammonia (50:0.75); C, methyl acetate/isopropanol/ammonia (45:35:20).

[b] Determined on Silica Gel G (Analtech).

[c] Determined on Silica Gel G (Brinkmann); authentic sample was mixed with extract of incubation mixture before chromatography.

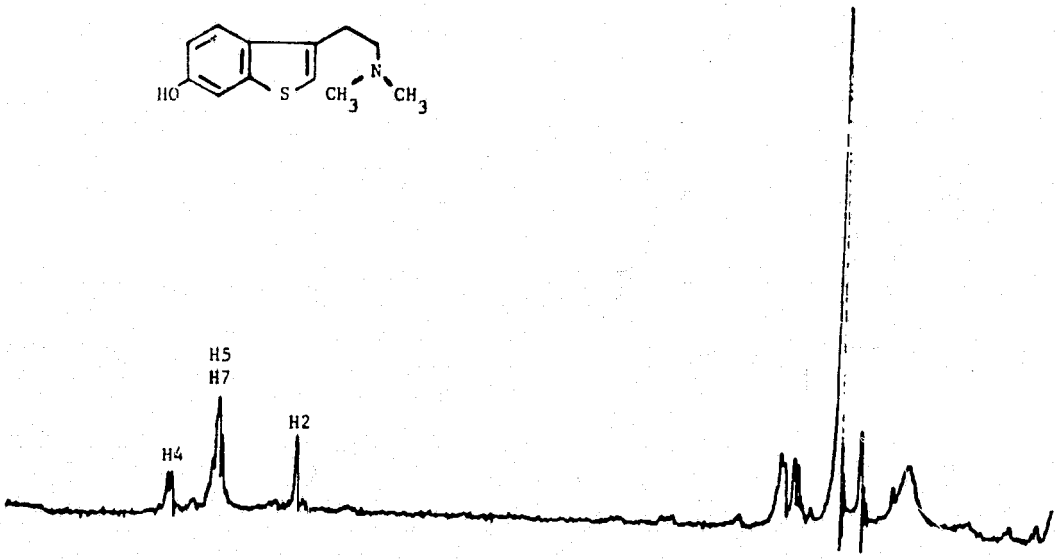


FIG. 1 *NMR spectrum (220 M Hz) of 6-hydroxy-3-(2-dimethylaminoethyl)benzo[b]thiophene.* Solvent: $CDCl_3$; interval ref.: $CHCl_3$; No. of scans: 61. Chemical shifts of aromatic protons are presented in table 4.

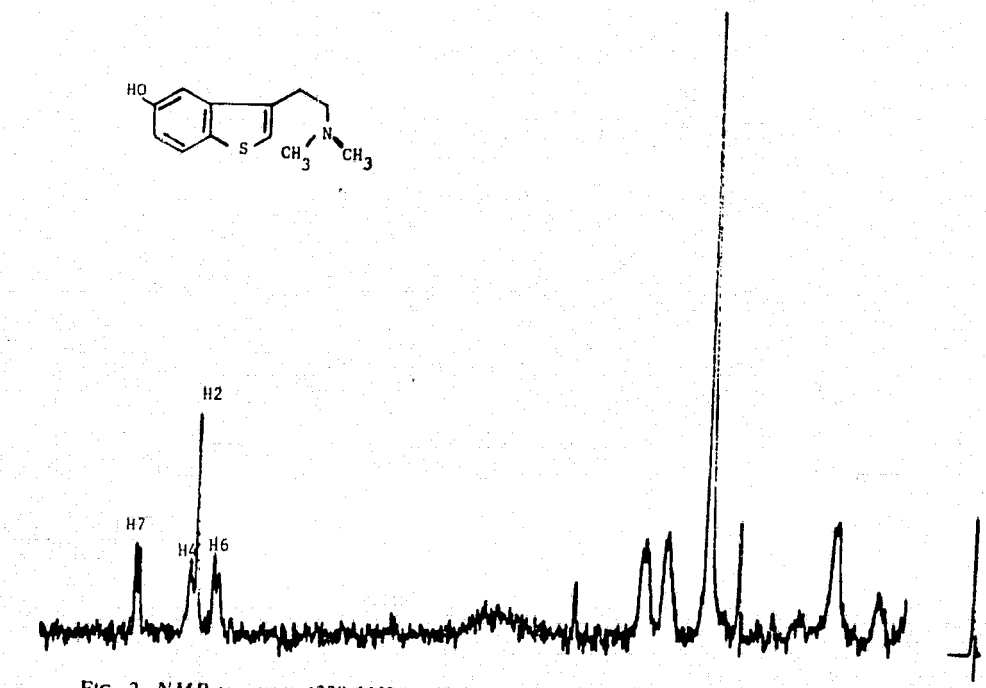


FIG. 2. *NMR spectrum (220 MHz) of 5-hydroxy-3-(2-dimethylaminoethyl)benzo[b]thiophene.* Solvent: $CDCl_3$; interval ref.: trimethylsilyl; No. of scans: 1. Chemical shifts of aromatic protons are presented in table 4.

port the conclusion that the major amine metabolite of I is 6-hydroxy-3-(2-dimethylaminoethyl) benzo[b]thiophene.

The second amine metabolite was well separated from I and 6-hydroxy-I by solvent system B (table 1). Preparative TLC of an extract of the incubation mixtures yielded a sample of mass spectral analysis. Spectral data were consistent with 3-(2-

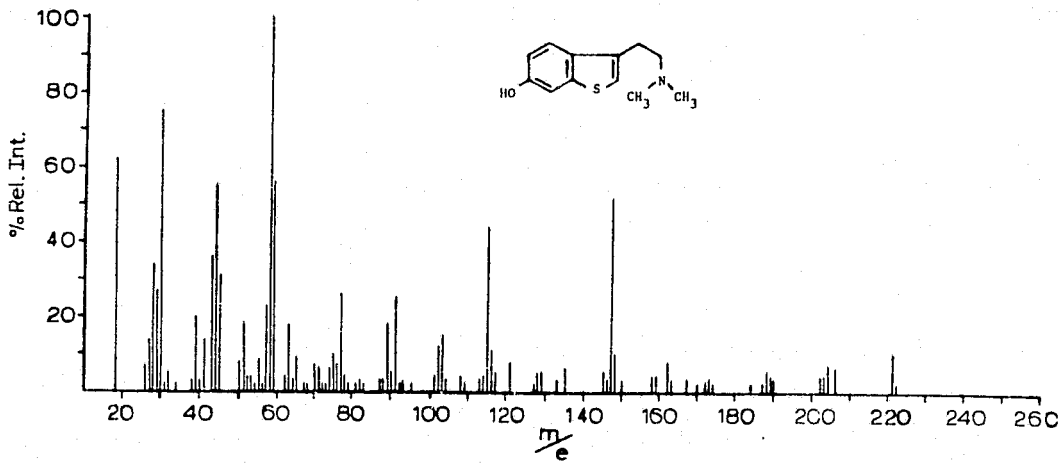


FIG. 3. *Mass spectrum of 6-hydroxy-3-(2-dimethylaminoethyl)benzo[b]thiophene.* This sample was obtained from a liver incubation mixture.

methylaminoethyl)benzo[b]thiophene. This conclusion was confirmed by chromatographic and spectroscopic comparison of synthetic 3-(2-methylaminoethyl)benzo[b]thiophene with the incubation mixture.

When ^{3}H-I was incubated with the 10,000*g* supernatant fraction of rat liver homogenate, radiochromatogram scans and thin-layer autoradiography revealed the presence of one acidic and one neutral metabolite. The mass spectra of these metabolites were consistent with benzo[b]thiophene-3-acetic acid and 3-(2-hydroxyethyl)benzo[b]thiophene. These conclusions were confirmed by comparison with authentic samples.

Quantitation of the *in Vitro* Metabolites of ^{3}H-I. The radioactivity present in a given incubation vessel was determined by digesting duplicate aliquots (0.1 ml) of incubation mixture with perchloric acid as described in Materials and Methods and counting. Aliquots of incubation mixture were diluted with water and subjected to fractional distillation to show that the tritium label was biologically and chemically stable. An aliquot (4 μl) of incubation mixture was then chromatographed in two dimensions, first in solvent system A, and then in solvent system B after rotation of the plate through a 90° angle. Histogram analysis of this chromatogram provided a direct determination of the amount of each metabolite produced. The results are presented in table 2. It was found that greater than 99% of the radioactivity applied to the plate was recovered in four spots.

Metabolism of ^{3}H-I *in Vivo*. The urinary excretion of radioactivity after an ip injection of ^{3}H-I (30 mg/kg and 125 μCi/kg) in the rat is presented in table 3. Urine was collected under toluene, and the possible loss of ^{3}H metabolites into the toluene was considered. The data indicate that 45% of the injected dose was excreted in the urine in 96 hr. From data derived as described in Materials and Methods, it was determined that 2% of the injected dose was excreted in the feces in 96 hr. An

TABLE 2

Production of [2-^{3}H]3-(2-dimethylaminoethyl)benzo[b]thiophene metabolites in vitro

Compound	μmol Product[a]/g Liver/hr	Per Cent[b]
3-(2-Dimethylaminoethyl)benzo-[b]thiophene	—	82.5
6-Hydroxy-3-(2-dimethylamino-ethyl)benzo[b]thiophene	2.3	8.5
3-(2-Methylaminoethyl)benzo-[b]thiophene	1.3	4.8
Benzo[b]thiophene-3-acetic acid	1.1	4.2

[a] Based on 10 μmol of substrate; not corrected for recovery.
[b] Percentage of total radioactivity present.

TABLE 3

Urinary excretion of radioactivity after ip administration of [2-^{3}H]3-(2-dimethylaminoethyl)benzo[b]thiophene in the rat

Time	Urinary Radioactivity
hr	*dpm/ml* × 10^3
5	473
24	564
48	14
72	8
96	7

examination of brain, liver, kidney, and plasma at 96 hr failed to reveal a concentration of radioactivity in these tissues. Radioactivity was uniformly distributed in brain, liver, and kidney to the extent of 13×10^3 dpm/g, whereas the plasma level was 6800 dpm/ml.

Radiochromatogram scans of urine extracts showed that although no radioactivity was extracted from pH 11 into ether, extraction from pH 1 yielded a single acidic metabolite. Its chromatographic characteristics, based upon previous experience with benzo[b]thiophene-3-acetic acid *in vitro*, suggested that the major *in vivo* metabolite was identical to the minor acidic metabolite produced *in vitro*. TLC comparison in all three solvent systems (A, B, and C) confirmed the identity of the urinary acid as benzo[b]thiophene-3-acetic acid. Histogram analysis of whole urine confirmed the presence of [2-^{3}H]benzo[b]thiophene-3-acetic acid as the major metabolite. The R_F by histogram was identical to that obtained by a radiochromatogram scan.

As described earlier, urine was collected under toluene, and in the first 5 hr of collection, 4% of the excreted radioactivity was found in the toluene. TLC examination of this toluene fraction revealed the presence of [2-^{3}H]-6-hydroxy-**I**. It accounts for 0.7% of the total radioactivity excreted in 96 hr. Alkaline permanganate revealed a trace of unchanged **I** that was not detected by a radiochromatogram scan.

Discussion

This study extends observations of the hepatic microsomal hydroxylation of 3-substituted indoles to the analogous benzo[b]thiophene ring system. High resolution NMR and mass spectroscopic methods were used to investigate hydroxylation and other biotransformations of 3-(2-dimethylaminoethyl)benzo[b]thiophene (**I**).

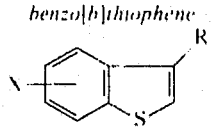
The production of benzo[b]thiophene-3-acetic acid reported in table 2 is a sum of the amounts of acid and 3-(2-hydroxyethyl)benzo[b]thiophene produced. Because the latter is not readily separated from the acid by TLC (19), direct quantitation of 3-(2-hydroxyethyl)benzo[b]thiophene was not accomplished. 3-(2-Aminoethyl)benzo[b]thiophene was not detected in this study. This is not surprising when one considers the comparative rates of oxidative N-demethylation of tertiary amines *vs.* secondary amines (20) and the substrate specificity of monoamine oxidase (21), a contaminant in crude liver preparations such as the 10,000*g* supernatant used in this study. Incubation of 3-(2-methylaminoethyl)benzo[b]thiophene and examination by TLC indicated that this compound is also hydroxylated, presumably in the 6-position, but no primary amine was detected. The occurrence of benzo[b]thiophene-3-acetic acid as the major urinary metabolite of **I** is similar to the case of DMT (22). No sulfoxidation was observed either *in vitro* or *in vivo*.

Failure to account for more than 47% of the administrated radioactivity in a 96-hr collection of excreta prompted an investigation of a limited number of tissues. After 96 hr, radioactivity was uniformly distributed in brain, liver, and kidney. The plasma level of radioactivity was about one-half that of the other three tissues examined. Fractional distillation of diluted plasma suggested that the plasma radioactivity was attributable to ^3H_2O. A definitive study of the tissue distribution of **I** and its metabolites has not been completed. Compounds of high lipid solubility are often sequestered in tissues other than brain, liver, or kidney (23). Robbins *et al.* (2) noted that 4-benzo[b]thiophene methylcarbamate was highly concentrated in rat lung.

Detailed analyses of the NMR spectra of a wide range of benzo[b]thiophene derivatives have been presented (24-26). A background for discussion is provided by the chemical shift data presented in figs. 1 and 2 and table 4. The entries in table 4 show that when the 5-position of the benzo[b]thiophene ring is substituted, the chemical shifts of the 4- and 6-protons are comparable and fairly constant. The signal from the 2-proton occurs downfield from the center of the 4,6-multiplet but overlaps the 4,6-multiplet. When the 6- position is substituted, the chemical shifts of the 5- and 7-protons are comparable and fairly constant, but the signal from the 2-proton is now shifted upfield and well separated from the 5,7-multiplet. This characteristic of the chemical shift of the 2-proton is well illustrated in figs. 1 and 2. The method by which the 6-hydroxy derivative was obtained is presented in Materials and Methods. The 5-hydroxy-3-(2-dimethylaminoethyl)benzo[b]thiophene was obtained by liberating the free base from the picrate according to the method of Fieser and Fieser (27). From a consideration of the data presented in table 4, it is clear that the microsomal enzymes hydroxylate **I** in the 6-position as they do the analogous indole. Hydroxylation in the 4- or the 7-position was ruled out in advance by considering the overall characteristics of the aromatic splitting pattern (see, for example, the NMR

TABLE 4

Chemical shifts (in ppm relative to trimethylsilyl) of aromatic protons in 3,5- and 3,6-disubstituted benzo[b]thiophene



X	R	H2	H4	H5	H6	H7	Oscillator Frequency (MHz)	Solvent	Ref
5-OH	$CH_2CH_2N(CH_3)_2$	7.07(s)	7.10(d)	—	6.90(dd)	7.62(d)	220	$CDCl_3$	—[a]
5-OD	$CH_2CH_2N(CH_3)_2$	7.41(s)	7.31(d)	—	7.02(dd)	7.68(d)	100	D_2O	17
5-OCH_3	$CH_2CH_2NHCOCH_3$	7.12(s)	7.22(d)	—	7.00(dd)	7.68(d)	100	$CDCl_3$	17
6-OH	$CH_2CH_2N(CH_3)_2$	6.84(s)	7.89(d)	7.49(m)	—	7.49(m)	220	$CDCl_3$	—[a]
6-OCH_3	$CH_2CH_2NHCOCH_3$	6.93(s)	7.60(d)	6.96(dd)	—	7.29(d)	100	$CDCl_3$	35
6-OCH_3	CH_3	6.70	7.38	6.85	—	7.14	100	—	—[b]

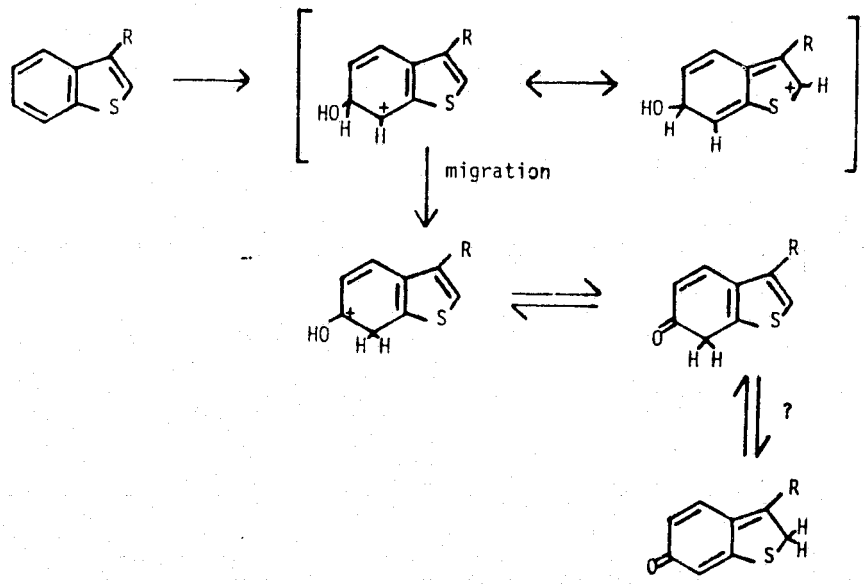
[a] S.D. Harrison, Jr., unpublished.
[b] A. Dinner, Department of Chemistry, Indiana University, unpublished.

spectrum of psilocin-S (18), a 3,4-disubstituted benzo[b]thiophene).

Very little systematic work has been done in the area of benzo[b]thiophene mass spectrometry. A brief review has appeared (24), but no work has been reported on derivatives similar to those of interest here, and one is forced to draw analogies with the mass spectrometry of indolealkylamines. Couch and Williams (28) recently reported work that provides an excellent summary of the mass spectrometry of tryptamines. Qualitative and quantitative differences in the mass fragmentation of indoles and benzo[b]thiophenes have been observed and have been presented in detail elsewhere (29). With few exceptions, the benzo[b]thiophene derivatives pertinent to the present work conformed to the fragmentation pathways that would be predicted from a consideration of indole mass spectrometry (28). The low relative abundance of *m/e* 163 in fig. 3 represents a possible exception, but the sample was not sufficient to yield a spectrum from which definitive conclusions about the mass fragmentation of 6-hydroxy-3-(2-dimethylaminoethyl)benzo[b]thiophene can be drawn.

Inasmuch as the 5-hydroxylation of tryptophan by a highly specific enzyme (30) is a widely known reaction, it seems appropriate to consider the apparently unique nature of the 6-position of indoles and benzo[b]thiophene. π-Electron densities (1) at positions 4, 5, 6, and 7 of benzo[b]thiophene decrease in the order $6 > 5 > 4 > 7$. The order for indole is $5 = 6 < 4 = 7$. In addition, evidence drawn from ultraviolet and NMR spectral characteristics of indole and benzo[b]thiophene derivatives suggests that π-electron conjugation into the ring may be more facile through the 6-position than through any other. Jepson *et al.* (31) have reported on the formation of a characteristic red color when a 6-hydroxy-3-substituted indole reacts with diazotized sulfanilic acid. They found that a free 2-position was necessary for the reaction to occur, and it appeared that the reaction product was a 2-azo derivative. Jaccarini and Jepson (12) reported absorption maxima for a series of hydroxytryptamines coupled with diazotized sulfanilic acid. The absorption maxima increase in the order (by position of hydroxyl group) $4 < 5 < 7 < 6$. It is well known (32) that π-electron conjugation shifts the absorption maximum of a given chromophore to a longer wavelength. The shift to longer wavelength that is apparent in the series of hydroxytryptamines (14) suggests that π-electron conjugation is maximum in the 6-hydroxytryptamine derivative.

Long-range coupling between the 2-proton and the 6-proton is a well documented characteristic of NMR spectra of benzo[b]thiophenes (24). Our NMR data revealed another consequence of the apparent electronic communication between the 2- and the 6-positions. Jackman and Sternhell (33) point out that the local diamagnetic shielding of a given proton is dependent upon the electron density about that proton. Ample evidence supports the conclusion that, in general, increasing the electron density about a proton will result in an upfield shift of that proton's resonance. This is exactly the behavior exhibited by the 2-proton when one progresses from 5-hydroxy-I to 6-hydroxy-I (figs. 1 and 2). This upfield shift of the 2-proton resonance in 6-hydroxy-I may be interpreted as resulting from an increased electron density at the 2-position as a consequence of an electron-donating group in the 6-position.



SCHEME 1. *6-Hydroxylation of a 3-substituted benzo[b]thiophene.*

The possibility of π-electron conjugation in indoles and benzo[b]thiophenes suggests a reason for hepatic mixed-function oxidation of these ring systems in the 6-position as opposed to any other. Resonance stabilization can contribute to a lower energy for 6-hydroxylation. This possibility is illustrated in scheme 1, derived from a consideration of the currently favored intermediates of microsomal aromatic hydroxylation (34). No comparable resonance stabilization can be envisioned for intermediates of 5-hydroxylation. Although electronic considerations may not be divorced from the steric and kinetic influences of an enzyme and its environment, the spectroscopic observations in our study that bear more directly on the question of positional specificity of aromatic hydroxylation are electronic in nature and are reported with the hope of stimulating further investigations of this question.

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BRIEF COMMUNICATION

Physiological Disposition of Atropine in the Rat[1]

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HARRISON, S. D., JR., T. R. BOSIN AND R. P. MAICKEL. *Physiological disposition of atropine in the rat.* PHARMAC. BIOCHEM. BEHAV. 2(6) 843–845, 1974. – The physiological disposition of atropine was studied in rats using ^{3}H-labeled drug and a specific assay method. At doses of 1.25 to 10 mg/kg, i.p., the greatest localization was seen in kidney and liver, with tissue: plasma ratios of >10 : 1. Tissue half-lives over the period 0.5 to 4 hr ranged from 40–46 min in plasma to 97–106 min in adipose tissue.

Atropine Tissue levels Physiological disposition

ALTHOUGH pure atropine was first isolated in 1931, and its medical use dates back many centuries, few detailed studies of the physiological disposition of this compound have been published. As mentioned by Evertsbusch and Geiling [3], suitable analytical methodology did not exist until the availability of radiolabeled atropine. These authors used randomly labeled ^{14}C-atropine and followed the time course of radioactivity in various tissues with no attempt to separate metabolites. Albanus *et al.* [1] used ^{3}H-atropine to study the disposition of the drug in mice, with a combination of radioautography and paper chromatography to correct for metabolites. Werner and Schmidt [12] used a combination of ^{3}H- and ^{14}C-atropine and specific procedures in a detailed study of the disposition and metabolism in mice. In addition, Albanus *et al.* [2] used ^{3}H-atropine to study the disposition of total radioactivity in the dog, again ignoring metabolites.

In addition to these studies, a number of reports have appeared describing the metabolic fate of atropine in a variety of animals. As early as 1949, Godeaux and Tonnesen [5] used a bioassay procedure to confirm the metabolism of atropine in rabbit blood and by cat, rabbit and rat liver in situ. Gosselin *et al.* [7] used ^{14}C-atropine to study disposition of radioactivity and excretion of the tropic acid metabolite in mice. Finally, Gabourel and Gosselin [4] examined the urinary excretion of ^{14}C-atropine in mice and rats, and Gosselin *et al.* [6] studied the urinary excretion of ^{14}C-atropine in man. Most recently, Kalser and McLain [9] have examined the excretion of ^{14}C-atropine in man, including studies of the plasma decay curves.

Despite the fact that atropine is widely used in behavioral research, primarily in rats [8,10], no attempts have been made to compare blood or tissue levels of the drug with behavioral effects. Indeed, the physiological disposition of atropine has not been reported in any detail in the rat. The present paper reports on the time course of physiological disposition of ^{3}H-atropine in the rat over a range of doses commonly used in behavioral studies.

METHOD

Adult, male Sprague-Dawley rats (290–350 g) were obtained from Murphy Breeding Laboratories, Plainfield, Indiana and maintained on Purina Lab Chow and tap water ad lib for 7–10 days prior to experimental use. Tritiated atropine (labeled in the para position of the phenyl ring of the tropic acid moiety) was obtained from New England Nuclear Corporation.

Groups of rats were stunned, then decapitated and exsanguinated at various times after i.p. administration of aqueous solutions of various doses of ^{3}H-atropine diluted with unlabeled atropine sulfate so that the dose of radioactivity per rat was 60 μc of ^{3}H. Blood was collected in heparin treated tubes and centrifuged immediately. Tissues were removed immediately; plasma and tissues were stored at −10° C until assay.

Radioactivity was measured in a Packard Tricarb Model

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TABLE 1

TIME COURSE OF LEVELS OF ^{3}H-ATROPINE IN VARIOUS RAT TISSUES

Tissue	Dose (mg/kg, i.p.)	0.05	1	2	4	$t_{1/2}$ min
Plasma	1.25	231± 37 (4)	91± 12 (4)	48± 10 (4)	5.6±1.9 (4)	43
	2.5	430± 78 (3)	163± 37 (3)	93± 12 (3)	11± 5 (3)	46
	5	857± 91 (4)	423± 82 (4)	209± 36 (4)	21± 5 (4)	46
	10	2243 (1)	1015± 96 (4)	375± 71 (3)	63± 9 (3)	40
Brain	1.25	329± 47 (3)	218± 39 (3)	85± 31 (3)	54± 17 (3)	82
	2.5	887± 42 (4)	539± 57 (3)	309± 37 (3)	145± 12 (3)	79
	5	1901± 76 (3)	1226± 109 (3)	794± 26 (3)	296± 20 (3)	71
	10	3639 (1)	2852± 191 (4)	1403±111 (3)	476± 28 (4)	71
Fat	1.25	944±111 (4)	453± 91 (4)	406± 47 (3)	209± 27 (4)	106
	2.5	1983±274 (3)	1645± 194 (3)	974± 93 (3)	511± 78 (3)	97
	5	3742±681 (3)	3444± 601 (3)	2191±278 (3)	1019± 93 (3)	100
	10	8011 (1)	7219± 697 (4)	4667±494 (4)	2312±174 (3)	103
Heart	1.25	601± 93 (3)	257± 33 (3)	102± 12 (3)	39± 13 (3)	53
	2.5	1210± 97 (4)	508± 66 (4)	219± 46 (3)	96± 41 (4)	54
	5	2514±271 (3)	997± 59 (4)	430± 58 (4)	207± 60 (3)	56
	10	5279 (1)	2360± 537 (4)	808±106 (4)	458± 57 (4)	52
Kidney	1.25	2917±168 (3)	1015± 57 (4)	488± 57 (4)	199± 27 (4)	58
	2.5	5612±409 (4)	2404± 360 (3)	995± 93 (4)	411± 78 (4)	56
	5	13206±930 (3)	5880± 397 (4)	1955±216 (4)	822±302 (4)	50
	10	26645 (1)	11681± 944 (4)	3674±378 (3)	1406±271 (4)	53
Liver	1.25	2606± 93 (3)	1178± 111 (4)	718± 76 (3)	346± 39 (4)	74
	2.5	5828±306 (4)	2407± 371 (4)	1631±134 (4)	649± 79 (4)	72
	5	10751±912 (4)	4911± 408 (4)	3079±475 (4)	1607±138 (4)	72
	10	26544 (1)	12988±1012 (4)	6214±612 (4)	3240±319 (4)	76

Each value (ng/g±S.D.) is the mean of values obtained from (N) rats.

4322 liquid scintillation spectrometer, with polyethylene vials and a cocktail consisting of 14 g of 2,5-bis-2-(5-tert-butylbenzoxazolyl)-thiophene and 280 g of naphthalene in a mixture of 2100 ml of toluene and 1400 ml ethylene glycol monomethyl ether [11]. A counting system of 15 ml of this cocktail and 0.5 ml of aqueous solution gives 18% efficiency for ^{3}H with a background of 10 to 15 cpm.

Atropine was determined as follows. Tissues were homogenized in 3 volumes of 0.05 N NCl, using a motor driven Teflon pestle and glass homogenizer. Aliquots of tissue homogenate (1.0 ml) or plasma (1.0 ml) were added to glass stoppered (g.s.) 50 ml shaking tubes containing 25 ml of benzene and 2.0 ml of a saturated aqueous solution of potassium carbonate. After mechanical shaking for 5 min, the tubes were centrifuged. Aliquots (10 ml) of the benzene phase were transferred to g.s. 50 ml shaking tubes containing 2.0 ml of 2.0 N HCl, and the tubes were stoppered, shaken for 5 min, and centrifuged. The benzene phase was removed and discarded. Aliquots (0.5 ml) of the acid phase were then transferred to polyethylene vials containing 15 ml of the liquid scintillation cocktail. The specificity of the method was confirmed by thin-layer chromatography using the system methanol: NH_4OH (100 : 1.5) and silica gel G plates. Under these conditions, only a single radioactive spot was found in the extracts, with an R_f corresponding to authentic atropine. Recovery of authentic atropine was 85–90%.

RESULTS AND DISCUSSION

The results obtained after administration of ^{3}H-atropine in i.p. doses of 1.25, 2.5, 5.0 or 10.0 mg/kg to rats are shown in Table 1. Half-life values were determined from the slopes of the decay curves as estimated by the method of least squares. Several facets are of particular interest. Plasma has the shortest half-life (40–46 min) while adipose tissue (97–106 min) has the longest. Localization is seen in all tissues with tissue: plasma ratios relatively constant over the first 2 hr, then increasing rapidly at 4 hr as plasma levels continue to fall rapidly. Tissue levels decay at a slower rate. In fact, the levels of atropine in tissues appear to have a biphasic decay curve on the basis of selected analyses done at 8 and 12 hr after the larger doses. Plasma values were below the level of detection at all doses after the 4 hr time point.

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reported that rabbit aldehyde oxidase (aldehyde: oxygen oxidoreductase, EC 1.2.3.1) has the ability to oxidize allopurinol to oxipurinol *in vitro*. Furthermore, Huff *et al.*[7] showed that there is a sex difference of aldehyde oxidase activity in mice when N-methylnicotinamide was used as substrate. Therefore we are now investigating the relationship between allopurinol oxidizing enzyme and aldehyde oxidase in mice.

Elion *et al.*[2,9] reported that allopurinol is rapidly oxidized *in vivo* to oxipurinol which also inhibits xanthine oxidase. Previously we reported that the ^{14}C-compounds disappear much slower in male than in female mice after labeled allopurinol treatment. Thus it seems that this sex difference in allopurinol oxidizing activity may, to some extent, be concerned with acute toxicity.

Further work is needed to clarify this problem.

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Inability of rat brain homogenate to oxidize amphetamine

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In a recent series of papers in this journal Guha and Mitra[1–3] have reported that homogenates of rat and guinea pig brain catalyze the reduction of neo-tetrazolium chloride (NTC) in the presence of amphetamine. The system has been described as an amphetamine dehydrogenase: the most recent report suggests that tranylcypromine is also actively dehydrogenated by this system.[3] However, the activity of the enzyme system has been estimated by measuring the production of diformazan from NTC. The authors have not determined that a degradation product of amphetamine is produced in the course of the reaction.

Hucker[4] and Parli and McMahon[5] have recently reported on the metabolism of amphetamine with no evidence for a dehydrogenase pathway. Earlier work from this laboratory, using [^{3}H]labeled amphetamine, had shown that rats convert the drug *in vivo* by *p*-hydroxylation and oxidative deamination.[6] The present communication reports on attempts to measure the production of metabolites of [^{3}H]amphetamine by the rat brain dehydrogenase system as described by Guha and Mitra.[1–3]

Brains were removed from adult (300–400 g) male, Sprague–Dawley rats purchased from Murphy Breeding Laboratories, Plainfield, Ind., and homogenates prepared according to Mitra and Guha.[3] *d*-Amphetamine phosphate was supplied by the Pennwalt Corp., [^{3}H]*d*-amphetamine sulfate was purchased from the New England Nuclear Corp., NTC from the Sigma Chemical Co. and diformazan from Nutritional Biochemicals Corp.

Incubations were performed according to Mitra and Guha.[3] Diformazan production was measured by the method of Lagnado and Sourkes[7] using a Gilford model 2000 absorbance recorder with a Beckman model DU monochromotor. [^{3}H]amphetamine was assayed as described by Maickel *et al.*,[6] using a Packard Tri-Carb model 2425 liquid scintillation system. Metabolites of [^{3}H]amphetamine were separated by TLC on Silica gel G plates (0·25 mm, Brinkmann MN-Polygram) in the system described by Hucker.[2] Phenyl-2-propanone was visualized by spraying with 1% $KMnO_4$ in 5% aqueous Na_2CO_3. TLC plates were scanned for radioactive spots in a Packard model 7200 radiochromatogram scanner.

The conversion of NTC to diformazan in the system of Guha and Mitra[3] occurred with or without the addition of amphetamine. In an attempt to prepare a standard curve for the absorbance of diformazan at 520 nm, it was found that absorbance is not linear over the concentration range reported by Guha and Mitra.[1–3] No amphetamine metabolites were observed when the incubate was extracted with methylene chloride and examined by TLC.[4] Incubation of [^{3}H]amphetamine with the homogenate and NTC, followed by extraction, TLC and radiochromatogram scanning indicated that greater than 95 per cent of the radioactivity present was contained in a spot corresponding to unchanged [^{3}H]amphetamine. In a definitive experiment, incubation mixtures were prepared with and without homogenate. Duplicate mixtures of each type were either incubated and extracted after 30 min or not incubated, i.e. extracted at $t = 0$. [^{3}H]amphetamine was extracted by a specific procedure and measured by liquid scintillation spectrometry.[6] Neither the presence of brain homogenate nor incubation at 37° for 30 min resulted in any reduction in the amount of [^{3}H]amphetamine that could be extracted. Based on the rate of diformazan production reported by Mitra and Guha,[3] one would have expected at least 85 per cent conversion of amphetamine. As little as 10 per cent metabolism of amphetamine would have been easily detected.

Many substances are known to reduce neo-tetrazolium chloride, ranging from simple alkaline pH to ascorbic acid, reducing sugars and flavoprotein enzymes.[8] Indeed, it was observed that during an early step in the amphetamine extraction procedure[6] at which the aqueous phase was made alkaline, the color characteristic of diformazan appeared in mixtures containing NTC and homogenate with or without amphetamine; mixtures containing only amphetamine and NTC developed no color. The reduction of NTC under these conditions appears to be a function of the presence of brain homogenate and not of amphetamine concentration.

In summary, an attempt was made to measure the production of [^{3}H]amphetamine metabolites by the rat brain amphetamine dehydrogenase system of Mitra and Guha.[3] No metabolic conversion of amphetamine was observed.

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DEVELOPMENT OF A MODEL STRESS SYSTEM FOR REPETITIVE DAILY STRESS

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INTRODUCTION

The concepts of homeostasis, which views living organisms as actively maintaining an optimal internal environment or "millieu interior", was largely developed from the work of Claude Bernard (1). Since the development of the various concepts of "stress" by Selye (2,3), many people have considered stress to represent any state of the organism in which reactions to stimuli have distributed the normal homeostasis by perturbation of biochemical or physiological mechanisms. Stress may thus be considered as an abnormal state of the organism, and, in the terminology of Selye (4) proceeds through three sequential phases: shock, resistance, and exhaustion. In this framework, shock is the initial phase, in which the organism is moved into a state of homeostatic imbalance by the action of the stressful stimuli. The second phase, resistance, is characterized by reactions of the organism as it attempts to regain its initial homeostatic state. The final phase, exhaustion, occurs when the organism is exposed to repeated or continued stressful stimuli, and, because of repeated or continued attempts to maintain homeostasis, the organism becomes unable to respond further or otherwise proceeds to a completely non-homeostatic state. The net result of the exhaustion state is generally expressed as one or more pathological phenomena; in the most severe case, irreversible damage or even death may ensue.

The general characteristics of mammalian response to stress include emergency responding of two body systems: the sympathomedullary system and the pituitary-adrenocortical system. The response of the sympathomedullary system is manifested in increased peripheral sympathetic nervous activity and consequent biochemical and physiological responses to the surging release of norepinephrine: increased heart rate and respiration, contraction of

blood vessels, piloerection, heat consevation, and kipolysis in adipose tissue. At the same time, adrenal medullary discharge of epinephrine evokes a mobilization of glucose from the liver glycogen stores. Meanwhile, the anterior pituitary is secreting large quantities of ACTH, causing increased production of glucocorticoids from the adrenal cortex and causing further imbalances in normal body functional systems.

In humans, elevated catecholamine outputs have been seen in response to a variety of psychological (5) or psychophysiological stimuli (6). Similarly, increased adrenal corticoid secretion has been evoked by fear (7), perceptual disturbances (8), emotional disturbances (9-10), and competitive sports (11). These findings seem most appropriate in view of the likelihood of similar types of stressful situations occurring on a repetitive basis during space flight - especially since the duration of manned space flights are getting longer.

Unfortunately, the ideal test system to examine the consequences of repetitive stresses does not as yet exist. Human test systems are extremely limited in that only a few measurement parameters can be used; no tissues are available, and samples other than urine are restricted in terms of the number that can be obtained. Animal test systems are readily available and permit ready sampling of almost anything, but, no appropriate animal model for repetitive stresses has yet been examined in sufficient details. In addition, while a large number of variables has been measured in a diversity of stresses and experimental animals, no one has been able to select the most appropriate variables to measure or the most useful stress to examine on a dose-response basis.

In this report, we present preliminary studies comparing three stresses: cold exposure (a predominantly physiological stress), immobilization (a predominantly psychological stress), and d-amphetamine (a predominantly

pharmacological stress). The experimental design permitted comparison of single stress with repetitive stress on primary (plasma corticosterone), secondary (plasma glucose, plasma FFA), and tertiary (brain norepinephrine and serotonin) parameters of the stress response.

METHODS

Adult, male, Swiss-Webster mice weighing 25-30 grams were used in all experiments. Cold exposure occurred in individual screen wire cages in a room at 4°C; immobilization was performed by restraining individual animals in wire mesh tubes; d-amphetamine was administered by i.p. injection at a dose of 4 mg/kg. Animals were killed by decapitation and blood was collected into heparinized tubes. Tissues were removed immediately and frozen; plasma was also prepared immediately by centrifugation and frozen. Plasma corticosterone, plasma glucose, and plasma FFA were determined as described by Maickel, et al (12); brain 5HT, 5HIAA, and NE by the method of Miller, et al (13).

RESULTS

Effects of a Single Stress on Parameters. The time course of an acute stress (4 hours) was measured; the data are shown in Figures 1, 2, and 3. At all times, and with all three stressful stimuli, the plasma corticosterone values were significantly elevated ($p < .05$) above control values (Fig. 1); cold exposure seems to reach the highest level at 0.5 hr, as well as to produce a longer elevation. The picture of plasma glucose is quite different. Only d-amphetamine evokes a significant elevation in this parameter, although this effect falls to non-significant values at the 4 hr. time point. In contrast, both cold exposure and immobilization show a pattern of an initial

insignificant rise, followed by a fall to lower than control values ($p < .05$) at 2.0 and 4.0 hours. In the case of plasma FFA, cold exposure produces a significant elevation at 1 to 4 hours, while immobilization causes a significant depression at 1 hour and amphetamine has no significant effects although the value at 0.5 hour is almost significantly ($.10 > p > .05$) lower than control.

Effects of Repetitive Stress on Parameters. The data presented in Figure 4 were those obtained by exposing mice to chronic daily stress: 2 hrs of cold exposure, 2 hours of immobilization, or 4.0 mg/kg, i.p. of d-amphetamine, for 14 days. The animals were sacrificed at the termination of the 14th stress session. Control animals were handled daily at the same times and to approximately the same extent as the experimental mice. The results indicate that cold exposure and amphetamine were similar in that plasma corticosterone values were significantly elevated after the 14th session, while immobilization animals had normal values. In the case of plasma glucose, levels were depressed in chronic immobilization and amphetamine, but were normal with repetitive cold exposure. No differences from control were seen in plasma FFA levels with any of the stressful stimuli.

Effects of Repetitive Stress on Response to Acute Stress. In order to determine whether repetitive exposures to stressful stimuli might influence the ability of an organism to respond to a subsequent acute stimulus, an experiment testing this situation was devised. Animals were repetitively stressed (as described in the preceeding section) by either cold exposure, immobilization, or d-amphetamine; on the next day, an acute "challenge" stress was applied. The results (Figures 5, 6, 7) indicate that the

repetitive stresses, in general, tend to reduce the ability of the animals to respond to a subsequent acute stress. This is most noticeable in the case of repetitive cold stress and least noticeable for repetitive amphetamine dosages.

Effects of Acute and Repetitive Stresses on Brain Biogenic Amines. In addition to the plasma biochemical parameters, measurements have also been made of the levels of brain serotonin (5HT), norepinephrine (NE), and 5-hydroxyindole-3-acetic acid (5HIAA) in the various stress conditions. The effects of an acute stress or repetitive stresses on these amines are shown in Figure 8. The acute cold and immobilization stresses evoke significant ($p < .05$) increases in both 5HT and 5HIAA, with increases, though not significant in NE; acute amphetamine fails to cause any significant changes. Repetitive stress by cold or immobilization evokes significant decreases in 5HT and 5HIAA, with no change in NE; repetitive amphetamine fails to influence 5HT or 5HIAA, but causes a fall in the level of NE.

In the case of an acute stress "challenge" tested after the 14th repetitive stress, the results differ with each stress. Repetitive cold exposure enhanced the increased 5HT and 5HIAA caused by all three acute stresses (Figure 9). No effect was seen with NE except in the case of acute amphetamine, where the repetitive cold exposures caused the animals to be less responsive. Repetitive immobilization had virtually no effect on the brain amine alterations caused by a subsequent acute stress (Figure 10), while repetitive amphetamine dosages enhanced the effects of a subsequent cold exposure on NE, and decreased the responsivity of all three brain substrates to a subsequent dose of amphetamine (Figure 11).

DISCUSSION

The results presented here should be considered as only preliminary in that they are studies in a single species of animal, with a minimal number of variables and parameters. However, several interesting points for further study have been clearly demonstrated. First of all, the effects of an acute stress of limited duration must be considered in the framework of a subject's prior experience as well as in the light of the sensitivity of the parameter of interest. Thus, a "classical" stress marker such as plasma corticosterone, while extremely sensitive and useful as an indicator of acute stress, becomes almost valueless when the same stress is applied repetitively (cf. Figures 1, 4, 5). Plasma glucose responds nicely to cold exposure or immobilization in both acute and repetitive situations (Figures 2, 4), but is not suitable as an indicator of a subsequent acute stress (Figure 6). Plasma FFA responds in a useful manner only to acute cold exposure; however, in the repetitive situation (Figure 7), the effects are useful only in the cold exposure situation.

In summary, these preliminary studies indicate the need for more detailed examination of potential stress "markers". An animal model would be most useful; if space flights of long duration are to be successful, repetitive stress must be considered. However, the search for a suitable animal model must be expanded to examine new possibilities for measurable markers.

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LEGENDS

FIGURE 1: Mean values of plasma corticosterone in control mice ☐ (N=50), at various times of cold exposure ☰ (N=20), immobilization ☷ (N=10), or after administration of d-amphetamine ▨ (N=10). Vertical bars indicate ± S.D.

FIGURE 2: Mean values of plasma glucose in control mice ☐ (N=200) at various times of cold exposure ☰ (N=20), immobilization ☷ (N=20), or after administration of d-amphetamine ▨ (N=10). Vertical bars indicate ± S.D.

FIGURE 3: Mean values of plasma FFA in control mice ☐ (N=200), at various times of cold exposure ☰ (N=20), immobilization ☷ (N=20), or after administration of d-amphetamine ▨ (N=10). Vertical bars indicate ± S.D.

FIGURE 4: Mean values of plasma corticosterone, glucose, and FFA in control mice ☐ (N=24), or mice after repetitive cold exposure ☰ (N=12), immobilization ☷ (N=12), or amphetamine ▨ (N=12). Vertical bars indicate ± S.D. Animals were killed 2 hrs after the last dose of amphetamine or immediately following the final 2 hr immobilization or cold exposure.

FIGURE 5: Mean values of plasma corticosterone response ratios (repetitive stressed ÷ non-stressed × 100%) to acute cold exposure ☰ (N=12), immobilization ☷ (N=12), or amphetamine ▨ (N=12) in animals subject to repetitive prior stresses. Vertical bars indicate ± S.D. Acute stress was applied 24 hrs after the 14th repetitive stress. Animals were killed as in Figure 4.

FIGURE 6: Mean values of plasma glucose response ratios (repetitive stressed ÷ non-stressed x 100%) to acute cold exposure  (N=12), immobilization  (N=12), or amphetamine  (N=12) in animals subject to repetitive prior stresses. Vertical bars indicate ± S.D. Acute stress was applied 24 hrs after the 14th repetitive stress. Animals were killed as in Figure 4.

FIGURE 7: Mean values of plasma FFA reponse ratios (repetitive stressed ÷ non-stressed x 100%) to acute cold exposure  (N=12), immobilization  (N=12), or amphetamine  (N=12) in animals subject to repetitive prior stresses. Vertical bars indicate ± S.D. Acute stress was applied 24 hrs after the 14th repetitive stress. Animals were killed as in Figure 4.

FIGURE 8: Mean values of brain 5HT  , 5HIAA  , and NE  in animals after a 2 hr acute stress or at the end of the 14th repetitive stress, expressed as percent of control values of non-stressed animals. Vertical bars are ± S.D.; N=24 (controls), N=12 (each experimental group).

FIGURE 9: Mean values of response ratios (repetitive stressed ÷ non-stressed x 100%) of brain 5HT  , 5HIAA  , and NE  in animals after a 2 hr acute stress 24 hrs after the 14th repetitive cold exposure. Vertical bars are ± S.D. Animals were killed as in Figure 4.

FIGURE 10: Mean values of response ratios (repetitive stressed ÷ non-stressed x 100%) of brain 5HT □ , 5HIAA ▦ , and NE ▩ in animals after a 2 hr acute stress 24 hrs after the 14th repetitive immobilization. Vertical bars are ± S.D. Animals were killed as in Figure 4.

FIGURE 11: Mean values of response ratios (repetitive stressed ÷ non-stressed x 100%) of brain 5HT □ , 5HIAA ■ , and NE ▩ in animals after a 2 hr acute stress 24 hrs after the 14th repetitive amphetamine dose. Vertical bars are ± S.D. Animals were killed as in Figure 4.

