Title
Ketamine

Author
Cohen, Marlene Lois

Publication Date
1973

Peer reviewed|Thesis/dissertation

KETAMINE: CEREBRAL DISPOSITION AND INTERACTION WITH BRAIN ACETYLCHOLINESTERASE

by

Marlene Lois Cohen
B.S. University of Connecticut, 1968

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACOLOGY

in the

GRADUATE DIVISION

(San Francisco)

TO: Archives Division, The Library DATE: 11-15-74

FROM: **Diane M. Pitre, Graduate Division**

The following theses/dissertations are submitted for deposit:

NAME (Last name first)	DEG	DEPT/FIELD	DATE CONFER'D	BIRTH DATE	U.M. No.
COHEN, Marlene Lois	**Ph.D.**	**Pharmacology**	**6-21-73**	**5-5-45**	**7424463**

Each Mss. includes a copy of the author's reproduction release form, signed & dated.

TOT MSS. SUBMITTED TO LIBRARY: 1 (____ Theses; 1 Dissertations)

FOR LIBRARY USE

TOT MSS. REJECTED & RETURNED: _____ (____ Theses; _____ Dissertations) Date _____
(Lined out above; to be resubmitted under new cover letter)

TOT MSS. ACCEPTED & SIGNED: _____ (____ Theses; _____ Dissertations)

DATE SENT TO B.P.D. _____ s/ _____

gsh Archives, r8/21/74

ROCHE INSTITUTE OF MOLECULAR BIOLOGY

NUTLEY, NEW JERSEY 07110

November 11, 1974

Ms. Diane M. Pitre
Graduate Division
Office of the Dean
University of California
San Francisco, California 94122

Dear Ms. Pitre:

In response to your letter of November 4, regarding rejection of my dissertation by the University of California Library, I am sorry about the deficiencies which have just been called to my attention. When my dissertation was completed, signed and given to the library, it had been examined and conformed to the necessary requirements at that time. It appears to me that these requirements have since changed during the last year and a half and that my dissertation should be accepted under the requirements stated at the time my degree was conferred. Unfortunately, it is not possible for me to send the original manuscript to you since I have been unable to locate it since my relocation from California.

Please inform me of the disposition of this matter.

Sincerely yours,

Marlene L. Cohen, Ph.D.

MC:pt

TABLE OF CONTENTS

Page

Page

ACKNOWLEDGEMENTS

Appreciation and gratitude are extended to the following people for their assistance in various aspects of my graduate training, research, and dissertation writing:

Dr. Anthony J. Trevor, for accepting me as his student, for permitting me independence in the selection of a research topic, and most importantly, for his advice and assistance during my research training and during the preparation of this document.

Dr. Neal Castagnoli, for his encouragement of and interest in this research project as well as for his discussion and advice on many aspects of this research.

Dr. Robert Featherstone, for serving on my committee, and for his valuable suggestions concerning the writing of this dissertation.

Dr. Alan Burkhalter, for introducing me to ketamine hydrochloride, and for his discussion, advice, and general helpfulness during the entire course of my graduate training and research.

Dr. Walter Way, for his enthusiasm and encouragement to pursue the initial cerebral disposition studies with ketamine hydrochloride.

Dr. Sin-Lam Chan, for his exceptional patience, technical assistance, and advice primarily on those aspects of this dissertation relating to acetylcholinesterase.

Chuck Pudwill, for preparing the gas chromatographic column and conditions, and for his valuable discussion concerning the analysis procedure for ketamine.

Dr. John Davisson, for his assistance with enzyme assays, and for his helpful information on some of the chemical aspects of this research.

Glauce Viana and John Meyer, for their technical assistance with enzyme assays, and Matt Numes, for his assistance with the pK_a determination.

Chuck Lee, for his assistance with procuring supplies, equipment, and technical information.

Claudia Trevor, for her efficiency and conscientiousness in typing the final copy of this dissertation.

Nick Proctor, Sue Duckles, and Barbara Fink, for their friendship, discussion, moral support, and welcome commiseration at critical times during the course of this research.

My family, for their general assistance during the entire course of my life, and more specifically,during the past four years of my graduate training.

This work was supported, in part, by a National Science Foundation Research Traineeship, NSF-G2-1150 and a National Institutes of Health Training Grant, USPHS GM-00475.

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

CHAPTER 3

CHAPTER 4

FIGURE PAGE

CHAPTER 5

ABSTRACT

KETAMINE: CEREBRAL DISPOSITION AND INTERACTION WITH BRAIN ACETYLCHOLINESTERASE

A sensitive gas chromatographic procedure was adapted for the measurement of ketamine and two of its metabolites in brain and tissues other than plasma. Intravenous injection of a concentration of ketamine shown to produce a 6-8 minute loss of righting reflex in rats resulted in maximal brain levels of ketamine within one minute. Concentrations of ketamine were higher in cerebral tissue as compared to plasma at all time intervals measured for periods up to 60 minutes, although brain:plasma concentration ratios did decline from 7.1:1 at 30 seconds to 2.8:1at 60 minutes. During the first minute following ketamine administration, there was a small but significantly higher concentration of ketamine in the cortical brain region as compared to the midbrain, brain stem, and cerebellum. The N-demethylated metabolite of ketamine (I) was detectable in plasma and brain within 60 seconds with higher concentrations found in cerebral tissue at later time intervals. No differences in regional brain concentrations of metabolite I were detected. The N-demethylated and oxidized product of ketamine (II) was found in plasma at 30 and 60 minutes following intravenous injection of ketamine in the rat.

Further studies of metabolite I involved *in vitro* metabolic experiments showing that the high cerebral levels of metabolite I following *in vivo* administration of ketamine

resulted primarily from extracerebral degradation of the parent compound. Following intravenous administration in the rat, metabolite I was found to produce anesthetic effects similar to ketamine, although it was less potent as an anesthetic and slightly more toxic than the parent compound.

Repeated administration of ketamine on gain of righting reflex in the rat resulted in cumulative anesthetic effects with each subsequent injection. These effects were correlated with brain and plasma levels of ketamine and its metabolites.

Mode of cerebral uptake of ketamine was investigated with in vitro techniques. Ketamine accumulation in rat cerebral cortical slice preparations was rapid and was not altered by metabolic inhibitors. This is consistent with a cerebral uptake of ketamine which is independent of an active transport mechanism. Equilibrium dialysis experiments using subcellular brain fractions indicated that cellular integrity was not necessary for the association of ketamine with brain particulate material. Studies with particulate brain subcellular fractions, brain slices, and brain tissue from rats previously injected with ketamine, produced consistent equilibrium ratios of ketamine associated with brain tissue to external media (or plasma). On the basis of this data, the partitioning characteristics and the pk_a of ketamine, tissue distribution of this anesthetic appears dependent primarily on lipid solubility, blood flow, and organ mass, similar to that of the short acting barbiturates.

Biochemical correlates of ketamine action included the mixed inhibition of multiple forms of mammalian brain

acetylcholinesterase *in vitro*. This inhibition was pH dependent and reversible on removal of the anesthetic. Inhibition occurred at concentrations commensurate with the concentrations available in brain tissue following the *in vivo* administration of a dose of ketamine which produced a 6-8 minute loss of righting reflex in rats.

CHAPTER 1. GENERAL INTRODUCTION

CRITIQUE OF AVAILABLE ANESTHETICS

Anesthesia is a term introduced by Oliver Wendell Holmes to signify a general loss of the ability to perceive and to respond to stimuli from the external environment. This physiological state of negligible pain perception is usually associated with loss of consciousness and achieved during surgical operations by the administration of drugs commonly referred to as general anesthetics. Ideally, an anesthetic should not be flammable, explosive, expensive, noxious, or toxic and should permit adequate ventilation, muscle relaxation and provide a rapid and pleasant induction and recovery.

Many structurally diverse chemicals are capable of producing general anesthesia. This heterogeneity of structures ranges from small, relatively non-chemically reactive molecules, such as xenon through small chain hydrocarbons, ethers, and halogenated compounds to reactive organic chemicals such as the barbiturates. The diversity of these agents with respect to structure and physical state is reflected in the possible routes of anesthetic administration which include inhalation, intravenous, and intramuscular methods.

Since this heterogeneity exists, it has not been possible to predict specific structural features essential to anesthetic activity. For this reason, there is no single unifying concept of anesthetic action although a variety of theories have been proposed (3). Suggestions for a mechanism of anesthetic action have been based largely on the physico-

chemical properties of anesthetics and have included solubility, thermodynamic, and molecular size considerations. Relatively little is known about the mode of action or biochemical effects of structurally nonspecific chemicals, although many of these chemicals have been shown to affect brain metabolism in a number of systems (146). Since there may not be a universal mechanism of anesthetic action, it is possible that a more precise understanding of the biochemical effects of specific anesthetic agents could offer a more sophisticated insight into potential anesthetic mechanisms. Differences in the alteration of biochemical parameters may also help to explain both central and peripheral actions of anesthetic agents other than the induction of anesthesia itself. For example, the inhibition of a discrete enzyme system (e.g., acetylcholinesterase) by one group of agents may prove responsible for certain actions (e.g., salivation or miosis) which do not occur with anesthetics unable to affect that particular enzyme. Differences in the degree to which certain biochemical parameters are altered by anesthetics may account for the wide variation (both qualitatively and quantitatively) in pharmacological effects of this group of drugs.

Anesthesia was introduced in the 19th century with the discovery that nitrous oxide inhalation obliterated pain. Shortly after this, ether was administered for the first surgical operation initiating the era of inhalation anesthesia. Gaseous anesthetics such as nitrous oxide, ethylene, and cyclopropane proved to be valuable agents, although not

perfect. Use of nitrous oxide has been restricted by its lack of potency increasing the danger from hypoxia. Ethylene and cyclopropane are both explosive gases while cyclopropane suffers from the additional disadvantage of flammability.

Some of the disadvantages of gaseous anesthetic agents have been eliminated with the use of liquid volatile anesthetics. Although gaseous anesthesia is still administered, volatile liquid anesthetics have virtually replaced these agents for extensive surgical procedures. Of these agents, halogenated compounds have obtained the widest use, although these anesthetics also have some serious limitations. For example, there has been substantial evidence to link certain of these halogenated compounds to liver disease (104, 169) and to a depression of cardiovascular status (128, 143). By virtue of the physical properties of this group of anesthetic agents, administration necessarily involves elaborate equipment, and the need for constant monitoring of the anesthetic state. Induction has been characterized by a delayed onset which may be shortened by the initial administration of a high anesthetic concentration, although this may complicate control of anesthetic status. In addition, a lengthy undesirable emergence from anesthesia may occur.

The introduction of intravenous anesthetics solved some of the problems associated with inhalation anesthesia. Practical intravenous anesthesia did not occur until the introduction of the rapid acting barbiturates in the 20th century. Use of intravenous agents has been especially

applicable to induction of anesthesia because of their rapid onset and short duration. Rapid onset of the intravenous barbiturates has been attributed primarily to their lipid solubility and a high cerebral blood flow permitting immediate penetration of the agent into brain tissue.

Thiopental is the prototype of the rapid acting intravenous anesthetic agents, although methohexital has achieved some popularity. Methohexital has been found to be more potent permitting the use of a more dilute solution, and to have a slightly shorter duration than thiopental (59). However, use of both of these agents has been associated with undesirable features including certain adverse clinical effects such as cough and hiccough, and hypotension. While accidental intraarterial injection of thiopental has resulted in severe arterial problems (20, 60), this difficulty has been reduced with the use of a dilute solution of methohexital (59). One of the more important limitations of this group of anesthetic agents is their apparent lack of any strong analgesic potency. In fact, these agents have been shown to decrease the threshold to certain painful stimuli at subanesthetic doses (59, 60). Termination of the anesthetic effects of barbiturates has been shown to result primarily from their redistribution into "neutral tissues" (75, 144). Although metabolism may exert some influence on the short duration of action (60, 151), a substantial amount of barbiturate is thought to remain in body tissues for some time following its administration. Because the potential for drug interactions and cumulative effects is increased

by the long term retention of barbiturates, it might be advantageous to produce an anesthetic agent with a short duration of action resulting from metabolism and rapid elimination of the drug from the body.

One such group of compounds is the derivatives of eugenol which, in addition to rapid detoxification, have advantages as intravenous anesthetics including stimulation rather than depression of respiration and no increased sensitivity to pain (60). However, the rapid hydrolysis of these agents results in part from serum cholinesterase activity (53) suggesting a rather high potential for the occurrence of interactions with other cholinesterase inhibitors, or agents similarly hydrolyzed by cholinesterase (e.g., succinylcholine). The most extensively studied drug of this group is propanidid which in the usual dose range (5 to 10 mg/kg) produces immediate loss of consciousness for 3-6 minutes followed by complete recovery in 5-10 minutes (33). This is considerably faster than recovery following the intravenous barbiturates. Propanidid is similar to the barbiturates in that it produces cardiovascular depression leading to hypotension (15), and damage to the arterial wall following intraarterial injection. Unfortunately, a high incidence of postoperative nausea and vomiting limits the widespread use of propanidid.

The search for better anesthetic agents has included a re-evaluation of drugs currently used for other purposes. This group includes the non-barbiturate sedative-hypnotic,

diazepam, which can produce anesthesia when administered by intravenous injection. Diazepam is an alternate drug to thiopental or propanidid with the sole advantage being the possible production of slight analgesia in low doses indicating the absence of antianalgesic effects. However, maximum response is somewhat delayed in onset (1 to 2 minutes), and anesthetic effects may be prolonged suggesting a lack of flexibility in anesthetic control (62). Diazepam also produces slight hypotension and definite respiratory depression. Although diazepam has been used as a muscle relaxant there are reports of a lack of abdominal relaxation following its intravenous administration (85). The primary application of diazepam has involved its use in combination with other analgetic agents.

Attempts at improvement in anesthetic agents have included the use of gammahydroxybutyric (60, 185) acid which may be utilized as an energy producing substrate by the body. The state of unconsciousness produced by this hypnotic is associated with random clonic movements, phonation, and some retention of reflex activity. Its principle action does not appear to involve alteration of the reticular activating system. Cardiovascular effects include a bradycardia with no remarkable alteration in blood pressure. However, application of a surgical stimuli may result in tachycardia, hypertension and increased cardiac output. Although this agent may produce unconsciousness with a slightly delayed

onset following intravenous administration, it does not offer complete protection from surgical pain. For this reason, it will certainly not replace any of the previously mentioned anesthetic methods, although it may be employed as an anesthetic supplement because of its low toxicity.

Ethanol has also been re-evaluated as an intravenous anesthetic agent. However, clinical experience with this agent has been disappointing (61). Steroidal anesthetics have been introduced as short acting intravenous agents with hydroxydione considered the prototype of this group of drugs (60). Although this agent appears to have a wide margin of safety, it is limited by a slow induction (15 to 20 minutes) and vascular disturbances associated with its intravenous injection.

For completeness of this brief evaluation of agents used to obliterate pain, mention must be made of combination drug therapy which has achieved substantial popularity in anesthesia (131). Neuroleptanalgesia is defined as the painless state induced by intravenous administration of a suitable dose of a neuroleptic drug (usually restricted to a non-phenothiazine major tranquilizer) and an analgesic drug (usually a narcotic analgesic). This state of "wakeful anesthesia" has been employed during major surgery, although it is probably best suited for procedures necessitating retention of consciousness when patient cooperation is required. If a general anesthetic is employed along with the neuroleptic and analgetic agent, the anesthetic state

is called neuroleptanesthesia. The most common conventional anesthetic agents used for this purpose include nitrous oxide and thiopental. Unnecessary side effects and the increased potential for drug interactions present the primary arguments against this method of analgesia and anesthesia, although it may offer certain advantages under specific circumstances.

DISSOCIATIVE ANESTHESIA

Benzylcyclohexylamine derivatives were introduced in 1959 with the demonstration that phencyclidine produced an anesthetic state in animals (141). These chemicals were claimed to produce a new, unique, and potentially improved anesthetic state termed "dissociative anesthesia" which was characterized by catalepsy, catatonia, amnesia, and analgesia. Dissociative anesthesia was originally defined on the basis of electroencephalographic (EEG) patterns, next on the clinical appearance of the anesthetic state, and lastly, as a description of the post-anesthetic effects. Studies of EEG changes induced by ketamine suggested the following descriptions of dissociative anesthesia:

> "Selective effect..... on the neocortex with delta-wave electroencephalographic activity elicited in the somatosensory and association areas along with theta-like activity in the frontal cortex." (40)
>
> "Functional dissociation between the thalamoneocortical and limbic systems..."(42)

> "..... sensory input may reach cortical receiving areas but fail to be perceived in some of the association areas because these are depressed. This interference in proper association of afferent impulses results in 'dissociation'." (39)

Observations of the symptoms of anesthesia suggested that dissociative anesthesia best be described as follows:

> "Profound analgesia is coupled with a somnolent state in which the patient does not appear to be asleep or anesthetized, but rather 'disconnected' from his surroundings." (42)

Since there is a report of phencyclidine induced anesthesia in decorticated monkeys (31) and since little is known of the actual mechanism of anesthesia, more appropriate definitions of "dissociative" have been suggested:

> "..... to describe the post-anesthetic effects rather than to imply the nature and cause of anesthesia." (31)

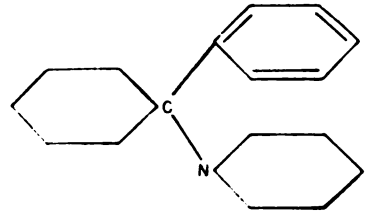
> "..... peculiar state of altered consciousness." (39)

In summary, although dissociative anesthesia was introduced as a singularly unique and specifically defined form of anesthesia, current evidence resulting from further clinical and experimental experience with this form of anesthesia has minimized this distinction. However, dissociative anesthetic agents have exhibited some marked differences in pharmacological actions as compared to conventional hydrocarbon and barbiturate anesthetics.

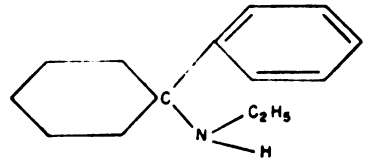
Clinically, dissociative anesthesia in man was first accomplished with the use of phencyclidine (see Figure 1-1). Shortly after its introduction, the disadvantages of phencyclidine anesthesia discouraged further attempts to use this agent clinically. Difficulties with phencyclidine included production of an inadequate anesthesia in some patients, convulsive seizures, and unmanageable postoperative manic behavior in a rather large proportion of patients. It is of interest to note that although unacceptable as an anesthetic agent, phencyclidine has achieved some measure of success as an illegal psychotomimetic. In this respect, it has been referred to as PCP, peace pill, and synthetic marijuana and may even be substituted for THC (tetrahydrocannabinol) on the street (100, 195).

Following the lack of clinical acceptance of phencyclidine, investigation was begun with another dissociative anesthetic agent, cyclohexamine (see Figure 1-1). This derivative suffered from disadvantages similar to phencyclidine including a high incidence of unsatisfactory anesthesia and excessive undesirable emergence reactions, so the compound did not achieve extensive clinical use.

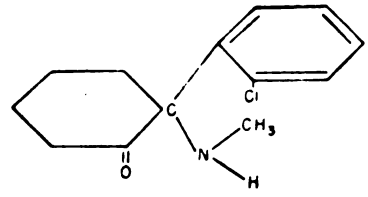
Search was continued for a more suitable derivative of phencyclidine with better anesthesia and less psychotomimetic properties. Ketamine (see Figure 1-1) hydrochloride (d,l-2-(o-chlorophenyl)-2-methylaminocyclohexanone-HCl) proved to be more satisfactory for the production of clinical anesthesia in man than phencyclidine or cyclohexamine and



Phencyclidine Hydrochloride [CI-395]



Cyclohexamine Hydrochloride [CI-400]



Ketamine Hydrochloride [CI-581]

Figure 1-1. Structural formulas of three dissociative anesthetic agents. Notation in parentheses refer to investigational code assignment for each structure.

has since achieved rather wide clinical use. Ketamine was synthesized in 1961, but the first major clinical investigation did not occur until 1965. Ketamine became commercially available in 1970 and is currently marketed by two pharmaceutical companies, Parke, Davis and Co., which introduced the compound, and Bristol Laboratories. Ketamine offers two principal advantages over phencyclidine: (a) absence of convulsions in the vast majority of people and (b) a decreased duration of action permitting better control of anesthesia (31, 54).

PHARMACOLOGY OF KETAMINE

In contrast to the barbiturates, administration of ketamine may be accomplished dependably by either the intravenous or intramuscular route. Intravenous administration has been shown to produce almost immediate anesthesia of short duration while onset and duration have been prolonged following intramuscular administration (39, 100, 116).

This anesthetic state may be characterized by varying degrees of hypertonus and involuntary muscular movement (13, 39, 99, 100, 130, 141). Eyelids have been reported to remain open and nystagmus to occur during induction and recovery (13, 99, 130, 141). Although laryngeal incompetence has been demonstrated (173), there may also be some retention of laryngeal and pharyngeal reflexes tending to maintain a patient airway during anesthesia (14, 39, 42, 100, 141).

Salivation and lacrimation have been notable especially during recovery from ketamine anesthesia (42, 116, 156, 186) and for this reason, an anticholinergic agent, usually atropine, has been a routine premedicant.

In doses employed for anesthesia, profound analgesia is produced by ketamine (39, 55, 101) in contrast to phencyclidine, and for this reason, ketamine may by used as the sole anesthetic in some surgical procedures. One distinct advantage of ketamine compared to the barbiturates is the analgetic efficacy of subanesthetic concentrations of ketamine (156).

Given slowly, ketamine has not appeared to depress respiration following therapeutic doses (39, 42). In fact, bronchodilating properties have been suggested, indicating a potential use for this agent in asthmatic patients (41). Although apnea and respiratory depression have occurred with rapid administration or overdosage of ketamine (42, 101, 141, 186), these effects were considerably less frequent than the respiratory depression which has resulted from barbiturate administration.

Increased arterial blood pressure and heart rate have been associated with the ketamine-induced anesthetic state, indicating a major difference from the hydrocarbon and barbiturate anesthetics which, in general, have been found to depress cardiovascular status. Attempts to localize the mechanism of the cardiovascular stimulatory properties have not been successful. Beta adrenergic blockade with

propranolol in man (13) and both propranolol and practolol in dogs (182) had no effect on the blood pressure or cardiac output response to ketamine, and this suggested the absence of sympathetic augmentation as a principle factor in this response. Mediation of this response by a peripheral action on the autonomic nervous system is also doubtful since hexamethonium administration (181) and epidural anesthesia (179) in dogs virtually abolished the pressor response to ketamine. To further complicate the situation, ketamine has been demonstrated to exert a direct negative chronotropic effect on an in situ dog heart-lung preparation (180). Stimulation of cardiac output has been postulated to involve a mechanism dependent on a direct action of ketamine on the myocardium (183), and a depression of baroreceptor reflex activity (57). However, increasing studies have suggested that a predominant effect of ketamine on the central nervous system may account almost entirely for its cardiovascular stimulatory effects (166, 179, 183).

Because of the demonstration of psychotomimetic properties with phencyclidine, recovery from the ketamine-induced anesthetic state has become a matter of primary interest. While initial observations on the ketamine-induced anesthetic state indicated only minor and relatively controllable psychotomimetic effects during emergence (39), emergence disorientation has appeared to be the drug's most severe limitation (99). The incidence of psychotomimetic reactions has varied widely from 5 to over 30 percent of

patients (99). These effects have been relegated to vivid dreams and illusions sometimes accompanied by delerium (14) which has not always proved to be disagreeable (130). True visual hallucinations have been rare (70, 130) with ketamine and the psychic phenomena have not been found to result in any permanent psychological changes (44, 130).

Although ketamine-induced anesthesia is of short duration following a single intravenous injection, certain parameters have not returned to normal for up to 3 hours. This has led some investigators to conclude that ketamine is not short acting since recovery has appeared extremely prolonged (63, 133, 167). Many attempts have been made to minimize the emergence reactions. Incidence of emergence phenomena have been diminished if the patient is not stimulated or aroused during recovery (42, 116). Pre-anesthetic medication of sedative-hypnotics, analgesics, and tranquilizers have been administered in attempts to reduce the psychotomimetic effects of ketamine (8, 14, 39, 66). In this regard, droperidol, a butyrophenone tranquilizer has shown the most potential usefulness (8, 155). Although the psychotomimetic properties of ketamine have reduced the initial enthusiasm surrounding the use of this anesthetic, it should be remembered that certain forms of dreaming also occur with the more conventional anesthetic agents (17).

With respect to the fate of ketamine in the body, preliminary studies have indicated that there are at least two biotransformation products of ketamine (27, 31).

Ketamine has been shown to be initially N-demethylated to form the free amino acompound which has been conventionally called metabolite I (see Figure 1-2). Further oxidation of this product to the cyclohexene derivative, known as metabolite II (see Figure 1-2), has been demonstrated. These two metabolites appear to be the predominent breakdown products of ketamine in man (27, 28, 115, 186) by available detection procedures. Two other potential intermediate metabolites have also been identified (see Figure 1-2) as the hydroxylated derivatives of metabolite I and have been detected in rat urine (27, 31). From this preliminary description of the metabolism of ketamine, it is apparent that very little information on the metabolism or metabolic products is available. In fact, in one study only 23 percent of the injected ketamine could be accounted for in human urine within 24 hours (186) suggesting the need for further investigation into the metabolic fate of ketamine.

It is important to emphasize the initial enthusiasm towards ketamine when it was first introduced by Parke-Davis researchers. At that time, this drug was thought by a number of anesthesiologists to be an "ideal" anesthetic possessing none of the negative traits of its predecessors. For example, it was claimed to preserve protective reflexes (42), never to obstruct the airway (42), to lack cumulative effects (31, 39, 124), to have minimal psychotomimetic properties (100, 147), and to be useful in neurosurgical procedures (42), ophthalmic procedures (42) and with a full

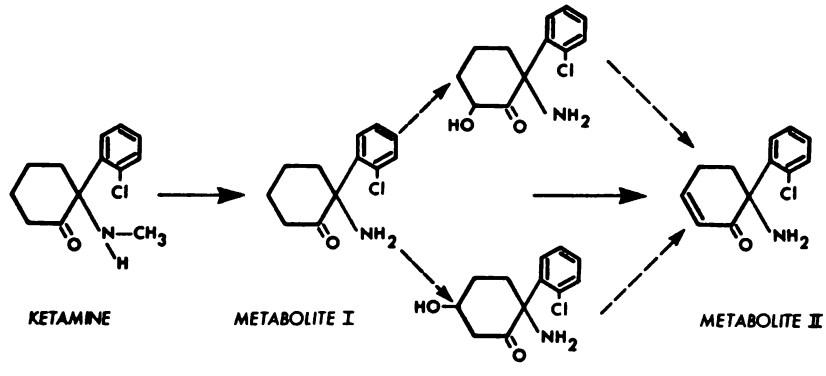


Figure 1-2. Proposed biotransformation pathways for ketamine. Predominent metabolites are indicated with solid arrows while dashed arrows indicate postulated hydroxylated intermediates.

stomach (42). Further clinical experience with this agent indicated that these specific favorable indications were premature, and the superiority of ketamine in those respects is doubtful. However, it is apparent that while ketamine has not solved all the problems of anesthesia and has produced some additional adverse properties, this anesthetic is a useful addition to the armamentarium of the anesthetist. It offers the advantages of dependable induction by intramuscular administration (116), cardiovascular stimulation rather than depression, no reported hepatic or renal toxicity, ease of administration, and only minimal respiratory depression. Cardiovascular stimulation suggests potential uses for this agent in cases of preexisting hypotention (116), for cardiac diagnostic procedures (14, 42, 166), and for shock patients because of the absence of any strong vasoconstricting action. It has wide application in pediatric procedures due to the availability of intramuscular administration, and during burns surgery (43) since it can eliminate the need for an inhalation mask or for locating a vein. For these reasons, ketamine is a valuable addition to the conventional hydrocarbon and barbiturate anesthetic agents and has the potential for widespread use for a variety of specific indications.

INTRODUCTION TO RESEARCH PROBLEM

Since ketamine is a relatively new anesthetic, research involving this agent has been directed primarily toward

obtaining a better appreciation of its clinical usefulness. Such investigations have served to establish and clarify the multiple actions produced by this agent with particular reference to its diverse central effects including analgesia, anesthesia, psychotomimetic properties and nystagmus. There is also the suggestion that some of the peripheral effects of ketamine may originate via a central influence of the drug (e.g., cardiovascular stimulation) while the physiological location of other actions such as salivation, lacrimation and hypertonus have not been examined and may result from either peripheral or central actions.

It would be exceedingly useful to define more precisely the specific mechanisms responsible for the diverse pharmacological properties of ketamine. Knowledge of the central mechanisms might permit more rational pharmacological control of various undesirable features of ketamine such as the emergence phenomenon. For example, clinical use of a specific antagonist to the psychic effects of ketamine might be possible if the mechanism of these effects were known. Information on the cerebral biochemical actions of ketamine also would be potentially useful in determining structural requirements for specific actions, thus enabling a more rational approach to the synthesis of similar but better anesthetic agents. Since ketamine does exhibit a wide variety of effects, and since it has been recommended for use in neuroleptanesthesia, there is a large potential for drug interactions. A better understanding of its biochemical actions might also be useful

in predicting the consequences of its combined use with other agents in order to prevent potential clinical problems.

