AN INVESTIGATION INTO THE EFFECTS OF INORGANIC TOXINS AND TRYPTOPHAN METABOLITES ON THE FOREBRAIN CHOLINERGIC SYSTEM AND THE PINEAL GLAND OF THE RAT

THESIS

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RAJESHREE MAHABEER

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ABSTRACT

As soon as the building of the body is completed, the ageing process begins. In the natural course of events, the functioning of some organ systems finally ebbs below the threshold necessary to maintain the body, resulting in death. This occurrence is relatively rare, because diseases superimpose themselves upon the ageing process, bringing premature death resulting from pathological causes.

This study focused on the cholinergic system of the rat forebrain. The cholinergic neurons in the brain are said to be involved in memory and learning, and a decrease in the activity of its enzymes has been reported in certain diseases, such as Alzheimer's disease.

In the present study, the in vitro effects on the cholinergic system, of aluminium and mercury and tryptophan metabolites, kynurenic acid and quinolinic acid, are determined. Aluminium has been considered as a possible factor in Alzheimer's disease. Mercury in high concentrations is toxic, and its use in amalgam for dental treatment is under consideration with regard to its possible role in promoting neurological disease. The tryptophan metabolites increase in the brain with age and may have a role in pathological diseases. Quinolinic acid, when administered in toxic concentrations produces a possible model for Huntington's disease.

This study investigated the effects of the above mentioned toxins on:
(1) The synthesis of acetylcholine by choline acetyltransferase;
(2) The specific binding of acetylcholine muscarinic receptors;
(3) The degradation of acetylcholine by acetyl cholinesterase,

Choline acetyltransferase activity did not change in the presence of aluminium chloride, kynurenic acid and quinolinic acid from 1 nM to 1 mM. Mercuric chloride had no significant effect on the enzymes activity from a concentration of 1 nM- 1 μM. At 10 μM there was a significant decrease in cholineacetyltransferase activity ($P < 0.001$). Enzyme activity continued to decrease at 100 μM ($P < 0.0002$). At 1 mM, enzyme activity was virtually non existent ($P < 0.0001$).

Acetyl cholinesterase activity was not affected by aluminium chloride, kynurenic acid and quinolinic acid. Mercuric chloride from 1μM - 1 mM significantly reduced the enzyme activity ($P < 0.05$).

The binding of the antagonist, [^{3}H] quinuclidinyl benzilate (QNB), to acetylcholine muscarinic receptors, revealed that aluminium chloride did not affect the binding of the antagonist, in the concentration range of 1 nM - 100 μM, to the receptors. At 1 mM, aluminium chloride appears to increase the sensitivity of the receptors for the ligand ($P < 0.01$). Mercuric chloride also does not appear to have any significant effect on receptor binding in this range. However, at 1 mM there appears to be a very significant decrease in receptor binding ($P < 0.01$). This decrease may be attributed to the interaction of mercury with the sulfhydryl groups in muscarinic receptors. Kynurenic acid had no effect on the receptor binding. Quinolinic acid, in the concentration range from 10 nM - 1 mM increased the binding of the receptor to [^{3}H] QNB significantly ($P < 0.001$).

The study also investigated the effect of the tryptophan metabolites of the kynurenine pathway on pineal indole metabolism. The kynurenine pathway is a major route of tryptophan metabolism in the pineal gland, along with indole metabolism. Investigations showed that kynurenic acid produced a decrease in N-acetylserotonin concentrations ($P < 0.001$) and melatonin concentrations ($P < 0.003$). Further experiments using quinolinic acid produced a similar decrease in N-acetylserotonin ($P < 0.001$) and melatonin ($P < 0.015$). A decrease was also noted in the level of 5-methoxytryptophol ($P < 0.0005$).

These findings suggest that aluminium chloride, kynurenic acid and quinolinic acid have no possible role in the decrease of activity of cholinergic enzymes which is observered in diseases such as Alzheimer's disease. The results regarding the effect of mercury chloride on the cholinergic system suggest that low exposure to the toxin will not adversely effect the enzymes. The decrease in N-acetylserotonin and melatonin concentrations reported here, may be a result of kynurenic acid and quinolinic acid having an inhibitory effect on the enzyme, serotonin N-acetyltransferase, which is responsible for the conversion of serotonin to N-acetylserotonin.

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LIST OF ABBREVIATIONS

%	Percent
α	Alpha
β	Beta
δ	Delta
µCi	Microcurie
µg	Microgram
µl	Microlitre
µM	Micromolar
µWatts	Microwatts
τ	Tau
^{14}C	Carbon-14 radiolabel
^{3}H	Tritium radiolabel
ACar	Acetylcarnitine
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
AD	Alzheimer's disease
aHT	N-Acetylserotonin
Al^{+}	Aluminium ion
aMT	Melatonin
ANS	Autonomic nervous system
B_{max}	Receptor density
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
ChA	Choline acetylase
ChAT	Choline acetyl transferase
ChE	Cholinesterases
Ci	Curie

cm	Centimetre
CNS	Central nervous system
CO_2	Carbon di oxide
CoA	Coenzyme A
CSF	Cerebrospinal fluid
DAG	Diacylglycerol
DPM	Disintergrations per minute
DTNB	5,5 Dithiobis-(2-nitrobenzoic acid)
EAA	Excitatory amino acids
EDTA	Ethylenediamine tetra-acetic acid
g	Gram
GTP	Guanine triphosphate
h	Hour
HA	5-Hydroxyindole acetic acid
HAS	Health and Human Services
HCl	Hydrochloric acid
HD	Huntington's disease
HIOMT	Hydroxyindole-O-methyltransferase
HL	5-Hydroxytryptophol
HP	Hepatic encephalopathy
HT	Serotonin
IP_3	Inositol trisphosphate
K^+	Potassium ion
KA	Kynurenic acid
KAT	Kynurenine aminotranferase
K_D	Equilibrium dissociation constant
K_m	Michaelis-Menten constant
M	Molar
m-AChR	Muscarinic acetylcholine receptor
MA	5-Methoxyindole acetic acid
MAO	Monoamine oxidase
mg	Milligram

min	Minute(s)
ml	Millilitre
ML	5-Methoxytryptophol
mm	Millimetre
mM	Millimolar
mmol	Millimoles
MT	5-Methoxytryptamine
n-AChR	Nicotinic acetylcholine receptor
Na^+	Sodium ion
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NAT	N-Acetyltransferase
NE	Norepinephrine
NFTs	Neurofibrillary tangles
nm	Nanometre
nM	Nanomolar
NMDA	N-methyl-D-aspartate
O_2	Oxygen
°C	Degree celsius
PD	Parkinson's disease
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol 4,5-bis-phosphate
pmole	Picomole
PZ	Pirenzepine
QA	Quinolinic acid
QNB	[^{3}H] quinuclidinyl benzilate
r^2	Correlation coefficient
rpm	Revolutions per minute
S	Substrate
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulfate

SI	Substantia innominata
SNAT	Serotonin *N*-acetyltransferase
SPs	Senile plaques
TLC	Thin layer chromatography
TPB	Tetraphenylborate
TRP	Tryptophan
V	Velocity
V_{max}	Maximum initial velocity at a particular concentration of enzyme

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CHAPTER ONE

LITERATURE REVIEW

1.1. INTRODUCTION

The view held by most neurobiologists, is that all behaviour is a reflection of brain function and the mind represents a range of functions carried out by the brain. The action of the brain underlies not only relatively simple behaviour such as walking and smiling, but also elaborate functions such as feeling, learning, thinking and writing. As a rule, the disorders of affection (emotion) and cognition (thought) that characterize neurotic and psychotic illness, must result from disturbances in the brain (Kandel and Schwartz, 1986).

The advent of neuroscience has been a great source of excitement in attempts to understand the biological basis of brain function and abnormal behaviour. Neuroscience is a vast area of biological sciences, and consists of several approaches to the study of the brain. The views currently held regarding nerve cells, the brain and behaviour have emerged from a fusion of neuroanatomy, physiology, biochemical pharmacology and the study of behaviour (Kandel and Schwartz, 1986).

The anatomical complexity of nervous tissue was first thought to be glandular in function, where the nerves were ducts conveying fluid secreted by the brain and the spinal cord to the periphery of the body. During the nineteenth century it was discovered that the nervous system is not a mass of fused cells, but an intricate network of discrete cells. This was shown by using the method of silver impregnation to label individual cells (Kandel and Schwartz, 1986).

Neurophysiology began in the eighteenth century when Luigi Galvani demonstrated

that nerve cells produce electricity. During the nineteenth century nerve cells were found to use electricity to signal information from one cell to another (Kandel and Schwartz, 1986).

The third discipline, biochemical pharmacology was realized when it was found that drugs interact with specific receptors on the surface of the cells. This insight became the basis of the modern study of chemical synaptic transmission (Kandel and Schwartz, 1986).

Psychology, the last of the four disciplines, studies the connection between the brain and behaviour, and has the longest history (Kandel and Schwartz, 1986). Since before recorded history, the brain has been associated with abnormal behaviour. Theories about the biological basis of abnormal behaviour were based on speculation, as the brain was not easily accessible (Bootzin and Acocella, 1988). Behaviour as the manifestation of the mind was only approached scientifically in the nineteenth century. Charles Darwin worked on evolution of behaviour, allowing psychology to develop as a discipline (Kandel and Schwartz, 1986).

The neuroscience perspective is directed to the organic determinants of behaviour regarding the physical and psychological aspects of our functioning (Bootzin and Acocella, 1988).

Interest in the brain also stems from questions regarding the development, evolution and function of the brain. Today such speculation is being informed by concrete evidence. The brain is no longer the dark territory that it used to be (Bootzin and Acocella, 1988).

The pace of neuroscience accelerated greatly at the end of the nineteenth century. After World War II new biochemical technology lead to significant progress in mapping neural pathways, understanding neural organization in the cell and at molecular levels (Bradford, 1986).

The brain is made up of individual units, the nerve cells (neurons) and glial cells. The purpose of neuroscience is to explain how the brain marshalls these units to control behaviour, and how, in turn, the functioning of the constituent cells in an individual brain is influenced by the behaviour of other people, and by a host of environmental factors.

As our understanding of the physical basis of brain function increases, it is hoped that substantial progress will be made in the alleviation of mental illness and in the search to understand man as a cognitive individual. Brain research is nonetheless only at its beginning.

1.2. NEUROANATOMY

1.2.1. Introduction

An understanding of the anatomy of the nervous system is important to studies of neurobiochemistry. The nervous system is a vast electrochemical conducting network that extends from the brain through the rest of the body. The nervous system has many divisions (**Figure 1.1.**), with its two basic parts being: 1) The central nervous system that consists of the brain and the spinal cord. 2) The peripheral nervous system that consists of the cranial and spinal nerves and other neuronal processes and cell bodies lying outside the central nervous system. This division in no way means that the two systems are completely separate. They are interconnected and parts of the same neurons may be found in both systems (Puri and Tyrer, 1992).

1.2.2. The Central Nervous System

The central nervous system (CNS) is responsible for the storage and transmission of information (nerve impulses). Instructions are sent through the spinal cord to the muscles. The muscles and the skin relay information about the outside world to the

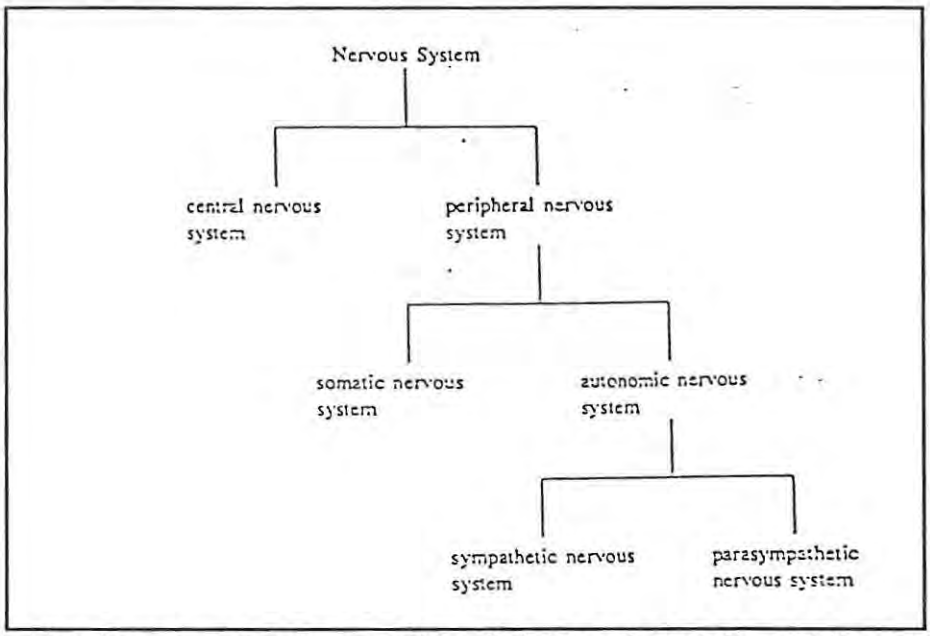

Figure 1.1. The Nervous System and its divisions (Bootzin and Acocella, 1988).

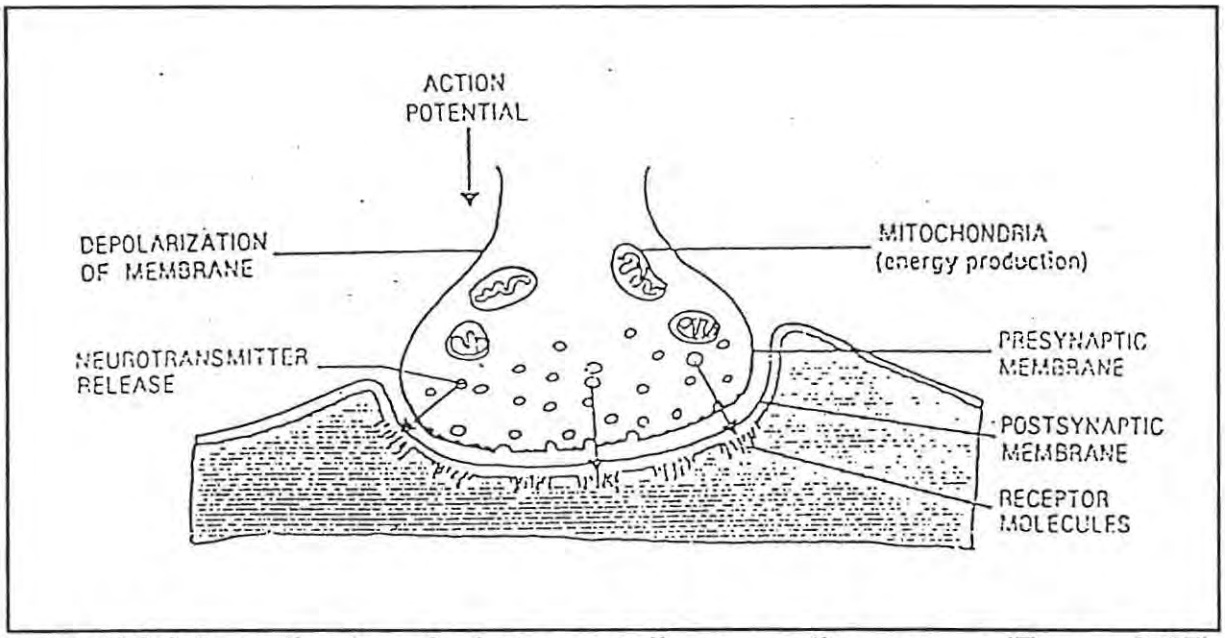

Figure 1.2. Information transfer between cells across the synapse (Tyson, 1987).

brain via the spinal cord. The information received by the brain is then processed. In this way the central nervous system controls all human behaviour (Bootzin and Acocella, 1988). The transmission of the nerve impulses between the nerve cells occurs across the synapse (i.e. the area between adjacent nerve cells). Chemical substances that are known as neurotransmitters are responsible for getting the impulse across the synapse. See **1.2.** (Tyson, 1987).

1.2.2.1. The Spinal Cord

The spinal cord is a complex nerve cable located in the vertebral column and connects the brain to most of the body. It is responsible for the upward and downward conduction of neural impulses and the integration of specific reflex actions (Louw and Edwards, 1993). In the spinal cord there is an orderly arrangement of motor and sensory nuclei. There are afferent and efferent pathways. The afferent pathways carry sensory information to the brain and the efferent pathways carry motor control from the brain to the motor neurons. The spinal cord is comprised of three main divisions where it enters the brain: the medulla, the pons, and the main brain.

1.2.2.2. The Brain

The brain is probably the most complicated organ of the body. It contains about 90% of all body neurons; although the brain represents approximately 2% of the total mass of the body and receives one sixth of the blood supply (Louw and Edwards, 1993).

1.2.2.2.1. The Structure of the Brain

Covering the brain is an intricate, convoluted outer layer of "grey matter" called the cerebral cortex. See **Figure 1.3.** The external surface of the cerebral cortex has many sulci (fissures) and gyri (ridges between the sulci). A major sulcus called the longitudinal fissure divides the brain along the midline into two symmetrical mirror image cerebral hemispheres, the right and the left brain, connected by the corpus

callosum, a band of nerve fibres. The central sulcus (or fissure of Rolando) divides the cortex into the frontal and the receptive cortex, made up of the parietal, temporal and occipital lobes. Another fissure, the lateral sulcus (or fissure of Sylvius), runs along the side of each hemisphere separating the temporal lobe from the frontal and parietal lobes (**Figure 1.3.**) (Bootzin and Acocella, 1988).

A cross section of the brain reveals further important structural features: the hypothalamus, the thalamus, the cerebellum, the hippocampus etc. Several of these brain structures are the foci of intense research, owing to their possible association with neurological diseases, for example Alzheimer's Disease and Huntington's Disease (Bootzin and Acocella, 1988).

1.2.2.2.2. The Medulla

The medulla can be described as a thickening at the top section of the spinal cord where it enters the brain (See **Figure 1.3.**). It contains both ascending and descending nerve tracts. In the medulla, the nerve tracts cross over so that the right side of the brain receives information from and transmits impulses to the left side of the body. The left side of the brain receives information from and transmits impulses to the right side of the body. The medulla also has an important role in essential bodily functions such as blood pressure, heart rate and breathing. Damage to the medulla will most often result in death (Louw and Edwards, 1993).

1.2.2.2.3. The Pons

The pons is located above the medulla, and contains a massive set of neurons that relay information from the cerebral hemispheres to the cerebellum (Kandel and Schwartz, 1986). See **Figure 1.3.**

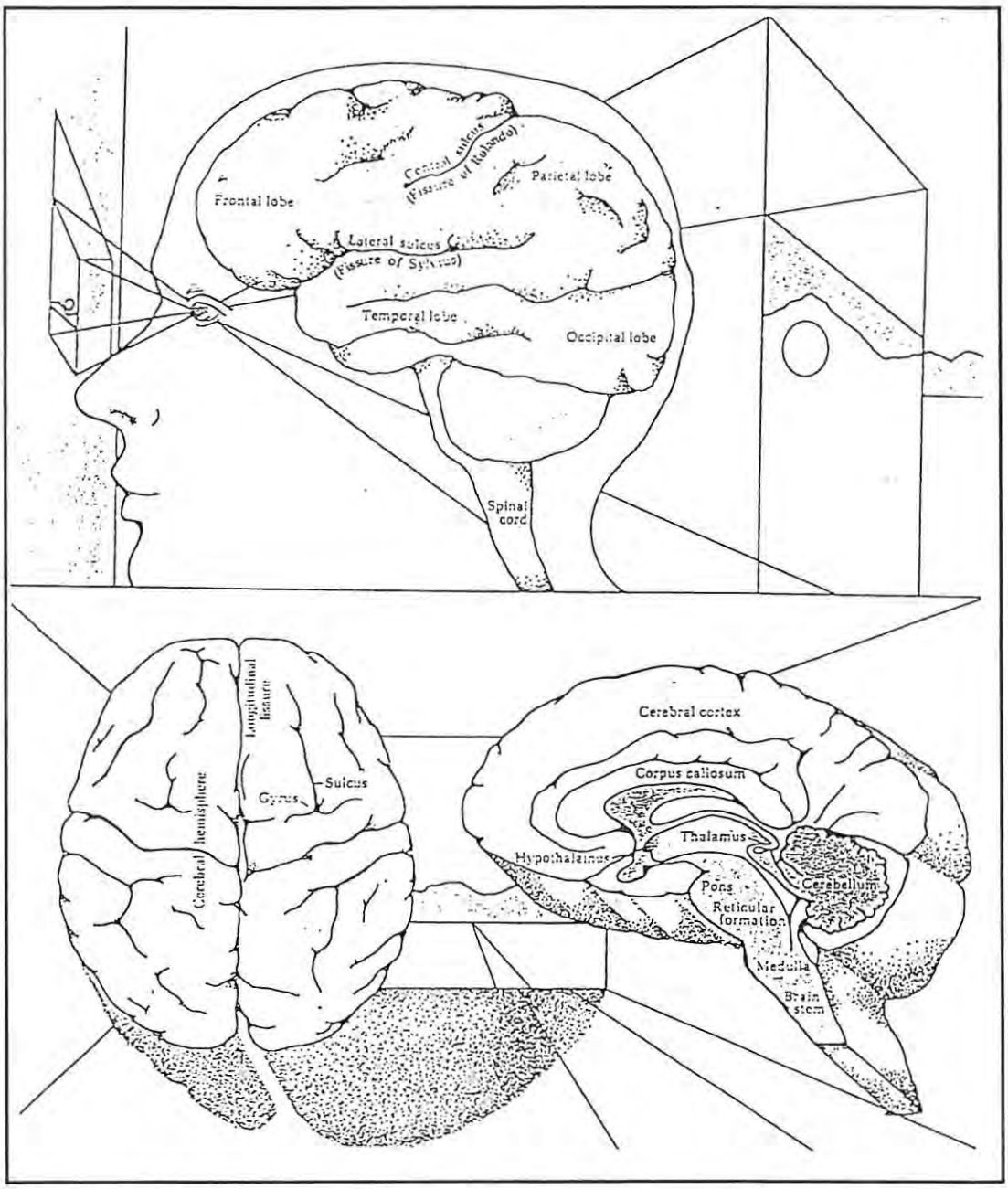

Figure 1.3. The Anatomy of the Brain (Bootzin and Acocella, 1988)

1.2.2.2.4. The Midbrain

The midbrain lies above the pons. It has an important role in the control of eye movement and contains an essential relay in the auditory pathway. The midbrain is also involved in the control of skeletal muscles (Kandel and Schwartz, 1986).

1.2.2.2.5. The Hypothalamus

This is an important area of the brain especially in the control of the autonomic processes, emotional behaviour and motivation. The hypothalamus is located below the thalamus. Damage to certain areas of the hypothalamus leads to apathetic behaviour. The hypothalamus, along with the pituitary gland, has a role in the function of the endocrine system (Louw and Edwards, 1993; Puri and Tyrer, 1992).

1.2.2.2.6. The Cerebellum

The cerebellum consists of two lateral hemispheres (Puri and Tyrer, 1992) and is a highly regular structure. This regular organization suggests that all areas of the cerebellum perform a set of similar functions, but that each area performs that function on a different set of structures. The cerebellum also plays an important, but indirect role in movement and posture, by adjusting the output of the major descending motor systems of the brain (Kandel and Schwartz, 1986).

1.2.2.2.7. The Cerebral Cortex

The cerebral cortex has already been mentioned, and it is known that there are four anatomically distinct lobes: the frontal, parietal, occipital and temporal lobes (See **Figure 1.3.**).

The functions of these different lobes of the brain have been the subject of much research and debate. The **frontal lobes** contain the motor area, and are involved in

voluntary movement. They also contain the Broca-area (in the left hemisphere) which is important in speech production (Louw and Edwards, 1992). In addition, the frontal lobes serve as a comparator organ.

This allows an individual to view and analyse his or her behaviour and perceive how others respond to the behaviour. Damages to these structures usually result, in odd behaviour (Bootzin and Acocella, 1988).

The **parietal lobes** are the centres of intersensory integration (for example, the ability to visualize a dog upon hearing a bark) and of the motor and sensory-somatic functions (Bootzin and Acocella, 1988). Damage to the parietal lobes results in spatial disorientation and in loss of control over gross-motor behaviour, for example walking.

The **occipital lobes** appear to control vision. They process and analyze the information received from the eyes (Bootzin and Acocella, 1988).

The **temporal lobes** control auditory perception, and are also involved in memory and vision. Damages to these lobes generally involves memory loss and result in difficulties in understanding the spoken language (Bootzin and Acocella, 1988; Louw and Edwards, 1993).

1.2.3. Pineal Gland

The pineal gland has been termed corpus pineale, glandula pinealis, pineapple or pine-cone gland (Pineapple gland, because its shape resembles a pineapple, especially in man). Ancient Greek literature termed the gland konareion (cone-shape). In Latin it was known as conarium. In the west, Herophilos of Alex (325-280 B.C.) first termed the organ *epiphysis cerebri* and said that the pineal functioned as a tap regulating the stream of "spiritus" from the third to the fourth ventricles of the brain. Twenty centuries later this view was elaborated on by Descartes (1596-1650) who was a philosopher, physicist and mathematician. He regards the epiphysis as the seat of the soul, as well

as the seat of imagination and consciousness. Further study on the pineal organ was neglected until the nineteenth century, when some interest was revived (Kappers, 1976).

Until the late 1950's and early 1960's, most scientists did not take research on the pineal gland seriously (Reiter, 1991b). The pineal gland is located between the cerebral cortex and the cerebellum. In the rat, brain the gland can be found between the two hemispheres just forward of the cerebellum (See **Figure 1.4a.** and **1.4b.**) (Rowett, 1962). In man, the gland weighs about 100 mg and 1 mg in the rat. The pineal gland originates in the brain of the developing mammalian embryo, but it loses direct nerve connection with the brain soon after birth (Axelrod, 1977).

Since 1956, several discoveries has been made that laid the foundation for what has become a very active area of investigation. These important early observations included the findings that, (1) the physiological activity of the pineal is influenced by the photoperiodic environment; (2) the gland contains a substance, *N*-acetyl-5-methoxytryptamine or melatonin, which has obvious endocrine capabilities; (3) the function of the reproductive system in photoperiodically dependent rodents is inextricably linked to the physiology of the pineal gland; (4) the sympathetic innervation to the pineal is required for the gland to maintain its biosynthetic and endocrine activities; (5) the pineal gland can be rapidly removed from rodents with minimal damage to adjacent neural structures using a specially designed trephine (Reiter, 1991b).

The pineal gland synthesizes melatonin (N-acetyl-5-methoxytryptamine) from serotonin. Melatonin is phylogenetically a very old molecule and has been remarkably well conserved during evolution. The hormone is known to exist in the dinoflagellate *Gonyaulax polyedra* and may be produced by most if not all organisms in the animal kingdom (Reiter *et al.* 1994).

The steps in the synthesis of melatonin from the amino acid tryptophan (TRP) are

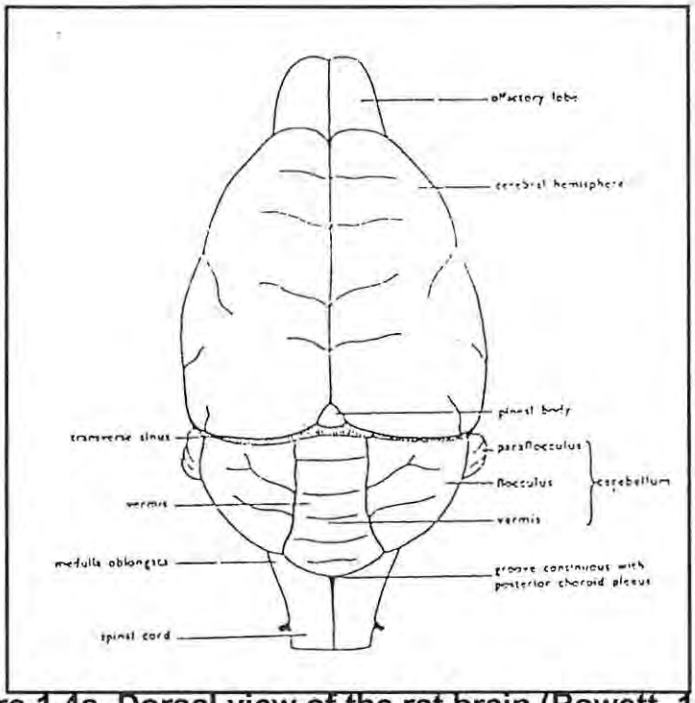

Figure 1.4a. Dorsal view of the rat brain (Rowett, 1962).