FIGURE I

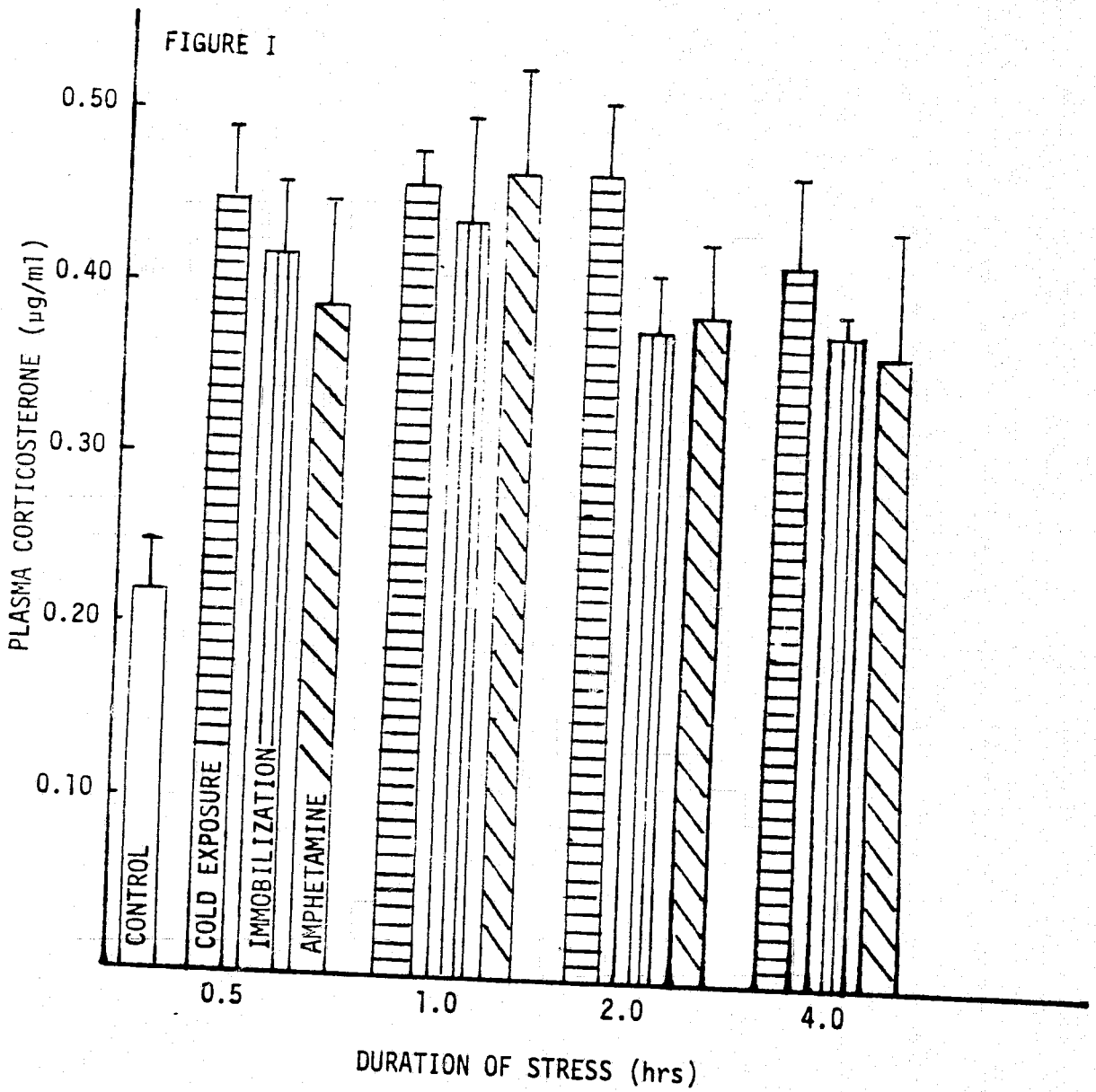

PLASMA CORTICOSTERONE (µg/ml)
0.50
0.40
0.30
0.20
0.10
CONTROL
COLD EXPOSURE
IMMOBILIZATION
AMPHETAMINE
0.5
1.0
2.0
4.0
DURATION OF STRESS (hrs)

FIGURE II

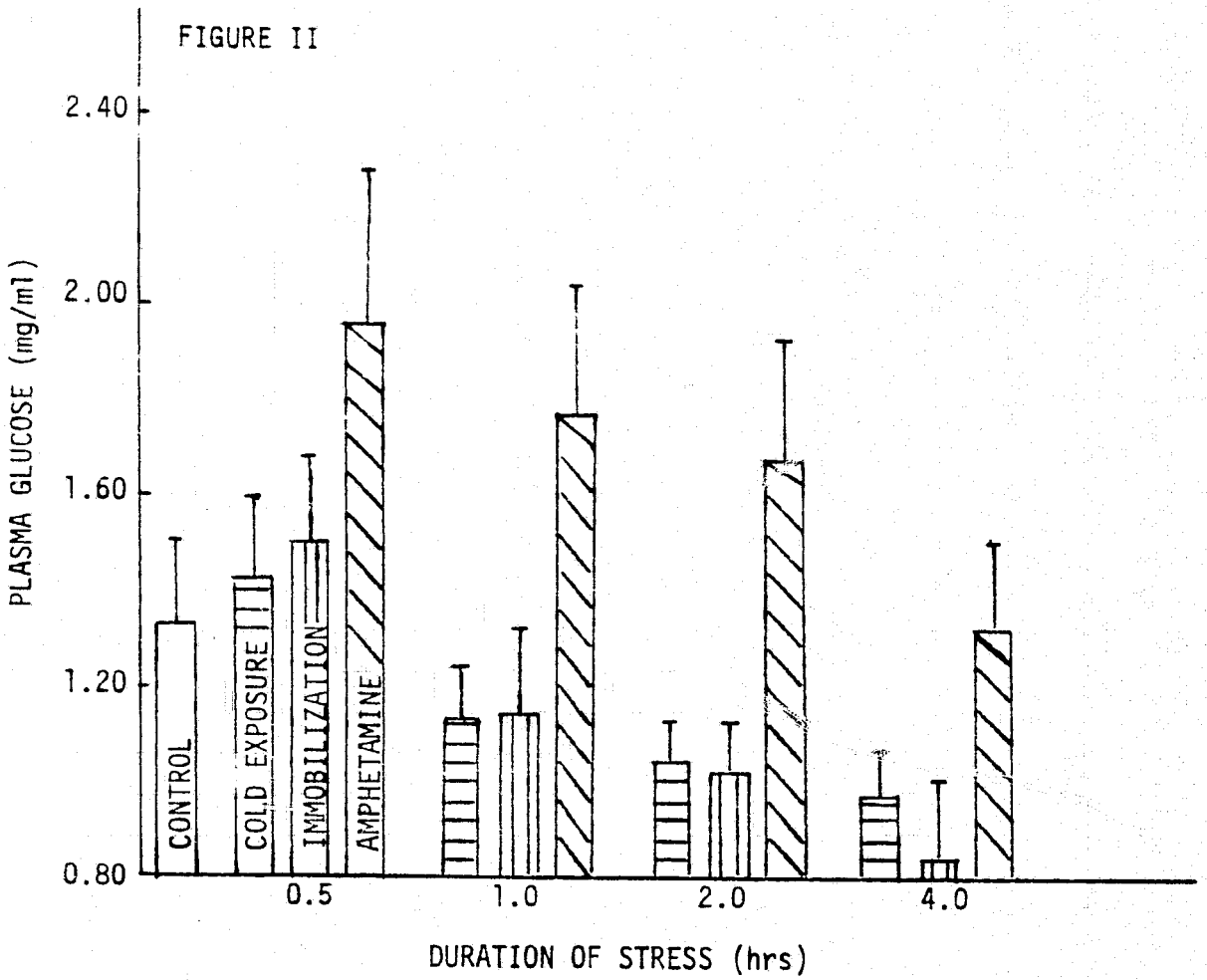
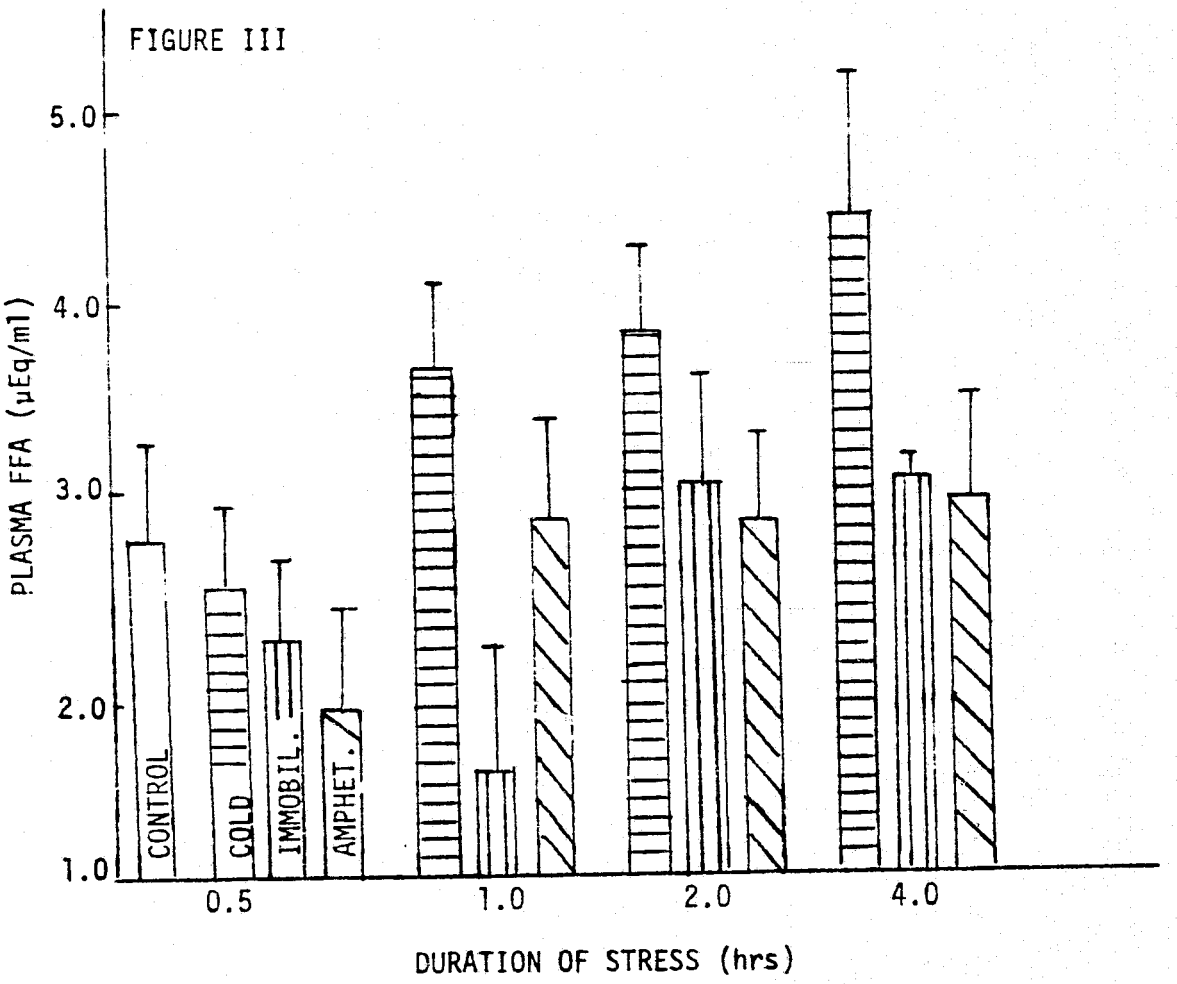

2.40
2.00
1.60
1.20
0.80
PLASMA GLUCOSE (mg/ml)
CONTROL
COLD EXPOSURE
IMMOBILIZATION
AMPHETAMINE
0.5
1.0
2.0
4.0
DURATION OF STRESS (hrs)

FIGURE III


PLASMA FFA (µEq/ml)
5.0
4.0
3.0
2.0
1.0
CONTROL
COLD
IMMOBIL.
AMPHET.
0.5
1.0
2.0
4.0
DURATION OF STRESS (hrs)

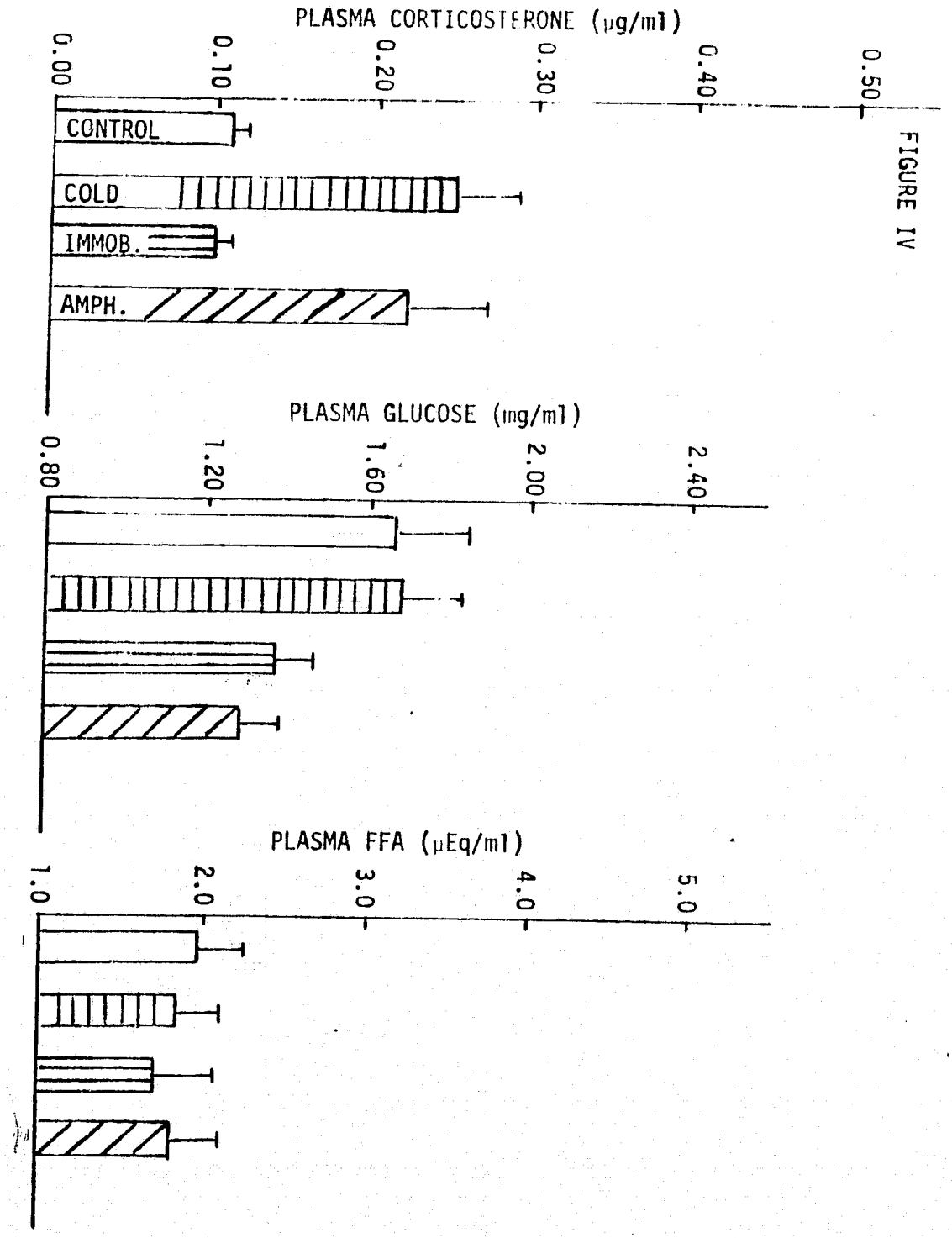
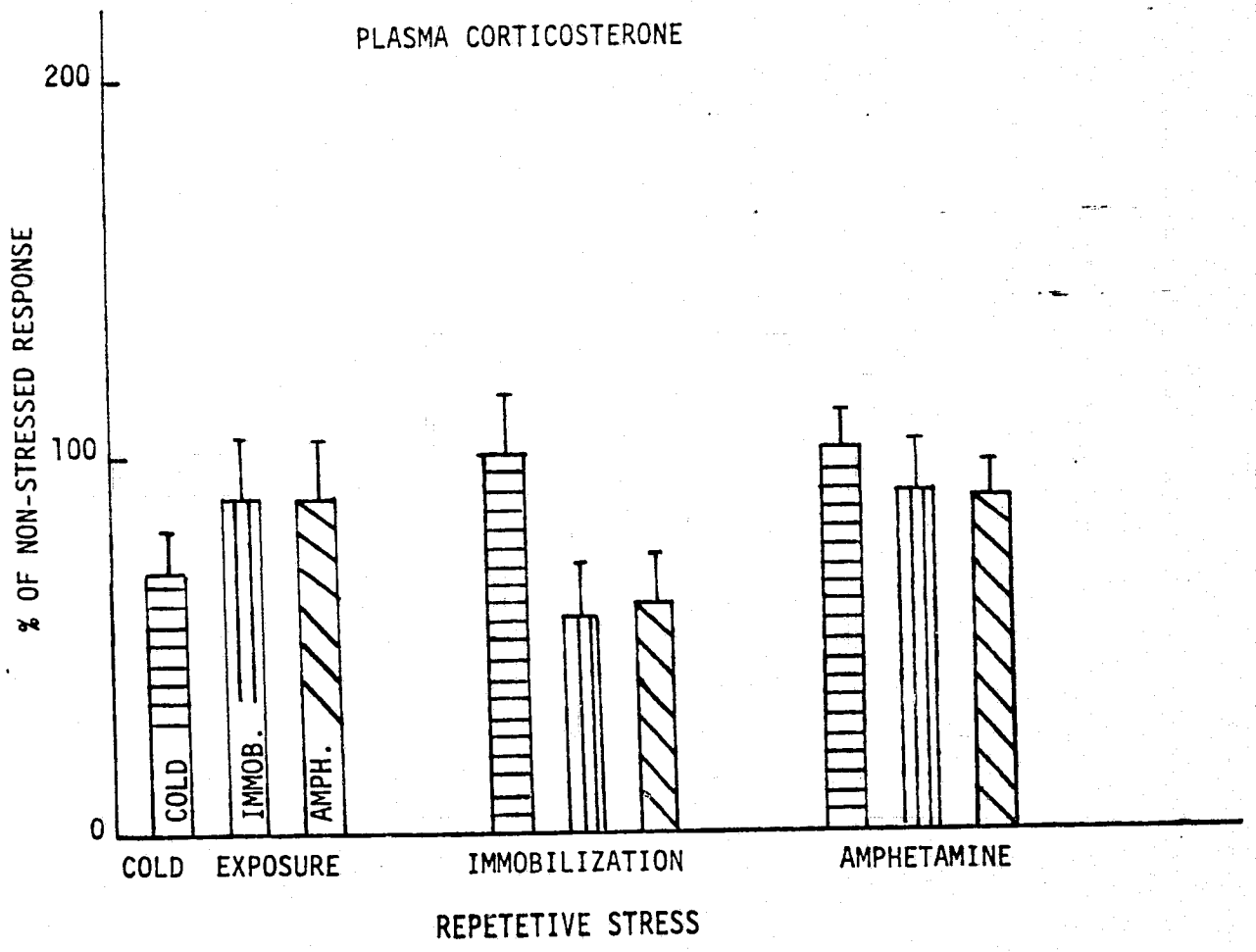

PLASMA CORTICOSTERONE (µg/ml)
0.00
0.10
0.20
0.30
0.40
0.50
CONTROL
COLD
IMMOB.
AMPH.
PLASMA GLUCOSE (mg/ml)
0.80
1.20
1.60
2.00
2.40
PLASMA FFA (µEq/ml)
1.0
2.0
3.0
4.0
5.0

FIGURE IV

FIGURE V


PLASMA CORTICOSTERONE
200
100
0
% OF NON-STRESSED RESPONSE
COLD
IMMOB.
AMPH.
COLD EXPOSURE
IMMOBILIZATION
AMPHETAMINE
REPETETIVE STRESS

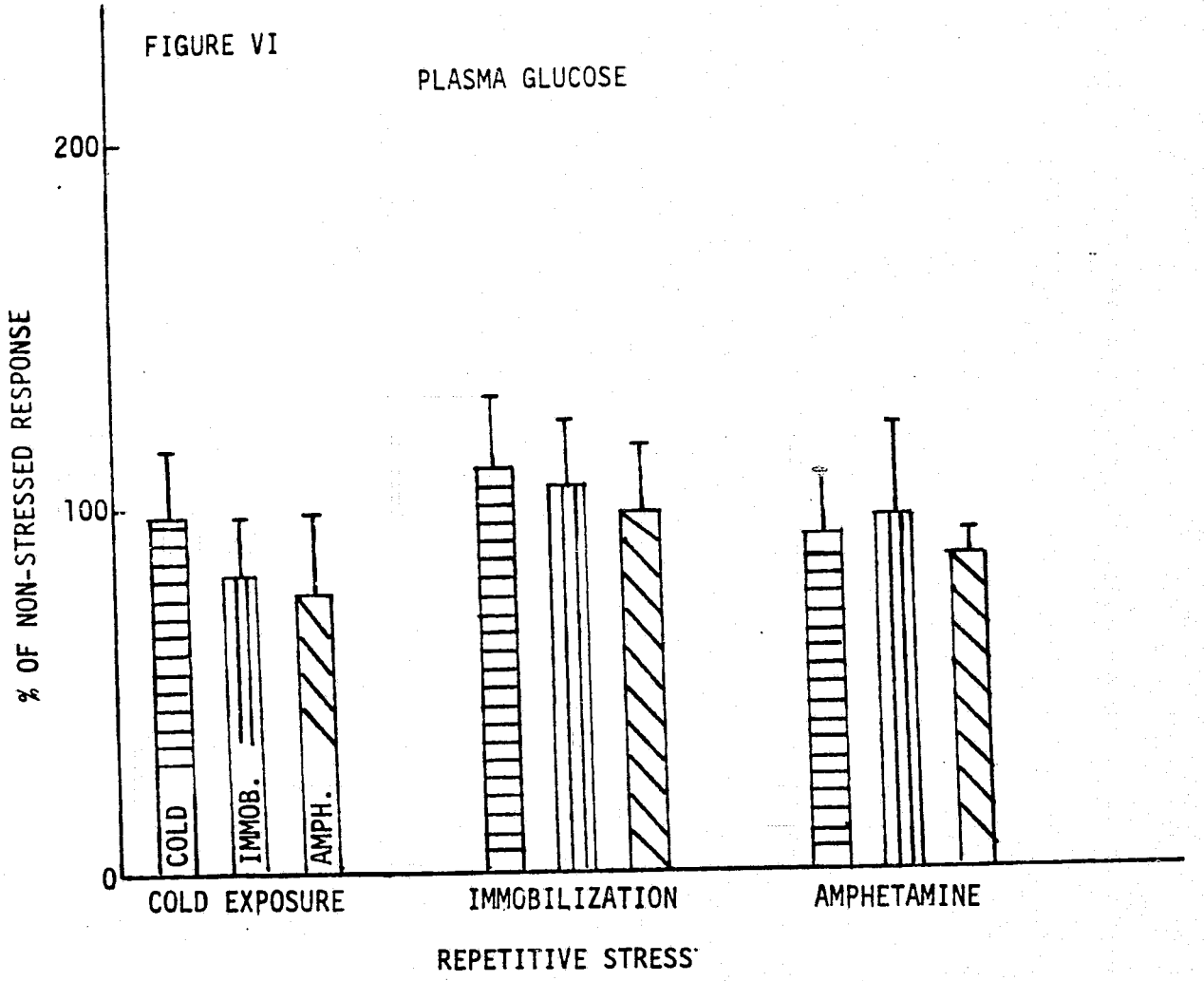


FIGURE VI
PLASMA GLUCOSE
200
100
0
% OF NON-STRESSED RESPONSE
COLD
IMMOB.
AMPH.
COLD EXPOSURE
IMMOBILIZATION
AMPHETAMINE
REPETITIVE STRESS

FIGURE VII

PLASMA FFA

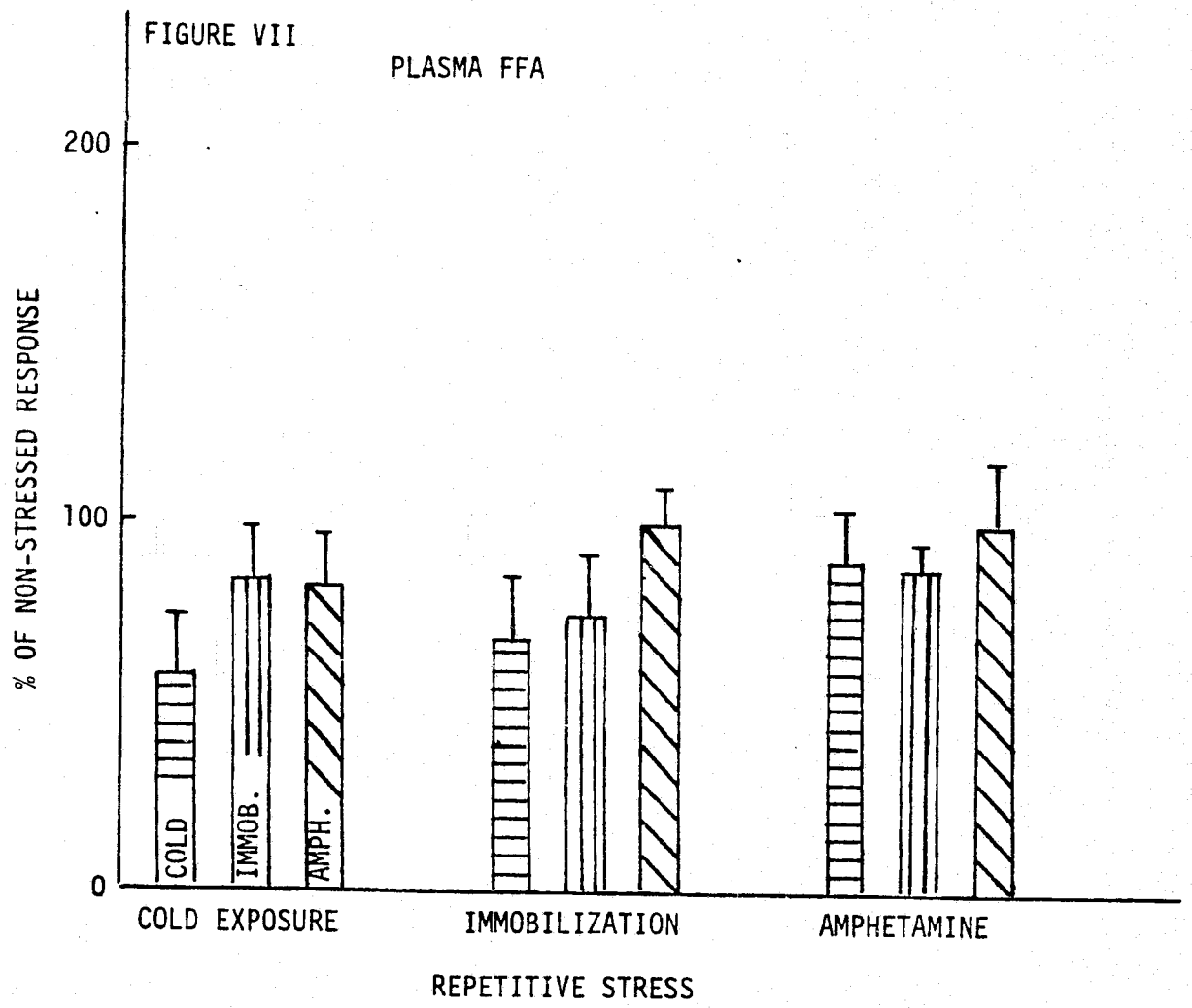


FIGURE VIII

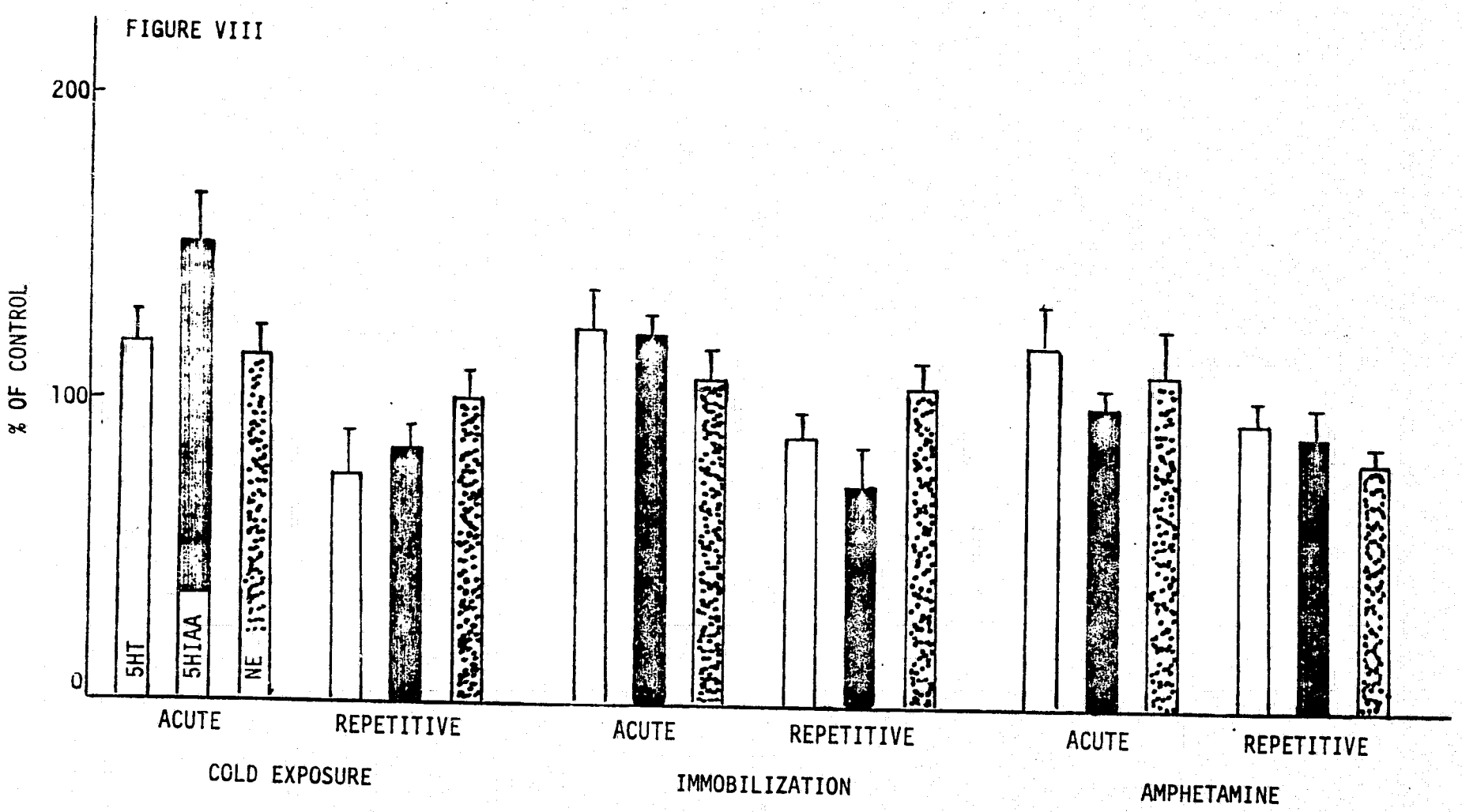

200
100
0
% OF CONTROL
5HT
5HIAA
NE
ACUTE
REPETITIVE
ACUTE
REPETITIVE
ACUTE
REPETITIVE
COLD EXPOSURE
IMMOBILIZATION
AMPHETAMINE

FIGURE IX

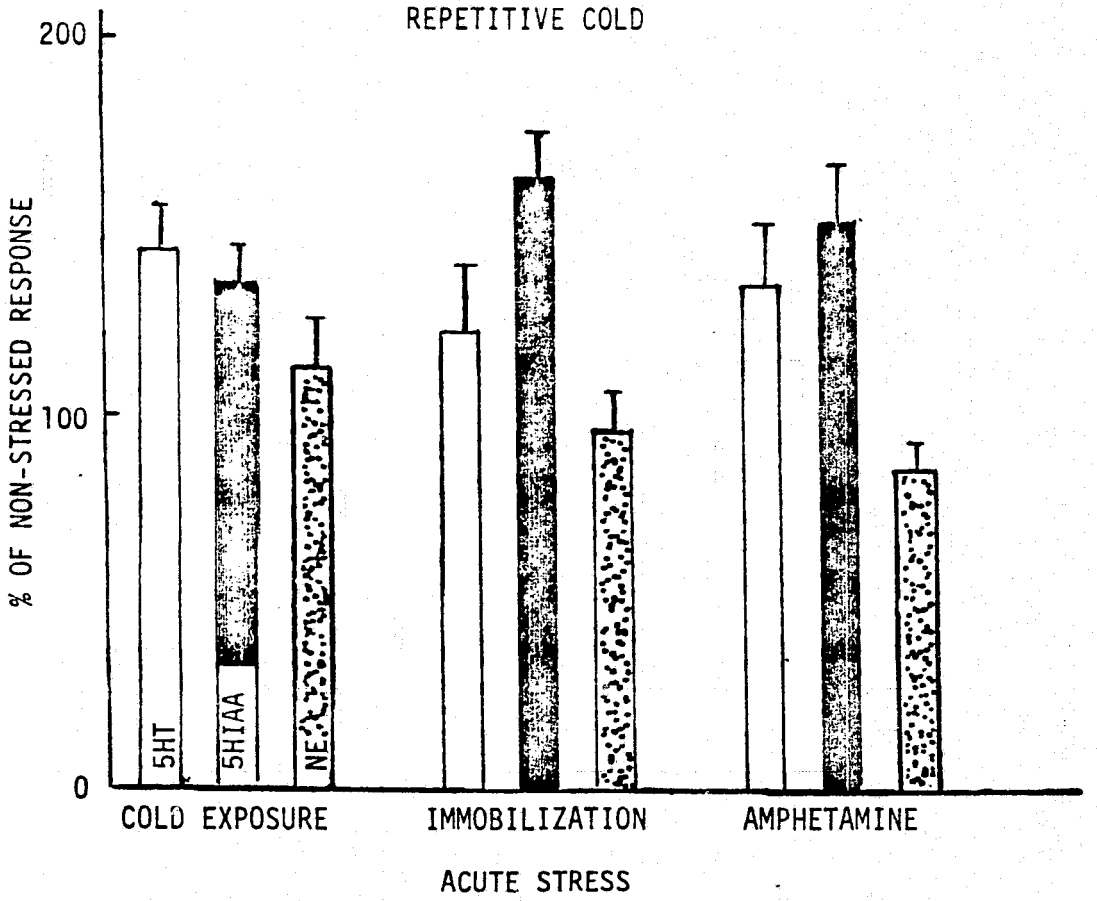
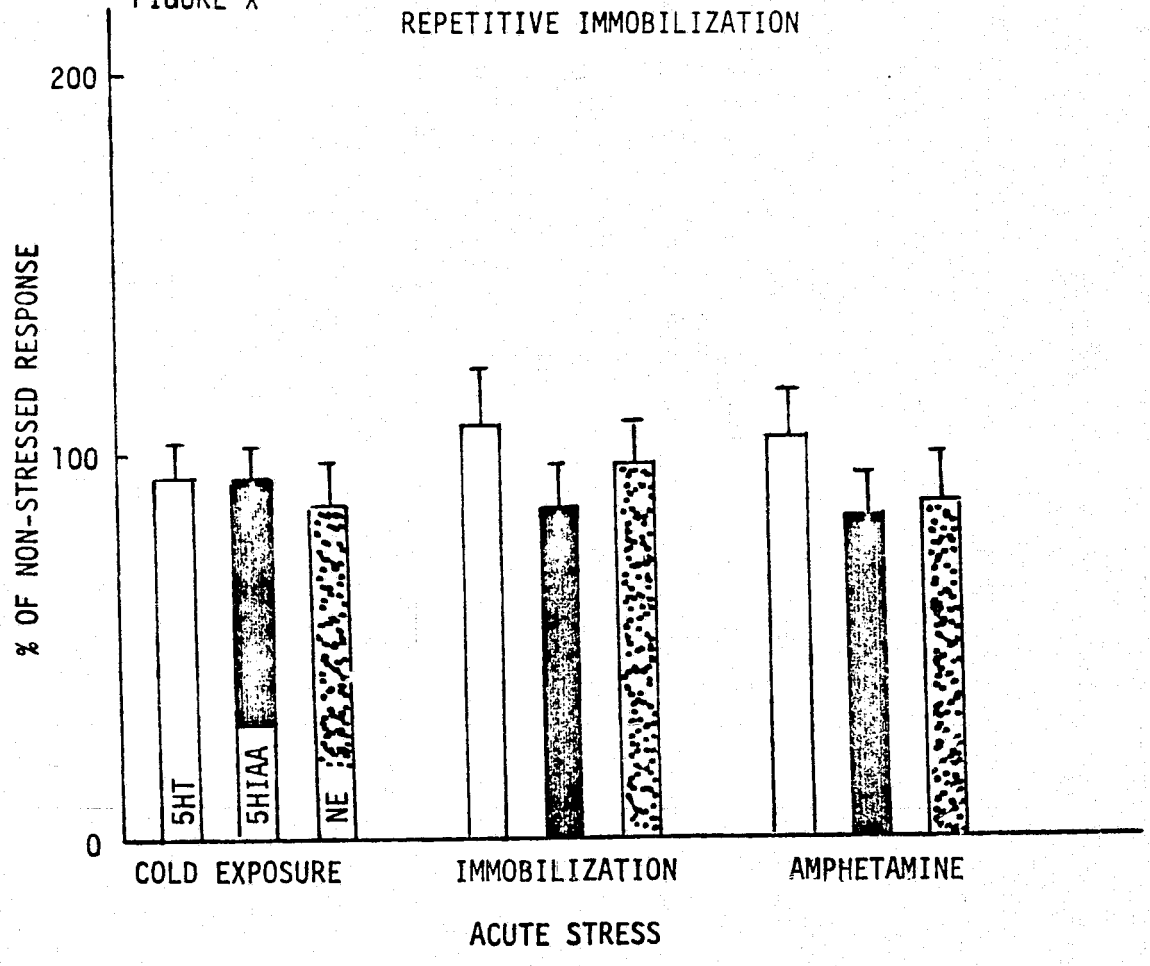

REPETITIVE COLD
200
100
0
% OF NON-STRESSED RESPONSE
5HT
5HIAA
NE
COLD EXPOSURE
IMMOBILIZATION
AMPHETAMINE
ACUTE STRESS

FIGURE X


REPETITIVE IMMOBILIZATION
200
100
0
% OF NON-STRESSED RESPONSE
5HT
5HIAA
NE
COLD EXPOSURE
IMMOBILIZATION
AMPHETAMINE
ACUTE STRESS

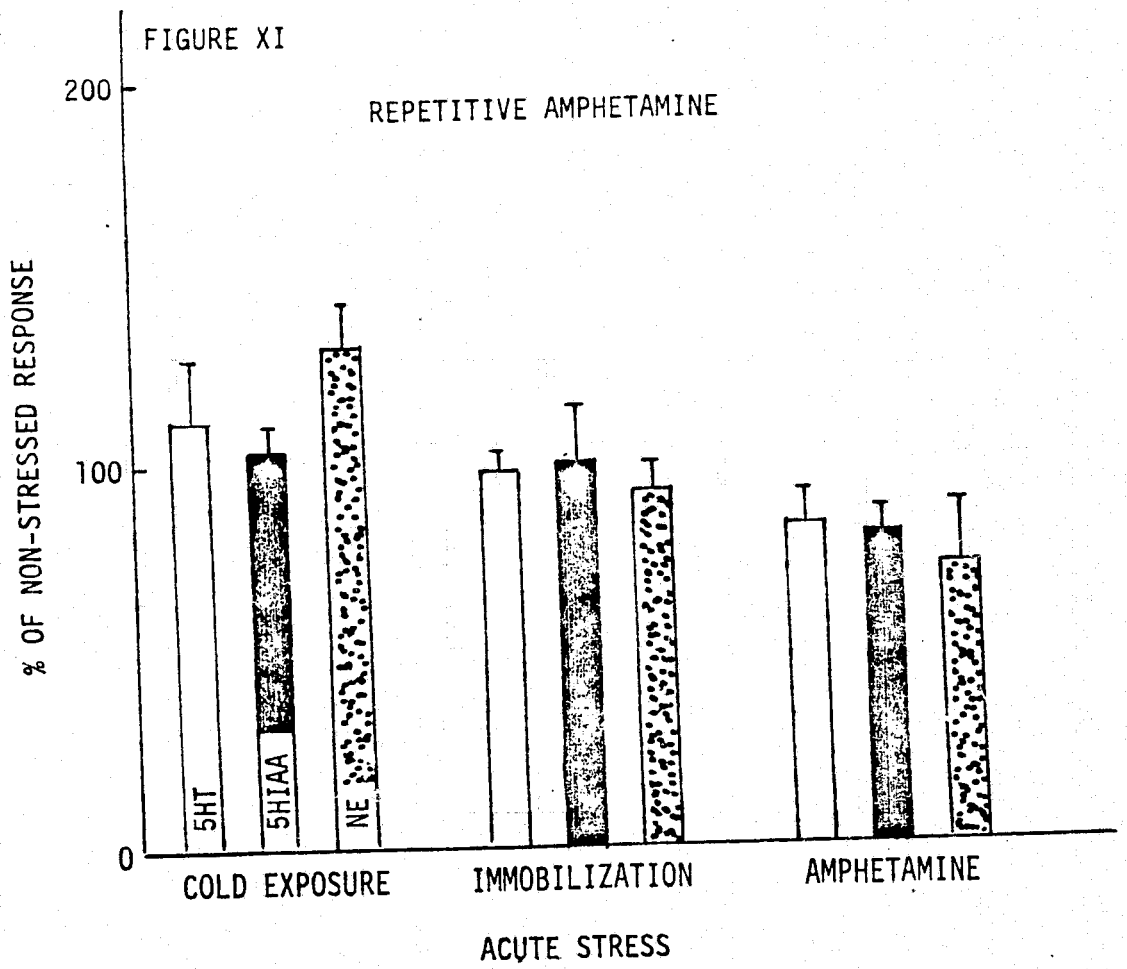


FIGURE XI
REPETITIVE AMPHETAMINE
200
100
0
% OF NON-STRESSED RESPONSE
5HT
5HIAA
NE
COLD EXPOSURE
IMMOBILIZATION
AMPHETAMINE
ACUTE STRESS

Pharmacology Biochemistry and Behavior, Vol. 2, pp. 431–434. ANKHO International Inc., 1974. Printed in the U.S.A.

BRIEF COMMUNICATION

Lack of Tolerance Development to the Dipsogenic Actions of Barbital[1]

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(Received 19 February 1974)

MAICKEL, R. P. AND G. J. MALONEY. *Lack of tolerance development to the dipsogenic actions of barbital.* PHARMAC. BIOCHEM. BEHAV. 2(3) 431–434, 1974. – Chronic dosage of rats with barbital produced a dipsogenic state with overhydration. No tolerance could be seen to this effect in fourteen days; on withdrawal of drugs, the water intake of the rats fell to below normal levels, on a weight basis. When daily intake of fluid was restricted during the period of barbital dosage, overhydration did not occur and drinking during the withdrawal phase was increased.

Barbital Drug induced drinking Tolerance

SCHMIDT and co-workers [11–19] have studied the effects of a variety of barbiturates on water consumption induced in rats by various means. In general, the compounds had a dipsogenic action, a finding corroborated by us for a variety of depressant drugs [7]. In addition, this laboratory recently reported an exhaustive study of factors involved in the dipsogenic actions of barbital [6].

Schmidt, *et al.* [16,18] administered phenobarbital to deprived rats daily for 30 days and measured water intake; no indication of tolerance to the dipsogenic effect of the drug was observed. Upon withdrawal of the drug from these animals, and also upon withdrawal of a group given drug for 14 days, the rats consumed less water than a saline control group for several days. This was later interpreted as the development of a hypersensitivity to the dipsogenic effect after a 15 day phenobarbital treatment and a 10 day withdrawal [19]. As evidence of hypersensitivity the authors cited the fact that the response to the second phenobarbital treatment, that is, after 15 days treatment and 10 days withdrawal, was greater than the initial response to the same dose.

The present paper reports on the effects of chronic daily dosage of barbital on the fluid intake of rats with restricted or unlimited access to water.

MATERIALS AND METHOD

Adult (280–290 g) male, Sprague-Dawley rats (Murphy Breeding Laboratories, Plainfield, Indiana) were used in all experiments. The animals were maintained on tap water and Purina Rat Chow ad lib for at least 1 week after arrival in the laboratory. All solutions for injection were made in glass distilled water such that a dose volume of 0.1 ml per 100 g body weight contained the desired dose; all injections were given intraperitoneally, 15 min prior to placing the animals in the drinking cages.

The procedures used to measure deprivation-induced water consumption were basically those of Gerald and Maickel [5]. Rats were placed in cages, 7 × 7 × 14 in., identical to the home cages of the animals. The cages were suspended in individual compartments of a sound-proofed box with uniform fluorescent lighting. Constant circulation of air by blowers maintained uniform temperature in the compartments; the noise level of blowers served as a white noise. Each cage contained a drinking tube connected to an external 50 ml buret filled with distilled water at the start of each test run and stoppered. As the animal consumed water, the change in volume was measured visually to the nearest 0.1 ml. The front door of each compartment was equipped with an eye-piece lens to permit visual observation of the rats without disturbing their behavioral performance. For at least 1 week prior to testing drug effects, rats were deprived of water daily for 23 hr prior to testing, then placed in the drinking cages and allowed to drink for 1 hr. Food was available ad lib in the home cages but was not available in the drinking cages. In the restricted intake studies, rats were given only a fixed volume of water, 14.0 ml.

Drug trials were started only after the animals demonstrated stable baselines (less than 5% daily variation) of

[1] Supported in part by USPHS Grants MH-18852 and KO2-MH-41083 and by NASA Grant NGL 15-003-117.
[2] Taken in part from a thesis submitted by Greg J. Maloney for the M.S. degree in Pharmacology, Indiana University, 1972.

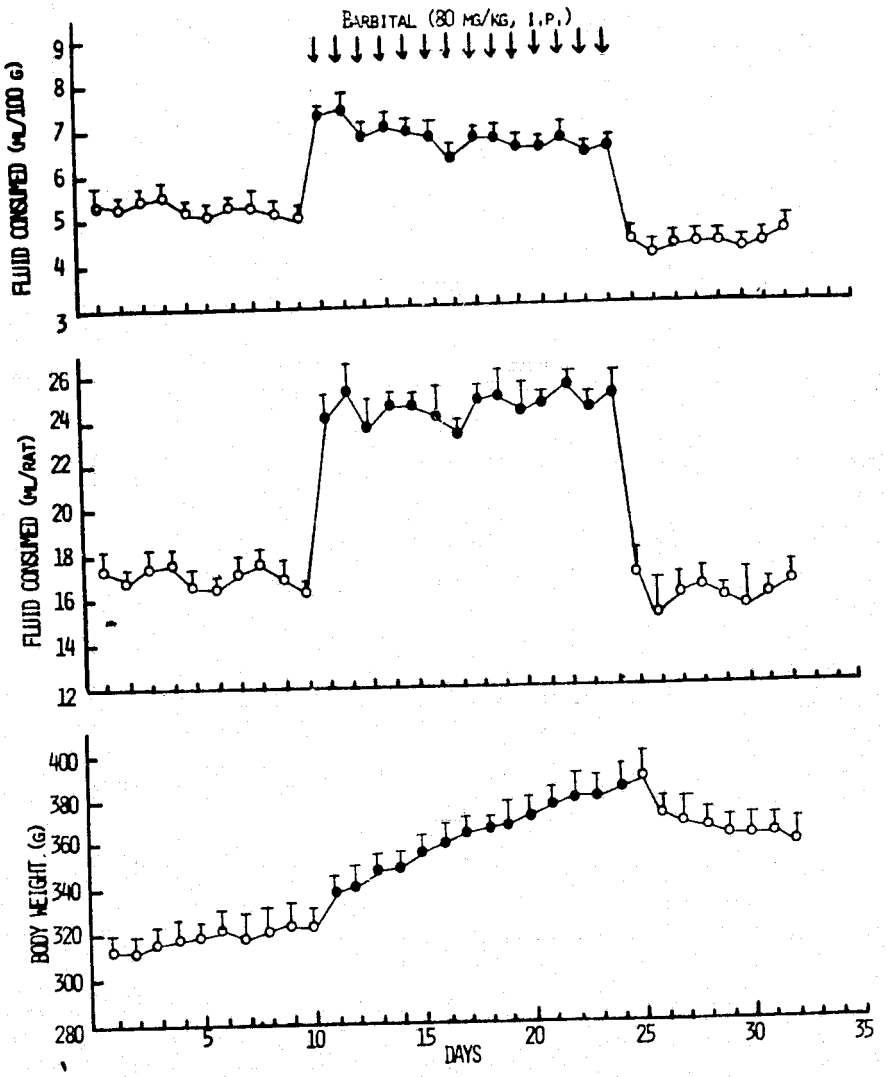

FIG. 1. Effects of barbital on fluid consumption-unrestricted intake. Each symbol is the mean of values obtained from 8 rats as described in Materials and Method. Vertical bars indicate 1 S.D. Solid circles indicate days of dosage with barbital (80 mg/kg, i.p.); open circles are placebo dosage.

water intake. The schedule for drug studies was arranged so that the rats were run daily. Water intake was recorded at 15, 30, and 60 min of the consummatory sessions, the greatest proportion of drinking occurred in the first 15 min in all cases.

Barbital sodium was used in all experiments, dosages are reported as weight of barbital administered in mg/kg body weight. Groups of 8 rats were run in each test system; data are reported as mean ± S.D.

RESULTS

Effect of Chronic Daily Dosage of Barbital on Fluid Consumption of Rats with Unlimited Volume Available

As shown in Fig. 1, chronic barbital (80 mg/kg, i.p.) increased daily fluid consumption above baseline for the duration of drug administration. There is a trend suggesting development of tolerance to the dipsogenic effect over the 14 days of drug treatment, seen when consummatory volume was plotted as ml/100 g body weight. The animals gained an average of 100 g of body weight during the course of this experiment. Substitution of saline for barbital resulted in a decrease in fluid consumption on the second day after drug withdrawal with a return to normal on the third and subsequent days. A weight loss of 18 g was also observed during this withdrawal period. Of particular interest is the fact that administration of a single dose of barbital on the ninth day after drug withdrawal (Day 33) gave a normal dipsogenic response (Table 1).

Effect of Chronic Daily Dosage of Barbital on Fluid Consumption of Rats with Limited Volume Available

Figure 2 shows the effect of limiting fluid intake during the drug treatment period of 14 ml/rat/day. Here, overdrinking and the weight gain during the drug period are

TABLE 1

DIPSOGENIC EFFECTS OF BARBITAL BEFORE AND AFTER CHRONIC TREATMENT

Day of Schedule	Drug	Volume Consumed	
		total ml	ml/100 g
1–10	Saline	16.9 ± 1.0*	5.3 ± 0.4*
11–24	Barbital	24.5 ± 1.7*	6.7 ± 0.6*
25–32	Saline	16.0 ± 1.5*	4.3 ± 0.4*
33	Barbital	23.2 ± 1.8†	6.4 ± 0.7†

Data are presented as volume consumed by 8 animals. Barbital (80 mg/kg, i.p.) was given as described in Materials and Methods.
*mean ± S.D. of 8 animals per day for number of days indicated
†mean ± S.D. of 8 animals on a single day

prevented with the result that now there is no withdrawal decrease in fluid intake nor weight loss. The period of withdrawal shows a significantly greater fluid intake by the animals.

DISCUSSION

From a consideration of the data presented in this paper, it seems likely that the earlier work of Schmidt and coworkers [16, 18, 19] suffers from several defects. First, no recognition was made of the large weight gain during the period of the study. It is possible that the increased intake attributed to hypersensitivity after a period of drug trials [19] may merely reflect the fact that heavier animals consume more fluid. When fluid consumption by our animals is expressed in ml consumed/100 g body weight, the daily intake decreases slightly over the drug period of 14 days. A transient weight loss and decrease in fluid consumption are present during the withdrawal period as previously reported by others [14,15].

Schmidt *et al.* [12,15] also considered the chronic overhydration of the animals during the drug period as inconse-

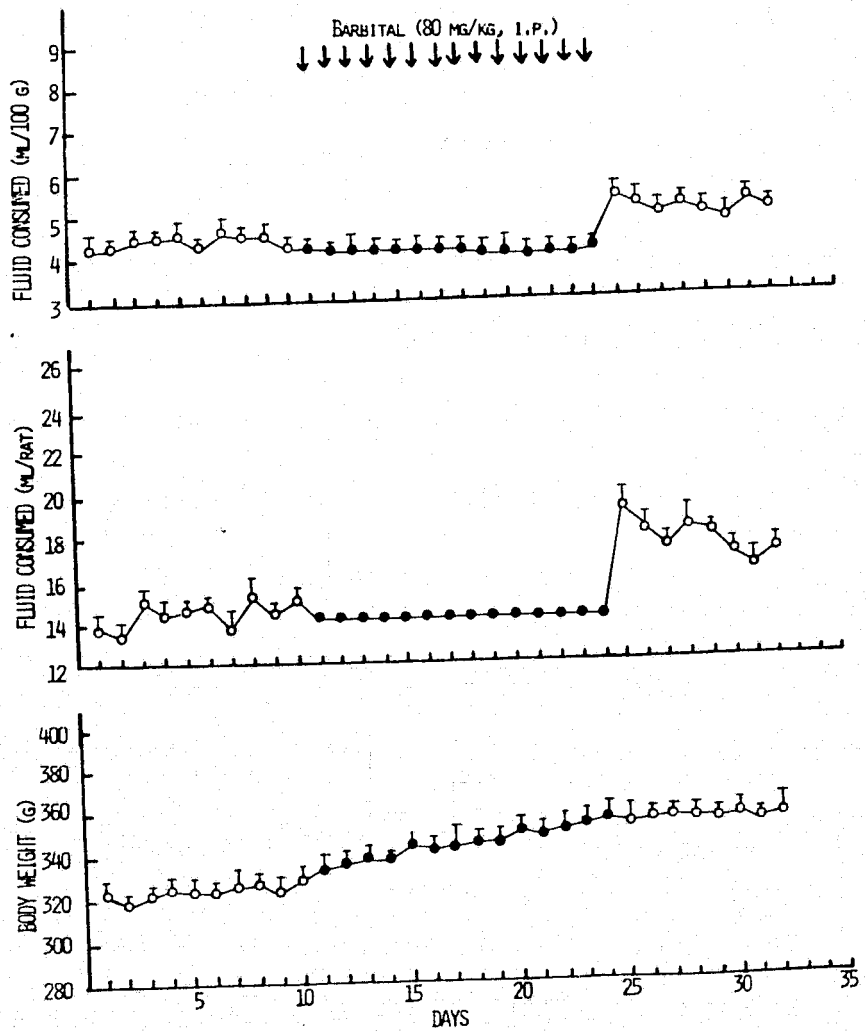

FIG. 2. Effects of barbital on fluid consumption-restricted intake. Each symbol is the mean of values obtained from 8 rats as described in Materials and Method. Vertical bars indicate 1 S.D.; no S.D. is seen for total fluid consumed on Days 11–24 when fluid was restricted. Solid circles indicate days of dosage with barbital (80 mg/kg, i.p.); open circles are placebo dosage.

quential to the characteristics of the withdrawal state. In the present study, when overhydration was prevented, the decrease in fluid consumption and body weight during withdrawal did not occur. The increase in fluid intake over baseline on the first day of withdrawal may reflect the presence of barbital in the animal since the drug is not appreciably metabolized and is excreted slowly [6]. It is unlikely, however, that this drug carryover would explain the increased consumption on subsequent days.

It is reasonable, in light of the changes in neurosecretory material in the neurohypophyseal system during overhydration [1], that prolonged overhydration in itself could lead to a decrease in fluid consumption upon removal of the stimulus for overdrinking. The animal is in a state of positive water balance and will restore his fluid balance by consuming less fluid. The post-drug weight loss appears directly related to the decrease in fluid consumption since the animals did not lose weight if they were prevented from overdrinking prior to withdrawal. The dipsogenic response during the post-drug treatment period by the limited-volume animals may reflect an alteration in fluid balance systems induced by the drug treatment.

A direct central action upon neurons involved in the regulation of water balance may cause the dipsogenic effect by depression of inhibiting centers. Animals with lesions in the septal area and in the anterior hypothalamus show an increased fluid consumption [2,10]. These drug effects could be due to chemical lesions via a depression of the septal area or hypothalamus. Interference with the hypothalamus neurohypophyseal system may also be involved in the dipsogenic action. Barbiturates are known to release ADH from the posterior pituitary [3]. The depression of osmoreceptor cells in the CNS, possibly those near the ADH secreting neurons, may alter an animals preception of his actual state of hydration and cause him to continue drinking longer than usual. The consummatory act itself may become pleasurable if the drug has depressed inhibitory brain areas.

In the rat satiation precedes restoration of the osmotic imbalance and comes about via impulses from nerves in the oral cavity traveling along cranial nerves to brain regions involved in fluid consumption [4]. If drugs were to interfere with the transmission of information as to the nature and amount of the fluid the rat is consuming, the animal might ingest larger amounts or cease earlier depending on the nature of the interference. Evidence for the involvement of the peripheral nervous system [5] suggests that depression of these neurons may interfere with the regulation of fluid intake. Another peripheral action could be drug interaction with the renin-angiotensin system. The release of ADH by barbiturates could also be a manifestation of the mediation of angiotensin since angiotensin itself releases ADH [8].

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DIFFERENTIAL EFFECTS OF d- AND l-AMPHETAMINE ON SPONTANEOUS MOTOR ACTIVITY IN MICE

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ABSTRACT

The effects of single doses of d- and l-amphetamine on motor activity in mice differed both in quantitative and qualitative aspects. At low doses (0.5 mg/kg, i.p.) and at high doses (8.0 mg/kg, i.p.), both isomers were stimulants of SMA, differing only in potency. However, at intermediate doses (2.0, 4.0 mg/kg, i.p.) the l-isomer caused a significant depression of SMA while the d-isomer was stimulatory.

INTRODUCTION

Since the first reports (Alles, 1933) of the stimulant actions of amphetamine, a large volume of literature has accumulated on the differential actions of the d- and l-isomers. While the generally held opinion is that they differ merely in quantitative potency (Goodman and Gilman, 1970), several recent reports support the possibility of qualitative differences as well. For example, Bainbridge (1970) reported that d,l-amphetamine, in doses of 1-5 mg/kg, had a depressant effect on spontaneous motor activity (SMA) in mice, while doses

of 10 mg/kg or greater were stimulatory. Snyder, et al (1970) and Taylor and Snyder (1970) reported differential effects of the d- and l-isomers on norepinephrine and dopamine in brain areas. Phillips and Fibiger (1973) showed that d-amphetamine was seven to ten times more effective than the l-isomer in inducing self-stimulation when electrodes were located in the hypothalamus of rats, while the two isomers were equipotent when electrodes were in the substantia nigra. The present paper compares the effects of the two isomers of SMA in mice, and shows both qualitative and quantitative differences.