Research on the cerebral biochemical actions of ketamine has not been abundant. Preliminary studies by Parke-Davis researchers on the peripheral effects of ketamine have suggested that it is devoid of alpha and beta blocking, ganglionic blocking, anticholinergic, antihistaminic and neuromuscular blocking activity (31). However, other preliminary studies have suggested a possible role of ketamine in the inhibition of norepinephrine uptake in smooth muscle (135), cardiac tissue (129) and cerebral tissue (35). Phencyclidine has perhaps been more completely studied with respect to its hallucinatory properties (111, 112, 177), although no definitive mechanisms have been proposed.

Naturally the demonstration of any biochemical actions of ketamine is insufficient in itself for the establishment of a clinically relevant mechanism of action. Proposed biochemical mechanisms observed in vitro would have little clinical relevance unless the necessary concentration range for the effect can be achieved under in vivo conditions. For this reason, information on the cerebral disposition of ketamine would be critical to any assessment of the clinical relevance of proposed biochemical mechanisms. Information on the cerebral disposition of ketamine may also be useful in deriving an anesthetic index for this agent. Recently the validity of minimum alveolar concentration has been

questioned as an index of anesthetic depth for inhalation anesthetics (194). These investigators have proposed a measurement based on the actual brain anesthetic concentration in small animals which may prove more useful for comparison among anesthetic agents.

Some extremely scanty, preliminary data on the disposition of ketamine indicated that highest concentrations were found in body fat of the rat at an unspecified time after administration (27). However, personal communication with these investigators indicated that precise cerebral levels were not available at any time interval following intravenous administration of ketamine in any animal species (72).

In attempting to assess a central mechanism of action for ketamine it would also be useful to have knowledge relating to the extent of metabolite formation and concentration in cerebral tissue along with an activity index for any metabolites present. This information is necessary to evaluate the potential role of biotransformation products in the production of some of the central effects observed following ketamine administration. The only available information in this regard was a preliminary report suggesting that metabolites I and II were weakly anesthetic, possessing 0.1 and 0.01 of the activity of ketamine respectively (31). No mention was made of the species or route of administration which could alter the apparent activity by factors relating to physico-chemical properties.

SCOPE OF RESEARCH PROBLEM

The purpose of the present research was to define clearly the cerebral disposition of ketamine and its two primary metabolites. A precise examination of the pharmacological activity of the N-demethylated product (metabolite I) was included in order to assess its potential contribution to the pharmacological actions of ketamine. Cerebral disposition of ketamine was further characterized with respect to its mode of entry into brain tissue. Finally, this study investigated the extent to which potential cholinergic manifestations of the ketamine-induced anesthetic state may be explained by inhibition of brain acetylcholinesterase.

Rats were selected as the experimental animal for the disposition studies for rather obvious considerations. Small animal species such as the rodent provide easy handling, are convenient to house, and are relatively inexpensive. Initial reports indicated the ability of rodents to respond to ketamine in a manner similar to primates (124), supporting the validity of this animal species as a model.

Ketamine was obtained from Parke-Davis Laboratories as the crystalline hydrochloride salt. Crystalline ketamine as well as the marketed product is a racemic mixture of the two possible enantiomers of ketamine. Although these isomers have been resolved (see Figure 1-3), there is not sufficient amount available for research purposes (73). These isomers both appear active but with quantitative differences (73). Because of the lack of availability of the separate isomers,

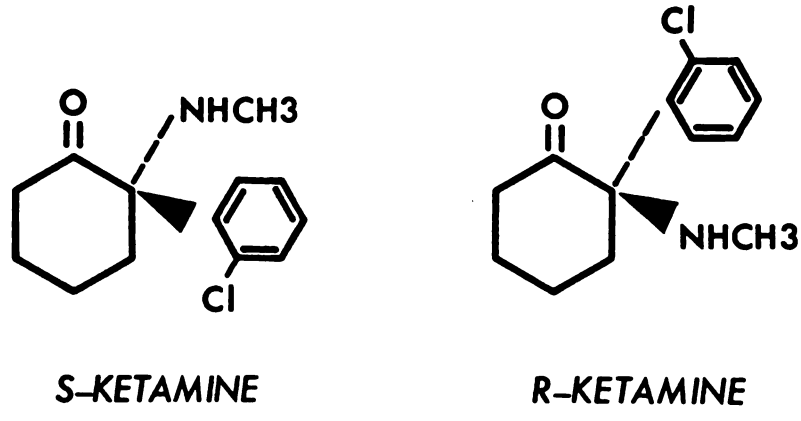


Figure 1-3. Enantiomeric forms of ketamine. Configuration about the asymmetric center is denoted by S and R as assigned by the Cahn-Ingold-Prelog convention.

it was not possible to examine the extent to which differences in distribution properties and metabolism might account for the differences in activity of the isomers. However, the present studies with the racemic mixture are relevant to the clinical administration of ketamine and permit disposition information which may be related to proposed biochemical actions of the anesthetic agent.

ORGANIZATION OF RESEARCH

Before a study of the cerebral disposition of ketamine could be undertaken, it was necessary to establish a method for the estimation of ketamine and its metabolites in biological tissue. Chapter 2 deals with the adaptation to brain tissue of a reported procedure for ketamine analysis from human plasma. This chapter also includes a discussion of certain problems encountered in the extraction of ketamine and its metabolites from a highly lipid tissue.

After establishing the analysis procedure, the pharmacological effects of ketamine and its N-demethylated metabolite (metabolite I) were studied in vivo following intravenous administration in the rat. After selecting a suitable dose level for ketamine, the distribution of ketamine and two of its metabolites was examined in plasma and brain for periods up to 60 minutes following the in vivo administration of ketamine. Distribution of ketamine and its metabolites was also analyzed regionally in four areas

of the brain to examine any possible differences in accumulation. Finally, cumulative effects were observed following the repeated administration of ketamine and these results were correlated with brain and plasma levels of ketamine and its metabolites. These in vivo studies are documented in Chapter 3.

Chapter 4 concentrates on the mode of entry of ketamine into cerebral tissue. For this investigation, the viability of the brain slice preparation was characterized utilizing the parameters of lactate production and inulin space. These parameters were further examined in the presence of ketamine. Accumulation of ketamine by cortical slices was observed in the presence and absence of metabolic inhibitors. Accumulation of ketamine was further characterized by examination of its release from the slice preparation. The requirement for cellular integrity in the cerebral accumulation of ketamine was assessed by measuring its association with subcellular fractions of brain tissue. Data highly suggestive of pronounced lipid solubility for ketamine at pH 7.4 were confirmed by an analysis of the distribution of ketamine into organic solvents and a determination of its pk_a value. The in vitro procedures described in Chapter 4 were also utilized to examine the potential contributory roles of brain and liver in the biotransformation of ketamine.

Finally, Chapter 5 contains data on the inhibition of mammalian forms of brain acetylcholinesterase by ketamine. Methods are discussed for the solubilization and purification

of three forms of this enzyme from ox caudate nucleus tissue. A membrane bound form of the enzyme was isolated from synaptic membranes for comparative studies. The kinetics of inhibition of these enzyme forms were characterized by determination of inhibitory constants, reversibility of inhibition, species sensitivity, and pH dependence. The inhibition of the enzyme forms was also examined with the N-dealkylated metabolic product of ketamine. Finally, this in vitro biochemical effect of ketamine is discussed with reference to its potential clinical relevance in terms of the cerebral disposition of ketamine.

CHAPTER 2. METHODOLOGY FOR GAS CHROMATOGRAPHIC ANALYSIS OF KETAMINE AND METABOLITES IN BIOLOGICAL TISSUES

INTRODUCTION

Methods permitting the analysis of ketamine and its metabolites have been restricted primarily to the estimation of plasma concentrations in man (28, 29, 51). Initially, these methods included the use of a relatively insensitive modified methyl orange procedure (27), a more sensitive fluorimetric assay which suffered from the presence of high blank values and metabolite interference (51), and the use of an apparently limited supply of tritium-labeled ketamine (29). Recently, reports have appeared describing highly sensitive gas chromatographic procedures for ketamine estimation in plasma (28, 81). Although rapid and consistent results may be obtained utilizing a flame ionization detector (81), this gas chromatographic technique permitted identification only of ketamine. However, a procedure based upon gas-liquid chromatography of a heptafluorobutyryl derivative utilizing an electron capture detector proved highly sensitive not only for ketamine, but for two of its metabolites as well (28). This procedure has been adapted for the estimation of ketamine and its metabolites from cerebral tissue in the present study.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade. Chemicals were obtained as follows: ketamine hydrochloride, metabolite I, metabolite II and the internal standard, 2-amino-2-

(o-bromophenyl)-2-methylamino-cyclohexanone, Parke, Davis & Co. (Detroit, Michigan); pyridine, benzene, nanograde, Mallinckrodt (St. Louis, Missouri); sodium sulfate, anhydrous, Allied Chemical (Morristown, New Jersey); heptafluorobutyric anhydride in sealed glass ampules, Pierce Chemical Co. (Rockford, Illinois); pretested 3 percent OV-17 on GasChrom Q, 100/120 mesh, Applied Science Laboratories (College Station, Pennsylvania).

Solutions

A. Borax Alkali: A solution of 10 percent borax ($Na_2B_4O_7 \cdot 10H_2O$) was made up in 5N sodium hydroxide.

B. Pyridine: Pyridine was dried over KOH pellets.

C. Internal Standard: Stock solution of 100 ug/ml in 0.1N HCl was prepared. Stock solution (0.5 ml) diluted to 10 ml with 0.1N HCl (5 ug/ml) became the working solution.

D. Ketamine Standard: Stock solution of 200 ug base/ml was prepared in 0.1N HCl.

E. Metabolite I Standard: Stock solution of 100 ug base/ml was prepared in 0.1N HCl.

F. Metabolite II Standard: Stock solution of 200 ug base/ml was prepared in 0.1N HCl.

G. Working Solution of Standards: Ketamine and metabolite II stock solutions (1.0 ml) and 0.4 ml of metabolite I stock solution was pipetted into a 25 ml volumetric flask. Enough saline

(0.9% NaCl) was added to make 25 ml in order to prepare the saline working standard solution which contained 8.0 ug/ml of ketamine and metabolite II and 1.6 ug/ml of metabolite I. The water working standard solution was prepared by pipetting 0.5 ml of ketamine and metabolite II stock solutions and 0.2 ml of metabolite I stock solution into a 25 ml volumetric flask. Enough deionized water was added to make 25 ml of working solution containing 4.0 ug/ml of ketamine and metabolite II and 0.8 ug/ml of metabolite I.

Gas Chromatographic Apparatus and Conditions

Analyses were conducted on a Varian aerograph series 1200 gas chromatograph with an electron capture detector of titanium tritide foil. The column used was a 1.8 meter (3 mm internal diameter) coiled glass tube packed with OV-17 (3 percent) on Gas Chrom Q (100/120 mesh). The column temperature was maintained at 190°C with the flash heater at 220°C and the detector at 225°C. The carrier gas (20 ml/min) consisted of 5 percent methane in argon. Standing current approximated 3 x 10^{-8} amperes and electrometer attenuation was set at 32 or 64.

Extraction Procedure

Aliquots of samples (.01 to 1 ml) in either deionized water or 0.1N HCl were measured by pipette into 15 x 135 mm glass stoppered conical centrifuge tubes. Additional deionized water was added to 1 ml and acid (0.1N HCl) was added to obtain a final volume of 2 ml and a final acidity

of 0.5N HCl. The internal standard (0.1 ml) was added to a final concentration of .25 ug/ml and the tubes were agitated on a vortex mixer. After addition of .1 ml borax buffer and 5 ml benzene, tubes were hand shaken for 30 seconds. After centrifugation in an International centrifuge at 1,750 RPM for 30 minutes (685 g) 4 ml of the benzene layer was transferred to clean glass-stoppered conical centrifuge tubes. Heptafluorobutyric anhydride (0.1 ml) and pyridine (0.1 ml) were added and the stoppered tubes were thoroughly mixed on a vortex mixer and allowed to stand at room temperature for 60 minutes with agitation every 10 minutes. The contents were then shaken for 5 minutes with 2 ml of 0.5N NaOH to remove excess reagent. The aqueous phase (bottom) was removed with a pasteur pipette. Two ml of 0.25N HCl were added and the resulting mixture was shaken for 5 minutes. After centrifugation on an International centrifuge at 1,750 RPM for 5 minutes (685 g) the aqueous phase (bottom) was again removed with a pasteur pipette. Approximately 1 g of anhydrous sodium sulfate was added to the benzene layer and the tubes were agitated on a vortex mixer and stoppered.

Retention Times

From .5 to 2 ul of the benzene solution was injected via a 10 microliter syringe directly into the column. Retention times were 1 min 5 sec for metabolite I, 1 min 35 sec for metabolite II, 2.0 min for ketamine and 2 min 45 sec for internal standard. The retention times were short enough to permit repeated injections of samples at 12 minute intervals.

Preparation of Tissue Samples for Recovery Determination

Male Sprague-Dawley rats (100-120 g) were decapitated and exsanguinated, and the brains were removed to ice. Blood from the neck region was collected in a heparinized beaker (50 ml). The brain was cleaned of blood vessels and homogenized in a glass homogenizer fitted with a teflon pestle in 4 volumes of 0.9% saline. In experiments to study the effect of pH, the saline had been previously adjusted to pH 7.4 and pH 9.0 from its initial pH of 5.5. For the brain tissue, an aliquot of the homogenate (1.0 ml) was transferred to 4 polycarbonate centrifuge tubes (13 ml capacity). To each tube, an aliquot (.1, .2, .5 and 1 ml) of standard saline working solution of ketamine (8.0 ug/ml), metabolite I (1.6 ug/ml), metabolite II (8.0 ug/ml), and sufficient saline was added to make a final volume of 2 ml resulting in a final 10% tissue homogenate. From each sample an aliquot (0.25 ml) was removed for assessment of recovery directly from brain homogenate. Samples were then centrifuged in the Spinco Model L Centrifuge using a 40 rotor at 100,000 g (40,000 RPM) for 30 minutes. Aliquots (0.25 ml) of the resulting supernatant were removed for assessment of recovery.

For recovery from blood samples, 0.5 ml of the collected heparinized blood was transferred to each of 4 conical glass centrifuge tubes (15 ml). To each tube an aliquot (.05, .1, .25, .5 ml) of the standard saline working solution was added with sufficient saline to make a final volume of 1.0 ml.

Aliquots (0.25 ml) of each sample were removed for assessment of recovery directly from blood. Samples were then centrifuged in the International centrifuge at 1,000 RPM for 15 minutes (225 g). Aliquots (0.25 ml) of the plasma were removed for assessment of plasma recovery.

Calculations and Statistics

Concentrations of ketamine and its metabolites were determined from the gas chromatogram by measuring the peak heights of these compounds from a baseline and dividing this figure by the peak height of the added internal standard. Since column conditions, detector responses and injection technique into the column may vary, individual variation was minimized by relating the peak heights of standards to the internal standard. For graphic representation, lines were determined by regression analysis forcing through the origin and correlation coefficients were obtained as an index of the fit of the line to the points. In tissue recovery experiments lines were compared to water standards by an analysis of covariance with significance reported on the basis of differences in elevation.

RESULTS

Gas chromatographic tracings from plasma or brain tissue supernatant revealed no interfering peaks due to naturally occurring constituents or resulting from the extraction procedure itself. A typical chromatogram (Figure 2-1) indicates the clarity and separation of peak heights obtained

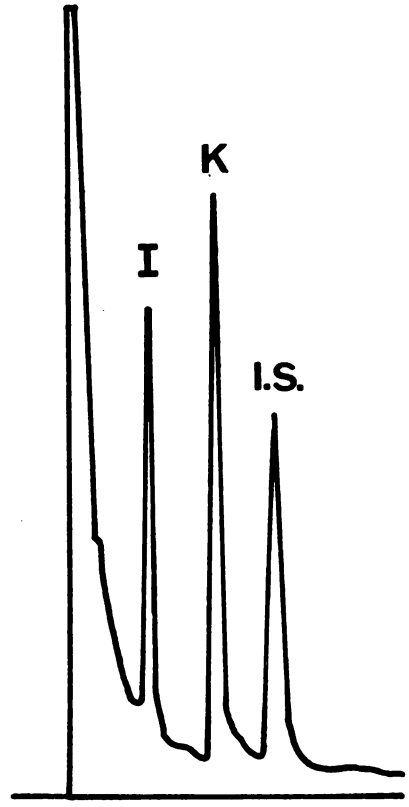


Figure 2-1. Representative gas-liquid chromatogram tracing of ketamine (K), metabolite I (I), and internal standard (I.S.) resulting from extraction of rat brain tissue. Brain was removed 5 minutes after intravenous administration of 20 mg/kg ketamine HCl. Column conditions and extraction procedure are described in Chapter 2, Methods.

from brain tissue following intravenous injection of 20 mg/kg ketamine in the rat.

Extraction of known amounts of ketamine, metabolite I and metabolite II from deionized water indicated the linearity of response of the electron capture detector (Figure 2-2), since all correlation coefficients were $> .9100$. This method of detection proved reliable and reproducible as indicated by the reasonably small standard errors (Figure 2-2) produced from averages of replicate assays done on three separate days. Variability was greater for the metabolites as compared to ketamine.

Recovery of standard solutions of ketamine, metabolite I and metabolite II added to and extracted from whole blood, was not significantly different from the recovery of each component added to water (Figures 2-3, 2-4, 2-5). However, standard solutions of ketamine and metabolite II added to heparinized whole blood and subsequently assayed from the plasma after centrifugation, resulted in a significantly lower recovery as compared to extraction from water (ketamine, $p < .005$; metabolite II, $p < .025$).

When all three standards were added to and immediately extracted from brain tissue homogenates prepared in normal saline (Figures 2-6, 2-7, 2-8), the resultant recovery was significantly lower than recovery of standards added to water (ketamine, $p < .005$; metabolite I, $p < .025$; metabolite II, $p < .05$). However, if this same tissue homogenate with the added standards was centrifuged to remove particulate material

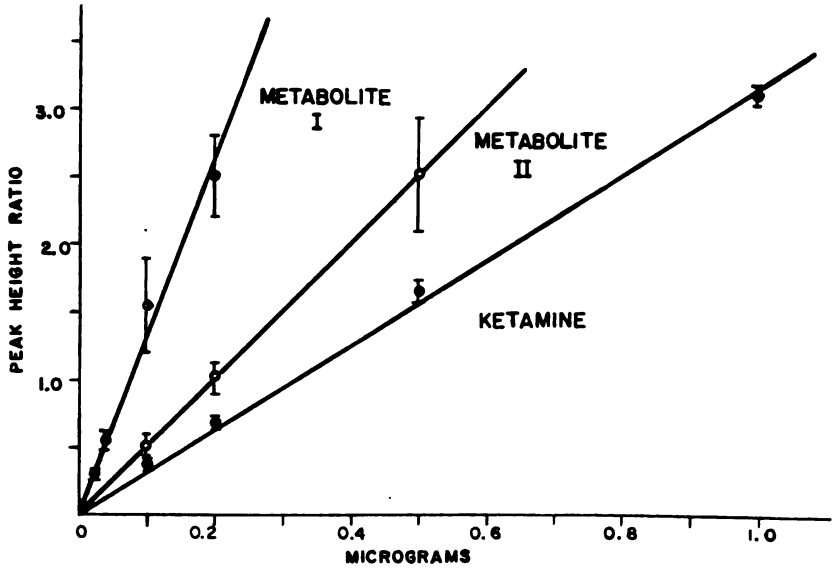


Figure 2-2. Linearity and variability of standard aqueous extraction curves for ketamine, metabolite I, and metabolite II. Peak height of standards divided by peak height of internal standard is plotted against known concentrations. Each point represents the mean ± standard error of three individual assays, extracted and analyzed on three separate days. Lines were calculated by regression analysis forcing through the origin and resulted in correlation coefficients of .9962, .9241, and .9137 for ketamine, and metabolites I and II, respectively.

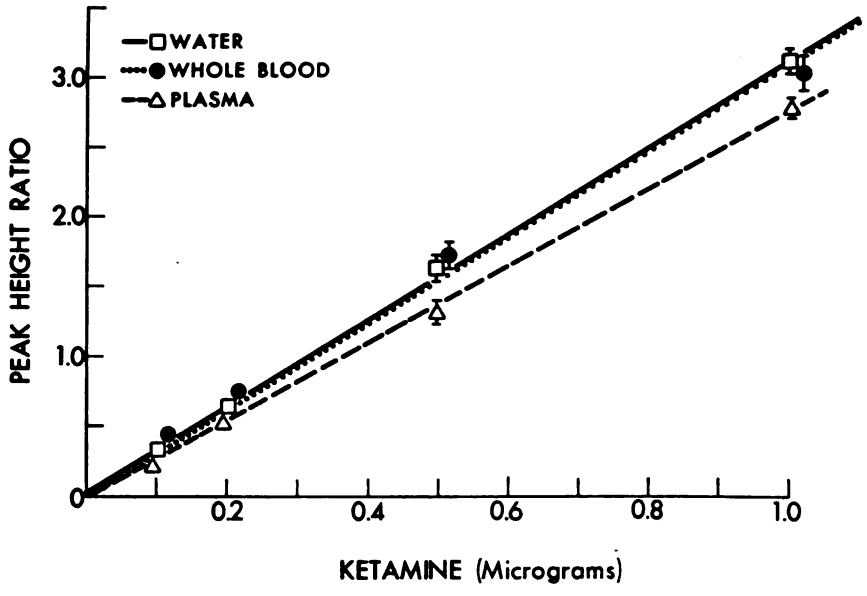


Figure 2-3. Comparison of standard curves for ketamine extracted from whole blood or separated plasma to aqueous standard curve. Each point represents the mean ± standard error of three individual assays extracted and analyzed on three separate days. Lines were calculated by regression analysis forcing through the origin.

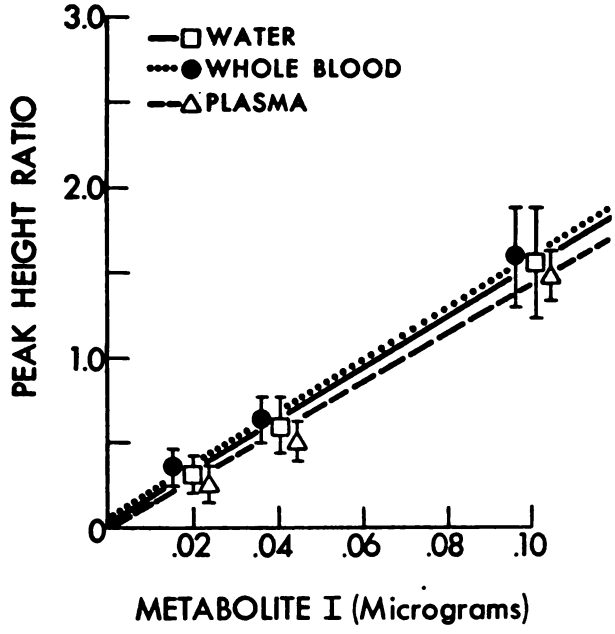


Figure 2-4. Comparison of standard curves for metabolite I extracted from whole blood or separated plasma to aqueous standard curve. Each point represents the mean ± standard error of three assays extracted and analyzed on three separate days. Lines were calculated by regression analysis forcing through the origin.

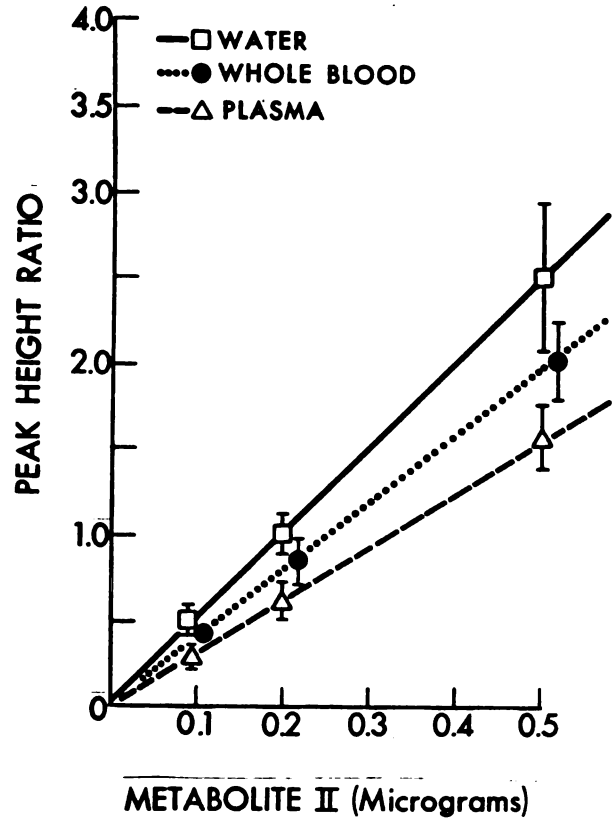


Figure 2-5. Comparison of standard curves for metabolite II extracted from whole blood or separated plasma to aqueous standard curve. Each point represents the mean ± standard error of three assays extracted and analyzed on three separate days. Lines were calculated by regression analysis forcing through the origin.

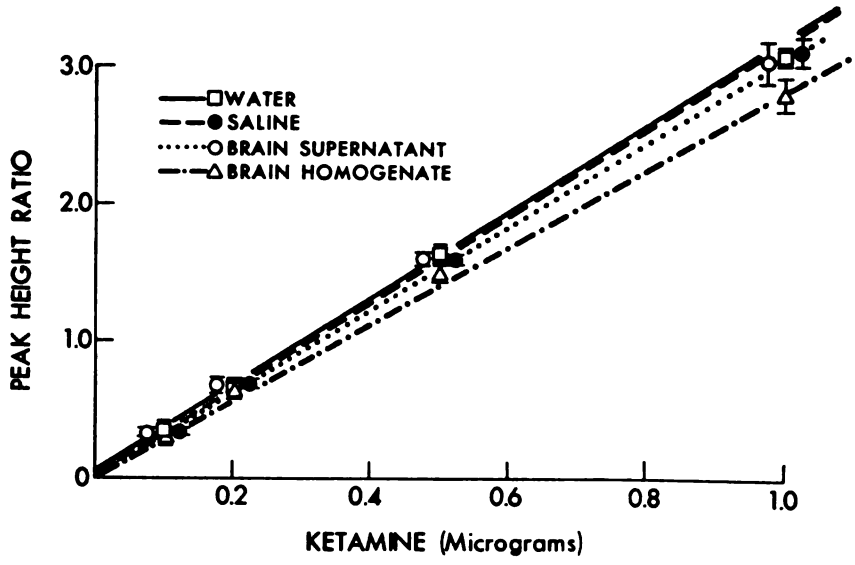


Figure 2-6. Comparison of standard curves for ketamine extracted from brain tissue homogenate, brain tissue supernatant, saline, and water. Each point represents the mean ± standard error of three assays extracted and analyzed on three separate days. Lines were calculated by regression analysis forcing through the origin.

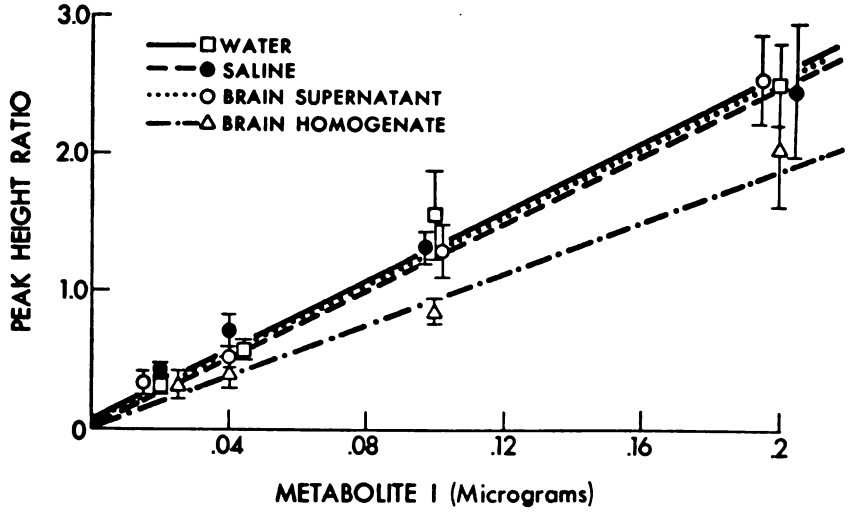


Figure 2-7. Comparison of standard curves for metabolite I extracted from brain tissue homogenate, brain tissue supernatant, saline and water. Each point represents the mean ± standard error of three assays extracted and analyzed on three separate days. Lines were calculated by regression analysis forcing through the origin.

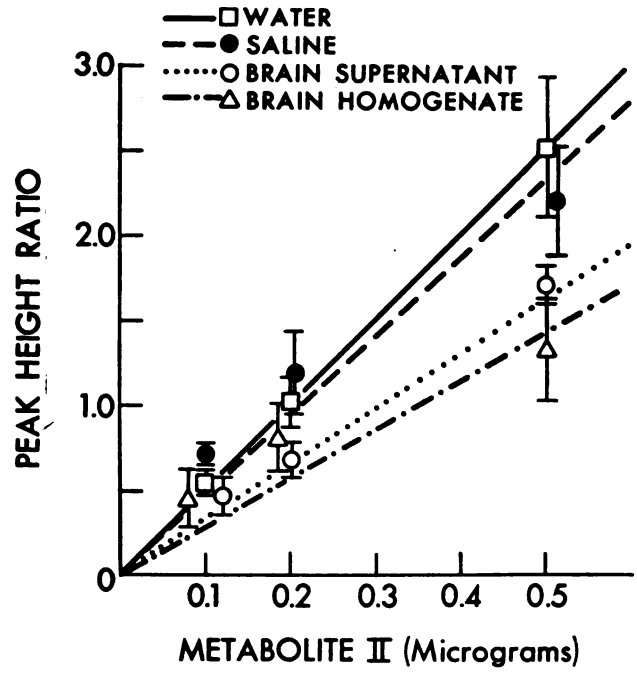


Figure 2-8. Comparison of standard curves for metabolite II extracted from brain tissue homogenate, brain tissue supernatant, saline and water. Each point represents the mean ± standard error of three assays extracted and analyzed on three separate days. Lines were calculated by regression analysis forcing through the origin.

virtually 100% of ketamine and metabolite I could be recovered by assay of the supernatant material. Recovery of metabolite II by either procedure resulted in approximately 70% of the recovery obtained from water.