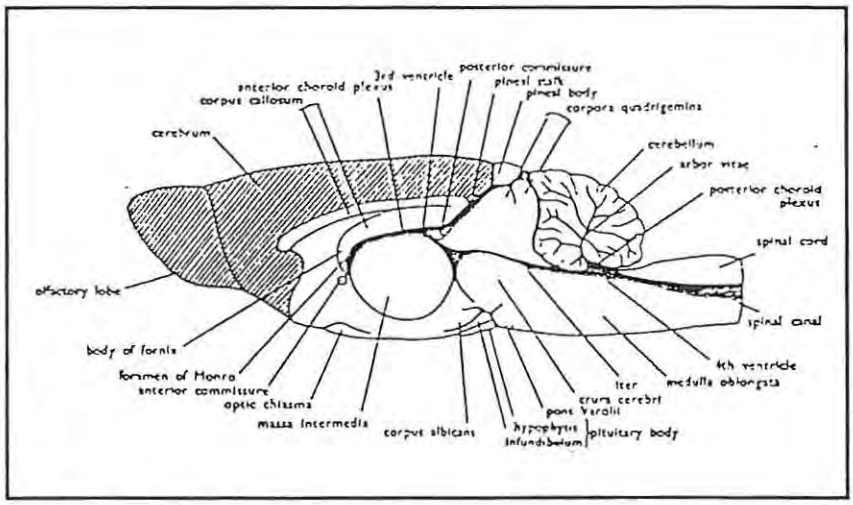

Figure 1.4b. Median sagittal view of the rat brain (Rowett, 1962).

summarized in **Figure 1.5.** The ability of the pineal gland to produce large quantities of methoxyindoles is related to the high levels of TRP hydroxylase within the gland. After the uptake of the amino acid into the gland, the hydroxylating enzyme converts it to 5-hydroxytrytophan. Following this, the 5-hydroxytrytophan is decarboxylated by the enzyme L-aromatic amino acid decarboxylase, resulting in the formation of serotonin. Serotonin is the common precursor of several indoles formed within the pineal gland. In a two-step process, serotonin is converted to melatonin. First, the serotonin is N-acetylated by the enzyme serotonin N-acetyltransferase (NAT), resulting in the product N-acetylserotonin. This step requires an acetyl group that is provided by acetyl CoA. The formation of melatonin from N-acetylserotonin is catalysed by the enzyme hydroxyindole-O-methyl-transferase (HIOMT). During the conversion the methyl group is provided by S-adenosylmethionine (Reiter, 1981).

As far as it can be determined, the melatonin produced is not stored in the pineal gland. Shortly after its synthesis, it is released, primarily into the blood vascular system (Reiter, 1981).

In the biological activity of the gland, the light: dark cycle is an important factor, i.e. the circadian rhythm. The rhythmic synthesis and secretion of the pineal hormone, melatonin, is suggested as the mechanism by which the pineal controls circadian oscillators in lower vertebrates (Underwood, 1990).

The conversion of serotonin to melatonin is under the control of the light: dark cycle to which the animals are exposed via its action on the peripheral sympathetic nervous system (Reiter, 1981). The pineal gland in the rat is innervated by fibres from the sympathetic nervous system (Kappers (1965) cited by Daya, 1989). Characteristically, periods of light are associated with high pineal levels of serotonin and low NAT activity. Conversely, during the daily period of darkness, the amount of serotonin diminishes because it is converted to N-acetylserotonin by the increased activity of the acetylating enzyme (Reiter, 1981). Mammals utilise their retinas to provide the brain with information about changes in light. The pineal gland is an end organ of the visual

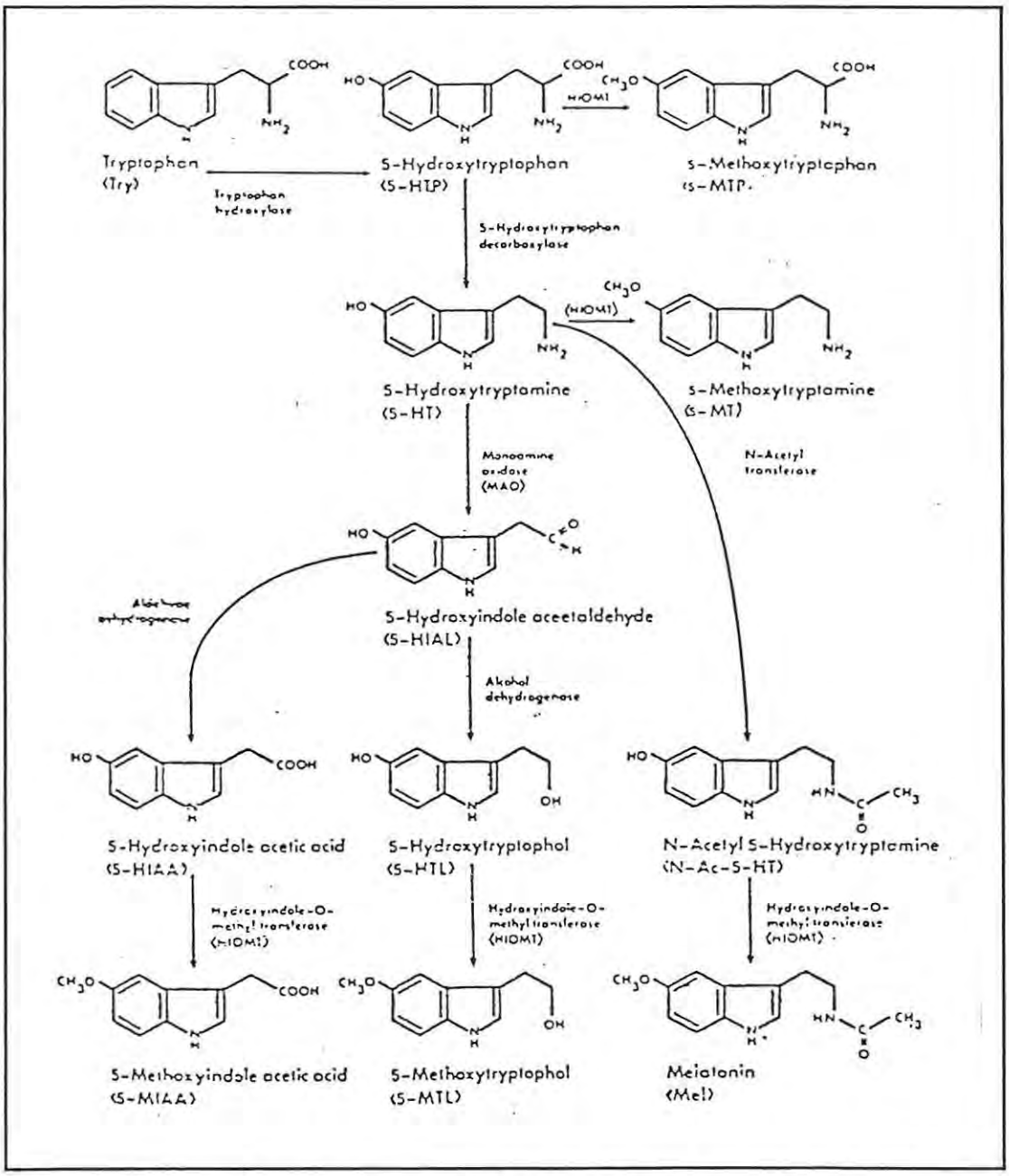

Figure 1.5. The Pathway of Pineal Indole Metabolism

system. It translates the photoperiodic information into a chemical signal which serves as a messenger to every organ in the body (Reiter, 1991a). The production and release of melatonin from the pineal is controlled by the action of the photoperiod by way of the suprachiasmatic nuclei (SCN) of the hypothalamus. At night, the SCN sends a neural signal to the pineal gland through the peripheral autonomic nervous system, resulting in the postganglionic sympathetic neurons releasing norepinephrine (NE) onto the pinealocytes (Reiter, 1991c). This results in the activation of the adenylate second-messenger system which stimulates an increase in NAT activity which, with HIOMT, converts serotonin to melatonin. The conversion of serotonin to N-acetylserotonin by NAT is the rate-controlling step in the production of melatonin (Moore and Klein, 1974). In the endocrine system, the pineal functions as an inhibitor of most other endocrine glands (Wilson *et al.*, 1989). Both light and temperature cycles can entrain the pineal melatonin rhythm. In this way, the pineal gland acts as a photo and thermoendocrine transducer, which functions to synchronize internal cycles with cycles in the environment (Underwood, 1990). In other words, the pineal gland is the intermediary between the external photoperiod and the internal environment (Reiter, 1991a).

Apart from melatonin, there are a number of other indoles produced in the pineal gland of mammals (**Figure 1.5.**). Some of these have been shown to have biological activity (Reiter, 1981).

1.2.4. Peripheral Nervous System

The peripheral nervous system can be divided into two functional parts: the somatic nervous system that is responsible for the innervation of voluntary structures, and the autonomic nervous system which is concerned with the innervation of the involuntary structures (Puri and Tyrer, 1992).

The autonomic nervous system is divided into the sympathetic and parasympathetic parts, each of which has afferent and efferent fibres (Puri and Tyrer, 1992).

1.2.5. The Autonomic Nervous System

The autonomic nervous system (ANS) also merits discussion in order to put into perspective the role of the cholinergic system in neurotransmission. The ANS (also known as the motor system) provides the innervation for the endocrine and exocrine glands, for the viscera and for the smooth muscles in all organs of the body.

1.2.5.1. Anatomy and Physiology of the Autonomic Nervous System (ANS)

There are two divisions of the ANS: the sympathetic (also referred to as the orthosympathetic) and the parasympathetic nervous systems. See **Figure 1.1.** The reasons for the divisions are: Firstly, the autonomic distributions of the nerve fibres in the two divisions are distinct from each other. Secondly, the effects of the two divisions on the organs are often antagonistic to each other. Thirdly, the types of hormones secreted at the nerve endings are usually different in the two systems (Guyton, 1969). Also, sympathetic and parasympathetic activity is predominantly, but not exclusively, reflex in nature (Appenzeller, 1982).

Both the divisions are important in mediating motivational and emotional states as well as in monitoring the body's basic physiology. The cell bodies that give rise to the sympathetic division lie in the thoracic and lumbar regions of the spinal cord. The neurons that give rise to the parasympathetic division lie above this region of the spinal cord in several brain stem nuclei associated with the cranial nerves, and below it in the sacral region of the spinal cord. The two divisions of the ANS are each made up of preganglionic and postganglionic neurons. The cell bodies for the preganglionic neurons lie in the brain or in the spinal cord and those of the postganglionic are found in the autonomic ganglia (Appenzeller, 1982). The autonomic ganglia are in the brain stem and the spinal cord (Kandel and Schwartz, 1986).

1.2.5.2. Characteristics of the Autonomic Nervous System (ANS)

The ANS functions are regulated by centres in the brain. There are also divisions in the hypothalamus that have a role in the regulation of certain functions of the ANS. Examples of the ANS functions regulated by the hypothalamus are cardiovascular regulation, regulation of body temperature, control of body water, regulation of feeding, control of excitement and rage and regulation of endocrine functions.

The hypothalamus receives information from many other structures, including higher levels of the motivational systems, for example the cerebral cortex and the reticular formation (Kandel and Schwartz, 1986). Stimulation of the ANS by the cerebral cortex occurs during emotional states. Many discrete centres of the brain, especially the prefrontal lobes and temporal regions of the cortex, can increase or decrease the degree of excitation of the hypothalamus centres. Both the conscious and the subconscious portions of the cerebellum can cause autonomic effects (Guyton, 1969).

1.2.5.3. Neurotransmission in Autonomic Nervous System (ANS)

In the ANS, nervous impulses are transmitted across synapses and neuroeffector junctions by a chemical mediator, known as the neurotransmitter. The neurotransmitter in all autonomic ganglia, the neuroeffector junction of postganglionic parasympathetic fibres and effector cells on smooth muscles, glands and visceral organs and the junction of autonomic nerve effector cell union on sweat glands and vasodilator vessels is Acetylcholine (ACh). Therefore the fibres have been termed Cholinergic (i.e. the release of ACh when a nerve action potential passes) (Bowman and Rand, 1990).

1.3. CHOLINERGIC TRANSMISSION SYSTEM

1.3.1. Introduction

The basic sequence of events in neurotransmission is synthesis, storage, release, postsynaptic reception and breakdown of the neurotransmitter. Since the cholinergic transmission is of primary concern here, the neurotransmitter, Acetylcholine (ACh), will be dealt with in detail.

All neural pathways that use acetylcholine as their neurotransmitter are referred to as cholinergic. ACh was the first neurotransmitter to be isolated and characterized in both function and structure. Also, many basic neurotransmitter system features were first investigated using cholinergic systems.

1.3.2. Acetylcholine

The neurophysiological activity of ACh has been known since the turn of the century. See **Figure 1.6.** The neuro-transmitter was first identified as a possible mediator of cellular function by Hunt and Taveau in 1906, and in 1914, Dale pointed out that ACh controls muscle contraction. Each movement (for example, walking, talking) depends on the secretion of ACh by the motor neurons and its subsequent effect on muscles (Louw and Edwards, 1993).

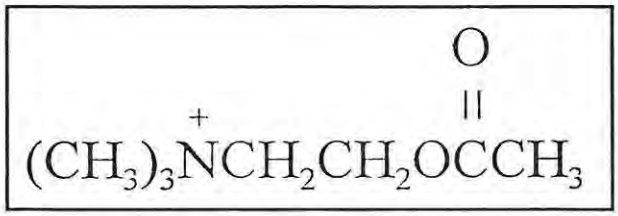

Figure 1.6. Structure of Acetylcholine.

The molecular structure of ACh consists of three planes:(1) the plane of the ester oxygen carboxyl group; (2) the plane of one methyl carbon atom, the nitrogen atom and the two CH_2 group carbon atoms and (3) the plane of the ester oxygen-CH_2 bond and one methyl carbon-nitrogen bond (Canepa *et al.*, 1966).

The presence of ACh in cholinergic tracts and terminals, have only in recent years been determined by the development of modern technology. There is considerable evidence that acetylcholine arrived within the evolutionary scheme long before the design of the nervous system as functional synapses. Bacteria, fungi, protozoa and plants store ACh and possess biosynthetic and degradative capacities for turnover of the molecule. Even in higher systems, ACh distribution is far wider than the nervous system. For example, ACh is found in the cornea, certain ciliated epithelia, the spleen of ungulates and the human placenta (Taylor and Brown, 1989).

ACh also plays a role in psychological processes such as memory, learning, attention and motivation. Alzheimer's disease, a disorder that appears to occur usually in old people has as its main characteristics, severe memory difficulties, disorientation, restlessness, faulty judgement and delusions of persecution. Research indicates that people who die as a result of Alzheimer's show abnormally low levels of ACh in areas of the brain (Louw and Edwards, 1993).

1.3.3. Synthesis of Acetylcholine

ACh is synthesized by the enzyme choline acetyltransferase (EC. 2.3.1.6) (ChAT), which employs two substrates, choline and acetyl coenzyme A (acetyl CoA) (Lopez *et al.*, 1993). ACh levels in intact nervous structures are generally well maintained during any one physiological activity. The mechanism for this constancy may be that the release of the neurotransmitter triggers an increase in the rate of synthesis by ChAT (Kaita and Goldberg, 1969). The reaction is reversible with its equilibrium shifted to the right. K_{eq} represents the equilibrium constant which is a measure of the equilibrium position for a reaction and its units of measure is in molar concentrations. Values of

K_{eq} have been reported by Pieklik and Guynn (1975) as 12.3. Hersh (1982) also determined the equilibrium as described by Pieklik and Guynn and found a result of K_{eq}= 13.3 which agrees with that of Pieklik and Guynn. The enzyme carnitine acetyltransferase can also produce ACh (White and Wu, 1973). Carnitine acetyltransferase is a mitochondrial enzyme involved in the metabolism of fatty acids and is not associated with synaptic transmission (Tucek, 1985).

Before any further discussion on ACh synthesis, it is important that the possible sources of acetyl CoA and choline be noted (See **Figure 1.7.**). The acetate moiety is derived from glucose via pyruvate and the mitochondrial pyruvate dehydrogenase complex generates acetyl CoA (Bradford, 1986). The route in which the intramitochondrial acetyl CoA reaches the extramitochondrialy located ChAT is undefined and three mechanisms are considered: (1) The transfer of acetyl groups after their incorporation into citrate or other compounds. Citrate is produced from intramitochondrial acetyl CoA and oxaloacetate. This moves across the inner mitochondrial membrane aided by tricarboxylate carriers. Then it passes the outer mitochondrial membrane and is transformed back to acetyl CoA and oxaloacetate by ATP citrate lyase. (2) The other way in which intramitochondrial acetyl CoA may become available for ACh synthesis is by direct passage of ACh through the mitochondrial membranes. The inner mitochondrial membrane is generally impermeable to large and charged molecules like ACh, but the passage of ACh out of the mitochondria has been observed in *in vitro* experiments. Also, increases in ACh moving out of the mitochondria were reported when Ca^{2+} ions were added. (3) It has also been suggested that extramitochondrial pyruvate dehydrogenase is present either in the membranes or the cytosol of cholinergic nerve terminals. This means that pyruvate generated acetyl CoA can be directly available for ACh synthesis without permeability problems. There is no evidence to support this idea (Tucek, 1985).

There is possibly more than one mechanism responsible for providing the required acetyl CoA. It is possible that combinations of citrate, acetylcarnitine and direct passage of acetyl CoA ensures the availability of intramitochondrial acetyl CoA outside

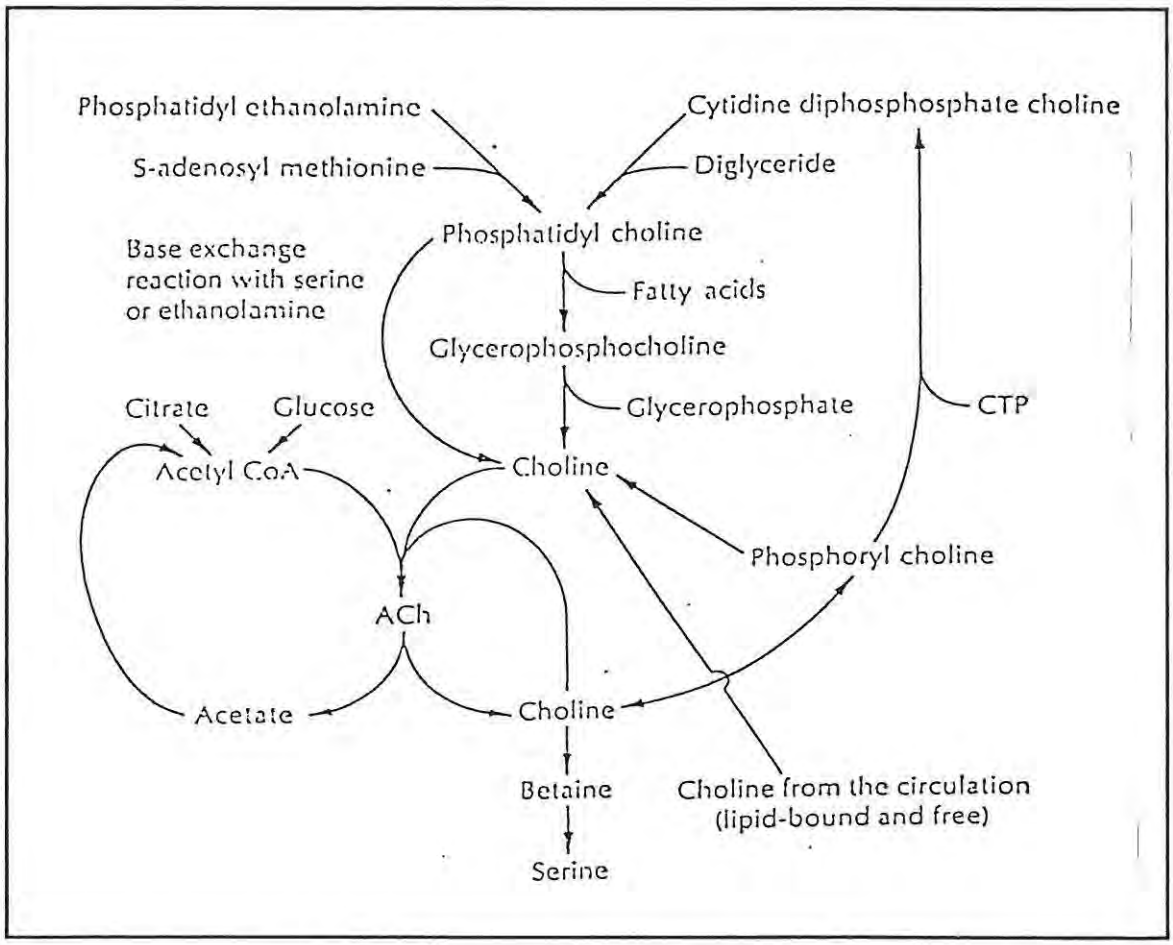

Figure 1.7. Acetylcholine Metabolism (Cooper *et al*, 1978).

the mitochondria for the synthesis of ACh (Tucek, 1985).

The other important substrate for ACh synthesis is Choline. Choline is a ubiquitous substrate (Bradford, 1986). It is not synthesized in the brain, but primarily in the liver. Choline is then transported to the brain in a free or phospholipid form by the blood (Cooper, 1978; Cornford *et al.*, 1978). Choline cannot penetrate the brain barrier and requires a specific uptake mechanism to enter the brain. A substantial increase in plasma choline concentration only slightly increases the choline content of various brain nuclei (Brunello *et al.*, 1982). Brunello *et al.* cites the following references, Racagni *et al.* (1975); Cohen and Wurtman, (1976); and Eckernas *et al.* (1977), as support for this notion. However, other authors differ. In 1975 Cohen and Wurtman reported that choline chloride administration to rats caused a dose-dependent increase in the brain concentration of the ACh, thereby suggesting that the availability of the precursor may influence brain ACh synthesis. Schuberth and Jenden (1975) have shown that labelled choline given *in vivo* to mice as a pulse injection is rapidly incorporated into the brain and is partly transformed to ACh. These findings of Schuberth and Jenden (1975) supported similar results of Haubrich *et al.* in 1972 and Jenden *et al.* in 1973. Free choline is specifically taken up into the cholinergic nerve terminals by a "high affinity" pump (Bradford, 1986). This choline uptake system is linked to both ACh synthesis and release. How this link is maintained is unclear. In all tissues, there is also a "low affinity" uptake system present (Taylor and Brown, 1989).

1.3.4. Storage of Acetylcholine

Synthesized ACh is stored in synaptic vesicles that possess a carrier substance for ACh. In the vesicle is a protein, vesiculin, which helps to stabilize the stored ACh, so that high concentrations of the neurotransmitter can be obtained. Stored ACh in vesicles is called "stable" or "bound", whereas ACh found in the cytoplasm is called "labile" or "available" (Lopez *et al.*, 1993).

Vesicle bound ACh is not accessible to attack by Acetylcholinesterase (AChE). AChE

is the enzyme responsible for the degradation of acetylcholine and will be discussed later. Whether synthesis occurs in the synaptoplasm before transfer to vesicles or whether ChAT bound to the vesicles fill these directly, is not clear. What is consistent is the finding that "newly synthesized" - meaning the most recently synthesized ACh is always the first to be released during nerve activity (Bradford, 1986).

1.3.5. Release of Acetylcholine

A nerve impulse arriving at the presynaptic membrane triggers the release of acetylcholine found in the synaptic vesicles. This occurs by a wide range of depolarizing agents (substances that cause depolarizing of cell membranes). ACh release is induced by mechanisms that require the presence of Ca^{2+} ions (Bradford, 1986). During the release of ACh, the enzyme ATPase (Na^+ and K^+ dependent) is inhibited. This causes a change in the ionic steady state. Once the release of the enzyme stops, the correct concentrations of sodium and potassium are re-established. ATP is needed for all the activities required for the release of the neurotransmitter (Taylor and Brown, 1989).

ACh release is usually followed by the onset of the neurotransmitter's resynthesis in the tissue to replenish depleted stores. Richter and Crossland have shown that a sixty percent decrease of ACh levels in the brain is achieved by electrical stimulation on rat heads. The ACh content returns to prestimulation values within a few seconds after the end of stimulation (Richter and Crossland, 1949). This demonstrates that the synthetic machinery is highly controlled and usually efficient and able to adapt very quickly to changes in ACh levels.

1.3.6. Action of Acetylcholine on the Postsynaptic Receptors

It is known that synapses operated by ACh fall into subcategories. They are distinguished by their different responses to two drugs, which are nicotine, a substituted pyrrolidine compound from the tobacco plant, *Nicotiana tabacum* (**Figure 1.8.**), and

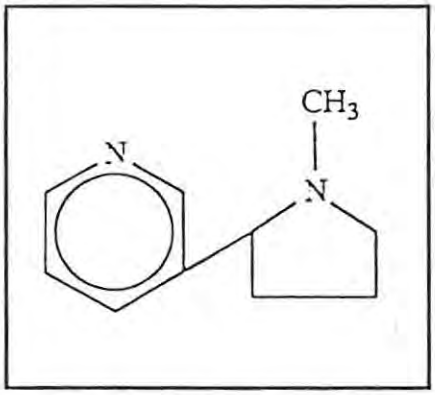

Figure 1.8. The Structure of Nicotine

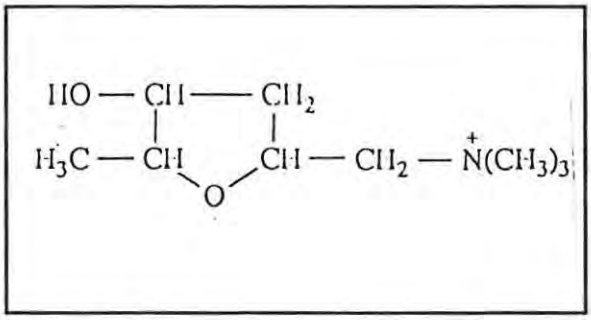

Figure 1.9. The Structure of Muscarine

muscarine, a tetrahydrofuran substituted tertiary amine from the fly agaric mushroom (*Amanita muscuria*) (**Figure 1.9.**).

Each stimulates different categories of cholinergic function, termed nicotinic and muscarinic, which are mediated by different subcategories (Bradford, 1986). The effects of nicotine and muscarine are similar to those of acetylcholine. Therefore ACh is said to have two groups of properties (Lopez *et al.*, 1993).

The interaction of acetylcholine with the postsynaptic receptor provokes configurational changes in the receptor molecules. This leads to the opening of the ionic channels and so to a flow of ions across the postsynaptic membrane, resulting in the depolarization of the postsynaptic terminal. Very large numbers of ACh molecules can be detected at the binding sites of peripheral synapses (Lopez *et al.*, 1993).

1.3.7. The Breakdown of Acetylcholine

After ACh has been released and has acted on the postsynaptic membrane receptors, it rapidly dissociates from the receptor. ACh is then broken down by the enzyme AChE to produce choline and acetate. A number of things can happen to the choline, but the greater part is taken up into the presynaptic neuron to be reutilized in the synthesis of Ach. This enzyme, AChE, is synthesized in the rough endoplasmic reticulum and transported to the terminals. It is an allosteric enzyme. The action of AChE on ACh produces choline and acetate (Lopez *et al.*, 1993).

1.4. CHOLINE ACETYLTRANSFERASE

1.4.1. Introduction

The enzyme responsible for the synthesis of acetylcholine is Choline acetyltransferase

(ChAT). This enzyme is the most reliable for cholinergic neurons (Ichikawa and Hirata, 1986). The enzyme was first discovered in the rabbit brain by Nachmansohn and Machado in 1943 and was then named Choline acetylase (ChA). Nachmansohn and Machado observed in a cell free system, the formation of acetylcholine, i.e. the acetylation of choline, in the presence of ATP. It was the first time that it was noticed that ATP could be used for biosynthesis. This was also the first demonstration of enzyme acetylation in a cell free system. The scientific community was extremely unbelieving and various journals, namely:- Science, Journal of Biological Chemistry, Proceedings of Society of Experimental Biology and Medicine, all refused to publish their findings (Mautner, 1977).

Intracellularly (i.e. within cholinergic neurons) ChAT is concentrated in nerve terminals. It is also present in axons, where it is transported from its site of synthesis in the soma (perikaryon) (Taylor and Brown, 1989). Investigations into the subcellular distribution by Fonnum (1970) revealed that ChAT was mainly found in the synaptosomal fraction, and only a small proportion of ChAT was recovered in the soluble fraction that consists of cytoplasmic constituents from disrupted cell bodies, axons and dendrites (Fonnum, 1970).

With respect to the cellular localization of ChAT, the highest activity is found in the caudate nucleus, retina, corneal epithelium and central spine roots and putamen of mammalian species. In contrast, the dorsal spinal roots contain only trace amounts of the enzyme (McGeer and McGeer, 1976; Cooper *et al.*, 1978).

Since ChAT was first described by Nachmansohn and Machado, various attempts have been made to purify the enzyme without success. However, partially purified preparations have been obtained from squid head ganglia, human placenta, fly, guinea pig, rabbit brains etc. (Cooper *et al.*, 1978; Chao and Wolfgram, 1973).