MATERIALS AND METHODS

Adult, male Swiss-Webster mice (Murphy Breeding Laboratories, Plainfield, IN) were used in all experiments. Drugs were administered as aqueous solutions by i.p. injection in volumes such that the required dose was contained in 0.1 ml/10 g body weight. All doses were given 5 min prior to placing the animals (3 per cage) in Woodward Actophotometers. Counts were recorded at 10 min intervals for 40 minutes. Statistical comparisons were made by multiple "t" tests.

RESULTS AND DISCUSSION

The data, as presented in Table 1 clearly demonstrate that the actions of the two isomers of amphetamine on SMA are both qualitatively and quantitatively different. Thus, d-amphetamine, at all doses tested and in all time periods, caused a significant increase in SMA when compared to animals given distilled water. The results with l-amphetamine were quite different. At the lowest dose (0.5 mg/kg) and the highest dose (8.0 mg/kg), the l-isomer significantly increased SMA over all time points, although the magnitude of the increase was less than that seen with the corresponding dose of the d-isomer. At a dose of

TABLE 1

Effects of Amphetamine Isomers on SMA of Mice

Drug	Dose mg/kg	N	Counts per Interval + S.D. 0-10 min	11-20 min	21-30 min	31-40 min
Control	-	21	896±147	609± 60	590± 58	348± 42
d-Amphetamine	0.5	4	1232±191[b]	758±113[c]	1007±161[a]	764±129[a]
l-Amphetamine	"	8	1177±104[a]	762± 62[a]	634± 62[d]	456± 55[a]
d-Amphetamine	1.0	4	1387±156[a]	1320±147[a]	1606±152[a]	1470±209[a]
l-Amphetamine	"	6	756±142[g]	617± 81	690± 60[b]	451± 44[a]
d-Amphetamine	2.0	4	1932±192[a]	2350±263[a]	1697±224[a]	1461±157[a]
l-Amphetamine	"	8	550±107[e]	395± 87[e]	464±100[f]	506± 81[e]
d-Amphetamine	4.0	4	1641±284[a]	1852±320[a]	1545±257[a]	1767±232[a]
l-Amphetamine	"	8	739±185[g]	331±109[e]	425±123[f]	480±104[b]
d-Amphetamine	8.0	4	1609±255[a]	1448±210[a]	1440±143[a]	1430±144[a]
l-Amphetamine	"	6	1189±210[c]	1069±125[a]	1340±334[a]	672± 86[a]

Values significantly greater than control: [a] = $p < .001$, [b] = $p < .005$, [c] = $p < .02$, [d] = $p < .05$.

Values significantly less than control: [e] = $p < .001$, [f] = $p < .005$, [g] = $p < .05$.

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1 mg/kg, l-amphetamine caused a significant decrease of activity relative to control in the first 10 minute period; by the 20-30 minute period, this became a stimulatory effect. At doses of 2 and 4 mg/kg, the effects of l-amphetamine were clearly depressant over the first three 10 minute periods. While the 30-40 minute period showed SMA to be increased relative to control mice, the activity did not differ significantly from that seen in the 20-30 minute period with the l-isomer. At a dose of 8 mg/kg, the l-isomer was clearly stimulatory at all time intervals.

From these data it seems evident that the isomers of amphetamine differ in both qualitative and quantitative characteristics. In view of the recent report by Phillips and Fibiger (1973) on differential behavioral potencies of

the isomers, it seems possible that this effect on SMA may reflect actions on differing neurochemical systems, possibly norepinephrine and dopamine (Snyder, et al, 1970; Taylor and Snyder, 1970). Other possibilities must also be considered. For example, Siva Sankar (1970) showed that l-amphetamine was a potent inhibitor of liver alcohol dehydrogenase, while the d-isomer was, at best, a weak inhibitor. In conclusion, the possibilities of pharmacological differences because of isomeric differences in the amphetamines warrants further study.

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DIFFERENTIAL EFFECTS OF MONOAMINE OXIDASE INHIBITORS

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ABSTRACT: Chronic dosage of rats for 20 days with non-toxic doses of different monoamine oxidase inhibitors had clearly differential effects on brain biogenic amines and spontaneous motor activity. No correlation could be seen between behavioral and biochemical effects of the drugs.

INTRODUCTION

Although the enzyme monoamine oxidase [MAO, E.C. 1.4.3.4, monoamine:O_2 oxidoreductase (deaminating)] has been known for almost 50 years, the role(s) it plays in various body functions are still only poorly understood, as evidenced by a recent sympsoium (Costa and Sandler, 1972). The discovery of the antidepressant potency of iproniazid, and its subsequent introduction into clinical medicine in the therapy of depressed states was a major pharmacological development of the 1950's (Pletscher, 1968), leading to the production of a host of drugs acting as monoamine oxidase inhibitors (MAOI).

One of the most perplexing aspects of the MAOI drugs has been an inabiiity to explain their actions on behavioral phenomena in terms of the changes they produce in brain levels of serotonin (5HT) and norepinephrine (NE) (Longo, 1972). In the present paper, we have compared the effects of 20-day chronic dosage to rats of four chemically different MAOI's on brain levels of NE, 5HT, and 5-hydroxyindole-3-acetic acid (5HIAA). In addition, behavioral activity in an open field was sampled throughout the dosage period.

MATERIALS AND METHODS

Experimental subjects were 20 adult, male Sprague-Dawley rats

(Hormone Assay Laboratories, Chicago, IL) weighing 320-350 grams at the start of the experiment, and maintained in single cages on Purina lab chow and tap water ad libitum.

Drugs were administered as solutions in distilled water made to concentrations such that 0.1 ml/100 g. body weight contained the desired dose. Injections were made subcutaneously at the same time (0930-1030 hrs) each day.

Body weights were recorded daily prior to each dosage. Estimation of open-field activity was made in 15 minute periods prior to dosage, using the procedure of Maickel and Johnson (1973). Animals were sacrificed by decapitation on the 21st day, 24 hours after the last dose of MAOI. Brains were removed and stored at -10^{o} C until assayed for NE, 5HT and 5HIAA by the method of Miller et al (1970). All data is reported as mean $\pm$ S.E.M.; statistical comparisons are by the student "t" test.

RESULTS

The doses of drugs were selected for use based on lack of toxicity observed in other studies in this laboratory. Body weight measurements over the course of the experiments show no significant effects, nor did gross observation of the body organs at sacrifice show any obvious pathology.

Effects of the chronic dosage with MAOI's on open field behavioral activity are demonstrated by the data shown in Table 1. Animals treated with isocarboxazid exhibited significantly greater activity than control animals from the 6th day of treatment until the end of the experiment. Pargyline-treated animals showed elevated activity on the 6th and 9th days of dosage only, while the animals treated

with SU-11,739 were unaffected. Tranylcypromine-treated rats showed a generally decreased activity as compared to controls, significantly lower only on the 9th and 18th days.

Table 1. Behavioral and Biochemical Effects of Chronic Dosage of MAOI's to Rats.

Open field activity measured as described in Materials and Methods. Animals were tested on days 3, 6, 9, 12, 15, 18 prior to daily dosage, and killed 24 hours after the last (20th) dose of drug. Values are mean ± S.E.M. of values for 4 animals.

Test Day	Control	Isocarboxazid	Pargyline	Su-11,739	Tranylcypromine
3	106± 7	80±14	105± 4	103± 6	88±14
6	79± 4	110±14[a]	129±10[a]	95±18	86±10
9	77± 4	106± 9[a]	128±15[a]	77± 5	59± 2[b]
12	62± 8	97±12[a]	69± 5	72± 5	55± 9
15	66± 8	103± 5[a]	66±10	69± 4	54± 4
18	65±10	98± 9[a]	66± 6	73± 4	47± 3[b]
Brain					
NE	0.52±.02	0.71±.04[a]	0.95±.04[a]	1.04±.09[a]	0.95±.03[a]
5HT	0.63±.03	1.03±.06[a]	1.17±.13[a]	1.77±.18[a]	1.86±.09[a]
5HIAA	0.62±.04	0.62±.03	0.56±.09	0.36±.02[b]	0.30±.02[b]

[a] = Significant elevation ($p < .05$) compared to control.
[b] = Significant depression ($p < .05$) compared to control.

The brain levels of NE, 5HT and 5HIAA on the 21st day are also shown in Table 1. Isocarboxazid and pargyline both elevated brain NE and 5HT significantly with decrease in 5HIAA. In contrast, both Su-11,739 and tranylcypromine not only elevated brain levels of NE and 5HT but also significantly reduced the levels of 5HIAA. Of particular interest is the observation that an increase of brain 5HT of 60-90% (as produced by isocarboxazid and pargyline) is not reflected in a significant decrease in 5HIAA, while increases of brain 5HT of 180-200% (as produced by Su-11,739 and tranylcypromine) are accompanied by reductions of 40-50% in 5HIAA levels.

DISCUSSION

The behavioral effects of the drugs were quite dissimilar and

could not be correlated to changes in brain amines. The effects of isocarboxazid and pargyline on 5HT and 5HIAA were not significantly different; however, pargyline-treated animals had a significantly higher level of NE. While the increases in NE produced by pargyline, Su-11,739 and tranylcypromine were virtually identical, the latter two compounds had a markedly greater effect on 5HT. In fact, while isocarboxazid and pargyline did cause a significant elevation of brain 5HT, neither compound showed an associated depression of 5HIAA, as did Su-11,739 and tranylcypromine. Even though the inhibition of MAO was only sufficient to reduce 5HIAA levels by 50% or less, elevations of as much as 200% in 5HT levels were observed.

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TASTE PHENOMENA INFLUENCES ON STIMULATION OF DEPRIVATION-INDUCED FLUID CONSUMPTION OF RATS*

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Summary—The dipsogenic actions of barbital and chlordiazepoxide are selectively influenced by the taste of the consummatory fluid presented to deprived rats. The potency of barbital as a consummatory stimulant is reduced relative to distilled water or tartaric acid by the use of a pleasant tasting fluid, saccharin. The potency of chlordiazepoxide as a dipsogenic agent was reduced by the use of either tartaric acid or saccharin as a consummatory fluid as compared to water. Promazine depressed deprivation-induced fluid consumption with a similar potency regardless of the consummatory fluid used.

A previous paper from this laboratory (MAICKEL and WEBB, 1972) described the influence of consummatory fluid taste on the reduction of fluid consumption by deprived rats. Both (+)-amphetamine and methapyrilene were less effective in reducing consumption of a pleasant-tasting fluid (saccharin) and an unpleasant tasting fluid (tartaric acid), compared with the reduction in consumption of "neutral" tasting distilled water. In contrast, atropine was more effective against the pleasant taste and less effective against the unpleasant taste when compared to distilled water. In all cases the drugs had a similar action, that is, a reduction in the volume of fluid consumed as compared to baseline consumption, regardless of the taste of the consummatory fluid.

More recently, this laboratory reported on the effects of a variety of depressant drugs on deprivation-induced fluid consumption (MAICKEL and MALONEY, 1973). In contrast to the earlier studies with cholinergic agents (GERALD and MAICKEL, 1969), antihistamines (GERALD and MAICKEL, 1972), amphetamines (MAICKEL, COX, KSIR, SNODGRASS and MILLER, 1970), and phenothiazine tranquilizers (MAICKEL, GERALD, WARBURTON and MAHJU, 1968), the depressant drugs stimulated consumption of water by deprived rats. The present paper describes studies of the actions of three depressant drugs; barbital, chlordiazepoxide, and promazine, as influenced by the taste of the consummatory fluid.

MATERIALS AND METHODS

Adult male Sprague–Dawley rats (300–350 g) (Murphy Breeding Laboratories, Plainfield, Indiana) were used in all experiments. The animals were maintained on tap water and Purina Rat Chow *ad lib.* for at least 1 week after arrival in the laboratory. All solutions for injection were made up in glass distilled water such that a dosage volume of 0·1 ml

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† Taken in part from a thesis submitted by G. J. Maloney for the M.S. Degree in Pharmacology, Indiana University (1973).

per 100 g body weight contained the desired dose: all injections were given intraperitoneally, 15 min prior to placing the animals in the drinking cages.

Fluid consumption was measured basically as described by GERALD and MAICKEL (1969). Rats were placed in cages, 17·6 × 17·6 × 35·2 cm, identical to their home cages. The test cages were suspended in individual compartments of a sound-proofed box with uniform fluorescent lighting. Constant circulation of air by blowers maintained uniform temperature in the compartments; the noise output of the blowers served as uniform white noise. Each cage contained a drinking tube connected to an external 50 ml burette filled with the appropriate fluid at the start of each run. As the animal consumed the fluid, the change in volume was recorded by visual observation to the nearest 0·1 ml. The front door of each compartment was equipped with an eyepiece lens to permit visual observation of the rats without disturbing their behavioural performance. Rats were deprived of water for 23 hr prior to testing and then placed on the drinking cages and allowed to drink for 1 hr. Food was available *ad lib.* in the home cages, but was not available in the drinking cages.

Drug trials were started only after the animals demonstrated stable baselines (less than 5% daily variation) of water intake. The schedule for drug studies was arranged so that the rats were run daily, with drugs administered every 4th day and placebo doses on the intervening days. Fluid intake was recorded at 15, 30 and 60 min of the consummatory sessions; the greatest proportion of drinking occurred in the first 15 min in all cases.

Consummatory test fluids were distilled water or solutions of saccharin (0·2%), or tartaric acid (0·5%) in distilled water. Three groups of eight rats were run first on distilled water, and all three drugs were tested. When a baseline had been re-established for each group on one of the other test fluids, the effects of all three drugs were tested on that consummatory system. For each drug, $n = 6$–8; an n of less than the maximum value indicates a malfunction of the apparatus or sickness or death of an animal. All results are expressed as the mean ± S.D. of the volume consumed in the drug test session divided by the volume consumed on the pre-drug day × 100, i.e. the percent consumption. Thus, each animal served as its own control.

Dose–response curves were fitted for each drug on the test fluids by the method of "least squares". The slopes of the lines were determined by linear regression and the value of the RD_{25} was obtained. This value is an estimate of the dose of a particular drug which would be required to produce a 25% change in fluid intake during the test session. Values for slopes and RD_{25} values and statistical comparisons were determined as described by COX (1970).

RESULTS

Effects of consummatory fluid taste on barbital action

The data in Table 1 are those obtained when dose–response studies of the dipsogenic actions of barbital were performed using water, saccharin, or tartaric acid. All three doses of drug exerted a significant dipsogenic effect, regardless of the consummatory fluid. The RD_{25} values for barbital were similar with values for water or tartaric acid, but were significantly higher ($P < 0.05$) for saccharin. The slopes of the log dose–response curves for water and tartaric acid were similar; that for saccharin was significantly lower ($P < 0.05$).

Effect of consummatory fluid taste on chlordiazepoxide action

In this test system, chlordiazepoxide, like barbital, also had significant dipsogenic action at all doses tested. The data in Table 2 show that chlordiazepoxide was most effective as

Table 1. Effect of taste phenomena on barbital effects on deprivation-induced fluid consumption

Fluid consumed	*n*	Barbital (mg/kg, i.p.)	Pre-drug (ml ± S.D.)	Drug (ml ± S.D.)	Percentage of Pre-drug	RD_{25} (mg/kg)	Slope
Water	8	20	15·5 ± 1·5	18·5 ± 3·2	119 ± 12·2*		
	8	40	17·7 ± 2·1	23·0 ± 2·6	130 ± 8·4*	27·3	+78·1
	7	80	14·6 ± 2·7	22·7 ± 1·5	166 ± 12·8*		
Tartaric acid (0·5%)	8	20	11·4 ± 2·2	14·0 ± 1·7	125 ± 18·0*		
	8	40	11·4 ± 2·3	15·1 ± 2·8	136 ± 33·4*	24·1	+63·1
	7	80	10·2 ± 0·83	16·5 ± 2·3	163 ± 32·3*		
Saccharin (0·2%)	8	20	23·7 ± 3·6	28·5 ± 3·1	120 ± 9·9*		
	8	40	23·9 ± 3·7	30·0 ± 5·3	126 ± 13·1*	35·6	+23·3
	7	80	21·8 ± 3·1	27·7 ± 6·3	134 ± 28·5*		

Animals were treated as described in Methods section: drug was administered 15 min prior to start of drinking session. Results are expressed as mean ± S.D. of the values obtained. Statistical comparisons were made by the correlated *t*-test for paired scores; significant differences from the corresponding pre-drug values ($P < 0·05$) are indicated by*.

Table 2. Effect of taste phenomena on chlordiazepoxide effects on deprivation-induced fluid consumption

Fluid consumed	*n*	Chlordiazepoxide (mg/kg, i.p.)	Pre-drug (ml ± S.D.)	Drug (ml ± S.D.)	Percentage of Pre-drug	RD_{25} (mg/kg)	Slope
Water	8	3·75	14·0 ± 2·4	18·2 ± 4·0	130 ± 18·4*		
	8	7·5	14·8 ± 2·0	21·5 ± 2·1	145 ± 25·8*	2·6	+38·2
	8	15·0	15·3 ± 1·8	23·4 ± 1·8	153 ± 21·1*		
Tartaric acid (0·5%)	8	3·75	10·0 ± 2·5	12·0 ± 2·3	120 ± 17·4*		
	8	7·5	10·4 ± 1·5	13·3 ± 2·0	128 ± 24·1*	5·5	+33·2
	8	15·0	10·3 ± 1·8	14·4 ± 3·0	140 ± 13·5*		
Saccharin (0·2%)	8	3·75	21·8 ± 2·6	26·8 ± 3·2	124 ± 18·0*		
	8	7·5	21·9 ± 5·6	29·6 ± 6·2	145 ± 14·9*	4·7	+91·4
	8	15·0	22·9 ± 2·8	40·6 ± 5·7	179 ± 30·1*		

Animals were treated as described in Methods section: drug was administered 15 min prior to start of drinking session. Results are expressed as mean ± S.D. for the values obtained. Statistical comparisons were made by the correlated *t*-test for paired scores; significant differences from the corresponding pre-drug values ($P < 0·05$) are indicated by*.

Table 3. Effect of taste phenomena on promazine effects on deprivation-induced fluid consumption

Fluid consumed	*n*	Promazine (mg/kg, i.p.)	Pre-drug (ml ± S.D.)	Drug (ml ± S.D.)	Percentage of Pre-drug	RD_{25} (mg/kg)	Slope
Water	8	2·5	19·2 ± 2·6	17·0 ± 2·3	89 ± 8·5		
	8	5·0	19·2 ± 2·2	13·4 ± 2·6	74 ± 7·0*	4·7	−48·2
	8	10·0	16·7 ± 2·2	10·1 ± 3·6	60 ± 9·8*		
Tartaric acid (0·5%)	8	2·5	11·4 ± 2·0	10·0 ± 2·8	86 ± 14·4		
	8	5·0	11·7 ± 1·2	9·0 ± 1·6	77 ± 14·1*	5·0	−39·9
	8	10·0	12·4 ± 2·2	7·8 ± 1·4	64 ± 8·6*		
Saccharin (0·2%)	8	2·5	24·2 ± 4·1	22·5 ± 4·6	93 ± 10·1		
	8	5·0	24·4 ± 4·5	20·7 ± 5·4	85 ± 8·6*	6·4	−40·7
	8	10·0	23·1 ± 2·8	14·5 ± 6·7	62 ± 2·5*		

Animals were treated as described in Methods section; drug was administered 15 min prior to start of drinking session. Results are expressed as mean ± S.D. of the values obtained. Statistical comparisons were made by the correlated *t*-test for paired scores; significant differences from the corresponding pre-drug values ($P < 0·05$) are indicated by*.

a stimulus for water and considerably less effective for solutions of tartaric acid and saccharin. The RD_{25} values for tartaric acid and saccharin do not differ significantly from each other, but both are significantly different from that of water. As with barbital, the slopes of the dose–response curves were parallel for water and tartaric acid; saccharin solutions were significantly different ($P < 0.05$).

Effect of consummatory fluid taste on promazine action

In the case of promazine, the drug depressed deprivation-induced fluid consumption, regardless of the fluid offered (Table 3). The efficacy for all three consummatory fluids is basically similar. Slopes of the dose–response curves are parallel, and RD_{25} values are not significantly different for all three fluids.

DISCUSSION

A number of previous reports from this laboratory have indicated that a variety of drugs depress deprivation-induced fluid consumption by rats (MAICKEL *et al.*, 1968; GERALD and MAICKEL, 1969; MAICKEL, *et al.*, 1970; GERALD and MAICKEL, 1972). More recently, we have shown that a variety of sedative, hypnotic and anxiolytic drugs have an opposite effect, i.e. they stimulate fluid consumption by deprived rats (MAICKEL and MALONEY, 1973). In addition, a previous report also described the effect of taste phenomena on the action of several drugs that depress deprivation-induced fluid consumption.

In the present report, the effects of three drugs are examined for their ability to alter the deprivation-induced consumption of distilled water, 0·5% tartaric acid or 0·2% saccharin. These three consummatory fluids represent a range of tastes from pleasant (saccharin) through neutral (distilled water) to unpleasant (tartaric acid); the volume consumed by deprived rats is increased about 42% (relative to water) by the use of saccharin, and decreased about 33% (relative to water) by the use of tartaric acid. The drugs chosen for this study also represent a diversity of pharmacological activities ranging from barbital, a long acting sedative, through chlordiazepoxide, an anxiolytic agent, to promazine, a mild antipsychotic.

The results obtained with the various drugs on deprivation-induced consumption of distilled water are similar to those previously reported, i.e. barbital and chlordiazepoxide stimulate consummatory behaviour, while promazine depresses it. When barbital was tested with other consummatory fluids, the results (see Table 1) demonstrated that the effects of the drug were influenced by pleasant taste. Thus, the slopes of the dose–response curves for barbital with water and tartaric acid solution was similar; the doses required to elicit at 25% increase in volume consumed were virtually identical. By contrast, when the pleasant tasting saccharin solution was used as a consummatory fluid, the dose–response curve was much shallower, and consequently the RD_{25} dose was higher, suggesting that the stimulatory action of the drug was less effective.

In the case of chlordiazepoxide, the dose–response curves for water and tartaric acid were again parallel, although the RD_{25} dose required for tartaric acid was more than double that required for water (Table 2). However, when the saccharin solution was used, the slope of the dose–response curve was much steeper, although the RD_{25} dose fell between those for water and tartaric acid. In the case of promazine, all three consummatory fluids behaved in a similar manner, with dose–response slopes and RD_{25} values not differing from each other.

In view of the previous report from this laboratory on taste phenomena (MAICKEL and WEBB, 1972), the present work adds a further dimension emphasizing the importance of standardizing conditions when using behavioural test systems involving fluid consumption or the thirst drive. While these drug effects do not delineate gustatory phenomena as opposed to drug effects on the thirst drive or on satiety centre, they certainly demonstrate the necessity for uniformity in test systems. GROSSMAN (1967) reported a number of divergent conclusions on thirst-associated phenomena or consummatory behaviour: a variety of consummatory fluids of varying tastes were used.

Of particular interest is the observation (Table 2) that chlordiazepoxide works best when the consummatory fluid is the pleasant-tasting saccharin solution. This must be considered in discussing the punishment attenuation hypothesis proposed for screening anxiolytic agents (GELLER and SEIFTER, 1960, 1962; GELLER, HUKAK and SEIFTER, 1962). In view of the dipsogenic action of anxiolytic agents (MAICKEL and MALONEY, 1973), and this stimulatory effect of chlordiazepoxide on a pleasant tasting fluid, the possibility of a simple override of the punishment must be considered.

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COMPARATIVE PHYSIOLOGICAL DISPOSITION OF MELATONIN AND ITS BENZO[b]THIOPHENE ANALOG IN THE RAT[1,2]

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SUMMARY

Melatonin and its benzo[b]thiophene analog were labeled by acetylation of the corresponding 5-methoxyarylethylamines with ^{3}H-acetic anhydride. The benzo[b]thiophene analog had a much higher lipid solubility. When administered to rats, both compounds disappeared from plasma and tissues by first-order decay. The dispositions were similar, with the higher lipid solubility of the benzo[b]thiophene analog resulting in higher tissue: plasma ratios, especially in adipose tissue, kidney and liver, and longer half-lives in plasma and tissues.

Melatonin (N-acetyl-5-methoxytryptamine, MLT) was first identified by Lerner and collaborators in 1959 (1). It has been found in the pineal glands of a variety of mammalian and avian species (2) and in the peripheral nerves and urine of humans (3). More recently, several papers have reported the fate of exogenously administered MLT in cats (4) and rats(5). In the present paper, we report on the comparative physiological disposition of MLT and its benzo[b]thiophene analog (5-methoxy-3-(β-acetaminoethyl)benzo-[b]thiophene, MLT-S) in rats.

MATERIALS AND METHODS

MLT and MLT-S were prepared from their respective 5-methoxyarylethylamines

[1] A preliminary report has been presented. The Pharmacologist 15:257 (1973).

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by reaction with ^{3}H-acetic anhydride as described by Campaigne and Dinner (6); the specific activities obtained were 4.1 mCi/mmole. The compounds were administered by i.v. injection in aqueous solution (0.5 ml/rat, containing 50 uCi of ^{3}H and 12 umoles of compound) to 16 adult, male, Sprague-Dawley rats weighing 300 grams. Four animals were sacrificed at 0.25, 0.5, 1.0 and 2.0 hrs. after dosage, and blood and tissues were collected and frozen until analysis.

^{3}H-MLT and ^{3}H-MLT-S were determined by a modification of the procedure described by Kopin, et al (4). Tissues were homogenized in 3 volumes of 0.05 N NaOH; plasma was diluted 1:1 with 0.05 N NaOH. Homogenate or diluted plasma (2.0 ml) were added to 50 ml glass-stoppered centrifuge tubes containing 15 ml of reagent grade toluene. The tubes were stoppered, shaken for 5 min. then centrifuged for 10 min. The aqueous layer was removed and discarded, and duplicate 5.0 ml aliquots of the toluene were removed and added to scintillation vials containing 7 ml of toluene, 8 ml of ethylene glycol monomethyl ether, 1.6 g of naphthalene and 80 mg of BBOT. The samples were counted in a Packard TriCarb Model 4322 Liquid Scintillation System. Results were converted to ng/g tissue. Decay curve slopes were determined by linear regression.

Randomly selected aliquots of the final toluene phase from various tissues and times were evaporated to dryness, the residues were taken up in a small volume of methanol and applied to Analtech Silica Gel G precoated plates. One-dimensional TLC was carried out using the solvent system B as reported by Bosin and Wehler (7). Radiochromatogram scanning of the plates, using a Packard Model 7200 Scanner, revealed that > 85% of the radioactivity applied to the plate resided in a single spot corresponding in R_f to authentic MLT or MLT-S. No other peaks of radioactivity were observed that were significantly greater than the background level of noise.

TABLE 1

Time Course of Disposition of MLT and MLT-S in Rat Tissues

Tissue	Compound	ng Compound/g Tissue±S.D. 0.25 hr	0.5 hr	1.0 hr	2.0 hr	$t_{1/2}$ min.
Plasma	MLT	1806± 120	746±121	82± 14	6.9± 2.8	12
	MLT-S	1650± 223	731±152	228± 39	21 ± 6.4	18
Adrenal	MLT	2036± 123	1096±258	294± 42	33 ±10	18
	MLT-S	3970± 579	1911±293	803±159	81 ±15	20
Brain	MLT	1311± 200	707± 82	104± 20	8.2± 2.6	14
	MLT-S	2467± 218	927±194	348± 90	34 ± 9.1	16
Fat	MLT	613± 106	334± 97	37± 16	2.1± 0.5	13
	MLT-S	1988± 354	1713±318	764±116	158 ±16	29
Heart	MLT	1840± 136	856±102	113± 18	6.9± 2.3	13
	MLT-S	2671± 490	1156±260	484± 97	44 ± 9.7	17
Intestine (Duodenum)	MLT	2064± 300	1194±205	285± 77	16 ± 5.0	15
	MLT-S	2140± 281	1167±267	628±118	166 ±29	20
Kidney	MLT	3606± 230	2281±390	245± 41	20 ± 5.8	14
	MLT-S	11694±1713	5168±762	2566±440	240 ±76	19
Liver	MLT	2270± 332	1272±208	192± 47	18 ± 4.6	16
	MLT-S	6313± 524	2878±367	1097±184	196 ±48	35
Lung	MLT	1388± 262	847±125	145± 11	33 ± 5.1	18
	MLT-S	2736± 361	1201±135	505±108	78 ±16	21
Muscle	MLT	2212± 241	788±155	112± 12	5.5± 2.1	13
	MLT-S	1543± 229	750±132	229± 37	25 ± 3.5	19
Spleen	MLT	1286± 190	770±241	82± 16	7.2± 3.5	14
	MLT-S	1350± 153	662±127	317± 50	26 ± 7.8	19
Testes	MLT	1687± 179	1230± 32	321± 17	45 ± 5.6	15
	MLT-S	2101± 379	1487±294	558± 35	73 ±19	22

RESULTS AND DISCUSSION

The data obtained for the tissues examined are presented in Table 1. Of particular interest is the fact that the tissue levels achieved by the benzo[b]thiophene analog at 15 minutes after dosage are higher for every tissue except muscle; this is reflected in a lower plasma level at that time. Kidney, liver and fat show the highest MLT-S/MLT ratios at 15 minutes. The half-life values are longer for MLT-S than for MLT in plasma and all tissues. Statistical comparison of the slopes of the decay curves for MLT and MLT-S indicate that fat, liver, muscle and plasma are different for the two analog.

These results suggest that both the endogenously occurring indolic compound and the foreign benzo[b]thiophene analog are handled in a similar manner by the body, the differences presumably being due to the higher lipid solubility (benzene: pH 7.4 distribution values were MLT = 8.2, MLT-S = 610) of the MLT-S. Ruffin, et al (8) have shown that MLT can be bioassayed by its ability to produce distinctive night coloration in the pencil fish, Nannostomus beckfordi anomalus. More recently, Reed (9) found that MLT-S was about one-tenth as potent as MLT in this test system, although both compounds had a similar toxicity. The biological activities of the two analogs have not yet been examined in mammals.

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PHARMACOLOGICAL AND TOXICOLOGICAL STUDIES ON 1,4-BUTANEDIOL[1,2]

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ABSTRACT

1,4-Butanediol had a significant sedative effect on rats at i.p. dosages >300 mg/kg. The LD_{50} was 1328 mg/kg. Effects of the drug on spontaneous motor activity was biphasic; doses of 50-200 mg/kg significantly reduced activity, while doses of 300-400 mg/kg produced loss of righting reflex. No increase in liver triglycerides was seen even at doses of 1000 mg/kg/day for 14 days.

INTRODUCTION

The compound 1,4-butanediol (1,4-BD) has numerous uses, for example, in the manufacture of polyurethanes and polyvinylpyrrolidone (Freifeld and Hart, 1966) as a curing agent in vulcanization (Athey, 1959), as a plasticizer

[1] A preliminary report of this material has been presented to the Society of Toxicology (Toxicol. Appl. Pharmacol. 25:461, 1973).

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for thermoplastic polymers (Farbenindustrie, 1943) as a softener and moistener for gelatin, cellophane and speciality papers (Aniline, 1964), as a mold-inhibiting humectant in tobacco products (Drucker, 1962), a component of cigarette filters (Toney, 1969), and in the preparation of flavoring and perfume solutions (Levinson, 1968) and suppositories (Kawamura et. al., 1968). In fact, 1,4-BD has such widespread use that it has been considered by Knyshova as an environmental pollutant (1968).

Despite this widespread usage, the literature contains little information on the pharmacology and toxicology of 1,4-BD. Sprince et. al. (1966) have reported the i.p. LD_{50} in rats to be 1.00 g/kg, while Rowe (1963) reported a p.o. LD_{50} in rats of 1.78 g/kg. Hinricks et. al. (1948) reported toxic effects of 1,4-BD in man, but Sprince et. al. (1966) suggest that this may have been due to a contaminant, 2-butyne-1,4-diol, the raw material used in the manufacture of 1,4-BD.

1,4-BD has been demonstrated to have a significant depressant action on the central nervous system, presumably through the action of a metabolite, α-hydroxybutyric acid (GHB) (Maxwell and Roth, 1972; Roth and Gairman, 1968); both the parent compound and GHB have effects on the rat EEG (Sprince et. al., 1966). Guidotti and Ballotti (1970) have reported that loss of righting reflex correlated better with brain concentrations of GHB than 1,4-BD.

The present paper reports on some preliminary pharmacological data obtained with 1,4-BD in rats. The results indicate that 1,4-BD is a unique sedative drug, quite dissimilar to ethanol.

MATERIALS AND METHODS

Adult, male Sprague-Dawley rats (300-350 g) were obtained from Murphy Breeding Laboratories, Plainfield, Indiana. Animals were maintained for 7-10 days prior to experimental use on an ad lib diet of Purina Lab Blox and

tap water.

1,4-BD (Research Grade) was purchased from Matheson Coleman and Bell and administered by i.p. injection as the pure compound or as solutions in distilled water such that the volume of injection was 0.1 ml per 100 g body weight.

Spontaneous motor activity was measured in Woodard Actophotometers. Liver triglycerides were determined using Harleco kits.

RESULTS

Preliminary results in the rat indicated the i.p. LD_{50} to be 1328 mg/kg, a value somewhat higher than that reported by Sprince et. al. (1966). No deaths were seen at doses below 1000 mg; doses of 1600 mg/kg or greater were an LD_{100}.

The loss of righting reflex after a single dose was dose dependent, as shown by the data in Table 1.

TABLE 1

Loss of Righting Reflex in Rats Given Various Doses of 1,4-BD

Dose mg/kg,i.p.	N	Duration of L.R.R. min±S.E.M.
250	8	0
500	12	105± 4.6
1000	12	318±21.8

While no loss of righting reflex was observed at 250 mg/kg, a significant effect was seen on rotorod performance at doses as low as 200 mg/kg (Table 2).

TABLE 2

Rotorod Performance in Rats Given Various Doses of 1,4-BD

Dose mg/kg, i.p.	N	Failures %
50	15	37.4
100	11	36.8
200	11	55.4
300	6	100.0
400	6	100.0

Similarly, significant effects were seen on spontaneous motor activity at doses as low as 50 mg/kg, i.p., although the dose-response curve was clearly biphasic as shown in Figure 1, with virtually a complete abolition of activity at doses of 300 or 400 mg/kg.

When rats were dosed with 1,4-BD daily for periods up to 14 days, a peculiar adaptation to the effects of the drug were observed (Table 3). The duration of loss of righting reflex was significantly lower with the fourth dose; further dosing did not lower the duration of action at all.

Measurement of liver triglycerides in the rats treated with daily doses of 1,4-BD as described in Table 3 indicated no significant change from control levels, a sharp contrast to the actions of ethanol under a similar dosage regimen. In addition, the effects of 1,4-BD, in combination with pentobarbital or chlorpromazine, on loss of righting reflex, are merely additive.

FIGURE 1

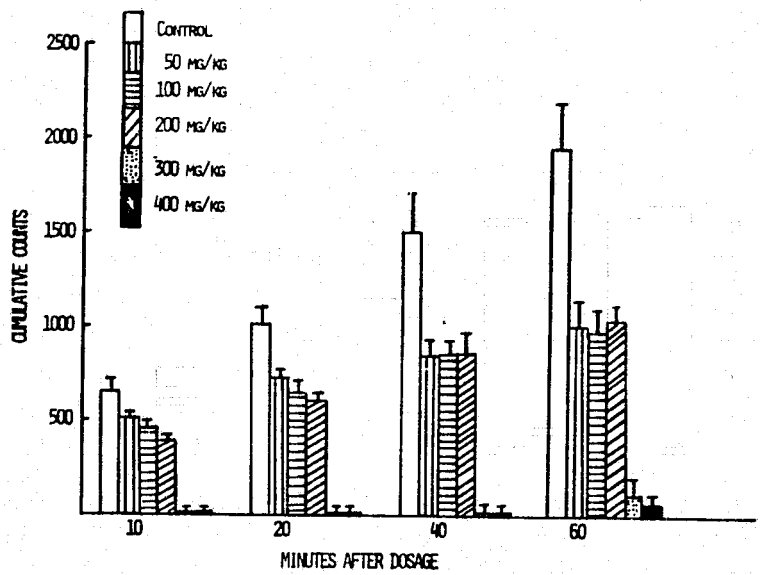

FIGURE 1: Effects of 1,4-Butanediol on Spontaneous Motor Activity of Rats. Each value is the mean of 6-8 rats; vertical bars indicate ± S.D.

TABLE 3

Variation in Duration of 1,4-BD Narcosis with Repeated Dosage

Day # of doses	Duration of Loss of Righting Reflex: 500 mg/kg, i.p. min±S.E.M.	(N)	1000 mg/kg, i.p. min±S.E.M.	(N)
1	105± 4.6	(12)	318±21.8	(12)
4	78±10.3	(7)*	191± 4.5	(8)*
7	87± 5.6	(6)*	210± 6.0	(5)*
14	79± 5.8	(3)*	189± 8.5	(2)*

* Significantly different from day 1 ($p < 0.05$)

DISCUSSION

Although 1,4-BD appears structurally to be a dimer of ethanol, the only pharmacological similarity between the two compounds appears to be in their depressant potency. 1,4-BD is active at doses of >50 mg/kg, i.p.; the activity depends on the parameter being measured. Thus, a reduction in spontaneous motor activity is seen at 50 mg/kg; retorod performance is imparied at 200 mg/kg; and loss of righting reflex occurs at 250 mg/kg. The dose-response data for rats treated with 1,4-BD is most intriguing, reflecting a biphasic type of response (Figure 1). At doses of 50, 100 or 200 mg/kg, 1.4-BD causes a significant decrement in activity; at doses of 300 or 400 mg/kg, activity levels are zero since the animals lose their righting reflex.

Another interesting phenomena was observed when rats were given daily doses of 1,4-BD for 14 days. Under these conditions, the duration of loss of righting reflex was significantly reduced by the 4th dose, although no further reductions in effect were observed over the remainder of the dosage period. In this regard, it was of interest that no significant elevations of liver triglycerides were observed over this period of treatment, a striking contrast to many reports on the actions of ethanol on liver lipids. In this regard, it is also interesting that the effects of combined dosages of 1,4-BD with pentobarbital or chlorpromazine have a merely additive effect on loss of righting reflex. Thus, it seems that the CNS actions of 1,4-BD may be sufficiently unique to justify further examination as a potential pharmacological agent. In this regard, the studies of Sprince et. al. (1966) and Roth and Gairman (1968) indicating that the CNS depressant effects of 1,4-BD may be mediated by its conversion to α-hydroxybutyric acid are most interesting. Certainly, the Therapeutic Index in rats as a potential anesthetic (LD_{50}/ED_{50} for loss of right reflex >60 minutes) is intriguing since it appears to be <4.0.

In combination with the demonstrated lack of hepatotoxicity, even with chronic dosage, this possibility seems to be worthy of further study.

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Intestinal transport of tryptophan and its analogs

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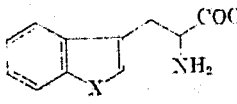
BOSIN, TALMAGE R., DAVID R. HATHAWAY, AND ROGER P. MAICKEL. *Intestinal transport of tryptophan and its analogs.* Am. J. Physiol. 228(2): 496–500. 1975.—A comparative study of the intestinal transport of DL-tryptophan and its 1-methylindole (tryptophan-1-*Me*) and benzo[b]thiophene (tryptophan-*S*) analogs has been carried out in vitro, using the everted intestinal sac of the rat and hamster. Both tryptophan and tryptophan-*S* are actively transported across the intestine, while tryptophan-1-*Me* is not actively transported. The active transport of tryptophan is competitively inhibited by tryptophan-*S*, suggesting a similar carrier, while tryptophan-1-*Me* is not an inhibitor of tryptophan transport, suggesting little or no interaction with the carrier. The transport of tryptophan and tryptophan-*S* is depressed at concentrations (10 mM), and all three amino acids produce subtle alterations in the barrier properties of the sacs, as evidenced by increased tetraethylammonium bromide-^{14}C diffusion.

active transport; aromatic amino acids

FOLLOWING THE REVIVAL by Wilson and Wiseman (22) of the everted intestinal sac techniques, developed by Reid (18), a great body of literature has accumulated concerning the carriers involved in the active transport of amino acids across the intestinal epithelium. Antedating methodology for elucidating active processes, Berg and Bauguess in 1934 (1) were first to note differences in absorption among tryptophan isomers and acetylated derivatives in the rat. Wiseman (24), using the everted sac technique, was unable to demonstrate active accumulation of L-tryptophan at a concentration of 20 mM. However, Spencer and Samily (20) showed active transport of L-tryptophan to occur in hamster intestinal sac at low concentrations, with uptake dropping off to zero at higher concentrations. This phenomenon has been confirmed by other investigators and attributed to toxicity (20), depression (7), and most recently, to a bidirectional flux characteristic of the carrier mechanism (16).

Despite 20 yr of study of the active transport of amino acids in the intestine, a satisfactory understanding of the role of structural factors in tryptophan transport has not been forthcoming. At present, investigations have demonstrated the primary role of the α-carboxyl and α-amino groups (19) and have shown rather conclusively that highly polar or charged substitutions on the aromatic ring reduce transportability (11). These findings prompted Wiseman (23) to state, in a recent review of amino acid transport, that the aromatic system seems to be of comparatively little importance, provided that it does not bear a charge.

As part of a continuing study of tryptophan (Ia) and its 1-methylindole (tryptophan-1-*Me*) (Ib) and benzo[b]thiophene (tryptophan-*S*) (Ic) analogs, we have studied the role of the indole nucleus in active intestinal transport in the rat and golden hamster.



Ia, X = NH
Ib, X = NCH$_3$
Ic, X = S

MATERIALS AND METHODS

Materials. Animals were adult male Sprague-Dawley rats, 280 g (Murphy Breeding Laboratories, Plainfield, Ind.) and adult male golden hamsters, 150 g (Engle Laboratory Animals, Inc., Farmersburg, Ind.). DL-Tryptophan [side chain-2,3-^{3}H] and tetraethylammonium bromide-1-^{14}C (TEA) of specific activities 1 Ci/mmol and 1 mCi/mmol, respectively, were purchased from New England Nuclear Corp., Boston, Mass. DL-Tryptophan-1-*Me* and DL-tryptophan-*S* were uniformly tritium labeled by New England Nuclear Corp., with respective specific activities of 193 mCi/mmol and 3 Ci/mmol. The purity of all labeled compounds was confirmed by thin-layer chromatography (2), scanning the plates with a Packard model 7200 radiochromatogram scanner. Unlabeled DL-tryptophan was purchased from Sigma Chemical Co., Inc., St. Louis, Mo., and DL-tryptophan-1-*Me* and DL-tryptophan-*S* were prepared by standard procedures (4, 25). The purity of all unlabeled compounds was confirmed by thin-layer and gas chromatography.

Methods. In all everted sac transport studies a modification of the method of Wilson and Wiseman (22) was employed. Animals were anesthetized with Nembutal (8–10 mg/kg, ip), laparotomy was performed, and 4-cm segments of ileum beginning 5 cm from the ileocecal valve were removed sequentially and individually with ligation of neighboring blood vessels to prevent excess blood loss. The modification insured the elimination of temporal incubation differences among sacs, i.e., the first sac was away from an intact blood supply the same length of time as the last. Typically, five sacs were prepared from each animal. Eversion of sacs was performed in isotonic saline at 23°C on a glass rod; any adventitious succus was gently washed from the mucosal surface. All sacs were filled with 0.4 ml of test solution, ligated, and placed in incubation vessels containing 5 ml of identical test solution. Incubations were carried out in an Aminco-Dubnoff metabolic shaker at 37°C and 30 oscillations/min. A direct oxygen

supply was provided for each incubation vessel by continuous bubbling of 100% oxygen through a Pasteur pipette at an approximate rate of 20 ml/min per vial. Incubations were carried out for 30 min.

Everted sacs employed in viability investigations were prepared and incubated as above. At 15 min 2 μCi of tetraethylammonium bromide-1-^{14}C (0.1 ml) were added to each vessel.

All solutions were prepared in a Krebs-Henseleit bicarbonate buffer stock, pH 7.4. To various concentrations of unlabeled amino acid, tracer amounts of the labeled compounds were added to achieve 0.1 μCi/ml. Nonabsorbable phenolsulfonphthalein, at a final concentration of 0.014 mM, was added to the test solution for purposes of volume corrections. Upon termination of the experiment, 0.2-ml aliquots of serosal and mucosal fluids were removed and added to counting vials containing the scintillation solution previously described (12) and counted in a Packard Tri-Carb model 4322 liquid scintillation system. Aliquots of serosal fluid, 0.1 ml, were diluted to 2.0 ml with 0.5 N NaOH and read on a Beckman DU spectrophotometer at 554 nm. Similar spectrophotometric readings were obtained on the original stock solution, and the ratios of initial and final absorbance values were used for volume correction. Tissue accumulation of amino acid was measured and used only for purposes of determining percent recovery of the labeled amino acid. Typical recoveries were 96–103%. The rates of transport were calculated as described by Lin et al. (11).

Slopes were determined by linear regression analysis. Comparisons of individual values were performed by the Student t test; slope and intercept comparisons were performed as reported by Cox (8).

RESULTS

The data obtained from studies of the everted sac of rat or hamster, using DL-tryptophan and its analogs, are presented in Table 1. Transport of tryptophan-1-*Me* was not observed. For both tryptophan and tryptophan-*S*, the data do not follow standard Michaelis-Menten kinetics, primarily due to a drastic fall in transport rate at 10 mM. This phenomenon has been reported for tryptophan (7, 20) and for other neutral amino acids at higher concentration (13, 14).

For tryptophan and tryptophan-*S*, K_m and V_{max} were determined by the Hofstee method in which the V_{max} is represented as the y-intercept and $-K_m$ is represented by the slope. Figure 1*A* presents results for the rat where a K_m of 1.70 mM and a V_{max} of 2.91 μmol/g per 30 min for tryptophan and K_m = 2.03 mM and V_{max} = 4.10 μmol/g per 30 min for tryptophan-*S* were determined. Statistical analysis of the differences in K_m and V_{max} values indicated that they were not significant at the 0.05 level.

A similar plot for the hamster appears in Fig. 1*B* where the K_m of tryptophan was found to be 1.96 mM and the V_{max} was found to be 3.33 μmol/g per 30 min, whereas for tryptophan-*S*, K_m was determined to be 2.49 mM and V_{max} was determined to be 4.63 μmol/g per 30 min. No statistically significant difference at the 0.05 level was found between the K_m and V_{max} values or between respective

TABLE 1. *Transport of tryptophan and analogs by everted sac preparations*

Compound	Species	Initial Serosal Concn, mM	Transported, μmol/g per 30 min
DL-Tryptophan	Rat	0.1	0.16±0.02
		0.5	0.65±0.03
		1.0	1.11±0.03
		3.0	1.85±0.21
		5.0	2.18±0.05
		10.0	1.90±0.09
DL-Tryptophan-*S*	Rat	0.1	0.19±0.02
		0.5	0.82±0.05
		1.0	1.28±0.04
		3.0	2.54±0.14
		5.0	2.42±0.21
		10.0	1.67±0.12
DL-Tryptophan-1-*Me*	Rat	0.1	−0.01±0.003
		1.0	−0.06±0.02
		5.0	−0.46±0.14
DL-Tryptophan	Hamster	0.1	0.17±0.01
		0.5	0.63±0.03
		1.0	1.15±0.10
		3.0	2.02±0.18
		5.0	2.45±0.09
		10.0	2.03±0.10
DL-Tryptophan-*S*	Hamster	0.1	0.18±0.01
		0.5	0.76±0.17
		1.0	1.30±0.06
		3.0	2.60±0.08
		5.0	2.72±0.21
		10.0	1.94±0.13
DL-Tryptophan-1-*Me*	Hamster	0.1	−0.02±0.005
		1.0	−0.06±0.03
		5.0	−0.88±0.11

Each value is the mean ± SD of five sac preparations. Transport (in μmol/g per 30 min) was calculated as described in MATERIALS AND METHODS.

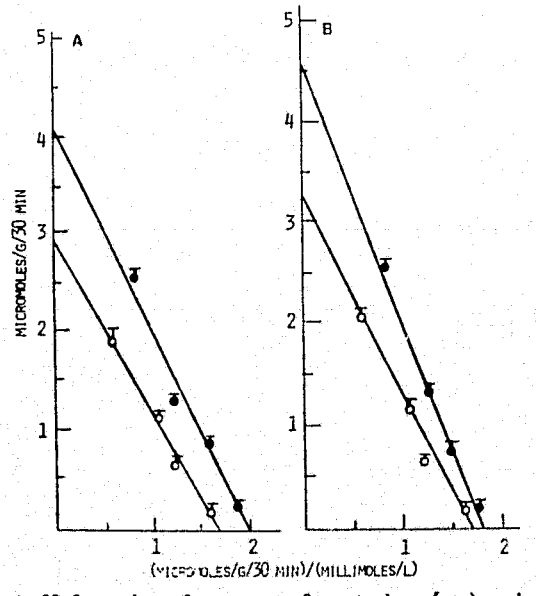


FIG. 1. Hofstee plots of transport of tryptophan (-○-) and tryptophan-*S* (-●-) by isolated everted sacs of rat (Fig. 1*A*) and hamster (Fig. 1*B*). Each point is mean of 5 sacs; vertical bars are ± SD.

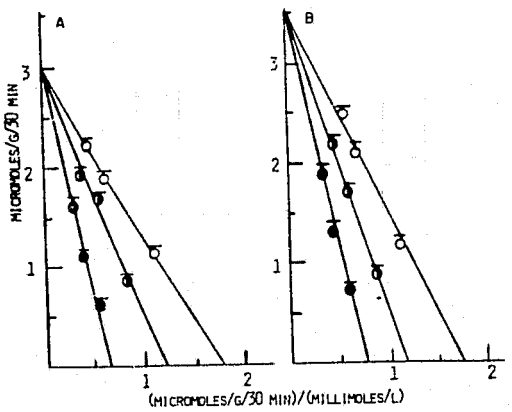


FIG. 2. Hofstee plots of transport of tryptophan in presence of varying concentrations: 0 (-○-), 1 mM (-◑-), and 3 mM (-●-) of tryptophan-*S* by isolated everted sacs of rat (Fig. 2*A*) and hamster (Fig. 2*B*). Each point is mean of five sacs; vertical bars are ± SD.

TABLE 2. *Transport of tryptophan in the presence of tryptophan-1-Me in the rat and hamster*

Species	Tryptophan Concn, mM	Tryptophan-1-*Me* Concn	
		1 mM, μmol/g per 30 min	10 mM, μmol/g per 30 min
Rat	0.1	0.11 ± 0.03	0.15 ± 0.02
Rat	1.0	1.15 ± 0.13	1.09 ± 0.10
Hamster	0.1	0.15 ± 0.01	0.14 ± 0.02
Hamster	1.0	1.11 ± 0.10	1.09 ± 0.14

Each value is the mean ± SD of five sac preparations. Transport (in μmol/g per 30 min) was calculated as described in MATERIALS AND METHODS.

K_m and V_{max} values of the tryptophans when compared between the two animals.

Figure 2*A* presents the kinetics of tryptophan transport in the rat in the presence of tryptophan-*S*. Competitive inhibition is observed, with the V_{max} remaining constant at all inhibitor concentrations (indicated by I values on the graph) while K_m changes. Similar results are obtained in the hamster and appear in Fig. 2*B*.

Tryptophan-1-*Me* was unable to inhibit tryptophan transport (Table 2). In the presence of 1 or 10 mM tryptophan-1-*Me*, the rate of tryptophan transport at 0.1 or 1.0 mM does not change. Statistical analysis of these values compared to tryptophan at zero inhibitor concentration indicates no statistical difference at the 0.05 level.

Considering the problem of decremental transport at high initial concentrations, Table 3 lists experimental velocities of tryptophan and tryptophan-*S* and the corresponding predicted values obtained by the use of the Michaelis-Menten equation. The last column expresses the percent predicted value of the experimentally determined velocity. It can be seen that deviation from predicted velocity occurs for tryptophan-*S* at 5 mM and for tryptophan at approximately 10 mM. Statistical comparison of the deviation at the 5 mM and 10 mM points indicated a significant difference ($P < .05$) between the two compounds.

Viability studies, at high concentration-induced decremental transport, were conducted by measuring the percent

TABLE 3. *Comparison of experimental and predicted transport velocities*

Species	Compound	Concn, mM	Exptl Velocity (V_E), μmol/g per 30 min	Predicted Velocity (V_p), μmol/g per 30 min	$V_E/V_p \times 100$
Rat	Tryptophan	5	2.18±0.05	2 17	100±2.3
Rat	Tryptophan	10	1.90±0.09	2.50	76±3.8
Rat	Tryptophan	3	2.54±0.14	2.46	103±5.7
Rat	Tryptophan-*S*	5	2.42±0.20	2.92	83±6.8
Rat	Tryptophan-*S*	10	1.67±0.12	3.40	49±3.5
Hamster	Tryptophan	5	2.45±0.09	2.38	103±3.1
Hamster	Tryptophan	10	2.03±0.10	2.78	73±3.7
Hamster	Tryptophan	3	2.60±0.08	2.51	104±3.2
Hamster	Tryptophan-*S*	5	2.72±0.21	3.06	89±6.9
Hamster	Tryptophan-*S*	10	1.94±0.13	3.68	53±3.4

Each value is the mean ± SD of five sac preparations.