Alteration of hydrogen ion concentration of the saline used to prepare the brain tissue homogenate resulted in a significant alteration in recovery of ketamine and its metabolites. When brain tissue was homogenized in saline which was previously adjusted to either pH 7.4 or pH 9.0 and a standard solution of ketamine was added, extraction of ketamine (Figure 2-9) after centrifugation resulted in 12% and 18%, respectively, lower recovery as compared to a water extraction or extraction from a homogenate prepared in normal saline. These differences were significant at $p < .005$.

DISCUSSION

The gas-liquid chromatographic procedure for estimation of ketamine and its metabolites from plasma (28) appeared suitable for determinations from other tissues, including the brain. The extraction procedure was found to be rapid and reproducible resulting in excellent separation of peak heights of ketamine and two of its metabolites. Other potential hydroxylated metabolites of ketamine were not extractable into benzene and were found not to interfere with the assay (28). This technique allowed the estimation of as low as .01 micrograms of ketamine and metabolite II

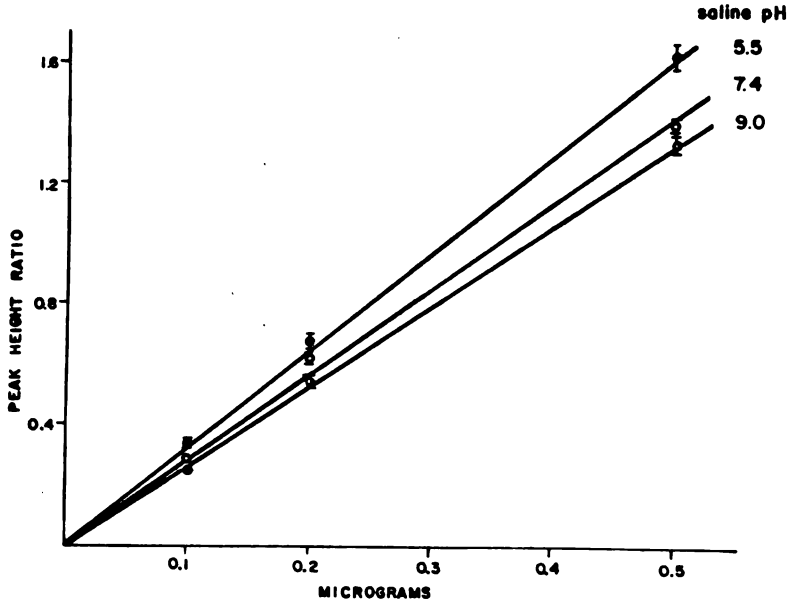


Figure 2-9. Effect of saline pH on standard curves for ketamine extracted from brain tissue supernatant. Brain tissue was homogenized in saline at pH 5.5, 7.4 or 9.0 and subsequently extracted and analyzed for ketamine. Each point represents the mean ± standard error of duplicate assays and lines were calculated by regression analysis forcing through the origin.

and .002 micrograms of metabolite I indicating greater sensitivity than previously reported gas chromatographic procedures (28, 81).

Since it was difficult to run standards in fresh plasma or brain homogenate for each daily assay procedure, it was necessary to determine mean recovery from these tissues. Knowledge of the degree of recovery from tissue as compared to water, permitted standard curves for every assay determination to be constructed from an aqueous extraction. Experiments to determine recovery of compounds from tissue, of necessity, involved the in vitro addition of known concentrations of standard solutions to blood, plasma, or tissue homogenates. This procedure was limited by the assumption that extraction of standards added to homogenized tissue accurately represented extraction of these same compounds following their in vivo tissue association. Although this presented a certain limitation to the usefulness of recovery experiments, experiments of this nature were the best available index for assessing the extraction and the quantitative determination of compounds from tissue.

Virtually 100% of ketamine and its metabolites added to whole heparinized blood was recovered. Although standards were never added directly to plasma, the total recovery from blood argues for a similar recovery from standards added directly to plasma. However, when plasma was separated from blood to which the standards had been added, less than

100% recovery resulted for ketamine and metabolite II. This indicates that a small but significant amount of ketamine and metabolite II may be accumulated and retained in red blood cells. While this may also be the case for metabolite I, recovery of metabolite I from plasma obtained from standards added to whole blood was not significantly reduced.

Initial attempts to extract standard amounts from brain tissue were unsuccessful due to the use of relatively large amounts of whole homogenate (.5 to 1.0 ml). This resulted in the formation of an emulsion following mixing with benzene on a mechanical shaker. In order to avoid this difficulty, it was necessary to use smaller aliquots of the homogenate and to resort to shaking the test tubes by hand which was somewhat less vigorous. This problem was entirely eliminated by centrifuging the homogenate to precipitate all particulate material and then removing an aliquot sample from the resulting supernatant. Recovery from both these procedures, whole brain homogenate and brain tissue supernatant, has been described.

Recovery of ketamine and metabolite I from brain tissue supernatant was no different than recovery following the addition of standards to water or saline. However, results from assay of the whole homogenate indicated a significantly lower recovery. A possible explanation for this difference involves the extraction procedure, in which the sample is diluted in water and acid and then made alkaline (approximate

pH 11.0) with a borax alkali reagent. At this pH, virtually all the ketamine and metabolite I are in the unionized form and should be preferentially soluble in the benzene, which is the situation for the aliquot of brain tissue supernatant. However, when tissue is present, as is the case with a sample aliquot of the entire homogenate, the basic forms of the drugs were also able to associate with tissue and consequently less drug was available for extraction into benzene. Further information on the association of ketamine with brain tissue (Chapter 4) substantiated this explanation. Only 70% of added metabolite II could be recovered when brain tissue supernatant or homogenate was extracted.

Since ketamine is an ionizable compound and may be associated with particulate material under certain conditions of the extraction procedure, it was reasonable to suspect that changes in pH would alter its extractibility. Utilizing saline of pH 5.5 as the brain tissue homogenization medium, resulted in complete recovery of ketamine from the supernatant material. At this pH value, ketamine would be expected to be almost completely ionized and virtually none of it would be associated with particulate material. However, homogenization of brain tissue in saline adjusted to pH 7.4 or pH 9.0, did result in a decrease in recovery from the supernatant. At these pH values, a greater amount of the ketamine would be expected to exist as the free base and therefore could associate with and dissolve in particu-

late material, thereby decreasing the amount remaining in the supernatant after centrifugation. Since the exact extent of ionization of ketamine was unknown, these conclusions regarding the ionic state of ketamine at various pH values were confirmed by a determination of its pKa (Chapter 4). As a result of this study with pH, brain tissue was homogenized in normal saline pH 5.5 for all subsequent assays.

CHAPTER 3. IN VIVO STUDIES ON THE PHARMACOLOGICAL EFFECTS AND CEREBRAL DISPOSITION OF KETAMINE AND METABOLITES IN THE RAT

INTRODUCTION

Attempts to relate any observed *in vitro* biochemical actions of ketamine to its clinical effects would be facilitated by information concerning the disposition of ketamine and its metabolites, particularly their distribution to the brain. Information on the concentration of ketamine and its metabolites in brain tissue following *in vivo* administration of ketamine would indicate a suitable concentration range for the study of biochemical parameters. Disposition studies in man have been limited to the estimation of plasma and urine concentrations of ketamine and its metabolites (28, 51, 81, 115, 186). In laboratory animals preliminary reports have appeared on tissue distribution (27) but no precise data are available on distribution of ketamine and its metabolites to cerebral tissue (72). Earlier studies of ketamine disposition were severely limited by lack of a sensitive detection method (27, 51, 186) which is not a problem at present (see Chapter 2).

There is evidence based on electroencephalographic patterns (40, 42, 186) and the failure to induce anesthesia with ketamine in patients with decreased cortical function (58, 88, 130) to suggest that ketamine exerts a preferential and highly specific action on the frontal cortical regions of the brain. Although it is difficult to relate concentration to activity, this specific action may be the result of a highly preferential and selective distribution of ketamine to the cortex compared to other regions of the

brain. In order to investigate this possibility, the distribution of ketamine and its metabolites was examined in whole brain as well as in four distinct regions of the brain.

Metabolite I has been found to be the primary biotransformation product of ketamine in human plasma (28, 115). In man, plasma levels of this metabolite approached 0.5 ug/ml following 2 mg/kg intravenous ketamine, close to the plasma concentration of ketamine at approximately 30 to 60 minutes. In the rat, preliminary studies suggested that metabolite I was also the major biotransformation product (27) following ketamine administration. Since N-demethylation reactions have produced active metabolic products of pharmacological agents such as dimethadione (69) and desmethylimipramine (168), the pharmacological characteristics and activity of metabolite I were investigated following its intravenous administration in the rat.

A common procedure for the administration of ketamine in preparation for surgery involves the administration of repeated doses (39) with up to nine consecutive doses being administered in some cases (130). Initial reports suggested no alteration of anesthetic duration in monkeys (124) and man (39) following a second injection of ketamine. The anesthetic duration following repeated doses of ketamine in the rat was investigated and correlated with brain and plasma levels of ketamine and its metabolites.

MATERIALS AND METHODS

Male Sprague-Dawley rats (100-130 g) were immobilized in cylindrical wire containers (4 1/2 cm diameter) fitted with rubber stoppers which were cut to allow the tail to extend outside the cylinder. Ketamine or metabolite I was injected (10 to 15 seconds) into the tail vein after enlarging the veins by application of hot water. The appropriate concentration of either ketamine or metabolite I was prepared in saline and was injected in a total volume of 0.2 ml by means of a disposable syringe (1 ml) fitted with a 26 gauge 1/2 inch needle. Animals were observed for two hours following injection (zero time was taken from the end of the injection period) to assess the duration of anesthesia and other pharmacological effects.

Blood samples during the first ten minutes were obtained by cardiac puncture through the intact skin. This permitted the withdrawal of 1 to 3 ml of blood into a 3 ml disposable syringe containing 0.1 ml heparin and fitted with a 23 gauge 1 1/2 inch needle. Immediately following the cardiac puncture the animals were decapitated, exsanguinated, and the brains placed on ice, cleaned of external blood vessels, and weighed. Blood samples determined at times later than ten minutes following the <u>in vivo</u> injections and determined after repeated doses of ketamine were obtained from the exsanguinated blood following decapitation.

In some experiments, the brain was dissected on ice into four main regions. This was accomplished by a transverse

slice caudal from the brain stem and cerebellum. The cerebellum was separated from the brain stem by incision of the cerebellar peduncles. The cerebral hemispheres were separated by incision through the corpus callosum and the cortical region was peeled at the corona radiata from the midbrain section.

A 10% brain tissue homogenate was prepared in 0.9% saline pH 5.5. The homogenate was centrifuged at 100,000 g for 30 minutes (40 rotor, 40,000 RPM) in the Spinco Model L centrifuge while the blood was centrifuged for 15 minutes in the International centrifuge to obtain tissue supernatant and plasma. Aliquots of the tissue supernatant and plasma were extracted and analyzed for ketamine and its metabolites (see Chapter 2).

RESULTS

Times to recovery of righting reflex and the cessation of ataxia and agitation as a function of ketamine dosage following intravenous injection in the rat are indicated in Table 3-1. Signs of increased purposeless movements and nystagmus were evident at doses above 10 mg/kg during the period of loss of righting reflex. At all doses, eyes remained open while corneal reflexes remained intact at doses up to 40 mg/kg but were lost at the higher dose used (60 mg/kg). After regaining the righting reflex, animals exhibited marked salivation and were agitated but ataxic. They frequently moved backwards in circular movements and appeared to suffer

TABLE 3-1. DURATION OF KETAMINE EFFECTS AS A FUNCTION OF DOSE

Ketamine Hydrochloride mg/kg	Number of Deaths	Time to Recovery of Righting Reflex (Minutes)	Time to End of Ataxia (Minutes)	Time to End of Agitation (Minutes)
5	0/4	-	5.13±0.31	12.5±1.26
10	0/4	3.94±0.41	12.00±0.71	23.8±1.38
20	0/4	7.50±0.35	30.00±1.68	40.5±1.32
40	1/5	13.00±1.22	39.50±1.55	54.5±3.33
60	2/5	32.67±5.91	63.3±14.83	69.3±9.26
80	4/4	-	-	-

Ketamine hydrochloride in the indicated doses was injected into the tail vein of rats. At doses greater than 10 mg/kg, loss of the righting reflex occurred during injection (15 seconds). Times to recovery of the righting reflex and to cessation of ataxia and agitation were estimated from the end of injection time. Data represent mean values ± standard errors.

from impairment of balance. Agitation, as judged by increased motility, continued after the disappearance of ataxia. By this mode of administration a dose of 60 mg/kg was found to be close to the LD_{50}. Since ketamine hydrochloride at 20 mg/kg provided a loss of righting reflex (6-8 minutes) close to the duration of anesthesia produced with therapeutic doses of ketamine in man, this dosage was chosen for subsequent studies on brain distribution.

Following intravenous administration, ketamine rapidly entered the brain and maximal levels occurred with 1 minute (Figure 3-1). Brain levels then declined, paralleling the fall in plasma concentration, and recovery of righting reflex occurred when brain ketamine levels were approximately 30 ug/g tissue and plasma levels were approximately 4.5 ug/ml. Despite the rapid fall of brain ketamine levels, CNS tissue retained the compound preferentially as evidenced by brain: plasma level ratios of approximately 7:1 at 30 seconds, 6.5:1 at 5 minutes, and 5.5:1 at 10 minutes following <u>in vivo</u> administration of ketamine.

The distribution of ketamine with time was examined in four regions of the rat brain; cerebellum, brain stem, midbrain and cerebral cortex (Figure 3-2). At time periods of 30 seconds and 1 minute after injection, the concentration of ketamine was significantly higher in the cerebral cortex than in the other brain regions ($p < 0.05$, paired Student's "t" test). No marked differences in regional brain distri-

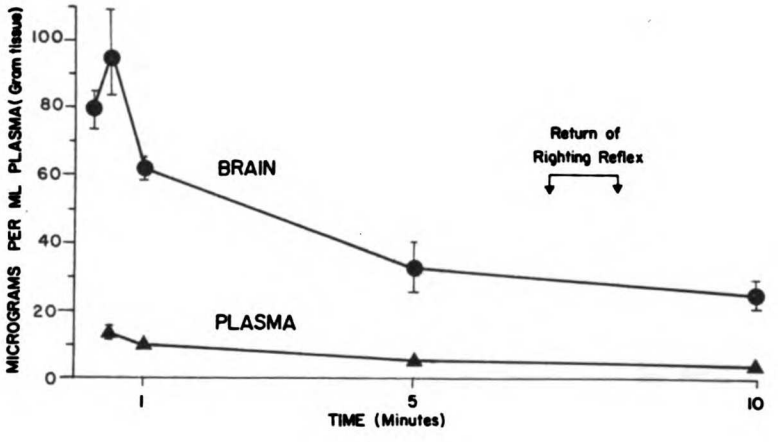


Figure 3-1. Plasma and brain levels of ketamine following intravenous administration. Ketamine hydrochloride (20 mg/kg) was injected into the tail vein of each rat. Samples of plasma and whole brain were assayed for ketamine at indicated times (see Chapter 2). Each point represents the mean value from four animals $\pm$ standard error.

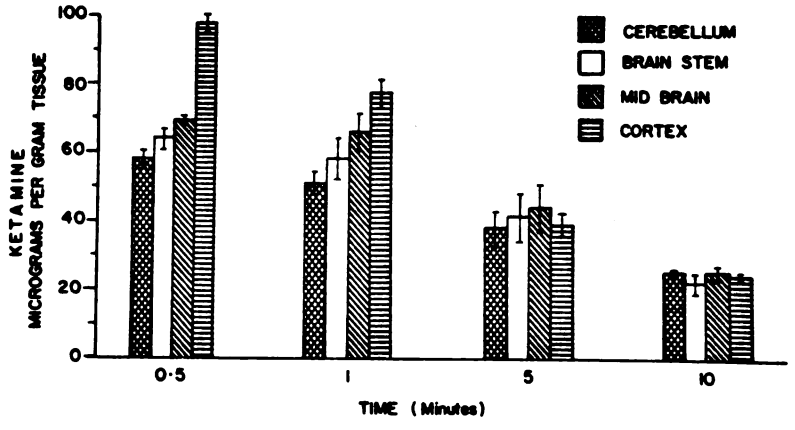


Figure 3-2. Distribution of ketamine in four regions of the rat brain following intravenous injection of 20 mg/kg ketamine hydrochloride. Brains were removed at appropriate times, dissected into the indicated regions, and assayed for ketamine (see Chapter 2). At each time interval, data represent mean values from three animals ± standard error.

bution were apparent at later times and the decline in regional levels of ketamine was similar to that observed for whole brain (Figure 3-1).

The N-demethylated metabolite (I) was detectable in plasma at 30 seconds and reached a plateau level of approximately 2.25 ug/ml between 5 and 10 minutes (Figure 3-3). Metabolite I was first detectable in brain tissue at 1 minute and continued to increase in concentration in the CNS during the 10 minute time period of the experiment. As with the parent compound, the rat brain appeared to retain metabolite I, with a brain:plasma concentration ratio reaching 2.5:1 at 10 minutes. Metabolite I was found in all regions of the brain at 5 and 10 minutes, but with no significant difference in concentrations among the regions studied (Figure 3-4). Metabolite II, the cyclohexanone oxidation product, was not detectable in plasma or brain tissue during the short time intervals studied in these initial experiments.

During longer time intervals after the in vivo administration of ketamine (Figure 3-5), metabolite I brain levels did not continue to increase. Metabolite I was found in brain tissue at 30 and 60 minutes in concentrations approximating 2-3 ug/g brain tissue. At these same time intervals, brain levels of ketamine were falling from the initially high levels to levels close to the concentration of its N-demethylated product (metabolite I) at 30 and 60 minutes.

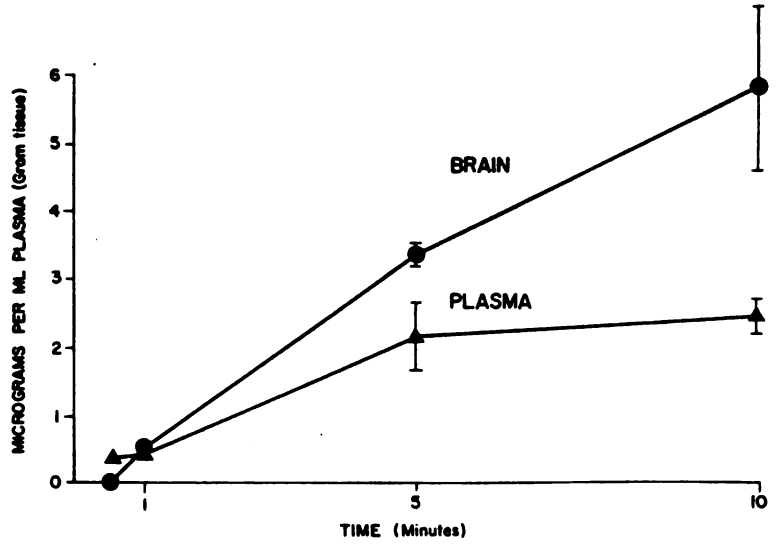


Figure 3-3. Plasma and brain levels of metabolite I following intravenous administration of ketamine hydrochloride (20 mg/kg) in the rat. Samples of plasma and whole brain were assayed for metabolite I at indicated times (see Chapter 2). Each point represents the mean value from four animals ± standard error.

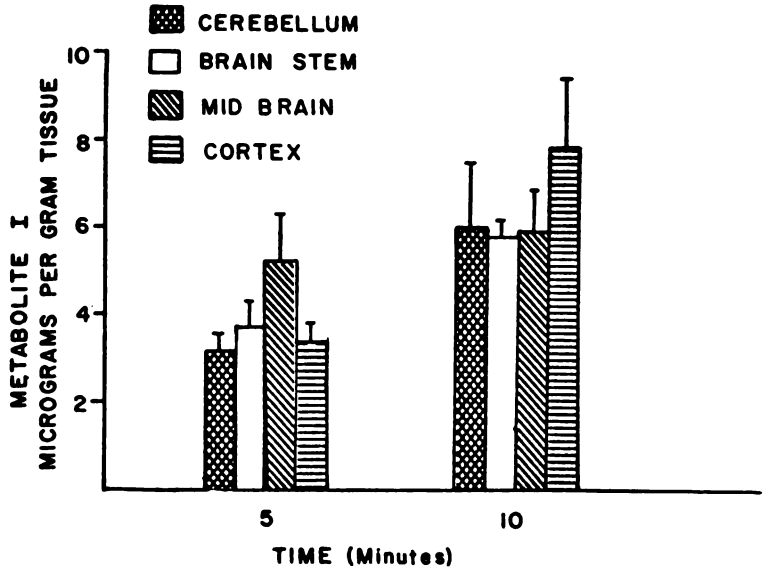


Figure 3-4. Distribution of metabolite I in four regions of the rat brain following intravenous injection of 20 mg/kg ketamine hydrochloride. Brains were removed at appropriate times, dissected into the indicated regions, and assayed for metabolite I (see Chapter 2). At both time intervals measured, data represent mean values from three animals $\pm$ standard error.

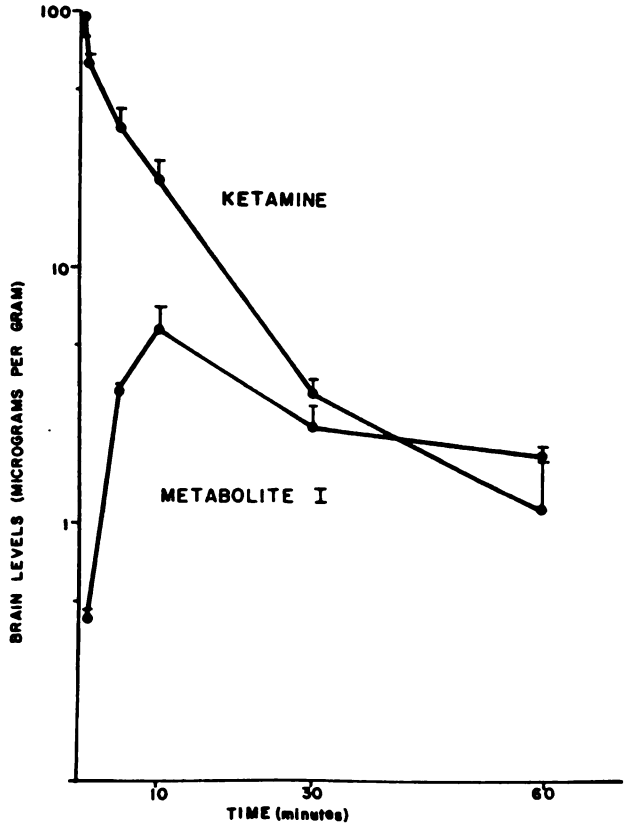


Figure 3-5. Brain levels of ketamine and metabolite I following intravenous injection of 20 mg/kg ketamine hydrochloride in the rat. Data for initial 10 minutes (Figures 3-1 and 3-3) is included for completeness. Each point represents the mean value from four animals ± standard error.

Plasma levels of ketamine declined following its intravenous administration (Figure 3-6) again paralleling the decline in brain levels observed at 30 and 60 minutes. At these longer time intervals, metabolite I plasma levels increased to concentrations greater than plasma levels of ketamine.

Metabolite II was detected in very low concentrations in plasma at both 30 and 60 minutes following ketamine administration (Figure 3-6). No metabolite II was detectable in brain tissue at these time periods. However, due to the presence of high concentrations of ketamine and metabolite I in brain tissue, analyses had to be restricted to quite small aliquots, and it is possible that small quantities of metabolite II would have been undetected.

Ketamine was also preferentially retained in brain tissue at these longer time intervals of 30 and 60 minutes when brain:plasma level ratios were approximately 3.5:1 and 2.8:1, respectively. It should be emphasized that while brain:plasma ratios did decrease with time following intravenous injection, they did not fall below 2.8:1 in 60 minutes.

Since metabolite I appeared in brain and plasma tissue at all time intervals measured following ketamine administration, the pharmacological properties of metabolite I were examined following its intravenous injection in the rat (Table 3-2). Anesthetic properties qualitatively similar to ketamine were seen with metabolite I (Table 3-1). Animals lost the ability to right themselves at doses greater than

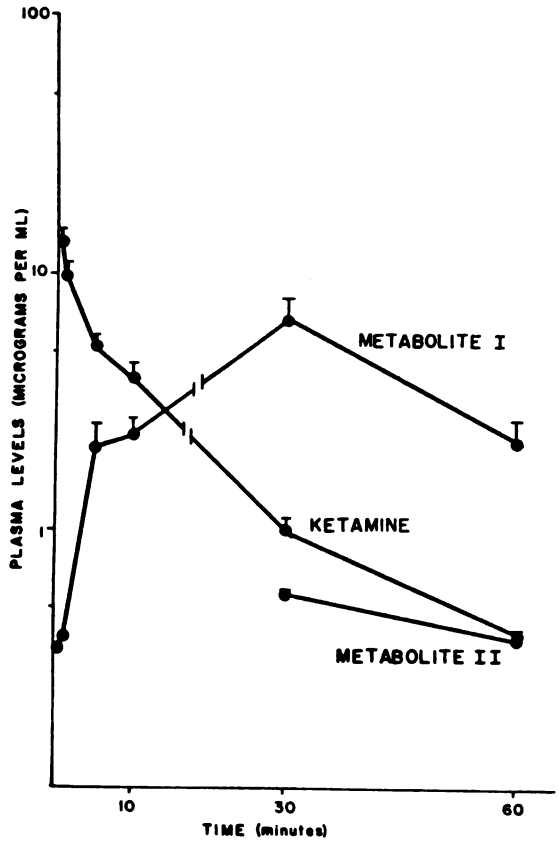


Figure 3-6. Plasma levels of ketamine, metabolite I, and metabolite II following intravenous injection of 20 mg/kg ketamine hydrochloride in the rat. Plasma was obtained by cardiac puncture during initial 10 minutes and by exsanguination at 30 and 60 minutes which is indicated by the discontinuity of the lines. Each point represents the mean value from four animals $\pm$ standard error.

TABLE 3-2. DURATION OF METABOLITE I EFFECTS AS A FUNCTION OF DOSE

Metabolite I mg/kg	Number of Deaths	Number Losing Righting Reflex	Time to Recovery of Righting Reflex (Minutes)	Time to End of Ataxia & Agitation (Minutes)
5	0/3	0/3	-	2.0
10	0/6	3/6	0.45±0.06	6.08±.33
20	0/5	3/5	2.50±0.76	8.6±1.21
40	0/5	5/5	5.5±0.82	17.4±1.5
50	3/6	3/6	7.18±1.18	25.0±5.0
60	4/4	-	-	-
100	2/2	-	-	-

Metabolite I in the indicated doses was injected into the tail vein of rats. At doses greater than 10 mg/kg loss of righting reflex occurred during injection (15 seconds). Times to recovery of the righting reflex and to cessation of ataxia and agitation were estimated from the end of injection time. Data represent mean values ± standard errors.

10 mg/kg and exhibited ataxia and agitation. However, metabolite I produced a shorter duration of loss of righting reflex than observed with ketamine at equivalent weight doses. Nystagmus and exopthalmus were predominent, although purposeless movements appeared to a lesser extent than following ketamine administration. During the period of ataxia, subjective evaluation of agitation indicated less central excitation than observed after ketamine, and the agitation disappeared with the disappearance of ataxia. By intravenous injection, a dose of 50 mg/kg was the approximate LD_{50} for metabolite I in the rat, a dose slightly lower than the LD_{50} for ketamine. It is of interest that two animals receiving metabolite I died within a week after recovering from the injection, while no such deaths occurred following injection of ketamine.

Repeated injections of ketamine (20 mg/kg) following the gain of righting reflex in the rat did prolong the duration of loss of righting reflex resulting from each subsequent injection (Table 3-3). The pharmacological effects observed were similar to those seen following a single injection, except that nystagmus, purposeless movements, and sialogogic actions were more pronounced following subsequent doses. Not only was the loss of righting reflex more prolonged, but the phase of ataxia and agitation did not terminate until approximately 90 minutes after the last injection, lasting almost twice as long as that following a single injection. On gain of righting reflex after the

TABLE 3-3. CUMULATIVE EFFECT OF REPEATED DOSES OF KETAMINE

Time of Injection (Minutes)	Duration of Loss of Righting Reflex (Minutes)	Brain Concentration (ug/gm)		Plasma Concentration (ug/ml)	
		Ketamine	Metabolite I	Ketamine	Metabolite I
0	6.65±0.58	95.7±12.6	0	13.35±.17	0.35±.003
9.0±.35	9.55±1.26**	-	-	-	-
22.1±1.63	13.69±.95**	120.5±9.8	8.25±1.45*	22.23±.94*	6.23±1.00*

Ketamine hydrochloride (20 mg/kg) was injected into the tail vein of each rat at the indicated times and the animals were observed for duration of loss of righting reflex. Other animals were decapitated 30 seconds after first and third injection. Plasma samples were obtained by cardiac puncture following the first injection and from exsanguinated blood following the third injection. Brain and plasma were analyzed for ketamine and its metabolites (see Chapter 2). Data at each time interval represent mean values from four animals ± standard errors.