In 1973 Chao and Wolfgram attempted to purify the enzyme from bovine brain. These authors found that ChAT dissociated from haemoglobin by chloroform-butanol

extraction, suffered an extensive loss of activity. Haemoglobin was a major contaminant. Chao *et al.* also cite a similar occurrence with attempts to purify bacterial ChAT (Chao and Wolfgram, 1973). Wenthold and Hahler attempted to purify rat brain ChAT. They found that the enzyme was relatively stable until the chromatography step on a second G-150 Sephadex column. The enzyme subsequently lost up to fifty percent of its activity in twenty-four hours at 4 °C (Wenthold and Mahler, 1975). Rossier concluded, after attempting to purify the ChAT enzyme from rat brain, that the mammalian brain is not a practical source for the purification of ChAT in vertebrates. This author suggested that immature human placenta or torpedo electric organs might yield better results (Rossier, 1976a).

Further attempts to purify the ChAT enzyme by using the *Torpedo californica* as a source was made by Brandon and Wu (1978). These investigators used the methods of ion-exchange, gel filtration and hydroxyapatite chromatography. There were several reasons for using the *Torpedo* electric organ: (1) Its innervation is exclusively cholinergic. (2) A partial characterization of crude homogenates of the enzyme had been reported by Bull *et al.*(1969) (cited by Brandon and Wu, 1978). (3) The biochemistry of the synaptic transmission in the electric organ had been extensively studied. When the results obtained were analyzed, it was suggested that the final enzyme preparations were essentially homogeneous and had a specific activity of 30 µmol/min/mg of protein at 37 °C (Brandon and Wu, 1978).

Later, the Slemmon (1982) group incorporated the two protein purification techniques of hydrophobic chromatography and group specific affinity chromatography using solid phase organic dye. The authors were able to purify *Drosophilia* ChAT to a final specific activity of 500 µmol/min/mg of protein. This specific activity was significantly higher than that previously reported from any other source. The high value was attributed to Drosophila ChAT possibly being less labile than the enzyme from other sources and easier to isolate in an active form (Slemmom et al, 1982).

Apart from Ostermann and colleagues (1990), only the Braun group (1987) have thus

far described the isolation of ChAT in large quantities. Braun *et al.* were able to isolate 66 micrograms of ChAT from 10 kilograms of starting material, which was pig brain. Ostermann's group reported isolating 50-100 micrograms of active ChAT per kilogram of porcine brain. This was achieved by immunoaffinity chromatography using a covalently immobilized monoclonal antibody. The purification yield was achieved routinely.

1.4.2. Properties of Choline Acetyltransferase

Since 1964 when Bull *et al.* first determined the molecular weight of ChAT, a number of researchers have reported a range of molecular weights for ChAT. Bull *et al.* determined the weight of human placental ChAT to be 59 000 daltons and 67000 daltons for rabbit brain enzyme (Bull *et al*, 1964). Chao and Wolfgram reported that ChAT could be dissociated into two non identical units with one of the units having a molecular weight of 69 000 daltons. The second subunit was apparently of a smaller molecular weight (Chao and Wolfgram, 1973). In a later study, Chao (1975) reported that the molecular weight of ChAT is approximately 87 000 - 89 000 daltons. This author further suggests that ChAT contains six identical subunits each of a molecular weight of approximately 14 700 daltons (Chao, 1975). All other findings that have been reported tend to disagree with Chao. Rossier's results put the molecular weight of the enzyme at about 68 000 daltons (Rossier, 1976c). Banns (1976) estimated the molecular weight to be in the range of 58 000 - 62 000 daltons. Diez *et al.* (1980) reported the molecular weight to be in the region of 67 000 daltons. Slemmon *et al.* (1982) used molecular exclusion chromatography and determined the weight to be approximately 67 000 daltons. Slemmon also found another peptide of 54 000 daltons and proposed that this peptide may be a proteolytic degradation product of the 67 000 dalton enzyme. Cozzari and Hartman (1983a/b) purified two active molecular forms, with molecular weights of 72 000 and 76 000. Numerous studies have been involved in purifying the enzyme. Therefore, there have been many conflicting reports regarding the properties of the enzyme. The majority of the conflicting reported data, has been generated, possibly, during the purification procedures. These problems have been

shown to be a result of the property of ChAT to interact with other protein factors in the brain (Cozzari and Hartman, 1983a)

1.4.3. Immunological Properties of Choline Acetyltransferase

Antisera so far produced against ChAT have the following properties:
(1) They inhibit the enzyme activity and precipitate an active enzyme-antibody complex that can be used to study the properties of the enzyme ChAT.
(2) The antisera is able to react with inactivated enzyme as it does with active enzyme.

These properties suggested by Rossier need to meet two fundamental requirements. Firstly, the antiserum must have a high titre (approximately 1 mg/ml) and secondly, the antiserum must be monospecific, i.e. directed only against ChAT (Rossier, 1976b).

A number of attempts at producing monospecific antisera have failed, owing to very low yields of the enzyme. One technique that appears to have some success is monoclonal antibody technology. This technique uses partially purified antigen and is particularly suitable for macromolecules that are difficult to purify. Crawford *et al.* applied this to the product of monoclonal antibodies to *Drosophila melanogaster* ChAT. These authors were able to derive two monoclonal antibodies specific to *Drosophila melanogaster* ChAT. Both anti-bodies were found to react at or near the same determinant in a manner that interfered with the catalytic activity of the enzyme. It is possible that the site of the antibody-ChAT interaction includes the acetyl CoA binding site of the enzyme but not the choline binding site since co-incubation of the antibody with acetyl CoA affords some protection from the antibody inactivation while choline has no such effect (Crawford *et al.*, 1982).

Rossier (1976b) observed a very wide cross-reactivity from fish to mammals, and suggested that the poor cross-reactivity previously observed was due to the antiserum having low activity. The Crawford group produced two antibodies that was found to be highly selective for *Drosophila melanogaster* ChAT and did not cross-react with ChAT

present in vertebrate or other insect neural tissue (Crawford *et al.*, 1982). This limits the use of antibodies.

In 1987, Ishida *et al.* reported the production of monoclonal antibodies to bovine ChAT, which recognized sodium dodecyl sulfate (SDS)-denatured ChAT protein and a rapid and efficient immunoaffinity purification procedure of bovine ChAT using the monoclonal antibody-Sepharose 4B column. Previously, the group had produced antibodies but these antibodies did not react with SDS-denatured ChAT. Ishida *et al.* (1987)state that their purification procedure for ChAT results in a good recovery, and it would also be applicable to immunoaffinity purification of other antigen proteins.

1.4.4. Kinetic Mechanism of Choline Acetyltransferase

Choline acetyltransferase has been demonstrated and studied in a variety of animal tissues. The activity of the enzyme has been demonstrated in most human tissues too. Enzymatic activities in human brains determined by Mahoney *et al.* (1971) were lower than that determined by Bull *et al.* (1970). Nevertheless, Mahoney *et al.* (1970) found that high enzyme activities in the putamen, caudate nucleus, globus pallidus and thalamus existed.

There have been a number of investigations into the mechanism of the action of ChAT isolated from the rat brain, bovine caudate nucleus, human placenta and ox brain (Sastry and Henderson, 1972; White and Wu, 1973; Currier and Mautner, 1974; Hersh and Peet, 1977; Hersh, 1982). Studies on the kinetic mechanism have lead to several different mechanisms being proposed: sequential ordered, double displacement mechanism, Theorell-Chance mechanism and a sequential random mechanism (Hersh, 1982). The ChAT enzyme used in all these studies was obtained from various sources. Therefore there is the possibility that the enzyme from different mammalian sources would exhibit different mechanisms of action. Though it is known among well-known cases that the enzymes from different tissues usually have the same kinetic mechanism (Sastry and Henderson, 1972).

The one mechanism that appears to come up constantly, is the Theorell-Chance mechanism. See **Figure 1.10.** The mechanism can be defined as a type of ordered Bi Bi mechanism, which is different from the usual case as the steady-state concentration of a central enzyme-substrate complex is very low (White and Wu, 1973).

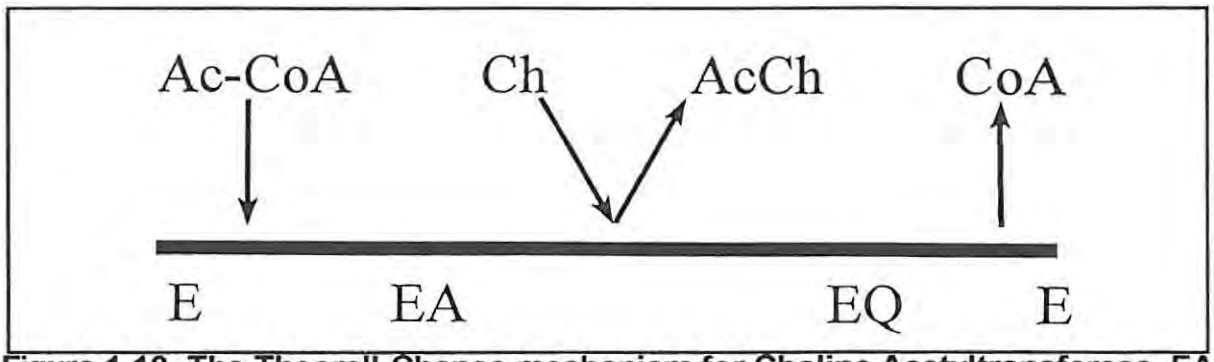

Figure 1.10. The Theorell-Chance mechanism for Choline Acetyltransferase. EA and EQ represent the transitory enzyme complexes. E represents the free enzyme (White and Wu, 1973).

Sastry and Henderson (1972) claim that the mechanism is Theorell-Chance because: (1) There is the formation of ternary intermediates that suggest an ordered mechanism. (2) Product inhibition patterns suggest a ping pong mechanism. These are the requirements for a special case of a Theorell-Chance mechanism. The experimental results that support this case are: (1) Reciprocal plots show the pattern of an ordered mechanism. (2) Competitive inhibition of the first substrate by the second product and of the second substrate by the first product. In other words Coenzyme A (CoA) competitively inhibits acetyl CoA and non-competitively inhibits choline. Also, ACh is a competitive inhibitor of choline and a non-competitive inhibitor of acetyl CoA, thus indicating the mechanism to be Theorell-Chance. In these experiments, Sastry and Henderson (1972) used human placentae as the enzyme source.

White and Wu have suggested that ChAT from rat brain may obey a different mechanism. These authors results show that inhibition by ACh of choline was partially

non-competitive, which is more consistent with an ordered Bi Bi mechanism. White and Wu state that a Theorell-Chance mechanism is distinguishable from an ordered Bi Bi mechanism, on the basis of whether ACh as a product inhibitor is competitive or non-competitive with varying concentrations of choline. Also, if the inhibition is competitive, then it should disappear with saturating concentrations of choline when acetyl CoA is varied. A non-competitive inhibition would become uncompetitive when choline is saturating (White and Wu, 1973). These authors found that there was only partial non-competitive inhibition. This was explained by ACh bearing a structural resemblance to choline. Therefore, it acts as a substrate analogue and a product inhibitor. Consequently, competitive inhibition is introduced to an otherwise non-competitive product inhibition when choline is varied, thereby resulting in a mixed type of inhibition (White and Wu, 1973).

Hersh and Peet (1977) using ChAT from human placenta, found that their results were not consistent with a Theorell-Chance mechanism. These researchers proposed that, apart from looking at initial velocity and product inhibition, one would have to examine the kinetics of dead end inhibitors and alternate substrates to determine the mechanism of the reaction. The results of Hersh and Peet led to the conclusion that ChAT reactions involve a random addition of substrates. Hersh and Peet go on to state that more work is needed to clarify this (Hersh and Peet, 1977).

Hersh (1982) later generated theoretical curves by substituting the appropriate substrate concentrations, V_{max} and previously determined kinetic constants into equations for a Theorell-Chance mechanism. This author then compared experimental data to the theoretical results, and found that there were large variations in the Michaelis constants from that predicted. This suggests that the reaction is not a simple Theorell-Chance mechanism, but a random mechanism in which a low steady state level of ternary complexes exist, and the kinetically predominant pathway is the one in which acetyl CoA or CoA binds before choline (See **Figure 1.10.**).

Therefore, the reaction will occur primarily through an ordered addition of substrate and

a low steady state of ternary complexes exists, and the mechanism approaches that of a Theorell-Chance type when the concentration of the ternary complexes is zero (Hersh, 1982).

As far as the debate over which of the two substrates, acetyl CoA or choline, is the lead substrate in ACh synthesis, most researchers believe it to be acetyl CoA. Sastry and Henderson (1972) give several reasons as to why acetyl CoA is most likely the lead substrate. Acetyl CoA is a coenzyme and coenzymes are usually the leading substrates in reactions. The K_m of acetyl CoA was found to be thirty times lower than that of choline, suggesting that acetyl CoA binds more strongly than choline. Furthermore, CoA inhibited the enzyme a hundred times more than ACh, indicating that CoA is the last product to dissociate from the enzyme. Also, CoA competitively inhibits acetyl CoA, suggesting that acetyl CoA is the first substrate to bind (Sastry and Henderson, 1972). White and Wu (1973) also suggest that acetyl CoA is the lead substrate since enzyme acetylation does occur in the absence of choline. Finally, Hersh (1982) proposed that while the kinetic mechanism for ChAT is random, the pathway whereby acetyl CoA binds first, is predominant.

1.4.5. Inhibition of Choline Acetyltransferase

One of the major gaps that exists regarding available information on ChAT is that there is no known useful direct inhibitor of the enzyme (Cooper *et al.*, 1978).

In a study by Giarman and Pepeu (1964), it was found that some centrally acting cholinolytic drugs cause a fall in total brain levels of acetylcholine in the rat. The synthetic cholinolytic agents in this study, hyoscine and atropine however do not cause this reduction in acetylcholine by inhibiting ChAT (Giarman and Pepeu, 1964).

ChAT is an enzyme that contains sulfhydryl groups. Chao and Wolfgram (1974) demonstrated that the enzyme is inhibited by heavy metal ions that have a high affinity for sulfhydryl groups. They tested a number of inorganic ions and found that mercury

chloride, silver acetate and copper sulphate gave a 100 % inhibition at a concentration of 10^{-4}M. Cadmium chloride and zinc acetate, at the same concentrations, are reported to cause approximately 50 % inhibition (Chao and Wolfgram, 1974).

Hemicholinium -3 is also known to inhibit acetylcholine synthesis. However, it does not inhibit the activity of the enzyme directly. It prevents ACh synthesis by preventing the uptake of choline into membrane-enclosed systems (Ansell and Spanner, 1975).

Numerous attempts have been made to prepare analogs of choline designed to label ChAT by forming covalent bonds within the active site. 3-bromoacetonyl trimethylammonium bromide had a low potency for causing a 50 % inhibition at a concentration of 5 X 10^{-4} M. The compound appears to bind irreversibly to the enzyme. Haloacetyl-cholines also inhibit ChAT, but there is only a 77 % inhibition with the most potent derivative, codoacetylcholine. Also, the inhibition was not irreversible (Mautner, 1977).

Bromoacetylcholine and bromoacetyl coenzyme A are also known inhibitors of ChAT and are reversible inhibitors (Roskoski, 1974). Another inhibitor reported by Malthe-Sorenssen *et al.* (1974), was acrylocholine. It is reported to inhibit rat and pigeon ChAT at concentrations of 5 x 10^{-5} and 2.5 X 10^{-5} M respectively. Inhibition is uncompetitive regarding both inhibitors (Malthe-Sorenssen *et al.*, 1974).

Several styrylpyridine analogs also inhibit ChAT but are light-sensitive, insoluble and can also inhibit acetylcholinesterase. Styrylpyridines have a vinyl group linking the aromatic rings which is essential for its inhibitory action. Only the trans vinyl compounds are active inhibitors (Maunter, 1977).

Many chemicals have been examined as potential inhibitors of this enzyme. Weak inhibitory activity *in vitro* (less than 50 % enzyme inhibition at 1 mmol/l) has been found with α-keto acids, naphtoquinones, nicotine, barbiturates, tetraethyl ammonium, acetylcholine, neostigmine, decamethonium and suxamethonium. ChAT inhibition by

these inhibitors has only been shown *in vitro*.

1.5. ACETYLCHOLINESTERASE

1.5.1. Introduction

Acetylcholinesterase (AChE) is the enzyme responsible for the termination of the neurotransmitter ACh after it has exerted its action on the post-synaptic membrane. The enzyme is synthesized in the rough endoplasmic reticulum and then transported (in association with the microtubules or smooth reticulum) to the terminals by axoplasmic flow. The enzyme is believed to be associated with nerve membranes and muscle cells. However AChE's precise association with the membrane is not well understood (Dudai and Silman, 1974). AChE is nature's most active enzyme. Hydrolysis of ACh by AChE occurs within seconds (Bradford, 1986).

AChE is also thought to have non-cholinergic functions along with its action at cholinergic synapses. In the central nervous system AChE has been shown to produce synaptic responses that are evoked by excitatory amino acids. This action is unrelated to cholinergic transmission (Appleyard, 1995). AChE has been found to occur in areas of the brain and in erythrocyte membranes where there are no cholinergic functions known to take place (Balasubramanian *et al.*, 1993).

Cholinesterases (ChE) are enzymes that hydrolyse a wide range of choline esters (for example, butyryl and propionyl choline). ChE are found in serum, erythrocytes, placenta, etc. AChE (EC 3.1.1.7), is a globular protein, which exists in multiple forms and is present in vertebrate nerves, muscle and erythrocytes. AChE is specific for ACh (Bradford, 1986).

1.5.2. Active Site of Acetylcholinesterase

The active site has two substrates 4-5 Å apart and are termed the anionic and esteratic sites. The anionic site is thought to consist of a carboxylate ion derived from the side chain of a glutamic or aspartic acid residue. It attracts the positive charge on ACh, which is the N atom in the choline portion. See **Figure 1.11.** The esteratic site is supposed to be more complex.

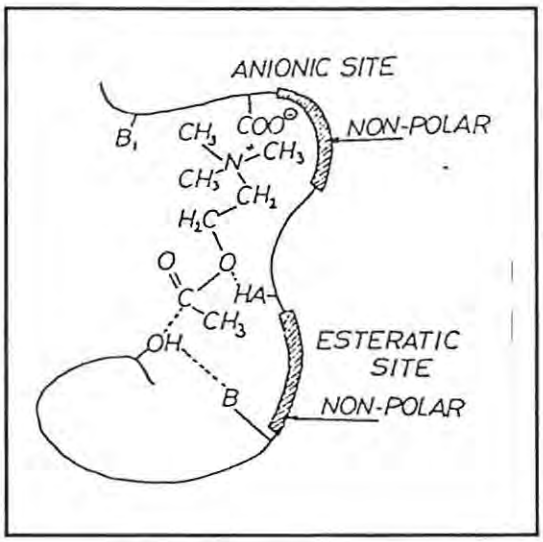

Figure 1.11. The Active Site of Acetylcholinesterase (Galley *et al*, 1973).

There is a serine hydroxyl present at this site. This serine hydroxyl becomes acylated when it interacts with the carbonyl carbon of ACh at the acetate portion of the neurotransmitter. The hydroxyl group is thought to be activated by the imidazole of a histidine residue (Bradford, 1986; Cooper *et al.*, 1978; Shuttleworth *et al.*, 1990). This leads to the temporary formation of an acetyl-enzyme complex that is hydrolysed.

1.5.3. Properties of Acetylcholinesterase

AChE is an allosteric enzyme and binds its substrate and other ligands in a complex

oligomeric form. The enzyme is a complex molecule with two to four or more different subunits. The smallest subunit has a molecular weight of between 62 - 64 000 daltons. Therefore the complete complex must have a very high molecular weight (Lopez *et al.*, 1993; Bradford, 1986).

Depending on the form of solubility, AChE can be isolated in various molecular forms of different molecular weights (Lopez *et al.*, 1993). Two classes of molecular forms of AChE exist. One form exists as a homologous assembly of catalytic subunits (Taylor and Brown, 1989). Solubilization of AChE with high ionic strength buffers produces several of these homologous globular forms that are monomers, dimers and tetramers of catalytic subunits (Lopez *et al.*, 1993). The dimers are linked by a single disulfide bridge and two dimers form a tetramer, probably by binding via Van der Waals forces. The tetramers are usually joined to three stranded "tails" by disulfide bonds. This is likely the basic unit of the enzyme with a molecular weight of around 330 000 daltons (Bradford, 1986). They differ in their degree of hydrophobicity. The hydrophobic character comes from a post translation addition of glycophospholipid on the C-terminal carboxyl group. The glycophospholipid is responsible for the enzyme attaching to the external surface of the cell membrane. The hydrophobic form can be found in excitable and non-excitable tissues (Taylor and Brown, 1989).

The second class exists as assemblies of heterologous subunits. One form consists of catalytic subunits (up to twelve) linked by a disulfide bond to filamentous collagen containing structural subunits. They are termed asymmetric as the tail unit imparts substantial dimensional asymmetry to the molecule. The asymmetry species appear on synapse formation and are localized to the synaptic areas. The collagenase tail unit is responsible for this molecular form being associated with the basal lamina (i.e. a mesh of connective tissue present in the gap between the pre- and postsynaptic membranes at the neuromuscular junctions (Kandel and Schwartz, 1986)) of the synapse instead of the plasma membrane. Evidently, this asymmetric form is particularly abundant in the neuromuscular junction (Taylor and Brown, 1989).

The precise biological reasons for the existence of all these forms of the same enzyme are not clearly understood, but their arrangement in different synapses, at neuromuscular junctions and ganglionic or central cholinergic synapses provides versatility in the control and efficacy of interaction of acetylcholine with its receptors in the small space of the synaptic cleft (Bradford, 1986).

1.5.4. Distribution of Acetylcholinesterase

Studies regarding intracellular distribution of AChE in the neurons of the central nervous system have shown that specific AChE (i.e. AChE responsible for Acetylcholine degradation) is concentrated in the cell membrane and continues into the dendrite and axonal prolongations. Koelle (1955) also cites reports of similar results regarding the giant axon of the squid by Boell and Nachmansohn. In the individual nerve cell, AChE is not uniformly distributed (Okinaka *et al.*, 1961).

Distribution of AChE in the human brain has been widely studied. Okinaka *et al.* (1961) cites a number of references, that conclude that the general distribution of the enzyme has not been clearly understood. These authors then proceed to investigate the AChE distribution by quantitative chemical methods and by histochemistry of the human cerebral cortex. Results imply that AChE activity in the cerebral cortex is weak compared with that in basal ganglia. Distribution of AChE in the cortex is not uniform. AChE activity is strong in areas such as the motor area, premotor area, auditory area, and the temporal lobe. It is also very strong in the hippocampus region compared to other areas of the cortex. AChE activity appears to be extremely weak in areas of the visual cortex. The activity of AChE found in the cerebrum is predominantly of the specific type (Okinaka *et al.*, 1961).

Other areas which have been researched for the presence of AChE activity are the anterior pituitary, posterior pituitary and the pineal gland. All these bodies are associated with the brain both functionally and anatomically. Studies by LaBella *et al.* (1968) on bovine and rat brains and pineal glands have demonstrated that there is less

AChE activity in the anterior pituitary, posterior pituitary and the pineal gland than in the whole brain. Of these three bodies, the posterior pituitary contained the highest AChE activity and anterior pituitary the lowest (LaBella *et al.*, 1968).

1.5.5. Inhibition of Acetylcholinesterase

Substances that inhibit or inactivate AChE are called anticholinesterases (anti-ChE) agents. These agents result in the accumulation of ACh at cholinoreceptive sites. This results in the production of effects equivalent to continuous stimulation of cholinergic fibres throughout the central and peripheral nervous systems. As the distribution of cholinergic neurons is widespread, anti-ChE agents have extensive practical applications as toxic agents, in the form of agricultural insecticides and potential chemical-warfare "nerve gas", than as drugs (Koelle, 1975).

1.5.5.1. Mechanism of Acetylcholinesterase Inhibition

Anti-ChE agents are among the few drugs in which the mechanism of action can presently be described in molecular terms. The mechanism of action of anti-ChE can be classified into three classes: Compounds that inhibit the enzyme reversibly by combining with it only at the anionic site thereby preventing the substrate from attaching. A more potent reversible inhibitor will also combine with the imidazole nitrogen atom of the esteratic site by hydrogen bonding. In some cases, inhibition is rapidly reversible and such drugs have a brief action following administration. Other inhibitors in this class, for example physostigmine (eserine), are thought to attach to the enzyme at both the anionic and esteratic sites, and then begin to undergo hydrolysis. The alcoholic moiety is split off, leaving a carbamylate enzyme that reacts with water to release a substituted carbamic acid and the regenerated enzyme (Koelle, 1975).

The reaction between AChE and most organic phosphorous inhibitors occurs only at the esteratic site. A phosphorylated enzyme is extremely stable. If the attached alkyl groups are methyl or ethyl, then a significant regeneration of the enzyme by hydrolytic

cleavage requires several hours. If the groups are iso-propyl, virtually no hydrolysis occurs and the return of AChE activity is dependent upon the synthesis of new enzyme (Koelle, 1955).

1.6. ACETYLCHOLINE RECEPTORS

1.6.1. Introduction

The notion of a receptor was a mental construction to explain the site on a membrane that is sensitive to a drug. Paul Ehrlich, a German biological chemist, proposed the idea of receptors to account for the selective action of toxins and other pharmacological agents, and the specificity of immunological reactions. In 1900, at a lecture, he said "chemical substances are only able to exercise an action on the tissue elements with which they are able to establish an intimate chemical relationship...[The relationship].. must be specific. The chemical groups must be adapted to one another.. as lock and key" (Kandel and Schwartz, 1986).

The ACh regulator is said to carry the receptor site for ACh, and the site for ion translocation (i.e. it is made up of two structural elements: the receptor unit (ACh receptor) and the biologically active unit). ACh binds to the receptor site and thereby has a regulating function, whereby it controls the opening of a nearby and closely linked ion channel (Giraudat and Changeux, 1980).

The acetylcholine receptor (AChR) has been under intense study for many years. The first success in extracting a functional receptor from intact membranes of the electric organs of *Electrophorus* and *Torpedo* fish was achieved in 1970, and highly purified preparations were obtained in 1973. Since then, much has been learned about its structure and function, and its relationship to various physiologically active substances (Bohinski, 1987).

As alluded to in section [1.3.6.] there are two categories of AchR - nicotinic and muscarinic cholinergic receptors. Each of these categories will be discussed further, with a greater emphasis on muscarinic AChR as the work done for this thesis concerned the muscarinic receptors.

1.6.2. Nicotinic Receptors

The nicotinic acetylcholine receptor (n-AChR) is a ubiquitous neurotransmission receptor and is the best described ligand-gated ion channel (Barrantes, 1993). The n-AChR is a 290 K protein that consists of 5 similar subunits ($\alpha\ \alpha\ \beta\ \tau\ \delta$) (Unwin, 1995). The subunits display partially homologous amino acid sequences. There is a 30 to 40 percent identification of amino acid residues. Structurally the subunits are arranged around a central cavity. This cavity is believed to be the ion channel, which (when in a state of rest) is impermeable to ions. When it is activated, it then opens to a diameter of 6.5 Å. The open channel is selective for cations. Permeation of the channel by particular cations appears to be limited primarily to the diameter of the open channel. Agonists and competitive antagonists bind to the alpha subunit. This is also the primary binding site with which alpha toxins of snakes associate. The duration of channel opening is dependent on the particular agonist, whereas the conductance of the open channel is agonist independent (Taylor and Brown, 1989).

The activation of the channel can be described as follows. Two ligands (L) associate with the receptor (R). Isomerization then follows to form the L_2R. The binding of the two ligands is not identical. When n-AChRs are continually exposed to agonists, a decrease in receptor response occurs. This occurs regardless of the available concentration of the agonists. This loss of response is known as desensitization.

1.6.3. Muscarinic Receptors

Muscarinic acetylcholine receptors (m-AChR) are found in neurons of the central and peripheral nervous systems, cardiac and smooth muscles, and a variety of exocrine

glands. Muscarinic receptors play a key role in regulating the functions of the target organs of the autonomic nervous system. Acetylcholine mediates a diverse range of responses, excitatory and inhibitory, *via* muscarinic receptors in the central nervous system and periphery. Centrally, activation of muscarinic receptors mediates many effects, including learning, memory, attention, mood, motor function, emotion, sleep, etc (Watson *et al.* 1986a). Therefore, they are important in the maintenance of the homeostasis of the organism. Activation of these receptors decreases the rate and force of contraction of the heart, constricts the airways, increases motility and secretions in the gastrointestinal tract, and increases secretions from salivary and sweat glands. The majority of the cholinergic synapses in the central nervous system are muscarinic (Nathanson, 1987).