TABLE 4. *Transintestinal diffusion of tetraethylammonium-1-^{14}C (TEA) in the presence of varying concentrations of tryptophan and its analogs*

Species	Concn, mM	Tryptophan		Tryptophan-1-*Me*		Tryptophan-*S*	
		% Dose	% Dose / % Control	% Dose‡	% Dose / % Control	% Dose	% Dose / % Control
Rat	0*	0.65 ±0.07	1.0	0.65 ±0.07	1.0	0.65 ±0.07	1.0
Rat	1	0.66 ±0.04	1.0	0.64 ±0.06	1.0	0.64 ±0.07	1.0
Rat	5	0.64 ±0.07	1.0	0.64 ±0.05	1.0	1.03 ±0.09	1.6†
Rat	10	1.48 ±0.22	2.3†	0.70 ±0.13	1.1	2.01 ±0.30	3.1†
Rat	20	2.31 ±0.34	3.6†	1.67 ±0.11	1.6†	4.13 ±0.45	6.3†
Hamster	0*	3.69 ±0.89	1.0	3.69 ±0.89	1.0	3.69 ±0.89	1.0
Hamster	1	3.46 ±1.08	1.0	3.63 ±0.91	1.0	3.51 ±0.80	1.0
Hamster	5	3.60 ±0.78	1.0	3.38 ±0.64	1.0	6.56 ±0.78	1.8†
Hamster	10	8.29 ±0.77	2.2†	4.02 ±1.02	1.1	10.46 ±1.09	2.8†
Hamster	20	12.85 ±1.00	3.5†	5.86 ±0.95	1.6†	24.30 ±2.51	6.6†

Each value is the mean ± SD of five sac preparations. * Control = diffusion of TEA when amino acid concentration = 0. † Significant difference ($P < 0.05$) from control and significant difference ($P < 0.05$) from other values in horizontal row. ‡ % Dose = % total TEA diffusing from mucosal to serosal compartment in 15 min.

total dose diffusion of the relatively nondiffusable and inert compound, TEA, from the mucosal to serosal compartment during the last 15-min period of a 30-min incubation in the presence of varying concentrations of the amino acids. These data were then correlated with histological evidence of sac disintegration. The premise was that a nondisrupted lipid epithelial border should serve as an effective barrier to transepithelial diffusion of the highly polar TEA; this has recently been confirmed (17). Similar methodology has been employed by Gibaldi and coworkers (9) in evaluating everted sac viability with good results.

Table 4 shows the results of the investigation. The column percent dose percent control is a ratio of the mean values of TEA diffusion at various amino acid concentrations to the mean diffusion value at zero amino acid concentration, the control. Statistical comparisons were made between the actual percent dose values shown in the first column under each compound. Ratios greater than 1 indicate a net increase in diffusion over the control. For tryptophan-*S*, a net increase in diffusion is seen to begin at 5 mM with progressive increase in direct correspondence to concentration. Significant diffusion in the presence of tryptophan does not begin until 10 mM and for the 1-methyl derivative, increased diffusion is first noted at 20 mM. Histological evidence of sac disintegration, observed as epithelial swelling, disruption of cell borders, and sloughing, was noted only at the 10 and 20 mM concentrations for tryptophan and tryptophan-*S*. This will be described in detail in a future report.

DISCUSSION

Selective molecular alterations might alter transportability in several ways. First, there may be stereochemical and electronic effects. The replacement of the indole nucleus by benzo[b]thiophene results in a molecule that is similar sterically to indole but electronically distinct due to the involvement of 3d orbitals on the sulfur atom (3), while the 1-methylindole analog resembles indole electronically but differs sterically due to the presence of the bulky methyl group. Additionally, indole possesses the capability of hydrogen bonding at the 1-position which is absent in the analogs.

A second way in which substitution might alter transport would be through alteration of the acid/base properties of the α-COOH and α-NH_2 groups. Studies by Christensen and Oxender (6) have indicated that modest alterations of pK_a values have slight effect on transport. Tryptophan and its sulfur and 1-methyl analogs do not differ greatly with respect to pK_a values of the α-carboxyl and α-amino groups (5).

Finally, transportability may be affected through a polarity change in the aromatic system, leading to differences in lipophilicity. Partition coefficient determinations have shown that the order of lipophilicity of the compounds is in the order: tryptophan-*S* > tryptophan-1-*Me* > tryptophan (5).

It is evident that steric bulk at the 1-position of the indole nucleus differentiates tryptophan and tryptophan-*S* from tryptophan-1-*Me*. The fact that tryptophan-*S* and tryptophan-1-*Me* are more lipophilic than tryptophan as indicated by their partition coefficients, yet the 1-methyl derivative is not actively transported, gives greatest support to steric factors in the transport mechanism. These findings contradict earlier thinking emphasizing the importance of aromatic ring system lipophilicity and the unimportance of nonpolar structural changes in determining transportability (11).

When compared to the studies of Cohen and Huang (7) who found transport of the 4-, 5-, and 6-methyl derivatives of tryptophan at a single concentration, our results would tend to suggest the importance of the 1-position of the indole nucleus as a site for steric interaction of tryptophan with the carrier molecule.

The results of the inhibition studies support the contention that tryptophan and tryptophan-*S* share the same carrier and interact in a competitive fashion. On the other hand, the absence of any competitive behavior of tryptophan-1-*Me* with tryptophan for transport would suggest a rather dramatic inertness of this compound with respect to carrier interaction. It must be remembered that all of the data reported in the present paper are for the racemic mixtures. While the D-isomer of tryptophan is presumably not transported (7), the assumption that it has no effect on the transport of the L-isomer when both are present cannot be taken as an absolute phenomenon.

The depression of transport observed at high concentrations of tryptophan has been reported by several groups (7, 16, 20). Our results confirm this phenomenon for tryptophan and tryptophan-*S* as well. In addition, by comparison of actual and predicted velocities, we have shown that depression of transport tends to occur earlier for the sulfur analog. Spencer and Samily (20) first speculated that damage to sacs might account for the inhibition of transport. Cohen and Huang (7), on the other hand, thought it represented some sort of depression but did not suggest a specific mechanism. Most recently Munck (16) has reopened the issue, and based on the theoretical work of Wilbrandt and Rosenberg (21), has attributed the decline in transport of tryptophan at high concentrations to a bidirectional flux characteristic of the transport mechanism (15).

In support of the data presented here, Laster et al. (10) reported light and electron microscopic evidence of cellular damage to hamster everted sacs incubated in vitro with 40 mM L-tryptophan. These lesions neither occurred with the D-isomer nor with 11 other amino acids tested at the same concentration (10). While we also examined histological samples and noted gross evidence of destruction at 10-20 mM DL-tryptophan and DL-tryptophan-*S* in both the rat and hamster, the measurement of TEA diffusion over a range of concentrations, when compared to a control, indicated that a more subtle alteration in the barrier properties of the sacs was occurring at tryptophan-*S* concentrations as low as 5 mM and tryptophan-1-*Me* concentrations as low as 20 mM. This we take as evidence of toxicity, although the exact nature of the toxic effect is at the present time unknown.

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DIFFERENTIAL EFFECTS OF αMT ON ANORECTIC AND STIMULATORY ACTION OF AMPHETAMINES

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ABSTRACT

Pretreatment of rats with a dosage regimen of αMT that has no effect on the anorectic action of a single dose of 2 mg/kg of d-amphetamine or methamphetamine causes a marked reduction in the rate of continuous avoidance responding evoked by that same dose. Similar pretreatment followed by a dose of 8.0 mg/kg of benzphetamine was without effect on the drug's action on both systems.

INTRODUCTION

Since the first studies of the action of d-amphetamine on anorectic and central stimulatory behavior (Lesses and Myerson, 1938; Prinzmetal and Alles, 1940), a large amount of research effort has been placed in attempts to separate the two actions of drugs in the amphetamine series. A recent symposium contains a variety of papers reviewing various aspects of this problem (Costa and Garattini, 1970). In addition, Cox and Maickel (1972) have reported a detailed comparison of the two activities of a large number

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of phenylethylamines, using reduction of deprivation-induced food consumption as a measure of anorectic potency and increase of continuous avoidance responding as a measure of central stimulatory potency. Since Miller, et al (1970) had reported that αMT pretreatment could reduce the stimulatory effect of d-amphetamine, it seemed reasonable to do a comparative study of several amphetamines in animals pretreated with αMT.

MATERIALS AND METHODS

Adult, male Sprague-Dawley rats (Hormone Assay Laboratories, Chicago) were used in all experiments. Details of animal handling, drug dosage, behavioral testing, and statistical processing of data may be found in the paper by Cox and Maickel (1972). Treatment with αMT was as reported by Miller, et al (1970). Brain NE and 5HT were determined as described by Maickel, et al (1968).

TABLE 1

Effect of αMT Pretreatment on Brain Levels of NE and 5HT

Each value is the mean ± S.D. of results obtained from 4 animals given 150 mg/kg (i.p.) of αMT as a suspension in peanut oil at 48, 36, 24 and 12 hours prior to the zero time point.

Hours After Last Injection	Brain NE μg/g	Brain 5HT μg/g
Control	0.46 ± .05	0.63 ± .06
6	0.28 ± .09*	0.70 ± .09
12	0.20 ± .03*	0.68 ± .09
24	0.21 ± .04*	0.64 ± .04
48	0.30 ± .03*	0.62 ± .07

*Significantly different from control ($p < .05$) as determined by the Student "t" test.

RESULTS

The effects of pretreatment with αMT in the dosage regimen described by Miller, et al (1970) are shown in Table 1. As can be seen from the data, a maximal reduction (to approximately 45% of control values) was maintained over the period 12-24 hours after the last dose. Accordingly, all behavioral testing was performed during this time period.

TABLE 2

Effect of αMT Pretreatment on Anorectic Action of Amphetamines

Drugs were administered 12 hours after the final dose of αMT. Each value is the mean ± S.D. of values obtained from 6 animals, each animal serving as its own control.

Compound (dose-mg/kg)	No Pretreatment (%)	αMT Pretreatment (%)
Saline	100 ± 8	87 ± 11#
d-Amphetamine (2.0 mg/kg)	60 ± 7*	58 ± 12≠
Methamphetamine (2.0 mg/kg)	53 ± 13*	55 ± 15≠
Benzphetamine (8.0 mg/kg)	66 ± 16*	68 ± 12≠

*Significantly different from saline ($p < .05$) as determined by the Student "t" test.

#No signigicant difference from non-pretreated animals ($p > .05$) as determined by the Student "t" test.

≠No significant difference from non-pretreated animals ($p > .05$) as determined by the Student "t" test, but significantly different from saline with αMT pretreatment ($p < .05$).

When animals were pretreated with αMT, then given a single dose of d-amphetamine, methamphetamine, or benzphetamine, the results obtained on deprivation-induced food consumption are shown in Table 2. It is clear that the αMT pretreatment had no effect on the anorectic activity of the amphetamines. The pretreatment itself had a slight, but non-significant, action on food consumption.

TABLE 3

Effect of αMT Pretreatment on Stimulatory Action of Amphetamines

Drugs were administered 12 hours after the final dose of αMT. Each value is the mean ± S.D. of values obtained from 6 animals, each animal serving as its own control.

Compound (dose-mg/kg)	No Pretreatment %	αMT Preatment %
Saline	100 ± 17	94 ± 11
d-Amphetamine (2.0 mg/kg)	259 ± 49*	67 ± 20#
Methamphetamine (2.0 mg/kg)	287 ± 51*	63 ± 23#
Benzphetamine (8.0 mg/kg)	97 ± 12	93 ± 17

*Significantly different from saline ($p < .05$) as determined by the Students "t" test.

#Significantly different from non-pretreated animals ($p < .05$) as determined by the Students "t" test.

In sharp contrast to these results are those obtained for continuous avoidance responding. The data obtained (in response to amphetamines) when animals were pretreated with αMT are presented in Table 3. Pretreatment with αMT had no significant effect on the rate of avoidance responding per se, but such pretreatment completely abolished the increased responding usually evoked by d-amphetamine and methamphetamine. Benzphetamine did not evoke stimulation in control animals; αMT pretreatment did not alter these results.

DISCUSSION

The ability of amphetamines to produce two different pharmacological effects: appetite depression and central stimulation, has been known for many years. The precise role(s) of norepinephrine in these actions of

amphetamines has also been a continuing area for research. Weissman, et al (1966) have shown that αMT pretreatment antagonized the effect of amphetamine on conditioned avoidance behavior. Moore, et al (1967) in a subsequent publication, attributed some effects of αMT to non-specific toxicological phenomena of the agent, especially when given in massive single doses. A previous publication from this laboratory (Miller, et al, 1970) has shown that use of a multiple-dosage schedule of more modest doses of αMT permits marked reduction in brain NE levels to be achieved, with no toxicity and only a slight perturbation of normal behavior.

The possible role(s) of brain NE in mechanisms related to food consumption have also been the subject of some study. Many of the studies have been reviewed in the publications by Morgane (1969), Grossman (1967), and Myers (1974). In this regard, it is of interest to note that the actions of amphetamine on food consumption can be altered by hypothalamic lesions (Cole, 1966) and that αMT treatment can reduce the rate of lever pressing for food by rats (Beaton and Crow, 1969), although this latter effect may be confounded by a concommitant decrease in motor activity.

The data presented herein show that a pretreatment regimen of αMT that lowers brain NE by approximately 55% has clearly separable effects on the anorectic and stimulatory actions of several amphetamines. Under these conditions, the central stimulatory actions of d-amphetamine and methamphetamine are completely abolished, while benzphetamine, which has no stimulatory activity at the dose tested, is not influenced by the αMT treatment. In contrast, the lowered brain NE levels have no effects on the anorexigenic actions of all three agents. The obvious question to be raised with regard to these results is whether they point to the possibility of developing new anorectic agents making use of this differential effect.

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We wish to thank Smith Kline and French Laboratories for supplying the d-amphetamine sulfate (Dexedrine^{R}) and the Upjohn Company for supplying the benzphetamine hydrochloride (Didrex^{R}) used in these experiments. This research was supported in part by NASA grant NGL 15-003-117.

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ON THE ROLE OF BRAIN BIOGENIC AMINES IN THE CONTROL OF PITUITARY-ADRENOCORTICAL ACTIVITY[1/]

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ABSTRACT

Pretreatment of rats with the MAO inhibitor pargyline prevents the metabolic destruction of 5HT and NE released in the brain by a subsequent dose of reserpine, and high levels of the amines remain in the brain. Under these conditions, the hypersecretion of ACTH usually evoked by reserpine does not occur. The elevated levels of brain 5HT and NE produced by MAO inhibition do not cause ACTH hypersecretion nor do they prevent the ACTH hypersecretion caused by exposure to cold or administration of sedative doses of chlorpromazine.

Previous reports have indicated that sedative doses of reserpine evoke a hypersecretion of ACTH in rats, similar to that produced by exposure to low environmental temperatures (Maickel et al, 1961; Westermann et al, 1962). The characteristic response includes decreased adrenal ascorbic acid and increased plasma corticosterone, liver tryptophan pyrrolase (TPO) and adrenal gland weight. Similar effects were produced in rats by sedative doses of the Rauwolfia alkaloids raunescine and rescinnamine, the reserpine analogue syrosingopine, or the benzoquinolizine Ro 4-1284 (2-hydroxy-2-ethyl-3-isobutyl-9, 10-dimethoxy-1,2,-3,6,7-hexahydrobenzo(a)quinolizine). In contrast, isoreserpine, the pharmacologically inactive stereoisomer of reserpine, failed to evoke a pituitary-adrenal response.

The ACTH hypersecretion evoked by reserpine was shown to be independent of the sedation caused by the drug (Martel et al, 1962). For example, pretreatment of animals with desmethylimipramine antagonized the reserpine-induced sedation without preventing the decline in brain amines or the hypersecretion of ACTH. The current paper explores the antagonism of reserpine-induced ACTH hypersecretion by the monoamine oxidase (MAO) inhibitor pargyline (MO 911,

N-methyl-N-benzyl-2-propynylamine). Evidence is presented that this antagonism is related to the level of brain biogenic amines maintained during the course of action of the drug. Pretreatment with MAO inhibitors does not affect the ACTH hypersecretion evoked by cold-exposure or chlorpromazine, lending further support to the hypothesis that reserpine-induced ACTH hypersecretion is related to brain amine changes.

MATERIALS AND METHODS

Experimental procedures were performed with unanesthetized adult male Sprague-Dawley rats (250-300 g). The animals were allowed food and water ad lib. and maintained as previously described (Maickel et al, 1961). Reserpine, as the lyophilized phosphate salt, was dissolved in 0.5 ml water and injected into the tail vein. Chlorpromazine, pargyline, and Ro 4-1284 were dissolved in distilled water and injected intraperitoneally. Control animals were injected with saline or distilled water. Animals were stunned and then immediately decapitated. Blood was collected into beakers containing heparin, transferred to tubes, and centrifuged immediately. Plasma samples for corticosterone assay were stored at -10°C. For the assay of liver TPO, adrenal ascorbic acid, and brain 5HT, NE and MAO, tissues were removed and stored at -10°C.

Chemical methods. Adrenal ascorbic acid was determined by the method of Maickel (1960); plasma corticosterone by the method of Guillemin (1959) and liver TPO Knox (1951); brain 5HT and NE by the method of Maickel et al (1968); and brain MAO activity by the method of Weissbach et al (1960).

Parameters of pituitary-adrenal stimulation. The following indices of pituitary-adrenal stimulation were measured; a fall in the level of adrenal ascorbic acid, a rise in the level of plasma corticosterone, and an increased

activity of liver TPO. The limitations and advantages of each of these indices have been discussed in a previous paper (Maickel et al, 1961). In some experiments the changes in plasma corticosterone levels were used as the sole index of pituitary-adrenal hyperactivity.

RESULTS

Effects of pargyline on brain levels of 5HT and NE and on pituitary-adrenal system. The administration of a single dose of pargyline (25 mg/kg, i.p.), or two doses 18 hours apart, did not affect the levels of adrenal ascorbic acid, plasma corticosterone, or liver TPO (Table 1). Brain levels of 5HT and NE were markedly increased and brain MAO activity was inhibited by > 95%, as determined by the method of Weissbach et al (1960).

Effects of pretreatment with pargyline on reserpine-induced ACTH hypersecretion. A previous communication reported that pretreatment of rats with pargyline prevented both the sedation and the ACTH hypersecretion induced by reserpine (Martel et al, 1962). The results in Table 2 show that administration of reserpine to rats pretreated with pargyline (25 mg/kg, i.p.) caused little change in brain 5HT and NE levels in 6 hours. The animals did not show the sedation characteristic of reserpine and there was no evidence of ACTH hypersecretion.

When rats were pretreated with smaller doses of pargyline, the subsequent administration of reserpine produced a variety of results (Table 3). In some animals, MAO inhibition was not sufficient to prevent the immediate metabolism of the amines released by reserpine. These animals became deeply sedated in 3 hours. At that time, brain levels of 5HT and NE had fallen markedly and there was a pronounced hypersecretion of ACTH. In other animals, the MAO inhibitor was only partially effective in protecting the released amines;

in these animals, the sedative action of reserpine was delayed. In 6 hours, sedation was apparent; however, the brain levels of 5HT and HE had not fallen to minimal values and no ACTH hypersecretion was observed. Finally, a third group of animals was observed in which the MAO inhibitor protected most of the released amines from destruction; 6 hours after reserpine, the animals showed only a slight sign of sedation. In these animals, reserpine had no significant effect on pituitary-adrenocortical activity.

Dose-response relationship of pargyline antagonism of reserpine-induced ACTH hypersecretion. Previous papers have shown that a number of reserpine-like compounds lower brain amine levels and cause ACTH hypersecretion in rats (Maickel et al, 1961; Westermann et al, 1962). One striking similarity in these data is that the brain levels of amines must be depleted below 50% of the normal level in order to observe hypersecretion of ACTH. When rats pretreated with varying doses of pargyline were given reserpine, only those animals in which the level of brain amines fell below 50% of the control animals showed ACTH hypersecretion (Table 4). If the dose of pargyline was sufficient to protect the reserpine-released amines to the extent that the levels of 5HT did not fall to values below 50% of normal, there was no measurable pituitary-adrenal stimulation.

Lack of effect of pargyline pretreatment on ACTH hypersecretion induced by other stressful stimuli. Previous papers from this laboratory have described the ACTH hypersecretion evoked by cold-exposure or administration of chlorpromazine (Maickel et al, 1961; Smith et al, 1963). From the data in Table 5 it is evident that pretreatment of rats with pargyline had no effect on the ACTH hypersecretion induced by cold-exposure or chlorpromazine. This is in striking contrast to the antagonism of reserpine action produced by pargyline and lends further weight to the hypothesis that the action of reserpine on the pituitary-adrenocortical system is mediated through an effect of the drug

on brain amines.

DISCUSSION

The studies presented in this paper lend further evidence that the ACTH hypersecretion caused by reserpine and reserpine-like drugs is related to the effects of the drugs on brain monoamine storage. A previous paper (Westermann et al, 1962) has noted that these drugs produce ACTH hypersecretion only when given in doses that lower the amine stores below 50% of normal, regardless of whether this was achieved by administration of a single large dose or repeated smaller doses. In animals pretreated with pargyline (in doses which block brain MAO activity by 95%) administration of reserpine does not lower the level of brain amines. Although reserpine impairs the storage of 5HT and NE, the liberated amines are protected from the degradative action of MAO; due to their poor lipid solubility they can diffuse across the blood-brain barrier into the blood stream only very slowly. Thus, high levels of free amines remain in the brain for a long period of time.

The relationship of pituitary-adrenal hyperactivity to depletion of brain amines to values below 50% of normal, first noted with reserpine alone, has also been observed in pargyline-treated animals. The possible significance of this is not immediately apparent. In the rat, the whole brain is used for the determination of 5HT and NE; the assay method measures the amines as if they were uniformly distributed throughout the entire brain. Perhaps this level of amines in a brain homogenate is equivalent to a complete depletion of the amines in one or more specific areas of the brain, or perhaps it is related to an imbalance of amines and consequently of neural function in some specific neuronal circuits.

The observation that pretreatment of rats with an MAO inhibitor does not prevent the stimulatory effects of cold-exposure or chlorpromazine on the pituitary-adrenal axis may also suggest that the hypersecretion of ACTH evoked by reserpine is due to interference with storage processes for brain biogenic amines.

TABLE 1

Effects of Pargyline on Brain Levels of 5HT and NE and On Pituitary-Adrenal Parameters

Numbers in parentheses represent number of animals studied. All values are means ± S.D.; underlined values are significantly different from controls ($p < .05$). Measurements were made 18 hours after the last dose of pargyline.

	Adrenal Ascorbic Acid	Plasma Corticosterone	Liver TPO	Brain	
				5HT	NE
	mg/100 g	µg/ml	µmol/g liver/hr	µg/g	µg/g
Control (20)	447 ± 41	0.15 ± .05	3.2 ± .36	0.45 ± .04	0.46 ± .04
Pargyline (13) one dose 25 mg/kg	451 ± 40	0.16 ± .05	2.9 ± .38	0.69 ± .06	0.60 ± .04
Pargyline (15) two doses 25 mg/kg i.p. 18 hours apart	453 ± 46	0.19 ± .04	3.0 ± .27	0.80 ± .06	0.72 ± .07

TABLE 2

Antagonism of Reserpine-Induced Sedation and ACTH Hypersecretion by Pargyline

Numbers in parentheses represent number of animals studied. All values are mean ± S.D.; underlined values are significantly different from controls ($p < .05$). Animals were pretreated with pargyline (25 mg/kg i.p.) at 36 and 18 hours prior to administration of reserpine (1 mg/kg i.v.). Brain amines and plasma corticosterone were measured 6 hours after reserpine.

	Plasma Corticosterone	Brain		Sedation
		5HT	NE	
	µg/ml	µg/g	µg/g	
Control (20)	0.15 ± .05	0.45 ± .04	0.46 ± .04	--
Pargyline (15)	0.19 ± .04	0.80 ± .06	0.72 ± .07	No
Reserpine (11)	0.44 ± .04	0.08 ± .02	0.07 ± .03	Yes
Pargyline + Reserpine (9)	0.12 ± .03	0.76 ± .03	0.55 ± .05	No

TABLE 3

Effect of a Borderline Dose of Pargyline on Reserpine-Induced Sedation and ACTH Hypersecretion

Numbers in parentheses represent number of animals studied. All values are mean ± S.D.; underlined values are significantly different from controls ($p < .05$). Animals were given a single dose of pargyline, 18 mg/kg i.p., 18 hours before administration of reserpine (1 mg/kg i.v.). All animals were killed 6 hours after reserpine.

	Sedation		Plasma Corticosterone	Brain	
	3 hours after Reserpine	6 hours after Reserpine		5HT	NE
			µg/ml	µg/g	µg/g
Control (20)	-	-	0.15 ± .05	0.45 ± .04	0.46 ± .04
Pargyline (6)	-	-	0.16 ± .03	0.62 ± .03	0.57 ± .04
Reserpine (11)	Deep	Deep	0.44 ± .04	0.08 ± .02	0.07 ± .03
Pargyline + Reserpine*					
I (10)	Deep	Deep	0.39 ± .03	0.20 ± .03	0.19 ± .04
II (10)	Slight	Deep	0.18 ± .05	0.40 ± .03	0.34 ± .03
III (11)	None	Slight	0.17 ± .05	0.53 ± .03	0.45 ± .05

* Groups I, II and III were selected by visual observation of degree of sedation at 3 hours after reserpine

TABLE 4

Dose-response of Pargyline Antagonism of Reserpine-induced ACTH Hypersecretion

Numbers in parentheses represent number of animals studied. All values are mean ± S.D.; underlined values are significantly different from controls ($p < .05$). Reserpine was administered 18 hours after pargyline. Animals were killed 6 hours after reserpine.

Pargyline	Reserpine		Plasma Corticosterone	5HT	NE	Sedation
mg/kg, i.p.	mg/kg, i.v.		µg/ml	µg/g	µg/g	
-	-	(20)	0.15 ± .05	0.45 ± .04	0.46 ± .04	-
10	-	(6)	0.14 ± .03	0.58 ± .03	0.50 ± .02	-
10	1	(6)	0.37 ± .03	0.21 ± .02	0.16 ± .02	Yes
25	-	(13)	0.13 ± .03	0.69 ± .04	0.60 ± .02	-
25	1	(9)	0.15 ± .04	0.57 ± .03	0.49 ± .03	No
25 x 2 days	-	(15)	0.14 ± .04	0.80 ± .06	0.72 ± .07	-
25 x 2 days	1	(9)	0.12 ± .03	0.76 ± .03	0.55 ± .05	No

TABLE 5

Failure of Pretreatment with Pargyline to Block ACTH Hypersecretion Induced by Cold-exposure or Chlorpromazine

Numbers in parentheses represent number of animals studied. All values are mean ± S.D.; underlined values are significantly different from corresponding controls ($p < .05$). Animals were exposed to cold (4°C) or given chlorpromazine (15 mg/kg, i.p.) 18 hours after pretreatment with pargyline (2 doses, 25 mg/kg, i.p., 18 hours apart), then killed 3 hours after chlorpromazine or after 2 hours of cold-exposure.

		Plasma Corticosterone μg/ml
Control	(20)	0.15 ± .05
" + Cold-Exposure	(7)	0.42 ± .04
" + Chlorpromazine	(10)	0.39 ± .04
Pargyline	(15)	0.14 ± .04
" + Cold-Exposure	(13)	0.40 ± .03
" + Chlorpromazine	(9)	0.38 ± .03

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PRESSOR EFFECTS OF TRYPTAMINE ANALOGUES

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1 Methylation of tryptamine in the 1-position had little effect on the potency of the drug as a pressor agent in the intact anaesthetized rat.

2 In contrast, substitution of a benzo[b]thiophene ring system for the indole ring decreased the pressor activity.

3 Pretreatment of the animals with reserpine reduced the pressor effect of tryptamine and its benzo[b]thiophene analogue while increasing the effect of the 1-methylindole analogue.

4 Pretreatment with phenoxybenzamine reduced the pressor effect of all three compounds.

Introduction

Since the effects of tryptamine on guinea-pig isolated ileum were not inhibited by atropine or antihistamines, and, since a strip desensitized by tryptamine was insensitive to 5-hydroxytryptamine and vice versa, Gaddum (1953) concluded there must be a specific tryptamine receptor. Rocha e Silva, Valle & Picarelli (1953) defined a 5-hydroxytryptamine receptor in guinea-pig ileum differing from those for acetylcholine, bradykinin, histamine, nicotine and pilocarpine. Gaddum & Hameed (1954) reported similar receptors in rat uterus and in the vasculature of the rabbit isolated ear. Gaddum & Picarelli (1957) defined two types of tryptamine receptor in the guinea-pig ileum, one blocked by morphine and the other by phenoxybenzamine.

The effect of tryptamine on blood pressure was first noted by Laidlaw (1912). The effects of tryptamine as a pressor substance have been compared to those of 5-hydroxytryptamine by Reid (1951), Reid & Rand (1951) and Page (1952) and ourselves (Hixson, Bosin, Zabik & Maickel, 1973). More recently, Winter, Gessner & Godse (1967) examined the effects of a series of amines on the rat isolated fundus preparation, and found that tryptamine and its benzo[b]thiophene analogue had higher relative intrinsic activities but lower affinities than 5-hydroxytryptamine.

Methods

Adult, male Sprague-Dawley rats (300–350 g) were obtained from Murphy Breeding Laboratories, Plainfield, Indiana, U.S.A. and maintained on Purina Lab Chow and tap water *ad lib* for 7–10 days before experimental use. Pentobarbitone sodium (Nembutal), reserpine (Serpasil), heparin, and tryptamine hydrochloride were purchased from commercial sources. Phenoxybenzamine hydrochloride (Dibenzyline) was kindly supplied by Smith-Kline and French Laboratories. 3-(2-Aminoethyl)benzo[b]thiophene (tryptamine-S), and 1-methyl-3-(2-aminoethyl)-indole (tryptamine-1-Me) were synthesized and supplied by Dr E. Campaigne, Department of Chemistry, Indiana University.

Surgical procedure

Animals were anaesthetized with pentobarbitone, intraperitoneally as follows: controls—60 mg/kg; reserpine pretreated—50 mg/kg; phenoxybenzamine pretreated—45 mg/kg. When pinching the tail elicited no response, the rats were placed on their backs on a small animal operating board (Interex Corp.). The trachea was cannulated to insure a free airway and the common carotid artery was ligated at the superior end then clamped at the inferior end. A cannula of PE 50 tubing was inserted into the artery through a small incision, attached to a 23 gauge needle, and connected via a three-way stopcock to a Statham strain gauge model P23AA and to the pressure bottle of a mercury manometer system. The system was filled with 0.93% w/v NaCl solution containing heparin 1000 u/ml. The strain gauge was connected to the coupler of an Offner Type RB Dynograph (Beckman Instrument, Inc.), or, via a Bridge Preamplifier (Narco Biosystems, Inc.) to a Physiograph model PMP-4A (Narco Biosystems, Inc.). The system was flushed briefly with the heparin/saline solution, and blood pressure recorded to confirm the intact system.

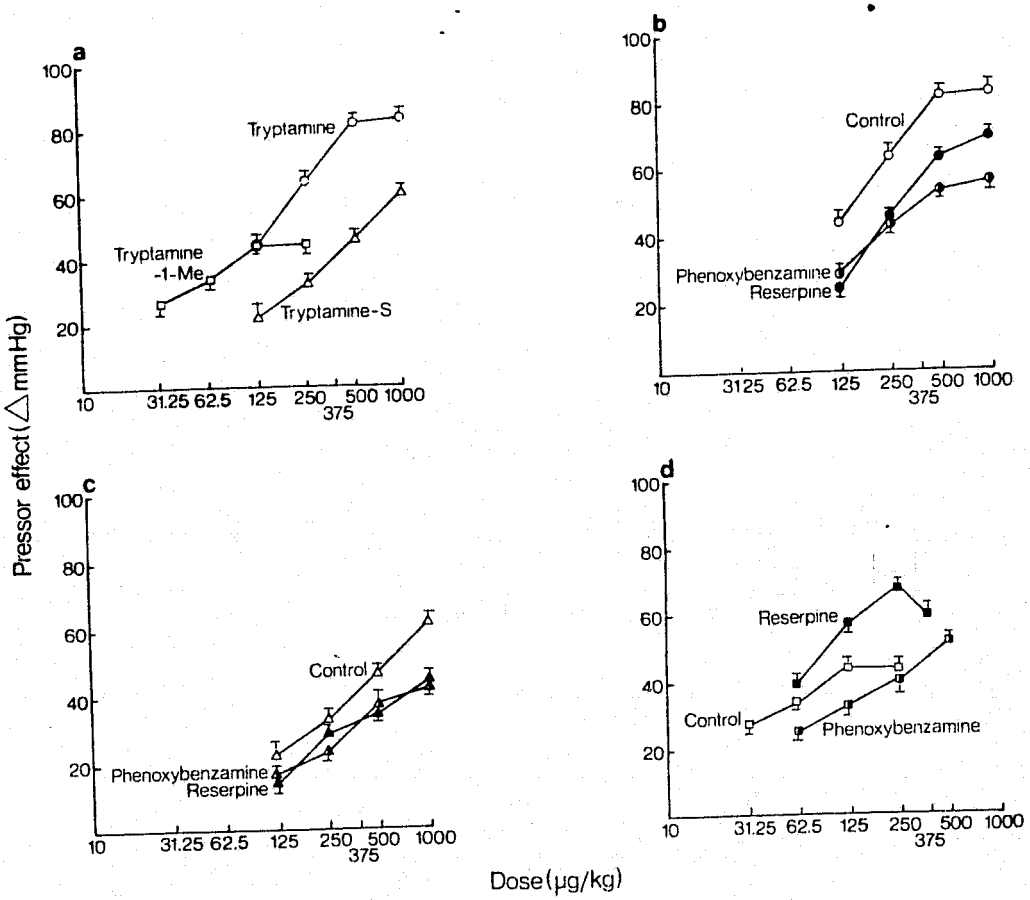

Figure 1 Pressor effects of tryptamine and analogues. Baseline blood pressure values were 110 ± 5 mmHg in control rats, 60 ± 4 mmHg in reserpine-pretreated rats, and 86 ± 5 mmHg in phenoxybenzamine-pretreated rats. (a) Effects of tryptamine and analogues in control rats; (b) effects of pretreatments on pressor responses to tryptamine; (c) tryptamine-S, and (d) tryptamine-1-Me. Vertical bars indicate s.e. mean.

The external jugular vein was then exposed for a distance of 1 to 1.5 cm. A cannula of PE 50 tubing was inserted, attached to a 23 gauge needle and connected via a three-way stopcock to a 3 ml syringe containing a flushing solution of the heparin/saline solution used above, and a 1 ml syringe containing the test drug.

Testing method

Test drugs were made up in the heparin/saline solution such that a volume of 0.1 ml contained a specific dose of the drug. Each of four animals was given four doses of drug four times, in a Latin square design, over a period of 1–2 hours. In addition, a Latin square was designed based on the sequence of the four cycles, so that each animal received a different sequence of cycles. Reserpine (10 mg/kg i.p.) was given 22–26 h before testing. Phenoxybenzamine (5 mg/kg i.p.) was given 1.5 h before testing.

Results

In control animals tryptamine caused a significant pressor response over a range of 125–1000 μg/kg. Both of the analogues also showed a pressor effect; tryptamine-S was less potent than tryptamine, while tryptamine-1-Me had a similar potency to tryptamine (Figure 1a). Transitory respiratory paralysis was seen occasionally after 500 μg/kg and usually after 1000 μg/kg of tryptamine and tryptamine-S. Tryptamine-1-Me was more toxic; 1000 and

500 μg/kg invariably caused a fatal cessation of respiration.

Pretreatment of rats with a single dose of reserpine reduced the pressor effects of typtamine and trypamine-S significantly (Figures 1b, c). Respiratory paralysis of brief duration occurred after the higher doses with both tryptamine and tryptamine-S. In contrast, the pressor responses to the doses of tryptamine-1-Me given to reserpine-treated animals, 62.5, 125 and 250 μg/kg, were greater in the same doses than in normal animals (Figure 1c).

In animals pretreated with phenoxybenzamine, the pressor effects of all three compounds were significantly reduced when compared to their actions in control animals (Figures 1b, c, d). Of particular interest is the fact that respiratory paralysis due to the pressor agents was a rare occurrence after phenoxybenzamine pretreatment.

Discussion

The scientific literature contains a variety of references to the existence of tryptamine receptors (Erspamer, 1954). However, it is difficult to extrapolate from responses of isolated preparations to those of the vasculature in the intact animal. While tryptamine may interact with its own specific peripheral receptor(s) to increase blood pressure, one cannot rule out the possibility of a centrally-mediated component.

Since the dose-response curves were not parallel the relative potencies of the analogues depended to some extent on the dose. Tryptamine-S was less potent than the other two compounds. Both pretreatments (reserpine and phenoxybenzamine) reduced the pressor effects of tryptamine and tryptamine-S. In contrast, reserpine pretreatment increased the pressor response to tryptamine-1-Me, while phenoxybenzamine caused a decrease in the pressor activity of this analogue. These results argue against the three agonists acting on a single receptor.

Chiu, Harrison, Maickel & Bosin (1973) have reported that the tryptamine analogues have lipid solubilities in the order: tryptamine-S > tryptamine-1-Me > tryptamine; the pK_a values and percent ionized at pH 7.4 for the three agonists are virtually identical. Thus, the differences in pharmacological activity described in this paper cannot be due merely to physicochemical differences; therefore, the differences in potency must be due to differences in the mechanism(s) by which these agonists exert their effect on blood pressure. It has been shown that tryptamine does cross the blood-brain barrier (Erspamer, 1961). The physicochemical characteristics of the two analogues indicate that they should also cross the barrier. Thus, a central component may be involved. From the data presented here, it is impossible to speculate as to what type of receptor or combination of receptors is involved.

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INTERACTIONS OF CAFFEINE WITH VARIOUS AMPHETAMINES ON RAT FOOD CONSUMPTION AND AVOIDANCE RESPONDING*

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Summary—Pretreatment of rats with caffeine potentiated the actions of the *p*-chloro- and *p*-methyl analogues of amphetamine and benzphetamine as depressants of deprivation-induced food consumption, although no such effects were seen with the unsubstituted compounds. Similar caffeine pretreatment completely antagonized the stimulant effect of amphetamine and *p*-chloroamphetamine on continuous avoidance responding. In contrast, combined dosage of caffeine potentiated the actions of *p*-methylamphetamine, or benzphetamine, drugs normally showing decreased avoidance responding. Caffeine, in combination with benzphetamine, caused a slight decrease in avoidance responding.

Caffeine is generally considered to be a stimulant drug, with actions not too dissimilar in man from those of the amphetamines (Weiss and Laties, 1962). Since caffeine is chemically a member of the structural series known as xanthines, it also has the ability to inhibit the phosphodiesterase enzyme known to be responsible for the degradation of cyclic 3′5′-adenosine monophosphate (cyclic AMP) (Robison, Butcher and Sutherland 1971a; Robison, Nahas and Triner, 1971b). A previous report from this laboratory (Cox and Maickel, 1972) compared the effects of a variety of phenylethylamines on deprivation-induced food consumption and continuous avoidance responding in rats, Substitution of a chlorine atom in the para-position of amphetamine increased the anorexigenic potency with little effect on avoidance responding. A *p*-methyl group reduced anorexigenic potency and changed the effects on avoidance responding from stimulant to depressant. Substitution of either a chlorine or methyl group in the para-position of benzphetamine reduced the anorexigenic potency and conferred significant depressant activity on the compound. Thus, it seemed of interest to examine the effects of the combination of caffeine and several amphetamines on the dual test system of deprivation-induced food consumption and continuous avoidance responding.

MATERIALS AND METHODS

All experiments were performed using adult, male Sprague–Dawley rats (Hormone Assay Laboratories, Chicago) weighing 250–300 g and maintained on a diet of Purina laboratory chow and tap water *ad lib.* for at least 10 days prior to the beginning of experimental use. The behavioural methods and statistical procedures used were identical to those previously reported (Cox and Maickel, 1972).

Free-feeding

The rats were housed in individual metabolism cages (20.3 × 24.1 × 17.8 cm) with continuous access to water. Rats were deprived of food for 22 hr; thus, being required to consume their total daily ration in the remaining 2 hr. Dry Purina chow was available during this time in spill-proof jars and the rats were required to put their heads through a small hole in order to obtain the food. This feeding schedule was used for the duration of a particular experiment (8–10 weeks). The establishment of a consistent day-to-day intake (15–20 g) took 10–14 days, during which time the rats lost 15–20% of their initial body weight. After base-line weights were attained, the weight of the rats did not change significantly for the duration of the experimental session.

Groups of 24 rats were conditioned to the feeding schedule as described above. After base-line weights were established, the rats were divided into four groups of six rats each. Each group was tested with four doses of a single drug. All drugs were administered by intraperitoneal injection in volumes of 0.1 ml/100 g of body weight. 30 min before the scheduled feeding time in order to allow sufficient time for onset of action of the drugs. Solutions of the drugs were prepared in distilled water in concentrations such that 0.1 ml/100 g of body weight resulted in the correct dose in terms of milligrams of free base per kilogram of body weight.

Results were calculated in terms of percentage of control with each animal serving as its own control. The control value was the mean intake for the three sessions immediately prior to drug administration. Dose–responses curves were determined with each data point representing mean ± S.E.M. of five or six animals.

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Continuous avoidance

Experiments were carried out in four boxes with sides, back and top of metal and a Plexiglas door to permit viewing of the subject. Each box had a grid floor 28.0 cm long and 26.7 cm wide with 1.0 cm grids placed 2.0 cm apart (centre to centre) for delivering shock to the feet and was equipped with two levers on the wall at one end, mounted 7.6 cm above the floor and 14.0 cm apart (centre to centre). Pressing the lever near the front wall postponed shock (avoidance response); pressing the lever near the back wall terminated the shock (escape response). Shock (350 V and 0.6 mA short-circuit current provided by a Grason–Stadler E-1064 GS shock generator) was delivered to the grids, walls and levers via a shock scrambler circuit. The boxes were enclosed in insulated shells to isolate them from external visual stimuli. Extraneous auditory stimuli were masked by continuous 'white' noise in each box and in the room. Programming and recording of experiments were accomplished by automatic switching and timing circuits, cumulative recorders and electrical impulse counters.

The response–shock interval was 40 sec: shock onset was delayed for 40 sec each time the rat pressed the avoidance lever when the shock was not on. Whenever the time since the last avoidance response exceeded 40 sec, shock came on and remained on until the rat pressed the escape lever or until the shock terminated automatically after 3 sec. The shock–shock interval was 20 sec: after a shock further shocks were presented at 20-sec intervals until the rat once again pressed the avoidance lever, when the shock was not ongoing, to return to the response–shock interval.

Individual rats were trained and tested at five-day intervals at the same time of day and in the same experimental box. The duration of each session was 4.5 hr with the initial 30 min serving as a 'warm-up' period. Approximately 30% of the rats were discarded because they failed to acquire the appropriate responses; i.e. escape and avoidance by the third session. The performance of the remaining rats was stable enough for drug testing after six sessions, which included two saline treatment sessions. Base lines were considered stable when the avoidance rate was consistent (160 ± 30 responses/hr) for each animal over the period $t = 1$ hr to $t = 3$ hr of three consecutive sessions. All drugs were administered by intraperitoneal injection, in solutions as described above, immediately following the 30 min 'warm-up' period.

Dose-response curves were determined; each curve was based on four rats with each data point representing mean ± S.E.M. of four or five replications. The dose for each rat on a particular session was dictated by a balanced 4×5 Latin square. With this technique, each rat received each dose of the drug in a different sequence. As a result, carryover effects could be determined if they existed. The results were calculated as percentage of control avoidance rate with each animal serving as its own control. The control avoidance rate was the mean of the avoidance rates for the three sessions immediately prior to the drug administration. The period of time selected was that equivalent to the time in the free-feeding situation (i.e. 30 min–2.5 hr after drug).

Statistical methods

With the determined data points, linear dose–response curves were fitted for each drug, in both the free-feeding and the continuous avoidance situation, by the method of least squares.

In order to compare the various drugs, a response dose 50 (RD_{50}) has been estimated for each drug. This is the estimated dose of a particular drug which would be required to produce a 50% decrease in food intake or a 50% change in avoidance rate. The more familiar term is the effective dose 50 (ED_{50}) which is an estimate of the dose which is effective in 50% of the animals. The ED_{50} estimate is based on quantal data and is therefore amenable to statistical manipulations. However, the RD_{50} estimate is based on a graded response and cannot be compared directly to determine significant differences. However, it does permit comparison of the relative potency of the drugs being studied.

Caffeine (free base) was dissolved in 1.0 M aqueous solutions of sodium benzoate (both obtained from commercial suppliers and of reagent grade) such that injections of 0.1 ml of the resulting solution per 100 g body weight resulted in the appropriate dose in mg/kg. The effect of caffeine on food intake was determined by administering the drug intraperitoneally, 30 min prior to the point of food access. In the continuous avoidance situation, caffeine was administered immediately after the 30 min 'warm-up' period.

RESULTS

Effects of caffeine on food intake and avoidance responding

The data obtained on food intake with doses of caffeine over the range 12–108 mg/kg are presented in Figure 1A. As can be seen, caffeine had no significant effect on food intake at doses less than 60 mg/kg; above this dose, a rapid decrement in food consumption was observed. In the continuous avoidance situation, no evidence for stimulation (in the form of increased avoidance responding) was seen at intraperitoneal doses of caffeine as low as 8 mg/kg (Fig. 1B). In fact, doses of less than 16 mg/kg significantly reduced the rate of avoidance responding in a dose-dependent manner. This effect was not accompanied by decreased escape responding or gross overt sedation as would be expected for a typical depressant drug.

Effects of caffeine pretreatment on anorectic action of amphetamines

An intraperitoneal dose of caffeine of 45 mg/kg was chosen as the interaction dose since it had no signifi-

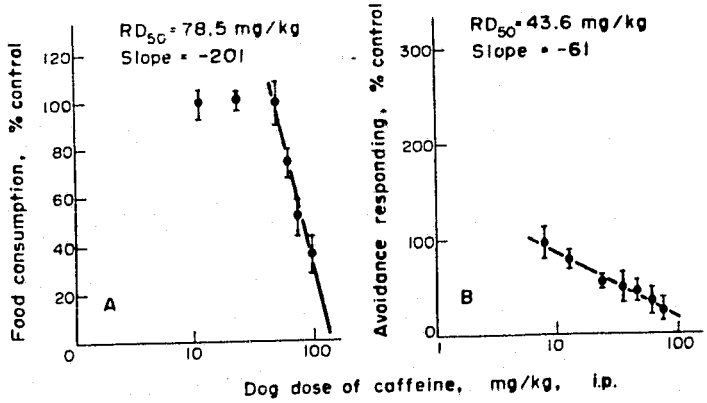

Fig. 1. Dose–response data for caffeine on food consumption (A) and avoidance responding (B) test systems. Each point is the mean value of 5–6 rats treated as described in the text; vertical bars are S.E.M. Lines drawn are those obtained from linear regression analysis.

cant action on food consumption while causing a significant reduction (approx. 50%) in avoidance responding. This dose was administered to groups of rats 60 min prior to exposure to food in the consummatory system previously described (Cox and Maickel, 1972). The amphetamines were given 30 min after the caffeine at 3 dose levels, in the ratio n:2n:4n. This permitted comparison with noncaffeine-pretreated animals over a similar dose–response range. The results obtained, presented in terms of RD_{50} values and slopes of the dose–response curves, are shown in Table 1. For both amphetamine and benzphetamine, caffeine pretreatment had no significant effect on anorectic activity. However, in the case of all four para-substituted compounds, the caffeine pretreatment produced a significant potentiation of anorectic activity. Pretreatment with a dose of caffeine (9 mg/kg, i.p.) that was ineffective on both food consumption and avoidance responding had no effects on the actions of a subsequent dose of any of the agents. Pretreatment with intraperitoneal doses of caffeine larger than 45 mg/kg could not be tested due to obvious signs of toxicity.

Effects of caffeine pretreatment on avoidance responding action of amphetamines

Pretreatment of rats with a dose of 45 mg/kg of caffeine prior to administration of the amphetamines was also tested on continuous avoidance responding. This dose of caffeine alone would cause an approximately 50% reduction in the rate of avoidance responding (see Fig. 1). The results obtained with the drug combinations are shown in Table 2. These results are more complex to interpret than the anorectic data, since the slopes change significantly for all of the compounds. Both amphetamine and its *p*-chloro analogue increased the rate of avoidance responding, and these actions were completely antagonized by caffeine pretreatment. In contrast, *p*-methylamphetamine caused a decreased rate of avoidance responding, an effect that was slightly potentiated by caffeine.

Table 1. Effects of caffeine pretreatment on anorectic action of amphetamines

Treatment	n	RD_{50} (95% C.L.) (mg/kg)	Slope + S.E.
Amphetamine	6	1.9 (1.7–2.0)	−84 ± 6
Amphetamine + Caffeine	6	1.4 (1.0–2.0)	−103 ± 23
p-Chloroamphetamine	6	1.5 (1.3–1.7)	−178 ± 26
p-Chloroamphetamine + Caffeine	6	0.6 (0.4–0.8)†	−93 ± 23
p-Methylamphetamine	5	8.8 (7.3–10.6)	−111 ± 16
p-Methylamphetamine + Caffeine	6	2.7 (2.2–3.3)*	−125 ± 20
Benzphetamine	6	12.5 (10.8–13.9)	−103 ± 8
Benzphetamine + Caffeine	6	12.2 (6.7–18.2)	−93 ± 27
p-Chlorobenzphetamine	5	19.7 (16.0–23.7)	−138 ± 20
p-Chlorobenzphetamine + Caffeine	6	3.4 (2.8–3.9)*	−103 ± 12
p-Methylbenzphetamine	6	27.8 (19.4–39.7)	−106 ± 22
p-Methylbenzphetamine + Caffeine	5	15.3 (10.8–21.8)*	−85 ± 13

* Caffeine pretreatment significantly different from no pretreatment ($P < 0.05$) and slopes are parallel.

† Slopes of drug and drug + caffeine are not parallel. Student t-comparison of all three doses with and without caffeine pretreatment were significant ($P < 0.05$).

Drugs were administered as indicated in the text. RD_{50} values, 95% confidence limits (C.L.) and slopes ± S.E. were determined as described by Cox and Maickel (1972).

Table 2. Effects of caffeine pretreatment on avoidance responding action of amphetamines

Treatment	n	RD_{50} (95% C.L.) (mg/kg)	Slope ± S.E.
Amphetamine	5	0.8 (0.3–2.3)	+232 ± 82
Amphetamine + Caffeine	5	32.5 (27.2–37.7)	−33 ± 12
p-Chloroamphetamine	5	0.8 (0.7–0.9)	+186 ± 41
p-Chloroamphetamine + Caffeine	5	20.6 (17.7–23.3)	−9 ± 5
p-Methylamphetamine	5	2.4 (1.3–4.5)	123 ± 39
p-Methylamphetamine + Caffeine	4	1.8 (1.3–2.9)	−66 ± 20
Benzphetamine	5	>32.0	0
Benzphetamine + Caffeine	4	6.2 (5.1–7.4)	+58 ± 17
p-Chlorobenzphetamine	5	25.1 (22.3–28.1)	−246 ± 35
p-Chlorobenzphetamine + Caffeine	4	16.0 (11.1–21.1)	−17 ± 11
p-Methylbenzphetamine	4	38.5 (20.3–72.9)	−70 ± 15
p-Methylbenzphetamine + Caffeine	5	2.4 (1.7–3.0)	−17 ± 9

Drugs were administered as indicated in the text. RD_{50} values, 95% confidence limits (C.L.), and slopes ± S.E. were determined as described by Cox and Maickel (1972)

In the benzphetamine series, the parent compound had no effect on the rate of avoidance responding at doses up to 32 mg/kg. Pretreatment with caffeine caused a decrement in avoidance response rate; however, the positive slope creates the possibility for two RD_{50} values, one of 6.2 mg/kg for a 50% decrease in responding and a theoretical additional one of 326 mg/kg for a 50% increase in responding. Both *p*-chlorobenzphetamine and *p*-methylbenzphetamine depressed avoidance responding rates themselves; these effects were potentiated by the caffeine pretreatment. As with the food consumption studies, attempts to utilize a lower dose of caffeine (9 mg/kg) were ineffective. Similarly, the combination of amphetamines with doses of caffeine of 60 mg/kg or higher caused severe toxicity and some deaths.