* $p < .05$ compared to initial injection as determined by Student's "t" test for unpaired data.

** $p < .05$ compared to initial injection as determined by Student's "t" test for paired data.

third injection, these animals appeared flaccid and inactive for an approximate duration of 8 minutes before entering the phase of agitation. This sequence was markedly different from the immediate occurrence of ataxia and agitation observed following a single injection of ketamine.

Brain and plasma levels of ketamine and metabolite I measured 30 seconds after the first and third injection of ketamine are compared in Table 3-3. Although brain levels of ketamine were increased following the third injection, these levels were not statistically different from levels obtained following a single injection. These data were derived from 4 animals and the concentration of ketamine in the brain of 1 of these animals was markedly lower than the other 3 (94 ug/g tissue). The lack of statistical significance may be due to this potentially erroneous value which could not be excluded for any obvious reason. Plasma levels of ketamine were significantly different at the 30 second time interval measured after the repeated injections, and the brain:plasma level ratio was approximately 5.5:1.

Metabolite I was not detectable in brain tissue at 30 seconds following a single injection but did reach levels of 8 ug/g brain tissue at this time following the third intravenous injection of ketamine. Plasma levels of metabolite I were approximately 5 times higher after the repeated doses as compared to the levels after a single injection.

DISCUSSION

These studies on the pharmacological effects of ketamine indicated that ketamine produced diverse central effects in the rat which may be comparable to the various behavioral manifestations reported in man. Specifically, eyes remained open, nystagmus occurred, and purposeless movements were observed during the period of loss of righting reflex. In the lowest dose employed and following gain of righting reflex for the other doses, the rat exhibited marked ataxia, agitation, and what appeared to be an impairment of balance. These behavioral effects may be analogous to the disorientation phenomena which are pronounced during emergence from ketamine-induced anesthesia in man. Convulsions were not observed during these studies even at the highest doses used, consistent with the finding that convulsions have not appeared, in general, to be any major problem following ketamine administration (55, 124, 141), although there has been a recent example of the occurrence of convulsions in man (174).

Given by the intravenous route, ketamine produced loss of righting reflex during the 10 seconds of the injection administration. This immediate loss of righting reflex correlates quite well with the availability of surgical anesthesia only 30 seconds after intravenous injection of ketamine in man (39). By intravenous administration, loss of righting reflex occurred at doses lower than necessary for loss of righting reflex following the intraperitoneal

route of administration in rats (124). In man, a dose of 2.2 mg/kg of ketamine produced a duration of anesthesia of approximately 6 to 10 minutes by intravenous administration (42, 186). In the rat, 10 times this dose of ketamine was necessary to produce a corresponding duration of loss of righting reflex. The lower sensitivity of the rat may be attributed to differences in the percent of cardiac output to the brain which would be especially important with drugs administered by the intravenous route. In man, approximately 15% of the cardiac output has been reported to be to the brain (22, 159, 190) while in the rat this value is approximately 2% (157, 159), a value not far from the 10 fold difference necessary to explain the discrepancy of different sensitivities. This explanation is supported by the similarity of ketamine doses in man, monkey, dog, and cat which have relatively similar distributions of cardiac output (159, 190). However, other explanations for this lowered sensitivity to ketamine in the rat include both the possibility of differences in rates of metabolism and in functioning or permeability of the CNS compared to man.

It is of interest that in initial toxicity studies in rats (124), repeated daily intravenous injections of 2.5, 5.0, and 10 mg/kg ketamine were given. It is obvious from the present studies that these dose levels produced only minimum effects in the rat. While in the toxicity studies a different strain of rat (Holtzman) was used, there are grounds for suspicion regarding their conclusion of lack of toxicity in this species, since such low doses were employed.

Following intravenous administration, 40 percent of the animals died at 60 mg/kg ketamine, a dose very close to the previously reported LD_{50} of 58.9 mg/kg (124). Death from high doses of ketamine has been attributed to respiratory arrest (123, 124).

In the present studies, the earliest plasma level estimated (30 seconds) represented a total plasma ketamine content of 40 ug per 100 g of rat if a plasma volume of 3 ml (4) is assumed, or approximately 2 percent of the injected dose. This observation indicates extremely rapid distribution of ketamine to tissue compartments which may well be the case in man. For example, from a similar calculation based on reported plasma levels in man 2 minutes after intravenous administration (115), it appeared that at this time, the total plasma content represented only 5 to 6 percent of the injected dose. In this respect ketamine appeared to be similar in terms of tissue distribution to the short acting barbiturates such as thiopental (75).

In the rat, rapid distribution to the brain occurred with brain levels of ketamine at 30 seconds representing about 150 ug/100 g of rat or approximately 7.5 percent of the injected dose. The rapid occurrence of peak brain levels in the rat corresponded extremely well with the onset of surgical anesthesia in man (39) and alterations in behavior and electroencephalograms observed in the cat (96). This, along with the observed rapid onset of anesthetic

effect in the rat is consistent with the absence of a blood-brain barrier to this agent. The fact that ketamine has been shown to increase cerebral blood flow in dogs (45) and in man (158) almost immediately after administration may be contributory to the rapid achievement of high brain levels. Preliminary reports have appeared noting that tissue concentrations of ketamine may exceed plasma levels in laboratory animals (27) but no precise data on this aspect of disposition of the drug have been published.

From studies of the regional CNS distribution of ketamine (Figure 3-2) higher levels were found in the cerebral cortex than in other regions during the early time periods after intravenous administration. Blood flow is a primary factor in the distribution of agents which rapidly enter the brain following a bolus injection. Wide variations in blood flow to different regions of the brain (163) have been reported in the cat, with cortical areas receiving one of the highest flow rates. High cortical blood flow may account for the initial differences in regional accumulation of ketamine. The initial cerebral disposition of gaseous anesthetics has also been shown to be a function of regional blood flow (34). At time periods greater than 60 seconds after intravenous injection of ketamine, there were no marked differences in regional brain distribution. This does not preclude the possibility of preferential accumulation of ketamine in more discrete regions of the brain than those examined in this study.

However, these data did indicate that any selective cortical effect of ketamine was not a result of the inability of other brain regions to accumulate the drug. Although it is recognized that studies of the distribution of ketamine in specific regions of the brain would not necessarily imply a cerebral site of action for ketamine, these studies were important in establishing the availability of ketamine in all areas of the brain.

The N-demethylated product of ketamine (metabolite I) has been reported to be a major metabolite in man (28, 115) and in our studies, was found to appear quite rapidly in the plasma and brain of the rat (Figure 3-3). Metabolite I also accumulated in CNS tissue of the rat to give brain levels greater than those of plasma. In fact, rapid distribution of metabolite I into body tissues may account for the plateauing of the plasma levels of this metabolic product during the early time periods following ketamine injection. A situation quite similar is suggested in man by the occurrence of relatively low plasma levels of metabolite I (115) although this may not necessarily be the case. The relatively high and prolonged brain levels of metabolite I raised the possibility that certain of the diverse and prolonged central effects observed following ketamine administration could be due to this compound. This was probably not the case with respect to ataxia and agitation. These effects were observed to occur rapidly in animals that did

not lose their righting reflexes, 1 or 2 minutes after injection and prior to the establishment of significant plasma or brain levels of metabolite I. However, it cannot be ruled out that other central effects, perhaps more delayed in onset, could be due to metabolic products of ketamine. Examination of brain levels of metabolite I at 30 and 60 minutes (Figure 3-5) following ketamine administration revealed the persistence of metabolite I in the brain, but indicated no further increase in the accumulation of this metabolic product. This may be a reflection of further rapid metabolism of metabolite I or possibly its preferential accumulation in other tissues.

During the earliest time periods investigated, metabolite II could not be detected in brain or plasma tissue of the rat. This is consistent with the previous suggestion that this N-demethylated and oxidized derivative of ketamine was not produced in the rat (27). However, at longer time intervals following ketamine administration, metabolite II was detected in low concentration in rat plasma. This metabolite appeared in plasma at times (30 minutes following ketamine administration) commensurate with its appearance in plasma of man (115). It appears that the rat is similar to man not only in the behavioral manifestations of the ketamine-induced anesthetic state, but also in its modes of ketamine biotransformation, on the basis of similar metabolite formation.

Ketamine exhibited a rapid, preferential accumulation in cerebral tissue resulting in an initial (30 seconds) 7:1 ratio of brain:plasma levels. In this respect, ketamine is similar to other drugs preferentially accumulating in highly perfused tissue such as thiopental (19, 75, 144) and imipramine (52) but different from certain analgetic agents (121) found not to accumulate appreciably in brain tissue. Although ketamine was retained by brain tissue at concentrations higher than plasma levels during the entire 60 minute period studied, the brain:plasma ratios did decline with time. This was probably best explained by the initial (30 seconds) rapid equilibration of ketamine with brain tissue due to the fairly rapid intravenous injection resulting in a transient and extremely high carotid arterial concentration. Following this rapid equilibration of brain at a high ketamine concentration, the drug level in plasma fell as the drug was both metabolized and distributed to more poorly perfused tissues. During long term equilibration, the initial high brain levels decreased as the brain, plasma and other tissues approached equilibrium with each other. The approximation of brain:plasma level ratios at 60 minutes of 2.8:1 may be considered close to an equilibrium (steady state) value under these dynamic in vivo conditions, based on subsequent in vitro studies (reported in Chapter 4).

Investigation of the pharmacological properties of metabolite I indicated that it was anesthetic with properties similar to ketamine but on a weight basis was less potent

and more toxic than the parent compound. Although brain levels following the intravenous injection of metabolite I were not examined, it must tentatively be concluded on the basis of its pharmacological actions and its cerebral accumulation following ketamine administration, that it exerted only a minor contribution, if any, to the effects observed from ketamine. There have been precedents for marked contributions of pharmacological actions by metabolic products to the overall actions of certain parent drugs. For example, the demethylated metabolite (dimethadione) of trimethadione has been estimated to contribute 85 percent of the total anticonvulsant activity observed following continuous treatment with trimethadione. However, dimethadione exhibited properties different from metabolite I, in that it was accumulated in high concentration in the CNS and was not further metabolized. Thus, there is the possibility that under certain circumstances unusual accumulation of metabolite I could promote the observed effects following ketamine.

In general, it is thought that repeated administration of a drug at short time intervals will alter the drug response in terms of intensity, duration of action, or both (154). Ketamine appeared to be unique with reference to the claimed lack of cumulative effect with repeated dosing immediately following return to consciousness in man (39) and return of righting ability in monkeys (124). While plasma levels in the rat were 4.5 ug/ml, brain concentration was 30 ug/g tissue at the time of gain of righting reflex in these

animals. Thus, at least in this species some cumulative effect on repeated administration of ketamine could be anticipated. In fact, a 50 percent increase over the initial duration of loss of righting reflex occurred with the second and third administration of ketamine, so that by the third dose there was a complete doubling of the duration of loss of righting reflex. It is highly likely that this phenomenon also occurs in other species since the inability of previous workers (39, 124) to detect this may be a reflection of the extremely favorable bias which surrounded the initial impact of this anesthetic agent.

Although brain levels of ketamine appeared to be higher following the third injection (Table 3-3), this difference was not statistically significant. This may be due to biological variability rather than a lack of difference. It is of interest to note that on the basis of brain levels of ketamine following a single dose (Figure 3-5), at the time of the third injection (22 minutes), the theoretical brain levels of ketamine resulting from the first and second dose approximated 7 and 16 ug/g tissue. The sum of these concentrations plus 96 ug/g tissue accumulated at 30 seconds following the last injection provided a theoretical cerebral concentration almost exactly equal to the observed 121 ug/g brain tissue. Theoretical plasma levels were approximately 18 ug/ml, again remarkably close to the 22 ug/ml actually found.

It is possible that under conditions of repeated administration of ketamine, the relatively high levels of metabolite I detected in brain may contribute to certain effects observed. This may have special relevance in the prolongation of ataxia and agitation occurring in this situation since exceptionally high levels of the N-demethylated metabolite were found.

CHAPTER 4. *IN VITRO* STUDIES ON THE DISPOSITION OF KETAMINE IN CEREBRAL TISSUE

INTRODUCTION

The *in vivo* studies detailed in Chapter 3 demonstrated that ketamine is rapidly accumulated and retained in brain tissue at concentrations above those in the plasma at all time intervals studied. This phenomenon might be explained by a number of possible mechanisms, three of which were examined in the present study: (a) active transport, (b) preferential affinity for specific cerebral components, and (c) partitioning as a result of lipid solubility of the drug.

The active transport of compounds into brain tissue can be conveniently examined using the *in vitro* brain slice preparation which has been employed extensively for such studies (1, 79, 87, 94, 109, 113) and which has certain properties similar to cerebral tissue *in vivo* (68, 80, 97, 125, 127, 134, 150). The possibility that ketamine uptake into brain tissue could be mediated to some extent by a transport system was based largely on the observed inhibition by ketamine of norepinephrine uptake into both cerebral (35) and peripheral tissues (129, 135). Since norepinephrine uptake is primarily a result of active transport mechanisms (46) and since ketamine may block this uptake competitively, it appeared reasonable to consider that the anesthetic agent could be a "substrate" for the same carrier mechanism. This phase of the investigation involved the establishment of brain tissue slice viability *in vitro* by examination of lactic acid production rate and by

examination of the ratio of intracellular:extracellular fluid in this preparation. Changes in these parameters in the presence of ketamine were followed to determine any metabolic actions of the anesthetic. Subsequently, the rate of ketamine accumulation (and release) was examined in control slices and in the presence of certain metabolic inhibitors which have been established to interfere with ATP dependent active transport mechanisms.

Accumulation and retention of ketamine in the brain might also be the result of a preferential affinity of the drug for specific cellular components of neural tissue. This possibility was examined with _in vitro_ techniques involving the subcellular fractionation of cerebral homogenates by centrifugation and subsequent equilibrium dialysis of separated components against ketamine. The binding of ketamine to such subcellular fractions was quantitated and related to the observed association of the drug with cerebral tissue _in vivo_ and brain slices _in vitro_.

The tissue distribution of conventional anesthetic agents has been well established to be a function of tissue mass, blood flow, and the partitioning characteristics of the compound in terms of its relative affinities for water and lipids (34, 75, 121, 144). By analogy with the intravenous barbiturates which have been considered to accumulate rapidly in the brain by virtue of extreme lipid solubility (121, 144), it appeared possible that ketamine association with cerebral tissue may be dependent on its partition

coefficient. This possibility was examined by estimation of the partitioning of ketamine (and metabolite I) in 3 organic solvents, heptane, benzene, and chloroform. Partitioning phenomena and cell membrane permeability, in general, are influenced by the state of ionization of a particular molecule, especially in the case of weak acids or bases. Knowledge of the degree to which ketamine (and metabolite I) exists in either the protonated or the basic form at physiologic pH was necessary for a precise understanding of its distribution properties. For this reason, pK_a values were determined for ketamine and its major metabolite.

In the studies detailed in Chapter 3, a relatively high concentration of metabolite I occurred in brain tissue after ketamine administration. This raised the possibility of a contributory role of the brain as a site of biotransformation of ketamine. This possibility was examined by estimation of ketamine metabolism in vitro utilizing brain tissue and liver homogenates. Additional data on this question were also obtained using the brain slice technique.

MATERIALS AND METHODS

Preparation and Incubation of Brain Slices

Male Sprague-Dawley rats (100-130 g) were decapitated and exsanguinated and the brains rapidly removed to a chilled filter paper moistened with incubation media. The hemispheres were separated and cleaned of external blood vessels with a moistened cotton swab. Either one or two cortical tissue

slices (30-50 mg) per hemisphere were cut as described by McIlwain and Rodnight (127) using a stainless steel blade and a 0.3 mm glass guide. Slices were floated free of the blade and guided into incubation media contained in a petri dish placed on ice. The tissue was trimmed, lifted on wire riders, blotted on a clean glass surface and weighed on a tared torsion balance. The slices were directly transferred to 30 ml beakers containing 5 ml of oxygenated incubation media at 37°C.

Incubation media consisted of: NaCl 127 mM, KCl 5.1 mM, $MgCl_2$ 1.3 mM, $CaCl_2$ 0.75 mM, glucose 10 mM and either sodium phosphate buffer 25 mM pH 7.4, or KH_2PO_4 1.3 mM with sodium bicarbonate buffer 26 mM. Bicarbonate buffered media was oxygenated with 95% O_2, 5% CO_2 and sodium phosphate buffered media was oxygenated with 100% oxygen. In both cases, pH was maintained at 7.4.

In all studies, slices were preincubated for 15 to 30 minutes which was sufficient time for the regeneration of ionic gradients (127). At the end of the experiment, slices were lifted on wire riders and rinsed through incubation media or 0.32 M sucrose contained in a petri dish on ice. Slices were then blotted on the edge of a clean glass petri dish and transferred to ground glass homogenizers containing 2 ml of 0.9% saline. Homogenization was accomplished with 10 vertical strokes of the pestle. The homogenate was transferred to polycarbonate centrifuge tubes (13 ml capacity) and centrifuged at 100,000 g for 30 minutes in the Spinco

Model L centrifuge. Appropriate aliquots of the incubation media and/or the brain tissue supernatant were used for assay of ketamine and its metabolites (see Chapter 2) and lactate. A similar procedure was followed for estimation of inulin space except that the tissue was homogenized in 10% trichloroacetic acid (2 ml).

Lactic Acid Analysis

Lactic acid which had accumulated in the incubation media following a 30 minute incubation period was assayed enzymatically by the method of Hohorst (83). The conversion of lactate to pyruvate by lactate dehydrogenase resulted in the simultaneous reduction of NAD. Pyruvate was trapped as the hydrazone and NADH accumulation was followed with the Unicam SP 500 spectrophotometer at 340 nm. Samples of 0.2 or 0.3 ml of the media were sufficient to give an optical density change of approximately 0.25 units.

Inulin Space Determination

Inulin carboxyl-C-14 was added to the incubation media (0.02 ug/ml) to allow estimation of the extracellular space of the tissue. Aliquots of the trichloroacetic acid extract and the incubation media were placed in 8 ml Aquasol and counted with a Packard Tricarb Model 3375 liquid scintillation counter. Efficiency of counting was determined by use of the automatic external standardization ratio (AES ratio) which had previously been calibrated for varying degrees of quenching produced by media and 10% trichloroacetic acid.

Inulin space was calculated from the inulin content of the slice in relation to the concentration in the incubation media, assuming the density of tissues and fluid to be 1 (184). Inulin space has been defined as the volume of water that is calculated from the amount of inulin taken up by the slice (68) and is expressed per gram initial wet weight of the slice.

Equilibrium Dialysis Procedure

Male Sprague-Dawley rats (100-130 g) were decapitated and exsanguinated and the brains rapidly removed, placed on ice and cleaned of external blood vessels. After weighing, each brain was minced and homogenized in 4 volumes of 0.9% saline containing 10 mM sodium phosphate buffer at pH 7.35 (20% homogenate). In some experiments, the homogenate preparation was transferred to polycarbonate centrifuge tubes (13 ml capacity) and centrifuged at 100,000 g for 30 minutes in the Spinco Model L centrifuge (40,000 RPM using 40 rotor). The supernatant was separated from the pellet and the pellet was resuspended in the original volume of buffered saline.

Tissue homogenate, tissue supernatant, resuspended particulate material and buffered saline (5 to 7 ml) were transferred into dialysis tubing (5/8 inch wide, 12 inches lengthwise and previously boiled for 15 minutes and subsequently cooled and washed with deionized water) placed U shaped in a 500 ml beaker containing 50 volumes (250 to 350 ml) of ketamine hydrochloride (10 ug/ml) in buffered

saline at room temperature. Solutions were agitated with magnetic stirrers and the solution outside and inside the dialysis tubing was sampled at 1, 3, and 6 hours. Samples were subsequently extracted and assayed for ketamine and its metabolites (see Chapter 2).

Partition Coefficient Determination

Organic solvent to water distribution ratios were determined by the procedure of Mayer et al (121). Heptane, benzene, and chloroform were washed 3 times with equal volumes of 0.1 M phosphate buffer, pH 7.4. Distribution ratios were measured by shaking solutions (2 ml) of drug (100 ug/ml) dissolved in 0.1 M sodium phosphate buffer, pH 7.4, with the organic solvent (2 ml) for 30 minutes in glass stoppered test tubes (15 ml capacity) on a mechanical shaker. The test tubes were centrifuged at 1,750 RPM on the International centrifuge for 20 minutes and the phases were separated with a pasteur pipette. Aliquots of the aqueous phase were extracted and assayed for ketamine or metabolite I (see Chapter 2). The partition coefficient was calculated as the ratio of the concentration assumed to be dissolved in the organic solvent divided by the concentration measured in the aqueous phase.

pKa Determination

pK_a was estimated from construction of a potentiometric titration curve (192). A dilute solution (7 ml) of ketamine hydrochloride or metabolite I (1×10^{-3}M) was neutralized with sodium hydroxide (2×10^{-3}M) added in increments of

0.2 ml delivered from a 5 ml glass syringe. Changes in emf (pH) were automatically recorded following each addition of base. Estimation of the inflection point of the resultant titration curve was used to determine the pK_a of each compound. Equipment for this procedure was made by the Radiometer Company in Copenhagen, Denmark and included a Radiometer pH meter 28, Radiometer titrigraph type SBR 2C, Radiometer syringe burette type SBU 1a fitted with a 5 ml capacity syringe type B104 and a Radiometer electrode GK 2301C.

Estimation of In Vitro Metabolism

Male Sprague-Dawley rats (100-130 g) were decapitated and exsanguinated and the brain and portions of the liver were immediately removed, placed on ice and cleaned of external blood. A 10% tissue homogenate was prepared in 0.9% saline and used immediately. Tissue homogenates (0.5 ml) were placed in Erlenmeyer flasks containing 0.5 ml sodium phosphate buffer with nicotinamide (30.5 mg/ml), $MgSO_4$ (6.2 mg/ml) and glucose -6-phosphate (10 mg/ml), 0.1 ml freshly prepared NADP (2 mg/ml) and 1.15 ml of 0.1 M sodium phosphate buffer, pH 7.35. To start the reaction, 0.25 ml of ketamine hydrochloride (1 mg/ml) was added to each flask, mixed, and a 0.1 ml sample was transferred to 0.9 ml of 0.1 N HCl for assay of initial ketamine concentration. The remaining reaction mixtures (2.4 ml) were incubated in a Dubnoff metabolic shaker for 30 minutes at which time 0.1 ml was transferred to 0.9 ml of 0.1 N HCl for assay of ketamine and its metabolites (see Chapter 2).

Chemicals

All chemicals used were of analytical grade. Chemicals were obtained as follows: glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate (NADP), hydrazine sulfate, L(+) lactic acid, 2,4 dinitrophenol, Sigma Chemical Co. (St. Louis, Missouri); sodium chloride, dextrose anhydrous, sodium hydroxide, benzene, Mallinckrodt Chemical Works (St. Louis, Missouri); magnesium sulfate, calcium chloride, magnesium chloride, potassium phosphate, potassium chloride, Allied Chemical (Morristown, New Jersey); sodium bicarbonate, chloroform, sodium phosphate, J.T. Baker Chemical Co. (Phillipsburg, New Jersey); normal heptane, Phillips Petroleum Co. (Bartlesville, Oklahoma); nicotinamide, Eastman Organic Chemicals (Rochester, New York); lactate dehydrogenase, Boehringer Mannheim Corporation (San Francisco, California); Aquasol,^{TM} New England Nuclear (Boston, Massachusetts); inulin (carboxyl-C14) s.a. 2-3 uc/mg, International Chemical and Nuclear Corporation (Irvine, California); iodoacetic acid, sodium salt, Calbiochem (Los Angeles, California).

RESULTS

The effect of ketamine (1.83×10^{-4}M) on lactic acid production, as an index of its gross effect on brain metabolism, was examined in the brain slice preparation (Table 4-1). Incubation of the slices in the presence of ketamine did not alter the formation of lactate by brain tissue. The effect of iodoacetate, a known inhibitor of glycolysis was

TABLE 4-1. EFFECT OF KETAMINE AND IODOACETATE ON LACTIC ACID FORMATION IN RAT CEREBRAL CORTICAL SLICES

Treatment	Lactate Formed umoles/g tissue/30 minutes
Control (4)	33.6±1.55
Ketamine (4)	34.6±2.76
Iodoacetate (2)	2.5±2.50*

Following a 15 minute preincubation in phosphate buffer, slices were transferred to media containing ketamine (1.83×10^{-4}M) or iodoacetate (5×10^{-4}M) and incubated for 30 minutes. Lactate released into the media was assayed by the method of Hohorst (83). Data are expressed as mean values ± standard errors for the number of slices indicated in parentheses.

* $p < .05$ as determined by Student's "t" test for unpaired data.

also examined. Although these data are less complete, iodoacetate (5×10^{-4}M) virtually abolished all lactate production under similar conditions (Table 4-1).

Since water content of the brain slice preparation may be altered by a variety of agents, the effect of ketamine on this parameter was investigated (Figure 4-1). The extracellular fluid compartment of the slice was estimated by means of the space occupied by the large molecular weight molecule, inulin. Although there was a tendency for the inulin space to increase, ketamine (2.1×10^{-5}M) did not significantly alter this parameter during any of the time intervals studied.

Uptake of ketamine hydrochloride (10 ug/ml) into the cortical slice preparation proved to be extremely rapid with measurable concentrations occurring following only a 10 second contact with the media (Figure 4-2). Accumulation appeared to be maximal at 5 minutes with no significant change in ketamine concentration at 10 minutes. At equilibrium (5 and 10 minutes) the ratio of ketamine brain concentration:external media concentration was approximately 2.3:1, indicating the occurrence of a preferential accumulation of ketamine into brain tissue similar to the *in vivo* situation. A 5 fold increase in ketamine concentation (50 ug/ml) in the media provided a similar maximal accumulation with approximate ratios of 2.28 at 5 minutes and 2.30 at 10 minutes.

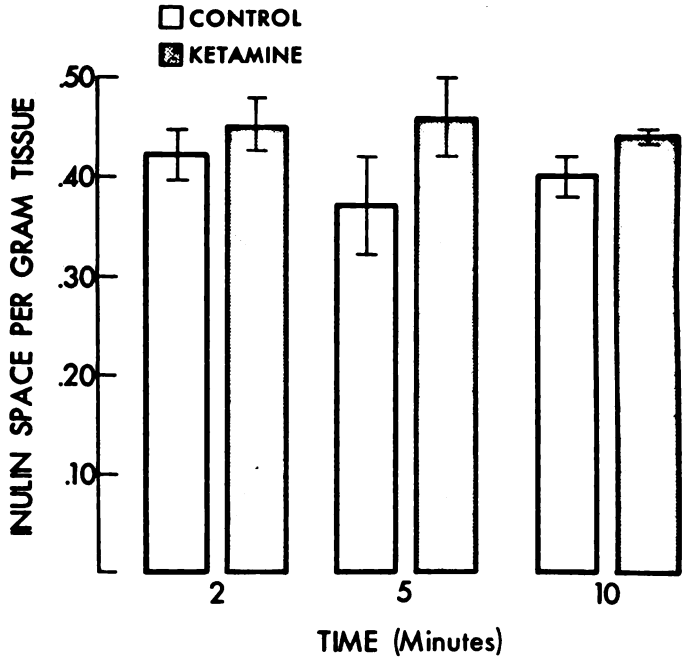


Figure 4-1. Effect of ketamine on inulin space of rat cerebral cortical slices. Slices were preincubated in bicarbonate buffer with inulin carboxyl-C-14 for 30 minutes. Ketamine (2.1×10^{-5}M) was added for the indicated times and inulin uptake was estimated by scintillation counting (see Chapter 4, Methods). Each bar represents the mean of four slices $\pm$ standard error.

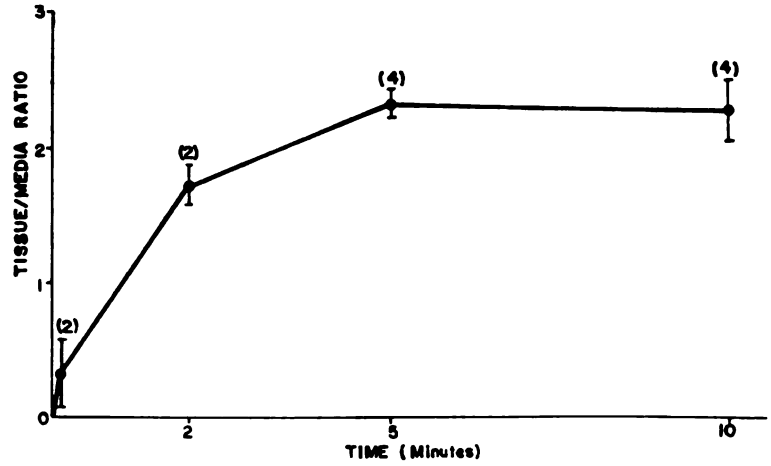


Figure 4-2. Rate of uptake of ketamine hydrochloride into rat cerebral cortical slices as a ratio of tissue:media concentration. Following a 15 minute preincubation in phosphate buffer, slices were incubated with ketamine hydrochloride (10 ug/ml). At the indicated times, the media and slice were assayed for ketamine (see Chapter 2). Each point represents the mean ± standard error. Number of slices at each point is indicated in parentheses.