1.6.3.1. Muscarinic Receptor Subtypes

The differences observed in the manner in which agonists and antagonists bind to m-AChR and the biochemical and physiological responses invoked, raises the question of whether more than one type of receptor exists. In 1980 Hammer *et al.* were able to differentiate between the binding properties of muscarinic receptors by using an antagonist, pirenzepine (PZ), a tricyclic compound. PZ (**Figure 1.12.**) is known to inhibit gastric secretion in animals and man, and this is supposed to be controlled by the m-AChR, thereby making PZ an antimuscarinic drug. The drug also antagonises the cholinergically stimulated contraction of the smooth muscles of the ileum and the urinary bladder, causes tachycardia (abnormally rapid heart beat), and blocks salivation. Hammer *et al.* concluded from their work that PZ is able to discriminate differences in subtypes of muscarinic binding sites that are not detectable by classical antagonists (for example, *N*-methylscopolamine) (Hammer *et al.*, 1980).

Szelenyi (1982) has questioned whether PZ is able to distinguish between subtypes of muscarinic receptors, as he found no evidence to confirm that PZ can differentiate between the subtypes of the receptor. This author also cites Daly *et al.* (1982) as support for his argument, since Daly's group had demonstrated that inhibitory doses

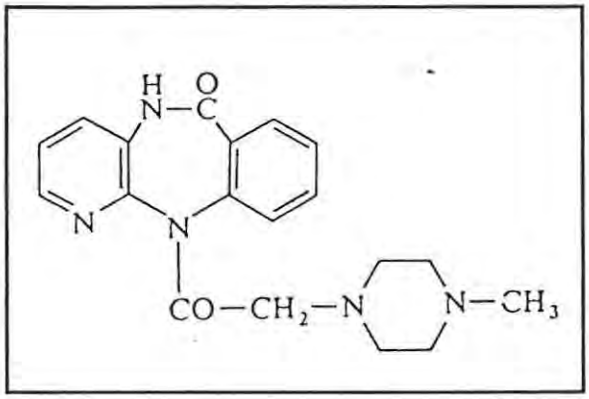

Figure 1.12. Structure of pirenzepine.

of PZ did not differentiate between muscarinic receptors of gastric acid and salivary secretions (Szelenyi, 1982).

There have, however, been other reports that support the fact that PZ differentiates between subsets. Binding studies by Watson *et al.* (1983) and Luthin and Wolf (1984) demonstrate that high affinity binding sites for PZ are subtypes of available muscarinic receptor binding sites .

The two subtypes of m-AChR, known as M1 and M2, were defined on the basis of their affinities, high and low respectively, for PZ. Muscarinic receptors have a variety of heterogenous properties that do not always correlate with this classification, and this suggests that there may be other classes (Bonner *et al.*, 1987). Recent works on brain cholinergic systems have shown that m-AChR belongs to five subtypes, instead of the two subtypes of the previous classification. Currently, pharmacological (radioligand binding) techniques characterise four subtypes of the receptor (M1, M2, M3, M4). Molecular biology techniques, however, identify five different subtypes of m-AChRs (m1, m2, m3, m4, m5) (Tata *et al.*, 1995). The m-AChRs are members of a huge superfamily of plasma membrane receptors that regulate cellular activity via coupling

to guanine nucleotide-binding proteins (G protein). These receptors are surmised to share a similar three-dimensional structure that consists of a tightly packed bundle of several transmembrane helices, linked by three extracellular and three intracellular loops (Wess *et al.*, 1995).

1.6.3.2. Regulation of Muscarinic Acetylcholine Receptors

The m-AChRs are found close to the cell surface proteins, and conduct their signals across the membranes by interacting with guanine triphosphate (GTP)-binding proteins (G proteins). G proteins are oligomers of three nonidentical subunits, and link various neurotransmitters to the regulation of adenylate cyclase. The initial event after ligand binding to the muscarinic receptor is its interaction with the G protein. This interaction initiates several biochemical events: inhibition of adenylate cyclase, stimulation of phosphoinositide hydrolysis, or activation of an ion channel, for example, K^+ channel (Taylor and Brown, 1989).

1.6.3.2.1. Adenylate Cyclase Inhibition

Two types of G proteins influence cyclase activity: G_s that stimulates, and G_i that inhibits the enzyme activity (Narayanan *et al.*, 1988). Activation of m-AChR in a sufficient number of cells, frequently causes a decrease in the rate of formation of cyclic adenosine monophosphate (cAMP). The receptor does not directly interact with the enzyme. It associates with adenylate cyclase via the G protein (Nathanson, 1987). The receptor activates G_i proteins, which compete with the G_s proteins for regulation of adenylate cyclase (Taylor and Brown, 1989).

1.6.3.2.2. Stimulation of Phosphoinositide Hydrolysis

Studies have shown that a large number of hormones and neurotransmitters in many different cells and tissues are able to stimulate phosphatidylinositol (PI) metabolism. PI is phosphorylated to give phosphatidylinositol 4,5-bis-phosphate (PIP_2). Activated

m-AChRs activates phosphlipase C, which cleaves PIP_2, to give two intracellular secondary messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Nathanson, 1987). Activation of phospholipase C also involves GTP, suggesting that GTP-binding proteins also have a role (Taylor and Brown, 1989).

1.6.3.2.3. Activation of Potassium (K^+) Channels

Activation of muscarinic receptors in the heart, decreases beating rate due to an increase in the permeability of the cardiac muscle membrane to potassium ions (K^+). Once the channel is activated by ACh, it has a high conductance for K^+ moving inward and a low conductance for outward moving currents (Nathanson, 1987). Recent evidence has shown that purified G protein subunits regulate K^+ conductance. This supports the likelihood that a G protein links the muscarinic receptor to the K^+ channel. Other evidence that also lends support to this, is the fact that other receptor activated channels are likewise coupled to their receptors in a GTP-dependent fashion (Taylor and Brown, 1989).

1.7. INORGANIC METAL TOXICITY

1.7.1. Introduction

Many toxic substances have been created and distributed throughout the environment by man. Toxic metals, however, are natural elements. Most of them have been important and useful to man since the beginning of civilization. These metals have been mined, purified and made into a variety of products. Metals have been attached to organic chemicals through synthetic and natural processes. These processes have often resulted in an increase in the toxicity of the metals. Human exposure to the metals has increased as a result of wide-spread distribution of the metals in air, water and soil. While many metals can be toxic, the metals of significance in this study are aluminium and mercury. These will be discussed further (Carpenter, 1994).

1.7.2. Aluminium

One of the most abundant elements is aluminium, and it comprises 8% of the earth's crust. This metal is the third most abundant element - after oxygen and silicon (Giordano and Costantini, 1993). Aluminium is present in trace amounts in most plants and animal tissues. Although biological systems have most probably evolved in the presence of aluminium, a number of observations indicate that high concentrations of the metal can be toxic (Crapper *et al.*, 1973). Aluminium ion (Al^+) concentrations in surface waters have (until recently) been minimal, as the aluminium hydroxide is insoluble at neutral pH. Acidification of surface waters through precipitation, releases Al^+ (Macdonald and Martin, 1988). Acidified well water and the unintentional overdosing of aluminium sulphate during water treatment can result in concentrations of aluminium that are large enough to constitute a major oral intake of aluminium (Glynn *et al.*, 1995). Living organisms have developed at pH ranges around seven, and cannot cope with the Al^+ activity that proves to be highly toxic biologically (Macdonald and Martin, 1988).

Aluminium compounds are also widely used in consumer products such as cosmetics, processed foods and pharmaceuticals. Therefore, a certain amount of exposure to aluminium is unavoidable. Aluminium exposure is thought to be higher with the use of medications such as antacids and buffered aspirin. At present there is no known biological role for aluminium, and the majority of ingested aluminium is supposed to either not be absorbed or be rapidly excreted from the body. There has however, in recent years been suggestions that aluminium exposure under certain conditions may lead to CNS toxicity (Shafer and Mundy, 1995). Some toxic effects of aluminium are: damage to the nervous system, the heart, the bones, the parathyroid glands and metabolic processes (Hosovski *et al.*, 1990). Perl *et al.* (1982) found that patients that died from Amyotrophic Lateral Sclerosis and Parkinsonism-Dementia in Guam, had a very high intra-neuronal accumulation of aluminium. Aluminium accumulation in control subjects was very low by contrast. Analysis of the drinking water and soil in the area show high levels of aluminium (Perl *et al.* 1982).

The possible association of aluminium and Alzheimer's Disease originated with the reports of Klatzos and colleagues (1965) and of Terry and Pena (1965). These authors described the induction of neurofibrillary degeneration in rabbits following exposure of the central nervous system to aluminium salts (Perl and Pendlebury, 1986). Increased concentrations of aluminium have been found in regions of neurofibrillary changes in brains with Alzheimer's Disease. The observed aluminium concentrations in humans with the disease, has been found to be comparable to that found in experimental animals with aluminium-induced neurofibrillary degeneration (Krishnan *et al.*, 1987). These findings suggest that exposure to aluminium may be important in causation of the disease (Martyn *et al.* 1989). Although aluminium is a proposed risk factor in Alzheimer's Disease, it is a controversial topic. The central disagreement among researchers regarding aluminium in Alzheimer's Disease revolves around the issue of timing and causality. Is aluminium deposited in the brain as a result or cause of Alzheimer's Disease? There is some evidence suggesting that aluminium is involved in the etiology of the disease, such as the neurofibrillary degeneration induced by aluminium in animals, and the elevated aluminium concentrations found in some Alzheimer's Disease brains (Graves *et al.*, 1990).

One of the most characteristic biochemical symptoms of AD is the generally diminished function of the cholinergic system (Bilkei-Gorzo, 1993). What is the effect of aluminium on the cholinergic system? In the present study the influence of aluminium chloride on the rat forebrain cholinergic system will be investigated.

1.7.3. Mercury

Mercury poisoning has a long history. It is the cause of the "mad hatter" disease best known from Alice in Wonderland. The disease was a result of neurological and behavioural changes resulting from exposure to mercuric chloride ($HgCl_2$), in the felt hat industry more than 400 years ago. In 1972, 500 people died and 6530 people were hospitalized in Iraq, as a result of eating imported grains treated with methylmercury. The symptoms were mainly neurological, with visual disturbances, ataxia, paresthesias,

hearing loss, dysarthria, mental deterioration, paralysis and death in some patients (Carpenter, 1994). The toxic effects of mercury are pronounced at the neurological level. Movement disturbances such as poor coordination and tremors, suggest that mercury may exert its effects by way of interfering with nerve function (Bondy and Agrawal, 1980). In recent years, it has been noted that mercury and methylmercury interfere with the migration of neurons in the developing embryo (Carpenter, 1994). Mercury is generally toxic to many enzymes, with severe effects on the CNS. The effects are pronounced during cerebral maturation. Methylmercuric chloride has been found to cause a decrease in cerebral acetylcholine levels (Bondy *et al.*, 1979).

The distribution of mercury in mice, following exposure to the metal has been studied. High exposure was found to result in mercury being distributed throughout the brain. Decreased exposure resulted in mercury being found primarily in the neocortical layer, white matter, thalamus and brain stem. Further decreases in mercury exposure saw the white matter and the brain stem as targets for mercury distribution (Warfvinge, 1995).

Mercury is used in the manufacture of electronics, plastics, fungicides and in a form of amalgam in dentistry (Carpenter, 1994). In 1993, the U.S. Department of Health and Human Services' (HAHS) report dealt with the amalgam that is used in tooth restoration. It is known as "silver" fillings and contains 50 percent mercury by weight (Lorscheider and Vimy, 1993). Amalgam is the most frequently used substance in restorative dental treatment, and is the main source of inorganic mercury exposure (Halbach, 1995). Experimental evidence shows that the daily total mercury released from a single mercury filling is approximately 15 µg. Mercury fillings are supposed to contribute to about two-thirds of the total human body burden of mercury. Experimental evidence in monkeys has shown that the mercury absorbed from fillings can be readily visualized by a body image scan in a variety of body tissue (Lorscheider and Vimy, 1993).

On the basis of available data, no claims are made that mercury is the cause of any

specific disease. However, experimental evidence shows pathophysiological changes in animals. This leads to the deduction that continuous exposure to mercury could have serious effects. The role of dental mercury as a causative factor of Alzheimer's Disease has been suggested as a possibility (Lorscheider and Vimy, 1993).

This study will examine the effects of mercury on the cholinergic system in the rat forebrain.

1.8. EXCITATORY AMINO ACIDS (QUINOLINIC ACID AND KYNURENIC ACID)

1.8.1. Introduction

Excitatory amino acids (EAA) are believed to play a major role in a host of neuropsychiatric diseases (Schwarcz *et al.*, 1992). This role for EAA was originally proposed by the work of Lucas and Newhouse (1957). These authors showed that systemic administration of glutamate to mice resulted in retinal degeneration (Beal, 1992). Administration of EAA (otherwise known as excitotoxins) to animals, causes specific chemical, behavioral, and neuropathologic disturbances. It is therefore not surprising that excitotoxic insults can provide models for diseases of the central nervous system as diverse as Huntington's disease, epilepsy, Alzheimer's disease, cerebrovascular disease and schizophrenia (Schwarcz *et al.*, 1992).

The potent neuroexcitatory effects of excitatory amino acids were first described over thirty-five years ago (Coyle, 1980). In the nineteen-sixties, Professor Cutis and his associates carried out studies which demonstrated the molecular characteristics that amino acids need to possess to have excitatory action. Specifically, there must be an amino group present that is optimally situated "alpha" to a carboxyl group and spaced two or three carbon atoms distant from a second acidic site.

This suggests that all active compounds react with a single three-point attachment site

on the exterior of a neuronal membrane. Modification of the amino group by methylation, or substitution of the distal carboxyl moiety by a sulphonated group, could yield compounds of greater excitatory potency (Watkins and Olverman, 1988).

From the stereochemical viewpoint, L(+1) isomers of endogenous acidic amino acids are approximately twice as active as their D(-) isomers. However, both enantiomeric members are excitatory, and both react with the same receptor site. With synthetic amino acids, it was found that the D(-) isomer is many times more active than the L(+) isomer (Watkins and Olverman, 1988).

Neurochemists have attempted to identify neuronal pathways in the mammalian Central Nervous System (CNS) which may utilize excitatory amino acids. An overview of this research suggests that the excitatory amino acids may play a fundamental role in brain function, that is only beginning to gain wide spread appreciation (Stone and Perkins, 1981).

The EAA's of interest in this investigation are quinolinic acid (QA) and kynurenic acid (KA) (Watkins and Olverman, 1988; Schwarz *et al.*, 1992; Stone and Perkins, 1981; Davies and Stanley, 1988). These amino acids are trytophan metabolites that are synthesised via the kynurenine pathway (See **Figure 1.13.**). These compounds are worthy of serious consideration as possible causative factors in degenerative disorders of the nervous system (Stone and Connick, 1985).

This study is aimed at investigating the effects of KA and QA on the cholinergic system.

1.8.2. Kynureninic Acid

Kynurenic acid (KA) is a metabolite of the kynurenine pathway of TRP metabolism (**Figure 1.13.**). It is a potent antagonist of neurotransmission at the CNS synapses that use excitatory amino acids as transmitters, and blocks amino acid induced excitotoxins.

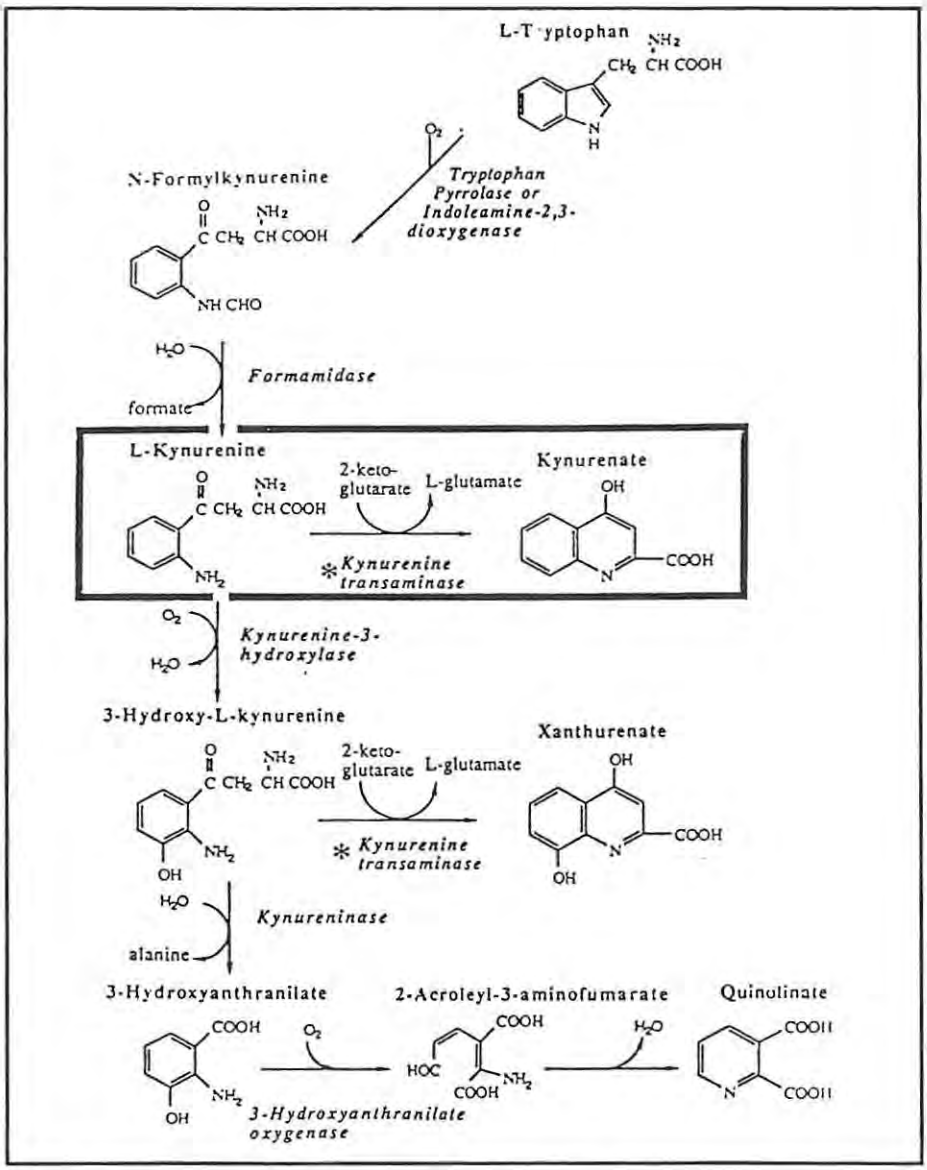

Figure 1.13. Kynurenine Pathway of Tryptophan Metabolism (Martin and Beal, 1992).

KA was first identified as a constituent of canine urine as early as 1853. Only in 1982 was KA recognised as having a possible biological function. KA is capable of preventing neurodegeneration induced by EAA receptor agonists. KA has been identified as a normal constituent of the human and rat brains (Lapin, 1982). *In vivo*, KA is especially potent as an antagonist of QA-induced neurotoxicity (Schwarcz *et al.*, 1992).

KA has a polar structure and therefore cannot penetrate the blood brain barrier under physiological conditions. Its bioprecursor, L-kynurenine, can however enter the rat brain via a transporter shared by other large neutral amino acids such as TRP and leucine (Schwarcz *et* al., 1992). In the mammalian brain, KA is produced irreversibly from L-kynurenine by the action of kynurenine aminotranferase (KAT). In the rat, only one type of KAT is known. Unlike the rat brain, the human brain has two types of KAT, termed KAT I and KAT II and synthesizes KA at physiological substrate concentration (Jauch *et al.*, 1995).

The metabolic pathway via which KA and QA are formed, is the result of oxidative cleavage of the indole nucleus between C-2 and C-3. The pathway is initiated by TRP pyrrolase, which is otherwise known as trytophan indoleamine-2,3-dioxygenase. TRP pyrrolase is an iron and copper dependent enzyme consisting of four subunits (Stone and Connick, 1985). The enzyme leads to the synthesis of N-formylkynurenine. See **Figure 1.13.** This enzyme is found in the liver and is enhanced by L-TRP feeding and the administration of corticosteroids. The existence of a cerebral enzyme was noted when [^{14}C]-L or D-TRP was incubated with rat brain homogenate supernatant, and the formation of kynurenine occurred (Gal and Sherman, 1978). The cerebral dioxygenase can be induced by TRP loading, but not by corticosteriods. Cerebral dioxygenase is similar to the TRP pyrrolase found in the intestine (Gal and Sherman, 1980). The metabolism of TRP via the kynurenine pathway is about 45 percent of the metabolism of TRP to 5-hydroxytryptamine. In the pineal gland, L-kynurenine accounts for about 32 percent of all TRP metabolites (Stone and Connick, 1985).

Studies by Jauch *et al.* (1995) have shown that in Huntington's disease (HD), the caudate nucleus and putamen show a decrease in KA levels and in the activities of the biosynthetic enzymes. This differs from previous findings. Jauch *et al.* (1995) cite a Connick *et al.* publication, which suggests increases in KA concentrations in HD. A possible explanation given by Jauch *et al.* (1995) for the differences in results obtained by researcher,s was differences in tissue dissection techniques (Jauch *et al.*, 1995). In principle, a decrease in KA concentrations, a neuroprotective agent, could be a causative factor in neuronal degeneration, and this could underlie a host of neurological diseases (Schwarcz *et al.*, 1992).

1.8.3. Quinolinic Acid

Quinolinic acid (2,3-pyridine dicarboxylic acid) (QA) is a heterocyclic amino acid (Wolfensberger *et al.*, 1983) which is also an endogenous metabolite of the kynurenine pathway of tryptophan (TRP) degradation (**Figure 1.13.**), and a precursor of nicotinamide adenine dinucleotide in mammalian peripheral organs. It is also a structural analog of N-methyl-D-aspartate (NMDA). QA was sought in the brain after an electrophysical demonstration of its activity (Stone and Connick, 1985).

The excitotoxin is synthesized in the liver and the CNS (Flanagan *et al.*, 1995). It is also present in the kidney of several animal species (Moroni *et al*, 1984a). The types of cells that synthesize QA have as yet not been identified, but cultures of human macrophages have been found to convert L-TRP to QA (Flanagan *et al.*, 1995). QA has been reported to be present in the brain in an uneven distribution pattern. The cortex and the hippocampus are the regions that appear to have the highest concentrations of the EAA molecule. Moroni *et al.* (1984b) suggest that the presence of QA in the brain implies that QA plays a specific role in brain function. However QA function and metabolic fate are still unknown (Schwarcz and Köhler, 1983). Stone (1984) cites Curtis and Johnston's suggestion that it is possible that QA may play a physiological role in determining the excitability of neurones bearing amino acid receptors. QA is known to be a potent endogenous excitotoxin capable of reproducing the

neuropathological features of several human neurologic diseases. It has been shown that QA-induced neurotoxicity closely mimics the patterns of selective nerve cell loss seen in Huntington's Disease, and temporal lobe epilepsy (Martin and Beal, 1992). QA treatment has been used to provide models for Huntington's disease. It is possible that QA has a role in the etiology of this disorder (Schwarcz *et al*, 1983).

There have been suggestions that QA's role in human neurodegenerative diseases may be based upon its neurotoxic and convulsant properties. The endogenous excitatory amino acids, aspartate and QA induces depolarization and is associated with epileptic seizures, either in focal epileptic neurons or electrical changes in limbic neurons during seizure activity (Patel *et al.*, 1988).

QA is a potent excitotoxin and has been suggested to induce neurodegenerative disorders in humans. The acid has been shown to have an excitatory effect on cortical neurons (Perkins and Stone, 1983). When it is injected intrastriataly, QA causes degeneration of striatal neurons (Woodruff *et al.*, 1988). Neurons in the spinal cord and the cerebellum have been found to be less sensitive to the effects of QA. QA is found in high concentrations in the cortex. It is more active in this region, than in the spinal cord. This difference in susceptibility to the acid, suggests that it is possible that two types of quinolinate-activated receptors exist. NMDA displayed no differences in its activity to the same receptors (McLennan, 1984).

QA concentrations also appear to change with age. Experiments with rats show that the concentrations of QA increase with age. TRP administration was also found to induce an increase in QA concentration in the brains of adult rats, but not in the brains of newborn rats (Moroni *et al.*, 1984a).

1.9. NEUROLOGICAL DISEASES

1.9.1. Introduction

Long before recorded history, people identified abnormal behaviour with events occurring inside the head. As the brain does not permit easy access, theories about the organic basis of abnormal behaviour remained for centuries in the realm of speculation. Today such speculation is beginning to be informed by concrete evidence.

The brain is no longer the dark territory that it used to be. Probably the greatest source of excitement in attempts to understand the biological basis of abnormal behaviour (physically and mentally), has been the advent of neuroscience.

The neuroscience perspective focuses on the organic determinant of behaviour with regard to the physical and psychological aspects of our functioning - the so- called mind-body problem, which was first discussed in the early seventeenth century by the French philosopher Descartés (Bootzin and Acocella, 1988).

1.9.2. Organic Brain Disorders

Disorders such as Schizophrenia, anxiety, depression, Alzheimer's disease, etc., have historically been traced to the individuals' relations to his or her experiences and environment. Current theories have now begun to recognize the role of biological factors in some of these disorders. For example, it has been shown that certain people may be genetically vulnerable to schizophrenia.

There are a number organic brain disorders characterized by a general deterioration of intellectual, emotional and motor functioning as a result of progressive pathologic changes in the brain, in other words as a result of neurodegeneration. Some of these disorders, for example Alzheimer's disease and Huntington's disease, which are connected with the cholinergic system, will be discussed further .

1.9.3. Mechanism of Pathogenesis

Degenerative diseases are characterised by histologic changes that are strangely nonspecific. Interpretations of findings with regard to the diseases are difficult. This has therefore forced researchers to speculate on the basis of background information of neurobiology on the possible mechanisms of pathogenesis. The theoretical choices are diverse:

(1) Toxic damage - There are two types of toxins argued to be linked with degenerative diseases. The first one is extrinsic toxin. Studies regarding the ability of individuals to detoxify noxious agents, indicate that disease might be derived from exceptional vulnerability to widespread substances. It is not known if acute, subacute, or chronic exposure might have an effect, or if damage is immediate or delayed. There is also no evidence to suggest whether organic or inorganic toxins are more likely to have an etiologic role. The second type of toxin to be proposed is intrinsic toxin. These are toxins normally present within the CNS. Two classes of endogenous agents have been implicated to play a part in pathogenesis - free radicals and excitatory amino acids. Although intrinsic toxins appear to play a role in neuron damage, there is no evidence to support this theory.

(2) Infective damage - Viruses are known to produce Parkinsonism, dementia and motor neuron death (poliomyelitis). Specific neuronal pathways undergo progressive degeneration after bacterial infection. There is however no evidence of past infection as a cause of neurodegenerative diseases.

(3) Failure of DNA repair - Errors are known to occur when DNA replicates, but central neurons do not replicate. Therefore, failure of DNA to repair correctly cannot be implicated in neurodegenerative diseases.

(4) Autoimmunity - There is much evidence available that demonstrates immunologically activated cells mounting an attack on tissue components, for example attacking thyroid cells in Hashimoto's thyroiditis, and destroying cholinergic receptors of the neuromuscular junction in myasthenia gravis. Activated microglia are known to accumulate at sites of nerve attrition in neurodegenerative disorders. There is nevertheless no known method, as yet, of distinguishing whether the activated

microglia present kill the nerve cells or simply remove the dead cells for some reason.
(5) Failure of a vital function - This theory encompasses the range of possible cellular disturbances that follow changes such as defective nutrient supply, defective removal of waste products, impaired activity of organelles such as mitochondria, and faulty production of a "growth factor". The only evidence to support these possibilities is the decrease in mitochondrial complex I reported in diseases such as Huntington's and Leber's diseases.
(6) Late expression of a lethal gene - There are several examples of programmed cell death, and the possibility that late expression of a gene promotes cell death cannot be ruled out (Calne *et al.*, 1992).

1.9.4. Alzheimer's Disease

Alois Alzheimer, a German neuropathologist, described in 1907 the case of a fifty-one year old woman who developed memory loss and personality deterioration. When the autopsy on the woman was done, the brain showed changes that today are known as neurofibrillary degeneration and senile plaques. In 1912, the condition was recognised as a clinicopathological disease by Kraepelin, and he applied the term "Alzheimer's disease" (Trapp *et al.*, 1978). Alzheimer's disease (AD) can be described as a slow, insidious onset, but progressive impairment of memory, reasoning, abstraction and language. It is also accompanied by personality changes (Shen, 1994), and these changes differ from person to person (McLean, 1993). The disease results in the disruption of mainly the frontal, temporal, and parietal cortical association areas and the hippocampus (Paulsen *et al.*, 1995). AD is a common and debilitating disease. It produces a marked disability in at least 2 percent of the population over sixty-five. The aetiology of the disease is unknown (King, 1984).