DISCUSSION

The mechanism(s) by which phenylethylamines such as amphetamine and benzphetamine produce anorexia in man and animals has been the basis for an extensive amount of research in recent years (Costa and Garattini, 1970). In particular, much emphasis has been placed on the possible role of brain norepinephrine in control of feeding behaviour (Morgane, 1969) and the likelihood that amphetamines may act by interacting with brain noradrenergic function (Modell, 1960). In addition, any consideration of biochemical phenomena related to catecholamines must consider the possibility that the adenyl cyclase–cyclic AMP system is somehow involved (Robison *et al.*, 1971a, b).

With this background in mind, the present paper reports on the interactions of caffeine and several phenylethylamines with deprivation-induced food consumption and continuous avoidance responding. The former may be considered as a measure of anoretic activity (Cox and Maickel, 1972; Heise, 1964), and the latter as a measure of central stimulatory and depressant activity (Heise and Boff, 1962).

Caffeine itself is anoretic, although the doses required are so large that the action of this drug may be considered as related, not to a specific depression of appetite, but, to a general depression of activity. This conclusion is supported by the data in Figure 1 which show that intraperitoneal doses of caffeine greater than 12 mg/kg caused a significant reduction in avoidance responding. This depressant action on responding resembles that of chlorpromazine rather than a barbiturate, since there is no concurrent impairment of escape responding (Heise and Boff, 1962). Caffeine has also been shown to have a depressant effect, alone or in combination with ethanol, on other behavioural test systems (Alstott and Forney, 1971).

The two parent compounds chosen for this study were amphetamine and benzphetamine (N-methyl-N-benzylamphetamine), both of which are clinically used as anoretic agents (Costa and Garattini, 1970). In addition, the *p*-chloro and *p*-methyl analogues of these compounds were also studied. The results obtained in the deprivation-induced food consumption test are clear cut. Caffeine pretreatment, at a dose which was, by itself, ineffective was without effect on the activity of the parent compounds while markedly potentiating the effects of the para-substituted analogues (Table 1).

The actions of amphetamine and its *p*-chloro analogue, normally stimulants of avoidance responding, were completely antagonized by caffeine pretreatment, while action of the *p*-methyl analogue, normally a depressant of avoidance responding, was slightly potentiated (Table 2). Benzphetamine, normally without effect on avoidance responding at doses up to 24 mg/kg, showed significant depression of avoidance responding at low doses (< 16 mg/kg) in caffeine pretreated animals. Extrapolation of the dose–response line would suggest that doses greater than 200 mg/kg would cause increased avoidance responding. A preliminary test of 45 mg/kg of caffeine plus 72 mg/kg of benzphetamine led to severe toxicity and death of 2 out of 6 animals. The *p*-chloro and *p*-methyl analogues of benzphetamine are both depressants of avoidance responding of themselves; caffeine pretreatment significantly potentiated this action (Table 2).

The results are both interesting and complicated. The lack of effects of caffeine on the non-para-substituted amphetamine and benzphetamine (in terms of appetite depression) is in sharp contrast to the actions of caffeine on all four para-substituted compounds where the dose–response curve is shifted markedly to the left. The effects on continuous avoidance responding suggest a preponderance of the caffeine effect to negate the potent stimulatory effect of amphetamine.

Alternatively, one may consider that amphetamine was able to partially overcome the depressant actions of caffeine. Attempts to confirm either of these hypotheses with other doses of caffeine were unsuccessful. This dissociation of avoidance and anorectic actions lends further credence to the hypothesis that they are under separate controlling systems. The specific differences between the amphetamine (primary amine) series and the benzphetamine (tertiary amine) series will obviously require additional study. In the case of the para-substituted compounds, the marked potentiation of their anorectic action by caffeine suggests a possible role for cyclic AMP and such a possibility also deserves further study.

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INTERACTIONS OF TRICYCLIC ANTIDEPRESSANT DRUGS WITH DEPRIVATION-INDUCED FLUID CONSUMPTION BY RATS*

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SUMMARY

Six tricyclic antidepressant drugs, representing both secondary and tertiary amines and four different ring systems, were examined for their effects on deprivation-induced fluid consumption by rats in a standardized test system. All compounds reduced fluid consumption with a potency ranking of: desipramine > amitriptyline > doxepin > nortriptyline > imipramine > protriptyline. In a similar test situation, pretreatment with PCPA markedly reduced fluid consumption at 24 hours; fluid consumption levels had not returned to normal by 15 days after dosage.

INTRODUCTION

The tricyclic antidepressant drugs have become very popular clinical agents since their introduction into therapeutic use some twenty years ago. Their clinical activity has often been attributed to interactions with central noradrenergic mechanisms, since they potentiate the peripheral response to exogenous catecholamines (Sigg, et al, 1963) and sympathetic nerve stimulation (Thoenen, et al, 1964). In addition, the tricyclic compounds have been shown to inhibit the uptake of exogenously administered NE (Hertting, et al, 1961). In this regard, Lahti and Maickel (1971) have shown that this effect is also shown by several phenothiazine tranquilizers and correlates with chemical structure rather than pharmacological activity.

A variety of behavioral actions have been shown to occur with tricyclic antidepressants. For example, Stein and Seifter (1961) showed that imipramine increased, in both magnitude and duration, the methamphetamine-induced increase in the rate of response of rats working for rewarding hypothalmic stimulation. In this test system, chlorpromazine had an opposite effect. Previous studies from this laboratory had demonstrated that phenothiazine tranquilizers (Maickel, et al, 1968a), as well as cholinergic blockers and cholinergic stimulants (Gerald and Maickel, 1969) were effective in reducing the volume of fluid consumed by deprived rats. Since some of the widely observed clinical side effects of the tricyclic antidepressants represent anticholinergic actions such as blurred vision, dry mouth, and urinary retention, it seemed of interest to test a variety of these agents for their effects on fluid consumption. In addition, the possibility of examining the role of biogenic amines in the actions of the tricyclic antidepressants by using drug interaction studies also seemed a feasible route for experimental design.

MATERIALS AND METHODS

Adult (300-350 g) male, Sprague-Dawley rats (Murphy Breeding Laboratories, Plainfield, Indiana) were used in all experiments. The animals were maintained on tap water and Purina Rat Chow ad lib. for at least 1 week after arrival in the laboratory. All solutions for injection were made in glass distilled water such that a dose volume of 0.1 ml per 100 g body weight contained the desired dose; all injections were given intraperitoneally, 15 min prior to placing the animals in the drinking cages.

The procedures used to measure deprivation-induced water consumption were basically those of Gerald and Maickel (1969). Rats were placed in cages, 7 x 7 x 14 inches, identical to the home cages of the animals. The cages were suspended in individual compartments of a sound-proofed box with uniform fluorescent lighting. Constant circulation of air by blowers maintained uniform temperature in the compartments; the noise level of blowers served as a "white noise". Each cage contained a drinking tube connected to an external 50 ml buret filled with distilled water at the start of each test run and stoppered. As the animal consumed water, the change in volume was measured visually to the nearest 0.1 ml. The front door of each compartment was equipped with an eye-piece lense to permit visual observations of the rats without disturbing their behavioral performance. For at least 1 week prior to testing drug effects, rats were deprived of water daily for 23 hr prior to testing, then placed in the "drinking cages" and allowed to drink for 1 hr. Food was available ad lib. in the home cages but was not available in the drinking cages.

Drug trials were started only after the animals demonstrated stable baselines (less than 5% daily variation) of water intake. The schedule for drug studies was arranged so that the rats were run daily, with drugs administered every fifth day, and placebo doses on the intervening days. Water intake was recorded at 15, 30 and 60 min of the consummatory sessions; the greatest proportion of drinking occurred in the first 15 min in all cases.

Brain levels of serotonin (5HT) and norepinephrine (NE) were determined in whole brain as reported by Maickel, et al (1968b). p-Chlorophenylalanine (PCPA) and α-methyltryosine (αMT) were purchased from Regis Chemical Company.

Groups of 8 rats were run in each system; an n of < 8 rats indicates that a value or values was discarded because of equipment malfunction. All data are reported as means ± S.D. Statistical comparisons were carried out by the correlated t-test for paired scores. The day prior to each drug day was used as the pre-drug value for that drug run. Each animal served as its own control; per cent of pre-drug values are the mean of values calculated for each animal. Dose-response curves were determined by linear regression analysis and slopes and RD_{50} values were calculated as described by Cox (1970) and Maickel and Webb (1972).

RESULTS

Effects of Tricyclic Compounds on Deprivation-Induced Fluid Consumption.

The results obtained from dose-response studies of single acute dosages of six tricyclic antidepressants are presented in Table 1. With the exception of the lowest doses of nortriptyline and protriptyline, all doses of each drug tested significantly decreased the volume of fluid consumed by the deprived rats. When the data were subjected to linear regression analysis, semilogarithmic plots yielded straight lines with slope and Rd_{50} values as presented

in Table 2; several striking relationships can be seen. First of all, although the potency ranking for the RD_{50} values shows a tenfold range (from 31.6 μmoles/kg for desipramine to 300.3 μmoles/kg for imipramine) the compounds fall into two groups. Four of the drugs (desipramine, amitriptyline, doxepin, nortriptyline) have molar RD_{50} values in the range of 31.6 to 59.7. In contrast, the molar RD_{50} values for protriptyline and imipramine are much higher, 158.2 and 300.3 μmoles/kg, respectively. Secondly, when the slopes of the dose-response curves are compared, the compounds fall into three distinct groups: imipramine and protriptyline (the most shallow), amitriptyline and desimpramine (intermediate), and doxepin and nortriptyline (the steepest slopes). Tests for slope parallelism indicate that the groups are significantly different from each other although the curves within a group are not significantly non-parallel.

In addition to these findings, one other aspect should be mentioned. On the post-drug day, a significant carryover effect, i.e., a reduction in fluid consumption, was seen with desimpramine (at the 20 mg/kg dose) and with protriptyline (at the 20 and 30 mg/kg doses).

Effects of αMT Pretreatment on Actions of Protriptyline.

In order to assess the effects of alterations in brain norepinephrine (NE) on the actions of protriptyline, rats were pretreated with αMT (150 mg/kg, in oil, i.p. at 12 hour intervals) with the last dose 6 hrs prior to testing, as reported by Miller, et al (1970). This regimen reduced brain NE values by 66%; the animals also showed a significantly reduced fluid intake as compared to pre-drug baseline (Table 3). Administration of protriptyline had no effects on brain NE or 5HT levels. The reduction in fluid consumption in αMT and protriptyline rats was approximately additive for the effects of the individual drugs.

Effects of PCPA Pretreatment on Actions of Protriptyline

The situation with PCPA was more complex, since Miller and Maickel (1969) have shown that differential effects on brain 5HT and NE with respect to time after dosage. Rats were treated with PCPA (400 mg/kg, i.p., in oil) as described by Miller and Maickel (1969) then dosed with protriptyline on days 1, 8, and 15. The results (Table 4) show that on day 1 there was a significant reduction in fluid consumption; this response was essentially normal on days 8 and 15 after PCPA treatment. The effects of protriptyline in the PCPA-pretreated animals were not significantly different from those seen in controls. On day 1, this means that the effect of protriptyline were antagonized by the PCPA. The effects of PCPA on brain biogenic amines were similar to those previously reported, namely a reduction in both 5HT and NE on day 1, a reduction in 5HT only on day 8, and a return of both amines to normal values by day 15.

DISCUSSION

The results presented herein lend further credence to the conclusion that the effects of drugs on deprivation-induced fluid consumption reflect the complexity of the thirst-consumption system. Certainly, the data presented in Tables 1 and 2 clearly demonstrate that all of the tricyclic antidepressant drugs tested are able to produce a dose-dependent decrement in the volume of water consumed by deprived rats. Several facets of the data, however are difficult to explain on the basis of known pharmacological actions of the drugs. For example, the dose-response curves for imipramine and protriptyline are parallel to each other; the dose-response curves for amitriptyline and desipramine, and for doxepin and nortriptyline represent similar parallel pairs.

The lack of overall parallelism makes it difficult to draw potency

comparisons within the series as has been done for cholinergic blockers by Gerald and Maickel (1969) or antihistamines (Gerald and Maickel, 1972). Nonetheless, the RD_{50} values presented in Table 2 indicate that imipramine and protriptyline are considerably less potent than the other four compounds tested. In this regard, it should be noted that, in these acute studies, no correlation could be observed between the three compounds that are tertiary amines (amitriptyline, doxepin, imipramine) or the three that are secondary amines (desipramine, nortriptyline, protriptyline) in terms of slope or RD_{50}.

Pretreatment of rats with PCPA caused a time-differential alteration of brain biogenic amines similar to that reported by Miller and Maickel (1969), that is, a significant reduction of both brain 5HT and brain NE at one day after administration of PCPA, a reduction only of brain 5HT at eight days, and normal brain levels of both amines at 15 days after PCPA. Tests of deprivation-induced fluid consumption over this period showed a marked reduction (to 58.9% of pre-drug levels) in fluid intake on the first day post PCPA, followed by a return to levels approaching normal (80-90% of pre-drug) by the third day post PCPA and a plateau effect at these levels through the 17th day (Table 4). Administration of protriptyline (20 mg/kg, i.p.) on days 1, 8, or 15 after PCPA evoked no additional reductions in fluid consumption; however, the values observed were not significantly different from protriptyline alone.

Pretreatment of animals with αMT led to a reduction in brain NE similar to that reported by Miller, et al (1970), and a concommitant reduction in deprivation-induced fluid consumption (Table 3). Administration of protriptyline (20 mg/kg, i.p.) to these animals caused a further reduction in the volume of fluid that was significantly greater than that seen with either

αMT or protriptyline alone. In fact, the reduction observed with the drug combination seemed to approximate what might be expected on the basis of a simple additive action of the two compounds.

Based on these studies, one may infer that the actions of protriptyline on deprivation-induced fluid consumption are not greatly influenced by modest alterations in brain NE caused by αMT. The results with PCPA are difficult to interpret. It does, however, seem that the PCPA effect is manifested by a reduction in the ability of protriptyline to reduce fluid consumption on day 1, that is, when both brain 5HT and brain NE are decreased.

Several recent reviews of the general area of thirst and fluid consumption have discussed the overall problems of drug effects on these systems (Epstein, et al, 1973; Fitzsimons, 1972). If one assumes that the deprivation schedule used has produced an extracellular dehydration with a consequent drinking elicited by the hypertensive response produced by the angiotensin system, subsequent administration of agents elevating blood pressure might be expected to increase fluid consumption, while hypotensive agents should cause a reduction in consumption. This might explain the reduced consumption caused by the αMT pretreatment, since lowered body NE stores should result in a modest hypotension. However, the effects of the tricyclic antidepressants should be hypertensive; nevertheless, these compounds also depress fluid consumption. The effects of PCPA at 1 day after dosage could also reflect decreased central and peripheral NE and a consequent hypotensive state. By 8 and 15 days, this would be expected to disappear as NE levels return to normal values. The inability to find simple explanations for the actions of the tricyclic antidepressants alone and their interactions with αMT and PCPA would seem to confirm the complexity of thirst mechanism(s), especially in terms of their neural control, as concluded by Fitzsimons (1972).

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Eli Lilly - nortriptyline hydrochloride (Aventyl®),

Merck, Shapr & Dohme - amitriptyline hydrochloride (Elavil®) and protriptyline hydrochloride (Vivactil®),

Pennwalt Corporation - doxepin hydrochloride (Adepin®)

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TABLE 1

Effects of Tricyclic Antidepressants on Deprivation-Induced Fluid Consumption.

Animals were treated as described in Materials and Methods section; drug was administered 15 min prior to start of drinking session. Results are expressed as mean ± S.D. of the values obtained. Statistical comparisons were made by the correlated t-test for paired scores; significant differences from the corresponding pre-drug values ($P < 0.05$) are indicated by*.

Drug	N	Dose mg/kg	Volume Consumed Pre-drug ml	Drug ml	% of Pre-drug
Amitriptyline	8	5	20.1 ± 2.1	14.9 ± 3.0	74.2 ± 15.6*
	15	10	22.6 ± 3.4	13.9 ± 3.8	60.5 ± 17.9*
	16	20	20.6 ± 4.4	7.1 ± 5.1	36.7 ± 27.1*
Desipramine	16	5	21.9 ± 3.0	13.1 ± 4.9	66.2 ± 18.5*
	16	10	21.0 ± 2.9	8.7 ± 4.1	40.8 ± 16.8*
	8	20	20.3 ± 2.7	5.8 ± 2.0	29.1 ± 11.8*
Doxepin	8	5	19.2 ± 3.0	15.2 ± 3.5	80.8 ± 17.0*
	8	10	22.7 ± 3.8	16.0 ± 4.1	70.3 ± 10.6*
	8	20	22.0 ± 3.2	10.8 ± 5.1	48.3 ± 17.1*
	8	30	21.2 ± 3.0	4.7 ± 3.5	21.7 ± 14.7*
Imipramine	16	5	21.1 ± 3.8	17.6 ± 4.3	83.8 ± 14.4*
	15	10	19.1 ± 3.4	13.1 ± 3.1	72.7 ± 11.3*
	8	20	19.2 ± 3.9	12.0 ± 2.1	62.4 ± 11.7*
Nortriptyline	16	5	20.5 ± 2.1	18.0 ± 3.6	88.7 ± 11.3
	16	10	21.1 ± 3.8	14.8 ± 2.4	70.7 ± 11.0*
	8	20	22.4 ± 4.3	7.9 ± 2.5	39.0 ± 16.2*
Protriptyline	8	5	18.8 ± 1.9	19.2 ± 3.1	102.1 ± 12.5
	8	10	19.2 ± 2.5	16.2 ± 1.8	84.1 ± 10.1*
	8	20	18.0 ± 4.4	12.8 ± 1.8	73.9 ± 16.1*
	8	30	15.1 ± 4.6	10.5 ± 2.3	69.5 ± 13.4*

TABLE 2

Analysis of Dose-Response Effects of Tricyclic Antidepressants On Deprivation-Induced Fluid Consumption

Slopes were determined by linear regression analysis of the data presented in Table 1. RD_{50} values represent the dose estimated to cause a 50% reduction in fluid consumption and have been derived mathematically from the linear regression analysis.

		RD_{50}	
Drug	Slope	mg/kg	μmoles/kg
Desipramine	-61.6	8.4	31.6
Amitriptyline	-62.3	13.0	48.0
Doxepin	-78.3	15.5	55.6
Nortriptyline	-82.5	15.7	59.7
Imipramine	-35.5	44.3	158.2
Protriptyline	-41.6	79.0	300.3

TABLE 3

Effects of αMT on Action of Protriptyline on Deprivation-Induced Fluid Consumption

Animals were pretreated with αMT as described in the text. Results are expressed as mean ± S.D. of the values obtained. Statistical comparisons were made by the correlated t-test for paired scores; significant differences from the corresponding control values ($p < .05$) are indicated by *. Animals were killed at the termination of the drinking session for brain amine determinations.

			Volume Consumed			Brain Amines	
Treatment I	Treatment II	N	Pre-Drug ml ± S.D.	Drug ml ± S.D.	% of Pre-Drug mean % ± S.D.	5HT ug/g	NE ug/g
Water	Water	8	21.7±2.9	21.4±2.1	97.4±5.5	0.70±.06	0.49±.05
Water	Protriptyline (20 mg/kg)	8	19.3±3.6	13.1±1.5	67.6±13.3*	0.68±.06	0.50±.04
αMT	Water	6	20.8±2.0	16.1±2.7	77.0±13.4*	0.71±.08	0.24±.05*
αMT	Protriptyline (20 mg/kg)	6	17.9±1.8	9.2±3.6	44.8±14.5*	0.69±.04	0.23±.05*

TABLE 4

Effects of αMT on Action of Protriptyline on Deprivation-Induced Fluid Consumption

Animals were pretreated with PCPA as described in the text. Results are expressed as mean ± S.D. of the values obtained. Statistical comparisons were made by the correlated t-test for paired scores; significant differences from the corresponding control values ($p < .05$) are indicated by *. Animals were killed at the termination of the drinking session for brain amine determinations.

			Volume Consumed			Brain Amines	
Treatment I	Treatment II	N	Pre-Drug ml ± S.D.	Drug ml ± S.D.	% of Pre-Drug mean % ± S.D.	5HT ug/g	NE ug/g
Peanut oil	Water	8	19.3±1.9	19.7±2.7	101.2±12.0	0.71±.04	0.48±.04
Peanut oil	Protriptyline (20 mg/kg)	8	19.6±3.1	12.8±2.1	65.8±10.1*	0.73±.08	0.49±.07
PCPA-1	Water	8	17.0±3.4	13.1±2.7	79.2±20.4*	0.29±.05*	0.32±.06*
PCPA-8	Water	8	19.9±2.9	19.2±2.5	97.0±10.4	0.37±.03*	0.47±.08
PCPA-15	Water	8	20.2±3.1	18.9±2.3	94.9±06.4	0.67±.07	0.51±.05
PCPA-1	Protriptyline (20 mg/kg)	6	14.3±3.1	9.1±2.0	66.7±19.7	0.30±.05	0.31±.07*
PCPA-8	Protriptyline (20 mg/kg)	6	20.8±1.3	13.5±5.1	65.3±24.8	0.39±.04*	0.46±.06
PCPA-15	Protriptyline	6	22.1±3.8	12.6±4.6	57.9±22.1*	0.69±.08	0.47±.03

STRUCTURE-ACTIVITY RELATIONSHIPS IN COMPOUNDS ANALAGOUS TO 5,6-DIHYDROXYTRYPTAMINE

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The disruption of normal neuronal transmission by interference with some aspect of neurotransmitter synthesis, uptake, storage, release, or metabolism is considered an important mode of drug action. Indeed, the use of drugs which block peripheral nervous transmission was indispensable in first establishing, then proving the very nature of neuromuscular transmission (1-3), as well as in the study of the autonomic nervous system (2,4). The method of blocking transmitter utilization has also been used in attempts to clarify the specific functions of putative neurotransmitters in the brain, and to substantiate hypotheses linking the effects of psychoactive drugs to their interactions with neurochemical systems (5,6).

Of greatest interest centrally have been the monoamines serotonin (5-HT) and norepinephrine (NE). Literally thousands of publications have covered their occurrence in nature; their distribution in various systems of vertebrate and invertebrate species; the nature of their nervous function; and the subcellular aspects of their utilization by

nerves (7-9). Their role, individually or together, in the neural processes underlying behavior remains controversial and largely unresolved (10). Present-day monoamine research seeks to provide the final, direct evidence confirming the status of NE and 5-HT as brain neurotransmitters, a goal heavily dependent on technological advances; and to link directly the central function of monoamine-containing neurons to behavior.

Hypotheses which emphasize the role of NE and 5-HT as neurotransmitters in the normal function of the CNS are of two varieties: I. Those which attempt to show evidence of this role, i.e., fulfill criteria necessary for the complete recognition of the compounds as neurotransmitters; and II. Those which, assuming NE and 5-HT to be neurotransmitters, postulate aberrations in their metabolism or neuronal release mechanisms as causes for mental disorders or drug effects.

The technique of selective depletion of central monoamines as a means by which their functional role in behavior may be studied presumably has its origin in traditional biology. The surgical destruction or extirpation of an organ as a method of investigating its physiological significance in a body has been a mainstay in biological research. The practical laboratory difficulties associated with surgical denervation in an organ as complex as the central nervous system encouraged a shift to the use of chemical means of differentiating the roles of the neurotransmitters (11).

In 1959, Senoh and co-workers (12-14) reported that the administration of DA to rabbits resulted in the auto-oxidative and enzymatic formation of 6-hydroxydopamine (6-HDA). The peripheral administration

of 6-HDA in the mouse led initially to the discovery that the compound produced a long-lasting depletion of NE in the heart (15). Further studies showed that while 6-HDA had a lower ED_{50} than either reserpine or metaraminol, its NE-depleting effects lasted considerably longer than those of the other compounds tested (16). It was originally suggested that 6-HDA damaged amine storage sites (15,17); electron-microscopic studies indicated that the long-term loss of NE resulted from the destruction of adrenergic nerve terminals (18) and concomitant loss of activity of NE-synthesizing enzymes was also found (19). 6-HDA's effects have been termed "chemical denervation" (20); the compound has been widely used in studies concerned with the synthesis, uptake, storage, and functional aspects of both peripheral and central catecholamines (21).

Following central administration of 6-HDA, long-lasting, selective decreases in catecholamine levels were observed without significant effect on 5-HT or GABA levels (19,22,23). Generally, behavior of animals given 6-HDA did not differ significantly from control (19,22,23). However, injections of 6-HDA into localized brain regions have been used to induce specific lesions of central monoamine neurons (24). Spontaneous rotational behavior was observed in rats with unilateral 6-HDA lesions in the nigro-striatal DA system (25,26). Bilateral injection of 6-HDA into the lateral hypothalamus caused adipsia and aphagia, while animals lesioned in the substantia nigra showed marked hyperactivity with behavioral reversal after one week (27).

In 1971, the apparent discovery of a "serotonergic 6-HDA" was

reported; intraventricular administration of 5,6-dihydroxytryptamine (5,6-DHT) resulted in the long-lasting depletion of brain 5-HT with only transient effects on the levels of DA and NE (28,29). The percentage loss of 5-HT and the dose-dependency of the effect varied in different brain regions. Subsequent fluorescent histochemical and electron-microscopic studies revealed selective degeneration of indoleamine terminals and axons similar to the cytotoxic effects of 6-HDA on adrenergic neurons (30,31). The tendency of 5-HT levels to recover after the initial phase of depletion led to the discovery of axonal regeneration by the damaged serotonergic neurons (32,33).

In contrast to 6-HDA, the intraventricular injection of 5,6-HDT caused dramatic behavioral changes (34). The acute disturbances have been described as "sedation during the first two hours after the injection (ether anesthesia), an increased irritability and hyper-reactivity against any type of sensory stimulation and bizarre social behavior (fighting in a stereotyped position and manner), and increased copulatory activity when kept in the same cage inbetween the 1st and 3rd day after 5,6-DHT." (35) Frequent fighting episodes as well as increased muricidal behavior have been reported, and decreased food consumption and loss of weight have also been observed. Intraventricular injection of 5,6-DHT produced significant hypothermia thirty minutes after administration (36).

The advent of a selective neurotoxic agent such as 5,6-DHT opens fresh avenues for the functional differentiation of central monoamines, and for possible discernment of their role in mediating behavior. The availability of such chemicals offers an alternative to the non-selective

destruction of brain regions with heterogeneous cell populations, e.g., by electrocoagulation or surgical lesioning. In addition, the functional significance of the depression of monoamine levels by selective synthesis inhibitors may be resolved through the use of cytotoxic agents. The use of chemicals to destroy monoamine nerve terminals may supplement brain amine concentrations as criteria for relating monoamine function to behavior.

The synthesis and pharmacological testing of structural analogues commonly employed to identify structural features responsible for a primary effect; to elucidate mechanisms of action; and/or to establish a rational basis for the development of more specific agents. The recent synthesis (37) of 3-(β-aminoethyl)-5,6-dihydroxybenzo[b]thiophene (5,6-DHT-S), the sulfur isostere of 5,6-DHT, offered the opportunity to investigate the relative importance of the indolic nitrogen in the action of 5,6-DHT. Benzo[b]thiophene isosteres of biologically active indoles have been studied previously by this laboratory in an attempt to estimate the pharmacological contribution of the indolealkylamine ring nitrogen (38-41). The availability of benzo[b]thiophene and 5,6-disubstituted tryptamines, permitted a study of the contributions of the chemical sub-groupings present in 5,6-DHT to its monoaminergic activity and general effects on behavior. The parallel neurochemical and behavioral structure-activity relationships make it possible to speculate on the relation of monoaminergic activity to behavior in the rat.

CONTRASTING BIOCHEMICAL EFFECTS OF 5,6-DHT AND 5,6-DHT-S IN THE RAT

NE levels in the heart and spleen were decreased significantly

by the interperitoneal injection of 30 mg/kg of each compound to adult male rats. Minimum levels in the heart were reached by the second hour; each compound produced a 55-60% depletion of NE. Values returned to control levels by 16 hours. In the spleen, NE levels were similarly reduced, differing only in that 5,6-DHT caused a longer lasting reduction (up to 16 hours) than in the heart. 5-HT levels in the spleen were reduced to 50% by 5,6-DHT at 16 hours, and were still decreased at 24 hours. 5,6-DHT-S, however, actually increased levels of 5-HT by 50% two hours after injection. No decrease in 5-HT levels was observed in the 24-hour period after 5,6-DHT-S. The data on spleen 5-HT and NE are presented in Figures 1a and 1b. In contrast to these effects, the i.p. dosages had absolutely no effects on the levels of 5-HT and NE in the brain of the animals.

The results obtained following intraventricular injection of 80 µg of the compound, were most dramatic. 5,6-DHT significantly decreased the 5-HT content of the three brain regions: cerebral hemispheres, cerebellum, and brainstem. The decline differing only in the time course of the effect. A rapid drop of 25-35% was observed in the cerebral hemispheres by 4 hours after dosage; this persisted for at least 16 days. Brainstem 5-HT was lowered by 20-30% in a similar fashion, while cerebellar 5-HT showed a 25% decrease only over the period 16 hours - 4 days after dosage. The data for the effects of 5,6-DHT and 5,6-DHT-S on brainstem 5-HT and NE are shown in Figure 2.

In contrast to their effects on 5-HT, <u>both</u> compounds decreased levels of NE in the three brain regions. The decline in the cerebral

hemispheres of approximately 30% was less sharp and more prolonged than in the brainstem, where similar decreases were last observed at 4 hours. Decreased levels of NE in the cerebellum reached 75% of control briefly, at 1 - 2 hours post-injection. (Figure 2).

Both 5,6-DHT and 5,6-DHT-S produced a transient increase in 5-HIAA levels. By eight hours, 5,6-DHT had reduced 5-HIAA levels 20-30% of control in all three brain regions, paralleling similar reductions of 5-HT. 5,6-DHT-S, on the other hand, demonstrated an ability to increase 5-HIAA levels, which returned to control values by 24 hours. 5,6-DHT's effects on 5-HIAA paralleled its decrease of 5-HT in the brainstem and cerebral hemispheres, and likewise in the cerebellum, where 5-HIAA levels returned to control levels after the 4th day. In contrast, 5,6-DHT-S caused a significant elevation in 5-HIAA levels 2 - 4 days after injection. Maximum increases of 40-50% were observed in the brainstem and cerebral hemispheres, 12-20% in the cerebellum. 5-HIAA levels returned to near normal levels by the 16th day. Lower doses (20 µg/rat or 40 µg/rat) of both 5,6-DHT and 5,6-DHT-S had similar effects though lesser in magnitude and duration.

BEHAVIORAL EFFECTS OF 5,6-DHT AND 5,6-DHT-S IN THE RAT

Peripheral injections of 5,6-DHT and 5,6-DHT-S in the rat elicited similar behavior. When administered intraperitoneally (30 mg/kg), both compounds produced peripheral signs such as exophthalamous, piloerection, rigid alert stance, and increased irritability, indicating sympathetic autonomic stimulation. No increase in motor activity, indicative of central activity, was observed.

When administered centrally, 5,6-DHT and 5,6-DHT-S differed in their behavioral effects. Injection of 80 µg 5,6-DHT-S into the lateral ventricle produced a behaviorally depressed animal; the sedated state could be described as a semi-conscious stupor, lasting 30-45 minutes. After recovery, animals could not be distinguished from controls. Central administration of 5,6-DHT in doses of 80 µg caused initial behavioral depression which, after 40 - 120 minutes, was usually succeeded by a period of sudden, intense activity.

The distinctive behavior after 5,6-DHT was characterized by explosive jumping (rats at times leapt through covered cages to heights of 18 inches); erratic running behavior, in which the back legs, in a flipping motion, propelled the rat in large circles in open space, or along the walls of an enclosure (the animal appeared to ignore barriers in its frantic movement and at times bloodied its head and cut its feet); vocalization was often present as high-pitched squeals or shrieks; convulsions during which the animal literally behaved as a fish out of water, unable to breathe or make sound; and death in 25-35% of the rats entering into this type of behavior. Each such outburst lasted about 30-90 seconds in confined spaces, but could continue much longer in open spaces. Milder episodes were interspersed among the more intense, and consisted of compulsive running and leaping at barriers, appearing to be a form of escape behavior. After each outburst the animal lay still, breathing deeply, but was alert and hyper-responsive to external stimulation. As many as thirty mild and intense outbursts have been recorded in the two hours following injection of 5,6-DHT; the period of 45-75 minutes after

injection contained the greatest frequency of such episodes. It was observed that those rats which did not enter into the second phase of behavior, but which remained behaviorally depressed and stuporous, were less likely to recover than those animals which underwent the violent behavioral spasms and lived.

Long-term behavioral effects were observed after central administration of 5,6-DHT, but not 5,6-DHT-S. Following the period of intense activity, rats given 5,6-DHT either recovered sufficiently to feed and water themselves, or remained adipsic and aphagic, dying within 2 - 4 days post-injection. Those which recovered tended to weigh less than control animals, and were often irritable and defensive when approached or handled. The long-term behavioral effects of 5,6-DHT were characterized by more frequent vocalization; unusual social interactions between cage-mates; and fighting episodes. One particular form of behavior was especially striking: two rats, standing on hind legs facing each other, would squeal, "hiss," or "spit" at each other, but would not make physical contact. At times, this behavior lasted as long as an hour or more.

BIOCHEMICAL AND BEHAVIORAL EFFECTS OF 5-HYDROXY-6-METHOXYTRYPTAMINE (5H6MT) AND 5-METHOXY-6-HYDROXYTRYPTAMINE (5M6HT) IN RATS

Each of these analogues was administered into the lateral ventricle of rats at a dosage of 100 µg/rat. No significant changes were seen in levels of 5-HT or NE in any of the brain regions over the period 1 - 24 hours after dosage. 5H6MT did cause a significant increase (20-25%) in 5HIAA levels at 2 hours after dosage, while 5H6MT did cause a significant increase (20-25%) in 5HIAA levels at 2 hours after dosage,

while 5M6HT caused a significant decrease (25-35%) in 5-HIAA levels at 1 hour post dosage.

Within 15 minutes following intraventricular injection of 5H6MT, a behavioral syndrome identical to that caused by 5,6-DHT was manifested. Brief, periodic (3 - 8 minute), convulsive episodes were combined with erratic running behavior and vocalizations. The behavioral effects differed from those of 5,6-DHT in that they were quicker in onset and lasted less than one hour. There was evident an increased alertness over control animals following this syndrome, then the animals could not be differentiated behaviorally.

In contrast, 5M6HT produced no dramatic behavioral effects. Some increased exploratory activity soon after injection was observed, as well as increased motor activity and alertness. However, these effects were slight, and the animals could not otherwise be differentiated from controls.

BIOCHEMICAL AND BEHAVIORAL EFFECTS OF 5,6-DIMETHOXYTRYPTAMINE (5,6-DMT) AND 3-(β-AMINOETHYL)-5,6-ISOPROPYLINDENDIOXYBENZO[b]THIOPHENE (IPST) IN THE RAT

Each compound was injected intraventricularly at a dose of 100 µg/rat. 5,6-DMT apparently elevated 5-HT levels, with no significant effect on 5-HIAA or NE. However, since 5,6-DMT is active in the method for the determination of 5-HT (42), the increased fluorescence persisting to 24 hours, suggests a continued presence of 5,6-DMT in significant amounts. IPST, which does not interact with the assay methods, elevated 5-HT levels at 2 hours (33%) and 24 hours (44%) in the cerebral hemispheres;

by 48 hours, however, these levels had declined to 59% of control. Brain-stem levels of 5-HT were increased by 20-30% at 24 hours post-injection with a concommitant decrease in 5-HIAA. In the cerebral hemispheres, 5-HIAA levels were depressed for 48 hours after injection. Levels of NE remained essentially unchanged throughout the 48 hour period after administration of IPST.

Both IPST and 5,6-DHT-S were behaviorally active. IPST, in contrast to its parent compound 5,6-DHT-S, increased motor activity; the most prominent feature of the behavioral effect was compulsive cage circling interrupted by brief pauses. The effect commenced shortly after injection and lasted nearly two hours. Paradoxically, IPST when administered peripherally (40 mg/kg, i.p.) depressed all behavior. The rat given IPST would lay prone and stuporous, but would lick a water bottle spout brought near to its mouth.

Rats given 100 µg 5,6-DMT intraventricularly exhibited a disoriented, groping behavior, while rolling over and over. The latter behavioral effect could be elicited upon moving the animal's cage. This behavior lasted up to 2.5 hours after injection, after which the animals could not be distinguished from controls. No convulsive behavior similar to that produced by 5,6-DHT was observed.

COMPARATIVE EFFECTS OF 5,6-DHT AND ANALOGUES IN THE RAT

A summary of the biochemical and behavioral effects of the various compounds tested is presented in Table 1.

Although 5,6-DHT is generally considered as a selective depletor of brain 5-HT, the results obtained from both peripheral and brain

tissues demonstrate an ability of the compound to lower both NE and 5-HT. That 5,6-DHT might share properties of both monoamines, and therefore have the potential to interact with either catecholaminergic or serotonergic systems, may be deduced readily from its structure. 5,6-DHT itself is the 6-hydroxy derivative of serotonin; the o-dihydroxyphenyl portion of the 5,6-DHT molecule is isomeric with catechol, a sub-group shared by the catecholamines norepinephrine and dopamine. In fact, prior to the discovery of its neurotoxic properties, 5,6-DHT was found to be as active at 5-HT in the contraction of smooth muscle preparations (43) as well as a potent releaser of heart NE (44). The diploid nature of 5,6-DHT's monoaminergic activity in vivo has therefore been confirmed here for peripheral and brain tissues.

Using 5,6-DHT's structure as the basis for making comparisons, the relation of chemical structure to monoaminergic activity may be summarized as follows. Replacement of the indolic nitrogen by sulfur abolishes 5-HT depleting activity in spleen and in brain, but does not alter NE depleting activity in the heart, spleen, or brain. Methylation of either the 5- or 6-hydroxyl groups of 5,6-DHT removes monoamine depletion activity. However, increased and decreased levels of 5-HIAA soon after injection indicate serotonergic interactions are not completely absent. Methylation of both hydroxyls on 5,6-DHT results in a complete abolition of monoaminergic depleting activity, although considerable behavioral activity is retained. In contrast, conversion of 5,6-DHT-S to its isopropylidene derivative produces a complex action on brain 5-HT and NE systems and causes a generalized stimulatory behavioral effect.

CONCLUSIONS

The indolic nitrogen of 5,6-DHT appears to be an absolute requirement for the 5-HT neurotoxic effects of the compound, at least insofar as substitution of a sulfur atom is concerned. Similar specificity appears to be demanded in terms of the 5- and 6-hydroxyl groups both existing in the phenolic state. In contrast, the similar ability of 5,6-DHT-S to lower stored NE levels for a transient period appears to be related only to the catechol characteristics associated with an arylethylamine. The overall behavioral actions of the compounds are complex and suggest a multiplicity of effects including amine release/depletion and direct receptor interactions.

ACKNOWLEDGMENTS

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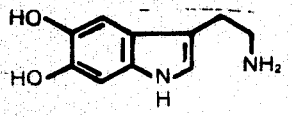
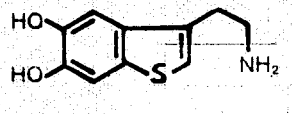
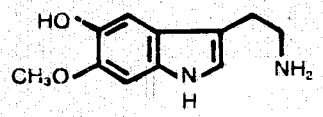
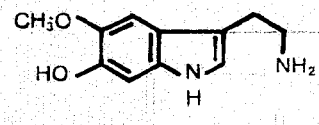
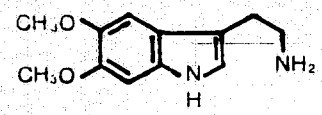
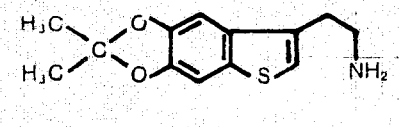
TABLE 1

EFFECTS OF 5, 6-DHT AND ANALOGUES ON BRAIN BIOCHEMISTRY AND BEHAVIOR IN THE RAT

COMPOUND	*BIOCHEMICAL EFFECTS*	*BEHAVIORAL RESULTS*
 5,6-DHT	Prolonged (20-30%) decrease in 5HT; initial increase, then prolonged decrease in 5HIAA; brief initial decrease in NE.	Initial behavioral depression, 30-45 min., then explosive jumping and erratic running behavior, convulsive episodes, vocalization.
 5,6-DHT-S	No effect on 5HT; increased 5HIAA levels at 2-4 days post-injection; brief initial decrease in NE.	Behavioral depression lasting up to 30 min. post-injection; sedation, animal lying prone.
 5H6MT	No effect on 5HT; brief initial increase in 5HIAA; no effect on NE.	Brief, periodic (3-8 min.) convulsive episodes within 15 min. injection, associated with erratic running behavior, vocalization; followed by period of increased alertness and responsivity.
 6H5MT	No effect on 5HT; brief initial decrease in 5HIAA; no effect on NE.	Some increased exploratory activity within 15 minutes of injection, but otherwise no difference from control animals.
 5,6-DMT	No significant effects on 5HT, 5HIAA or NE.	Disoriented, groping behavior; animals exhibit much rotational behavior and hyperreactivity to sensory stimulation.
 IPST	Initial increase, then prolonged decrease in 5HT; generally decreased 5HIAA; brief initial decrease in NE.	Increased motor activity; cage circling with periodic pauses, lasting two hours following injection.

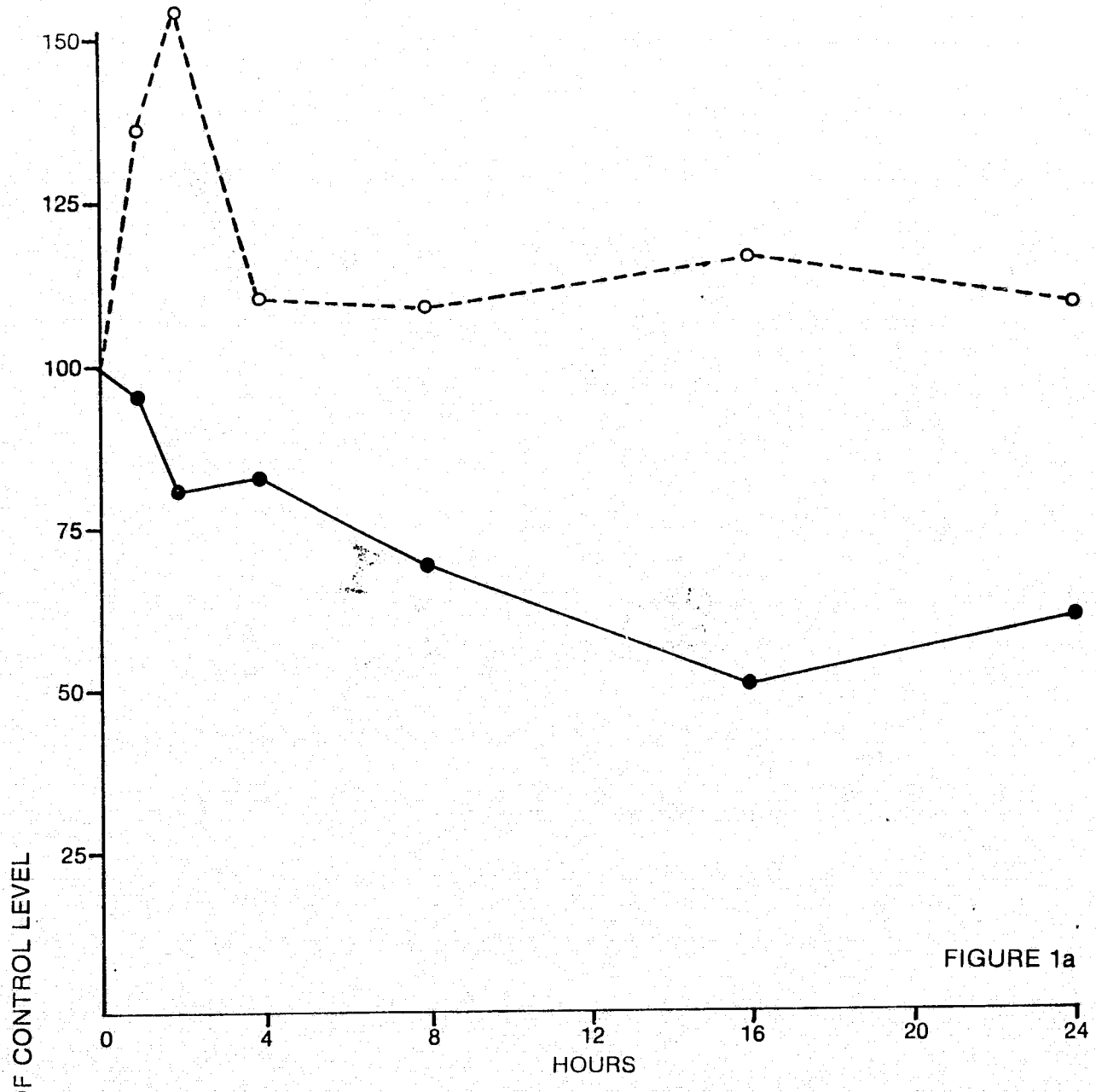


FIGURE 1a

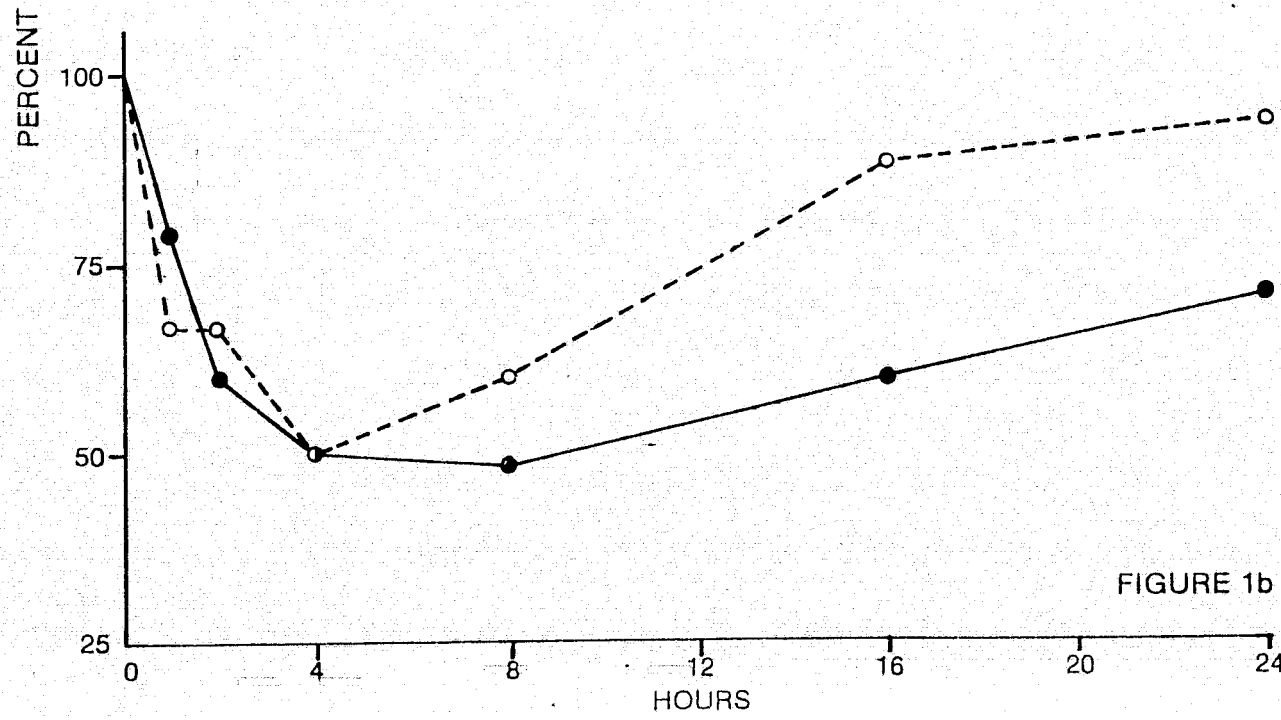


FIGURE 1b

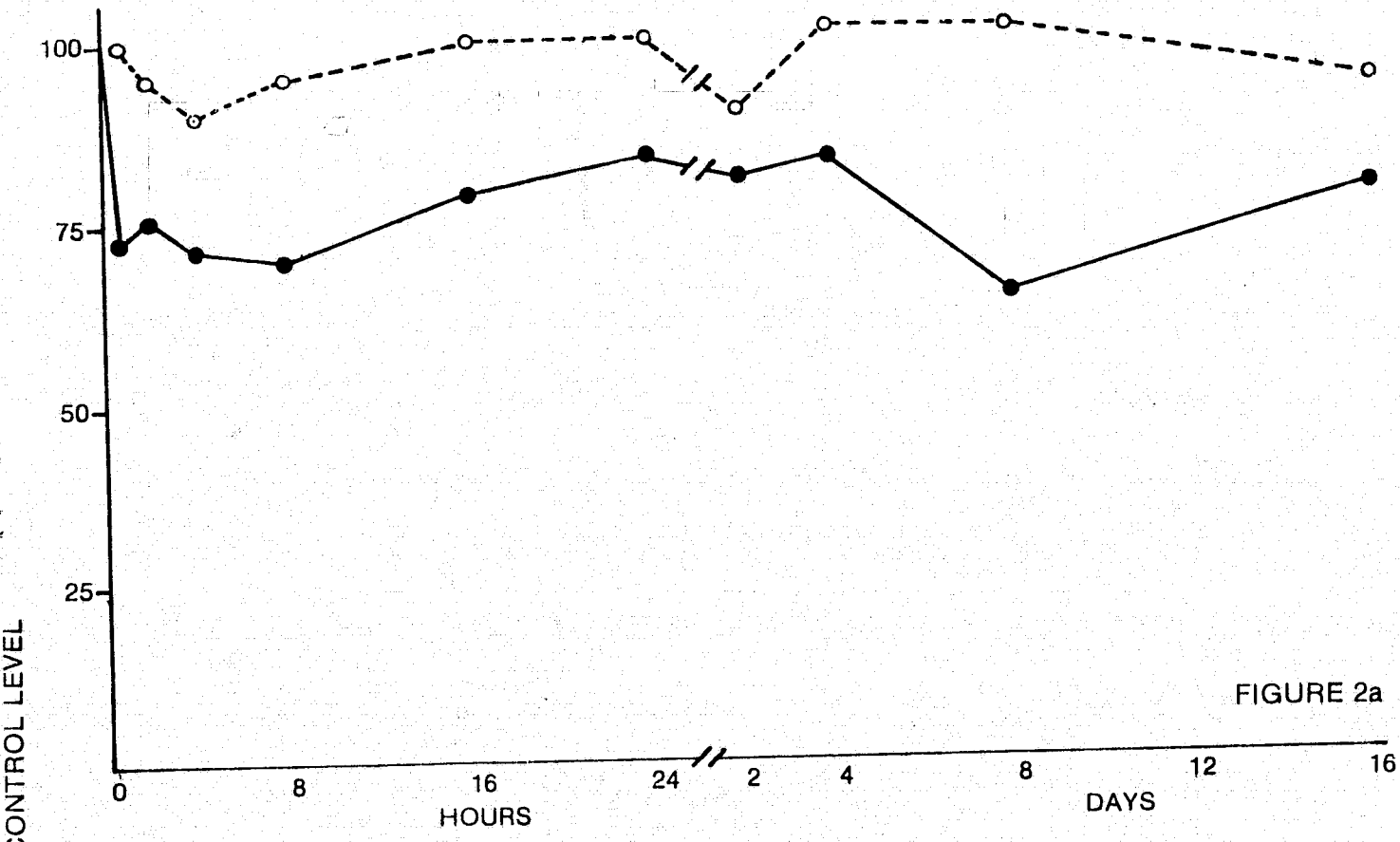


FIGURE 2a

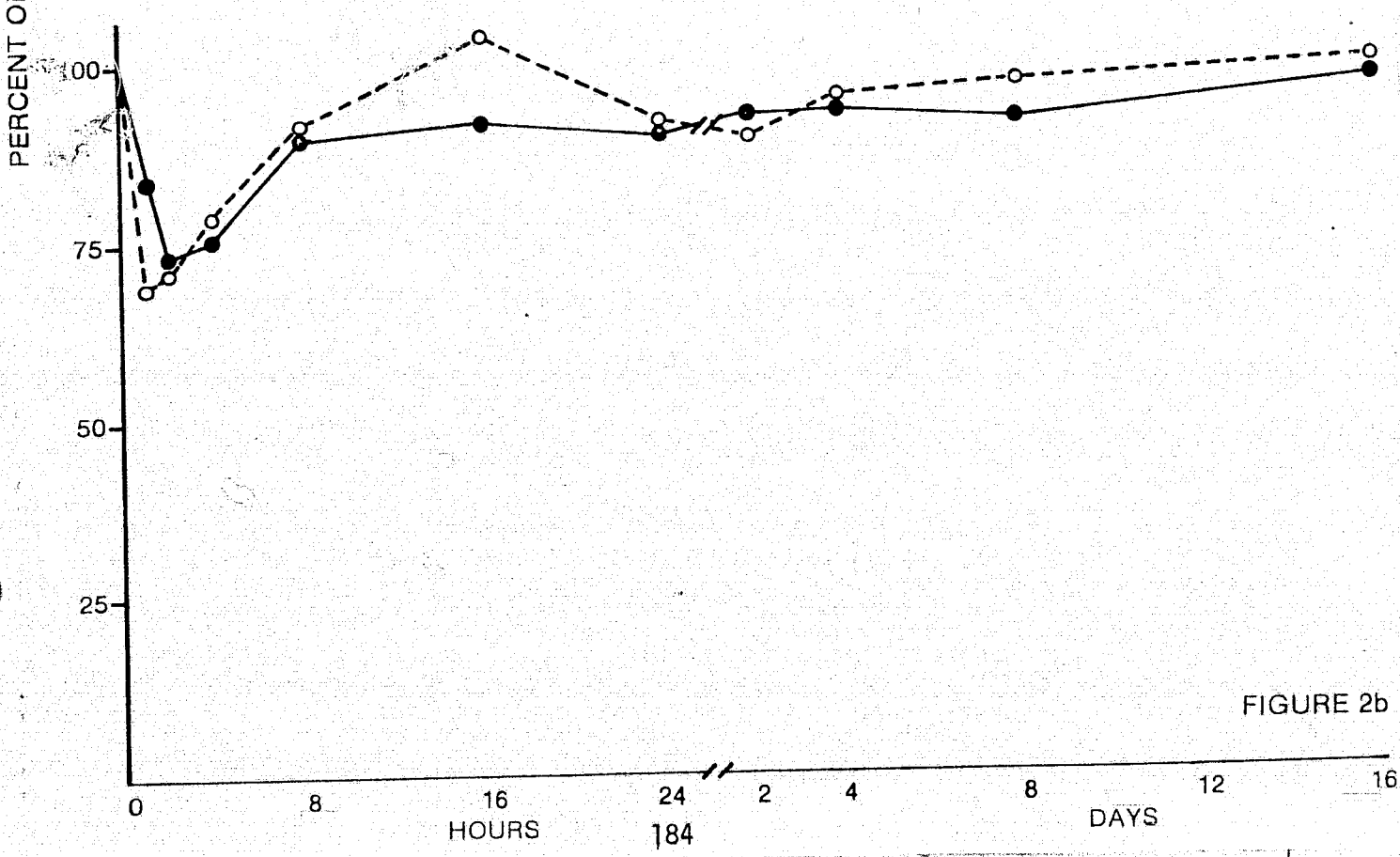


FIGURE 2b

LEGENDS

FIGURE 1a. Levels of 5-HT in rat spleen at various times after a single dose (30 mg/kg, i.p.) of 5,6-DHT (—●—) or 5,6-DHT-S (---○---). Each value is the mean of 4 animals; control values (N = 8) were 1.40 ± .09 (SD) µg/g.