Maximal accumulation of ketamine was observed in the presence of the conventional metabolic inhibitors (Figure 4-3), 2,4 dinitrophenol (5×10^{-5}M) and iodoacetate (5×10^{-4}M). Iodoacetate has been established to inhibit glycolysis principally by inhibiting phosphoglyceraldehyde dehydrogenase (191) while with dinitrophenol, oxidation has been shown to occur, but is uncoupled from the formation of ATP (38). Ninety five percent ethanol was included as the solvent control for the dinitrophenol experiments. None of these metabolic inhibitors altered the accumulation of ketamine in the brain slice, indicating that ketamine uptake is not dependent on glycolytic metabolism or ATP production.

In some experiments, ketamine retention by the brain slice was examined (Table 4-2) following a 10 minute incubation with ketamine (10 ug/ml) by transferring the slice to fresh media and observing the decrease in ketamine concentration in the slice and the appearance of ketamine in the fresh media for 5 or 15 minutes. Under these conditions, ketamine tissue concentrations fell from an initial 23 ug/g tissue to 3.2 and 1.6 ug/g tissue at 5 and 10 minutes, respectively. Despite this decline, there was still a considerable retention of ketamine in the slice as evidenced by the extremely high tissue:media ratios remaining after 15 minutes of release.

In order to evaluate the necessity of cellular integrity for ketamine accumulation and retention, the association of ketamine with subcellular brain fractions was examined

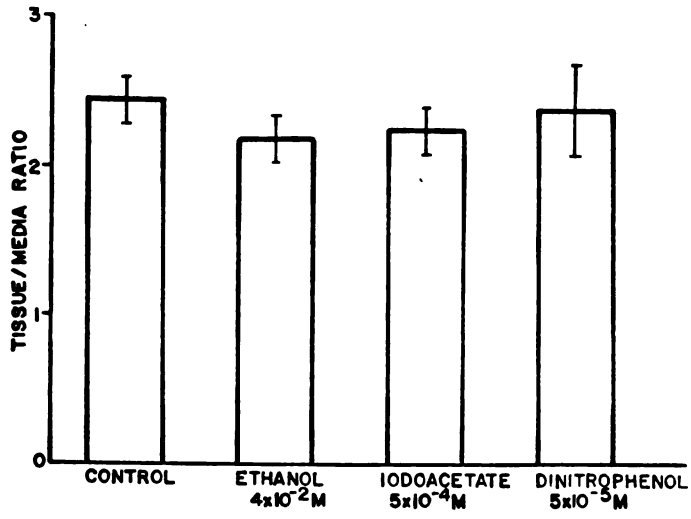


Figure 4-3. Effect of metabolic inhibitors on the uptake of ketamine into rat cortical slices as a function of the tissue:media ratio of ketamine. Slices were incubated in phosphate buffer with ketamine hydrochloride (10 ug/ml) for 5 or 10 minutes in the presence of either ethanol (4×10^{-2}M), iodoacetate (5×10^{-4}M) or dinitrophenol (5×10^{-5}M). Media and slices were assayed for ketamine as detailed in Chapter 2. Data for each treatment represent the mean of four slices $\pm$ standard error.

TABLE 4-2. RELEASE OF KETAMINE FROM RAT CEREBRAL CORTICAL SLICES AS A FUNCTION OF TIME

Time (Minutes)	Ketamine	
	ug/g tissue	tissue:media ratio
5	3.21±.35	22.35±6.05
15	1.63±.55	9.19±2.61

Following a 15 minute preincubation in phosphate buffer, slices were incubated with ketamine (1.83×10^{-4}M) for 10 minutes. Slices were then transferred to ketamine-free media and at 5 and 15 minutes the amount of ketamine was assayed in the tissue and in the media (see Chapter 2). There was approximately 23 ug ketamine/g tissue following the 10 minute incubation with ketamine. Data represent the mean of two slices for each interval ± standard errors.

(Figure 4-4) by an equilibrium dialysis method. Ketamine accumulation into dialysis tubing containing saline equaled the concentration in the external media between 3 and 6 hours. Thus, 6 hours was considered sufficient time for equilibrium in this system. At every time interval measured, there was a significantly greater concentration of ketamine in the homogenate preparation as compared to saline. Studies using soluble components of the homogenate indicated no greater concentration in this preparation as compared to saline. The concentration of ketamine associated with particulate material appeared to account almost entirely for the accumulation of ketamine in the homogenate preparation. At every time interval studied, there was no significant difference in these concentrations. At equilibrium, the ratio of ketamine concentration in tissue to its saline concentration was approximately 2.2:1. Thus, ketamine accumulation in neural tissue was similar in all three systems examined; brain homogenate, brain slice, and brain tissue at equilibrium following in vivo ketamine administration.

The partitioning characteristics of ketamine and its primary metabolite (I) were investigated using the conventional organic solvents, heptane, benzene, and chloroform (Table 4-3). Both ketamine and metabolite I were soluble in the organic solvents employed, although higher partitioning ratios were observed for ketamine than for its more polar metabolite.

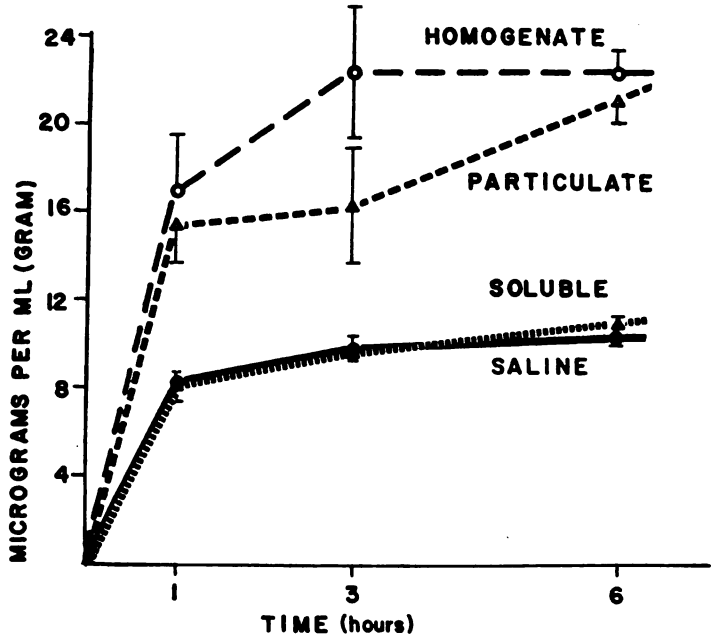


Figure 4-4. Association of ketamine with subcellular fractions obtained from rat whole brain by differential centrifugation. Fractions were dialyzed against 50 volumes of ketamine hydrochloride (10 ug/ml) in buffered saline. At the indicated times, external media and each fraction were sampled and analyzed for ketamine (see Chapter 2). Each point represents the mean ± standard error of four determinations for the homogenate fraction and saline and for three determinations for the particulate and soluble fractions.

TABLE 4-3. PARTITION COEFFICIENTS FOR KETAMINE AND METABOLITE I

	Organic Solvent/Buffer (pH 7.4) Distribution Ratio		
Compound	Heptane	Benzene	Chloroform
Ketamine	5.48±.10	97.8±11.2	1500
Metabolite I	1.12±.19	58.2±3.3	435±91

Distribution between organic solvents and 0.1M sodium phosphate buffer, pH 7.4 was measured by the procedure of Mayer et al (14). Ketamine and metabolite I were assayed as detailed in Chapter 2. Data represent the mean of three experiments ± standard errors. Data for solubility of ketamine in chloroform must be considered approximate due to the extremely low concentrations measured in the aqueous phase.

It has been generally considered that lipid solubility of an agent is dependent on the non-polar nature of the unionized species. Alterations of pH have the potential to affect greatly the distribution of agents in a manner dependent on the pk_a of the compound. A pk_a of 7.5 (Figure 4-5) was determined for ketamine, while the pk_a of its N-demethylated biotransformation product was found to be 6.65. At physiologic pH (7.4), ketamine would exist in a 1.26:1 ratio of the protonated molecule:basic form, while metabolite I would be approximately five times less protonated at the same pH.

Studies on the metabolism of ketamine indicated that N-dealkylation of ketamine occurred in liver homogenates, the only detectable product being metabolite I (Table 4-4). Under the in vitro conditions of this study, ketamine was calculated to be metabolized in liver at a rate of approximately 25 ug/g tissue/minute. The appearance of metabolite I accounted almost exactly for the quantity of ketamine which disappeared from liver homogenates and no metabolite II could be detected under these conditions.

Ketamine was not metabolized to any statistically significant extent in rat brain tissue homogenates, under similar conditions. Significance was determined by comparison of the amount of ketamine initially present to the amount remaining after the 30 minute incubation as well as by comparison of the ketamine removed by brain tissue to the same value derived from saline controls. As judged by both

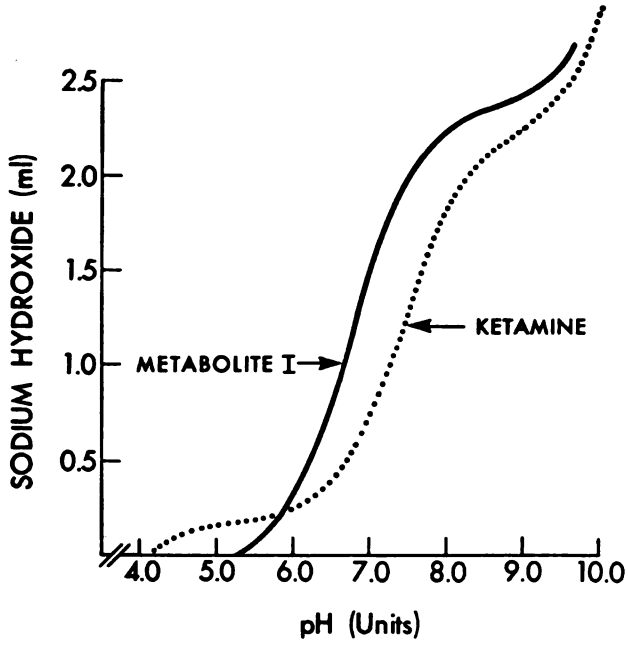


Figure 4-5. Representative potentiometric titration curves for ketamine and metabolite I. Ketamine and metabolite I (1 x 10^{-3}M) were titrated with sodium hydroxide (2 x 10^{-3}M) and changes in pH were directly recorded (see Chapter 4, Methods). Arrows indicate inflection point for each curve.

TABLE 4-4. METABOLISM OF KETAMINE IN RAT BRAIN AND LIVER HOMOGENATES

Tissue	Ketamine (umoles/gm)		Metabolite I (umoles/gm)
	t_0'	t_{30}'	t_{30}'
Brain	20.50±.12	19.60±.38	0.0
Liver	20.11±.52	16.99±.50*	3.70±.20*

Ketamine was added to rat liver and brain homogenates and incubated for 30 minutes at 37°C. Samples were taken at zero time (t_0') and 30 minutes (t_{30}') and analyzed for ketamine and metabolites (see Chapter 2). Data represent mean values ± standard error derived from 4 animal preparations.

* $p < .05$ as determined by Student's "t" test for unpaired data.

statistical methods, ketamine was not significantly metabolized in brain while it was in liver. Neither metabolite I nor II could be detected following incubation of brain tissue with ketamine.

DISCUSSION

Brain slice preparations have been established to possess many of the cellular characteristics of the brain in vivo including ionic gradients (98), action potentials (80, 150) and neurotransmitter uptake (23, 46, 87, 113), and release (6, 95, 137, 138). Many parameters have been employed to evaluate the viability and functioning of this preparation including phosphocreatine levels (171), oxygen consumption (2, 78, 126, 139), ATP production (7, 139), pyruvic acid formation (78), ionic concentrations (68, 97, 98, 165, 178), lactic acid production (21, 78, 126, 188), and aqueous compartments (36, 68, 140, 184). In the present studies, lactate production and the size of the extracellular compartment were measured as indices of the metabolic state of the slices used herein.

Close agreement for lactic acid production was obtained with levels reported by other workers (21, 78, 126, 188) indicating comparable viability of this preparation. Ketamine (1.83×10^{-4}M) did not affect lactate production suggesting the absence of any major action on cerebral metabolism. This conclusion was supported by the observation that ketamine did not alter glucose utilization in brain slices or homo-

genates (119) although these same authors did report a rather small non-dose dependent increase in oxygen consumption occurring only in the tissue slice preparation. Respiratory parameters of tissue slices have been modified by neuroleptic agents (24, 126, 188) and there has been the indication that lactate production is more sensitive than oxygen consumption as an index of metabolic alterations by depressant drugs in the unstimulated slice (126). These results did not exclude the possibility of other metabolic alterations by ketamine which would not be reflected in lactic acid measurements.

Metabolic parameters of the slice preparation were altered in the presence of iodoacetate, a known glycolytic inhibitor (191). In studies using iodoacetate all lactate production was virtually abolished, in agreement with reported results on guinea pig cortical slices (78). These studies on lactate production indicated that this preparation was similar to that of others in that modifying agents did alter lactate production, although ketamime had no gross effect on this parameter.

Water content and compartmentation of the brain slice preparation has been shown to increase with time (68) and may be regulated by brain respiration (139). Alteration of water content may be a reflection of changes in ionic distribution (139) and the resultant metabolic state of the slice. Determination of inulin space has been a conventional method for estimating water extracellular space (127) although

this technique is limited by many assumptions (68, 140), the primary one being that inulin is located only extracellularly. An approximate inulin space of 40 - 45 percent was observed in slice preparations used in these studies, values comparable to other brain slice preparations (21, 68, 98). Although other agents have been reported to alter inulin space (36, 140, 184), ketamine (2.1×10^{-5}M) had no significant effect on this parameter. Observations that lactate production and inulin space were not grossly affected by ketamine support the contention that ketamine did not exert any primary action on metabolism which would be reflected in alterations of these measurements.

Rapid accumulation of ketamine in the brain slice preparation paralleled the rapid in vivo brain accumulation observed following ketamine administration. The striking resemblance of the in vitro slice studies to the in vivo situation was strengthened by examination of the ratios of brain tissue concentration to extracellular concentration of ketamine. In vivo studies, although complicated by an inaccurate assessment of equilibrium, indicated an initial brain:plasma ratio of 7:1 which declined to approximately 2.8:1 as this dynamic system approached equilibrium at 60 minutes. In the slice preparation, which offered the advantage of precise knowledge of the extracellular concentration of ketamine at any given time, equilibration resulted in a brain:media ratio of 2.3:1. From these studies, ketamine entry into the brain slice preparation appeared analogous to its in vivo accumulation.

Dependence on a transport mechanism has been demonstrated for the uptake of some compounds not naturally present in the brain including metabolic inhibitors (142), and quaternary ammonium compounds (176). Although ketamine was accumulated in brain tissue to a greater extent than its extracellular concentration, the rapidity of equilibration and independence of uptake on concentration (although a complete dose curve was not obtained) argued against an active process. Active transport mechanisms have been shown to be dependent on extracellular concentration and rarely involve a tissue:media ratio of 1 within 10 minutes (46). In fact, the accumulation of ketamine was not affected in the presence of metabolic inhibitors, thus indicating the lack of dependence of ketamine uptake on metabolic processes or energy production. These data are consistent with the suggestion that active transport did not exert any major influence in the cerebral accumulation of ketamine.

Decline in ketamine concentration in equilibrated brain slices was found to be rapid, analogous to the decline in brain levels observed in in vivo studies. In the brain slice, equilibrium occurred when the rate of drug entry became equal to the rate of exit (142). Since equilibrium was rapidly attained, the rate of ketamine uptake into the brain slice was exceptionally rapid, and it could have been predicted that the rate of exit would be equally rapid. However, under the conditions of release, a considerable amount of ketamine was retained in brain tissue beyond the

concentration appearing in the incubation media. This correlated well with the high brain:plasma level ratios for ketamine, observed over long time intervals following its *in vivo* administration. This supported the suggestion that ketamine is preferentially associated with cerebral tissue.

Investigation of the association of ketamine with subcellular fractions derived from brain homogenates demonstrated that intact cellular morphology was not a requirement for accumulation of the anesthetic. These studies showed that ketamine did not bind to soluble components of the tissue, since this fraction exhibited concentrations similar to the saline control. Association of ketamine with homogenates resulted almost entirely from its interaction with particulate components of the cell. Achievement of a 2.2:1 equilibrium ratio of ketamine with such particulate material as compared to saline was close to the ratios obtained under similar conditions of equilibrium for the brain slice (2.3:1) and for the *in vivo* situation (2.8:1). No further examination of differential affinity of ketamine for specific particulate components was measured. On the basis of the distribution properties of other anesthetics as a function of physico-chemical properties including lipid solubility, it was deemed more profitable to examine the partitioning characteristics of ketamine.

Heptane, benzene and chloroform have been conveniently employed to assess the partitioning of agents into tissues

(18, 121, 148), although there has been some skepticism concerning the resemblance of organic solvents to neural tissue. It should be mentioned that in these studies, the amount of ketamine in the organic phase was not directly measured. It was highly unlikely that the ketamine molecule could be chemically altered in the organic solvents. For this reason, it was assumed that the difference between the amount of ketamine originally in the aqueous phase and the amount remaining after contact with the organic solvent was located entirely in the organic phase.

Ketamine exhibited exceptional lipid solubility on the basis of its partitioning characteristics in organic solvents. For comparisons, partition distribution ratios for thiopental under the exact same conditions were 0.95 - heptane, 2.0 - benzene, and 102 - chloroform (121). It is evident that ketamine was extremely lipid soluble, in fact, 5 to 10 times more so than a generally acknowledged highly lipid soluble substance, thiopental. Metabolite I which is more polar was also highly lipid soluble, although less so than ketamine (Table 4-3).

Conventional hydrocarbon and barbiturate anesthetic agents have been established to distribute in body tissues partly on the basis of their lipid solubility (18, 144) at physiologic pH. If it be permitted to extrapolate from intestinal absorption of drugs to brain accumulation, the rate of absorption appears to parallel the lipid solubility

of compounds (82, 160), and it may be suggested that rate of cerebral entry of drugs may be greater with higher lipid solubility. Rapid entry of ketamine into brain tissue may be well correlated with its physico-chemical property of exceptional lipid solubility at physiologic pH. Although ketamine may produce a unique type of anesthetic state, its tissue distribution appeared similar to the short acting barbiturate anesthetic agents in that it is dependent primarily on lipid solubility, blood flow, and organ mass.

Distribution of drugs into body tissues depends on the pk_a of the compound as well as its lipid solubility at physiologic pH. pk_a values of 7.5 for ketamine and 6.65 for its primary metabolite are in agreement with previously determined values (74). From data on the pk_a, ketamine exists in almost equal amounts in the basic and protonated forms at pH 7.4. Because the demonstrated pk_a (7.5) of ketamine is exceedingly close to the pH of its environment, any slight alteration of physiologic pH could potentially alter the distribution of ketamine to a considerable extent. It is also pertinent to mention here that alterations of urinary pH could influence the degree of ionization of ketamine in urine. Thus, lowering of urinary pH could potentially serve to hasten the excretion of ketamine in situations where this might be useful.

Intracellular pH has been established to be approximately 7.1 in peripheral (187) and rat cerebral (152) tissue, while extracellular pH has been considered to be 7.4. This pH differential may partly contribute to the persistently high

cerebral levels of ketamine found after its *in vivo* administration. Since ketamine is a weak base with a pk_a of 7.5, approximately twice as much of it will exist in the ionized form at pH 7.1 than at pH 7.4 when these two compartments equilibrate. Thus, it is apparent that there may be intracellular pooling of ketamine by virtue of the distribution properties of the protonated species. This phenomenon has been suggested as an explanation of the distribution of other weak bases into smooth muscle (11) and red blood cells (164).

Shortly after the intravenous injection of ketamine in man, the pH of arterial and venous blood as been repeatedly demonstrated to fall slightly (158, 172, 175, 183, 186). While it is doubtful that this drop in pH occurs rapidly enough to affect the initial entry of ketamine into cerebral tissue, it may serve to potentiate the decline in cerebral tissue concentration of ketamine and in this manner contribute to the short duration of anesthesia.

Since metabolite I was shown to accumulate in cerebral tissue following ketamine administration (Figure 3-5), it was possible that N-demethylation of ketamine by brain tissue was responsible for the decline of cerebral ketamine levels. Brain tissue is known to contain enzymes necessary for the demethylation of a variety of agents including morphine, meperidine, codeine (64) and aminopyrine (5). However, ketamine did not appear to be metabolized in rat brain tissue under conditions of the present *in vitro* experiments. Since demethylation reactions occur to a

lesser extent in brain as compared to liver tissue (64), the inability to detect metabolites in brain homogenates may be a reflection of limitations of measurement sensitivity. However, even if this were the case, it is rather doubtful that brain metabolism would be sufficient to account for the relatively high cerebral concentration of metabolite I. Lack of in vitro cerebral metabolism of ketamine was confirmed in the brain slice preparation since no metabolites could be detected under those experimental conditions either. It must tentatively be concluded that the appearance of the N-demethylated product in the CNS followed extracerebral degradation of the parent compound.

N-dealkylation of ketamine to metabolite I in rat liver homogenates occurred at a rapid rate with the sole appearance of metabolite I. It appeared that this was the major breakdown product of ketamine in the rat, consistent with conclusions previously drawn from the in vivo studies. On the basis of in vivo studies, the 30 minute incubation period in vitro may not have been sufficient for the further oxidation of metabolite I. It is also possible that further metabolism does not occur in liver, and the appearance of metabolite II in plasma may reflect oxidation in tissues other than brain and liver.

Drug metabolism is dependent on a wide variety of factors including the physico-chemical characteristics of the agent as well as factors related to the organism (37, 118). Although there are exceptions (122), there is a high

correlation of lipid solubility of drugs and their oxidation in liver (71). It has been suggested that lipid solubility is necessary for substances to gain access to smooth endoplasmic reticular oxidative systems. This suggestion of a requirement for high lipid solubility is strengthened by observations of the rapid metabolism of ketamine which exhibits exceptional lipid solubility (Table 4-4). Studies demonstrating sex differences in the N-dealkylation of drugs suggest that N-dealkylation occurs less in females than in males (64, 110, 145). Since N-dealkylation is the primary route of ketamine biotransformation it is highly possible that decreased metabolism of ketamine may account for the more predominant psychic effects occurring in women following ketamine administration (14).

While one must extrapolate from in vitro experiments to the intact animal situation with caution, it is of interest that the rate of hepatic degradation in the rat could account for almost 50 percent of the administered dose of ketamine (20 mg/kg) during the time of loss of righting reflex. This may indicate that, at least in this species, metabolic transformation of ketamine may play a significant role in the termination of certain of the drug's central actions. If this were the case, it represents a situation quite distinct from that for thiopental (75). In man, plasma ketamine levels fall rapidly after an intravenous injection of 2.2 mg/kg, consciousness returning with plasma levels in the range of 0.7 to 1.0 ug/ml (115). Since

metabolite I rapidly appears in the plasma in man, it is reasonable to suggest that metabolic degradation may also be significant here with respect to duration of action. To date, there is no reported evidence that might suggest unusual responses to ketamine in situations of gross hepatic impairment, but some degree of caution is suggested until such time as it becomes apparent that accumulation is not a problem in man.

CHAPTER 5. INTERACTION OF KETAMINE AND METABOLITE I WITH MULTIPLE FORMS OF BRAIN ACETYLCHOLINESTERASE

INTRODUCTION

There is experimental and clinical evidence indicating a potential interaction of ketamine with cerebral acetylcholinesterase (AChE). Experimentally, ketamine has been shown to desychronize and activate electroencephalograph patterns in subcortical nuclei including the hippocampus (42, 96). This closely mimicks the expected acetylcholine response in this area (16). Although not all cortical cells respond to the iontophoretic application of acetylcholine, of those which do respond, the majority exhibit neuronal excitation (149). Although there are limitations in relating ionotophoretic results and electroencephalographic recordings, ketamine has been demonstrated to activate the cortical EEG (96) which may be the equivalent of neuronal excitation and indicate an action similar to the effect of iontophoretic application of acetylcholine.

Clinically, ketamine-induced anesthesia is associated with extensive lacrimation and salivation (42, 96, 124), an increase in skeletal muscle (39, 101, 141) and uterine tone (89, 115) and a transient decrease in cardiac output and heart rate in some patients (42, 141) and animals (136) immediately following induction of anesthesia. The excessive sialogogic actions as well as certain central manifestations of disorientation on emergence from ketamine-induced anesthesia have been antagonized by atropine (130) and antipsychotic agents (8, 12, 14, 155) possessing anticholinergic activity. All of these effects may be indicative

of excess cholinergic involvement either centrally or peripherally and may be explained either by a direct interaction of ketamine with a cholinergic receptor or an increase in endogenous acetylcholine due to inhibition of AChE. Although ketamine may structurally resemble nicotine and could potentially interact with the cholinergic receptor, at physiological pH approximately 50 percent of the available ketamine is protonated (see Chapter 4), and available for interaction with anionic sites on AChE.

Metabolism of ketamine involves the formation of metabolite I which has been shown to accumulate in cerebral tissue (Figure 3-5) and to produce anesthetic effects similar to the parent compound (Table 3-2). Thus, it is possible that some of the multiple effects of ketamine are a result of the inhibition of AChE by its biotransformation product, metabolite I. Since both ketamine and its metabolite can occur in brain concomitantly, it would also be of importance to determine the result of a simultaneous interaction of these two agents with AChE.

An attempt to explain some of the cerebral actions of ketamine is central to this investigation of AChE. In the past, enzymes of non-mammalian origin (eel) have been applied almost exclusively to the study of drug modification of AChE, primarily because they have been more easily purified and more readily available than AChE from mammalian sources. Purification of brain AChE has been hindered by difficulty in solubilizing the enzyme from

particulate components. Recently, however, bovine caudate nucleus AChE has been solubilized and purified (25, 26).

Although in most studies of drug:enzyme interactions, it is necessary to use highly purified enzymes to formulate a precise understanding of the molecular nature of the interaction, it must be remembered that AChE normally exists as a membrane bound protein. Since during purification the conformation of the enzyme could have been altered, thus affecting drug interactions with AChE, a bound form of the AChE was isolated from synaptic membranes of bovine caudate nucleus tissue for comparative studies.

In attempting to achieve a detailed understanding of the nature of the cerebral actions of ketamine and its primary metabolite, actions of these compounds were studied on three purified forms of AChE as well as on an isolated membrane bound form of the enzyme. The kinetics of ketamine induced inhibition of AChE were characterized by type of inhibition, determination of I_{50} levels, calculation of K_i values, reversibility of the inhibition, species selectivity, and pH dependence.

MATERIALS AND METHODS

Assays

Acetylcholinesterase Activity -

AChE activity was determined by the thiocholine spectrophotometric method (65). The reaction mixture contained phosphate buffer, 0.1M; 5,5'-dithiobis-2-nitrobenzoic acid

(DTNB), 0.01M; acetylthiocholine iodide (ATC) at various concentrations, ketamine and/or metabolite I in indicated concentrations, enzyme, and deionized water to a final volume of 3 ml. The rate of reaction was determined in duplicate on a Norelco Unicam SP500 spectrophotometer at 412 mu. Temperature was monitored and ranged from 22° to 26°C. Specific activity is expressed as micromoles ATC hydrolyzed/mg protein/hr after correction for non-enzymatic hydrolysis of acetylthiocholine. Assays documenting AChE activity for isolation and separation purposes were conducted at optimum conditions: with 1mM ATC and phosphate buffer, pH 8.0.

Protein -

Protein was determined by the method of Lowry et al (117). Assays were conducted in duplicate and standards of bovine serum albumin were used for every determination.

Succinic Dehydrogenase Activity -

Measurements of succinic dehydrogenase activity were made utilizing the substrate-dependent reduction of a blue dye, sodium 2,6 dichlorophenolindophenol (DCPIP) to a colorless form (77). Enzymatic oxidation of succinate with indophenol in the presence of cyanide was measured in duplicate with a Hitachi Perkin-Elmer Model 139 spectrophotometer at 600 mu. The reaction mixture contained phosphate buffer, 0.1M pH 7.4 (2 ml); sodium cyanide (0.5mM), DCPIP (0.029mM), succinate (2.5mM), and sample. Decrease in absorbance was recorded after addition of substrate.

Sodium, Potassium Stimulated
Adenosine Triphosphatase Activity -

Assay of Na^+,K^+ stimulated adenosine triphosphatase activity involved a reaction mixture consisting of ATP (tris salt), 2.5mM; KCl, 10mM; NaCl, 110mM; $MgSO_4$, 5mM; imidazole buffer, 20mM pH 6.9; and the appropriate enzyme preparation in a total volume of 0.5 ml. Mg-stimulated activity was determined in a similar system containing choline chloride rather than NaCl and KCl. This activity was subtracted from the total activity to give values reported as Na-K-stimulated ATPase activity. The mixture, containing everything except ATP, was incubated at 37°C for 1 minute, and the reaction was allowed to run for 10 minutes after addition of substrate. It was terminated by the addition of 0.05 ml of 10 percent perchloric acid. A 0.25 ml aliquot was utilized for phosphate determination by the procedure of Martin and Doty (120). In addition to the experimental samples, one sample containing the entire reaction mixture, but no enzyme was treated simultaneously as a blank.

Chemicals

All chemicals used were of analytical grade. These chemicals were obtained as follows: acetylthiocholine iodide (ATC), tris (hydroxymethyl) aminomethane, Sigma Chemical Co. (St. Louis, Missouri); 5, 5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin); affinose-202, Bio-Rad Labs (Richmond, California); sephadex

G-200, Pharmacia (Uppsala, Sweden); sucrose, sodium succinate, Mallinckrodt Chemical Works (St. Louis, Missouri); ammonium sulfate, sodium cyanide, Allied Chemical (Morristown, New Jersey), 2,6-dichlorophenol indophenol, sodium salt, Calbiochem (Los Angeles, California).