Studies have been focused on animal models, humans and in areas of clinical, pathological and biochemical aspects, genetics, cellular and molecular biology. A large number of senile plaques (SPs) and neurofibrillary tangles (NFTs) are the hallmarks of AD. The NFTs are readily observed with the light microscope after silver

impregnation staining, and consist of parallel arrays of thickened coarse, argyrophilic fibres within the neuronal cytoplasm (Perl and Brody, 1980). There are, however, many questions that require answers. For example, what is the mechanism by which the SPs and NFTs are formed? Why are the progressive dysfunction and death of many neurons within the cortex and limbic-paralimbic, system and subcortical regions that project to cortical and limbic areas, associated with the development of SPs and NFTs? (Shen, 1994). Another characteristic associated with AD, is deficits in parameters of cortical cholinergic neural function. There is a widespread agreement that ChAT and AChE are markedly reduced in AD brains, especially in the hippocampus and cortical area (Whitehouse, 1982; Lang and Henke, 1983; Bogdanovic *et al.*, 1993). Choline transport is also known to be affected. The transport of choline into central cholinergic nerves is a rate-limiting step in the synthesis of ACh, and its inhibition reduces neuronal function (King *et al.* 1983).

Reductions in cholinergic systems have also been detected in normal old age but to a lesser extent than that in dementia. The regional distributions of cholinergic enzymes in normal aged brains and in AD were found to be similar (Perry *et al.*, 1977b). The degeneration of nucleus basalis cholinergic neurons with a subsequent reduction in ChAT in the cerebrocortical projection fields of these neurons, was the first specific neurochemical alteration discovered in AD research in 1976 (Bissette *et al.* 1996). ChAT activity has been found to be significantly reduced in the cerebral cortex and the hippocampus of the diseased brain, as compared with that of age-matched normal subjects (Perry *et al.*, 1977b). Age appears to influence ChAT in such a way that a marked decline in the enzyme is observed in most areas of the brain as age increases (Perry *et al.*, 1977a).

One of the main neuronal sources of cholinergic projections is the substantia innominata (SI). These regions have recently been shown to have pathological changes on AD. Nagai *et al.* (1983) cites Whitehouse *et al.* who have reported substantial reductions in large cells in the SI in AD, and has suggested that this reduction could account for the decrease in ChAT in the neocortex. Rossor *et al.*

(1982) found ChAT activity in the SI to be significantly reduced in AD as compared with the controls. The Rossor team also investigated surrounding areas of the brain, such as the amygdala, putamen, globus pallidus and hypothalamus, and found that only one other area, the amygdala, showed a significant decline in ChAT activity. Unlike Whitehouse (cited by Nagai *et al.*), Rossor does not believe that the loss in ChAT activity is a reflection of cell loss. Also, loss in ChAT activity in the SI but not in the hypothalamus and other adjacent areas, means that there is not a generalized metabolic deficit of cholinergic enzymes in AD. Nevertheless, it does support the view that dysfunction of discrete populations of cholinergic neurons is involved in the pathogenesis of the disease (Rossor *et al.*, 1982).

Another enzyme of the cholinergic system is AChE which is responsible for the breakdown of acetylcholine. This enzyme has been reported to be significantly reduced in AD. In a series of experiments carried out by Davies (1979) it was found that there is a significant decrease in AChE activity in AD. The caudate region of the brain showed the smallest reduction in AChE activity, while the cortex exhibited a major decrease of 80-90% in AChE activity. In the controls, there were age-related declines in cortical AChE activity, but there were no significant differences between young and older AD cases. Davies (1979) also found that there was no reduction in the enzyme activity in the Cerebrospinal fluid (CSF).

m-AChR studies in AD have produced conflicting reports regarding changes in the receptors. In several reports, m-AChRs are said to increase or be preserved. One argument in favour of an increase in receptors, is that receptor mediated signal transduction in AD is not functionally intact. Evidence shows that a direct interaction between muscarinic receptors and guanine nucleotide binding proteins, exists. The presence of GTP induces a dissociation of the G protein's α subunit, and the receptor obtains a low affinity. Warpman *et al.* (1993) report findings that support the assumption that muscarinic receptors mediated signal transduction mechanism via G proteins might be altered in AD. The Warpman group (1993) also support the reports stating an increase in the receptors, and suggest that a deficiency in G protein coupling

might induce an increase in muscarinic receptors, as a compensatory mechanism of transduction failure. Other reports state that there is a decrease in receptor numbers particularly for the M subtype (Svensson *et al.*, 1992). Lang and Henke (1983) found that there was no significant change in the concentration of m-AChRs in any regions of the AD brains they examined. In 1985, Mash *et al.*, decided to investigate the situation on the basis of there being subtypes, and have reported a loss of M2 muscarinic receptors in advanced AD. Mash *et al.* (1985) also maintain that the receptors diminish in proportion to the reduction in ChAT activity. The M1 receptors are evidently unchanged in cases of AD. Similar findings were also reported by Bogdanovic *et al.* in 1993.

In the past decade there have been significant advances in our knowledge of AD. It is now known that a major component of AD is loss of neurons and synapses, particularly in the cortex and the hippocampus (Katzman *et al.*, 1991). The cholinergic loss correlates strongly with the loss of memory and function during life (Katzman, 1986). However, the basis of this cholinergic abnormality is still unclear (Whitehouse *et al.*, 1982). The findings of this cholinergic abnormality have led to extensive trials of possible acetylcholine precursors, such as 2-dimethylaminoethanol ("Deanol"), lecithin and choline, for the possible improvement of memory (McGeer and McGeer, 1981). Several companies have been seeking effective drugs for AD. These have focused mainly on ACh replacement therapies. Some of the drugs are tacrine, which is approved by the Food and Drug Administration (Bethesda, MD) and the unapproved velnacrine. Cholinesterase inhibitors increase acetylcholine availability. Tacrine has so far been marginally useful, showing benefit in a small fraction of AD patients (Schehr, 1994).

1.9.5. Huntington's Disease

Huntington's disease (HD) is an inherited autosomal dominant neurodegenerative disorder, characterized by chorea and dementia with a progressive course leading to severe debilitation and death, usually within 15-20 years (DiFiglia, 1990). The disease

is characterized by a progressive movement disorder, personality change, and a "tendency to insanity". The clinical diagnosis may be confirmed by the detection of an abnormally expanded trinucleotide repeat sequence on chromosome 4 (Jackson *et al.* 1995). There is a loss of neurons in the striatum (caudate nucleus and putamen). In later stages of the disease, there is also severe loss of the cerebral cortical neurons (Martin and Beal, 1992).

Several investigators have explored the biochemical alterations in the processes mediating synaptic neurotransmission in the brains of patients with HD. There is a striking reduction in the activity of glutamic acid decarboxylase, the enzyme that synthesizes the neurotransmitter GABA. There is also a patchy but severe reduction in the activity of ChAT, the enzyme that is responsible for the synthesis of acetylcholine. Stahl and Swanson (1974) found a reduction in ChAT activity of 73 to 99 % in striatal tissue in some HD cases. In these cases, there was no apparent reduction in cerebral cortex. This ChAT activity decrease was also found to occur in the caudate and putamen (Stahl and Swanson, 1974).

Several neurotransmitter receptors are also altered in HD. The density of muscarinic m-AChRs shows a reduction of 40 to 60 % in the caudate, putamen, and globus pallidus in patients with HD. Other receptors that show major reduction are the serotonin receptor, especially in the globus pallidus. β-adrenergic receptor binding was also found to be significantly reduced, but only in the globus pallidus (Coyle *et al.*, 1977). The other cholinergic enzyme AChE showed no reduction of activity in HD cases (Stahl and Swanson, 1974).

CHAPTER 2

CHOLINE ACETYLTRANSFERASE

STUDIES

2.1. INTRODUCTION

Choline acetyltransferase (Acetyl CoA : choline *O*-acetyltransferase, E.C. 2.3.1.6) catalyses the synthesis of acetylcholine (ACh) from acetyl CoA and choline. This enzyme is of major interest in neurobiochemistry.

Investigations into choline acetyltransferase (ChAT) function have been limited, until recently, owing to the lack of a sensitive, quick and accurate assay for the enzyme. Hestrin (1949) designed a colorimetric method but it was not sensitive enough. In 1966, Schuberth developed a radiochemical assay for the enzyme. This assay required an acetyl CoA generating system since it could not distinguish acetyl CoA from ACh. Selective precipitation methods were developed by McCaman and Hunt (1965) and Alpert *et al.* (1966). In these assays, ACh is precipitated as reineckate. The methods are specific and sensitive, but the tedium of washing and centrifuging the precipitated product remains (Schrier and Shuster, 1967).

In 1967, Schrier and Shuster developed an assay method that took advantage of the fact that the enzyme transfers an acetyl moiety from an anionic substrate (acetyl CoA) to a cationic substrate (choline) resulting in a cationic product, namely, ACh. The two classes of ions could then be separated by anion exchange chromatography. Schrier and Shuster (1967) reported that the method was rapid and simple to perform, very reproducible and highly sensitive. It was also reported to be adaptable to assay either

the bacterial or mammalian ChAT enzyme. This method was however found to produce an artificially high value for ChAT activity, since it was unable to separate ACh from Acetylcarnitine (ACar). ACar is another product, apart from ACh, which is found to be produced in a cell homogenate (Hamprecht and Amano, 1974).

The requirements for a reliable radiochemical assay for ChAT are: reproducibility and rapid and specific procedures for isolating ACh from the incubation mixture. This was achieved by Fonnum (1969, 1975). In this assay, ACh is precipitated as a tetraphenylborate (TPB) salt. The ACh is isolated by liquid cation exchange, with TPB being the cation exchange group. The assay is also able to facilitate the isolation of ACh from carnitine, so that artificial values for ChAT activity are not obtained.

2.2. ChAT ASSAY TECHNIQUE

2.2.1. Theory of Assay

Very small amounts of ACh are present in biological preparations and it is relatively unstable. Therefore, the isolation of this compound is difficult to achieve. Column, paper, and thin-layer chromatography or electrophoresis can be used to isolate concentrated amounts of ACh. The problem of ACh isolation from dilute solutions, has become increasingly important with the introduction of radioisotopic ACh. Methods used prior to the introduction of the radiochemical assay were time-consuming, inefficient and could not be used in conjunction with liquid-scintillation counting, as they often produced coloured precipitates and solutions. The assay used in present studies is based on the observation that ACh-TPB complexes are insoluble in aqueous solutions, but soluble in some organic solvents such as nitriles and ketones. Very low concentrations of ACh can be isolated from biological preparations, and the radioactivity readily determined with high efficiency, by liquid scintillation spectroscopy (Fonnum, 1969).

The isolation of the radiolabelled ACh is achieved in a biphasic aqueous: toluene scintillation solution mixture. The biphasic aqueous solution used is acetonitrile. The procedure depends on two observations: (1) acetonitrile in toluene constitutes an efficient extraction solvent for liquid cation exchange extraction of acetylcholine with TPB; (2) liquid scintillation counting of the organic phase can proceed undisturbed in a biphasic organic aqueous solution mixture. The procedure allows the extraction of the radiolabelled ACh, but not the radiolabelled acetyl-CoA, which remains in the aqueous phase (Fonnum, 1975).

2.2.2. Methods and Materials

2.2.2.1. Chemicals, Drugs and Reagents

[^{3}H] acetyl coenzyme A (specific activity 3.3 Ci/mmol) was obtained from Amersham International, England. Acetyl CoA, physostigmine (eserine) and sodium tetraphenylboron (Kalignost) were purchased from Sigma Chemical Co., USA. Acetonitrile and butyronitrile were obtained from Merck (Germany). All other chemicals used were of the purest analytical grade available.

2.2.2.2. Animals

Inbred adult male albino rats of the Wistar strain were used in this study. All animals were kept in groups of 5 in opaque white plastic commercial cages. A metal grid served as the floor in each cage. Animals were maintained on a diet of standard rat pellets and tap water *ad libitum*. The animals weighed between 200-300 g at the time of sacrifice. The animals were maintained under artificial illumination, with a daily 12 hour light phase (lights on at 06h00) and a 12 hour dark phase. During the light phase, the light intensity was approximately 300 μWatts/cm^2. The temperature in the room was kept at a constant 24 °C. Stale air was continuously removed from the room by an extractor fan. Rats were killed swiftly by cervical dislocation and decapitation. After decapitation, an incision was made through the bone from the foramen magnum to the

area near the orbit, with a pair of scissors. A clean, sterile dissecting forceps was inserted into this incision and the skull was opened, lifted and folded back. The brain was gently peeled away from the dura and removed. The forebrain was dissected, frozen in liquid nitrogen and stored at -70 ^{0}C until required.

2.2.2.3. Preparation of Tissue Extract

The forebrain of the rat was allowed to thaw. The tissue was then homogenized with a glass homogenizer in 5-10 volumes of ice-cold sodium phosphate buffer (50 mM, pH 7.4), containing 1 mM EDTA. The homogenates were then centrifuged at 900 X g for 10 minutes at 4 ^{0}C. Triton X-100 [1% (v/v)] was added to each supernatant solution, which was kept on ice with occasional shaking.

2.2.3. Choline Acetyltransferase Assay

ChAT activity was assayed by a technique that measures the transfer of the [^{3}H] acetyl moiety from [^{3}H] acetyl CoA to choline, resulting in the formation of [^{3}H] acetylcholine. The labelled acetylcholine is then isolated from the aqueous solution by liquid cation exchange, where sodium tetraphenylboron (Kalignost) is the organic solvent (Phansuwan-Pujito *et al.*, 1990).

The incubation mixtures contain (final concentration) 10 mM choline chloride, 300 mM sodium chloride, 10 mM EDTA, 50 mM sodium phosphate buffer (pH 7.4), 0.1 mM eserine, [^{3}H] acetyl CoA (0.05 µCi - 0.1 µCi per assay tube) and unlabelled acetyl CoA to give a final concentration of 0.5 mM. Aliquots of 10 µl of the incubation mixtures were transferred to test tubes, which already contained 10 µl of the enzyme solution. In all studies, protein concentration ranged between 3.5-4.0 mg/ml. These reaction mixtures were then incubated at 37 °C for 15 minutes. The incubations were then terminated by adding sequentially and immediately, 2.5 ml of 10 mM sodium phosphate buffer (pH 7.4) and 1 ml of butyronitrile containing 15 mg of TPB. The solutions were then vortexed for 10 seconds and centrifuged at 900 X g for 5 minutes. Aliquots of 0.5

ml of the organic layers of the solutions were transferred to scintillation vials containing 2 ml acetonitrile and 3 ml of Emulsifier Scintillator Plus scintillation cocktail. The radioactivity was then measured using liquid scintillation spectroscopy at 63 % efficiency. The activity of choline acetyltransferase was expressed as pmol of [^{3}H] acetylcholine formed/mg protein/min. Using the Eadie-Hofstee and Hanes-Woolf plots, K_m and V_{max} values were estimated. The data were calculated and the line of best fit was ascertained, using the GraphPad InPlot computer program developed by Graph Pad Software, Inc, USA.

2.2.4. Protein Determination

The protein concentrations of the solutions were determined by Folin Ciocalteu Protein Assay, using bovine serum albumin as a standard (Lowry *et al.*1951). The protocol for this method is outlined in **Table 2.1.** and explained below. A typical protein standard curve obtained by the Folin Ciocalteu method is shown in **Figure 2.1.**

To determine protein concentrations using this assay, the following series of assay tubes were prepared: (1) a blank containing 1.0 ml H_2O; (2) a duplicate set of assay tubes containing appropriate aliquots of water and protein standard solution (eg. Bovine Serum Albumin (BSA) 1 mg/ml) to yield final protein concentrations of 50, 100, 200, and 300 µg in a final volume of 1.0 ml. (3). 100 ml of *fresh* alkaline copper reagent was prepared by *mixing in order* 1 ml of 1% $CuSO_4.H_2O$, 1 ml of 2% sodium tartrate and 98 ml of 2% Na_2CO_3 in 0.1 N NaOH. 6 ml of the alkaline copper reagent was added to each tube and mixed immediately. After 10 minutes, at room temperature, 0.3 ml of Folin-Ciocalteu reagent was added to each tube.

The test tubes were rapidly vortexed. The test tubes were allowed to stand for 30 minutes and the absorbance of the standards and unknown was determined at 500 nm against the blank.

TABLE 2.1. Scheme for the determination of protein concentration (Lowry *et al.*, 1951)

Protein Concentration (µg/ml)	Blank	50	100	150	200	250	300
Protein Volume (1 mg/ml) (ml)	0	0.05	0.1	0.15	0.2	0.25	0.3
Distilled Water (ml)	1.0	0.95	0.9	0.85	0.8	0.75	0.7
Alkaline Copper reagent (ml)	6	6	6	6	6	6	6

Stand for 10 minutes at room temperature

Folin-Ciocalteu reagent (ml)	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Total (ml)	7.3	7.3	7.3	7.3	7.3	7.3	7.3

Stand for 30 minutes at room temperature

Read absorbance of the standards at

500 nm against the blank which contained no protein.

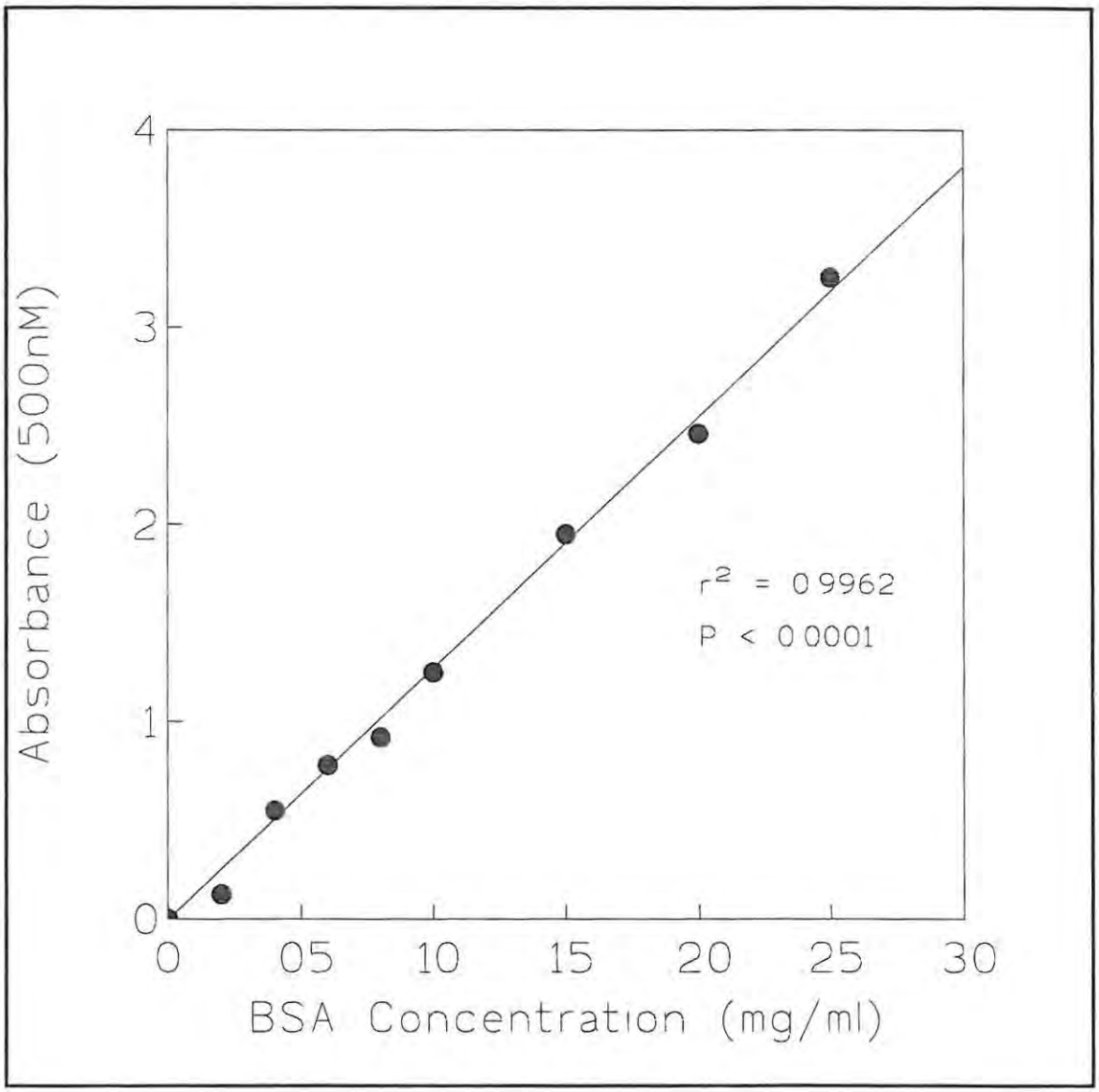

FIGURE 2.1. **A typical protein standard curve. Each point represents the mean of triplicate determinations.**

2.2.5. Kinetic Studies

For Michaelis-Menten studies, the final concentrations of choline chloride was varied from 0 - 20 mM, while keeping the concentration of "cold" acetyl CoA (i.e. unlabelled acetyl CoA) at a constant level of 0,5 mM and [^{3}H] acetyl CoA constant at 0.05 µCi. Conversely, the final concentrations of acetyl CoA were varied from 2.5 - 0 mM, while keeping the concentration of choline chloride at a constant concentration of 10 mM. The V_{max} (i.e. the maximum initial velocity at a particular concentration of the enzyme present) and K_m (i.e. the substrate concentration at which the initial velocity is equal to $½V_{max}$) were calculated.

2.3. RESULTS

The Michealis-Menten Curve for the variation of the substrate choline chloride for ChAT obtained from rat forebrain is shown in **Figure 2.2.** The correlation coefficient (r^2) was found to be 0.986. **Figures 2.3.** and **2.4.** represent the Eadie-Hofstee plots, [where velocity (V) versus velocity/ substrate (V/S)], and Hanes-Woolf plots,[where V/S versus S], respectively. These figures were generated from the data of **Figure 2.2.** The r^2 V_{max} and K_m values calculated from **Figures 2.3.** and **2.4.** are represented in **Table 2.2.**

TABLE 2.2. The V_{max}, K_m, P-value and r^2 calculated for choline acetyltransferase from rat forebrain when choline chloride concentration was varied and acetyl CoA concentration was constant.

PLOT	V_{max} (pmoles/mg/min)	K_m (mM)	r^2
Eadie-Hofstee	0.28 ± 0.10	0.42	0.888
Hanes-Woolf	0.26 ± 0.08	0.34	0.998

The mean K_m value when choline chloride was varied from 0-20 mM and acetyl CoA was fixed at 0.5 mM for rat forebrain ChAT was 0.38 ± 0.04 mM.

The Michealis-Menten Curve for the variation of the substrate acetyl CoA for ChAT obtained from rat forebrain is shown in **Figure 2.5.**, **Figure 2.6.** and **Figure 2.7.** represent the Eadie-Hofstee,[V versus V/S], and Hanes-Woolf plots,[where V/S versus S], respectively. These figures were generated from the data of **Figure 2.5.** The r^2, V_{max} and K_m values calculated from **Figures 2.6.** and **2.7.** are represented in **Table 2.3.**

TABLE 2.3. The V_{max}, K_m, P-value and r^2 calculated for choline acetyltransferase from rat forebrain when acetyl CoA concentration was varied and choline chloride concentration was kept constant.

PLOT	V_{max} (pmoles/mg/min)	K_m (mM)	r^2
Eadie-Hofstee	**0.10 ± 0.007**	**0.07**	**0.847**
Hanes-Woolf	**0.10 ± 0.140**	**0.08**	**0.999**

The mean K_m value calculated in this study for ChAT, when acetyl CoA was varied from 0-2.5 mM and choline chloride was fixed at 10 mM, was 0.075 ± 0.05 mM.

2.4. DISCUSSION

The protein concentration of the tissue extract was determined by the Folin-Ciocalteu assay (Lowry *et al.*, 1951). This method is considered to be one of the most sensitive assays for protein concentration determination, and is one of the most commonly used assays. The procedure utilises two colour forming reactions to assay protein concentration photometrically. Firstly, a low efficiency biuret assay results in alkaline copper ions producing a deep bluish colour in the presence of peptide bonds. This

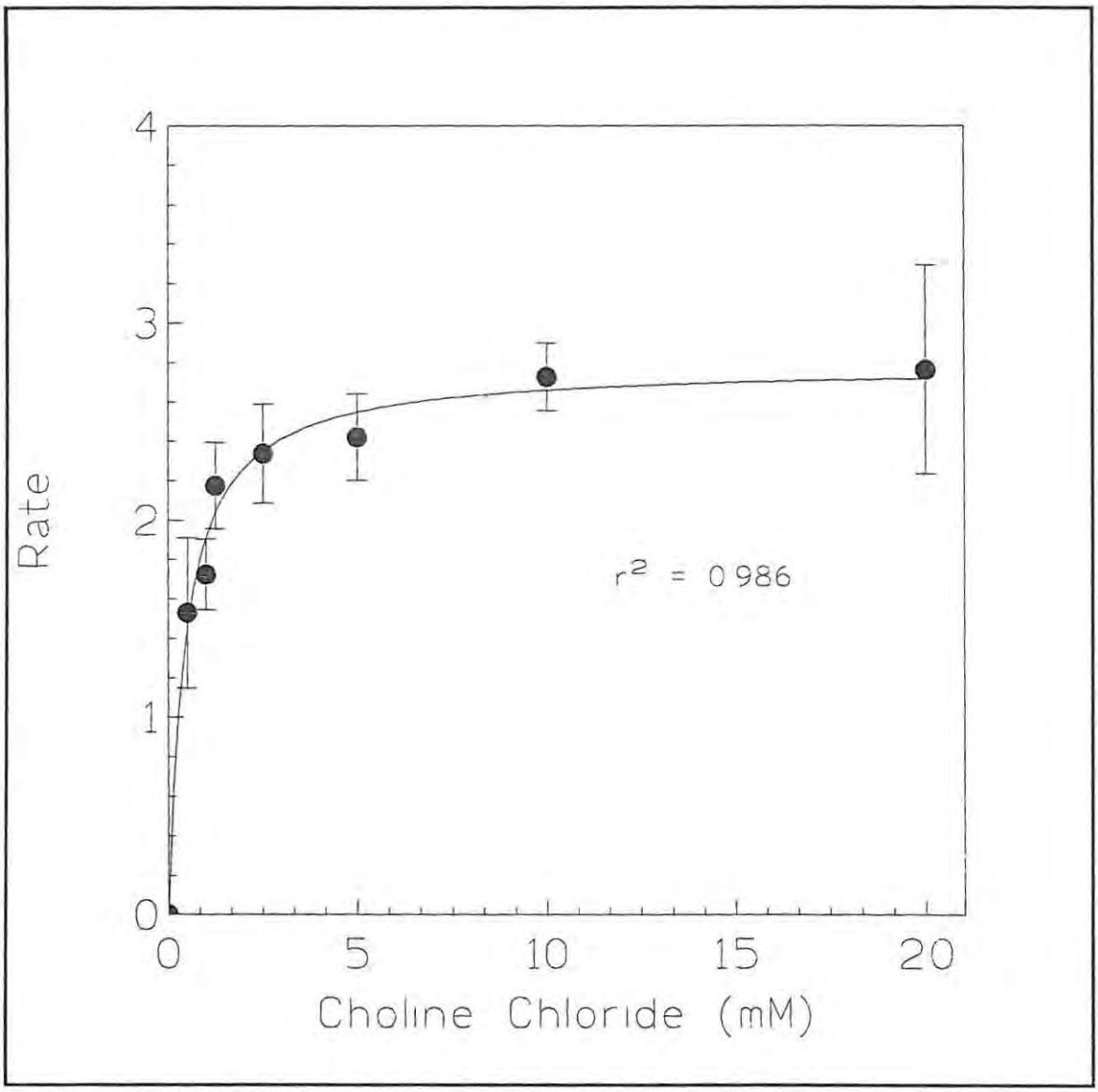

FIGURE 2.2. Michealis-Menten plot of choline acetyltransferase of rat forebrain for the substrate choline chloride. The study was carried out by varying the concentration of choline chloride from 1 mM - 20 mM and by fixing the concentration of acetyl CoA at 0.5 mM. The initial velocity (Rate) was expressed as pmoles of acetylcholine synthesised/mg protein/min according to the procedure described in ChAT ASSAY TECHNIQUE section [2.2]. Each value represents the Mean ± SEM (n=3).