FIGURE 1b. Levels of NE in rat spleen at various times after a single dose (30 mg/kg, i.p.) of 5,6-DHT (—●—) or 5,6-DHT-S (---○---). Each value is the mean of 4 animals; control values (N = 8) were 0.42 ± .07 (SD) µg/g.

FIGURE 2a. Levels of 5HT in rat brainstem at various times after a single dose (80 µg/rat, intraventricularly) of 5,6-DHT (—●—) or 5,6-DHT-S (---○---). Each value is the mean of 4 animals; control values (N = 8) were 1.13 ± .17 (SD) µg/g.

FIGURE 2b. Levels of NE in rat brainstem at various times after a single dose (80 µg/rat, intraventricularly of 5,6-DHT (—●—) or 5,6-DHT-S (---○---). Each value is the mean of 4 animals; control values (N = 8) were 1.01 ± .11 (SD) µg/g.

13.0 APPENDIX III

PAPERS SUBMITTED FOR PUBLICATION

Bioisosteric Inhibition of DOPA Decarboxylase*

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#Taken in part from a thesis submitted by John R. Baldwin in partial fulfillment of the requirements for the M.S. degree in Pharmacology, Indiana University, 1975.

ABSTRACT

In an *in vitro* system of purified hog kidney aromatic-L-amino acid decarboxylase, the benzo[b]thiophene analogue of 5-hydroxytryptophan was found to be an effective inhibitor of DOPA decarboxylation, with a potency almost twice that of α-methyl-DOPA. When administered to mice prior to a dose of ^{3}H-L-DOPA, the benzo[b]thiophene analogue of 5HTP led to significant elevations in brain levels of DOPA, dopamine, and metabolites one hour later.

INTRODUCTION

Aromatic-L-amino acid decarboxylase (EC 4.1.1.26) has been characterized as the enzyme responsible for the conversion of 3,4-dihydroxyphenylalanine (DOPA) to 3,4-dihydroxyphenylethylamine (DA), a reaction first demonstrated by Holtz, et al [1] in 1938 with kidney extracts of rabbits and guinea pigs. The enzyme has subsequently been shown to be widely distributed in various mammalian tissues, and to require pyridoxal phosphate as a co-enzyme [2]. The enzyme has been partially purified from hog kidney by Christenson, et al [3] and characterized in terms of optimal cofactor and substrate concentrations as well as medium pH by these authors and by Werle and Aures [4]. In addition, a variety of aromatic-L-amino acids (both natural and unnatural) have been shown to have activity as substrates for the enzyme [3,5-7].

One of the more interesting aspects of this enzyme has been the controversy surrounding its ability to act as the decarboxylation system for indolealanines such as tryptophan as well as for phenylalanines [8,10]. There is no doubt that aromatic-L-amino acid decarboyxlase preparations purified from hog kidney [3] have considerable activity towards a variety of aromatic-L-amino acids, including analogues of tryptophan [3,9,11-13]. Some analogues of tryptophan that are poor substrates for decarboxylation have been shown, not unexpectedly, to be inhibitors of the decarboxylation of tryptophan, as well as other amino acids [13,14].

The finding of Ehringer and Hornykiewicz [15] that patients with Parkinson's disease had sub-normal levels of DA in the substantia nigra and the subsequent discovery and development of L-DOPA as a therapeutic

agent have stimulated interest in inhibitors of the decarboxylation of this amino acid [16-18]. A variety of compounds have been found to be inhibitors of the decarboxylation system *in vitro*, including hydrazine derivatives [18-20], α-methyl amino acids [10,21], and by hydroxycinnamic acids [22-24]. A recent report from this laboratory has indicated that the benzo[b]thiophene and 1-methylindole analogues of tryptophan are effective inhibitors of the decarboxylation of tryptophan and phenylalanine *in vitro* [13]. The present paper reports on the activity of several analogues of tryptophan as inhibitors of the decarboxylation of L-DOPA.

MATERIALS AND METHODS

Materials

D,L-DOPA-2-^{14}C(256 μCi/mg) and DA-2-^{14}C(300 μCi/mg) were purchased from Amersham/Searle Corporation, Chicago, IL and L-DOPA-^{3}H (general label, 30 mCi/mg) and DA-^{3}H (general label, 61 mCi/mg) were purchased from New England Nuclear Corp., Boston, MA. DL-DOPA and DL-tryptophan and pyridoxal phosphate were purchased from Sigma Chemical Co., St. Louis, MO, DA was purchased from Nutritional Biochemical Corp., Cleveland, OH, and 5-hydroxytryptophan, α-methyltryptophan, L-DOPA, 3,4-dihydroxyphenyl-acetic acid, 3,4-dihydroxyphenylethanol, 3-methoxy-4-hydroxyphenylethanol, and homovanillic acid were purchased from Regis Chemical Co., Chicago, IL. The benzo[b]thiophene analogues of tryptophan, 5-hydroxytryptophan, and α-methyltryptophan and the 1-methylindole analogue of tryptophan were supplied as racemates by Dr. Ernest Campaigne, Chemistry Department, Indiana University. Pargyline hydrochloride was kindly supplied by Abbott Laboratories.

All chemicals and reagents used were purchased from commercial sources. Ion exchange resin (CG-50, Type 2, 200-400 mesh) was obtained from Mallinckrodt Chemical Co., St. Louis, MO. Adult, male, Swiss-Webster mice (18-25 g) were purchased from Murphy Breeding Laboratories, Plainfield, IN. Hog kidneys were obtained fresh from the Winterlein Meat Packing Co., Bloomington, IN.

Enzyme Preparation

The procedure of Christenson et al [3], was followed through the second ammonium sulfate precipitation with modifications. All work was done at 4° C. Defatted hog kidney (300 g) was homogenized with 900 ml 0.005 M NaH_2PO_4 pH 7.2 containing 0.01 M 2-mercaptoethanol in a Sorvall Omnimixer at high speed for one minute. This homogenate was centrifuged at 10,000 rpm (16,000 x g Sorvall RC2-B refrigerated centrifuge, rotor type GSA) for 30 min. The supernate was decanted and was brought to 37% saturation by the dropwise addition of pH 8.0 saturated ammonium sulfate solution. After stirring for 20 min., it was centrifuged as above for 20 min. The supernate was again decanted and was brought to 55% saturation with saturated ammonium sulfate. After stirring for a further 20 min., the solution was centrifuged under the above conditions for 20 min. The supernate was discarded, and the precipitate was dissolved in 150 ml of 0.01 M 2-mercaptoethanol. The enzyme was dialyzed overnight against 0.01 M 2-mercaptoethanol and then frozen until use. Enzyme protein was determined by the method of Lowry, et al [25].

Incubation Procedure

The frozen enzyme was allowed to thaw at room temperature prior to

use. All incubations were done in triplicate, using an incubation mixture modeled after Creveling and Daly [26] and consisting of: 0.2 ml 0.5M NaH_2PO_4 buffer (pH 7.0), 0.1 ml pyridoxal-5-phosphate (final concentration 10^{-4}M), 0.01 ml pargyline HCl (final concentration 1.25×10^{-5}M), 0.02 ml enzyme, 0.01 ml DOPA, and distilled water or inhibitor to a final volume of 1.0 ml. All incubations were done at 37° C for 10 min. under air in an Aminco constant temperature shaking incubator. The mixture was pre-incubated for 3 min. before addition of substrate. Each incubation vessle contained 0.2 μCi DL-DOPA-2-^{14}C. Inhibitors, when used, were added in 0.5 ml volume 1 min. prior to the addition of the substrate; 0.5 ml of H_2O was deleted from the original reaction mixture to allow the final volume to remain at 1 ml. The enzymatic reaction was stopped by the addition of 0.1 ml 50% TCA. After addition of 0.1 ml of 1×10^{-3} M DA as a carrier plus 10 mg ascorbic acid, 2 ml of 0.5 M KH_2PO_4 (pH 6.5) and NH_4OH to obtain a pH of 6.5 was then added. The mixture was transferred to centrifuge tubes and spun at 37,000 rpm in a IEC Model HN centrifuge for 15 min. One ml of the incubate was then added to the ion exchange columns. Zero time points were prepared by the addition of 0.1 ml 50% TCA just prior to substrate addition.

Analytical Procedures

The columns were 1 ml disposable pipets cut to 12 cm in length with a 12 cc disposable syringe used as a reservoir. A three-way plastic stopcock connected the reservoir to the pipet. The resin (Amberlite CG-50) 150 mg/column was prepared by the method of Hirs, et al [27] and used in the Na+ form at pH 6.5.

After the 1 ml sample loading, the columns were washed with 8.0 ml of distilled water, then with 1 ml 2N HCl and 0.5 ml of the eluant was pipetted into a polyethylene vial containing 15 ml scintillation fluid. Each sample was counted twice for 10 min. in a Packard Tricarb Liquid Scintillation Spectrometer, Model 2425. Scintillation fluid was prepared by mixing 14 g BBOT (2,5-bis-2-(5-tert-butylbenzoxazolyl)-thiophene, 280 g napthalene, 2000 ml toluene and 1400 ml 2-methoxyethanol.

The ability of the ion exchange columns to separate DOPA from DA was tested by adding aliquots of DOPA (^{14}C) to one set of columns and DA (^{14}C) to another set at concentrations of 1×10^{-4} M to 1×10^{-7} M. The columns were then eluted and aliquots were collected and counted as described above. Recovery of DA over the range 10^{-7} - 10^{-4}M was 82 - 93% under these conditions.

In Vivo Studies

For in vivo experiments, drugs were made up in 0.5% methyl cellulose suspensions. Pretreated mice received 300 mg/kg of inhibitor i.p. 1.5 hrs. prior to a dose of 200 mg/kg L-DOPA (spec. act. 1.97 mCi/mmole) p.o. Control mice received only 200 mg/kg L-DOPA (^{3}H-G) p.o. All mice were sacrificed by decapitation 1 and 2 hours after DOPA administration. Tissue samples were removed, rinsed with saline and frozen immediately. The samples were assayed by a procedure modified after Taylor and Laverty [28]. Tissues were homogenized (Sorvall Omnimixer) for one minute in 4 ml 0.4 M $HClO_4$ with 0.1 ml 10% EDTA and 5.2 mg ascorbate. The homogenate was centrifuged for 10 min. (IEC Model B-20) at 4° C and 10,000 rpm. The precipitate was then washed with 2 ml of 0.4 M $HClO_4$, centrifuged, decanted

and then washed again with 2 ml $HClO_4$. The two washes were combined with the first centrifugation and the pH was brought to 6.5 with 5N KOH. The sample was then centrifuged (IEC Model HN) for 5 min. at 3,700 rpm to remove $KClO_4$ precipitate. An aliquot (8 ml) was then added to an ion exchange column prepared as described above. The 8 ml load was collected for further analysis.

The column was washed with 8 ml H_2O and the rinse combined with the liquid collected from the load. This fraction contains DOPA, and its acid and neutral (alcohol) metabolites. This was taken to dryness and dissolved in 2 ml 0.01 N HCl. Sufficient 2 N HCl was added to bring the pH to 1 prior to washing with 3 volumes of chloroform. Aliquots of 0.5 ml of the aqueous phase (DOPA)were then pipetted to scintillation vials and counted.

The remaining aqueous phase was aspirated off and the chloroform phase containing the alcohol and acidic metabolites was taken to dryness and reconstituted in 2 ml of 0.01 N HCl; 0.5 ml of this fraction was counted. The rinsed columns containing the DA and NE were eluted with 1 ml 2 N HCl and 0.5 ml was transferred and counted.

This extraction process was tested with authentic DOPA, 3-methoxy-4-hydroxyphenylethanol, was chosen to represent the neutral (alcohol) metabolites, and homovanillic acid (HVA), chosen to represent the acidic class. Concentrated samples of each phase described above were spotted adjacent to these spots. The papers were developed using two solvent systems, butanol/acetic acid/H_2O (25:4:10) and isopropanol/2N HCl (65:35) and scanned (Packard Model 7200 Radio-chromatogram Scanner. Authentic

compounds were detected with iodine vapor. The scan from the water fraction contained one major peak at R_f ~ 0.5 while the scan from the chloroform phase also showed one peak but of much less intensity at R_f ~ 1. Authentic DOPA ran in the butanol/acetic acid/H_2O solvent system at an R_f of 0.29 and at an R_f of 0.85 in the isopropanol/HCl system. Authentic 3-methoxy-4-hydroxyphenylethanol and HVA ran at an R_f of 0.93 in both solvent systems.

Data Processing

Km, Vmax, and Ki were calculated using linear regression analysis of unweighted double reciprocal (1/V vs 1/[S]) data, using computerized procedures.

RESULTS

Initial Studies of DOPA Decarboxylation

Using this system and purified hog kidney enzyme, initial studies showed the reaction for the decarboxylation of DOPA to be linear over the period of 2 - 10 minutes of incubation. The K_m ranged from 1.96×10^{-4} to 2.38×10^{-4} when derived from data obtained over a range of substrate concentrations of 5×10^{-5} to 1×10^{-3} M. Using a substrate concentration of 5×10^{-5} M and inhibitor concentration of 10^{-3} M a preliminary series of inhibition studies was performed. The results, presented in Table 1, show that the benzo[b]thiophene analogue of 5-hydroxytryptophan was the most potent inhibitor of the test series, almost twice as effective as α-methyl DOPA, while 5-hydroxytryptophan itself was only slightly less potent.

Inhibition of DOPA Decarboxylation *In Vitro*

Detailed kinetic studies were performed with 5-hydroxytryptophan, α-methyl-5-hydroxytryptophan, and the benzo[b]thiophene analogues of these two compounds. The results, presented in Table 2, indicate that the benzo[b]thiophene analogue of 5-hydroxytryptophan was the most potent inhibitor of DOPA decarboxylation in the test system. In the case of α-methyl tryptophan, replacement of the indolic nitrogen with a sulfur atom led to a slight decrease in potency. The Lineweaver-Burke plot for the inhibition of DOPA decarboxylation by the 10^{-3} concentrations of the various compounds is shown in Figure 1.

Studies of *In Vivo* Inhibition of DOPA Decarboxylation

In an attempt to further test the efficacy of the most potent *in vitro* inhibitor, mice were pretreated with the benzo[b]thiophene analogue of 5-hydroxytryptophan prior to the administration of L-DOPA, and brain and kidney were removed at 1 and 2 hours for the assay of DOPA, DA and metabolites as described in Materials and Methods. Brain levels of total radioactivity were 44% higher after 1 hour in mice pretreated with the inhibitor; this was reflected in significantly elevated levels of DOPA, DA, and metabolites (33%, 58% and 32%, respectively). No significant elevations were seen in brain levels of radioactivity at 2 hours, nor were any of the kidney levels significantly elevated.

DISCUSSION

The initial screening results presented in this paper indicate that the benzo[b]thiophene analogue of 5-HTP is the most potent inhibitor of

DOPA decarboxylation (Table 1), followed in order by α-methyl-DOPA, the benzo [b]thiophene and 1-methylindole analogues of tryptophan, and tryptophan itself. These results are consistent with previous findings that a 5-hydroxy function considerably increases the affinity of tryptophan for the enzyme [5,16,29]. One can also compare this to the relative rates of decarboxylation of 5HTP, tyrosine isomers and tryptophan. 5HTP and tyrosine isomers are decarboxylated at a rate of 10 - 40% that of DOPA while tryptophan is decarboxylated 1 - 10% that of DOPA [5]. On this basis, the benzo[b]thiophene analogue of 5HTP should have the highest affinity followed by α-methyl DOPA, tryptophan, and derivatives not having the 5-hydroxy group. The fact that the benzo[b]thiophene analogue of tryptophan is considerably more lipid-soluble than tryptophan [30], combined with the fact that it is sterically similar to tryptophan may facilitate binding to the active site of the enzyme.

Lipid solubility would also explain why α-methyl tryptophan has more affinity for the enzyme than tryptophan. 1-Methyltryptophan is practically as lipid soluble as the benzo[b]thiophene analogue which would also facilitate binding to the enzyme. The fact that the bulk of the methylated indole nitrogen may prevent the compound from being as strongly bound to the enzyme probably dictates the order of affinity indicated in Table 1.

From the kinetic studies presented in Figure 1 and Table 2, it is clear that substitution of sulfur for the indolic nitrogen increases the affinity of the enzyme for the 5-hydroxylated compounds, with virtually the opposite effect for α-methyltryptophan. The bulkiness of the α-methyl

group may greatly effect the positioning of the structure within the active site thereby limiting the interaction of the benzo[b]thiophene nucleus with a specific area of the protein. Therefore, when looking at α-methylated compounds steric hinderance may be the limiting factor rather than the lipid solubility of the compounds.

The small duration of effect seen when the benzo[b]thiophene analogue of 5HTP was used as an *in vivo* pretreatment prior to administration of L-DOPA is somewhat disconcerting in view of the potency of this compound as an *in vitro* inhibitor. However, only a single dose combination was tested, and nothing is known of the rate of degradation and excretion of the inhibitor. Further studies are needed to explore the possible significance of these compounds as inhibitors of DOPA decarboxylation.

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TABLE 1

Inhibition of DOPA Decarboxylation by Various Compounds

Purified hog kidney enzyme was used as described in Materials and Methods. Inhibition values were calculated on the basis of 4 - 6 replicate runs with 10 mins. of incubation.

Compound	% Inhibition
Tryptophan	0.0%
1-Methyltryptophan	10.8%
β-(3-Benzo[b]thienyl)-α-alanine	31.1%
5-Hydroxytryptophan	83.2%
β-(4-Hydroxy-3-benzo[b]thienyl)-α-alanine	93.5%
α-Methyl DOPA	51.7%

TABLE 2

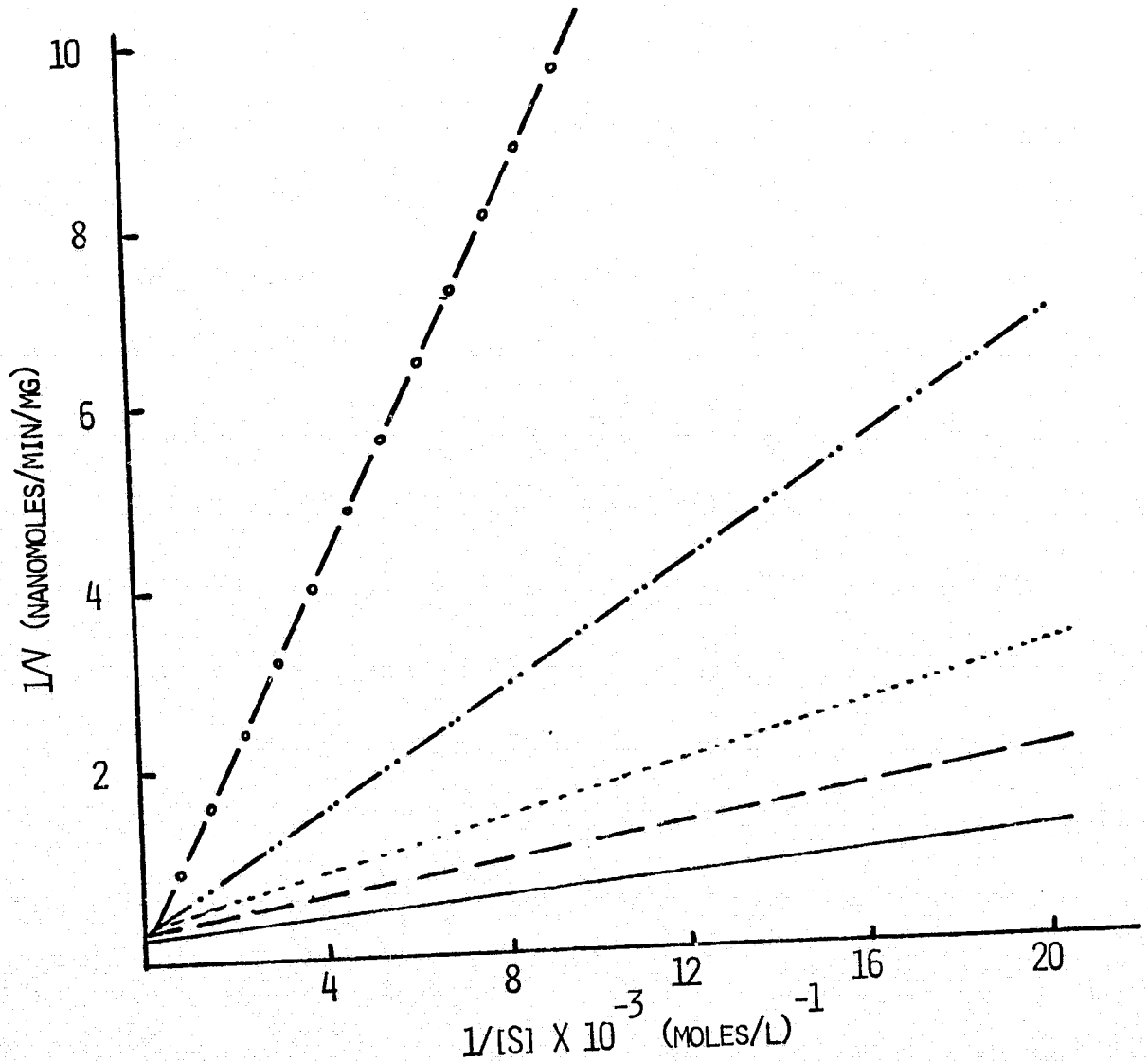
Inhibition of DOPA Decarboxylation by 5-OH and α-CH_3 Analogues

Values for KI were determined as described in Materials and Methods, using substrate concentrations from 5×10^{-5} M to 10^{-3} M and inhibitor concentrations of 10^{-4} and 10^{-3} M. Each point was derived from 4 - 6 replicate runs with 10 min. incubation.

Compound	KI
α-Methyl DOPA	7.7×10^{-4}
β-(3-Benzo[b]thienyl)-α-alanine	1.9×10^{-3}
α-Methyltryptophan	3.7×10^{-4}
α-Methyl-β-(3-Benzo[b]thienyl)-α-alanine	7.0×10^{-4}
5-Hydroxytryptophan	2.1×10^{-4}
β-(5-Hydroxy-3-benzo[b]thienyl)-α-alanine	5.8×10^{-4}

LEGEND

Figure 1: Lineweaver-Burke plots of inhibition of DOPA (5×10^{-5}M) decarboxylation by various compounds (10^{-3}M). DOPA alone (———), methyl-β-(3-benzo[b]thienyl)-α-alanine (— — —), α-methyltryptophan (·····), 5-hydroxytryptophan (—··—), and β-(5-hydroxy-3-benzo[b]-thienyl)-α-alanine (—○—).


10
8
6
4
2
1/V (NANOMOLES/MIN/MG)
4
8
12
16
20
1/[S] X 10^-3 (MOLES/L)^-1

The Metabolism of 2-(3-Benzo[b]thienyl)-l-aminopropionic Acid in the Rat

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Footnotes: (a) Unnumbered

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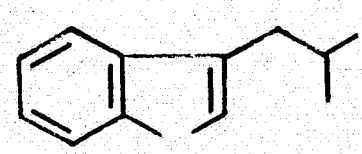
(b) Numbered

[1]Abbreviations used are: TLC, thin-layer chromatography; GC, gas chromatography; TMS, trimethylsilyl; GC/MS, coupled gas chromatography-mass spectrometry; UV, ultraviolet; BSA, N,O-bis(trimethylsilyl) acetamide.

Because of its unique structure, tryptophan occupies a central position among amino acids and has stimulated interest in the study of the role of aromatic and heteroaromatic amino acids in the living organism. Since its discovery as one of ten essential amino acids (1), numerous investigations of tryptophan metabolism have appeared in the literature, including one monograph (2). Considering only the initial reaction involved in tryptophan metabolism, these investigations have defined four distinct pathways: 1. conversion to N-formylkynurenine by tryptophan pyrrolase; 2. conversion to 5-hydroxytryptophan by tryptophan 5-hydroxylase; 3. conversion to tryptamine by aromatic L-amino acid decarboxylase; and 4. conversion to indole-3-pyruvic acid by tryptophan aminotransferase or D- and L-amino acid oxidase. The significance and scope of these pathways have been examined in relation to their role in carcinoid tumor syndrome, Hartnup's disease, phenylketonuria, and maple syrup urine disease (3,4).

An interesting aspect of tryptophan research has been the synthesis and biological evaluation of bioisosteric analogs of the molecule. The concept of bioisosterism (5) has provided a rationale for the design of compounds possessing similar spatial and electronic properties and has provided the basis for our study of bioisosteres of biologically active indole derivatives (6,7). As part of that study we have undertaken the investigation of the metabolic fate in the rat of DL-2(3-benzo[b]thienyl)-1-aminopropionic acid (I), the sulfur analog of tryptophan which constitutes the subject of this report.



Materials and Methods

Materials

Animals

Male Sprague-Dawley rats (Murphy Breeding Laboratories, Plainfield, IN), each weighing 350-400 g, were housed in separate metabolic cages (Maryland Plastics, Inc., New York, NY) designed to permit the separate collection of urine and feces. The animals were maintained on Purina lab chow and tap water *ad libitum* during the course of the experiment.

Chemicals

Compound I was synthesized by the method of Avakian, *et al* (8) and was tritium-exchange labeled (New England Nuclear Corp., Boston, MA) to give a specific activity of 3 Ci/mmole. The radiochemical purity of I was confirmed to be 98% by TLC[1] (9) and scanning with a Packard model 7200 radiochromatogram scanner.

Authentic samples of DL-2-(5-hydroxy-3-benzo[b]thienyl)-1-aminopropionic acid, 5-hydroxybenzo[b]thiophene-3-acetic acid, 3-(2-aminoethyl)-benzo[b]thiophene, 3-(2-hydroxyethyl)benzo[b]thiophene, and benzo[b]thiophene-3-acetic acid (II) were supplied by Dr. E. Campaigne, Department of Chemistry, Indiana University, Bloomington, IN. Benzo[b]thiophene-3-pyruvic acid (III) and DL-benzo[b]thiophene-3-lactic acid (IV) were prepared from I by known procedures (10,11). N-(Benzo[b]thiophene-3-acetyl)glycine (V) was synthesized by the following procedure:

N-(Benzo[b]thiophene-3-acetyl)-glycine (V).

In a 50 ml round bottom flask was placed 1.0 g (5.2 mmol) of II and 5.0 ml of thionyl chloride, and the resulting

solution gently refluxed for 2.5 hr. Excess thionyl chloride was removed under reduced pressure and the reaction mixture was dried by azeotropic benzene co-distillation. The resulting dark oil was dissolved in 4 ml of dry acetone and added to a cold solution of 1.5 g (20.0 mmol) of glycine in 30 ml of 1N NaOH. The reaction mixture was allowed to warm to room temperature, decolorized (Norit), filtered, and acidified with concentrated HCl to give a yellow oil which was extracted with 3 x 25 ml ether. The combined extracts were washed with 0.2N HCl (20 ml), H_2O (2 x 20 ml), dried ($MgSO_4$) and evaporated under reduced pressure to leave an amorphous yellow-white solid which was recrystallized from water to yield 0.47 g (36%) of white plates, m.p. 162-163° C. Molecular weight: Calculated: 249.0460, Found: m/e 249.0460. Chromatographic and mass spectral data: see Table 2. The identity of this material with metabolite V was based on the correspondence of their TLC, GC, and GC/MS properties.

Methods

In Vivo Metabolism

Five rats of equal age and weight were placed in individual metabolism cages inside a standard fume hood and were maintained on a light-dark schedule providing 0700 hr - 2300 hr light and 2300 hr - 0700 hr dark. At 0900 hr and 1500 hr animals were injected ip with a solution of I at a dose of (100 mg/kg) and (100 µCi); control animals were injected ip with an equal volume of 0.001N HCl.

Urine was collected under toluene for periods of 24 and 48 hr following

injection and was used immediately for the isolation and/or quantitation of metabolites, or was stored under N_2 at -5° C. For the determination of residual radioactivity at 48 hr, animals were sacrificed and exsanguinated, samples of plasma, liver, kidney, brain, heart, lung, spleen, testis, and epidymal fat were collected and frozen. Feces were also collected and frozen.

Radioactivity in each of these samples was measured in a Packard Tri-Carb model 2425 liquid scintillation spectrometer, with polyethylene vials and a cocktail composed of 14 g of 2,5 bis2-(-tert-butylbenzoxazolyl)-thiophene and 280 g of napthalene in a mixture of 2100 ml of toluene and 1400 ml of ethylene glycol monomethyl ether. Duplicate aliquots (0.1 ml) of the toluene phase were counted directly, while duplicate aliquots (0.1 ml) of urine was diluted to 100 ml prior to counting. Samples of feces or tissue were homogenized in 4 volumes of 0.01N HCl and duplicate aliquots (0.5 ml) of each homogenate were counted.

Isolation of Metabolites

Since the 24 hr urine contained the most concentrated source of metabolites, it was chosen for isolation and characterization of the individual metabolites arising from I. Metabolites were isolated by a differential pH extraction procedure which gave seven fractions. The procedure (Fig. 1) consisted of taking 1 ml of urine and diluting with 1 ml of distilled water. The pH was adjusted to 1.0 by the addition of approximately 0.3 ml of 2N HCl and the sample extracted with diethyl ether (2 x 4 ml). The combined ether fractions were extracted with 2 ml of 5% sodium bicarbonate solution and the remaining ether solution, which consisted of uncharged lipid soluble

and weakly acidic compounds (phenols), was designated Fraction A. The sodium bicarbonate solution was adjusted to pH 1.0 by the addition of approximately 0.6 ml of 2N HCl and extracted with diethyl ether (2 x 4 ml). The ether fraction which contained carboxylic acids was designated Fraction B.

The aqueous urinary fraction was next adjusted to pH 13.0 by the addition of approximately 0.70 ml of 2N NaOH and extracted with diethyl ether (2 x 4 ml). The resulting ether fraction which consisted of organic bases, was designated Fraction C.

The aqueous urinary fraction was finally adjusted to pH 6.8 by the addition of approximately 0.36 ml of 2N HCl and extracted with diethyl ether (2 x 4 ml). The ether fraction which consisted of weakly polar and neutral compounds, was designated Fraction D and the remaining aqueous phase, which consisted of polar non-extractable neutral and amphoteric compounds, was designated Fraction E.

In order to obtain a non-aqueous extract of Fraction E, non-ionic resin chromatography (NIRC) was employed. Pre-packed "Drug-Skreen®" XAD-2 resin cartridge columns (0.9 x 5.0 cm), containing 2.0 ± 0.1 g of resin and sample reservoirs were used (Brinkmann Instrument Co., Westbury, NY). Cartridges were sealed and stored at 5° C and brought to room temperature immediately prior to use. After assembly, columns were wetted with methanol (20 ml) and water (5 x 20 ml). A 2.0 ml aliquot of Fraction E was diluted to 10.0 ml with distilled water and passed through the column. The flow rate was adjusted to 0.2 ml/min and the sample recycled through the column three times to insure adequate adsorption. Columns were washed with water

(4 x 5 ml) and aspirated by means of a water aspirator for 5 min prior to the elution of the sample with methanol (2 x 5 ml). Column adsorption efficiency was 73%. The resulting methanol eluate was designated Fraction F and the aqueous phase Fraction G.

Duplicate aliquots (0.01 - 0.1 ml) of Fractions A - G were counted. Similar fractions were obtained from control urine, and were used as blanks.

Thin-layer Chromatography

All TLC was done using precoated, prescored uniplates containing 250 μ silica gel G or GF layers (Analtech, Inc., Newark, DL). Plates were developed in a solvent system prepared by shaking a mixture of chloroform/methanol/fornic acid (75:5:5) with 2.0 ml of 0.01 M sodium borate ($Na_2B_4O_7.10H_2O$) solution, followed by separation of the organic phase directly into the chromatography chamber (30 x 20 x 10 cm). The TLC plates were developed for a distance of 12 cm, air-dried for 30 min, then re-developed in the same direction and chamber for 12 cm. Since the plates were double-developed, the apparent retardation factor (R_f') were expressed as a function of the R_f as follows:

$$R_f' = 2R_f - R_f^2$$

Visualization of the TLC plates was accomplished by chemical, fluorescent or radiochemical methods. The chemical methods consisted of I_2 vapor, alkaline $KMNO_4$ and ninhydrin sprays (12) and fluorescent visualization was achieved by inspection of silica gel GF plates under 254 nm UV light. Radiochemical visualization of TLC plates was accomplished using a Packard model 7200 radiochromatogram scanner.

Quantitation of urinary metabolites was achieved by spotting 10 µl of unmanipulated urine on a silica gel G plate, developing as above, and scraping the TLC plate into 2 mm segments using an Isolab TLC plate scraper (Isolab, Inc., Akron, OH). The silica gel from each 2 mm segment was scraped directly into liquid scintillation vials containing 10 ml of liquid scintillation cocktail and the amount of radioactivity determined.

Silylation

Aliquots (2.0 ml) of Fractions A - F were evaporated to dryness under a stream of N_2 in teflon-lined screw-capped tubes. BSA (0.1 ml) was added to each tube which was then sealed under N_2 and heated at 50 - 60° C for 5 min (Fractions A - E) or 1 hr (Fraction F). Authentic samples were derivatized in the same manner.

Gas Chromatography

GC separations were carried out on a Varian model 1840-3 gas chromatograph equipped with a flame ionization detector and model 20 recorder. The column was a 6' x 1/8" silanized glass column packed with 3% OV-17 on Gas-Chrom Q (80/100 mesh). The injector temperature was 240° C, the detector temperature 280° C, the N_2 flow rate 30 ml/min, and the column oven temperature was programmed from 70 - 270° C, with 4 min pre-program and post-program periods run at constant temperature. The GC analyses were obtained by injecting 1 - 2 µl of silylation solution with the simultaneous initiation of the temperature program described above. Tracings were obtained with an attentuation setting of 16×10^{-10} amps/m volt.

Gas Chromatography - Mass Spectrometry

An LKB model 9000-S combination GC/MS instrument (LKB Instruments, Rockville, MD) interfaced with a computer (System Industries, Sunnyvale, CA)

was employed for structure determinations. The chromatographic columns used in this system were either a 3' x 1/8" silanized glass column packed with 2% OV-17 on Gas Chrom Q (80/100 mesh) at 170° C, or a 2' x 1/8 silanized glass column containing 3.8% W-98 on Chromosorb W-Hp (80/100 mesh) at 190° C. Operating conditions were as follows: H flow, ml/min; flash heater, ° C; column 170° C or 190° C; separator, °C; accelerating voltage, KV; ionization potential, 70 eV; trap current, amp; filament current, μ amp.

RESULTS

Metabolites of I were found in highest concentration (76%) in the urine collected during the first 24 hr following administration (table 1). An additional 4% of the injected dose was excreted in the 24 - 48 hr urine and 5% in the 0 - 48 hr feces. Analysis of residual tissue levels of radioactivity 48 hr following administration of I indicated that only kidney and liver contained significant levels of activity; total tissue content was less than 4% of the injected dose. Using urinary and fecal excretion data and the residual tissue levels of radioactivity, it was possible to account for 89% of the administered dose of I.

Since the 24 hr urine sample contained the highest concentration of metabolites it was selected for the isolation and identification of the individual metabolites. Isolation of urinary metabolites was accomplished by a differential pH extraction procedure (Fig. 1). Analysis of the radioactivity present in Fractions A - E indicated that Fractions B and E contained greater than 97% of the urinary radioactivity; Fraction B consisted of carboxylic acid metabolites and Fraction E consisted of non-extractable

and/or amphoteric compounds. Metabolites present in Fraction E could readily be isolated in methanolic solution (Fraction F) (16.5%) by means of XAD-2 column chromatography and the remaining aqueous phase was designated Fraction G (16.0%). Fractional distillation of Fraction G (13) indicated that approximately 10% of the urinary radioactivity of I was present in this fraction as [^{3}H] water. The only metabolite present in Fraction G was unchanged I which failed to be completely absorbed by the XAD-2 column. Only trace amounts of urinary radioactivity was found in Fractions A, C, and D.

The number of metabolites present in Fractions B and F were determined by evaluation of their GC profiles. The GC scans of compounds present in Fractions B and F, obtained from control and dosed animals, are presented in Fig. 2 and Fig. 3, respectively. Examination of Fig. 2 revealed the presence of four carboxylic acid metabolites, which when compared to authentic materials possessed retention times identical to metabolites II - V,(Table 2). Similarly, examination of Fig. 3 revealed the presence of a single metabolite which exhibited a retention time identical to I. When the metabolites present in Fractions B and F were examined by TLC, R_f values similar to authentic materials were obtained in each instance (Table 2). Complete identification of each urinary metabolite was accomplished by GC/MS. Total mass spectra were obtained for each metabolite and were computer matched against spectra obtained from authentic samples (Table 2); in each instance perfect agreement was obtained and the metabolites fragmented according to expected pathways.

The quantitation of urinary metabolites was readily achieved by quantitative TLC which depended upon development of a suitable solvent

tem (Table 2). The quantitative data (Table 3) indicated metabolite . .o be the major metabolite (46.1%), accounting for more than twice that of unchanged I (20.6%). Metabolites II - IV account for the remaining urinary radioactivity. Individual variation of metabolites was found to be minimal; no value for any metabolite level in any animal exceeded 1 S.D. from the mean level for the entire population.

DISCUSSION

The results presented in this report reveal significant differences in the metabolism of tryptophan when the indole nucleus is replaced by benzo[b]thiophene. In contrast to tryptophan metabolism where only small amounts of the administered dose are excreted in the first 24 hr (14), the bulk of I metabolites (76%) are excreted during this time period. An additional 4.2% of the injected I was excreted in the 24 - 48 hr period following administration and 4.8% appeared in the feces; thus, greater than 85% of the administered dose of I was excreted in the 48 hr following administration.

A review of the literature (14) on the quantitative aspects of tryptophan metabolism indicated that the major portion of administered tryptophan is metabolized by tryptophan pyrrolase along the hynurenine pathway. Metabolites derived from the tryptophan 5-hydroxylase pathway account quantitatively for only a small percent of the administered dose, while metabolites derived from the aromatic L-amino acid decarboxylase, tryptophan transaminase, and D- and L-amino acid oxidase pathways were insignificant. In light of the pathways available to I, it was surprising that its metabolism occurred by only one of these pathways; namely, the α-keto acid pathway

(Scheme 1). Thus, the immediate questions which can be posed are of two distinctly different types: 1. What are the characteristics of the α-keto acid pathway which make it the sole route of metabolism?; and 2. What are the characteristics of the other metabolic pathways, which, though anticipated, were apparently not involved?

The α-keto acid pathway of endogenous tryptophan metabolism is one of the more complex and least understood pathways. It can occur by either transamination or oxidative deamination; the former being a reversible process catalyzed by aminotransferase enzymes which require pyridoxal phosphate, and the latter being an irreversible process which liberates ammonia and is catalyzed by D- or L-amino acid oxidase (15).

The study of tryptophan animotransferase specificity and multiplicity has been actively pursued for nearly two decades. Early studies (16,17) presented evidence for the existence of at least two hepatic enzymes capable of transaminating tryptophan, which could be adaptively increased (18). Recent work (19,20) has both supported and expanded these earlier findings.

The role of transamination in the metabolism of I is difficult to access due to the existence of D- and L-amino acid oxidases. Even before transamination was recognized as a biochemical pathway, Neubauer had shown that aromatic amino acids could be ixidatively deaminated to the corresponding α-keto acids. Subsequently, Krebs showed that tissue slice preparations of liver of kidney from normal animals could catalyze the oxidation of either D- or L-isomers of as many as twenty-five amino acids (22,23). Little progress has been made in understanding the chemistry of these two enzymes; however, Freter et al. (24) did demonstrate that 5-hydroxytrypto-

phan could be oxidized to 5-hydroxyindole-3-pyruvic acid by L-amino acid oxidase and further broken down to 5-hydroxyindole-3-acetic acid, a pathway predicted by Blaschko & Hoge (25).

There remains, of course, the question of the metabolic fate of benzo-[b]-thiophene-3-pyruvic acid. α-Keto acids are known to be readily decarboxylated to arylacetic acids; a reaction demonstrated for indole-3-pyruvic acid. At least two enzyme systems requiring thiamine pyrosphosphate are known to catalyze this reaction: one oxidative and the other non-oxidative (15). Accordingly, they lead to different products: an acetyl-sCoA and acetaldehyde derivative, respectively, which may be hydrolyzed and/or oxidized to the corresponding arylacetic acid. In addition, α-keto acids are known to undergo non-enzymatic decarboxylative decomposition. In order to determine the stability of III under the conditions of urine collection, a sample of III was incubated in control urine and analyzed at frequent intervals between 2 - 36 hr. The results indicated that III undergoes spontaneous decarboxylation in urine at a sufficiently rapid rate to account for the total urinary content of II.

Compound III can also be reduced to IV in a reversible reaction catalyzed by lactate dehydrogenase. This enzyme is not highly specific and has been shown (16) to be capable of reducing arylpyruvic acids. More recently Zannoni and Weber (26) have isolated and characterized an aromatic α-keto acid reductase from dog heart which was NADH-dependent and capable of reducing indole-3-pyruvic acid.

The final biotransformation of III involves amino acid conjugation, or more specifically glycine conjugation to produce V which was the major

urinary metabolite to I. Elvins and Laidlaw (27) first observed N-(indole-3-acetyl)glycine (indoleaceturic acid) following the administration of tryptamine to dogs. The in vitro biochemistry of this reaction has been worked out in detail (28) and the enzyme which catalyzes the reaction, glycine N-acyltransferase, has been purified and characterized (29). Prior to glycine conjugation, however, II must be activated to the corresponding acyl CoA derivative, the intermediate in the oxidative decarboxylation of III acid.

Finally, one must consider the characteristics of the tryptophan pathways which are anticipated but apparently not involved. The first pathway was that of decarboxylation which was not highly anticipated since our laboratory (30) had shown that I was not a substrate for aromatic L-amino acid decarboxylase. A careful search for the sulfur analog of tryptamine in Fraction C failed to detect its presence at levels of 0.1% of the administered dose. Nevertheless, the presence of II and its glycine conjugate V made it difficult to definitively rule out that possibility; however, Buckpitt (30) using pargyline pretreated rats was unable to detect any amine following the administration of I.

While the absence of decarboxylation was expected, the complete absence of the tryptophan pyrrolase and 5-hydroxylase pathways was somewhat surprising. Partial explanation for this observation may be the higher electron density of the indole nucleus relative to benzo[b]thiophene and its propensity for electrophilic attack (6). Presumptive metabolites of the tryptophan pyrrolase pathway could not be assessed because potential metabolites are unknown compounds. In contrast, the availability of three important 5-hydroxy

derivatives, the benzo[b]thiophene analogs of 5-hydroxytryptophan, 5-hydroxytryptamine and 5-hydroxyindole-3-acetic acid, made it possible to demonstrate that none of these compounds were present as urinary metabolites of I at levels down to 1.0 μg/ml (0.1% of the administered dose).

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TABLE 1

Excretion and tissue disposition of tryptophan-S and metabolites

Sample	Radioactivity	% of Administered Dose[a]
Urine (0-24 hr)	152.8 µCi	76.4
Urine (24-48 hr)	8.4 µCi	4.2
Feces (0-48 hr)	9.6 µCi	4.8
Plasma	3.52 µCi/ml	1.76
Liver	1.60 µCi/g	0.80
Kidneys	0.9 µCi/g	0.45
Lungs	0.26 µCi/g	0.13
Heart	0.22 µCi/g	0.11
Testes	0.34 µCi/g	0.17
Spleen	0.12 µCi/g	0.06
Fat	0.16 µCi/g	0.08
Brain	0.18 µCi/g	0.09

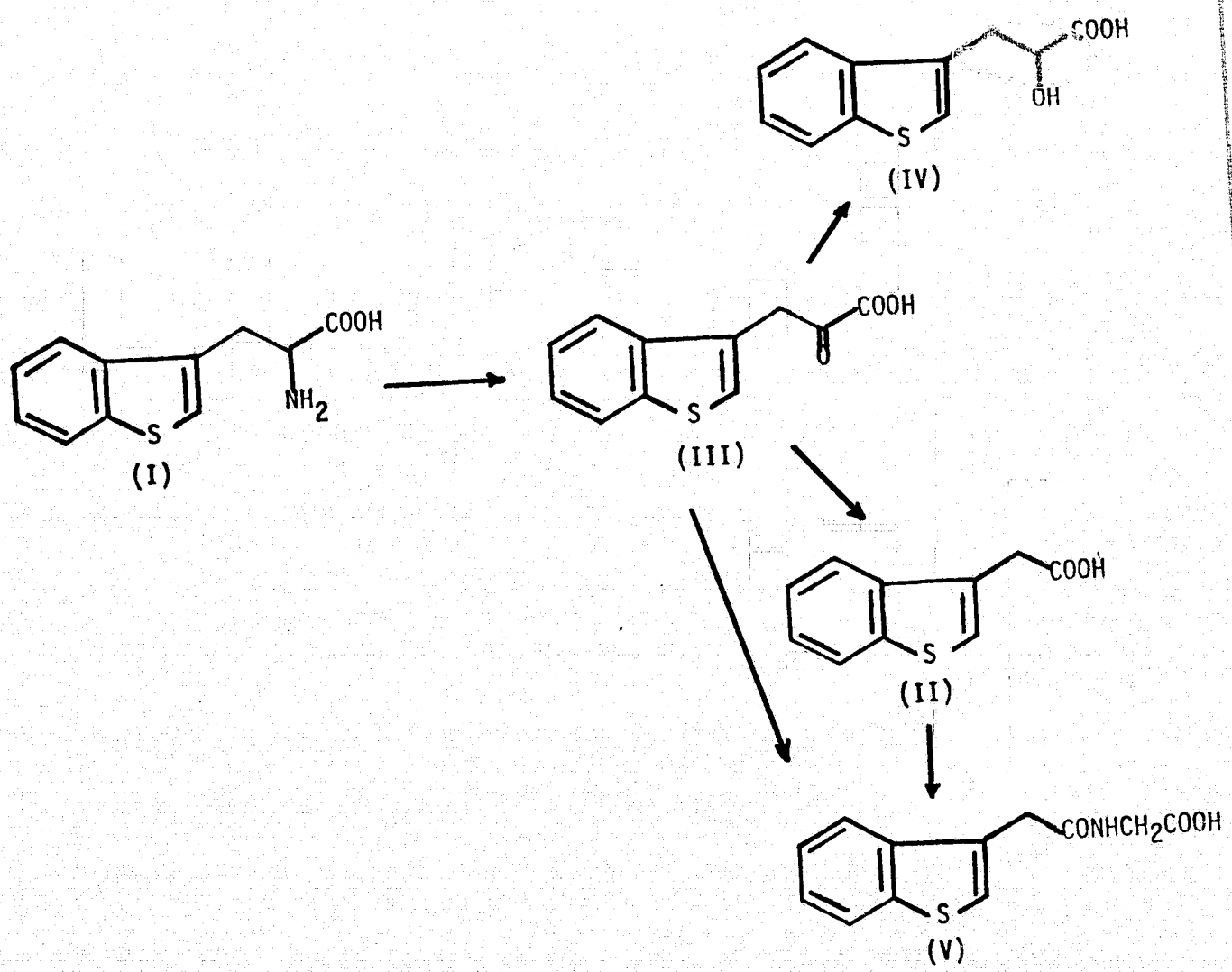
[a]Mean value of duplicate determinations are shown.

TABLE 3

Urinary excretion of tryptophan-S and metabolites by rats

The values shown are mean % of dose S.D. for a group of five rats.

Compound	% of dose
Tryptophan-S (I)	20.6 ± 0.8
Metabolites	
II	1.3 ± 0.1
III	14.2 ± 1.4
IV	4.9 ± 0.3
V	46.2 ± 1.7



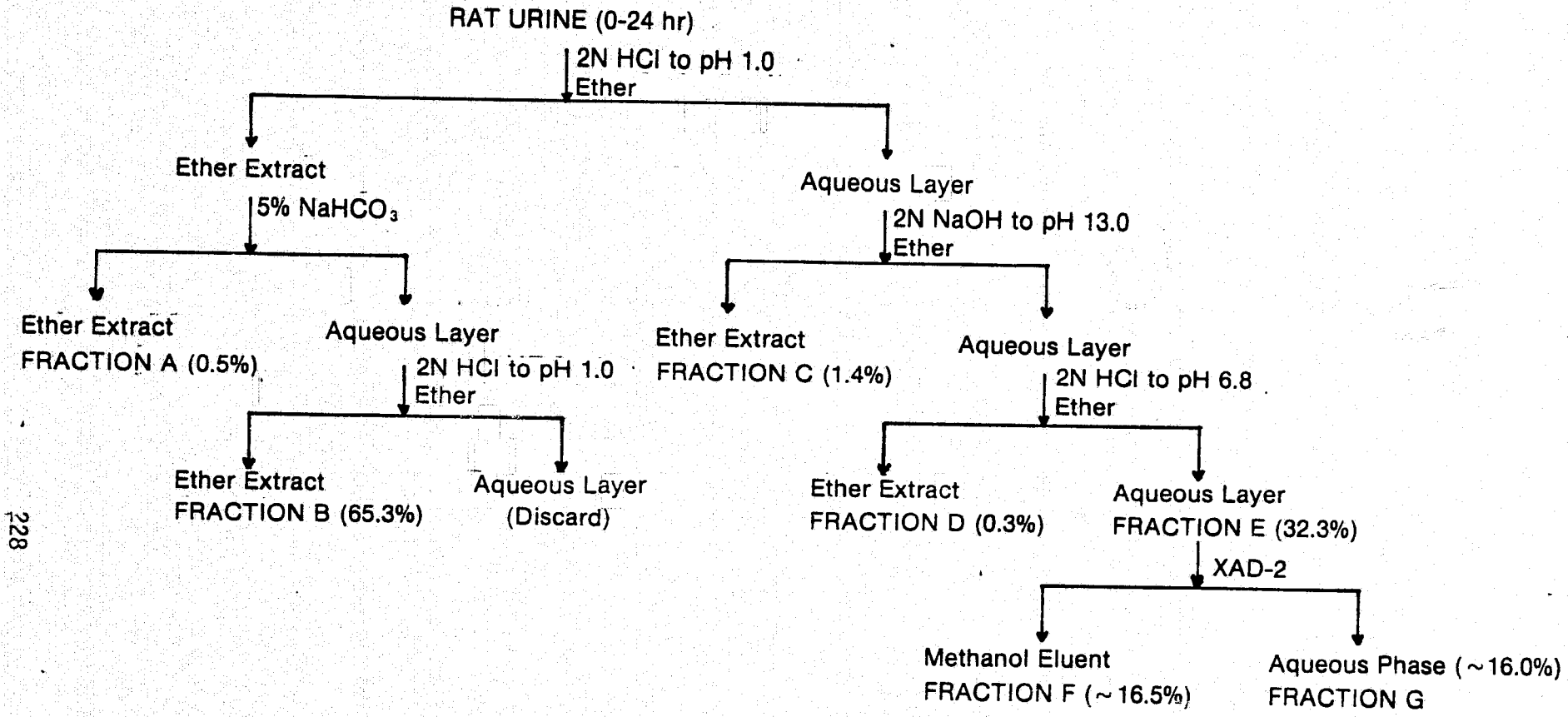
Scheme 1. The metabolic fate of tryptophan-S in the rat.