Preparation of Purified Forms of Brain AChE

Solubilization Process -

Bovine brains were obtained from a local slaughterhouse and transported on ice. Caudate nucleus tissue was removed and a 20 percent (w/v) homogenate was prepared in 0.32M sucrose containing 1mM EDTA at pH 7.0. Centrifugations were carried out at 0°-4°C in a Lourdes Model A2 Betafuge using the 9RA rotor and a Spinco Model L centrifuge using a 30 rotor according to the scheme recently reported (26) and indicated in Figure 5-1. Resuspended pellets were stirred overnight at 0-4°C. Supernatant fractions 2,3,4 were subsequently dialyzed and concentrated at 4°C against 1mM phosphate buffer pH 7.0 and then centrifuged at 80,000 g for 75 minutes. These supernatant fractions were then combined for further purification.

Purification Process -

The purification procedure was accomplished by affinity chromatography (25) according to the scheme indicated in Figure 5-2. Multiple forms of brain AChE (A,B,C) were obtained with this process, and these forms appear to be completely purified. A specific activity on the order of

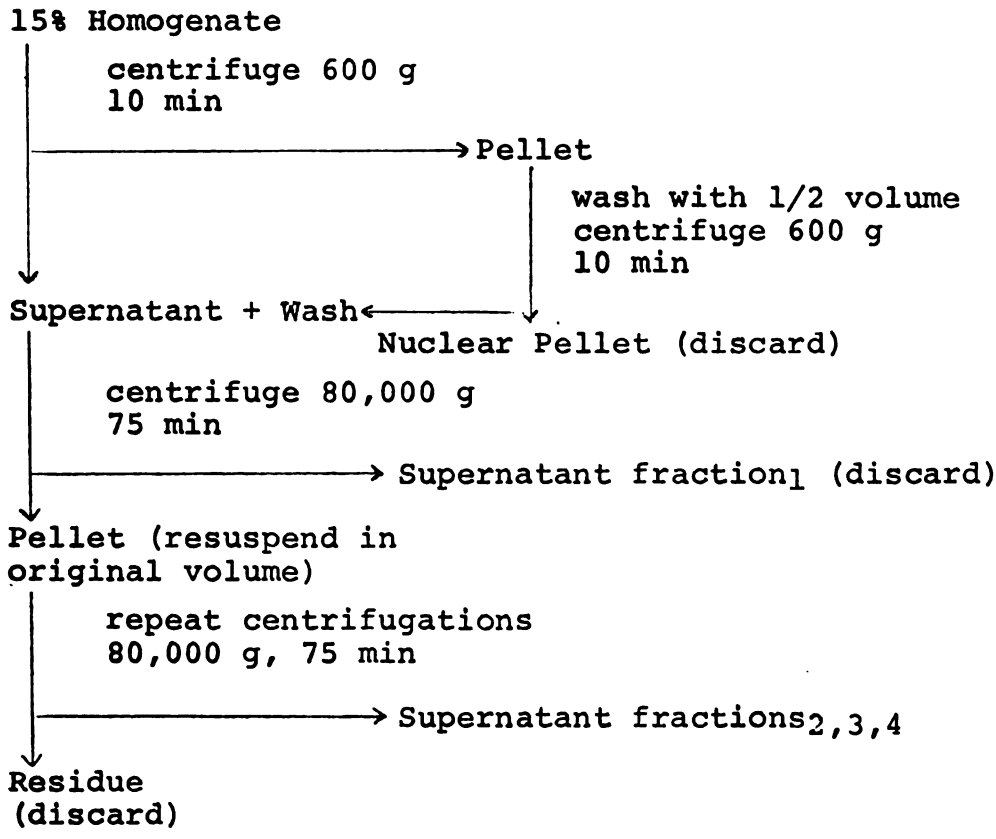


Figure 5-1. Fractionation scheme for solubilization of acetylcholinesterase (26).

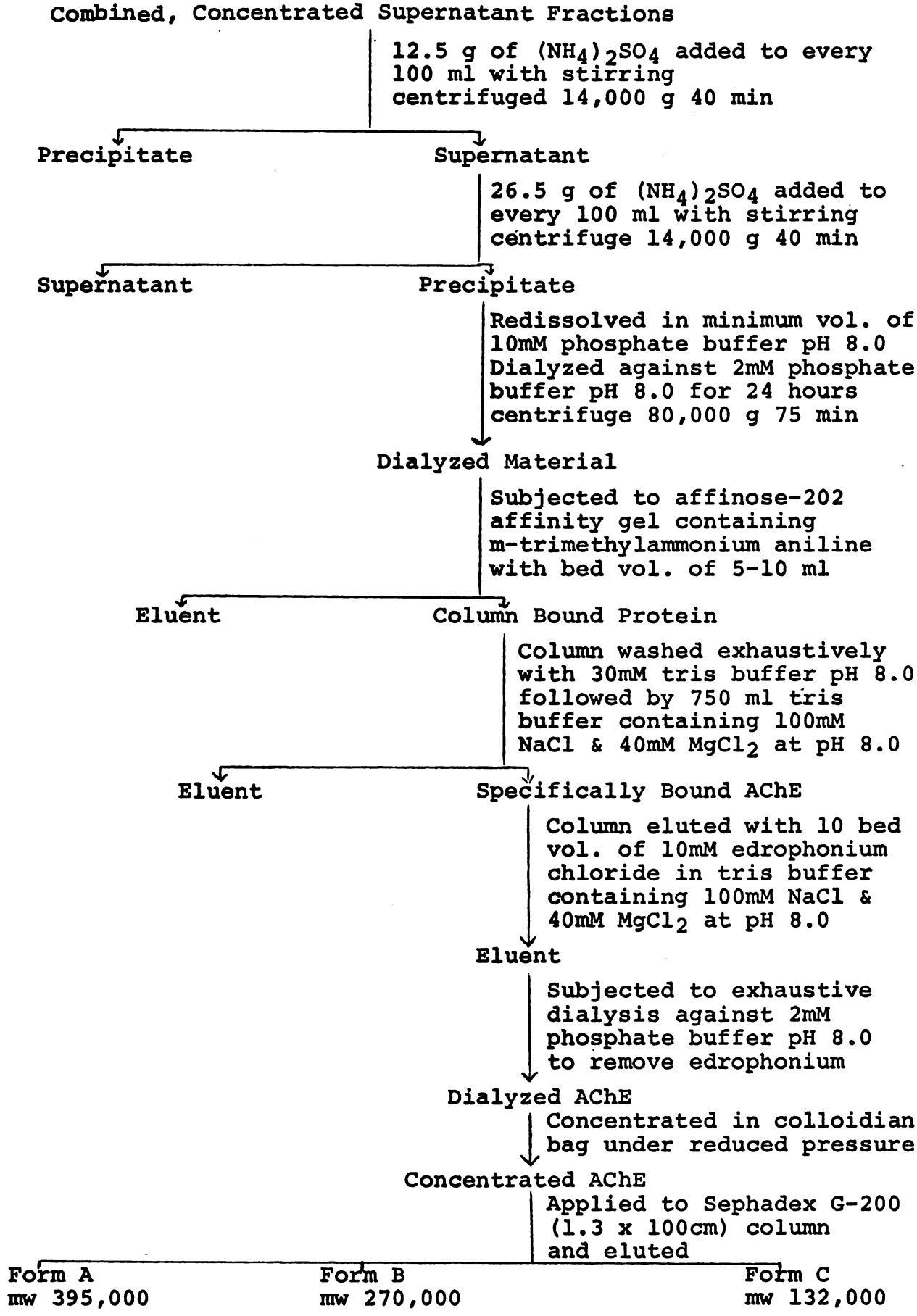


Figure 5-2. Fractionation scheme for purification of multiple forms of brain acetylcholinesterase.

400,000 to 600,000 umoles ATC hydrolyzed/mg protein/hr was observed for enzyme forms obtained by this method.

Preparation of Synaptic Membrane Bound AChE

Beef brains were obtained from a local slaughterhouse and were transported on ice. Caudate nucleus tissue was cleaned of white matter and blood capillaries and was homogenized in 9 volumes of .32M sucrose adjusted to pH 7.0 with tris buffer. Homogenization was accomplished with 10 vertical strokes in a glass-teflon homogenizer adjusted to a clearance of 0.25 mm. Subcellular fractionation of this homogenate was carried out at 0-4°C in a Lourdes Model A2 Betafuge using the 9RA rotor according to the fractionation procedure of De Robertis et al (47) which is presented in Figure 5-3. The distribution and yield of protein and AChE activity are indicated in Table 5-1.

Following the primary centrifugation, the crude mitochondrial fraction was submitted to osmotic shock in distilled water, after which it was centrifuged, resulting in a pellet (M_1) and a supernatant. The resuspended M_1 pellet was placed on a four layer sucrose density gradient (0.8M, 0.9M, 1.0M and 1.2M sucrose). After centrifugation for 2 hours in a Beckman L2-65B ultracentrifuge using a SW41 rotor at 50,000 g, six gradient layers were separated and pooled; a top clear layer, followed by five particulate layers. Purity of these fractions was evaluated by enzyme marker assays utilizing succinic dehydrogenase, sodium potassium stimulated adenosine triphosphatase and acetylcholinesterase,

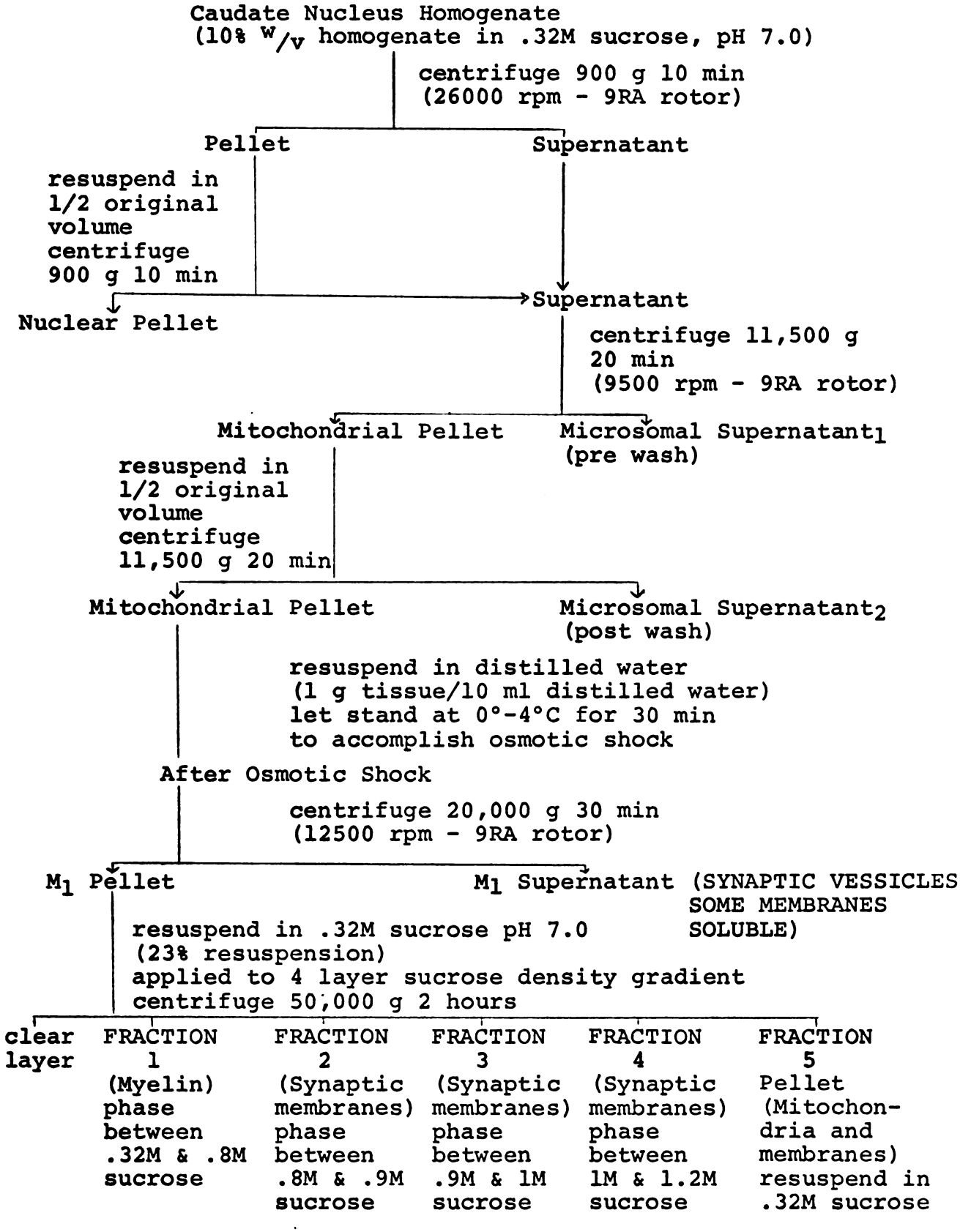


Figure 5-3. Subcellular fractionation scheme for isolation of membrane bound acetylcholinesterase.

TABLE 5-1. PRIMARY SUBCELLULAR FRACTIONATION OF OX CAUDATE NUCLEUS TISSUE

Fraction	Protein		Acetylcholinesterase		RSA
	mg/gm wt weight	%	umoles/mg protein/hr	%	
Homogenate	101.8±5.9	100	31.07±2.49	100	
Nuclear	10.4±1.73	10.1±1.7	12.65±1.89	3.4±0.6	.34
Mitochondrial	54.8±3.86	53.9±3.8	22.55±0.54	41.3±2.7	.767
Microsomal					
Supernatant$_1$	28.7±1.63	28.2±1.6	35.38±1.44	35.0±0.9	1.24
Supernatant$_2$	11.1±1.63	10.9±1.6	49.18±1.16	17.2±1.8	1.58
Recovery		102.5±5.2		96.8±3.6	
M_1 Pellet	43.2±2.04	42.5±2.0	22.85±0.91	32.5±2.3	.765
M_1 Supernatant	11.4±1.01	11.2±1.0	20.33±1.77	7.4±0.9	.66
Recovery		99.8±5.4		96.5±2.9	

A 10% homogenate was prepared as described in Chapter 5, Methods, and subjected to differential centrifugation as indicated in Figure 5-3. Protein and acetylcholinesterase activities were estimated by the methods of Lowry (117), and Ellman et al (65), respectively. Data represent the means ± standard errors of four separate fractionations. Relative Specific Activity (RSA) = % activity recovered/% protein recovered.

as indicated in Table 5-2. The isolation of AChE enriched synaptic membranes occurred in fractions 2 and 3, with fraction 1 consisting principally of myelin, fraction 4 containing synaptic membranes with some mitochondria contamination and fraction 5 being predominantly mitochondrial in nature (108).

Preparation of Rat Brain Acetylcholinesterase

Male Sprague-Dawley (100-120 g) rats were decapitated and exsanguinated. Whole brain was removed, placed on ice, cleared of external blood vessels, and homogenized in 9 volumes of .32M sucrose adjusted to pH 7.0 with tris buffer. The procedure indicated for preparation of synaptic membrane bound AChE (Figure 5-3) was followed to the formation of an M_1 pellet. This pellet was resuspended in .32M sucrose pH 7.0 to 23 percent of the original volume.

An aliquot of this M_1 fraction containing EDTA (.5mM) and imidazole (5mM) was mixed with a magnetic stirrer at 0-4°C for 36 hours. This preparation was then centrifuged in a Spinco Model L centrifuge using a 40 rotor at 100,000 g for 1 hour in order to obtain a soluble form of rat brain AChE. Ketamine inhibition was studied with AChE from the M_1 fraction (particulate) and the AChE solubilized from this preparation.

Determination of Reversibility of Ketamine Inhibition

The reaction mixture contained phosphate buffer .1M pH 8.0, enzyme preparation, appropriate concentration of ketamine and deionized water to a volume of 28 ml. An

TABLE 5-2. SUCROSE DENSITY GRADIENT SUBFRACTIONATION OF OX CAUDATE NUCLEUS TISSUE

Fraction	Protein		Succinic Dehydrogenase		Sodium, Potassium, Stimulated Adenosine Triphosphatase		Acetylcholinesterase	
	mg/gm wt	weight %	umoles/mg prot./hr	%	umoles/mg prot./hr	%	umoles/mg prot./hr	%
M_1 Pellet	43.9	100	489.0	100	10.2	100	25.1	100
Clear	1.5	3.5	109.9	0.7	8.7	.6	30.6	4.3
Fraction 1	9.8	22.4	16.4	0.9	9.9	18.5	32.1	28.7
Fraction 2	3.9	8.9	74.3	1.0	21.0	15.5	55.6	19.7
Fraction 3	3.6	8.1	141.9	2.5	20.6	13.9	42.1	13.6
Fraction 4	6.7	15.3	304.0	11.5	25.1	32.0	41.5	25.3
Fraction 5	12.2	27.8	1245.0	81.0	12.9	29.8	12.4	13.7
Recovery		86		97.6		110.3		105.3

M_1 pellet was centrifuged on a four layer sucrose density gradient resulting in a soluble top layer (clear) and five particulate fractions as defined in Figure 5-3. These data are representative of a typical experiment indicating protein and enzyme activities in each of the resultant fractions. Assays were conducted as described in Chapter 5, Methods.

initial aliquot of 2.8 ml was removed for assay of AChE activity after the addition of .1 ml DTNB (0.01M) and .1 ml ATC (1mM). The remainder of the reaction mixture was immediately inserted inside a 15 inch length of dialysis tubing (dialysis tubing was previously boiled in distilled water for 15 minutes, washed and cooled) placed U shaped into a 1000 ml beaker containing 600 ml of phosphate buffer, 0.1M pH 8.0 which was mixed with a magnetic stirrer at room temperature. At appropriate times, a 2.8 ml aliquot was removed from inside the dialysis tubing for assay of AChE activity similar to the initial assay prior to dialysis. In order to correct for any loss of activity due to instability of the diluted enzyme preparation with time, this procedure was repeated in the absence of ketamine. Percent activity was calculated at each time interval as the activity in the presence of ketamine divided by the activity in the absence of ketamine.

Calculations and Graphic Presentation of Kinetics

Ketamine inhibition of acetylcholinesterase was graphically represented by Lineweaver-Burk double-reciprocal plots (114) with lines calculated by the least squares method of linear regression analysis. Apparent dissociation constants (ki) were calculated from the slopes of the inhibited reactions at two different substrate concentrations (191). In most studies this was confirmed by direct graphical observation of Hunter and Downs plots (84). The concentration of inhibitor necessary to obtain 50 percent of the initial

control enzyme activity (I_{50}) was determined graphically from a plot of log (I) vs percent enzyme activity.

RESULTS

Ketamine was found to inhibit mammalian brain acetylcholinesterase and analysis of kinetic studies with the three purified and the membrane-bound form of AChE indicated mixed inhibition in all cases. Mixed inhibition was demonstrated at two inhibitor concentrations with each of the enzyme forms as determined by Lineweaver-Burk plots (Figures 5-4 thru 5-7) and confirmed by the characteristic downward turning of a Hunter and Downs plot. At the higher inhibitory concentrations, there was a shift of the substrate optimum to higher concentrations in the presence of ketamine. Inhibitory constants were calculated for each of the enzyme forms (Table 5-3). The apparent inhibitory dissociation constants ranged from 4.95×10^{-4}M for the membrane-bound preparation to 6.93×10^{-4}M for purified enzyme A. While the purified forms appeared to be slightly more sensitive, 50 percent inhibition occurred at a concentration of 2.6×10^{-3}M ketamine for the membrane-bound enzyme (Figure 5-8). Preincubation had no marked effect on this degree of inhibition.

Since it was possible that enzyme inhibition was due to some nonspecific effect involving irreversibility, especially at higher inhibitor concentrations, the reversibility of AChE inhibition by ketamine was investigated (Figure 5-9). For purified forms A and B and the membrane

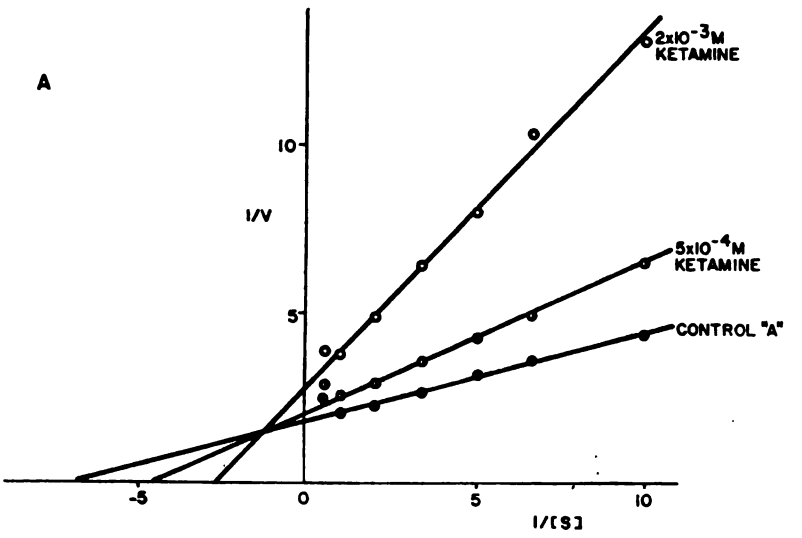


Figure 5-4. Lineweaver-Burk plot of inhibition of purified acetylcholinesterase, Form A by two concentrations of ketamine. Substrate concentrations ranged from 0.1 to 5mM ATC. Enzyme activity was determined by the method of Ellman et al (65) at optimum pH (7.9). Control enzyme activity was 480 mmoles ATC hydrolyzed/mg protein/hour at optimum substrate concentration (1mM). Lines were calculated by the least squares method of linear regression analysis.

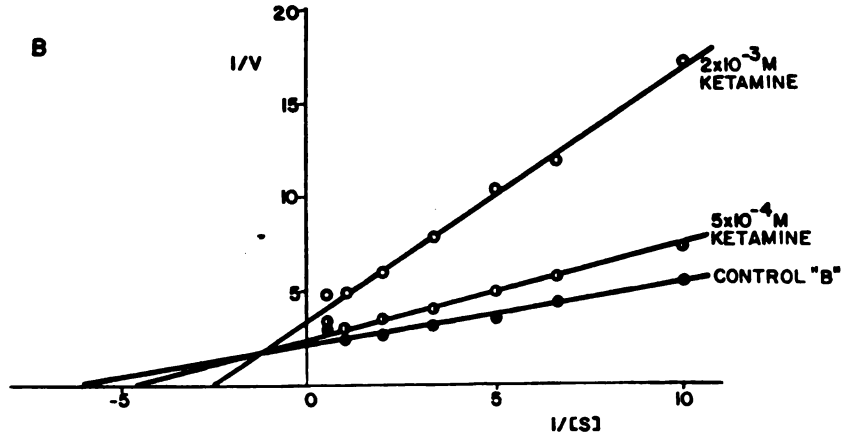


Figure 5-5. Lineweaver-Burk plot of inhibition of purified acetylcholinesterase, Form B by two concentrations of ketamine. Substrate concentrations ranged from 0.1 to 5mM ATC. Enzyme activity was determined by the method of Ellman et al (65) at optimum pH (7.9). Control enzyme activity was 400 mmoles ATC hydrolyzed/mg protein/hour at optimum substrate concentration (1mM). Lines were calculated by the least squares method of linear regression analysis.

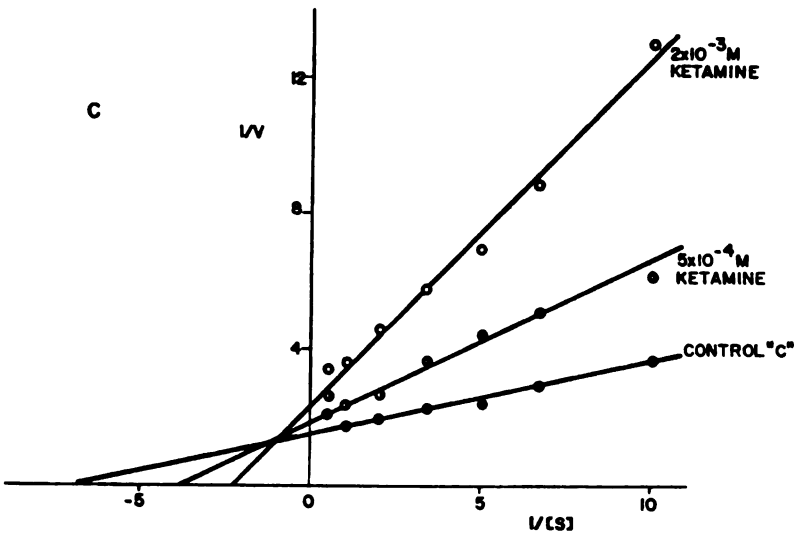


Figure 5-6. Lineweaver-Burk plot of inhibition of purified acetylcholinesterase, Form C by two concentrations of ketamine. Substrate concentrations ranged from 0.1 to 5mM ATC. Enzyme activity was determined by the method of Ellman et al (65) at optimum pH (7.9). Control enzyme activity was 575 mmoles ATC hydrolyzed/mg protein/hour at optimum substrate concentration (1mM). Lines were calculated by the least squares method of linear regression analysis.

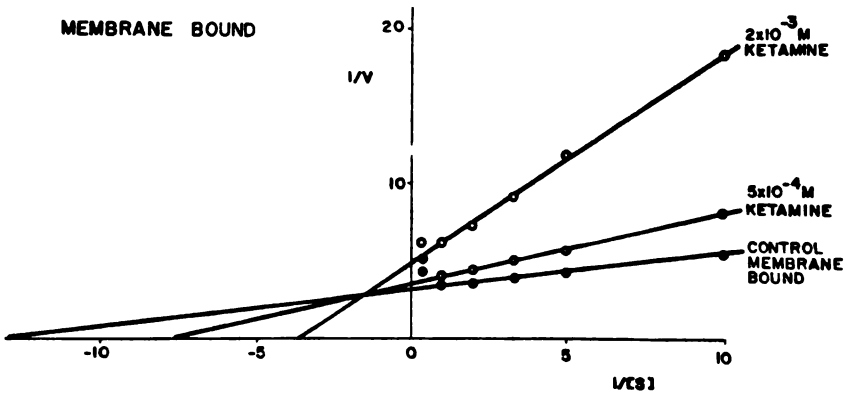


Figure 5-7. Lineweaver-Burk plot of inhibition of acetylcholinesterase isolated from nerve endings as a membrane bound form by two concentrations of ketamine. Substrate concentrations ranged from 0.1 to 5mM ATC. Enzyme activity was determined by the method of Ellman et al (65) at optimum pH (7.9). Control enzyme activity was 45 umoles ATC hydrolyzed/mg protein/hour at optimum substrate concentration (1mM). Lines were calculated by the least squares method of linear regression analysis.

TABLE 5-3. BRAIN ACETYLCHOLINESTERASE:

INHIBITORY CHARACTERISTICS OF KETAMINE

Acetylcholinesterase Form	Apparent K_i (Molar)	I_{50} (Molar)
A	6.9×10^{-4}	1.7×10^{-3}
B	6.65×10^{-4}	2.0×10^{-3}
C	5.06×10^{-4}	1.6×10^{-3}
Membrane bound	4.95×10^{-4}	2.6×10^{-3}

Inhibitory constants were calculated for each of the enzyme forms investigated as described in Chapter 5, Methods. The apparent K_i is an approximation of the dissociation constant of the enzyme:inhibitor complex. I_{50} is the inhibitor concentration necessary to reduce enzyme activity to half its control value.

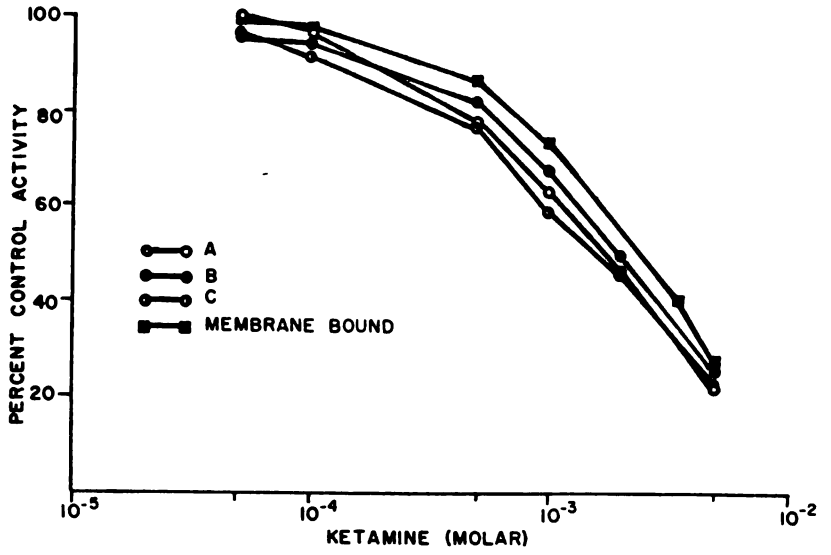


Figure 5-8. Effect of ketamine concentration on acetylcholinesterase activity of the three purified forms, A, B, and C, and the membrane bound form of the enzyme. Acetylcholinesterase activity was determined by the method of Ellman et al (65) at optimum pH (7.9) and optimum substrate concentration (1mM). Control activity expressed as umoles ATC hydrolyzed/mg protein/hour was: 480,000, Form A; 400,000, Form B; 575,000, Form C; and 45, membrane bound form.