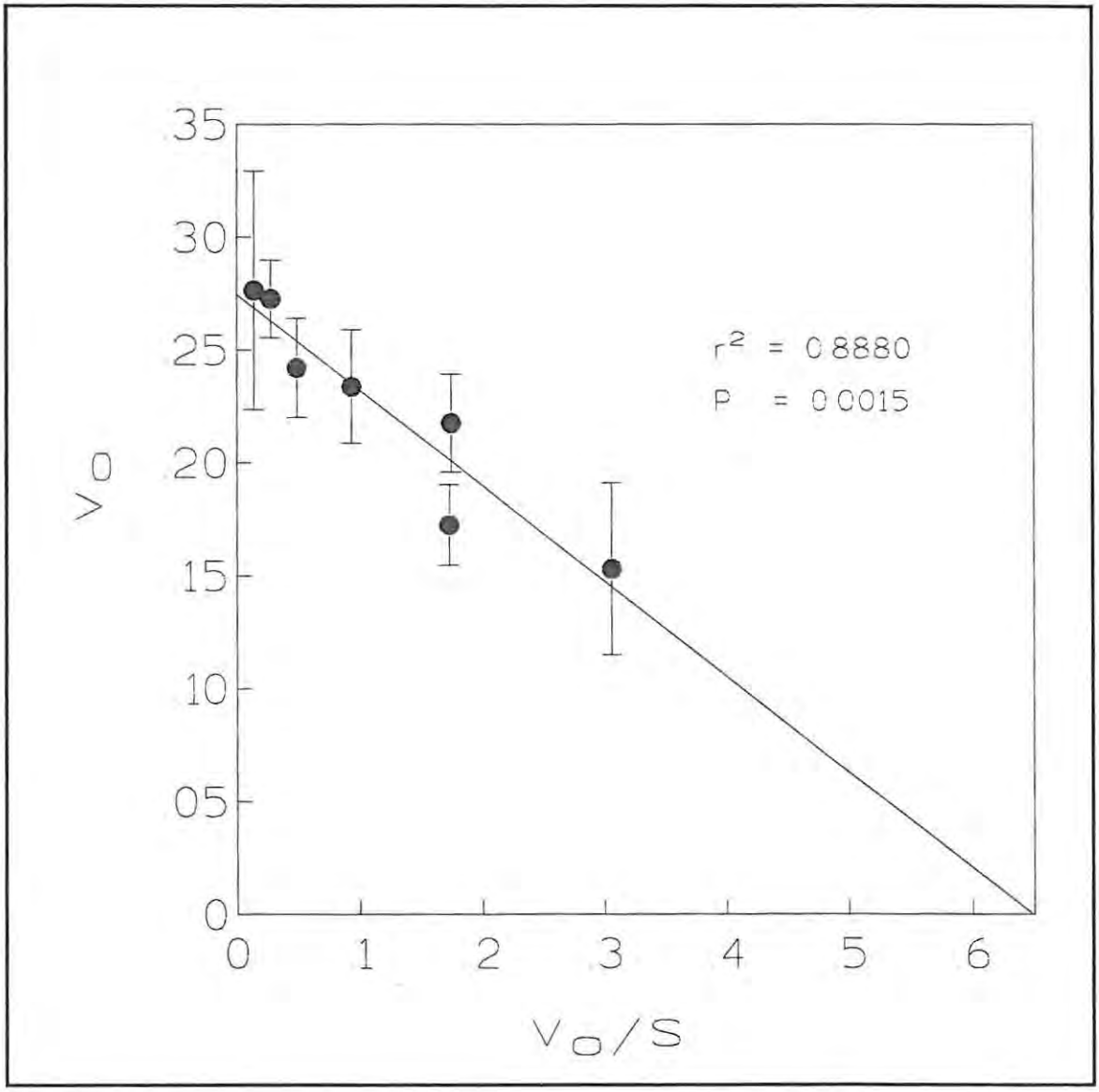

FIGURE 2.3. **Eadie-Hofstee plot of choline acetyltransferase of rat forebrain for the substrate choline chloride. The study was carried out by varying the concentration of choline chloride from 1 mM - 2 mM and by fixing the concentration of acetyl CoA at 0.5 mM. The initial velocity (Rate) was expressed as pmoles of acetylcholine synthesised/mg protein/min according to the procedure described in ChAT ASSAY TECHNIQUE section [2.2]. Each value represents the Mean ± SEM (n=3).**

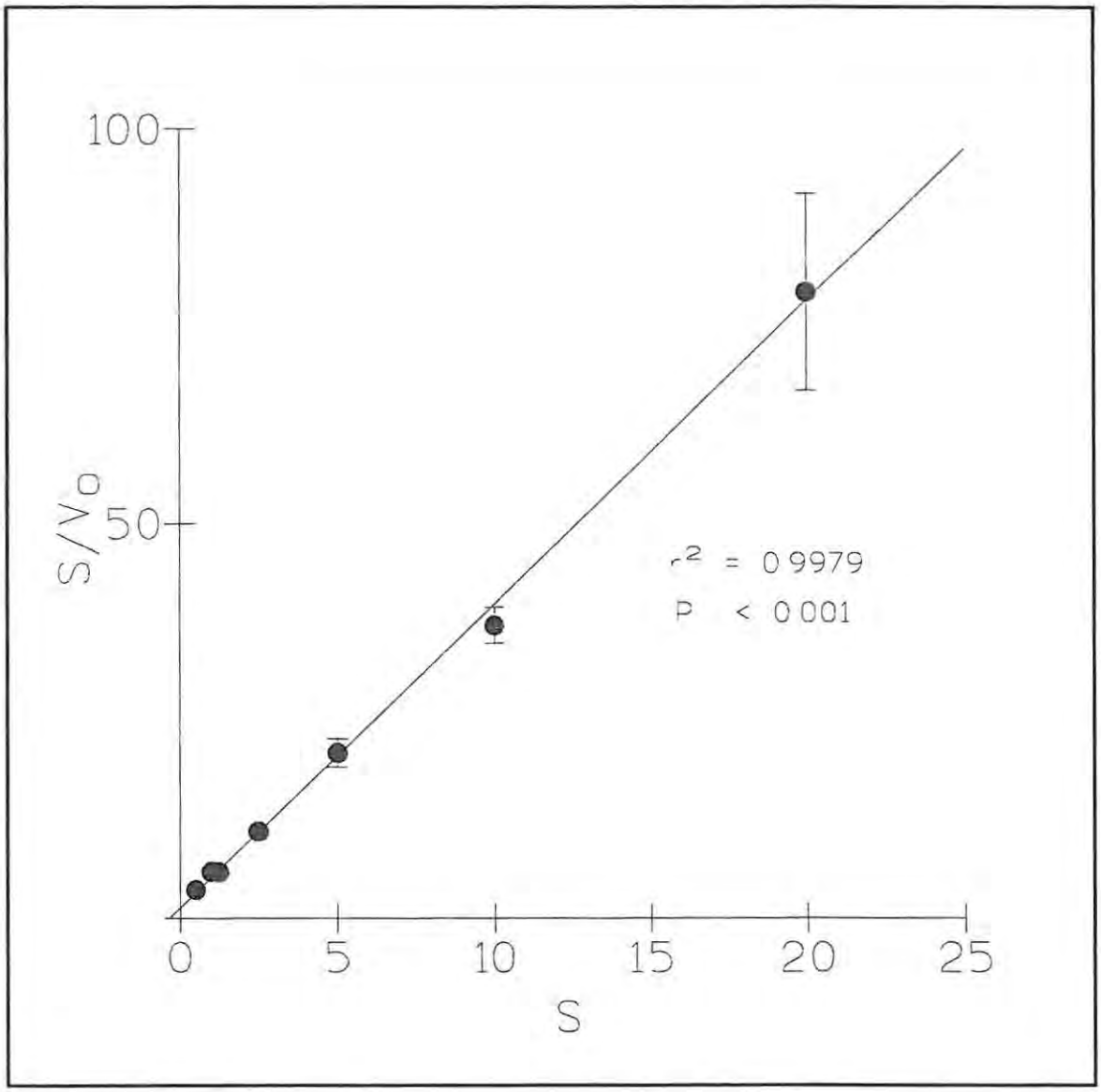

FIGURE 2.4. **Hanes-Woolf plot of choline acetyltransferase of rat forebrain for the substrate choline chloride. The study was carried out by varying the concentration of choline chloride from 1 mM - 20 mM and by fixing the concentration of acetyl CoA at 0.5 mM. The initial velocity (Rate) was expressed as pmoles of acetylcholine synthesised/mg protein/min according to the procedure described in ChAT ASSAY TECHNIQUE section [2.2]. Each value represents the Mean ± SEM (n=3).**

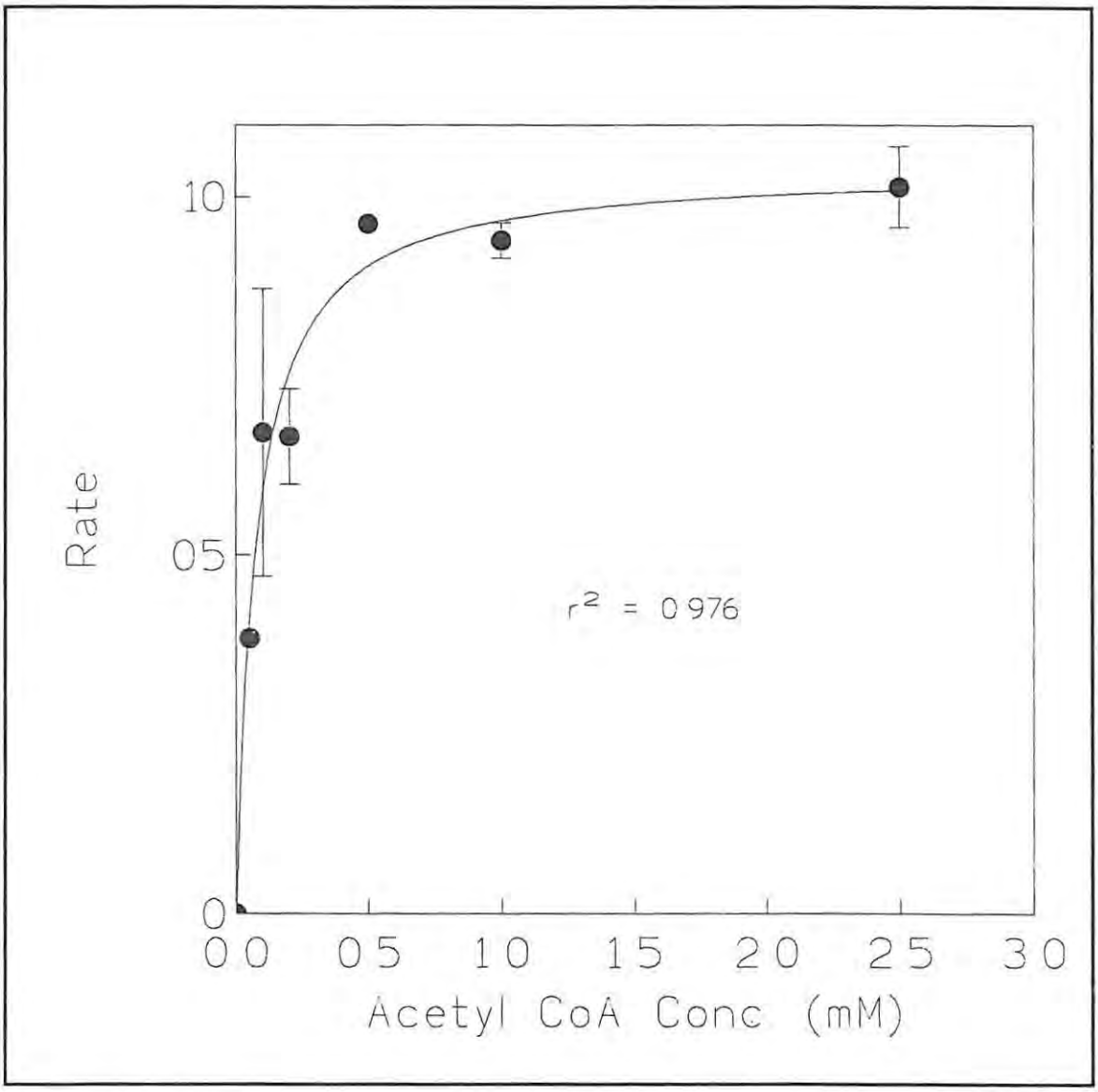

FIGURE 2.5. **Michealis-Menten plot of the rat forebrain choline acetyltransferase for the substrate acetyl CoA. The study was carried out by varying the concentration of acetyl CoA from 0 mM - 2.5 mM and by fixing the concentration of choline chloride at 10 mM. The initial velocity (Rate) was expressed as pmoles of acetylcholine synthesised/mg protein/min according to the procedure described in ChAT ASSAY TECHNIQUE section [2.2]. Each value represents the Mean ± SEM (n=3).**

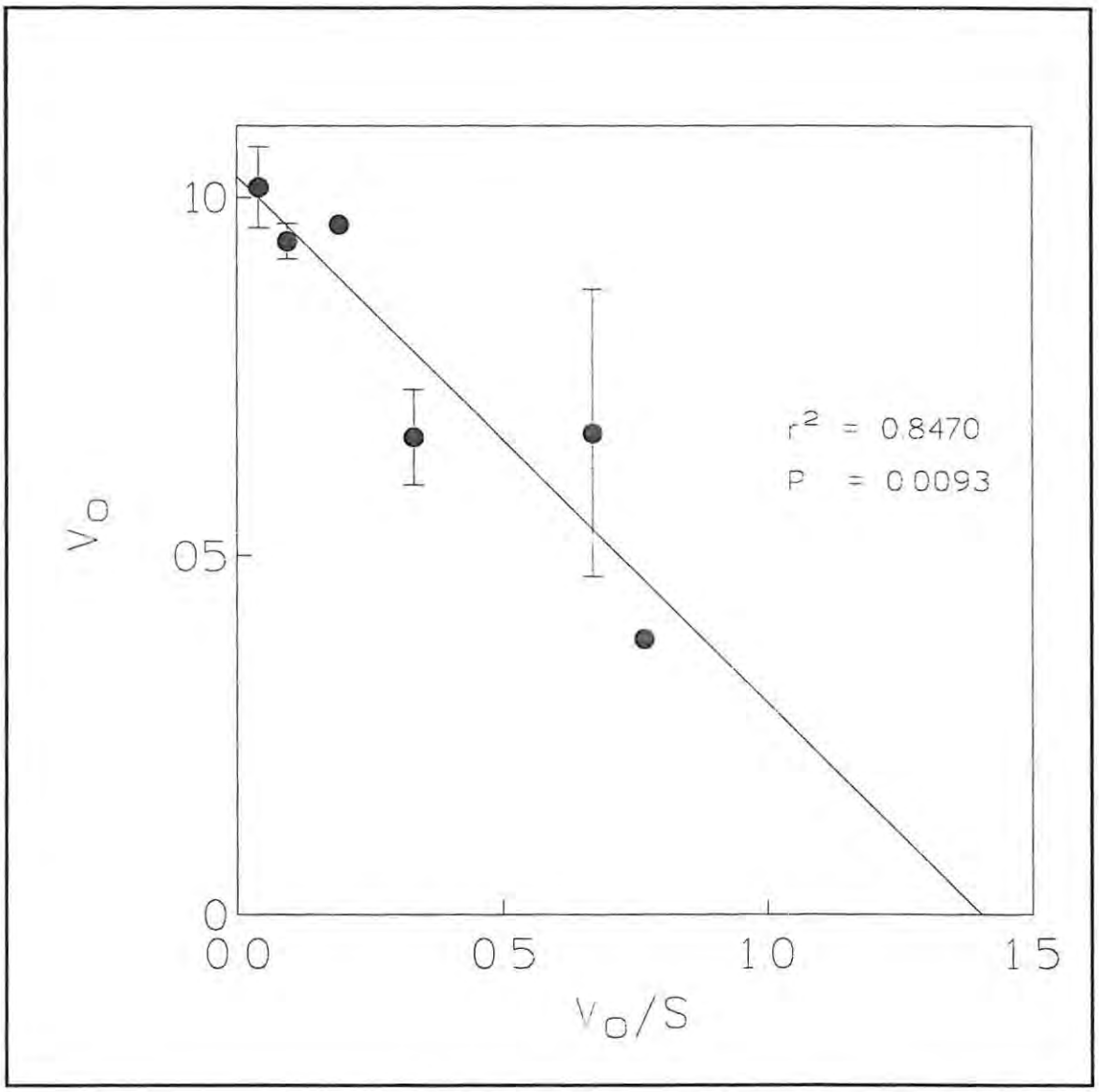

FIGURE 2.6. **Eadie-Hofstee plot of the rat forebrain choline acetyltransferase for the substrate acetyl CoA. The study was carried out by varying the concentration of acetyl CoA from 0 mM - 2.5 mM and by fixing the concentration of choline chloride at 10 mM. The initial velocity (Rate) was expressed as pmoles of acetylcholine synthesised/mg protein/min according to the procedure described in ChAT ASSAY TECHNIQUE section [2.2]. Each value represents the Mean ± SEM (n=3).**

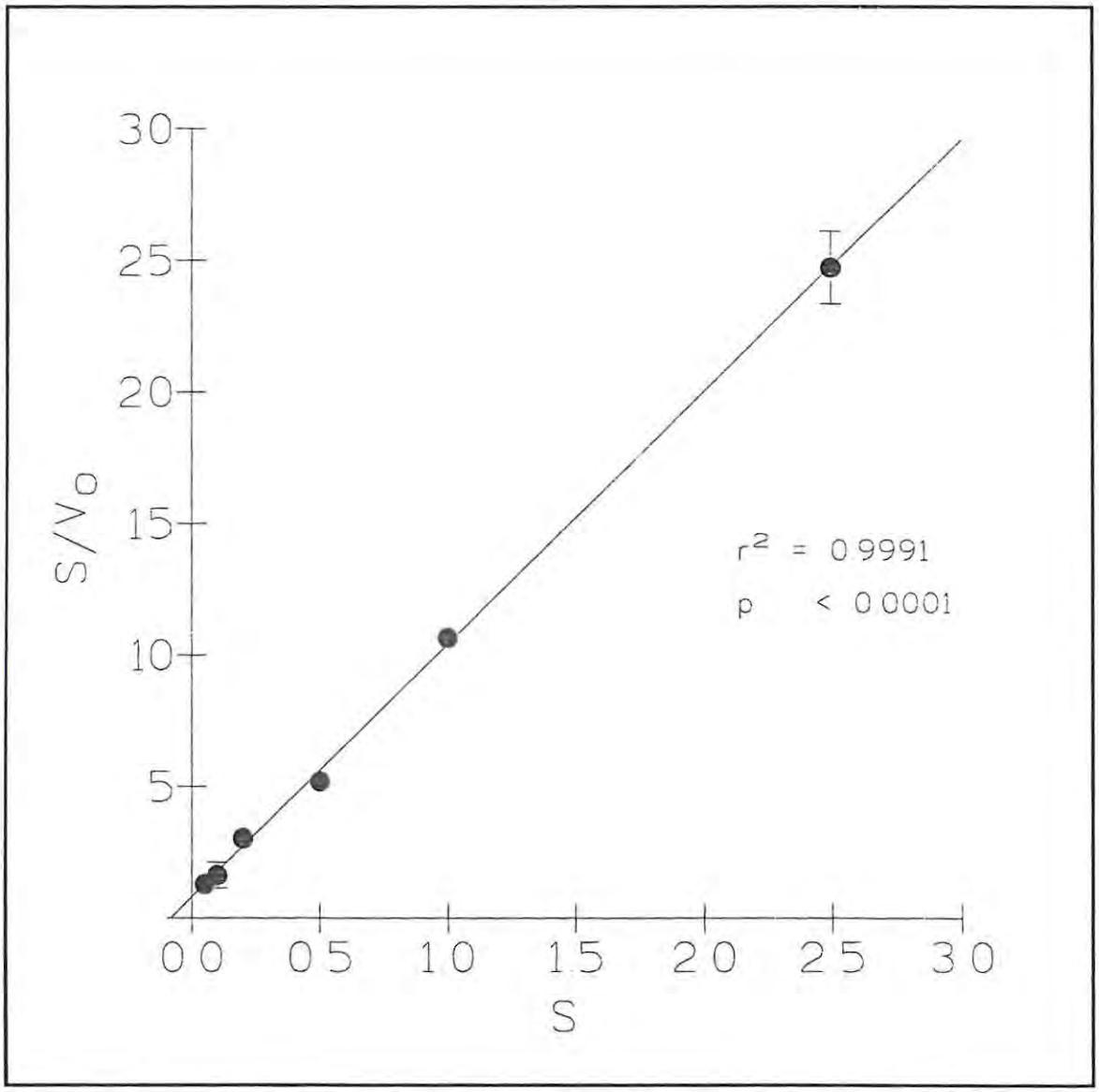

FIGURE 2.7. **Hanes-Woolf plot of the rat forebrain choline acetyltransferase for the substrate acetyl CoA. The study was carried out by varying the concentration of acetyl CoA from 0 mM - 2.5 mM and by fixing the concentration of choline chloride at 10 mM. The initial velocity (Rate) was expressed as pmoles of acetylcholine synthesised/mg protein/min according to the procedure described in ChAT ASSAY TECHNIQUE section [2.2]. Each value represents the Mean ± SEM (n=3).**

colour is a property of all proteins and forms a basic background colour for the Folin-Ciocalteu assay.

Also employed in the assay, is the Folin-Ciocalteu reagent which is a complex inorganic salt mixture, and in the presence of either free or peptide bound tyrosine and tryptophan, yields an intense bluish-green colour. The combined levels of these two amino acids are generally constant in soluble proteins, with a few exceptions (for example, BSA, which is deficient in these two amino acids). The intense bluish-green colour of the assay is indicative of, and proportional to the protein concentration (Clark and Switzer, 1977).

The amounts of ACh present in biological preparations are small and unstable, resulting in isolation of the compound being difficult. The radiochemical method developed by Fonnum (1969, 1975) to determine ChAT activity was used in the present study. The procedure extracts ACh selectively. Unlike the method by Schrier and Schuster (1967), Fonnum's (1975) assay is able to separate ACh from ACar and does not give an artificially high ChAT activity value (Hamprecht and Amano, 1974; Phansuwan-Pujito *et al.*, 1990). This assay by Fonnum (1975) is probably the most sensitive available. It is based on the formation of radiolabelled ACh from radiolabelled acetyl CoA. This makes the assay unsuitable for studies in which rapid measurements are used to probe the details of intermediate steps in the catalytic process (Maunter, 1977).

K_m and V_{max} determination cannot be obtained accurately from the graph of the Michaelis-Menten equation (**Figure 2.2.** and **Figure 2.5.**), as it is a curved graph. If there are at least three consistent points on the plateau of the curve at different substrate values, then a fairly accurate value of V_{max} can be obtained and hence the K_m. To overcome this problem, Lineweaver and Burk (1934) (Cited by Palmer, 1991) simply took the Michaelis-Menten equation

$$V_o = \frac{V_{max}[S]}{[S]+ K_m}$$

and inverted, it to give a plot of $1/V_o$ versus 1/S plot (Lineweaver-Burk plot). The graph is linear and therefore can be extrapolated. From this plot, the values of K_m and V_{max} can be determined. In this study, the Lineweaver-Burk plot was not used to determine the K_m and V_{max} values, as there are several criticisms against it. The least important one being that the extrapolation across the $1/V_o$ axis to determine the $-1/K_m$ sometimes reaches the edge of the graph before reaching the 1/[S] axis. Secondly, it is said to give undue weight to measurements made at low substrate concentrations, when results are likely to be most inaccurate. Finally, departure, from linearity are less obvious than in other plots, and this could be very important if a reaction mechanism was being investigated (Palmer, 1991).

In this study the Eadie-Hofstee (**Figure 2.3.** and **Figure 2.6.**) and Hanes-Woolf plots (**Figure 2.4.** and **Figure 2.7.**) are used to determine the values of K_m and V_{max} as these plots are considered to give accurate determinations.

ChAT is an enzyme that requires two substrates, acetyl CoA and choline chloride. Acetyl CoA is thought to be the leading substrate (i.e. the first substrate to bind to the enzyme) for several reasons: (1) Acetyl CoA is a coenzyme, and coenzymes are usually the leading substrate in a reaction. (2)The K_m value of acetyl CoA was found in a study by Sastry and Henderson (1972) to be 30 times lower than that for choline, suggesting that acetyl CoA binds with a higher affinity than choline. (3) Acetyl CoA was found to be a hundred times more potent than ACh in inhibiting ChAT. This indicates that CoA was the last product to dissociate from the enzyme (Sastry and Henderson, 1972).

The K_m value for choline chloride obtained in this study (See **Table 2.2.**) corresponds well with the published value of 0.41 mM for rat brain by White and Wu (1973). This value differed from that found by Kaita and Goldberg (1969). These authors determined the K_m for choline chloride to be 0.77 mM, which is twice the value calculated in this study. For acetyl CoA, the K_m value obtained in the present study was 4 times higher than that obtained by White and Wu, (1973) and Kaita and Goldberg

(1969). The K_m for acetyl CoA is nevertheless very low, thereby suggesting that there is a strong affinity for the enzyme (ChAT) substrate (acetyl CoA) complex to form.

2.5. EXPERIMENT: DETERMINATION OF THE EFFECTS OF INORGANIC TOXINS (ALUMINIUM AND MERCURY) ON CRUDE CHOLINE ACETYLTRANSFERASE (ChAT) FROM THE RAT FOREBRAIN.

2.5.1. INTRODUCTION

Choline acetyltransferase (ChAT) has been found to be reduced in activity in the brains of individuals with Alzheimer's disease (AD) (Cherroret *et al.*, 1994). Reduction in ChAT activity has been demonstrated in all areas of the cerebral cortex of these patients. Biopsy studies suggest that the decline in the enzymes' activity occurs relatively early in the onset of the disease (Palmer and Gershon, 1990). Morphologic alterations in the brains of AD have been associated with the cerebral cortex as well as the hippocampus and amygdala (Hooper and Vogel, 1976).

Over the last 20 years, there has been an increasing awareness of the neurotoxic effects of metal. Unlike organic pollutants, metals are not degraded in the environment. These agents are also not metabolized by the body (Carpenter, 1994). Metals affect many organ systems; for example, exposure to mercury vapour for several weeks has been noted to cause chronic renal failure in children (Herstrom *et al.*,1995), and studies have been done to investigate the factors affecting renal uptake of inorganic mercury (Endo *et al.*, 1995).

Aluminium intoxication has been implicated in the pathogenesis of dementia, AD, and microcytic aneamia (Andreoli *et al.*, 1984). Elevated levels of aluminium have been found in the postmortem brains of AD patients (Hetnarski *et al.*, 1980; Yokel *et al.*, 1995). Aluminium is a neurotoxin, both in experimental animals and humans. There are a number of questions regarding the relationship between increased levels of aluminium and AD, and whether the metal is an etiological factor in the disease (Glynn *et al.*, 1995). Aluminium has also been implicated in the aetiology of Parkinson's disease. Accumulation of aluminium salts in certain regions of the brain impairs

cognitive function in humans and other animals.

Another inorganic metal, mercury, is toxic to a number of enzymes, but has particularly severe effects on the CNS (Bondy *et al.*, 1979). Mercury is found in amalgam, which is the most frequently used material for restorative dental treatment, and represents the main source of exposure to inorganic mercury for the general population (Halbach, 1995).

At present the proposed acceptable daily intake of inorganic and organic mercury is 40 µg (WHO, 1972; WHO, 1989). The potential health risk of mercury exposure depends on the organisms period of exposure and the dose. According to Lorshceider and Vimy (1993), evidence exists that the daily total mercury released from a single dental mercury filling is approximately 15 μg. The average absorbed dose of mercury for human subjects with eight fillings is estimated to be 10 μg per day. Halbach (1995) found that the average daily dose of mercury from amalgam is about 4 to 5 µg. These values are below those of the acceptable intake values.

Researchers have not made any claims that amalgam mercury is the cause of a specific disease. However, the possibility that mercury may have a role in the aetiology of disease (for example in AD) has not been ruled out (Lorsheider and Vimy, 1993).

Thus, the present study investigated the effects of the two inorganic metals, mercury and aluminium, on the activity of ChAT.

2.5.2. MATERIALS AND METHODS

2.5.2.1. Chemicals, Drugs and Reagents

Aluminium chloride was obtained from Pal Chemicals, South Africa and mercuric

chloride was obtained from Merck (Germany). All the other chemicals and reagents used were outlined in section [**2.2.1.1**].

2.5.2.2. Method

To determine the effect of the inorganic metals on ChAT activity, the ChAT assay described in section [**2.2.3**] was used with the following changes. [^{3}H] acetyl CoA concentration was kept constant at 0.1 μCi, and choline chloride was kept constant at 10 mM. The inorganic metal in question was added to the incubation mixtures to reach final concentrations of, 1 nM, 10 nM, 1 μM, 10 μM and 1 mM. Statistical calculations and significance were determined using the *Unpaired Student's t test with a two-sided P-value.*

2.5.3. RESULTS

The rate of activity of ChAT from the forebrain of a rat, as determined *in vitro* in the presence of varying concentrations of aluminium chloride, is shown in **Table 2.4.** A graphical representation of these results is illustrated in **Figure 2.8.** The rate is expressed as pmoles of acetylcholine synthesised per mg of protein per minute. No significant change in the activity of ChAT was observed in the concentration range used.