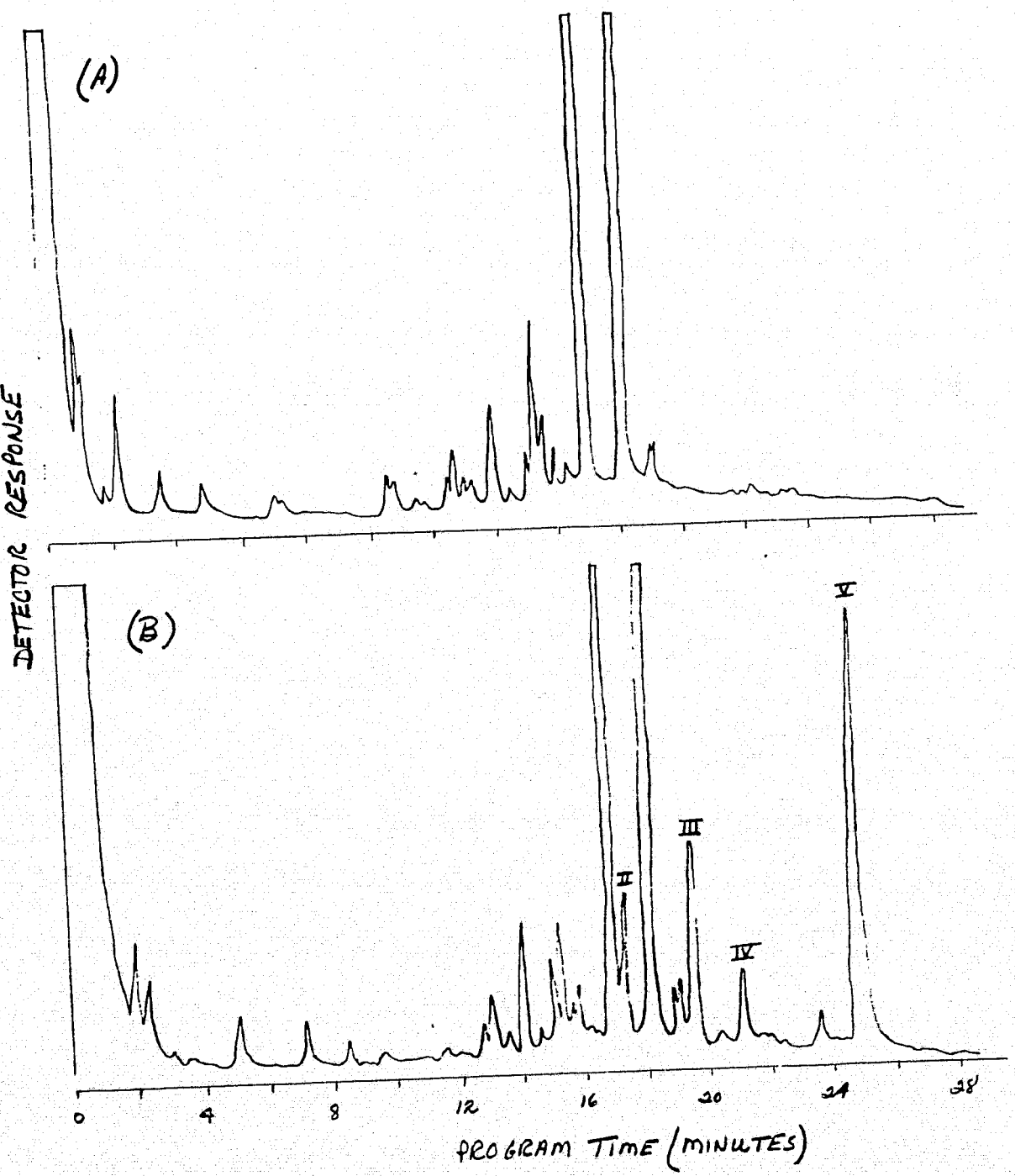
Legends

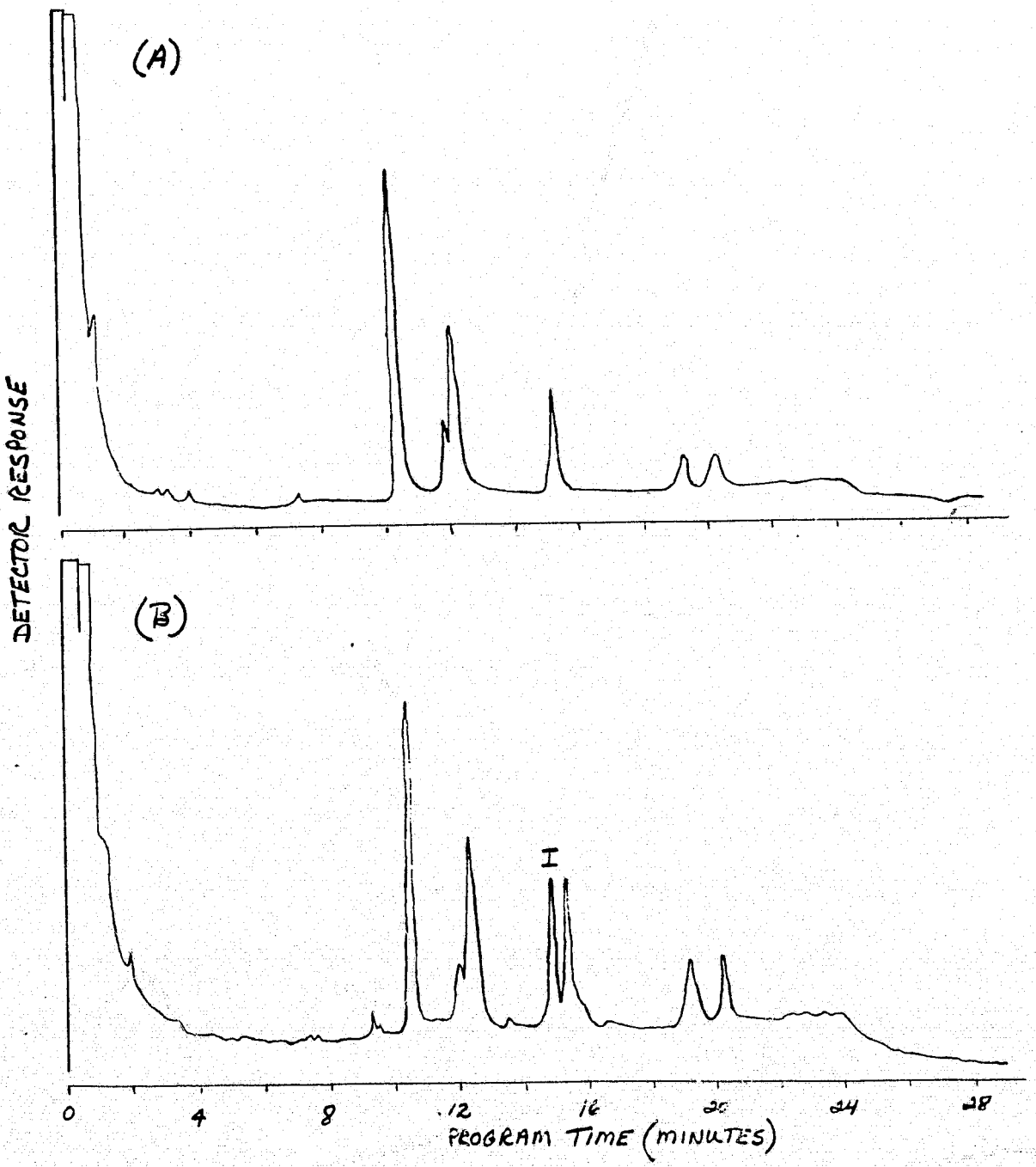
Fig. 1. Scheme for the isolation of metabolites from rat urine.

Fig. 2. GC separation of TMS derivatives present in Fraction B obtained from control (A) and drug-treated rats (B). Numbers II-V denote metabolites.

Fig. 3. GC separation of TMS derivatives present in Fraction F obtained from control (A) and drug-treated rats (B). Number I denotes the parent compound.


RAT URINE (0-24 hr)
2N HCl to pH 1.0
Ether
Ether Extract
5% NaHCO3
Aqueous Layer
2N NaOH to pH 13.0
Ether
Ether Extract
FRACTION A (0.5%)
Aqueous Layer
2N HCl to pH 1.0
Ether
Ether Extract
FRACTION C (1.4%)
Aqueous Layer
2N HCl to pH 6.8
Ether
Ether Extract
FRACTION B (65.3%)
Aqueous Layer
(Discard)
Ether Extract
FRACTION D (0.3%)
Aqueous Layer
FRACTION E (32.3%)
XAD-2
Methanol Eluent
FRACTION F (~16.5%)
Aqueous Phase (~16.0%)
FRACTION G


(A)
(B)
DETECTOR RESPONSE
II
III
IV
V
0
4
8
12
16
20
24
28
PROGRAM TIME (MINUTES)


(A)
(B)
I
DETECTOR RESPONSE
0
4
8
12
16
20
24
28
PROGRAM TIME (MINUTES)

COMPARATIVE EFFECTS OF 5,6-DIHYDROXYTRYPTAMINE AND ITS BENZO[b]THIOPHENE ANALOGUE ON BIOGENIC AMINES IN THE RAT*

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ABSTRACT

The effects of 5,6-dihydroxytryptamine (5,6-DHT) and its benzo-[b]thiophene analogue (5,6-DHT-S) have been compared with regard to their ability to influence tissue levels of 5HT and NE in rats. After i.p. administration, both compounds caused significant reduction in NE levels in heart and spleen, while only 5,6-DHT reduced spleen 5HT, and neither compound had any effect on brain NE or 5HT. When administered directly into the lateral ventricle, both compounds caused reduced NE levels; the duration of effect was less than 1 day. In contrast to the prolonged lowering of brain 5HT and 5HIAA observed after 5,6-DHT, the benzo[b]thiophene analogue was without effect.

INTRODUCTION

The search for compounds to use as biochemical tools for examining the function of neurotransmitter systems has seen a significant volume of publications in the past decade, especially in terms of those systems involving biogenic amines. For example, the initial discovery of the long-lasting depletion of heart norepinephrine (NE) following administration of a single dose of 6-hydroxydopamine (6-HDA) [1] led to hundreds of studies of the latter compound and culminated in its present characterization as an agent capable of producing a "chemical denervation" of adrenergic neurons [2]. Because of the poor ability of 6-HDA to penetrate the blood-brain barrier, it was soon found that injections into the lateral ventricles or other area(s) of the brain were necessary to obtain effects on central norepinephrine systems [3]; once the blood-brain barrier was bypassed, expected effects were obtained [4].

In 1971, Baumgarten and coworkers [5] published the first report of the actions of 5,6-dihydroxytryptamine (5,6-DHT), a compound apparently analagous to 6-HDA, but active only on those neuronal fibers containing serotonin (5HT). Since that first report, a number of papers have appeared confirming the fact that 5,6-DHT caused selective degeneration of indoleamine terminals in a manner similar to that caused by 6-HDA on adrenergic neurons [6-8]. As with 6-HDA, the poor ability of 5,6-DHT to cross the blood-brain barrier demanded that the compound be administered by intraventricular injection in order to obtain an effect on

brain 5HT [5-9].

Additional efforts by Baumgarten, et al [10] demonstrated that peripheral administration of 5,6-DHT produced a marked decrease of NE levels in heart and spleen of rats and mice, although the NE levels returned to normal in 24 hours. In this regard, it is of interest that Heikkila and Cohen [11] found that 5,6-DHT was an inhibitor of 5HT and dopamine uptake by brain slices; in contrast, 6-hydroxydopamine under similar conditions inhibited dopamine uptake but was without effect on 5HT uptake. Decreased uptake of 5HT by brain synaptosomes in vitro has been domonstrated by Baldessarini and Gerson [12] to occur after pretreatment of rats with 5,6-DHT; these authors found no reduction in NE uptake by spinal cord synaptosomes. Richardson, et al [13] found depletion of brain 5HT but not brain NE at 14 days after a single dose of 5,6-DHT. Against this background, one unusual report is that of Costa, et al [14] who found that samples of 5,6-DHT differed in potency as measured by their effects on 5HT.

This laboratory has been interested in the application of principles of selective molecular modification as a means of studying biological structure-activity relationships of indolic compounds for a number of years. In particular, efforts have centered on the pharmacological effects of the benzo[b]thiophene and 1-methylindole analogues of indolealkylamines [15-17], the intestinal absorption of similar analogues of tryptophan [18-19], and the substrate specificity of aromatic-L-amino acid decarboxylase with regard to tryptophan analogues

[20]. Recently, the synthesis of 5,6-DHT-S [21] made available sufficient material to permit a detailed study of the role of the indolic nitrogen in the action of 5,6-DHT on monoaminergic systems.

MATERIALS AND METHODS

Adult, male Sprague-Dawley rats (275 - 300 g) were obtained from Murphy Breeding Laboratories, Plainfield, Indiana and maintained for at least five days prior to use on a diet of Purina Laboratory Chow and tap water *ad lib*. All chemicals and reagents were purchased from commercial sources.

Drugs given intraperitoneally were made up as solutions in distilled water such that the specified dosage in mg/kg was contained in a volume of 1 ml/kg body weight. Drugs injected intraventricularly were dissolved in diluted mammalian Ringer's solution (1/3 distilled water, 2/3 Ringer's solution) containing 0.1 mg ascorbic acid per milliliter at concentrations such that the desired dose was given in a 10 μl volume.

Intraventricular injections of drugs were accomplished according to the method of Noble, *et al* [22] with the following modifications. Injection volumes were delivered by Hamilton 50 μl syringes and limited to 10 μl maxima. The syringes were equipped with 3/8" 27-guage needles and were stereotoxically placed to a depth of 3.75 $\pm$ .05 mm in the lateral ventricle. Following injection, the hole in the skull was closed with bone wax, the wound was dusted with sulfathiazole and closed with wound clips.

Rats were sacrificed by decapitation, and tissues were removed,

washed in cold 0.9% saline, blotted dry and stored at -20° C until assay. Brains were dissected just prior to assay into three sections: cerebral hemispheres, cerebellum, and the remainder (arbitrarily referred to as "brainstem"). Tissue levels of 5HT, NE, and 5HIAA were determined as reported by Miller, et al [23].

RESULTS

Effects of Parenteral Administration of 5,6-DHT and 5,6-DHT-S

The results obtained after administration of single i.p. doses of 5,6-DHT and 5,6-DHT-S (30 mg/kg) to rats are presented in Table 1. The results for heart NE show the compounds to be virtually identical; an initial decrease to minimal values of approximately 50% of zero time values is followed by a return to slightly above normal levels by 16 hours after dosage. The actions of the two analogues on spleen NE were slightly different. 5,6-DHT reduced spleen NE levels to a minimal value of less than 50% of control at 8 hours after dosage; at 24 hours values were still only 71% of control. The action of 5,6-DHT-S was slightly less effective (just reaching the 50% level of depletion at 4 hours) and shorter in duration, returning to normal levels by 16 hours after dosage.

The results for 5HT in spleen were quite different in terms of the two compounds. 5,6-DHT caused a significant and persistent decrease, reaching a minimal level of about 50% of control at 16 hours and lasting beyond the 24 hour time point. In contrast, 5,6-DHT-S produced an initial increase in spleen 5HT, peaking at

2 hours, and returning to normal levels by 4 hours. As might be expected, no effects whatsoever were seen with either compound in terms of brain 5HT or NE.

Time Course of Effects of Intraventricular Administration of 5,6-DHT and 5,6-DHT-S

Since the blood-brain barrier prevented the 5,6-dihydroxy compounds from having any effects on brain biogenic amines, a series of studies were performed with the injection of the compounds directly into the lateral ventricles of the rat brain. Because of the well-known differences in amine biochemistry in various brain areas, brains were divided into three parts prior to the assay. These results are presented in Tables 2, 3, and 4 for NE, 5HT and 5HIAA, respectively.

The effects on NE levels, as shown in Table 2, show that both the indole and benzo[b]thiophene compounds are completely inactive at the lowest dose of 20 μg/rat, while some slight but significant decreases are seen at the higher doses. In the cerebral hemispheres, 5,6-DHT causes a significant reduction of NE levels at both 40 and 80 μg doses from 2 to 16 hours. In contrast, 5,6-DHT-S significantly lowers NE at the 40 μg dose only at the 4 hour time point, while the 80 μg dose of the benzo[b]thiophene compound is effective in lowering NE levels at the 4, 8, and 16 hour points. Cerebellar NE levels were significantly lowered by 5,6-DHT at 1 and 2 hours after doses of 40 or 80 μg/rat; the only point showing a significant effect of 5,6-

DHT-S was at 1 hour after the 80 µg/rat dose, where a decreased NE level was observed. In the brainstem portion, neither compound had a significant effect on NE levels at the lowest (20 µg) dose; the 1 hour time point showed a modest, though not statistically significant lowering of NE. At a dose of 40 µg/rat, 5,6-DHT lowered brainstem NE significantly at the 1, 2, and 4 hour points, while at the 80 µg/rat doses, both 5,6-DHT and 5,6-DHT-S significantly lowered the NE at 1, 2, and 4 hours.

The effects of the two compounds on 5HT levels were distinctly different, as shown in Table 3. 5,6-DHT-S was completely ineffective; no significant alterations in 5HT levels were seen in any brain area, at any dose or time point. In contrast, the effects of 5,6-DHT were clearly unique to each brain area. In cerebral hemispheres, the lowest dose (20 µg) of 5,6-DHT was ineffective, the intermediate dose (40 µg/rat) caused significant lowering of 5HT levels from 2 hours through 2 days, and the high dose (80 µg/rat) was effective for at least 16 days. Cerebellar 5HT was relatively resistant to the effects of 5,6-DHT; a significant lowering was observed only at 16 hours, 1 day and 4 days after the highest dose (80 µg/rat). In the brainstem, the low dose (20 µg/rat) of 5,6-DHT caused a significant lowering of 5HT at the 4 hour and 1 day points, while at the highest dose (80 µg/rat), 5HT levels were significantly depressed from the 1 hour point to at least 16 days. The intermediate dose (40 µg/rat) decreased brainstem 5HT from the 1 hour point through 8 days; the lack of statistical significance at the

16 hour, 1 day and 4 day points may be attributed to the larger standard deviations obtained.

The effects on 5HIAA levels in the various brain areas are shown in Table 4. With the exception of the 2 hour point after the high (80 µg/rat) dose of 5,6-DHT-S in brainstem, no significant changes in 5HIAA levels were seen. A lowering of 5HIAA was seen after the high (80 µg/rat) dose of 5,6-DHT in cerebral hemispheres at all time points from 4 hours to 16 days. At the intermediate dose (40 µg/rat) a lowering of hemisphere 5HIAA was seen from 4 hours to 8 days, although only the 16 hour and 4 and 8 day points were statistically significant. No changes were observed after the low dose. Cerebellar 5HIAA was resistant to the effects of 5,6-DHT; only a few time points at the high dose were significantly lowered. In general, the effects of the high dose of 5,6-DHT on brainstem 5HIAA reflected the actions of the compound on 5HT, that is a lowering from 2 hours through 16 days, although the 4 hour time point was not statistically significant. The intermediate (40 µg/rat) dose showed a significant lowering of brainstem 5HIAA only at the 4, 8, and 16 hour time points, while the lowest dose had a significant effect only at 4 hours and 2 days.

DISCUSSION

Since the first report by Baumgarten and co-workers on the selective neurotoxicity of 5,6-DHT [5] this compound has been of wide interest to investigators dealing with various aspects of serotonergic function in animals. Of particular interest has been

the selectivity of the compound; its ability to cause damage to serotonergic stores without affecting catecholaminergic stores is unique, as is its exceptionally long duration of action [5-13]. Although some questions have been raised of its relative potency (perhaps due to an impurity in some preparations), 5,6-DHT has, in general been shown to be an effective and selective serotonin neurotoxin [14].

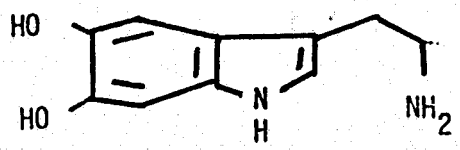
For the past 8 years, this laboratory has had a continuing interest in the comparative actions of benzo[b]thiophene analogues of indolic compounds having biological significance. Beginning with the comparative effects of serotonin and its benzo[b]thiophene analogue [15], and continuing through the pressor effects of tryptamine and its benzo[b]thiophene and 1-methylindole analogues [16] studies have shown that certain similarities and differences in pharmacologic potency exist with regard to the atom located at the 1-position of the heterocycle. For example, the substitution of a sulfur atom for the ring nitrogen in tryptophan has little effect on the active transport of the molecule across the intestine [19]; however, the slightly greater lipid solubility of the benzo-[b]thiophene analogue of tryptophan does lead to a slightly more rapid passive diffusion component in the in situ perfused intestine [18]. When the analogues of tryptophan were tested as substrates for aromatic-L-amino acid decarboxylase, however, replacement of the indolic nitrogen by a sulfur atom led to a complete loss of substrate activity [20].

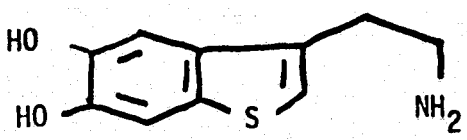
The possibility of examining the neurotoxic action of 5,6-DHT by use of its benzo[b]thiophene analogue led to its synthesis [21] and the comparative studies reported in the present paper. When administered parenterally to rats, 5,6-DHT-S had an effect on NE in heart and spleen basically similar to that of 5,6-DHT, that is, a modest depletion seen over 2 - 8 hours after dosage. No effects of either compound could be measured on brain NE or 5HT, nor did the benzo[b]thiophene have any effect on spleen levels of 5HT, other than a modest elevation at the 2 hour time point (Table 1).

When administered by direct injection into the lateral ventricle of the rat brain, 5,6-DHT-S had, in general, effects on NE similar to those of the indolic 5,6-DHT (Table 2), although the benzo[b]-thiophene analogue is only about half as potent. In sharp contrast, however, are the data presented in Tables 3 and 4, clearly demonstrating that the 5,6-DHT-S has no effects on brain 5HT whatsoever. The actions of 5,6-DHT, as reported in the present paper, are basically in agreement with previous reports, especially that of Costa, et al [14]. Thus the cerebellum is most resistant to the 5HT-depleting actions; no significant effects are seen until the dose of 80 µg is reached. Another similarity is the extent of 5HT depletion seen with 5,6-DHT. The material used in this study was obtained from the Regis Chemical Company, is presumably similar to that described as 5,6-DHT-II by Costa, et al [14], and therefore is slightly less potent than the material originally used by Baumgarten and co-workers [5].

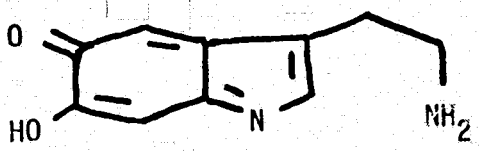
Perhaps the most significant aspect of the present report lies in

the inability of the benzo[b]thiophene analogue of 5,6-DHT to exert any significant effects on levels of 5HT. One explanation for this striking difference may lie in the possibility that the action of 5,6-DHT on 5HT stores requires an intermediate quinone-type structure as has been proposed for 6-hydroxydopamine [24]. If one considers the structures of 5,6-DHT and its benzo[b]thiophene analogue:





it is reasonable to assume that a likely quinone structure can be proposed for the indolic compound that would not be chemically feasible for the benzo[b]thiophene:



This may be the structure active in causing 5HT depletion and degeneration of serotonergic fibers. In this sense, both compounds might be expected to have a short-acting effect on NE stores, merely since they both contain a catechol function that could act as a non-specific substitute for catecholamines in their storage processes.

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TABLE 1

Effects of Parenteral Administration of 5,6-DHT and 5,6-DHT-S

Each value is the mean ± S.E.M. of values obtained from 4 animals, except that control values are obtained from 16 rats. Values that significantly differ from control ($p < .05$) are underlined.

Time (hrs)	Compound*	Heart NE µg/g	Spleen NE µg/g	Spleen 5HT µg/g	Brain NE µg/g	Brain 5HT µg/g
0		0.29±.02	0.42±.07	1.40±.09	0.49±.04	0.61±.04
1	N	0.19±.02	0.33±.02	1.35±.16	0.47±.03	0.61±.03
	S	0.24±.02	0.28±.01	1.92±.18	0.49±.05	0.64±.07
2	N	0.14±.01	0.25±.02	1.14±.08	0.48±.02	0.58±.07
	S	0.15±.01	0.28±.03	2.21±.28	0.51±.05	0.59±.04
4	N	0.17±.01	0.21±.01	1.16±.13	0.50±.03	0.60±.06
	S	0.18±.03	0.21±.04	1.56±.10	0.48±.03	0.58±.05
8	N	0.25±.02	0.20±.01	0.97±.07	0.51±.06	0.57±.03
	S	0.23±.01	0.25±.04	1.42±.25	0.46±.02	0.62±.02
16	N	0.33±.02	0.25±.04	0.72±.05	0.47±.03	0.65±.07
	S	0.30±.02	0.37±.02	1.64±.21	0.50±.05	0.58±.03
24	N	0.31±.01	0.30±.03	0.85±.06	0.48±.02	0.61±.02
	S	0.27±.03	0.39±.04	1.53±.04	0.45±.05	0.59±.05

*N = 5,6-DHT; S = 5,6-DHT-S

TABLE 2

Effects of Intraventricular Dosage of Compounds on Regional Brain NE

Time/Dosage*		Cerebral Hemispheres			Cerebellum			Brainstem		
		20µg	40µg	80µg	20µg	40µg	80µg	20µg	40µg	80µg
0 hrs	-	0.35±.06	0.43±.04	0.40±.05	0.14±.02	0.17±.01	0.17±.02	0.98±.13	1.05±.08	1.01±.11
1 hrs	N	0.34±.07	0.37±.06	0.36±.04	0.13±.02	0.12±.03	0.13±.02	0.76±.19	0.85±.06	0.85±.10
"	S	0.37±.07	0.33±.07	0.39±.07	0.16±.03	0.17±.03	0.13±.02	0.74±.19	0.99±.05	0.70±.13
2 hrs	N	0.32±.10	0.30±.04	0.28±.07	0.13±.03	0.14±.02	0.13±.02	0.81±.16	0.85±.09	0.74±.14
"	S	0.37±.04	0.37±.05	0.37±.04	0.13±.03	0.16±.02	0.15±.03	0.84±.11	0.99±.11	0.72±.14
4 hrs	N	0.32±.08	0.31±.07	0.29±.03	0.13±.04	0.17±.03	0.16±.02	0.93±.11	0.75±.10	0.77±.06
"	S	0.43±.12	0.35±.02	0.34±.04	0.15±.02	0.15±.02	0.16±.02	0.92±.06	0.91±.11	0.79±.10
8 hrs	N	0.41±.05	0.30±.09	0.30±.05	0.12±.04	0.16±.04	0.16±.02	1.03±.12	0.99±.08	0.91±.13
"	S	0.37±.03	0.37±.05	0.27±.08	0.15±.01	0.16±.02	0.16±.03	0.98±.11	0.92±.13	0.93±.16
16 hrs	N	0.42±.10	0.30±.09	0.27±.03	0.13±.03	0.15±.04	0.14±.03	0.88±.09	1.07±.17	0.93±.11
"	S	0.36±.09	0.43±.04	0.32±.04	0.12±.02	0.19±.02	0.15±.02	0.97±.09	1.07±.09	1.04±.11
1 day	N	0.36±.03	0.35±.05	0.34±.09	0.11±.03	0.15±.02	0.14±.03	1.02±.17	1.08±.06	0.94±.10
"	S	0.39±.06	0.39±.04	0.30±.09	0.13±.03	0.17±.02	0.17±.01	0.93±.15	1.03±.14	0.93±.11
2 days	N	0.33±.08	0.35±.05	0.43±.05	0.15±.02	0.17±.02	0.15±.03	0.90±.11	1.13±.14	0.93±.11
"	S	0.35±.11	0.34±.05	0.37±.02	0.13±.03	0.15±.03	0.16±.02	0.94±.15	0.97±.16	0.90±.17
4 days	N	0.37±.06	0.41±.06	0.41±.11	0.12±.02	0.18±.03	0.17±.01	0.92±.19	1.08±.11	0.93±.13
"	S	0.33±.06	0.36±.04	0.43±.09	0.11±.03	0.16±.02	0.17±.02	0.89±.17	1.09±.11	0.89±.14
8 days	N	---	0.40±.08	0.37±.05	---	0.18±.03	0.15±.01	---	1.13±.15	0.92±.09
"	S	---	0.41±.05	0.43±.04	---	0.18±.02	0.16±.02	---	1.12±.13	1.05±.11
16 days	N	---	---	0.35±.12	---	---	0.16±.02	---	---	0.98±.07
"	S	---	---	0.35±.08	---	---	0.15±.03	---	---	0.99±.05

*N = 5,6-DHT; S = 5,6-DHT-S

Each value is the mean ± S.E.M. of values obtained from 4 animals, except that control values are obtained from 16 rats. Values that significantly differ from control ($p < .05$) are underlined.

TABLE 3

Effects of Intraventricular Dosage of Compounds on Regional Brain 5HT

Time/Dosage*		Cerebral Hemispheres			Cerebellum			Brainstem		
		20µg	40µg	80µg	20µg	40µg	80µg	20µg	40µg	80µg
0 hrs	-	0.34±.04	0.31 .04	0.33±.02	0.20±.03	0.18±.01	0.19±.02	1.09±.11	1.02±.16	1.17±.13
1 hr	N	0.35±.03	0.29±.05	0.32±.04	0.19±.02	0.15±.02	0.16±.02	0.96±.16	0.84±.11	0.85±.13
"	S	0.31±.03	0.30±.03	0.33±.05	0.21±.02	0.18±.03	0.96±.17	0.96±.17	1.00±.11	1.18±.18
2 hrs	N	0.29±.03	0.26±.02	0.29±.03	0.20±.04	0.16±.02	0.18±.02	1.00±.17	0.80±.13	0.89±.06
"	S	0.31±.06	0.28±.05	0.31±.04	0.22±.05	0.16±.02	0.19±.03	1.17±.15	0.87±.13	1.11±.11
4 hrs	N	0.29±.04	0.24±.03	0.26±.03	0.17±.03	0.17±.02	0.17±.02	0.90±.08	0.75±.12	0.84±.16
"	S	0.34±.02	0.31±.16	0.31±.04	0.20±.03	0.17±.03	0.22±.04	1.13±.07	0.88±.15	1.05±.11
8 hrs	N	0.31±.02	0.25±.03	0.28±.03	0.21±.02	0.16±.04	0.19±.01	0.96±.09	0.80±.14	0.83±.12
"	S	0.32±.02	0.30±.04	0.30±.03	0.22±.04	0.19±.02	0.20±.03	1.04±.10	0.98±.14	1.13±.15
16 hrs	N	0.29±.03	0.24±.04	0.22±.06	0.20±.02	0.17±.02	0.14±.03	0.98±.12	0.89±.15	0.91±.07
"	S	0.34±.03	0.31±.04	0.37±.03	0.18±.03	0.19±.02	0.20±.03	1.04±.10	1.06±.17	1.17±.06
1 day	N	0.36±.03	0.25±.03	0.26±.02	0.19±.03	0.17±.02	0.14±.02	0.80±.14	0.89±.14	0.97±.10
"	S	0.34±.04	0.33±.04	0.30±.06	0.20±.03	0.17±.04	0.19±.03	1.00±.12	1.11±.11	1.18±.11
2 days	N	0.35±.03	0.24±.03	0.28±.01	0.19±.03	0.19±.03	0.16±.02	1.02±.12	0.71±.17	0.95±.11
"	S	0.36±.03	0.28±.04	0.32±.03	0.19±.03	0.18±.03	0.21±.02	1.19±.11	0.95±.13	1.05±.12
4 days	N	0.31±.03	0.32±.05	0.26±.03	0.18±.04	0.18±.04	0.16±.01	0.96±.11	0.92±.07	0.97±.14
"	S	0.37±.03	0.33±.03	0.32±.03	0.21±.03	0.20±.04	0.18±.02	1.04±.11	1.08±.11	1.19±.13
8 days	N	---	0.27±.04	0.27±.03	---	0.19±.03	0.21±.02	---	0.80±.07	0.74±.20
"	S	---	0.29±.04	0.33±.03	---	0.21±.05	0.18±.03	---	1.10±.10	1.19±.13
16 days	N	---	---	0.23±.03	---	---	0.18±.02	---	---	0.91±.08
"	S	---	---	0.33±.02	---	---	0.18±.03	---	---	1.09±.10

*N = 5,6-DHT; S = 5,6-DHT-S

Each value is the mean S.E.M. of values obtained from 4 animals, except that control values are obtained from 16 rats. Values that significantly differ from control ($p < .05$) are underlined.

TABLE 4

Effects of Intraventricular Dosage of Compounds on Regional Brain 5HIAA

Time/Dosage*		Cerebral Hemispheres			Cerebellum			Brainstem		
		20µg	40µg	80µg	20µg	40µg	80µg	20µg	40µg	80µg
0 hrs	-	0.52±.04	0.50±.07	0.56±.06	0.23±.01	0.21±.02	0.25±.02	0.76±.04	0.65±.10	0.77±.09
1 hr	N	0.56±.04	0.52±.12	0.62±.06	0.25±.04	0.21±.03	0.26±.04	0.82±.14	0.75±.16	0.86±.12
"	S	0.57±.04	0.42±.13	0.50±.09	0.24±.03	0.18±.03	0.24±.05	0.87±.15	0.88±.13	0.80±.12
2 hrs	N	0.47±.05	0.57±.11	0.52±.11	0.21±.02	0.22±.03	0.20±.06	0.86±.14	0.79±.12	0.97±.16
"	S	0.55±.06	0.47±.09	0.48±.12	0.26±.03	0.22±.03	0.27±.05	0.89±.14	0.69±.11	1.00±.09
4 hrs	N	0.51±.10	0.44±.13	0.43±.09	0.19±.06	0.21±.03	0.18±.03	0.55±.12	0.51±.07	0.87±.17
"	S	0.58±.13	0.46±.09	0.56±.06	0.26±.07	0.23±.05	0.28±.05	0.72±.06	0.58±.11	0.98±.12
8 hrs	N	0.54±.05	0.42±.11	0.45±.05	0.25±.03	0.21±.02	0.20±.04	0.65±.21	0.47±.07	0.57±.08
"	S	0.54±.04	0.43±.07	0.55±.10	0.25±.02	0.24±.04	0.27±.03	0.86±.15	0.68±.13	0.73±.11
16 hrs	N	0.44±.06	0.38±.07	0.45±.05	0.21±.02	0.18±.04	0.20±.05	0.71±.08	0.49±.08	0.59±.06
"	S	0.46±.08	0.48±.07	0.63±.08	0.20±.06	0.23±.02	0.23±.03	0.68±.07	0.67±.09	0.67±.09
1 day	N	0.45±.06	0.41±.13	0.38±.04	0.23±.02	0.19±.04	0.20±.04	0.72±.07	0.56±.12	0.62±.07
"	S	0.53±.08	0.55±.07	0.50±.07	0.22±.05	0.19±.04	0.28±.03	0.72±.09	0.68±.08	0.85±.08
2 days	N	0.55±.04	0.46±.07	0.44±.07	0.19±.03	0.21±.05	0.18±.03	0.59±.05	0.65±.12	0.55±.10
"	S	0.49±.04	0.53±.09	0.57±.08	0.23±.02	0.24±.03	0.28±.06	0.72±.04	0.63±.16	0.89±.07
4 days	N	0.53±.11	0.36±.11	0.39±.02	0.19±.04	0.24±.03	0.23±.04	0.79±.11	0.60±.12	0.59±.11
"	S	0.56±.09	0.48±.12	0.57±.06	0.25±.03	0.24±.04	0.23±.03	0.82±.12	0.70±.13	0.79±.15
8 days	N	---	0.40±.04	0.34±.11	---	0.21±.02	0.28±.03	---	0.62±.07	0.60±.05
"	S	---	0.51±.08	0.61±.11	---	0.24±.03	0.21±.06	---	0.73±.08	0.88±.09
16 days	N	---	---	0.46±.04	---	---	0.21±.04	---	---	0.52±.13
"	S	---	---	0.58±.12	---	---	0.25±.03	---	---	0.90±.13

*N = 5,6-DHT; S = 5,6-DHT-S

Each value is the mean ± S.E.M. of values obtained from 4 animals, except that control values are obtained from 16 rats. Values that significantly differ from control ($p < .05$) are underlined.

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CONTRACTILE RESPONSES TO TRYPTAMINE ANALOGUES IN ISOLATED SMOOTH MUSCLE

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Running Title: Effects of tryptamine analogues on smooth muscle.

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INTRODUCTION

In 1954, two reviews of the earlier literature on 3-(2-amino ethyl) indole (tryptamine) and its 5-hydroxylated analogue (serotonin) were published (1,2); both of these hinted at a unique set of physiological receptor systems for indolealkylamines. Most of the support for specific tryptamine or serotonin receptors came from two laboratories who had published the previous year (3,4). A variety of subsequent studies led to the conclusion that receptors with considerable specificity for tryptamine did exist in some peripheral smooth muscle sites (5) although there was some question of the existence of peripheral receptors specific for serotonin (6). Recently, Pinder, et al (7) examined some effects of the idene and benzo[b]thiophene isosteres of serotonin on the rat stomach fundus preparation. More recently, this laboratory has reported on the comparative pressor effects of the benzo[b]thiophene and 1-methylindole analogues of tryptomine in the intact rat (8). The present paper reports on the isolated rat aorta and stomach fundus preparations as a test system for comparing the isosteres of tryptamine.

MATERIALS AND METHODS

Adult, male Sprague-Dawley rats (180-220 g) were obtained from Murphy Breeding Labs, Plainfield, Indiana, U.S.A. and maintained on a diet of Purina laboratory chow and tap water ad lib for 7-10 days prior to experimental use. The animals were sacrificed by decapitation. Aortic strips (1.5 to 2 mm wide and 3 cm long) were prepared by the method of Furchgott and Bhadrakon (9) and suspended in a 60 ml organ bath. Stomach fundus strips (one strip 1.5 to 2.0 mm wide and 1.5 cm long from each stomach) were prepared by the method of Offermeier and Ariens (10), and likewise suspended in a 60 ml organ bath.

Drugs and solutions. The Kreb's bicarbonate solution in which the tissues were suspended contained the following compounds in m moles per liter: NaCl, 118.5; KCl, 4.8; $CaCl_2$, 2.5; NaH_2PO_4, 1.18; $MgCl_2$, 1.18; $NaHCO_3$, 24.87; D-glucose, 11.1. In addition 10 mg of disodium ethylenediamine tetraacetic acid (Na_2EDTA) were added per liter to protect the added amines from oxidation. The solution was stored at 4° C until use; during the experiment it was maintained at 37° C and bubbled with 95% O_2/5% CO_2. The pH of the solution at room temperature without bubbling was adjusted to 6.5 with concentrated HCl; after warming and bubbling for at least ten minutes the pH increased to 7.4.

The following drugs were used: l-norepinephrine hydrochloride (Regis Chemical Co.), 5-hydroxytryptamine creatinine sulfate (Regis Chemical Co.), tryptamine hydrochloride (Regis Chemical Co.), pargyline hydrochloride (Eutonyl^R, Abbott Laboratories), and methysergide tartrate (Sanfert^R, Sandoz Laboratories). In addition, the following compounds, prepared by Dr. E. E. Campaigne (Department of Chemistry, Indiana University, Bloomington, IN)

were used: 3-(2-aminoethyl)benzo[b]thiophene (tryptamine-S) and 1-methyl-3-(2-aminoethyl indole (tryptamine-1-Me). All drugs with the exception of methysergide were made up in distilled water and the pH adjusted to 6 with solid $NaHCO_3$. L-ascorbic acid (Fisher Scientific Co.), 0.2 mg/ml, was added to the serotonin solution to prevent oxidation. Methysergide was dissolved in 1N HCl; the pH was adjusted to 5 with 0.5N NaOH. All drugs were kept frozen until just prior to use; concentrations are expressed as weight of free base.

Procedure. A strip of either tissue, attached using silk suture thread to an isotonic lever under 2.5 g tension, was connected through an isotonic myograph transducer to a recording physiograph (Narco Biosystems, Inc., Houston, TX). Eight- to ten-fold magnification was used for the aortic strips, four-fold for the stomach fundus strips. Aortic tissue was allowed to rest for 2 hours prior to study with bath fluid changes at 15-minute intervals. Pargyline at 10^{-5}M was added to the bath during the first hour at rest. Stomach fundus strips were allowed to rest for 50 minutes; pargyline at 10^{-5}M was added to the bath four times during this period.

In all experiments the height of maximal contraction was first determined by the cumulative addition of increasing concentrations of the agonist. The preparations were then washed at 15-minute intervals until each had returned to its previous baseline. Cumulative dose-response curves for the agonist (3 or 4 responses, 20 to 80% maximal) were obtained until two successive curves were similar with respect to both the height of the response for each addition and the height of the total response. When the tissue had thus stabilized, cumulative dose-response curves for the agonist were determined

alternately with those for the reference standard, serotonin.

The tissues were preincubated with antagonist for 15 minutes. On each aortic strip, three concentrations of antagonist were tested alternately with the agonist alone; only one concentration of antagonist was tested on each stomach fundus strip.

Calculations. All responses were recorded as percentage of the maximal contraction. Log dose-response curves were calculated as the percent of response versus the log dose by the method of least squares. The degree of antagonism was calculated using the dose-ratio of Gaddum, et al (11), comparing the log dose-response curve in the presence of antagonist to the immediately preceding curve in the absence of antagonist. The pA_2 and pA_{10} were determined by the method of Arunlakshana and Schild (12). Student's independent t test was used for tryptamine-S and norepinephrine; Student's dependent t test was used to compare the intrinsic activities of the test compounds to that of serotonin. The 95% confidence limits for the pD_2, pA_2, and pA_{10} values were calculated as described in Documenta Geigy (1970). All calculations were performed using a Compucorp Model 445 Computer.

RESULTS

Rat Aorta Strips. Norepinephrine, serotonin, tryptamine, and tryptamine-1-Me elicited increasing contractile responses with the cumulative addition of increasing concentrations of agonist. The intrinsic activities of norepinephrine, tryptamine, and tryptamine-1-Me were the same as that of serotonin ($p > .40$); thus $\alpha = 1$ for these three test compounds. The pD_2 values for these three compounds are presented in Table 1. The intrinsic activity of tryptamine-S was 0.79 ± .03 (mean ± S.E.M.). Tryptamine-S did not elicit

increasing contractile responses with increasing concentration; for this reason, no attempt was made to calculate a pD_2 for tryptamine-S in this tissue. The log dose-response curves for norepinephrine, serotonin, tryptamine, and tryptamine-1-Me are shown in Figure 1; the slopes of these curves are given in Table 1. The curves for the indolealkylamine compounds are all parallel; the curve for norepinephrine is parallel to that of serotonin but not to those of the other two indolealkylamines.

Methysergide at concentrations of 5×10^{-8}M to 1×10^{-6}M had no significant effect on the responses elicited by norepinephrine. Methysergide exhibited a dose-dependent antagonism of the effects of serotonin, tyrptamine, and tryptamine-1-Me. The pA_2 and pA_{10} values calculated for methysergide against these three agonists are presented in Table 2; the pA plots of Arunlakshana and Schild (12) are shown in Figure 2; the slopes of these plots are given in Table 2. These slopes are parallel by statistical test, and all are significantly different from the theoretical value of (-1).

Rat Stomach Fundus Strips. Serotonin, tryptamine, tryptamine-1-Me, and tryptamine-S elicited increasing contractile responses with the cumulative addition of increasing agonist concentrations. The maximum responses elicited by the three test compounds were not significantly different from that of serotonin; thus the intrinsic activity $\alpha = 1$ for all three test agonists in this tissue. The pD_2 values for these four compounds and the slopes of their log dose-response curves are given in Table 3. The tryptamine curve is not parallel to any of the others; the serotonin, tryptamine-1-Me, and tryptamine-S curves are all parallel to each other. The log dose-response curves for the four compounds are shown in Figure 3.

Methysergide exhibited a dose-dependent antagonism of the effects of all four compounds tested. The pA_2 and pA_{10} values calculated for methysergide against the four agonists are given in Table 4. The pA plots are shown in Figure 4; the slopes of the plots for the four compounds are given in Table 4. The slope of the serotonin curve is not statistically significantly different from the theoretical value of (-1). The slopes of the curves for the other three compounds are parallel, and all are statistically significantly different from (-1).

DISCUSSION

The data in Table 1 show that norepinephrine had a much higher affinity for receptors on the aorta than does serotonin; in fact, the order of affinity for the indole compounds was: tryptamine-1-Me > tryptamine > serotonin. These three compounds apparently act by a similar mechanism, as the slopes of their log dose-response curves were parallel. Norepinephrine might be interpreted as acting via the same mechanism as serotonin, as these two curves had parallel slopes. However, the use of specific antagonists has shown that they act on different receptors (13), illustrating the necessity for the use of antagonists in addition to the study of the effects of the agonist alone. The intrinsic activities of the two indole test compounds did not differ from that of serotonin, suggesting that the 5-hydroxyl group and the methyl group on the indole nitrogen have no effect on the ability of the compound to elicit a response once it reaches the receptor. These changes do, however, alter the affinity of the drug for the receptor relative to that of the parent compound.

It is difficult to interpret the action of tryptamine-S on the aortic

strip, since the average threshold response was 65.9% and the average maximal response was 79.3%. This response range (13.4%) is too narrow to yield useful dose-response data. A decay in the response to tryptamine-S was usually seen; that is, after the contraction elicited by a particular concentration had stabilized, it decreased again toward the resting value. This type of effect is usually attributed to metabolic breakdown of the agonist; it could not have been due to breakdown by monoamine oxidase as serotonin did not produce this effect on the aortic strips. It is possible that tryptamine-S is broken down by a pathway for which serotinin is not a substrate. Another possible explanation for the decay seen with tryptamine-S would be a breakdown of the free amine by the oxygen dissolved in the buffer; this breakdown has been shown to take place in the presence of air (14). However, this decay was not seen using fundus strips with identical oxygen flow through the same buffer. The explanation that fits the results best is that tryptamine-S exhibits properties called "noncompetitive autoinhibition" by Ariens, et al (15). In this concept, a single compound might interact with the contractile receptor R and with an inhibitor "receptor" R'. This latter "receptor" is the site of action of noncompetitive antagonists, that is, the point at which these agonists disrupt the chain of events linking stimulus and response. A compound exhibiting this dualism would have a separate intrinsic activity and affinity for each of the two receptors. Depending upon the equilibrium constants for occupation of the two types of receptors, one or the other of the two effects, contraction or inhibition, may precede the other in time. These results have been observed on frog rectus abdominis muscle by Ariens, et al (16). In the present case, the results indicate that the agonistic effect of tryptamine-S precedes the antagonistic effect. The

affinity of such a drug for the contractile receptor may be calculated, although with difficulty, by the use of a competitive antagonist; this has not been attempted in the present case due to the narrow response range for tryptamine-S.

On stomach fundus strips tryptamine-S elicited increasing contractile responses characteristic of a simple agonist, that is, one which does not exhibit the dualism described above. This is seen in the case where α', the intrinsic activity of the compound on R', is equal to zero (15). However, on the aorta tryptamine-S has $\alpha' > 0$. Thus, either R' in stomach fundus is different from that in the aorta (more specifically, the mechanism for translation of stimulus into response is not the same in stomach fundus as in aorta), or tryptamine-S cannot gain access to R' in stomach fundus.

The intrinsic activities of the three test compounds, tryptamine, tryptamine-1-Me, and tryptamine-S on stomach fundus strips was the same as that of serotonin. It would appear that tryptamine does not act by the same mechanism as the other three compounds based on the fact that the slope of its log dose-response curve is not parallel to that of any of the others. The order of affinity of the four compounds, as seen in Table 3 was serotonin >> tryptamine-1-Me > tryptamine > tryptamine-S. Serotonin has the greatest affinity for receptors in the stomach fundus, while in the aorta it had the least. The differences in the two results may be due at least in part to the lipid solubilities of the various compounds versus the lipid content of the two tissues. Lipid partition determinations for the four compounds indicate the order of solubility in organic solvents to be tryptamine-S > tryptamine-1-Me > tryptamine >> serotonin (17). In the isolated aorta, exogenous compounds must diffuse through either of two layers of tissue which sandwich the smooth muscle fibers. The stomach fundus strip prepared by the method of Offermeier

and Ariens (10) has the mucous membrane portion removed, which might facilitate the diffusion of less lipid soluble compounds. Thus, more lipid soluble compounds might have an advantage in gaining access to the receptor on aortic smooth muscle, in contrast to the case of stomach fundus with the gastric mucosa removed. This would cause an apparently high affinity of the test compounds for aorta as compared to serotonin, whereas the results using stomach fundus may more accurately represent the "true" affinities of the four compounds for serotonin receptors.

From Tables 2 and 4 it is apparent that methysergide fulfills the criteria for competitive antagonism only against serotonin on stomach fundus, where the $pA_2 - pA_{10}$ difference is .92 and the slope is not significantly different from the theoretical value of (-1). When tested on the aortic strip, methysergide did not act competitively against any of the three agonists. The slopes of the curves give an n that is significantly less than 1 and the $pA_2 - pA_{10}$ differences are higher than .95 (Table 6). Marshall (18) has suggested that such a result may be due to the fact that the antagonist has some affinity for receptors for other compounds as well as those under study, so that at higher concentrations some of the antagonist is "wasted" by being adsorbed onto these other receptors. Gorlitz and Frey (13) have shown that methysergide has affinity for adrenergic receptors; it does block the effect of norepinephrine, although at higher concentrations than those required for serotonin blockade. The slopes of the three pA plots are parallel, therefore there is no statistically significant difference in the values of n for the three compounds. Although methysergide is not acting competitively against the three compounds, it must interact in the same way with each of the agonists,

as the values of n are the same for each.

The results using methysergide against tryptamine, tryptamine-1-Me, and tryptamine-S on stomach fundus are the opposite of those in aorta. The pA_2 - pA_{10} differences are less than .95, and the slopes give an n greater than 1 (Table 4). Marshall (18) suggested that pA_2 - pA_{10} differences less than .95 indicate that the antagonist has a general spasmolytic action on contraction rather than a specific action at the receptor. If this were the case, the methysergide should have also antagonized the effects of serotonin noncompetitively. Gaddum (19) and Arunlakshana and Schild (12) suggested that a value of n greater than 1 indicates that more than one molecule of antagonist is required in order to block each receptor. For example, the pA plot of tryptamine has a slope of (-1.77) (Table 4). The value of n is equal to (-slope), or 1.77. Then perhaps 1.77 molecules of methysergide must be present simultaneously in order to block one receptor.

Since the slopes of the curves for tryptamine, tryptamine-1-Me, and tryptamine-S are parallel, then the value of n for methysergide against all three compounds must be the same (The slopes of the three curves are -1.77, -2.22, and -2.19, respectively; the value of n probably falls near the mean of the three slopes, at approximately 2.00). The pA_2 and pA_{10} values for these three compounds are also quite similar (Table 4), so it is probable that the three test drugs interact with methysergide in the same (noncompetitive) fashion. Since methysergide interacted with serotonin competitively, the three test drugs, tryptamine, tryptamine-1-Me, and tryptamine-S do not exert their effects by the same mechanism as serotinin on rat stomach fundus. It is possible that, in addition to a direct effect on serotonin receptors,

tryptamine also has an indirect effect in eliciting a contraction. Indirect effects of serotonin have been observed in various portions of the gastrointestinal tract in several species (20-22). Perhaps the lipid solubility differences discussed above enable tryptamine and its isosteres to reach a site of indirect action more easily than serotonin, thus requiring more methysergide per receptor in order to antagonize their effects.