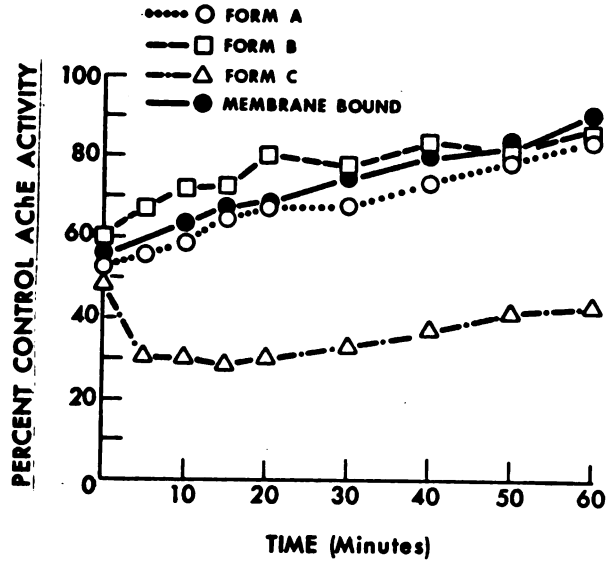


Figure 5-9. Return of acetylcholinesterase activity following removal of ketamine by dialysis. Enzyme forms were inhibited by 2 x 10^{-3}M ketamine. Control enzyme activity at pH 7.9 and 1mM ATC expressed as umoles ATC hydrolyzed/mg protein/hour was 480,000, 400,000, 575,000, and 45 for purified forms, A, B, C, and the membrane bound form, respectively. Data are plotted as the percent of the inhibited activity to the control activity determined at each time interval.

bound preparation, inhibition was reversible and enzyme activity returned almost completely to control levels after 1 hour of dialysis to remove ketamine (2×10^{-3}M). Preincubation had no marked effect on the reversal of inhibition of these forms. However, there was an increase in inhibition of purified form C during the initial removal of ketamine, after which there was a slow return to activity which did not exceed the initial inhibited activity in 1 hour. This unusual effect on form C was duplicated by a repetitive experiment on the same enzyme preparation.

Degree of inhibition of acetylcholinesterase was influenced to some extent by pH. Alterations in pH may result in changes in charge on the enzyme molecule itself and changes in the ionization state of the inhibitory agent. Enzyme inhibition with 1×10^{-3}M ketamine at optimum pH (7.9) was approximately 24 percent for the membrane bound preparation (Figure 5-10). While enzyme inhibition was less at the higher pH of 8.5, inhibition was found to be greater at pH 7.5 and pH 7.1 by approximately 7 percent. At the very low pH of 6.2, ketamine did not inhibit the enzyme as well as at pH 7.9.

To insure that inhibition of AChE derived from bovine caudate nucleus tissue was not peculiar to this cerebral tissue alone, the inhibition by ketamine of AChE derived from rat brain was also investigated. Both solubilized and particulate associated acetylcholinesterase from rat whole brain were inhibited by ketamine. Inhibition occurred at

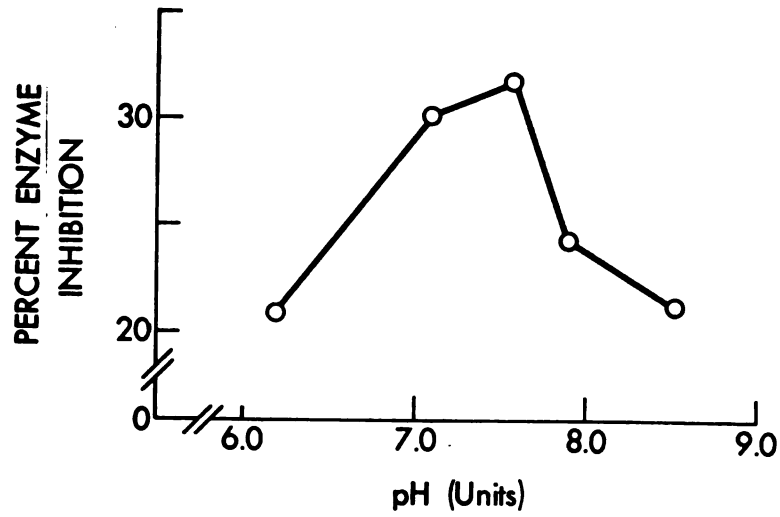


Figure 5-10. Effect of pH on the inhibition by ketamine (1×10^{-3}M) of membrane bound acetylcholinesterase. Enzyme activity was determined by the method of Ellman et al (65) at optimum substrate concentration (1mM). Control enzyme activity was 45 umoles ATC hydrolyzed/mg protein/hour at pH 7.9. Sodium phosphate buffers at various pH values were used to alter pH. The pH values reported are the final pH values which were measured for each reaction mixture.

concentrations similar to those necessary to inhibit the pure enzymes obtained from bovine caudate nucleus tissue (Figure 5-11).

A primary breakdown product of ketamine (metabolite I) was also found to inhibit the purified forms of AChE, but at concentrations approximately four times greater than those necessary for inhibition by ketamine (Figure 5-12). Metabolite I did not appear to potentiate or antagonize the inhibition of these enzymes by ketamine. When both inhibitors were simultaneously available for interaction with AChE the resulting degree of inhibition was not different from that which occurred with ketamine alone (Table 5-4). Increasing the concentration of metabolite I from 1 to 2mM did not effect the degree of enzyme inhibition in the presence of ketamine.

DISCUSSION

In mammals, acetylcholine (Ach) is established to be a neurotransmitter at skeletal myoneural junctions, in ganglia of the autonomic nervous system, at parasympathetic neuroeffector junctions, and at certain synapses in the spinal cord. If analogy to these sites is allowed, it is highly probable that Ach is also a neurotransmitter at synapses in higher cerebral centers. This conclusion is consistent with evidence of neuronal excitation and inhibition following the iontophoretic application of Ach to various brain areas (16, 149). Again, by analogy to peripheral and spinal sites,

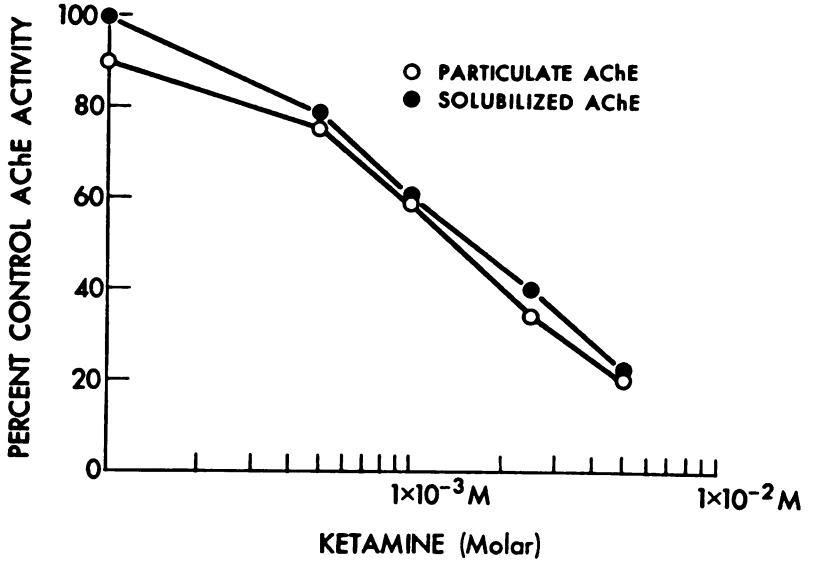


Figure 5-11. Effect of varying concentrations of ketamine on a particulate bound form and a solubilized form of rat brain acetylcholinesterase. Acetylcholinesterase activity was determined by the method of Ellman et al (65) at optimum pH (7.9) and optimum substrate concentration (1mM). Control activity was 9.38 and 14.30 umoles ATC hydrolyzed/mg protein/hour for the bound form and solubilized enzyme, respectively.

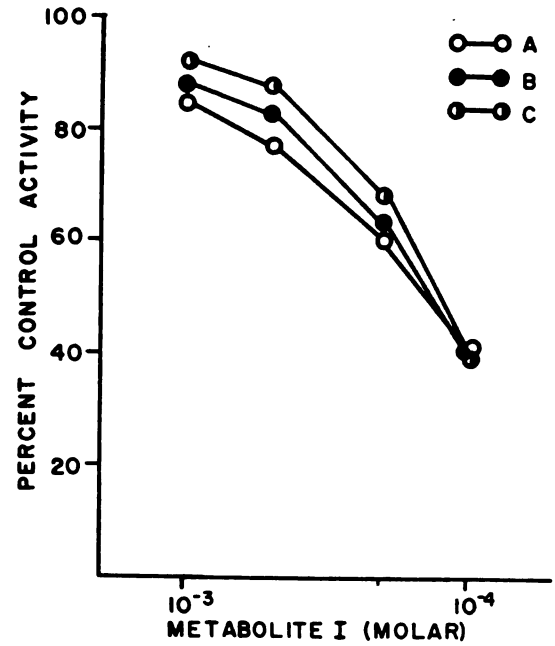


Figure 5-12. Effect of metabolite I on acetylcholinesterase activity of the three purified enzyme forms, A, B, and C as a function of concentration. Acetylcholinesterase activity was determined by the method of Ellman et al (65) at optimum pH (7.9) and optimum substrate concentration (1mM). Control activity expressed as mM ATC hydrolyzed/mg protein/hour was 480, 400, and 575 for forms A, B, and C, respectively.

TABLE 5-4. EFFECT OF KETAMINE AND METABOLITE I IN COMBINATION ON ACETYLCHOLINESTERASE PURIFIED FORM C

Concentration (Molar)		Percent Control AChE Activity
Ketamine	Metabolite	
1×10^{-3}	-	64
1×10^{-3}	1×10^{-3}	68
1×10^{-3}	2×10^{-3}	64
2×10^{-3}	-	48
2×10^{-3}	1×10^{-3}	53
2×10^{-3}	2×10^{-3}	54

The reaction mixture contained either ketamine, or ketamine and metabolite I in the concentrations indicated. Acetylcholinesterase activity was measured by the method of Ellman et al (65), and control enzyme activity was 575 mmoles ATC hydrolyzed/mg protein/hour at pH 7.9 with 1mM ATC. Inhibition with metabolite I was 9 and 12 percent at 1×10^{-3}M and 2×10^{-3}M, respectively. Data are averages of duplicate assays.

termination of the action of Ach centrally is presumed to occur via its inactivation by AChE. Consequently AChE probably plays a key role in the modulation of Ach levels available for post-synaptic interaction and subsequent information transfer between neuronal cells. Modification of the activity of AChE by drugs can indirectly affect the available concentration of Ach, and by this mechanism, could alter cerebral excitability. Attempts to define the precise molecular events of enzyme alteration by drugs must of necessity involve purified enzymes.

Since these studies were concerned with the potential interaction of ketamine and AChE in an attempt to understand more precisely some of the diverse central effects of ketamine, it was desirable to utilize an enzyme of cerebral origin. Although the non-mammalian eel enzyme was readily available, there was evidence for differences in eel and brain AChE (25) indicating a potential hazard in extrapolating data acquired utilizing the eel enzyme, to cerebral forms of AChE. Caudate nucleus tissue was chosen because of the high degree of enzyme activity located in this area (105) insuring sufficient yield of AChE following a multistep purification procedure.

Preparation of mammalian AChE by this procedure resulted in a 23,000 fold purification, isolating three active forms of the brain enzyme. Although stability of these preparations presented a minor difficulty due to rapid loss of enzyme activity, this was overcome by maintaining the

purified enzymes in a concentrated form and diluting with buffered sucrose immediately prior to the kinetic assays. The stability problem was presumably a result of the low protein concentration obtained in the final step of the purification procedure.

Drug modification of purified forms of mammalian brain AChE may have been altered by some artifact occurring during the purification procedure. To examine this, as well as the possibility that localization of AChE in the intact membrane influences its modification by drugs, nerve endings containing a synaptosomal membrane bound form of AChE were utilized for comparison of ketamine and metabolite I interactions with the purified forms.

Results following differential centrifugation procedures (Table 5-1) compare favorably with those reported by others (50, 108) with respect to protein and AChE activity. High protein and AChE activity were found in the mitochondrial fraction, while the high specific activity and yield of AChE in the microsomal supernatant may be due in part to nonspecific esterases (106). The primary mitochondrial fraction presumably contained presynaptic nerve endings enclosing mitochondria and synaptic vesicles, and an associated postsynaptic membrane (49). When this fraction was submitted to osmotic shock and subsequently centrifuged, the resultant M_1 pellet lacked synaptic vesicles but contained disrupted nerve endings, myelin, and mitochondria (49, 108). Although this procedure was originally developed as a technique to

isolate synaptic vesicles (49), this has been adapted in the present study for the further isolation of AChE enriched synaptic membranes.

Resuspension and application of the M_1 fraction to a four layer sucrose density gradient (Table 5-2) permitted the separation of nerve ending membranes (subfractions 2, 3, and 4) containing approximately 4-fold higher AChE activity (41 - 56 umole ATC/mg/hr) than that of the most dense particulate fraction, in close agreement to the results of others (50, 108, 161). Succinic dehydrogenase is considered to have a specific mitochondrial localization (50, 76, 161, 170) while sodium, potassium, adenosine triphosphatase appears to be localized in nerve endings (47, 170). These two enzyme markers were used as criteria for the purity of the synaptic membrane preparation. Eighty percent of the succinic dehydrogenase activity was found in the most dense particulate fraction while the isolated membrane fractions contained only 1-3 percent (Table 5-2). Sodium potassium ATPase activity was highest in the membrane fractions which also contained high AChE activity (Table 5-2).

Morphological studies have also been used to characterize and document the homogeneity of the fractions containing nerve ending membranes, prepared in the same manner (48, 76, 108). The existance of different types of synaptic membranes has been suggested. For example, the occurrance of high levels of C^{14}-d-tubocurarine in fractions 2 and 3 with considerably less d-tubocurarine occurring in fraction 4 (47) may be indicative of the cholinergic nature of membrane

fractions 2 and 3. On this basis, as well as the appearance of less mitochondrial contamination of fractions 2 and 3, these preparations were exclusively used for the subsequent kinetic analyses of ketamine and metabolite I.

Ketamine hydrochloride was found to inhibit all forms of mammalian brain acetylcholinesterase used in these studies. This inhibition may be important in elucidating the molecular nature of brain AChE and may aid in obtaining a more detailed knowledge of the events occurring during interaction of the enzyme with inhibitory agents. On the basis of Lineweaver-Burk plots (Figures 5-4 thru 5-7) and Hunter and Downs plots, the mechanism of inhibition may be classified as mixed, indicating in part, a competitive component which interferes with the binding of substrate. The simultaneous presence of a noncompetitive component is implied by the increase in 1/Vmax values. This is considered indicative of an interaction of ketamine with the enzyme in a manner which prevents breakdown of the active enzyme:substrate complex.

Many studies on the regulation of various forms of AChE have alluded to the existance of at least one non-catalytic site on the enzyme which is separate and distinct from the active site. Due, in part, to the cationic nature of certain weak inhibitors of AChE such as d-tubocurarine and gallamine (9,30,103,153,196), succinylcholine (103,196), decamethonium (103, 153, 196), TMA (86), TEA (153, and atropine (90, 91, 92, 93), the non-catalytic site (or sites) on AChE is thought

to consist of a negative charge. These cholinergic blocking agents appear to interact with this second, regulatory site on the enzyme which under certain circumstances may alter the catalytic power of the active site, and in this manner, produce mixed inhibition. Since ketamine is partially protonated at physiological pH, it is reasonable to assume that it also can interact with available regulatory anionic sites on the enzyme.

Interpretations of kinetic data derived from Lineweaver-Burk plots are limited with respect to the exact molecular events occurring. Kinetics defined as mixed inhibition may be explained by more than one potential type of interaction of the inhibitory agent with the macromolecule. Postulation of a regulatory anionic site which can alter the interactions at the active site implies a single site for binding of the inhibitor with the enzyme. Mixed kinetics could also result from a dual interaction of the inhibitor (ketamine in this case), with the active site (competitive component) as well as to another site (non-competitive component) on the enzyme. It is also possible that two molecules of the inhibitor bind at two sites distinct from the active site in order to alter the active site and thereby produce mixed inhibition.

The peak of the substrate inhibition curve for each of the enzyme forms was found to shift in the presence of ketamine. This is best explained as a reflection of the competitive aspect of inhibition resulting in a decrease in the affinity of the enzyme for the substrate. From structural

considerations regarding the cationic nature of ketamine, if substrate inhibition occurs at an allosteric anionic site (91) or at the anionic portion of the active site (193), competition for substrate binding at either of these sites may occur with ketamine.

Loss of enzyme activity following ketamine inhibition of purified forms A and B and membrane bound enzyme was not due to permanent inactivation of the enzyme as judged by the almost complete reversal of the inhibition following removal of ketamine by dialysis. The apparent potentiation of loss of enzyme activity with form C following removal of ketamine may be due to an alteration in C which promoted its inactivation. This difference between C and the other enzyme forms is consistent with other observations (26) suggesting differences among the multiple forms of purified mammalian brain AChE.

Since inhibition of AChE with ketamine is considerably more complex than inhibition with the conventional competitive and irreversible inhibitors, it is difficult to obtain an accurate assessment of K_i values. However, calculations of apparent K_i based on the slopes of Lineweaver-Burk plots of mixed inhibition resulted in similar values among the various forms used in this study. From this and the work of others (196) it may be concluded that the purification process did not alter the enzymes with respect to ketamine inhibition when compared to the more "physiologically located" membrane-bound form.

While inhibition of mammalian brain AChE differed from the classical competitive and irreversible inhibitors in that concentrations of ketamine in the millimolar range were required for enzyme inhibition, these concentrations were not markedly different from that of other inhibitors (91, 132, 153, 193) thought to bind to the enzyme at a regulatory site. The degree of enzyme inhibition with ketamine was dependent on substrate concentration. At lower substrate concentrations, such as may occur *in vivo* (102), enzyme inhibition by ketamine would be greater than at the 1mM concentration of ATC under the *in vitro* conditions of these studies.

Inhibition of AChE by ketamine was dependent on pH (Figure 5-10) in a manner similar to that previously observed for other ionizable drugs (107, 191). At pH values lower than pH 7.9 which was optimum for these *in vitro* studies, a higher concentration of ketamine would exist in the cationically charged form since the pka of ketamine (Chapter 4) is 7.45. At pH values of 7.0 and 7.5, there was greater inhibition with ketamine while at pH 8.5 inhibition was less when compared to the optimum condition of pH 7.9. This is consistent with the postulation of an interaction of ketamine with AChE which is, at least partially, dependent on the cationic form of ketamine. However, the changes in the degree of inhibition produced by these pH manipulations were not marked and did not appear to be stoichiometric with anticipated changes in the ratios of ionized:non-ionized species. This might be explained by the fact that changes

in pH affect not only the ionized state of ketamine, but also the charged areas on the acetylcholinesterase macromolecule. Indeed, brain AChE activity in the absence of inhibitory agents is markedly influenced by pH (26, 162). At low pH (below neutrality) it is also possible that protons (H^+ ions) could compete for anionic sites on the enzyme thus reducing the degree of ketamine inhibition. From the standpoint of physiological significance of ketamine interaction with AChE, it is relevant that the degree of enzyme inhibition is greater at physiological pH (7.4) than at the pH for optimum enzyme activity *in vitro*.

The physiological studies on ketamine disposition utilized animal tissue from the rat (Chapters 3 and 4) while this biochemical investigation utilized bovine neuronal tissue exclusively. In order to facilitate correlation of these two studies, ketamine inhibition of acetylcholinesterase was examined with enzyme preparations from rat brain. Inhibition produced with these preparations was not strikingly different from inhibition of bovine neuronal AChE (Figure 5-11) with respect to the degree of inhibition. The similarity of ketamine inhibition of bovine and rodent AChE is consistent with the possibility that this action is also likely in other species including man. Evidence does exist for differences in species sensitivity of the inhibition of diaphragm acetylcholinesterase of rat and guinea pig preparations to pralidoxime and tetraethylpyrophosphate (10). Species variation might also partially explain the different

inhibition effects of hemicholinium-3 and neostigmine observed between rat brain and bovine erthrocyte acetylcholinesterase (56).

Although there was inhibition of AChE *in vitro* with metabolite I, the inhibition occurred only at concentrations considerably higher than would be expected in the CNS following ketamine administration (Figure 5-12). In the concentrations used in these studies, there was no apparent effect of metabolite I on the interaction of ketamine with AChE (Table 5-4). If some of ketamine's actions are mediated via inhibition of AChE, it must be concluded that there is no involvement of metabolite I, and that competition with metabolite I is not directly responsible for the termination of any clinical actions resulting from ketamine inhibition of AChE.

The documentation of inhibitory actions of any drug on an enzyme *in vitro* has limited relevance unless inhibition occurs at concentrations which are achievable in the intact animal following conventional pharmacological doses. It is apparent that high brain levels of ketamine were rapidly achieved (Figure 3-1) and that ketamine is potentially available for cerebral enzyme inhibition. While doses, necessary for 50 percent enzyme inhibition *in vitro* are five times the peak levels found in rat brain, the *in vivo* levels of ketamine did result in a 20 percent reduction in *in vitro* enzyme activity. As discussed previously with respect to pH and substrate concentration, there is reason

to believe that ketamine inhibition of AChE *in vivo* may be greater than that achieved *in vitro*. It is possible that even a small degree of enzyme inhibition could facilitate neural transmission in the CNS since facilitation of neurotransmitter release has been shown to occur following as little as a 15 percent inhibition of AChE at a neuromuscular site under certain conditions (67).

Since ketamine inhibition of AChE has not been observed (or looked for) in man, the physiological significance of the above studies remains uncertain. However, in view of the documented cholinergic manifestations of ketamine following its clinical anesthetic use, it seems reasonable to postulate that such inhibition of AChE could occur in man. Certainly the potential for this interaction is increased on repeated dosing of ketamine and following overdosage of ketamine. Since other drugs not primarily used for their anti-AChE activity do interact with the enzyme (9, 30, 56, 93, 132, 153, 189), it also is possible that the observed inhibition with ketamine could result in certain drug interactions. Inhibition of AChE may also be contributory to the analgetic effects of ketamine similar to the correlation of analgesia with AChE inhibition induced by normeperidine congeners (32). There is also evidence for a tentative relationship of anticholinesterases and behavioral disturbances (197). Thus, it is possible that inhibition of AChE by ketamine may contribute to the psychotomimetic effects observed following ketamine administration.

Although primary interest in these studies was focused on the central properties of ketamine, the demonstration of AChE inhibition may have relevance in the periphery as well. It is possible that inhibition of AChE at a neuromuscular site could contribute to the muscular rigidity which is observed during the ketamine induced anesthetic state. Since salivation and lacrimation may also be peripheral manifestations of ketamine, these too, may result from peripheral AChE inhibition.

There is evidence in the literature documenting similarities between AChE and the cholinergic receptor. For example, neuromuscular blocking agents, parasympatholytics and ganglionic stimulators and blockers have been shown to inhibit the enzyme as well as to interact with the cholinergic receptor (9, 30, 86, 91, 92, 153, 196). Since ketamine inhibition of AChE strongly resembled the allosteric inhibitory properties of these other drugs, and ketamine shows some structural resemblance to nicotine, this interaction of ketamine and AChE may be a reflection of a more direct interaction of ketamine with the cholinergic receptor in the periphery or in the CNS.

SUMMARY

Ketamine is an anesthetic agent which produces a clinical state characterized by purposeless movements, open eyes, nystagmus, hypertension, tachycardia, and psychotomimetic effects. In order to facilitate the clinical management of the ketamine-induced anesthetic state, this study investigated the cerebral distribution and metabolism of ketamine with particular reference to the mode of entry of ketamine in cerebral tissue. In order to do this, a gas chromatographic procedure reported for the analysis of ketamine and two metabolites in plasma was modified and adapted for the measurement of ketamine and its metabolites in brain and tissues other than plasma.

Intravenous injection of ketamine in doses greater than 10 mg/kg in the rat resulted in an immediate loss of righting reflex. A dose of 60 mg/kg was found to be close to the LD_{50}. Following injection of 20 mg/kg, maximal brain levels of ketamine occurred within 1 minute. Brain levels then declined parallel to the decline in plasma levels during the 60 minute time period measured. Following injection of ketamine (20 mg/kg), recovery of the righting reflex (6 to 8 minutes) occurred when brain and plasma ketamine levels were approximately 30 ug/g tissue and 4.5 ug/ml, respectively. At all time intervals measured, higher concentrations of ketamine were found in CNS tissue, although brain:plasma level ratios declined from 7.1:1 at 30 seconds to 2.8:1 at 60 minutes. A study of four regional areas of the brain

indicated a small but significantly higher concentration of ketamine in the cortical region as compared to the midbrain, brain stem, and cerebellum at 30 seconds and 1 minute but not at later time intervals.

Following ketamine administration, the N-demethylated metabolite I was detectable in plasma at 30 seconds and in brain at 60 seconds. As with the parent compound, metabolite I occurred in higher concentrations in brain tissue, reaching a maximal brain:plasma level ratio of 2.5:1 at 10 minutes. No differences in regional brain distribution of metabolite I were detected. Metabolite II, the cyclohexanone oxidation product of ketamine was found in plasma in low concentration (approximately 0.5 ug/ml) at 30 and 60 minutes following in vivo ketamine administration. This metabolite was not detected at time intervals earlier than 30 minutes.

Metabolite I was further investigated with respect to its formation and pharmacological activity. On the basis of in vitro metabolic studies, no metabolism of ketamine was observed in brain homogenates or slices. Thus, it must be concluded that the high cerebral levels of metabolite I following in vivo administration of ketamine result primarily from the extra-cerebral degradation of ketamine and the subsequent cerebral uptake of the metabolic product. Other studies investigated the pharmacological activity of metabolite I following its intravenous administration in the rat. Metabolite I was found to produce anesthetic effects similar to ketamine, but was less potent than ketamine on a weight basis and slightly more toxic (LD_{50} was 50 mg/kg).

Contrary to data reported by other investigators, repeated administration of ketamine in the rat was found to produce cumulative anesthetic effects. These effects were then correlated with brain and plasma levels of ketamine and its metabolites following the repeated injections.

Potential mechanisms for the rapid occurrence of high brain levels of ketamine following its in vivo administration were further investigated using in vitro techniques. Ketamine was found to rapidly accumulate in rat cortical slices reaching maximal tissue:media ratios of 2.3:1 within 5 minutes following incubation. The presence of two metabolic inhibitors, dinitrophenol and iodoacetate had no effect on the accumulation of ketamine in rat cortical slices. This is consistent with the postulate that energy is not necessary for the uptake of ketamine into cerebral tissue and that ketamine uptake is not dependent on an active transport mechanism in the CNS.

Cerebral accumulation of ketamine was further investigated by measuring the association of ketamine with subcellular fractions of brain tissue. From these data, cellular integrity was not found to be necessary for the accumulation of ketamine in brain tissue. At equilibrium, the ratio of ketamine associated with tissue to external media (or plasma) was similar for the subcellular fraction consisting only of particulate material, the brain slice preparation, and the brain following in vivo administration of ketamine.

In further studies, ketamine was found to have a pk_a of 7.5 and to be highly lipid soluble as determined by measurement of its distribution into organic solvents from aqueous buffer at pH 7.4. On the basis of the distribution data obtained using the various cerebral preparations and the partitioning characteristics of ketamine, it would appear that tissue distribution of ketamine is similar to that of the short acting barbiturate anesthetic agents and is dependent primarily on lipid solubility, blood flow, and organ mass.

Since some of the clinical effects of ketamine may be parasympathomimetic in origin, and since ketamine is protonated at physiological pH, the interaction of ketamine with mammalian brain acetylcholinesterase was investigated. Ketamine was found to inhibit multiple forms of this enzyme under *in vitro* conditions. This inhibition occurred at concentrations commensurate with the concentrations available in brain tissue following the *in vivo* administration of a dose of ketamine which produced a 6-8 minute loss of righting reflex in rats. The interaction of ketamine with the multiple forms of brain acetylcholinesterase was further defined as mixed inhibition and was found to be pH dependent and reversible on removal of the anesthetic agent.