Table 2.5. represents the rate of activity of ChAT from the forebrain of a rat, as determined *in vitro* in the presence of varying concentrations of mercuric chloride. **Figure 2.9.** is the graphical representation of the results in **Table 2.5.** The rate is expressed as pmoles of acetylcholine synthesised per mg of protein per minute. At a concentration of 10 µM, there was a significant decrease in ChAT activity ($P < 0.001$). Further decreases occur in ChAT activity at 100 µM ($P < 0.0002$) and 1 mM ($P < 0.001$) mercuric chloride concentrations. At 1 mM mercuric chloride, virtually no ChAT activity was observed.

TABLE 2.4. Rate of activity of choline acetyltransferase *in vitro* in the presence of varying concentrations of aluminium chloride. Each value represents the mean ± SEM (n=3).

ALUMIMIUM CHLORIDE CONCENTRATION	RATE (pmoles of acetylcholine synthesised/mg protein/min)	SIGNIFICANCE
CONTROL	3.44 ± 0.11	-
1nM	3.15 ± 0.79	-
10nM	3.23 ± 0.007	-
100nM	3.05 ± 0.14	-
1µM	3.11 ± 0.10	-
10µM	2.82 ± 0.27	-
100µM	3.06 ± 0.63	-
1mM	2.84 ± 0.39	-

TABLE 2.5. Rate of activity of choline acetyltransferase *in vitro* in the presence of varying concentrations of mercuric chloride. Each value represents the mean ± SEM (n=3).

MERCURIC CHLORIDE CONCENTRATION	RATE (pmoles of acetylcholine synthesised/mg protein/min)	SIGNIFICANCE
Control	1.95 ± 0.12	-
1nM	2.07 ± 0.23	-
10nM	2.04 ± 0.06	-
100nM	2.07 ± 0.14	-
1µM	2.07 ± 0.15	-
10µM	0.61 ± 0.97	$P < 0.001$
100µM	0.21 ± 0.05	$P < 0.0002$
1mM	0.05 ± 0.008	$P < 0.0001$

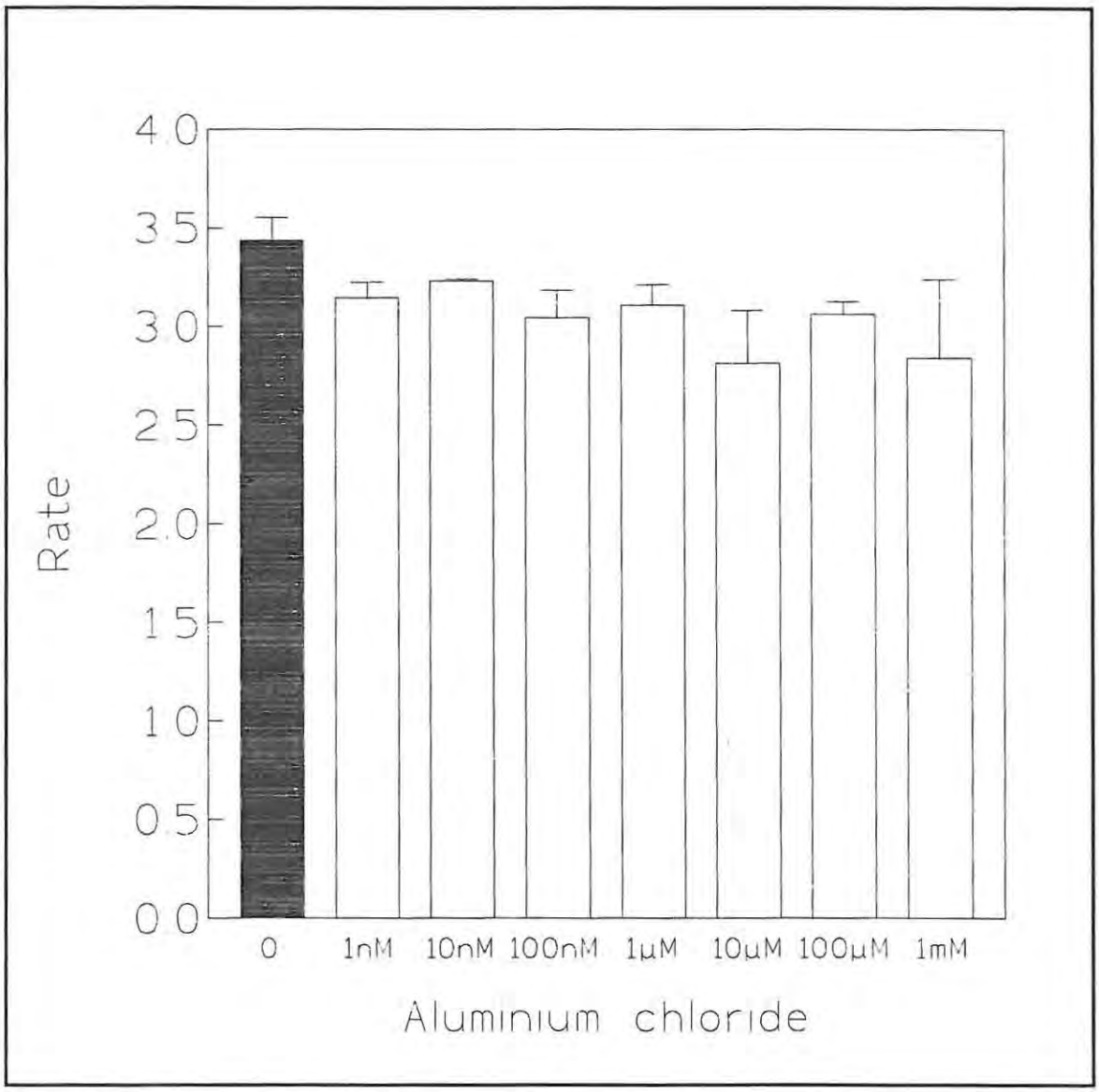

FIGURE 2.8. **The e ffect of different concentrations of aluminium chloride on the rate (pmoles of acetylcholine synthesised/mg protein/min) of activity of rat forebrain choline acetyltransferase according to the procedure described in ChAT ASSAY TECHNIQUE section [2.5.2.2.]. Each value represents the Mean ± SEM (n=3). In this study, the concentration of the substrates radiolabelled acetyl CoA and choline chloride, was kept constant at 0.1 µCi/assay tube and 10 mM, respectively.**

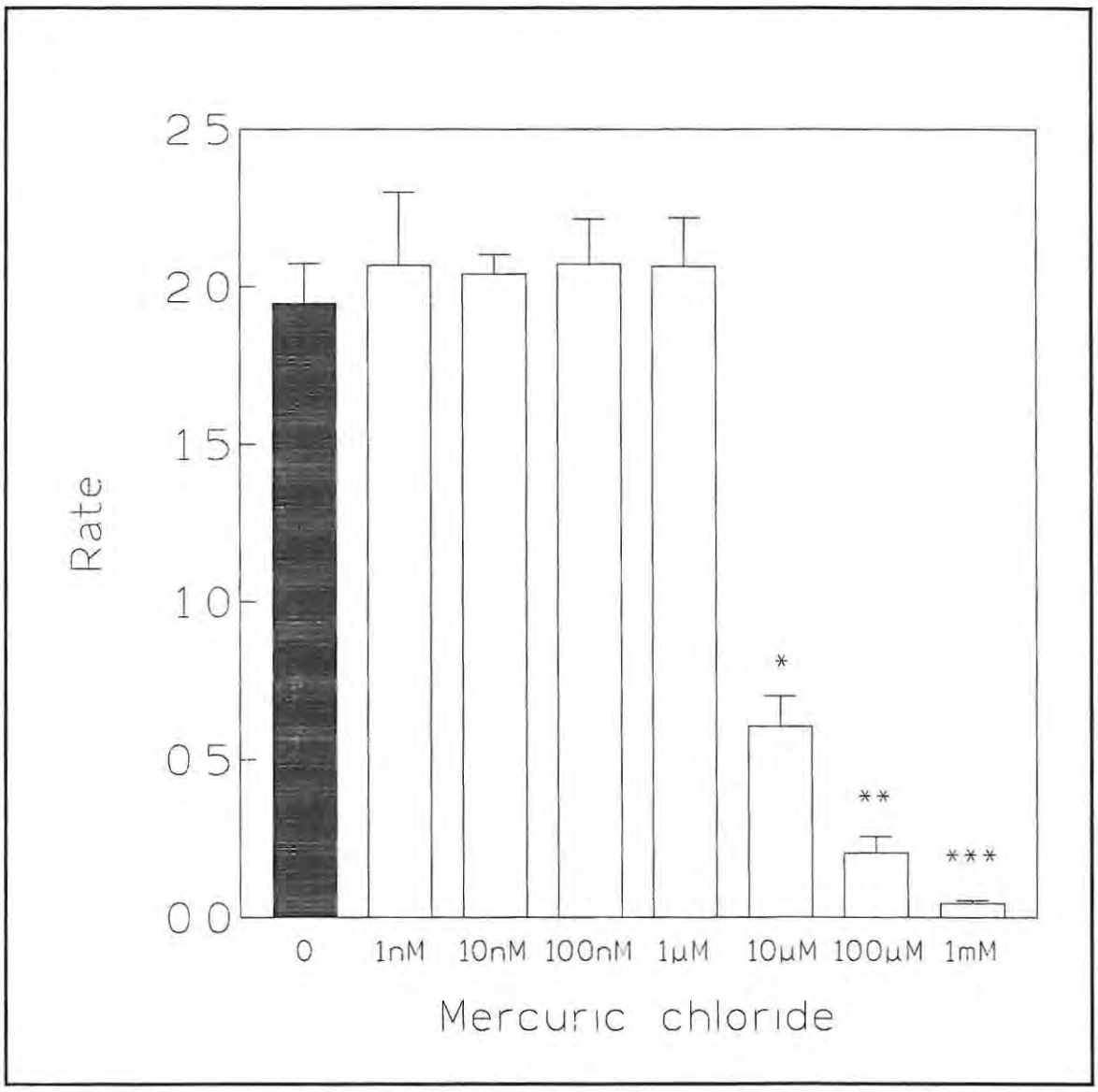

FIGURE 2.9. **The effect of different concentrations of mercuric chloride on the rate (pmoles of acetylcholine synthesised/mg protein/min) of activity of rat forebrain choline acetyltransferase according to the procedure described in ChAT ASSAY TECHNIQUE section [2.5.2.2.]. Each value represents the Mean ± SEM (n=3). In this study, the concentration of the substrates radiolabelled acetyl CoA and choline chloride, was kept constant at 0.1 µCi/assay tube and 10 mM, respectively.**

*** - P < 0.001, ** - P < 0.0002, *** - P < 0.0001**

2.5.4. DISCUSSION

Over the past twenty years, animal and human studies have demonstrated the neurotoxic nature of aluminium (Julka and Gill, 1995). The involvement of aluminium in the etiology of various dementias has been proposed by a number of authors (Bird *et al.*, 1983; McGeer, 1984).

ChAT activity has been reported to be affected by aluminium *in vivo.* Cherroret *et al.*(1994) cites Yates *et al.* (1980) and Hofstetter *et al.* (1987) as references. The results of this study show that aluminium chloride (0-1 mM) had no significant effect on ChAT activity *in vitro* (See **Figure 2.8.**). Similar results were also obtained by Cherroret *et al.* (1994), with aluminium concentrations of 100 μM and 1 mM. The experiments of Cherroret *et al.* (1994) determined the effect of aluminium on ChAT activity, in rats of both sexes at 2 and 7 post-natal days and on male rats only at 13, 15, 30, 60 and 90 days (adult stage). Cherroret (1994),found that at a concentration of 100 µM, no decrease in ChAT activity occurred, irrespective of age. At a concentration of 1 mM, it was shown that aluminium inhibits the activity of rat brain ChAT significantly in 15 day old rats. The inhibition decreases with the age of the rat. In adult rats there is insignificant inhibition of the enzyme. At concentrations above 1 mM, Cherroret (1994) found that ChAT activity was activated until about postnatal day 30. Thereafter the enzyme was inhibited with age.

King (1984) found that intracisternal injection of aluminium chloride into rabbits, caused a reduction in ChAT activity when measured seven days after injection. This author also found that in *in vitro* experiments the addition of 10 µM aluminium chloride showed no inhibition of the enzyme. Previously, Hetnarski *et al.* (1980) reported that aluminium incubated with rabbit thoracic spinal cord homogenate had an inhibitory influence on ChAT. The authors do not however give any indication of the aluminium concentration used for their *in vitro* experiments. In *in vivo* experiments, Hetnarski and his workers found no significant differences in the activity of ChAT. In another report, rats, male and female, treated with aluminium for ninety days, were found to show a decrease in

the enzyme's activity (Bilkei-Gorzo, 1993).

Toxic effects of mercury are known to be pronounced at the neurological level (Bondy and Agrawal, 1980). Mercury is generally toxic to many enzymes, and severely affects the CNS (Bondy *et al.*, 1979). Mercuric chloride appeared to insignificantly increase ChAT activity at concentrations between 0-1 μM (See **Figure 2.9.**). Significant decreases in ChAT activity occurred with mercuric chloride concentrations above 1 μM. At 1 mM there was almost no enzyme activity ($P < 0.0001$). Similar results were obtained by Chao and Wolfgram (1974) when it was demonstrated that ChAT is inhibited by heavy metal ions that have a high affinity for sulfhydryl groups which are present in the ChAT enzyme.

The results obtained in this study suggest that low exposure to mercury will not adversely affect the synthesis of the neurotransmitter acetylcholine by ChAT. However, an increase in exposure to mercury may result in very significant inhibition of the ChAT enzyme activity according to the results obtained (**Figure 2.9.**)

2.6. EXPERIMENT: DETERMINATION OF THE EFFECTS OF EXCITATORY AMINO ACIDS (KYNURENIC ACID AND QUINOLINIC ACID) ON CRUDE CHOLINE ACETYLTRANSFERASE (ChAT) FROM THE RAT FOREBRAIN

2.6.1. INTRODUCTION

Excitatory amino acids (EAA) of exogenous or endogenous origin, are thought to play a major role in a host of neuropsychiatric diseases, i.e. excitotoxins can cause neuronal dysfunction, and subsequent nerve death by turning physiological signals into a pathologic process (Schwarcz *et al.*, 1992).

The excitatory amino acids of interest in this study are quinolinic acid (QA) and kynurenic acid (KA). Both are metabolites of the kynurenine pathway of tryptophan degradation. QA has been reported to have excitatory effects in some areas of the CNS, such as the cortex and the hippocampus (Perkins and Stone, 1983). In the cortex, QA concentrations are much higher than in any other region of the brain.

QA accumulation in the brain is hypothesized to cause a broad spectrum of human neurological diseases. The amino acid is produced by macrophages, at substantially elevated levels, in patients with inflammatory neurological conditions (Blight *et al.*, 1995). QA inhibits several aminotransferases, namely ornithine, alanine and aspartate transferase (Hsu and Fahien, 1976), and is also proposed to play a role in the pathogenesis of hepatic encephalopathy (HP). L-Kynurenine, which is an intermediate precursor of QA, is also supposed to be associated with the pathogenesis of HP (Basile, 1995).

The concentration and metabolism of QA appears to depend on the age of the rat. This was concluded by Moroni *et al.* (1984a) from the observation that the administration of tryptophan to newborn rats showed no increase in QA levels, while administration to adult rats showed an increase. Another interesting observation is that the

concentration of QA in the brain of rats increased with age. Thus a progressive increase was found in rats of 3 days, 3 months, 9 months and 30 months of age. In the 30 month old rats studied, half of them were found to have QA concentrations approaching those which cause neurotoxicity (Stone and Connick, 1985). From observations such as these, the suggestion has been put forward that QA may be a causative factor in senile dementias such as Alzheimer's disease.

The other EAA, KA, was first reported in 1988 to be present in the mammalian brain (Moroni *et al.*, 1988). In the rat brain, it was noted that KA steadily increases with age, so that the brain cortex of a 2-year-old rat contains approximately ten times the amount of KA present in a 2-month-old rat (Schwarcz *et al.*, 1992). The amino acids concentration varies between brain regions and also between species. A human brain on average contains 10-20 times more KA than the rat brain. So far no evidence has been found to define the manner in which KA is degraded in the brain. There have been some reports suggesting the existence of specific KA-catabolizing enzymes, but nothing conclusive. There has also been no identification of a KA uptake mechanism. The fate of KA in the brain is therefore a mystery, and its role physiologically or pathologically has not been determined (Schwarcz *et al.* 1992).

Ageing of the brain is associated with a decrease of cholinergic neurochemical markers and cognitive ability. The causes and mechanisms for these deficiencies are unexplained. ChAT activity in the striatum and frontal cortex of aged rats, is reported to be lower than the levels in young rats (Fong *et al.* 1995). ChAT activity in the nucleus of Meynert, and in the temporal cortex, is reduced by at least 90% and 75% respectively. Neuron loss in the nucleus of Meynert does not reflect this loss. This suggests that a key pathological change in AD may be the "down regulation" of the ChAT enzyme, and that neuron loss is a secondary feature (Perry *et al.* 1982). In Huntington's disease (HD), a loss in ChAT activity has also been reported in certain areas of the brain (Stahl and Swanson, 1974). QA-induced neurotoxicity has been shown to closely mimic the patterns of selective nerve cell loss seen in Huntington's Disease and temporal lobe epilepsy (Martin and Beal, 1992), and has been suggested

as a possible animal model for the disease (Schwarcz *et al*, 1983). It is possible that excitotoxicity by EAA's might be involved in neurodegenration disorders such as HD and AD.

The aim of the present study is to determine whether QA and KA, which increase with age, have any effect on ChAT activity.

2.6.2. MATERIALS AND METHODS

2.6.2.1. Chemicals, Drugs and Reagents

QA and KA were purchased from Sigma Chemical Co., USA. All the other chemicals and reagents were obtained as outlined in section [**2.2.1.1**].

2.6.2.2. Method

To determine the effect of the inorganic excitatory amino acids on ChAT activity, the ChAT assay described in section [**2.2.3**] was used with the following changes. [^{3}H] acetyl CoA concentration was kept constant at 0.1 μCi and choline chloride was kept constant at 10 mM. The amino acid in question, was added to the incubation mixtures to give final concentrations of 1 nM, 10 nM, 1 μM, 10 μM, 100 µM and 1 mM. Statistical calculations and significance was determined using the *Unpaired Student's t test with a two-sided P-value.*

2.6.3. RESULTS

The results in **Table 2.6.** show the effect of varying concentrations of QA on the activity of ChAT *in vitro*. **Figure 2.10.** is a graphical representation of the results in **Table 2.6.** The rate of activity is expressed as pmoles of acetylcholine synthesised per mg of

protein per minute. No significant change in activity was observed at the various concentrations of QA tested. At concentrations below 100 nM, no decrease in activity was observed. At higher concentrations, a slight decrease in activity was noted, but it was not statistically significant.

Table 2.7. represents the rate of activity of ChAT from the rat forebrain in an *in vitro* experiment, in the presence of different concentrations of KA. **Figure 2.11.** is the graphical representation of the results in **Table 2.7.** The rate is expressed as pmoles of acetylcholine synthesised per mg of protein per minute. A slight, but insignificant increase in ChAT activity was noted at 1 nM KA concentration. At concentrations of 10 nM and 100 nM, the rate of ChAT activity was found to be equivalent to that of the control. An insignificant decrease in the rate of activity occurred at 1 μM KA, and this rate was maintained up to a concentration of 1 mM, with no further change.

2.6.4. DISCUSSION

Quinolinic acid (QA) is an endogenous, neuroexcitatory, tryptophan metabolite. It has been shown to cause neuronal lesions when injected into the brain (Farmer and Butterfield, 1984) and has been used to produce selective effects that closely mimic the neuronal loss in HD. The Huntington's chorea animal model with QA, involves the degeneration of cholinergic interneurons with a loss of ChAT activity. In the concentration range between 0 - 1 mM, no statistically significant change in ChAT activity was noted. KA also does not appear to have any effect on ChAT activity *in vitro*.

Loss in ChAT activity in AD, and the degeneration of cholinergic interneurons in HD do not appear to be a result of QA and KA toxicity causing a direct inhibitory effect on the enzyme ChAT. QA has been used to produce animal models of HD. If QA has a role in the pathogensis of HD, then the reported loss of ChAT (Stahl and Swanson, 1974) may be a secondary factor.

TABLE 2.6. Rate of activity of choline acetyltransferase *in vitro* in the presence of varying concentrations of quinolinic acid. Each value represents the mean ± SEM (n=3).

QUINOLINIC ACID CONCENTRATION	RATE (pmoles of acetylcholine synthesised/mg protein/min)	SIGNIFICANCE
Control	5.13 ± 0.35	-
1nM	5.06 ± 0.42	-
10nM	5.04 ± 0.59	-
100nM	4.56 ± 0.30	-
1µM	4.39 ± 0.41	-
10µM	4.36 ± 0.50	-
100µM	4.17 ± 0.06	-
1mM	4.37 ± 0.99	-

TABLE 2.7. Rate of activity of choline acetyltransferase *in vitro* in the presence of varying concentrations of kynurenic acid. Each value represents the mean ± SEM (n=3).

KYNURENIC ACID CONCENTRATION	RATE (pmoles of acetylcholine synthesised/mg protein/min)	SIGNIFICANCE
Control	0.75 ± 0.06	-
1nM	0.79 ± 0.02	-
10nM	0.76 ± 0.05	-
100nM	0.76 ± 0.26	-
1µM	0.69 ± 0.04	-
10µM	0.70 ± 0.02	-
100µM	0.69 ± 0.02	-
1mM	0.68 ± 0.01	-

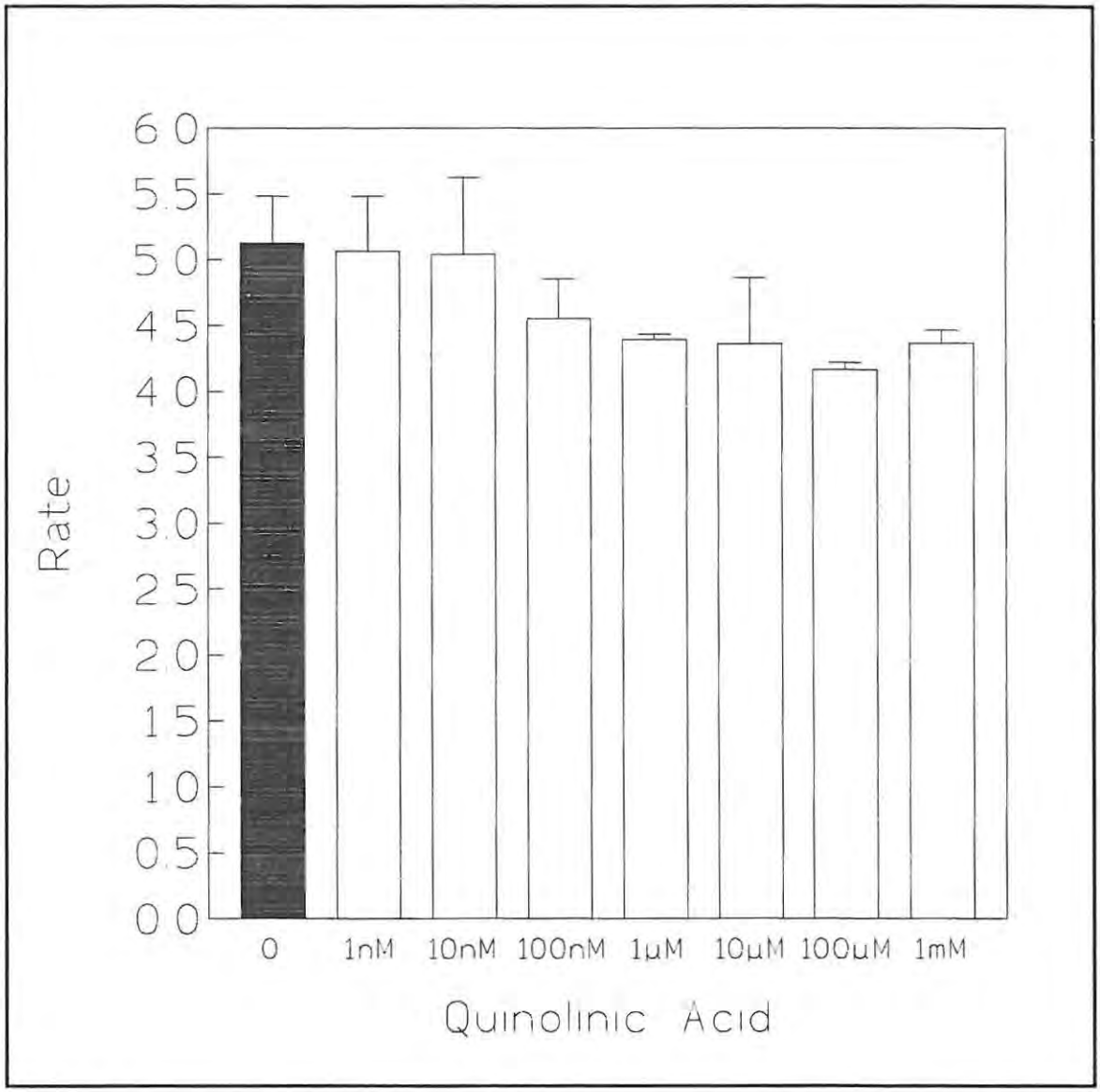

FIGURE 2.10. **The effect of different concentrations of quinolinic acid on the rate (pmoles of acetylcholine synthesised/mg protein/min) of activity of rat forebrain choline acetyltransferase according to the procedure described in ChAT ASSAY TECHNIQUE section [2.6.2.2.]. Each value represents the Mean ± SEM (n=3). In this study, the concentration of the substrates radiolabelled acetyl CoA and choline chloride, was kept constant at 0.01 μCi/assay tube and 10 mM, respectively.**

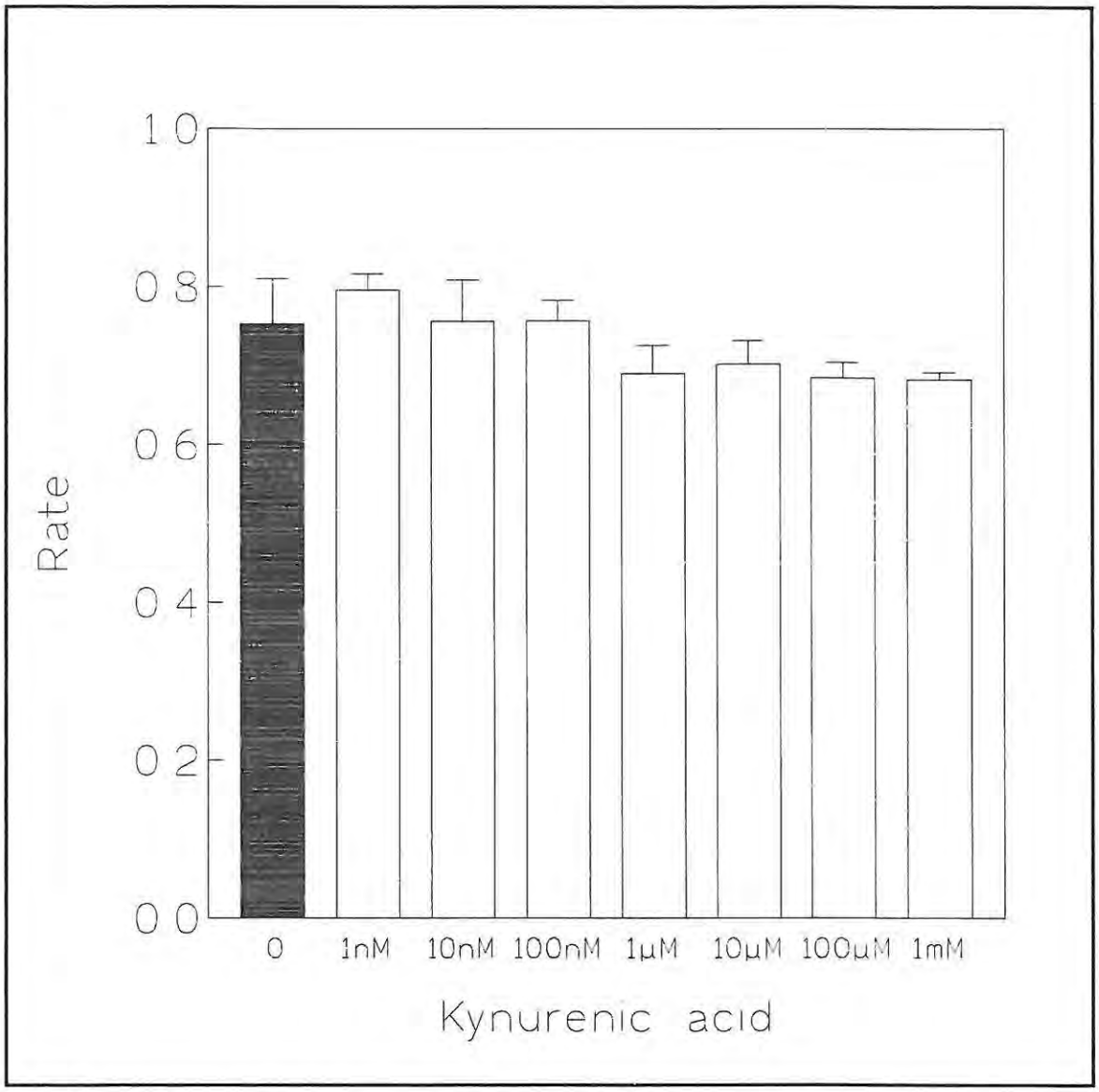

FIGURE 2.11. **The effect of different concentrations of kynurenic acid on the rate (pmoles of acetylcholine synthesised/mg protein/min) of activity of rat forebrain choline acetyltransferase according to the procedure described in ChAT ASSAY TECHNIQUE section [2.6.2.2.]. Each value represents the Mean ± SEM (n=3). In this study, the concentration of the substrates radiolabelled acetyl CoA and choline chloride, was kept constant at 0.01 μCi/assay tube and 10 mM, respectively.**

CHAPTER 3

ACETYLCHOLINESTERASE STUDIES

3.1. INTRODUCTION

Acetylcholinesterase [(E.C.3.1.1.7); (AChE)] is the enzyme that catalyses the hydrolysis of acetylcholine (ACh) (Stejskal *et al.*, 1985). Evidence has shown that generally, neurons that contain ChAT, are also rich in AChE. AChE has also been found to be associated with neurons that use catecholamines and other monoamines as neurotransmitters (Shen, 1994). Breakdown of ACh by the enzyme AChE is the major route for the termination of ACh action in the synaptic cleft.