It is not strictly possible to compare the receptors in the two different tissues by use of pA_2 and pA_{10} values if the antagonist does not act competitively against the compounds that activate those receptors. However, based on the values for n which a particular antagonist exhibits against a group of compounds in two different tissues, it should be possible to compare the members of the group against the standard. Serotonin, tryptamine, and tryptamine-1-Me interact with methysergide in the same fashion on the receptors of the aorta. On the stomach fundus, however, tryptamine and tryptamine-1-Me apparently do not act on serotonin receptors. Since the test drugs are the same and the antagonist is the same, on the basis of the experimental evidence presented herein it would seem that serotonin receptors in the aorta are not the same as those in the stomach fundus. It would appear that the receptors in the stomach are more specific for the serotonin structure than those in the aorta, as the loss of 5-hydroxyl group and addition of a methyl group to, or substitution of sulfur for the indole nitrogen leads to a change in the mechanism by which the compound elicits a response in the stomach. Thus, the differences in the relative affinities of the test agonists versus serotonin on the two tissues are not due to differences in lipid solubility alone, but rather reflect a true difference in the interaction of the drugs with the receptors in the two tissues.

Another difference in the contractile mechanism in the two tissues is shown by the interaction of tryptamine-S with methysergide on stomach fundus. We have seen that tryptamine-S exhibits autoinhibition with intrinsic activity $\alpha' > 0$ for the inhibitory receptor in aorta; on stomach fundus, however, no autoinhibition was observed, indicating that either α' equals zero, then tryptamine-S should act as a competitive antagonist of methysergide (19). This would result in a shift of the pA plot for tryptamine-S to the right, toward higher concentrations of methysergide, in order to obtain the desired dose-ratio. Tryptamine and tryptamine-S showed the same intrinsic activity and approximately the same affinity for the receptors. If methysergide interacts with both of these agonists in the same fashion, as it does, the pA curve for tryptamine-S should be shifted to the right of that for tryptamine if tryptamine-S is a competitive antagonist of methysergide. Figure 4 demonstrates that this is not the case. Therefore, no autoinhibition of tryptamine-S is seen in stomach fundus because the inhibitory receptors are not the same as those in aorta, indicating that the events linking stimulus and response are not the same in the two tissues.

In order to uphold these conclusions further experimental evidence is required. Most importantly, it would be desirable to obtain a compound which acts competitively toward serotonin in both aorta and stomach. This would provide more conclusive evidence regarding the identity of the receptors in the two tissues. Since methysergide did not act competitively, one cannot state conclusively whether the isosteres act on serotonin receptors in aorta. If indeed the two tissues have different serotonin receptors, it may be difficult to find an antagonist which is competitive against each. This difficulty is compounded by the fact that the competitive antagonists of serotonin are often

other indole analogs. The results of analog-analog interactions could be difficult to interpret. LSD and BOL are probably not the best candidates for further receptor studies, as they are so closely related to methysergide in structure that they may only give the same results as those presented herein. On the aorta, the analogs should also be tested against phentolamine (an α-adrenergic antagonist) and possibly cyproheptadine (an antagonist of serotonin and histamine H_1 receptors) in order to elucidate their mechanism more fully. It would also be desirable to test antagonists of a different class of compounds against the isosteres on stomach fundus in order to determine their mechanism; such antagonists should include atropine, and ganglionic blockers in order to examine the possibility that the isosteres are acting indirectly.

SUMMARY

Tryptamine and its benzo[b]thiophene and 1-methylindole analogues had a lower affinity for receptors in rat stomach fundus strips than did serotonin; the interaction between serotonin and methysergide in this system was competitive, while the interactions between tryptamine and its analogues and methysergide were noncompetitive. In contrast, the affinity of tryptamine and 1-methyltryptamine for receptors in rat aorta strips was greater than the affinity for serotonin; all three compounds showed noncompetitive interactions with methysergide. The results support the hypothesis of differing serotonin receptors in the two tissues.

ACKNOWLEDGEMENTS

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TABLE 1

pD_2 VALUES OF NOREPINEPHRINE AND INDOLEALKYLAMINES ON ISOLATED RAT AORTA STRIP

Compound	pD_2 ± C.L.*	n	Slope of Log Dose-Response Curve
Norepinephrine	8.10 ± .15	61	41.51
Serotonin	6.57 ± .08	166	34.38
Tryptamine	6.98 ± .21	59	27.69
Tryptamine-1-Me	7.13 ± .04	58	29.17

*pD_2 values ± 95% confidence limits are calculated as described in Methods; n is the total number of responses to all concentrations of each compound.

TABLE 2

ANTAGONISM OF EFFECTS OF INDOLEALKYLAMINES ON RAT AORTA STRIP BY METHYSERGIDE

Compound	pA_2 ± C.L.*	pA_{10} ± C.L.*	$pA_2 - pA_{10}$	Slope of pA plot
Serotonin	10.36 ± .2	8.57 ± .07	1.79	-.64
Tryptamine	9.44 ± .2	6.96 ± .2	2.48	-.45
Tryptamine-1-Me	10.09 ± .3	6.62 ± .2	3.47	-.38

*pA_2 and pA_{10} values ± 95% confidence limits are calculated as described in Methods.

TABLE 3

pD_2 VALUES OF SEROTININ AND TEST AGONISTS

ISOLATED RAT STOMACH FUNDUS STRIP

Compound	pD_2 ± C.L.*	n	Slope of Log Dose-Response Curve
Serotonin	9.34 ± .16	72	42.49
Tryptamine	7.60 ± .06	35	82.09
Tryptamine-1-Me	7.91 ± .08	71	47.77
Tryptamine-S	7.51 ± .08	66	47.35

*pD_2 values ± 95% confidence limits are calculated as described in Methods; n is the total number of responses to all concentrations of each compound.

TABLE 4

ANTAGONISM OF EFFECTS OF SEROTONIN AND TEST AGONISTS ON RAT STOMACH FUNDUS STRIP BY METHYSERGIDE

Compound	pA_2 ± C.L.*	pA_{10} ± C.L.*	$pA_2 - pA_{10}$	Slope of pA plot
Serotonin	8.80 ± .23	7.88 ± .30	.92	-1.26
Tryptamine	8.77 ± .09	8.22 ± .09	.55	-1.77
Tryptamine-1-Me	8.67 ± .06	8.24 ± .07	.43	-2.23
Tryptamine-S	8.85 ± .12	8.39 ± .09	.46	-2.19

*pA_2 and pA_{10} values ± 95% confidence limits are calculated as described in Methods.

LEGENDS

FIGURE 1. Effects of Agonists on Isolated Rat Aortic Strips.

Graphical presentation of data used to obtain the values presented in Table 1. The effects of norepinephrine (NE, —□—), serotonin (5HT, —●—), tryptamine (T, - -■- -), and 1-methyltryptamine (1MT, - · -○· - ·) are plotted as log agonist concentration (abscissa) against percent contraction (ordinate). Straight lines were determined by regression analysis using the method of least squares.

FIGURE 2. Effects of Methysergide on Agonist Action on Rat Aortic Strips.

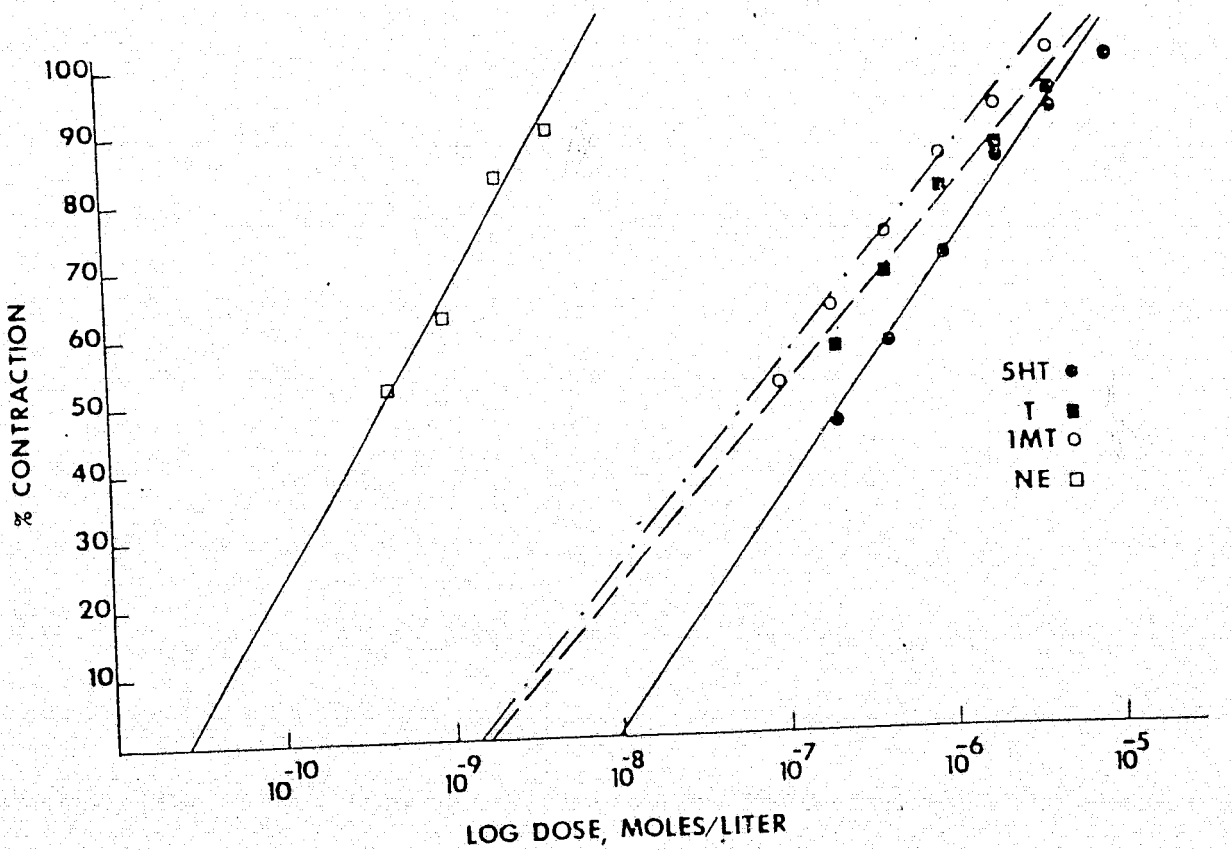
Graphical presentation of the data used to obtain the values presented in Table 2, plotted according to the method of Arunlakshana and Schild (1959). The negative log of the molar concentrations of methysergide (abscissa) are plotted against log X-1, where X is the dose ratio for serotonin (5HT, —●—), tryptamine (T, - -■- -) and 1-methyltryptamine (1MT, - · -○· - ·). Straight lines were determined by regression analysis using the method of least squares.

FIGURE 3. Effects of Agonists on Isolated Rat Stomach Fundus Strips.

Graphical presentation of data used to obtain the values presented in Table 3. The effects of serotonin (5HT, —●—), tryptamine (T, - -■- -), 1-methyltryptamine (1MT, - · -○· - ·), and 3-(2-aminoethyl)benzo[b]thiophene (ST, - - -□- - -) are plotted as log agonist concentration (abscissa) against percent contraction (ordinate). Straight lines were determined by regression analysis using the method of least squares.

FIGURE 4. Effects of Methysergide on Agonist Action on Rat Stomach Fundus Strips.

Graphical presentation of the data used to obtain the values presented in Table 4, plotted according to the method of Arunlakshana and Schild (1959). The negative log of the molar concentrations of methysergide (abscissa) are plotted against log X-1, where X is the dose ratio for serotonin (5HT, —●—), tryptamine (T, - -■- -), 1-methyltryptamine (1MT, - · -○· - ·), and 3-(2-aminoethyl)benzo[b]thiophene (ST, - - -□- - -). Straight lines were determined by regression analysis using the method of least squares.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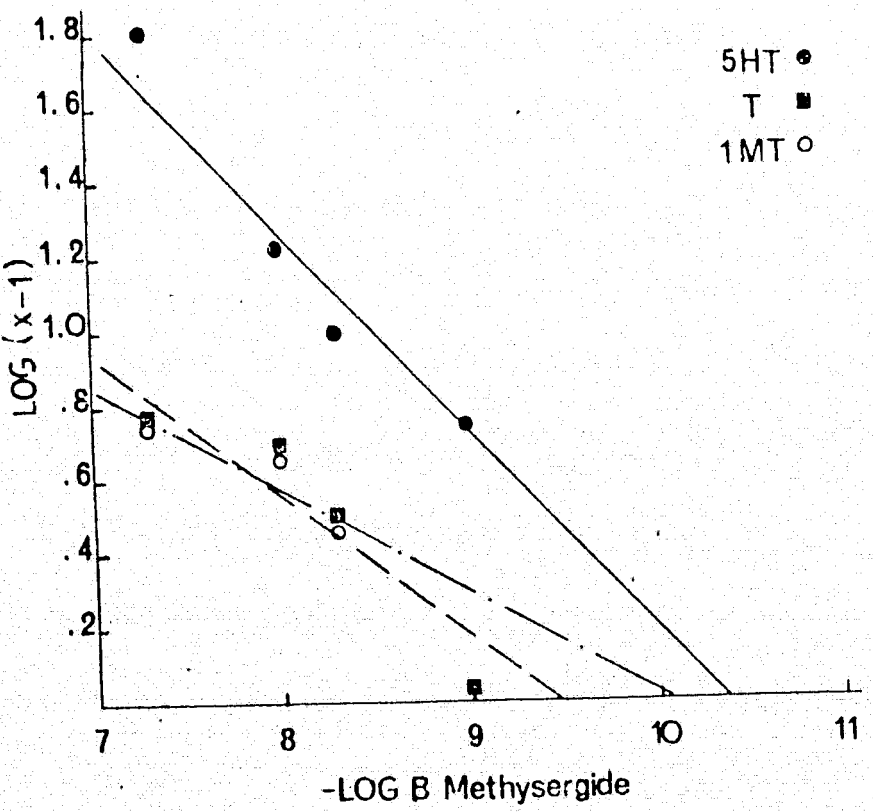
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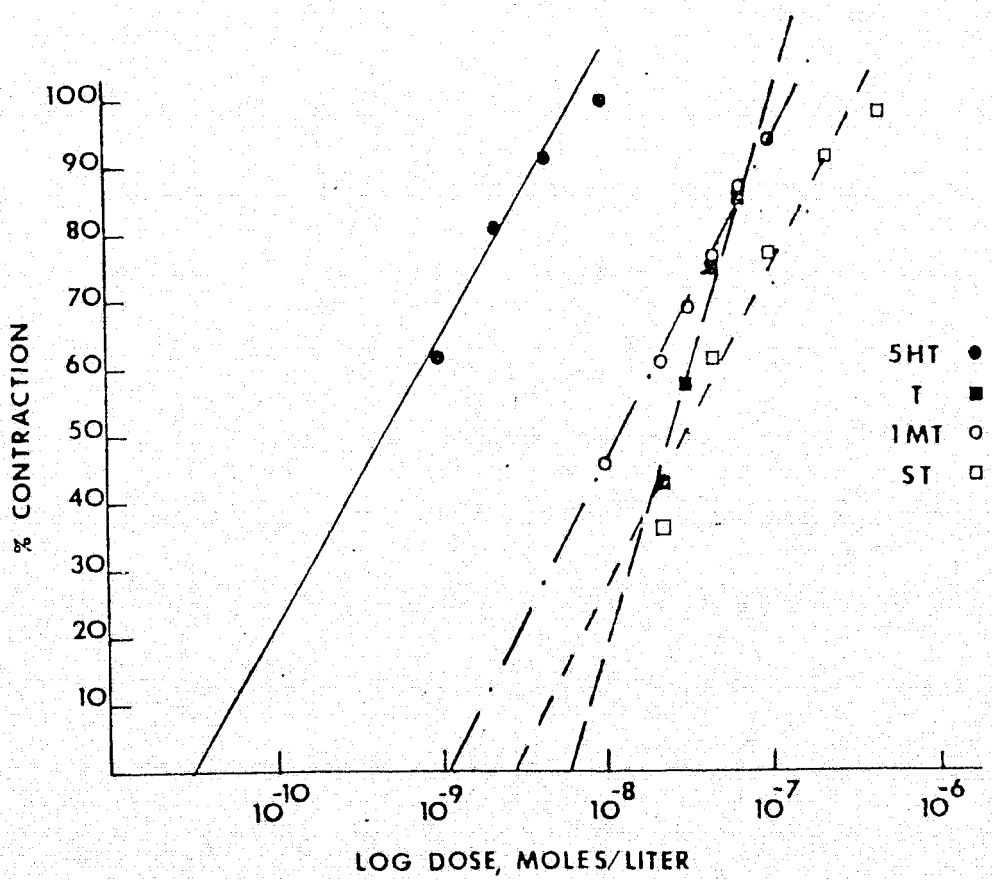
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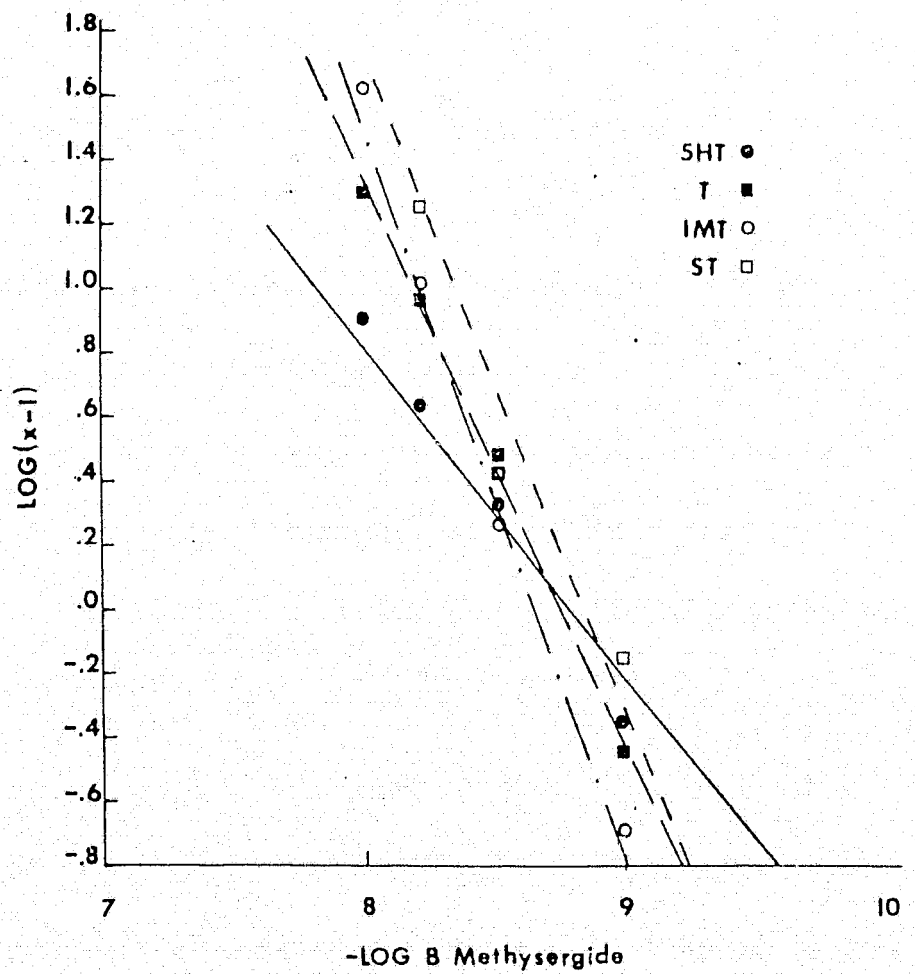
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DRUG INTERACTIONS ON BODY TEMPERATURE MAINTENANCE IN THE MOUSE*

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ABSTRACT

Exposure of mice to an environmental temperature leads to a loss of body temperature and death in 5 - 6 hours. Intraperitoneal injection of water or d-amphetamine exacerbates this effect, while caffeine has a modest antagonistic effect. The combination of caffeine and d-amphetamine is synergistic and leads to a rapid loss in body temperature with death occurring in less than 3 hours.

INTRODUCTION

When the mammalian organism is placed under environmental conditions other than those considered usual or normal, the effects of drugs may be considerably altered. Thus, a variety of stresses have been shown to significantly influence drug action (Rupe, et al, 1963; Barry and Buckley, 1966). One specific area in which drugs and abnormal environmental conditions are known to interact is that of body temperature maintenance. The homeothermic mammal maintains its core temperature within a relatively narrow range despite wide variations in environmental temperature; the obviously complex

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homeostatic mechanisms involved in this process present a wide range of targets for drug effects (Whittow, 1971; Weihe, 1973). Of particular interest is a previous study from this laboratory examining the ability of a variety of drugs to interfere with thermoregulation in the cold-exposed rat by actions presumed to involve central and peripheral monoaminergic pathways (Maickel, 1970).

A report by Muller and Vernikos-Danellis (1970) indicated that a decreased environmental temperature reduced the toxicity of d-amphetamine in mice but increased the toxicity of caffeine. Since this laboratory had a continuing interest in drug interactions with amphetamine in terms of behavioral (Cox and Maickel, 1976) and toxicological phenomena (Sokol and Maickel, 1972), a study of interactions of d-amphetamine and caffeine in cold-exposed animals was undertaken. The results, as presented herein, indicate that an interaction between the two drugs does indeed occur, but a confounding variable, the stress of an intraperitoneal injection, must be taken into account.

MATERIALS AND METHODS

Adult, male C57-B16 mice (22 - 25g) were obtained from Murphy Breeding Laboratories, Plainfield, IN and maintained on an *ad lib* diet of Purina laboratory chow and tap water for 7 - 10 days prior to experimental use. The animals were cold-exposed in individual screen-wire cages (10 x 10 x 15 cm) in a walk-in room at 4° C. All drugs were administered by i.p. injections in a volume of 0.1 ml/10 g body weight as solutions in distilled water; control animals were given distilled water. Rectal temperatures were measured using a YSI Telethermometer® with a small animal probe. Rate of heat loss (-dQ/dt) was calculated as described by Maickel, *et al* (1967) and slope comparisons for body temperature decay curves were made as described by Cox (1970). Statistical comparisons for -dQ/dt values were performed as in Maickel (1970).

RESULTS

Preliminary studies indicated that single i.p. doses of 50 mg/kg of caffeine and 10 mg/kg of d-amphetamine caused a 3 - 4 fold increase in the rate of body temperature decline in 2 hours of exposure of mice at 4° C, so these dosage levels were selected for the test. The data presented in Table 1 show the effects of single doses of the drugs. The results clearly demonstrate that a single i.p. injection of distilled water evokes a significantly greater rate of heat loss as compared to control (uninjected, but handled) animals. In fact, statistical comparisons of the -dQ/dt values and the slopes for the water and caffeine-treated groups indicate that both of these differ significantly from controls but not from each other. In contrast, dosage with d-amphetamine caused a significantly steeper temperature decay curve and significantly greater rate of heat loss than any other group. Of interest is the fact that caffeine seemed to afford some degree of protection in that 10 of 12 animals survived for 4 hours as compared to 0/12 for water-injected mice. The enhanced toxicity evoked by d-amphetamine is confirmed by the death of 2/12 mice by the 3 hour time point.

Because of the possible confounding variable of water injection, the drug interaction studies were run as a separate experiment; these data are presented in Table 2. The effects of two water injections were virtually identical to those of a single injection of d-amphetamine (Table 1) as well as to the combination of water plus amphetamine injections in terms of the rate of heat loss, as well as in the fact that 1/12 animals died by 3 hours and all 12 were dead by the 4 hour point. In contrast, the combination of water and caffeine injections showed a significant protective effect; 7/12 mice were still surviving at the 4 hour time point, and the rate of heat loss and decay slope for body temperature were significantly reduced. The combi-

TABLE 1

Effects of Single Drug Dosage on Body Temperature Maintenance

by Cold-Exposed Mice

Mice were given a single i.p. injection as described in Materials and Methods at 25 minutes prior to the start of cold exposure. Each value is the mean ± SEM of values obtained; the number in parenthesis indicates the N at each time point. Values for -dQ/dt and for the slope of the body temperature decay curves are for the period 0 - 2 hours.

	Body Temperature @ Hours of Exposure						
Treatment	0 C°	1 C°	2 C°	3 C°	4 C°	-dQ/dt cal/hr	Slope
Control	37.8±0.2(12)	36.1±0.2(12)	34.4±0.3(12)	29.4±0.3(12)	18.9±0.3(12)	35.5±2.7	-.588
Water	37.8±0.3(12)	33.9±0.4(12)	27.2±0.2(12)	16.7±0.4(12)	#	110.8±9.3	-.184
Caffeine	37.0±0.2(12)	32.7±0.3(12)	26.8±0.3(12)	17.4±0.4(12)	12.6±0.5(10)	115.0±6.1	-.194
d-Amphetamine	37.8±0.3(12)	31.1±0.4(12)	21.7±0.3(12)	13.9±0.3(10)	#	168.2±14.0	-.123

#All animals dead; body temperatures < 11° C.

TABLE 2

Effects of Drug Interactions on Body Temperature Maintenance of Cold-Exposed Mice

Mice were given i.p. injections as described in Materials and Methods, one at 27 minutes and the other at 23 minutes prior to the start of cold exposure. Sequencing was randomized so that half of each group received the reverse order. Each value is the mean ± SEM of values obtained; the number in parenthesis indicates the N at each time point. Values for -dQ/dt and for the slope of the body temperature decay curves are for the period 0 - 2 hours.

Treatments	Body Temperature @ Hours of Exposure						
	0 C°	1 C°	2 C°	3 C°	4 C°	-dQ/dt cal/hr	Slope
Water & Water	38.3±0.3(12)	32.8±0.4(12)	23.0±0.5(12)	14.4±0.5(11)	#	150.5±8.3	-.136
Water & Caffeine	37.0±0.4(12)	33.0±0.3(12)	27.5±0.7(12)	17.6±0.4(12)	12.5±0.9(7)	99.3±5.2	-.209
Water & d-Amphetamine	37.1±0.3(12)	29.4±0.5(12)	19.2±0.4(12)	12.5±0.6(8)	#	187.1±9.6	-.111
Caffeine & d-Amphetamine	37.8±0.2(12)	27.8±0.3(12)	16.1±0.3(11)	#	---	226.8±11.3	-.092

#All animals dead; body temperatures < 11° C.

nation of amphetamine plus caffeine had an enhanced toxicity, with the most rapid rate of heat loss of any tested group. In fact, the 1/12 mice died in 2 hours and all 12 were dead at the 3 hour time point.

DISCUSSION

The use of an excessive environmental temperature differential as a system to examine drug effects on the ability of a homeothermic animal to maintain body temperature within normal limits has been a common technique (Chen, et al, 1943; Maickel, 1970; Weihe, 1973). In homeothermic mammals, the processes involved in body temperature maintenance in the face of a low environmental temperature include both heat conservation and increased heat production (Jamira, et al, 1965; Maickel, et al, 1967). This entire area of temperature regulatory processes has been the subject of several extensive reviews (Hardy, et al, 1970; Cabanac, 1971).

The studies presented in the present report are of significant value in regard to two diverse findings. First of all, a significant potential artifact in thermoregulatory studies may exist with mice in that the animals lose body heat at a significantly faster rate merely due to an intraperitoneal injection of a small volume (0.1 ml/100 g body weight) of distilled water. This effect appears to be due to the injection process, since merely puncturing the abdomen or injection of a similar volume of physiological saline had a similar action. The effect does not occur in rats (Maickel, 1970) and may thus reflect a greater responsivity to stress in the mouse; the additional stress having a deleterious effect on thermoregulatory process.

Administration of caffeine has a modest antagonistic effect towards the injection stress per se, while administration of a single dose of d-amphetamine appears to exacerbate the stress effect on temperature maintenance. However, the combination of caffeine and d-amphetamine had a marked synergistic action

resulting in a rapid temperature fall and the deaths of one hundred percent of the animals in slightly more than 2 hours of exposure to a 4° C environmental temperature. This drug interaction must be considered in the light of previous studies from this laboratory showing that, in the rat, caffeine had no effects on the anorectic action of d-amphetamine although it did antagonize the stimulatory effect of the latter drug on continuous avoidance responding. It appears that the interactions of d-amphetamine (a stimulant of catecholaminergic function) and caffeine (a blocker of the phosphodiesterase system that destroys cyclic AMP) may be uniquely specific to the test system being measured.

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INFLUENCE OF DRUGS ALTERING BRAIN BIOGENIC AMINES ON THE EFFECTS OF AMPHETAMINE ISOMERS ON LOCOMOTOR ACTIVITY

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INTRODUCTION

The methyl group attached to the α-carbon of amphetamine confers on the molecule the property of stereoisomerism, giving rise to two enantiomers, both of which have significant pharmacological activity (1). For a number of years, the generally held belief was that d-amphetamine was 3 to 5 times more potent than the l-isomer as a stimulant of the central nervous system, while l-amphetamine was slightly more potent than the d-isomer as a peripheral pressor agent (7). However, studies reported in the past fifteen years give support to the hypothesis that the two isomers may have actions on various behavioral test systems that differ in both qualitative and quantitative aspects. Moore (15) has shown that the d-isomer is more toxic than the l-isomer in both isolated and aggregated mice; this toxicity difference paralled the potency of the isomers in reducing brain norepinephrine (NE). Several laboratories in studying the actions of amphetamine isomers on locomotor activity have concluded that the d-isomer is merely more potent than the l-isomer (17, 21, 23-25). It should be noted that some of these studies are confounded by the fact that animals were pretreated with a monoamine oxidase inhibitor (MAOI). Studies of the effects of the isomers on stereotyped behavior suggested that the effects of d-amphetamine could be correlated with actions on NE systems while those of l-amphetamine correlated better with actions on dopamine (DA) systems (5, 23, 24). Sparber and coworkers (18, 27) have reported differential actions of the amphetamine isomers in operant behavioral systems such as fixed-interval and fixed-ratio responding.

In addition to these behavioral studies, a number of reports have demonstrated differential actions of the amphetamine isomers on brain catecholamine uptake, release, and metabolism; these actions show both qualitative and quantitative differences in the isomers (4, 19, 26). Finally, several studies have suggested that a catecholamine-serotonin interaction may exist in the control of locomotor activity in the rat (11, 16), although this hypothesis has recently been challenged by Jacobs, et al (8).

In terms of the effects of amphetamine isomers on spontaneous motor activity in mice, Bainbridge (2) found that d,l-amphetamine had a dose-dependent effect on SMA; doses < 5 mg/kg were depressant, while doses > 10 mg/kg were stimulatory.

A previous publication from this laboratory (12) demonstrated that at low (0.5 mg/kg) and high (8.0 mg/kg) doses, both isomers were stimulants of SMA in mice, while at intermediate doses (1.0-4.0 mg/kg), the d-isomer was stimulatory while the l-isomer caused a significant depression.

The present paper examines the actions of d- and l-amphetamines on SMA in mice pretreated with agents known to cause alterations in brain biogenic amines: α-methyltyrosine (αMT), p-chlorophenylalanine (PCPA), pargyline, and reserpine.

MATERIALS AND METHODS

Adult, male, Swiss-Webster mice, weighing 25-30 grams were obtained from Murphy Breeding Laboratories, Plainfield, Indiana. The animals were maintained on ad lib diet of Wayne Lab Blox and tap water for 7-10 days prior to experimental use in an animal room with controlled temperature

and a 14:10 light-dark cycle. All testing was done between 1000 and 1600 hours, midway in the light cycle. Drugs were administered by i.p. injection as aqueous solutions (d-amphetamine, l-amphetamine, pargyline, reserpine) or peanut oil suspensions (αMT, PCPA) in a volume of 0.1 ml/10g body weight. All dosages were given and are reported as base weight.

αMT and PCPA were purchased from Regis Chemical Company and Pierce Chemical Company, respectively. d-Amphetamine sulfate was kindly supplied by Smith-Kline and French; l-amphetamine phosphate was kindly supplied by Pennwalt Corporation; pargyline hydrochloride was kindly supplied by Abbott Laboratories; and lyophylized reserpine phosphate was kindly supplied by CIBA-Geigy Corporation.

SMA activity was measured, using groups of 3 mice, in Woodward acto-photometers; the procedure was basically that described in a previous paper from this laboratory (12). The system as utilized has been considered as a standardized measurement of psychogenic spontaneous locomotion (9). It is especially reliable for stimulant drugs such as d-amphetamine, yielding inverted "U" dose-response curves similar to those obtained with continuous avoidance responding. Brain levels of 5HT and NE were determined by the method of Maickel, et al (13).

RESULTS

Standardization of the SMA Test System. Effects of Single Doses of Amphetamine Isomers.

In order to restandardize the test system and provide baseline data for interaction experiments, the experiments reported by Maickel, et al (12) were repeated, using doses of 1.0 or 4.0 mg/kg of d-amphetamine or l-amphetamine. The data obtained are presented in Table 1. As can be seen,

both doses of d-amphetamine caused a significant increase in SMA activity at all time points. The lower dose of l-amphetamine (1.0 mg/kg) caused a decrease in activity for the first 10 minutes and an increase in the third and fourth 10 minute periods, while the higher dose caused a decreased SMA activity in the first three 10 minute periods and an increase in the final period.

Effects of Drug Treatments and Pretreatments on Brain Levels of 5HT and NE.

In order to have some information on the effects of the various compounds studies on brain levels of 5HT and NE, assays for these amines were performed. The data are presented in Table 2. As can be seen, αMT reduced brain NE levels by 64% with no change in 5HT, while PCPA reduced 5HT levels by 67% with no significant change in NE. Pargyline increased 5HT levels by 89% and NE levels by 81%, while reserpine reduced 5HT levels by 82% and NE levels by 81%. Neither of the amphetamine isomers had any significant effect on either amine.

Effects of αMT Pretreatment on Actions of Amphetamine Isomers.

The data in Table 3 demonstrate the effects of αMT pretreatment on the actions of d- and l-amphetamine on mouse SMA activity. The αMT pretreatment itself caused a modest, but significant, reduction in SMA at all four time intervals. Both doses of d-amphetamine caused an increase in SMA in the αMT pretreated mice, although the characteristics of the effect differed from that seen in control animals (Table 1). For example, the magnitude of the stimulatory effect of the 1.0 mg/kg dose in the first activity interval in the αMT pretreated mice, when estimated by the ratio of counts, did not differ significantly from that seen in control mice;

however, in the final two intervals, the αMT pretreatment virtually abolished the stimulatory effect of the d-amphetamine. At the higher dose (4.0 mg/kg) of d-amphetamine, a similar pattern of reduction of response was seen over the final two intervals although in the first interval a greater increase was observed in the αMT pretreated animals than in the controls.

With the 1.0 mg/kg dose of the l-isomer, a slight increase in SMA (as compared to αMT alone) was observed in the first interval, followed by a decrease in the second interval and then two intervals where no drug effects were observed. When compared to this dose of l-amphetamine in untreated animals, an increased SMA ratio was seen in the first interval, followed by slightly lower values for the remaining intervals. The 4.0 mg/kg dose of l-amphetamine in αMT pretreated mice showed no intervals with SMA counts greater than those of pretreatment alone. When comapred to the effects of that dose of l-amphetamine in control mice, the only significant difference was a marked lessening of activity in the 31-40 minute interval.

Effects of PCPA Pretreatment of Actions of Amphetamine Isomers.

These data are presented in Table 4. Pretreatment with PCPA had no significant effects on SMA at any of the intervals. Both doses of d-amphetamine increased SMA significantly at all intervals as compared to PCPA pretreatment alone. The magnitude of stimulation evoked by the lower dose of d-amphetamine (1.0 mg/kg) was somewhat reduced by the PCPA pretreatment. In contrast the higher dose of d-amphetamine was slightly more effective in the animals pretreated with PCPA.

At the lower dose (1 mg/kg), l-amphetamine acted as a stimulant in PCPA pretreated animals; the effect was greater than that of the same dose in control animals. The higher dose of l-amphetamine (4.0 mg/kg) had no signifi-

cant effect on SMA in PCPA pretreated mice.

Effects of Pargyline Pretreatment on Actions of Amphetamine Isomers.

The data, as presented in Table 5, show that the pargyline pretreatment itself caused a significant decrease in SMA over the last three intervals. When compared to these values, both doses of d-amphetamine caused increased SMA at all intervals; only the first interval with the lower dose was not statistically significant. However, in comparison to the effects in control animals, the pargyline-pretreated animals were less responsive to the stimulatory action of d-amphetamine at both doses.

l-Amphetamine had no significant actions at the lower dose (1.0 mg/kg) when compared to pargyline pretreatment alone. At the higher dose (4.0 mg/kg), a complex pattern was seen, with significantly decreased SMA in the first and fourth intervals, a significant increase in the second, and no effect in the third when compared to pargyline pretreatment alone. When compared to the actions of the l-isomer in untreated animals, the effects of pargyline pretreatment seem to have been those of removal of any stimulatory component.

Effects of Reserpine Pretreatment on Actions of Amphetamine Isomers.

Table 6 shows that mice treated with a single dose of reserpine (10 mg/kg, i.p.) 24 hours prior to testing obviously displayed the typical sedation syndrome with a virtual absence of SMA. Administration of the low dose (1.0 mg/kg) of d-amphetamine evoked a small but significant increase in SMA which was far less than that produced by a similar dose in control animals. However, the larger dose of the d-isomer, when given to reserpine-pretreated mice, produced counts that were greater, at each interval, than those seen in control animals given the same dose.

Administration of the low dose (1 mg/kg) of l-amphetamine to mice pretreated with reserpine resulted in a brief stimulatory response of small magnitude followed by negligible activity over the last two intervals. At the higher dosage, the l-isomer was slightly stimulatory, with the effect decreasing with time.

DISCUSSION

The effects of amphetamine on various aspects of animal behavior have been the subject of literally thousands of experiments. Until about twelve years ago, most reports concluded that the two isomers of amphetamine differed only in terms of quantitative potency. This was especially true in terms of behavioral tests in which the drug effect was manifested as a central stimulatory activity, that is, one resulting in increases in rates of activity or responding. More recently, a variety of reports (18, 27), including one from this laboratory (12), have suggested that some basic qualitative differences may exist in the actions of the amphetamine isomers on animal behavior. In extending our previous work, the use of the simple actophotometer, with groups of 3 mice per unit, was continued as a measure of spontaneous motor activity. The use of naive mice in each test run insured a high component of exploratory activity in the overall SMA measurement; the use of 10 minute counting intervals permitted evaluation of varying duration of drug effects as well as enhancing the magnitude of specific phenomena of brief duration.

The data obtained in control (non-pretreated) animals, as presented in Table 1, were basically similar to those previously reported by this laboratory (12). Thus, d-amphetamine, at both dosages, (1.0 and 4.0 mg/kg), caused a marked increase in SMA over control values. The decrease in SMA during the

third interval at the higher dose of d-amphetamine was seen consistently and may reflect a temporary fatigue. In contrast to the d-isomer, l-amphetamine showed markedly differing dosage effects. At the lower dose (1.0 mg/kg), a significant decrease in SMA in the first interval was followed by a non-significant action in the second test interval, and a slight stimulatory action in the remaining two intervals. In contrast, the higher dose of l-amphetamine (4.0 mg/kg) caused a significant reduction in SMA activity over the first 3 test intervals. reverting to stimulatory actions only in the last ten minutes.

The first pretreatment studied was that of αMT which produced a 64% decrease in whole brain NE at the time of starting the test period with no significant alteration in brain 5HT (Table 2). Since αMT depletes NE by inhibition of tyrosine hydroxylase, it may be assumed that some reduction in brain dopamine levels also occurred under these conditions (22). With this pretreatment, the stimulatory effects of the low dose of d-amphetamine were markedly reduced both in magnitude and duration (Table 3), while the higher dose had an initial stimulatory effect greater than that seen in controls, followed by a rapid abatement of stimulatory activity. These results confirm the observations made by Miller, et al (14) that αMT pretreatment had a marked effect on the ability of d-amphetamine to increase continuous avoidance responding in rats. Thus, the stimulatory actions of d-amphetamine may be dependent upon the presence of a labile or newly synthesized catecholamine pool. In contrast to the actions on d-amphetamine, the αMT pretreatment generally caused the l-isomer to have a greater SMA depressant effect, especially in terms of duration of action. This would agree with the hypothesis that this depressant action may involve systems that are

functionally antagonistic in terms of controlling SMA; one involving catecholamines, and the other involving some other biogenic amine.

Pretreatment with PCPA, at a dosage regimen that reduced brain 5HT by 67% with only a slight and non-significant action on brain NE, (Table 2), had no significant effect on the SMA (Table 4). PCPA pretreatment caused a small reduction in the SMA stimulation evokec by the lower dose of d-amphetamine, but enhanced the stimulatory actions of the higher dose of d-amphetamine. The action of PCPA pretreatment, with its concommitant reduction of brain 5HT, was most dramatic on the effects of l-amphetamine. The depressant effects of both doses of the l-isomer was completely blocked; indeed the lower dose even showed a modest stimulatory effect on SMA. These results lend support to the hypothesis that the depressant actions of the l-isomer on SMA may involve action on a serotonergic system.

Pargyline pretreatment, elevating brain levels of 5HT by 110% and NE by 81% (Table 2), caused a modest decrease in SMA (Table 5) possibly due to an increased serotonergic tone. The stimulatory effects of both doses of d-amphetamine were reduced at all intervals; the most dramatic reductions were seen in the periods beyond 20 minutes, where the 3- to 5-fold increase in counts seen in control animals was reduced to 2- to 3-fold increases by the pargyline pretreatment. In contrast to these observations, the low dose of l-amphetamine was without a significant effect on SMA, while the high dose depressed SMA activity in the first and fourth intervals, but showed a significant paradoxical increment in the second interval.

Reserpine pretreatment, on the other hand, reduced brain 5HT levels by 82% and brain NE levels by 80% (Table 2); under these conditions, SMA

was virtually abolished (Table 6). Both doses of d-amphetamine had stimulatory activity in reserpine pretreated animals (Table 6). In fact, the effects of the 4.0 mg/kg dose were similar to or greater than the actions of the drug in non-treated controls. This may be a reflection of a central nervous system version of "supersensitivity" (28) or it may reflect a direct stimulatory action of d-amphetamine as proposed by Rech (20). At the lower dose, l-amphetamine had a very small stimulant effect in reserpine-pretreated animals, while at the higher dose the l-isomer had a modest stimulant action, approximately one-fourth as potent as that of the d-isomer. Of course, since the SMA after reserpine pretreatment was so low, it was impossible to elicit any further depression with l-amphetamine. Nevertheless, it was of interest to discover that the mixed activity of the l-isomer could be so easily converted to purely stimulatory action by virtue of reserpine pretreatment.

When the various results presented in this paper are recomputed in terms of "percent control" as described by Jacobs, et al (8), a most interesting set of numbers are generated; these are presented as Table 7. In no instance did any dose or count interval in animals treated with d-amphetamine show a decrease in SMA; in contrast, with the exception of the animals pretreated with reserpine or PCPA, the preponderant response to l-amphetamine was a decreased SMA.

An overview of these results leads to the conclusion that the d-isomer of amphetamine, at doses of 1.0 and 4.0 mg/kg, k.p. in mice, has a net stimulant action as reflected by increased SMA, and, that this effect is mediated by catecholamine release from freshly synthesized or labile pools.

In contrast, the l-isomer of amphetamine appears to have a dual nature. In addition to possessing a modicum of catecholamine releasing activity, it also has an ability to interact with serotonergic systems, perhaps by the very nature of its β-phenylethylamine structure. Thus, if one assumes that SMA reflects a net activity controlled by a "balance" of 5HT and catecholamine functions (3, 6), d-amphetamine will increase SMA by shifting the "balance" to the catecholamine side, while l-amphetamine will decrease SMA by shifting the "balance" in favor of the serotonin side. The possible role of other biogenic amine systems (such as dopamine) cannot be eliminated, although in the present work, the levels of amphetamines used may not have had a significant action on dopaminergic systems (10).

TABLE 1

Effects of Single Doses of Amphetamine Isomers on Mouse SMA

Isomer	Dose mg/kg	N	Counts per Interval ± SEM* 0-10 min	11-20 min	21-30 min	31-40 min	F#
--	--	15	818± 25	558± 16	393± 12	297± 10	--
d	1.0	12	1266± 46^{I}	1290± 42^{I}	1270± 31^{I}	1257± 45^{I}	<.01
d	4.0	12	1498± 43^{I}	1697± 47^{I}	1209± 37^{I}	1508± 41^{I}	<.01
l	1.0	12	690± 22^{D}	565± 14^{NS}	460± 17^{I}	385± 16^{I}	<.05
l	4.0	12	675± 19^{D}	303± 14^{D}	283± 15^{D}	409± 19^{I}	<.01

Data were obtained as described in Materials and Methods; each "N" represents a run of 3 mice.

*Superscript letters refer to individual comparisons of each interval to corresponding control interval by two-tailed "t" test:

I = increased counts ($p < .001$)
D = decreased counts ($p < .001$)
NS = non-significant

#One way analysis of variance for drug effect; p values are given for F (2,6).

TABLE 2

Effects of Drug Treatments on Levels of 5HT and NE in Mouse Brain

Drug Treatment	N	Brain Levels (µg/g ± SMA) 5HT	NE
None	12	0.73 ± .02	0.59 ± .02
αMT	8	0.77 ± .02	0.21 ± .01^{D}
PCPA	8	0.24 ± .02^{D}	0.51 ± .02
Pargyline	8	1.38 ± .05^{I}	1.07 ± .05^{I}
Reserpine	8	0.13 ± .01^{D}	0.11 ± .01^{D}
d-Amphetamine	6	0.71 ± .02	0.58 ± .02
l-Amphetamine	6	0.73 ± .03	0.59 ± .02

Each "N" value represents a pool of brains from 2 mice. Pretreatment schedules were as follows:

αMT - 150 mg/kg, i.p. (peanut oil suspension) at 24 hours and 4 hours prior to sacrifice.

PCPA - 400 mg/kg, i.p. (peanut oil suspension) at 48 hours prior to sacrifice.

Pargyline - 40 mg/kg, i.p. (aqueous solution) at 24 hours and 4 hours prior to sacrifice.

Reserpine - 10 mg/kg, i.p. (aqueous solution) at 24 hours prior to sacrifice.

d- or l-Amphetamine - 4 mg/kg, i.p. (aqueous solution) at 30 minutes prior to sacrifice.

Values differing significantly from control (two-tailed "t" test, $p < .05$) are indicated by D = decrease or I = increase.

TABLE 3

Effects of αMT Pretreatment on Actions of Amphetamine Isomers

Isomer	Dose mg/kg	N	Counts per Interval ± SEM* 0-10 min	11-20 min	21-30 min	31-40 min	F#
--	--	8	708± 39^{D}	429± 22^{D}	297± 17^{D}	237± 16^{D}	<.01
d	1.0	4	1157± 61^{I}	850± 55^{I}	408± 43^{NS}	317± 23^{I}	<.05
d	4.0	4	2062±119^{I}	1239± 86^{I}	309± 24^{NS}	301± 26^{NS}	<.05
l	1.0	4	856± 43^{I}	283± 23^{D}	296± 20^{NS}	276± 28^{NS}	<.05
l	4.0	4	628± 41^{NS}	329± 24^{D}	254± 27^{NS}	160± 16^{D}	<.05

Animals were treated with αMT as described in Table 2; each "N" represents a run of 3 mice.

*Superscript letters refer to individual comparisons of each interval to the corresponding control (for αMT alone) or αMT interval by two-tailed "t" test:

I = increased counts ($p < .05$)
D = decreased counts ($p < .05$)
NS = non-significant

#One way analysis of variance for αMT effect as compared to control data (Table 1) and for each drug effect as compared to αMT alone; p values are given for F (2,6).

TABLE 4

Effects of PCPA Pretreatment on Actions of Amphetamine Isomers

Isomer	Dose mg/kg	N	Counts per Interval ± SEM* 0-10 min	11-20 min	21-30 min	31-40 min	F#
--	--	16	832± 24^{NS}	559± 23^{NS}	427± 16^{NS}	310± 15^{NS}	NS
d	1.0	4	1167± 66^{I}	927± 52^{I}	867± 36^{I}	1008± 35^{I}	<.01
d	4.0	4	1742± 69^{I}	1724± 96^{I}	1824± 74^{I}	1883± 98^{I}	<.01
l	1.0	4	898± 39^{NS}	764± 46^{I}	767± 36^{I}	726± 48^{I}	<.01
l	4.0	4	809± 49^{NS}	497± 45^{NS}	392± 49^{NS}	357± 34^{NS}	NS

Animals were treated with PCPA as described in Table 2; each "N" represents a run of 3 mice.

*Superscript letters refer to individual comparisons of each interval to the corresponding control (for PCPA alone) or PCPA interval by the two-tailed "t" test:

I = increased counts ($p < .001$)
D = decreased counts ($p < .001$)
NS = non-significant

#One way analysis of variance for PCPA effect as compared to control data (Table 1) and for each drug effect as compared to PCPA alone; p values are given for F (2,6).

TABLE 5

Effects of Pargyline Pretreatment on Actions of Amphetamine Isomers

Isomer	Dose mg/kg	N	Counts per interval ± SEM* 0-10 min	11-20 min	21-30 min	31-40 min	F#
--	--	6	901± 32^{NS}	483± 24^{D}	340± 15^{D}	250± 11^{D}	<.05
d	1.0	3	972± 27^{NS}	1075± 58^{I}	657± 54^{I}	599± 55^{I}	<.01
d	4.0	3	1257±105^{I}	1099± 59^{I}	697± 62^{I}	797± 38^{I}	<.01
l	1.0	3	797± 44^{NS}	483± 46^{NS}	241± 53^{NS}	209± 42^{NS}	NS
l	4.0	3	722± 48^{D}	748± 73^{I}	357± 43^{NS}	128± 21^{D}	<.05

Animals were treated with partyline as described in Table 2; each "N" represents a run of 3 mice.

*Superscript letters refer to individual comparisons of each interval to the corresponding control (for pargyline alone) or pargyline interval by the two-tailed "t" test:

I = increased counts ($p < .02$)
D = decreased counts ($p < .02$)
NS = non-significant

#One way analysis of variance for pargyline effect as compared to control data (Table 1) and for each drug effect as compared to pargyline alone; p values are given for $F(2,6)$.

TABLE 6

Effects of Reserpine Pretreatment on Actions of Amphetamine Isomers

Isomer	Dose mg/kg	N	Counts per Interval ± SEM* 0-10 min	11-20 min	21-30 min	31-40 min	F#
--	--	6	6± 1^{D}	5± 1^{D}	11± 2^{D}	12± 4^{D}	<.01
d	1.0	3	191± 16^{I}	300± 28^{I}	354± 26^{I}	189± 20^{I}	<.01
d	4.0	3	1631±191^{I}	1799± 94^{I}	1838±114^{I}	1930± 59^{I}	<.01
l	1.0	3	68± 10^{I}	50± 12^{I}	18± 6^{NS}	8± 3^{NS}	<.05
l	4.0	3	485± 46^{I}	464± 58^{I}	330± 28^{I}	162± 24^{I}	<.01

Animals were treated with reserpine as described in Table 2; each "N" represents a run of 3 mice.

*Superscript letters refer to individual comparisons of each interval to the corresponding control (for reserpine alone) or reserpine interval by the two-tailed "t" test:

I = increased counts ($p < .01$)
D = decreased counts ($p < .01$)
NS = non-significant

#One way analysis of variance for reserpine effects as compared to control data (Table 1) and for each drug effect as compared to reserpine alone; p values are given for F (2,6).

TABLE 7

Effects of Various Pretreatments on SMA Actions of Amphetamine Isomers

Pretreatment	Isomer	Dose mg/kg	Percent Control Counts 0-10 min	11-20 min	21-30 min	31-40 min
None	d	1.0	155	231	323	423
		4.0	183	304	308	508
αMT	d	1.0	163	198	137	134
		4.0	291	289	104	127
PCPA	d	1.0	139	165	203	325
		4.0	208	308	427	607
Pargyline	d	1.0	108	223	193	240
		4.0	140	228	205	319
Reserpine	d	1.0	3183	6000	3218	1575
		4.0	27183	35980	16709	16083
None	l	1.0	84	101	117	130
		4.0	83	54	72	138
αMT	l	1.0	121	66	100	116
		4.0	89	77	86	68
PCPA	l	1.0	107	137	180	234
		4.0	96	89	92	115
Pargyline	l	1.0	88	100	71	84
		4.0	80	155	105	51
Reserpine	l	1.0	1133	1000	164	67
		4.0	8083	9280	3000	1350

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