REFERENCES

1. Abadom PN, Scholefield PG: Amino acid transport in brain slices. I. The relation between energy production and the glucose-dependent transport of glycine. Canad J Biochem & Physiol 40:1575, 1962
2. Abadom PN, Scholefield PG: Amino acid transport in brain cortex slices. III. The utilization of energy for transport. Canad J Biochem & Physiol 40:1603, 1962
3. Adriani J: The Pharmacology of Anesthetic Drugs. Charles C Thomas, Springfield, Illinois 1967
4. Altman PL: Blood and Other Body Fluids. Federation of American Societies for Experimental Biology, Washington, D.C., 1961
5. Arrigoni E, Manzo L, Sarti P: Metabolism in organs isolated in situ. III. Demethylation and acetylation of aminopyrine in the brain. Boll Soc Ital Biol Sper 43:1461, 1967
6. Baldessarini RJ, Kopin IJ: Tritiated norepinephrine: release from brain slices by electrical stimulation. Science 152:1630, 1966
7. Banay-Schwartz M, Piro L, Lajtha A: Relationship of ATP levels to amino acid transport in slices of mouse brain. Arch Biochem Biophys 145:194, 1971
8. Becsey L, Malamed S, Radnay P, Foldes FF: Reduction of the psychotomimetic and circulatory side-effects of ketamine by droperidol. Anesthesiology 37:536, 1972

9. Belleau B, DiTullio V: Specific labelling of the curare binding sites of acetylcholinesterase and some properties of the modified enzyme. Canad J Biochem 49:1131, 1971

10. Berry WK: Some species differences in the rates of reaction of diaphragm particulate acetylcholinesterases with tetraethyl pyrophosphate and pralidoxime. Biochem Pharmacol 20:1333, 1971

11. Born GVR: The fate of 5-hydroxytryptamine in a smooth muscle and in connective tissue. J Physiol 161:160, 1962

12. Bosomworth PP: Ketamine symposium - comments by moderator. Anesth Analg 50:471, 1971

13. Bovill JG, Dundee JW: Attempts to control the cardiostimulatory effect of ketamine in man. Anaesthesia 27:309 1972

14. Bovill JG, Dundee JW, Coppel DL, Moore J: Current status of ketamine anaesthesia. Lancet 1:1285, 1971

15. Bradburn CC: Severe hypotension following induction with propanidid. Br J Anesth 42:362, 1970

16. Bradley PB: Synaptic transmission in the central nervous system and its relevance for drug action. Intl Rev Neurobiol 2:1, 1968

17. Brice DD, Hetherington RR, Utting JE: A simple study of awareness and dreaming during anesthesia. Br J Anesth 42:535, 1970

18. Brodie BB, Kurz H, Schanker LS: The importance of dissociation constant and lipid-solubility in influencing the passage of drugs into the cerebrospinal fluid. J Pharmacol Exp Ther 130:20, 1960

19. Brodie BB, Mark LC, Papper EM, Lief PA, Bernstein E, Rovenstine EA: The fate of thiopental in man and a method for its estimation in biological material. J Pharmacol Exp Ther 98:85, 1950

20. Brown SS, Lyons SM, Dundee JW: Intraarterial barbiturates: A study of some factors leading to intravascular thrombosis. Br J Anesth 40:13, 1968

21. Bull RJ, Trevor AJ: Saxitoxin, tetrodotoxin and the cation flux and metabolism of isolated cerebral tissues. J Neurochem 19:999, 1972

22. Butler TC: Fundamentals of Drug Metabolism and Drug Disposition. Edited by BN LaDu, HG Mandel, EL Way. Baltimore, Williams and Wilkins Company, 1971, p 58

23. Carlsson A: Structural specificity for inhibition of [^{14}C]-5-hydroxytryptamine uptake by cerebral slices. J Pharm Pharmacol 22:729, 1970

24. Chan SL, Quastel JH: Effects of neurotropic drugs on sodium influx into rat brain cortex in vitro. Biochem Pharmacol 19:1071, 1970

25. Chan SL, Shirachi DY, Bhargava HN, Gardner E, Trevor AJ: Purification and properties of multiple forms of brain acetylcholinesterase (E.C.3.1.1.7). J Neurochem 19:2747, 1972

26. Chan SL, Shirachi DY, Trevor AJ: Purification and properties of brain acetylcholinesterase (E.C.3.1.1.7). J Neurochem 19:437, 1972

27. Chang T, Dill WA, Glazko AJ: Metabolic disposition of 2-(o-chlorophenyl)-2-methylamino-cyclohexanone-HCl (Cl-581) in laboratory animals and in man. Fed Proc 24:268, Abstr 770, 1965

28. Chang T, Glazko AJ: A gas chromatographic assay for ketamine in human plasma. Anesthesiology 36:401, 1972

29. Chang T, Savory A, Albin M, Goulet R, Glazko AJ: Metabolic disposition of tritium-labelled ketamine (Ketalar; Cl-581) in normal human subjects. Clin Res 18:597, 1970

30. Changeux J-P: Responses of acetylcholinesterase from torpedo marmorata to salts and curarizing drugs. Mol Pharmacol 2:369, 1966

31. Chen G: Ketamine, Anaesthesiologie und Wiederbelebung. Bd 40. Edited by H Kreuscher. Berlin-Heidelberg-New York, Springer-Verlag, 1969, pp 1-11

32. Christian St, Gorodetzky CW, Lewis DU: Structure-activity relationships of normeperidine congeners on cholinesterase systems in vitro and analgesia in vivo. Biochem Pharmacol 20:1167, 1971

33. Clarke RSJ: The eugenols-propanidid, Newer Intravenous Anesthetics. Edited by RSJ Clarke, Int Anesth Clin 7:43, 1969

34. Cohen EN, Chow KL, Mathers L: Autoradiographic distribution of volatile anesthetics within the brain. Anesthesiology 37:324, 1972

35. Cohen ML, Chan SL, Trevor AJ: In vitro inhibition of rat brain norepinephrine uptake and acetylcholinesterase by ketamine. Fed Proc Abstr, in press, 1973

36. Cohen SR, Blasberg R, Levi G, Lajtha A: Compartmentation of the inulin space in mouse brain slices. J Neurochem 15:707, 1968

37. Conney AH: Fundamentals of Drug Metabolism and Drug Disposition. Edited by BN LaDu, MG Mandel, EL Way. Baltimore, Williams and Wilkins Company, 1971, p. 253

38. Cooper C, Lehninger AL: Oxidative phosphorylation by an enzyme complex from extracts of mitochondria. IV. Adenosine triphosphatase activity. J Biol Chem 224:547, 1957

39. Corssen G, Domino EF: Dissociative anesthesia: Further pharmacologic studies and first clinical experience with the phencyclidine derivative CI-581. Anesth Analg 45:29, 1966

40. Corssen G, Domino EF, Bree RL: Electroencephalographic effects of ketamine anesthesia in children. Anesth Analg 48:141, 1969

41. Corssen G, Gutierrez J, Reves JG, Huber FC: Ketamine in the anesthetic management of asthmatic patients. Anesth Analg 51:588, 1972

42. Corssen G, Miyasaka M, Domino EF: Changing concepts in pain control during surgery: Dissociative anesthesia with CI-581. A progress report. Anesth Analg 47:746, 1968

43. Corssen G, Oget S: Dissociative anesthesia for the severely burned children. Anesth Analg (Cleve) 50: 95, 1971

44. Corssen G, Oget S, Reed PC: Computerized evaluation of psychic effects of ketamine. Anesth Analg 50:397, 1971

45. Dawson B, Michenfelder JD, Theye RA: Effects of ketamine on canine cerebral blood flow and metabolism: Modification by prior administration of thiopental. Anesth Analg 50:443, 1971

46. Dengler HJ, Michaelson IA, Spiegel HE, Titus E: The uptake of labeled norepinephrine by isolated brain and other tissues of the cat. Int J Neuropharmacol 1:23, 1962

47. DeRobertis E, Alberici M,Rodriguez G, Azcurra JM: Isolation of different types of synaptic membranes from the brain cortex. Life Sci 5:577, 1966

48. DeRobertis E, Rodriguez G: Structural components of the synaptic region. Handbook of Neurochemistry. Vol II. Edited by A Lajtha. New York, Plenum Press, 1969, p 365

49. DeRobertis E, Rodriguez G, Pellegrino A: Isolation of synaptic vesicles from nerve ending of the rat brain. Nature 194:794, 1962

50. DeRobertis E, Pellegrino A, Rodriguez G, Salganicoff L: Cholinergic and non-cholinergic nerve endings in rat brain - I. J Neurochem 9:23, 1962

51. Dill WA, Chucot L, Chang T: Determination of ketamine in blood plasma. Anesthesiology 34:73, 1971

52. Dingell JV, Sulser F, Gillette JR: Species differences in the metabolism of imipramine and desmethylimipramine (DMI). J Pharmacol Exp Ther 143:14, 1964

53. Doenicke A, Krumey I, Kugler J, Klempa J: Experimental studies of the breakdown of epontol: Determination of propanidid in human serum. Br J Anaesth 40:415, 1968

54. Domino EF, Chodoff P, Corssen G: Human pharmacologic effects of CI-581, a phencyclidine derivative. J Clin Pharmacol 6:279, 1965

55. Domino EF, Chodoff P, Corssen G: Human pharmacology of CI-581, a new intravenous agent chemically related to phencyclidine. Fed Proc 24:268, Abstr 771, 1965

56. Domino EF, Shellenberger MK, Frappier J: Inhibition of acetylcholinesterase *in vitro* by hemicholinium-3. Arch Int Pharmacodyn 176:42, 1968

57. Dowdy EG, Kaya K: Studies of the mechanism of cardiovascular responses to CI-581. Anesthesiology 29:931, 1968

58. Drury WL, Clark LC: Ketamine failure in acute brain injury. Anesth Analg 49:859, 1970

59. Dundee JW: Current views on the clinical pharmacology of the barbiturates. Newer Intravenous Anesthetics. Edited by RSJ Clarke. Int Anesth Clin 7:3, 1969

60. Dundee JW: Comparative analysis of intravenous anesthetics. Anesthesiology 35:137, 1971

61. Dundee JW, Isaac M: Intravenous ethanol. Newer Intravenous Anesthetics. Edited by RSJ Clarke. Int Anesth Clin 7:67, 1969

62. Dundee JW, Keilty SR: Diazepam. Newer Intravenous Anesthetics. Edited by RSJ Clarke. Int Anesth Clin 7:91, 1969

63. Eckart I: Ketamine, Anaesthesiologie und Wiederbelebung. Bd 40. Edited by H Kreuscher. Berlin-Heidelberg-New York, Springer-Verlag, 1969, pp 200-205

64. Elison C, Elliott HW: N- and O-demethylation of some narcotic analgesics by brain slices from male and female Long-Evans rats. Biochem Pharmacol 12:1363, 1963

65. Ellman GL, Courtney D, Andres V, Featherstone RM: A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7:88, 1961

66. Erbugth PH, Reiman B, Klein RL: The influence of chlorpromazine, diazepam, and droperidol on emergence from ketamine. Anesth Analg 51:693, 1972

67. Ferry CB, Marshall AR: Action of edrophonium on acetylcholinesterase at the mammalian neuromuscular junction. Br J Pharmacol 43:435P, 1971

68. Franck G, Cornette M, Schoffeniels: The cationic composition of incubated cerebral cortex slices. J Neurochem 15:843, 1968

69. Frey HH, Schulz R: Time course of the demethylation of trimethadione. Acta Pharmacol et Toxicol 28:477, 1970

70. Garfield JM, Garfield FB, Stone JG, Hopkins D, Johns LA: A comparison of psychologic responses to ketamine and thiopental-nitrous oxide-halothane anesthesia. Anesthesiology 36:329, 1972

71. Gaudette LE, Brodie BB: Relation between the lipid solubility of drugs and their oxidation by liver microsomes. Biochem Pharmacol 2:89, 1959

72. Glazko AJ: Personal communication. Parke, Davis and Company, June 28, 1972

73. Glazko AJ: Personal communication. Parke, Davis and Company, February 26, 1973

74. Glazko AJ: Personal communication. Parke, Davis and Company, March 15, 1973

75. Goldstein A, Aronow L: The durations of action of thiopental and pentobarbital. J Pharmacol Exp Ther 128:1, 1960

76. Gray EG, Whittaker VP: The isolation of nerve endings from the brain: An electron-microscopic study of cell fragments derived by homogenization and centrifugation. J Anat 96:79, 1962

77. Green DE, Mii S, Kohout PM: Studies on the terminal electron transport system. I. Succinic dehydrogenase. J Biol Chem 217:551, 1955

78. Heald PJ: The effect of metabolic inhibitors on respiration and glycolysis in electrically stimulated cerebral-cortex slices. Biochem J 55:625, 1953

79. Hendley ED, Taylor KM, Snyder SH: ^{3}H-Normetanephrine uptake in rat brain slices. Relationship to extraneuronal accumulation of norepinephrine. Europ J Pharm 12:167, 1970

80. Hillman HH, Campbell WJ, McIlwain H: Membrane potentials in isolated and electrically stimulated mammalian cerebral cortex. J Neurochem 10:325, 1963

81. Hodshon BJ, Ferrer-Allado T, Brechner VL, Cho AK: A gas chromatographic assay procedure for ketamine in plasma. Anesthesiology 36:506, 1972

82. Hogben CAM, Tocco DJ, Brodie BB, Schanker LS: On the mechanism of intestinal absorption of drugs. J Pharmacol Exp Ther 125:275, 1959

83. Hohorst HJ: Methods of Enzymatic Analysis. Edited by HU Bergmeyer. New York and London, Academic Press, 1962, p 266

84. Hunter A, Downs CE: The inhibition of arginase by amino acids. J Biol Chem 157:427, 1945

85. Hunter AR: Diazepam (Valium) as a muscle relaxant during general anaesthesia: A pilot study. Br J Anaesth 39:633, 1967

86. Iverson F: The influence of tetraethylammonium ion on the reaction between acetylcholinesterase and selected inhibitors. Mol Pharmacol 7:129, 1971

87. Iversen LL, Neal MJ: The uptake of [^{3}H] GABA by slices of rat cerebral cortex. J Neurochem 15:1141, 1968

88. Janis KM, Wright W: Failure to produce analgesia with ketamine in two patients with cortical disease. Anesthesiology 36:406, 1972

89. Jawalekar KS, Jawalekar SR, Mathur VP: Effect of ketamine on isolated murine myometrial activity. Anesth Analg 51:685, 1972

90. Kato G: Nuclear magnetic resonance study of the interaction between acetylcholine and horse serum cholinesterase. Mol Pharmacol 5:148, 1969

91. Kato G, Tan E, Yung J: Allosteric properties of acetylcholinesterase. Nature New Biology 236:185, 1972

92. Kato G, Yung J, Ihnat M: NMR studies of the interaction of eserine and atropine with acetylcholinesterase. Biochem Biophys Res Comm 40:15, 1970

93. Kato G, Yung J, Ihnat M: Nuclear magnetic resonance studies on acetylcholinesterase. The use of atropine and eserine to probe binding sites. Mol Pharmacol 6:588, 1970

94. Katz RI, Chase TN: Neurohumoral mechanisms in the brain slice. Adv Pharmacol Chemother 8:1, 1970

95. Katz RI, Kopin IJ: Release of norepinephrine-^{3}H and serotonin-^{3}H evoked from brain slices by electrical field stimulation - calcium dependency and the effects of lithium, ouabain and tetrodotoxin. Biochem Pharmacol 18:1935, 1969

96. Kayama V, Iwama K: The EEG, evoked potentials, and single-unit activity during ketamine anesthesia in cats. Anesthesiology 36:316, 1972

97. Keesey JC, Wallgren H: Movements of radioactive sodium in cerebral-cortex slices in response to electrical stimulation. Biochem J 95:301, 1965

98. Keesey JC, Wallgren H, McIlwain H: The sodium, potassium and chloride of cerebral tissues: Maintenance, change on stimulation and subsequent recovery. Biochem J 95:289, 1965

99. Ketamine - a new anaesthetic. Brit Med J 2:666, June 19, 1971

100. Ketamine hydrochloride: A new anesthetic. Medical Letter on Drugs and Therapeutics 12:70, 1970

101. Ketamine hydrochloride - a second look. Medical Letter on Drugs and Therapeutics 14:75, 1972

102. Kitz RJ: Molecular Pharmacology. Edited by RM Featherstone. New York, Marcel-Dekker, in print

103. Kitz RJ, Braswell LM, Ginsburg S: On the question: Is acetylcholinesterase an allosteric protein? Mol Pharmacol 6:108, 1970

104. Klatskin G, Kimberg DV: Recurrent hepatitis attributable to halothane sensitization in an anesthetist. New Eng J Med 28:515, 1969

105. Koelle GB: The Pharmacological Basis of Therapeutics. Edited by LS Goodman, A Gilman. London, The Macmillan Company, 1970, p 417

106. Koeppen AH, Barron KD, Bernsohn J: Redistribution of rat brain esterases during subcellular fractionation. Biochim Biophys Acta 183:253, 1969

107. Laidler KJ: The Chemical Kinetics of Enzyme Action. Oxford, Claredon Press, 1958

108. Lapetina EG, Soto EF, DeRobertis E: Gangliosides and acetylcholinesterase in isolated membranes of the rat-brain cortex. Biochim Biophys Acta 135:33, 1967

109: Lassanova M, Brechtlova M: Transport of L-glutamate and glycine in cells of rat cerebral cortex slices, kinetics of transport. Physiol Bohem 20:235, 1971

110. Lee CC, Anderson RC, Chen KK: Distribution and excretion of radioactivity in rats receiving N-methyl-C^{14}-erythromycin. J Pharmacol Exp Ther 117:265, 1956

111. Leonard BE, Tonge SR: The effects of some hallucinogenic drugs upon the metabolism of noradrenaline. Life Sci 8:815, 1969

112. Leonard BE, Tonge SR: Some effects of an hallucinogenic drug (phencyclidine) on neurohumoral substances. Life Sci 9:1141, 1970

113. Liang CC, Quastel JH: Uptake of acetylcholine in rat brain cortex slices. Biochem Pharmacol 18:1169, 1969

114. Lineweaver H, Burk D: The determination of enzyme dissociation constants. J Am Chem Soc 56:658, 1934

115. Little B, Chang T, Chucot L, Dill WA, Enrile LL, Glazko AJ, Jassani M, Kretchmer H, Sweet AY: Study of ketamine as an obstetric anesthetic agent. Am J Obstet Gynecol 113:247, 1972

116. Lotfy AO, Amir-Jahed AK, Moarefi P: Anesthesia with ketamine: indications, advantages, and shortcomings Anesth Analg (Cleve) 49:969, 1970

117. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the folin phenol reagent. J Biol Chem 193:265, 1957

118. Mannering GJ: Selected Pharmacological Testing Methods. Volume 3. Edited by A Burger. New York, Marcel Dekker, Incorporated, 1968, p 51

119. Martin AV, Alonso JMA, Casado JC: Effect of ketamine hydrochloride on oxygen and glucose uptake by brain tissue in vitro. Experientia 28:1070, 1972

120. Martin JB, Doty DM: Determination of inorganic phosphate. Anal Chem 21:963, 1949.

121. Mayer S, Maickel RP, Brodie BB: Kinetics of penetration of drugs and other foreign compounds into cerebrospinal fluid and brain. J Pharmacol Exp Ther 127:205, 1959

122. Mazel P, Henderson JF: On the relationship between lipid solubility and microsomal metabolism of drugs. Biochem Pharmacol 14:92, 1965

123. McCarthy DA, Chen GM: General anesthetic action of 2-(o-chlorophenyl)-2-methylaminocyclohexanone HCl (CI-581) in the Rhesus Monkey. Fed Proc 24:268, Abstr 769, 1965

124. McCarthy DA, Chen G, Kaump DH, Ensor C: General anesthetic and other pharmacological properties of 2-(o-chlorophenyl)-2-methylamino cyclohexanone HCl (CI-581). J New Drugs 5:21, 1965

125. McIlwain H: Metabolic response in vitro to electrical stimulation of sections of mammalian brain. Biochem J 49:382, 1951

126. McIlwain H: The effect of depressants on the metabolism of stimulated cerebral tissues. Biochem J 53:403, 1953

127. McIlwain H, Rodnight R: Practical Neurochemistry. London, J and A Churchill, 1962

128. Merkel G, Eger EI: A comparative study of halothane and halopropane anesthesia. Anesthesiology 24:346, 1963

129. Miletich DJ, Ivankovic AD, Albrecht RF, Zahed B, Ilahi A: Effect of ketamine infusion on the turnover rate of C^{14}-norepinephrine in the isolated, perfused rat heart. Abstracts of Scientific Papers. Amer Soc Anesth Annual Meeting, 1972, p 231

130. Morgan M, Loh L, Singer L, Moore PH: Ketamine as the sole anaesthetic agent for minor surgical procedures. Anaesthesia 26:158, 1971

131. Morrison JD: Drugs used in neuroleptanalgesia. Newer Intravenous Anesthetics. Edited by RSJ Clarke. Int Anesth Clin 7:141, 1969

132. Mounter LA, Ellin RI: The inhibition of acetylcholinesterase by 2-pyridinealdoxime methyl halide. Mol Pharmacol 4:452, 1968

133. Nagel F: Ketamine. Edited by R Frey, F Kern, O Mayrhofer. Berlin-Heidelberg-New York, Springer-Verlag, 1969, p 140

134. Nakazawa S, Quastel JH: Effects of inorganic salts and of ouabain on some metabolic responses of rat cerebral cortex slices to cationic and electrical stimulation. Canad J Biochem 46:355, 1968

135. Nedergaard OA: Ketamine induced potentiation of the contraction of vascular smooth muscle to sympathetic nerve stimulation and tyramine. Fifth International Congress on Pharmacology, Abstracts of Volunteer Papers #990, 1972

136. Newman WH, Valicenti JH, Bagwell EE, Pruett JK, Robie NW: Myocardial contractility during induction and steady-state ketamine anesthesia. Fifth International Congress on Pharmacology, Abstracts of Volunteer Papers #999, 1972

137. Ng KY, Chase TN, Colburn RW, Kopin IJ: Dopamine: Stimulation-induced release from central neurons. Science 172:487, 1971

138. Ng KY, Chase TN, Kopin IJ: Drug-induced release of ^{3}H-norepinephrine and ^{3}H-serotonin from brain slices. Nature 228:468, 1970

139. Okamoto K, Quastel JH: Water uptake and energy metabolism in brain slices from the rat. Biochem J 120:25, 1970

140. Pappius HM, Elliott KAC: Water distribution in incubated slices of brain and other tissues. Canad J Biochem Physiol 34:1007, 1956

141. Pender JW: Dissociative anesthesia. J Amer Med Ass 215:1126, 1971

142. Piccoli F, Lajtha A: Some aspects of uptake of non-metabolites in slices of mouse brain. Biochim Biophys Acta 225:356, 1971

143. Price HL, Dripps RD: The Pharmacological Basis of Therapeutics. Edited by LS Goodman, A Gilman. London, The Macmillan Company, 1970, p 79

144. Price HL, Kovnat PJ, Safer JN, Conner EH, Price ML: The uptake of thiopental by body tissues and its relation to the duration of narcosis. Clin Pharm Ther 1:16, 1959

145. Pscheidt GR: Demethylation of imipramine in male and female rats. Biochem Pharmacol 11:501, 1962

146. Quastel JH: Neurochemistry: The Chemistry of Brain and Nerve. Edited by KAC Elliott, IH Page, JH Quastel, CC Thomas. Springfield, Illinois, 1962, p 790

147. Question and Answer Period Following Symposium "Anesthetic Characteristics of CI-581." Anesth Analg 47:779, 1968

148. Rall DP, Stabenau JR, Zubrod CG: Distribution of drugs between blood and cerebrospinal fluid: General methodology and effect of pH gradients. J Pharmacol Exp Ther 125:185, 1959

149. Randic M, Siminoff R, Straughan DW: Acetylcholine depression of cortical neurons. Exptl Neurol 9:236, 1964

150. Richards CD, McIlwain H: Electrical responses in brain samples. Nature 215:704, 1967

151. Robertson JD: Recent Advances in Anaesthesia and Analgesia. Edited by CL Hewer. London, J and A Churchill, 1967, p 21

152. Roos A: Intracellular pH and buffering power of rat brain. Am J Physiol 221:176, 1971

153. Roufogalis, Quist EE: Relative binding sites of pharmacologically active ligands on bovine erythrocyte acetylcholinesterase. Mol Pharmacol 8:41, 1972

154. Rowland M: Clinical Pharmacology: Basic Principles in Therapeutics. Edited by KL Melmon, HF Morrelli. New York, The Macmillan Company, 1972, p 21

155. Sadove MS, Hatano S, Zahed B, Redlin T, Arastounejad P, Roman V: Clinical study of droperidol in the prevention of the side effects of ketamine anesthesia: A Preliminary Report. Anesth Analg 50:388, 1970

156. Sadove MS, Shulman M, Hatano S, Fevold N: Analgesic effects of ketamine administered in subdissociative doses. Anesth Analg 50:452, 1971

157. Sapirstein LA, Sapirstein EH, Bredemeyer A: Effect of hemorrhage on the cardiac output and its distribution in the rat. Circulation Res 8:135, 1960

158. Sari A, Okuda Y, Takeshita H: The effect of ketamine on cerebrospinal fluid pressure. Anesth Analg 51:560, 1972

159. Sasaki Y, Wagner HN: Measurement of the distribution of cardiac output in unanesthetized rats. J App Physiol 36:879, 1971

160. Schedl HP, Clifton JA: Small intestinal absorption of steroids. Gastroent 41:491, 1961

161. Sellinger OZ, Borens RN: Zonal density gradient electrophoresis of intracellular membranes of brain cortex. Biochim Biophys Acta 173:176, 1969

162. Shirachi DY, Chan SL, Bhargava HN, Gardner E, Trevor AJ: Multiple forms of brain acetylcholinesterase. Proc West Pharmacol Soc 15:132, 1972

163. Sokoloff L: The Effects of Anesthetic Agents, Uptake and Distribution of Anesthetic Agents. Edited by EM Papper, RJ Kitz. New York, McGraw-Hill Book Company, 1963, p 150

164. Stacey RS: Red cells and 5 hydroxytryptamine. J Physiol 132:39, 1956

165. Stahl WL, Swanson PD: Movements of calcium and other cations in isolated cerebral tissues. J Neurochem 18:415, 1971

166. Stanley V, Hunt J, Willis KW, Stephen CR: Cardiovascular and respiratory function with CI-581. Anesth Analg 47:760, 1968

167. Stöcker L: Ketamine. Anaesthesiologie und Wiederbelebung. Bd 40. Edited by H Kreuscher. Berlin-Heidelberg-New York, Springer-Verlag, 1969, p 236

168. Sulser F, Watts J, Brodie BB: On the mechanism of antidepressant action of imipramine like drugs. Ann N Y Acad Sci 96:279, 1962

169. Summary of the National Halothane Study. JAMA 197:775, 1966

170. Sun AY, Samorajski T: Effects of ethanol on the activity of adenosine triphosphatase and acetylcholinesterase in synaptosomes isolated from guinea-pig brain. J Neurochem 17:1365, 1970

171. Swanson PD: Effects of ouabain on acid-soluble phosphates and electrolytes of isolated cerebral tissues in presence or absence of calcium. J Neurochem 15:57, 1968

172. Takeshita H, Okuda Y, Sari A: The effects of ketamine on cerebral circulation and metabolism in man. Anesthesiology 36:69, 1972

173. Taylor PA, Towey RM: Depression of laryngeal reflexes during ketamine anaesthesia. Brit Med J 2:688, 1971

174. Thompson GE: Ketamine-induced convulsions. Anesthesiology 37:662, 1972

175. Thompson GE, Moore DC: Ketamine, diazepam, and Innovar^{R}: A computerized comparative study. Anesth Analg 50:458, 1971

176. Tochino Y, Shanker LS: Active transport of quaternary ammonium compounds by the choroid plexus in vitro. Am J Physiol 208:666, 1965

177. Tonge SR, Leonard BE: The effects of some hallucinogenic drugs upon the metabolism of 5-hydroxy-tryptamine in the brain. Life Sci 8:805, 1969

178. Tower DB: Ouabain and the distribution of calcium and magnesium in cerebral tissues in vitro. Exp Brain Res 6:273, 1968

179. Traber DL, Wilson RD: Involvement of the sympathetic nervous system in the pressor response to ketamine. Anesth Analg 48:248, 1969

180. Traber DL, Wilson RD, Priano LL: Differentiation of the cardiovascular effects of CI-581. Anesth Analg 47:769, 1968

181. Traber DL, Wilson RD, Priano LL: Blockade of the hypertensive response to ketamine. Anesth Analg 49:420, 1970

182. Traber DL, Wilson RD, Priano LL: The effect of beta-adrenergic blockade on the cardiopulmonary response to ketamine. Anesth Analg 49:604, 1970

183. Tweed WA, Minuck M, Mymin D: Circulatory responses to ketamine anesthesia. Anesthesiology 37:613, 1972

184. Varon S, McIlwain H: Fluid content and compartments in isolated cerebral tissues. J Neurochem 8:262, 1961

185. Vickers MD: Gammahydroxybutyric acid. Newer Intravenous Anesthetics. Edited by RSJ Clarke. Int Anesth Clin 7:75, 1969

186. Virtue RW, Alanis JM, Mashiro M, La Farque RT, Vogel JHK, Metcalf D: An anesthetic agent: 2-orthochlorophenyl, 2-methylamino cyclohexanone HCl (CI-581). Anesthesiology 28:823, 1967

187. Waddell WJ, Bates RG: Intracellular pH. Physiol Rev 49:285, 1969

188. Wallgren H, Kulonen E: Effect of ethanol on respiration of rat-brain-cortex slices. Biochem J 75:150, 1960

189. Waser PG, Schaub E: Action of some neuro- and psycho-pharmaca on membrane ATP-ase and acetylcholinesterase of cortical synaptosomes. Drugs and Cholinergic Mechanisms. Edited by E Heilbronn, A Winter. Stockholm, Sweden, Research Institute of National Defense, 1970, p 359

190. Webb JL: Enzyme and Metabolic Inhibitors. Volume I. New York, Academic Press, 1963

191. Webb JL: Enzyme and Metabolic Inhibitors. Volume III. New York, Academic Press, 1966

192. Willard HH, Merritt LL, Dean JA: Instrumental Methods of Analysis. 4th edition. New York, D Van Nostrand Company, Incorporated, 1965

193. Wilson IB, Alexander J: Acetylcholinesterase: Reversible inhibitors, substrate inhibition. J Biol Chem 237:1323, 1962

194. Wolfson B, Dorsch SE, Kuo, T-S, Siker ES: Brain anesthetic concentration - a new concept. Anesthesiology 36:176, 1972

195. Wolfson EA: Drug abuse: The doctor's role. J Med Soc N J 67:465, 1970

196. Wombacher H, Wolf HV: Regulation of membrane-bound acetylcholinesterase activity by bisquaternary nitrogen compounds. Mol Pharmacol 7:554, 1971

197. Wray SR, Cowan A: Correlation between animal and clinical findings with a psychotomimetic anticholinesterase. Neuropharm 12:397, 1973