Pharmacological studies support the theory that cholinergic systems are involved in memory and learning. Drachman (1977) tested this by inducing memory and cognitive impairment through the administration of scopolamine. Scopolamine blocks muscarinic ACh receptors. Physostigmine, an alkaloid (Aeschlimann and Reinert, 1931) and an anti-cholinesterase drug, was then administered to determine whether there was any improvement in memory and cognitive impairment. Physostigmine antagonized the scopolamine blockade by preventing the degradation of ACh. In addition, physostigmine largely reversed many of the features of scopolamine impairment by antagonizing the blockade of ACh receptors by scopolamine. Striking improvements were observed with respect to word storage. There was also improvement in mean digit storage, although this was not significant. Cognitive non-memory functions also improved significantly. This study shows that cholinergic systems are involved in memory and other cognitive functions (Drachman, 1977).

AChE is also thought to play a critical role in AD and other types of dementia. A marked reduction in the enzyme's activity has been consistently observed in AD. The AChE system has been proposed to be the most vulnerable and the earliest affected

system in the pathological processes of AD. It is also suggested that the senile plaque, which is characteristic of AD, may be formed within the terminals of AChE-containing neurons(Shen, 1994). Parkinson's disease (PD) patients with cognitive impairment have also been shown to have a decreased activity of AChE. This decrease however, is not apparent in PD patients without dementia (Konings *et al.*, 1995).

In the present study, the *in vitro* effects of aluminum, mercury, quinolinic acid and kynurenic acid on rat forebrain AChE were investigated. Aluminum, mercury, quinolinic acid and kynurenic acid have all been suggested to play a possible role in various neurological diseases (Andreoli *et al.*, 1984; Glynn *et al.*, 1995; Bondy *et al.*,1979; Schwarcz *et al.*, 1992; Blight et al., 1995).

3.2. ACETYLCHOLINESTERASE ASSAY TECHNIQUE

3.2.1. Theory of Enzyme Assay

Ellman *et al.* (1961) cite that Bonting and Featherstone (1956) proposed a modification of the Hestrin hydroxamic method for the determination of AChE activity. The modification proved successful in several studies, but did have a few disadvantages. One of the disadvantages was that the time course of the reaction could not be followed. Also, the colour measured was developed from the remainder of an added substrate, resulting in a high possibility of error when the enzyme activity levels are low i.e. lack of sensitivity.

Ellman *et al.* (1961) modified a method reported by Koelle (cited by Ellman *et al.*, 1961) that employed a sulfhydryl reagent. The method is sensitive and applicable to either small amounts of tissue, or low concentrations of the enzyme. Detailed kinetic studies of AChE are also possible with this method. Thus the present AChE studies were performed using the method of Ellman *et al.* (1961).

Acetylthiocholine is used as the substrate. The principle of the method is the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolysed. This is achieved by the continuous reaction of the thiol with 5:5-dithiobis-2-nitrobenzoate ion (I) to produce the yellow anion of 5-thio-2-nitro-benzoic acid (II) (**Figure 3.1.**). The rate of production of the yelllow colour is measured spectrophotometrically. The reactions with the thiol are rapid and not rate limiting in the measurement of the enzymic hydrolysis (Ellman *et al.*, 1961).

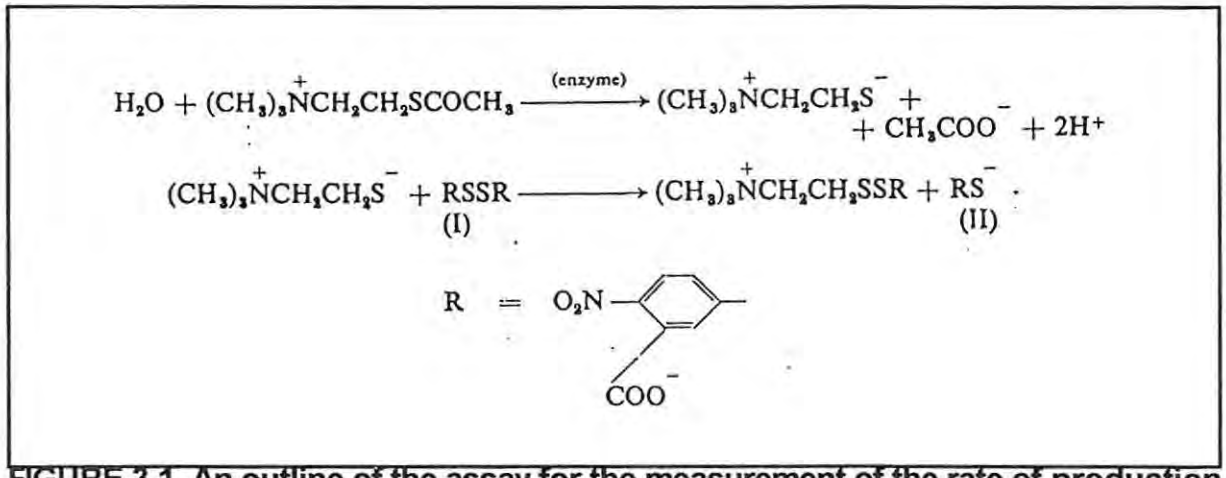

FIGURE 3.1. An outline of the assay for the measurement of the rate of production of thiocholine as acetylthiocholine is hyrolyzed (Ellman, *et al.*, 1961).

3.2.2. Materials and Methods

3.2.2.1. Chemicals, Drugs and Reagents

Acetylthiocholine iodide was purchased from Sigma Chemical Co., USA. 5,5 Dithiobis-(2-nitrobenzoic acid)(DTNB) was obtained from Boehringer Mannheim. All other inorganic and buffer chemicals were of reagent grade.

3.2.2.2. Animals

The rats used in this study were adult male rats of the Wistar strain and these were maintained and sacrificed as previously described in section [**2.2.2.2.**].

3.2.2.3. Preparation of Tissue Extract

The frozen forebrain of the rat which had earlier been stored at -70°C, was allowed to thaw. Then the tissue was homogenized in ice cold 0.1 M Sodium phosphate buffer (pH 8.0), to give a 2% (w/v) homogenate using a glass homogenizer.

Protein concentration of the total homogenate, i.e. crude enzyme, was determined by the Folin Lowry Protein Assay, as outlined in section [**2.2.4.**]. See **Figure 2.1.** for a typical protein standard curve.

The rate of enzyme activity (velocity) for the crude enzyme was then measured over a range of protein concentrations from 0 - 0.5 mg/ml according to the assay described below in section [**3.3.1.4**]. The substrate concentration was then varied in the range of 0 - 10 mM, and the rate of activity of the crude enzyme was measured.

3.2.2.4. The Acetylcholinesterase Assay

The assay mixture was made up as follows. In a cuvette, phosphate buffer (0.1 M, pH 7), buffered Ellman's reagent (DTNB 0.01 M) with 15 mg $NaHCO_3$, and 0.4 ml of the homogenate was made up to 3.12 ml. The absorbance was measured at 412 nm at 25 °C. When the absorbance stopped increasing, it was set to zero and the reaction was initiated by the addition of 20 µl substrate (acetylthiocholine iodide [0.075 M]). Change in absorbance per minute was calculated over a period of 5 minutes.

3.3. RESULTS

The rate of hydrolysis of acetylthiocholine by rat forebrain AChE is shown in **Figure 3.2.** and is expressed as the change in absorbance per minute at a wavelength of 412 nm. The data was calculated and the line of best fit was ascertained using the GraphPad InPlot computer program developed by Graph Pad Software, Inc, USA. The rate of hydrolysis was linear to protein concentration (r^2 is 0.96; P = 0.0001)

The rate of hydrolysis (velocity) of acetylthiocholine is shown in **Figure 3.3.** as a function of substrate concentration. The line of best fit was ascertained using the GraphPad InPlot computer program developed by Graph Pad Software, Inc, USA. The rate is expressed as moles/min/mg. **Figure 3.4.** is Eadie-Hofstee plot and **Figure 3.5.** is a Hanes-Woolf plot. The P-value = 0.0327 and r^2 = 0.56 for the Eadie-Hofstee plot. For the Hanes-Woolf plot, P < 0.0001 and r^2 = 0.9473. These are linear plots, and from here the V_{max} and the K_m for the substrate acetylthiocholine was calculated. These values are summarised in **Table 3.1.** below.

TABLE 3.1. V_{max} and K_m values for the hyrolysis of acetylthiocholine by crude rat forebrain acetylcholinesterase.

Plot	V_{max} (x 10^{-6}) (moles/min/mg)	K_m (mM)	P-VALUE	r^2
Eadie-Hofstee	15.46 ± 2.33	2.64	0.03	0.56
Hanes-Woolf	16.14 ± 0.006	3.05	0.001	0.95

The mean K_m value calculated from **Table 2.2.** is 2.84 ± 0.21 mM.

The Hill Plot (**Figure 3.6.**) was constructed using the V_{max} value, averaged from the Eadie-Hofstee Plot and Hanes-Woolf plot = 15.79917 ± 0.3425. The Hill coefficient (n), which is equal to the number of binding sites, was found to be 1.13 (r^2 = 0.907; P =

0.0003)

3.4. DISCUSSION

The assay used in the present study to determine AChE activity, was reported by Ellman *et al.* in 1961. It has several advantages:
(1) The assay is dependent on changes in the visible region of the spectrum. This means that any unusual changes in the absorbance can be detected and checked immediately, e.g. appearance of turbidity, spills on the photocell windows, etc.
(2) Measurements of the appearance of products are usually more sensitive than the disappearance of products.
(3) Homogenates of tissue require no special handling.
(4) The reagents required are commercially available.
The assay is also sensitive, and small amounts of tissue or low concentrations of enzyme do not constitute a problem (Ellman *et al.*, 1961).

The rate of enzyme activity was a linear function of protein concentration. Similar results were also reported by Ellman *et al.* (1961) for the hydrolysis of acetylthiocholine by bovine erythrocyte esterase.

Acetylcholinesterase is an allosteric enzyme (Lopez *et al.*, 1993). This means that the binding of a substrate molecule to an enzyme induces structural or electronic changes (conformational) in the enzyme, which affects the vacant substrate binding sites enabling the second substrate to bind faster. In other words, cooperative binding occurs (Palmer, 1991).

Therefore, the curve expected for the Michealis-Menten plot should be a sigmoidal curve, but was found to be hyperbolic (See **Figure 3.3.**). The Hill plot (**Figure 3.5.**) which was based on **Figure 3.2.** gave an n (number of binding sites) value of 1.13. If a sigmoidal plot had been obtained, the Hill coefficient would have been significantly

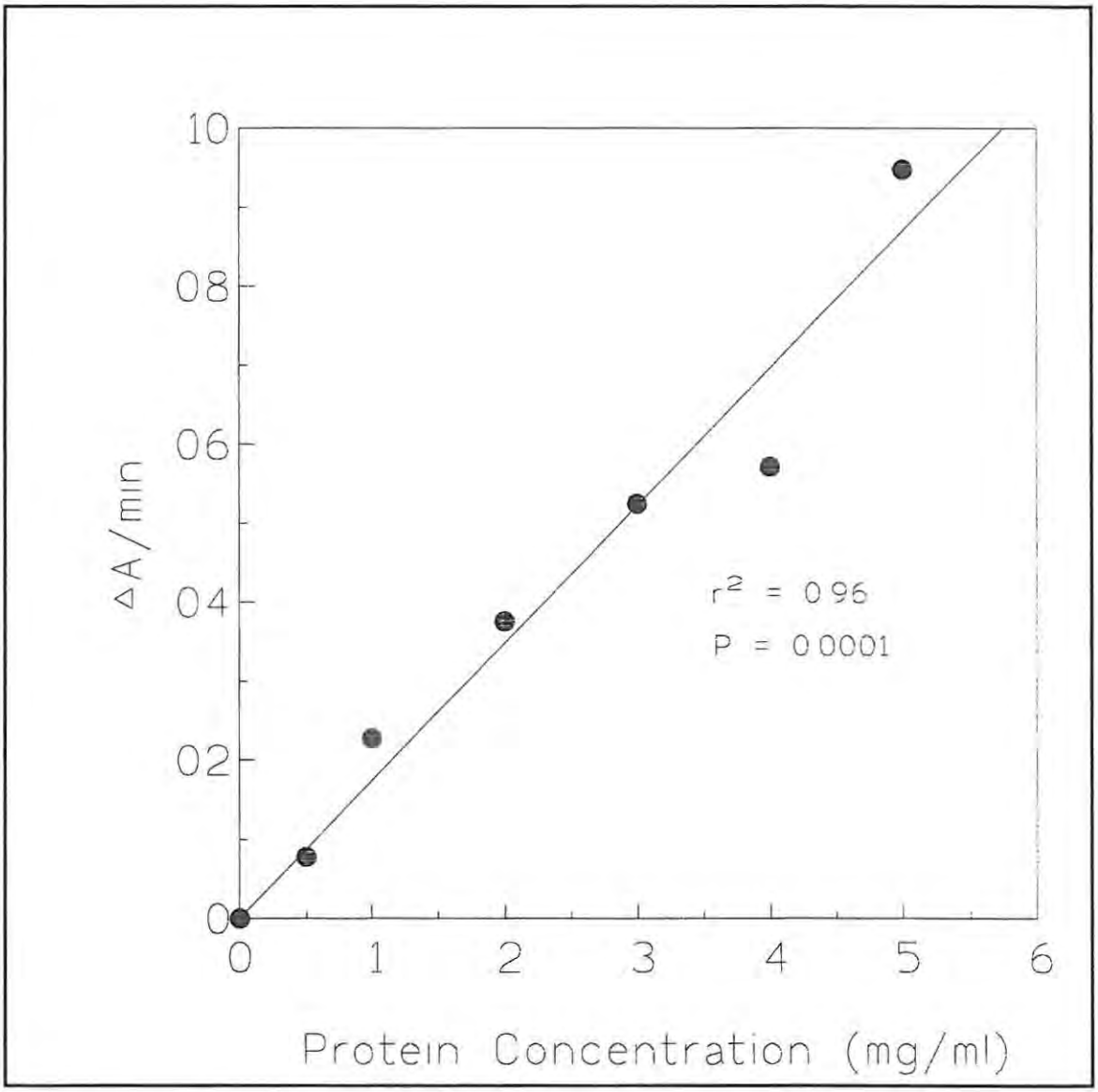

FIGURE 3.2. **The relationship between crude rat forebrain protein concentration and acetylcholinesterase enzyme activity. The substrate, acetylthiocholine, concentration was fixed at 0.075 M and the change in absorbance per min was calculated over a period of five minutes at a wavelength of 412 nm. Each point represents the Mean ± SEM (n=3).**

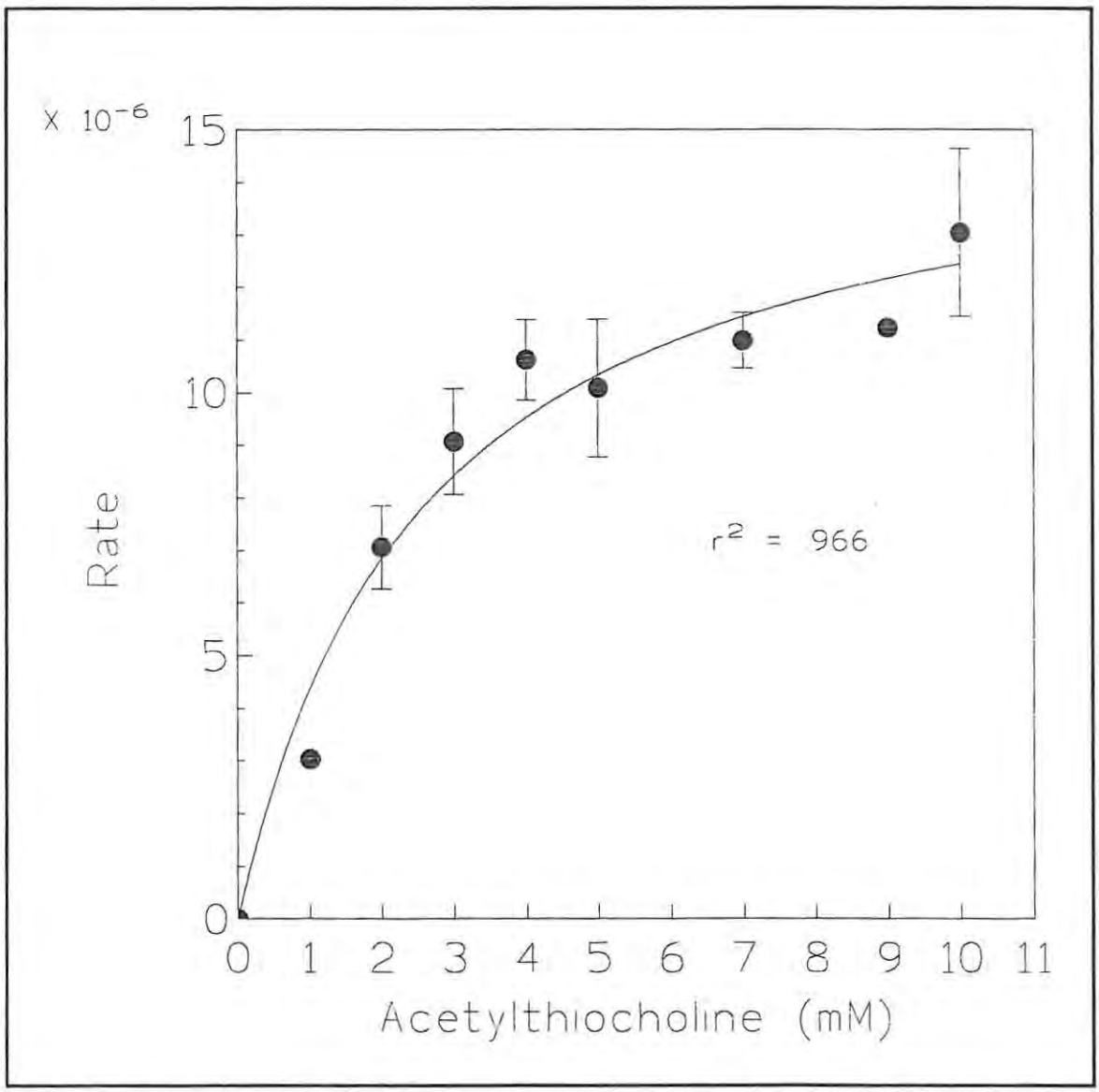

FIGURE 3.3. **Graph of rate (moles/min/mg protein) versus substrate (acetylthiocholine) concentration for the hydrolysis of acetylthiocholine by acetylcholinesterase. Acetylthiocholine concentration was varied from 0-10 mM and protein concentration was fixed at 0.4 mg/ml. The change in absorbance per min was calculated over a period of five minutes at a wavelength of 412 nm. The Rate was expressed as moles/min/mg according to the procedure described in section [3.2.2.4]. Each point represents the mean ± SEM (n=3).**

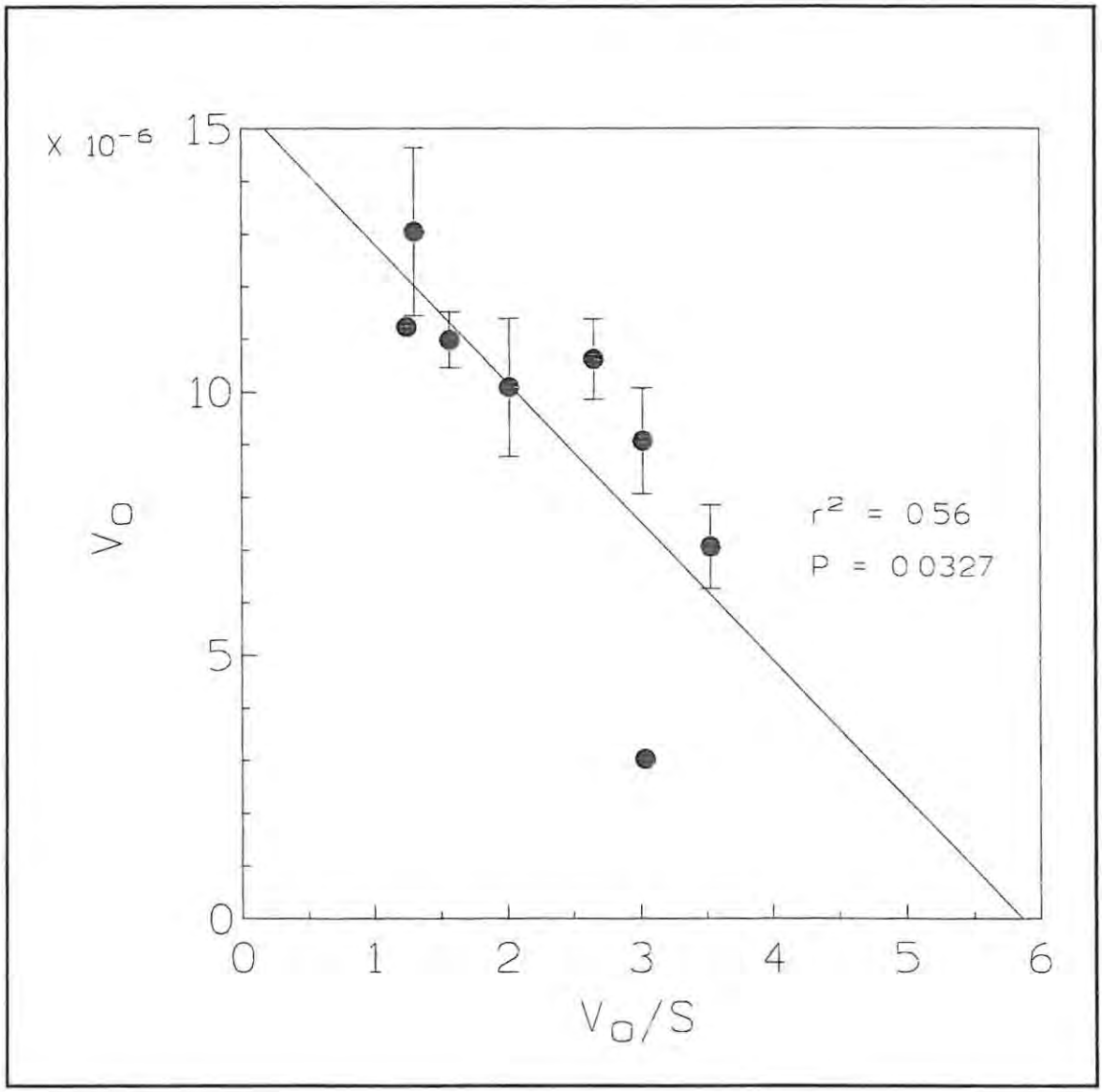

FIGURE 3.4. **Eadie-Hofstee plot for the rate (V_o) (moles/min/mg protein) of production of thiocholine as a function of substrate concentration. The plot is based on data from Figure 3.3. Acetylthiocholine concentration was varied from 0-10 mM and protein concentration was fixed at 0.4 mg/ml. The change in absorbance per min was calculated over a period of five minutes at a wavelength of 412 nm. The Rate was expressed as moles/min/mg according to the procedure described in section [3.2.2.4]. Each point represents the mean ± SEM (n=3). The experiment was carried out at 25 °C.**

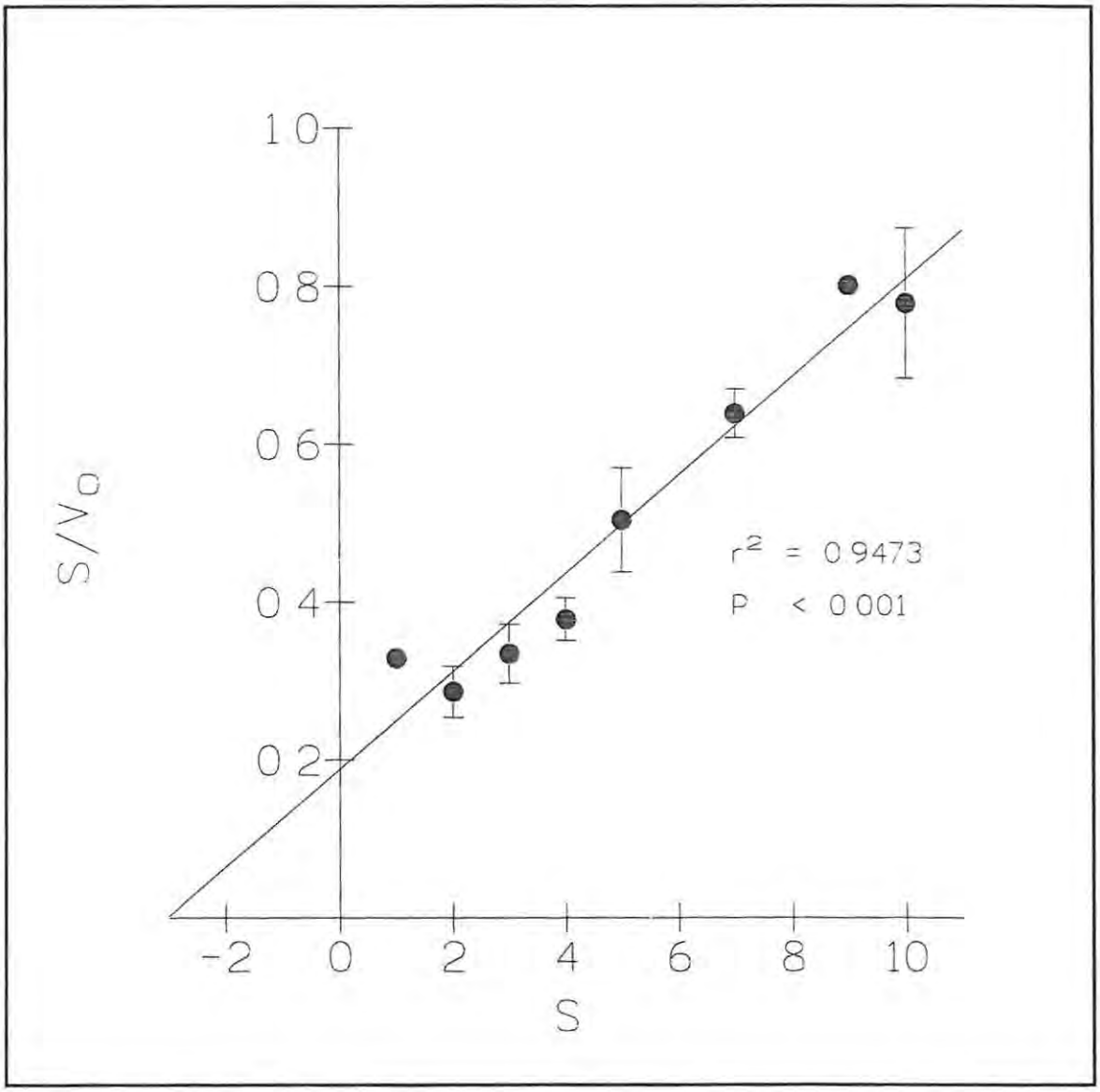

FIGURE 3.5. **Hanes-Woolf plot for the rate (V_0) (moles/min/mg protein) of production of thiocholine as a function of substrate concentration. The plot is based on data from Figure 3.3. Acetylthiocholine concentration was varied from 0-10 mM and protein concentration was fixed at 0.4 mg/ml. The change in absorbance per min was calculated over a period of five minutes at a wavelength of 412 nm. The velocity (Rate) was expressed as moles/min/mg according to the procedure described in section [3.2.2.4]. Each point represents the mean ± SEM (n=3). The experiment was carried out at 25 °C.**

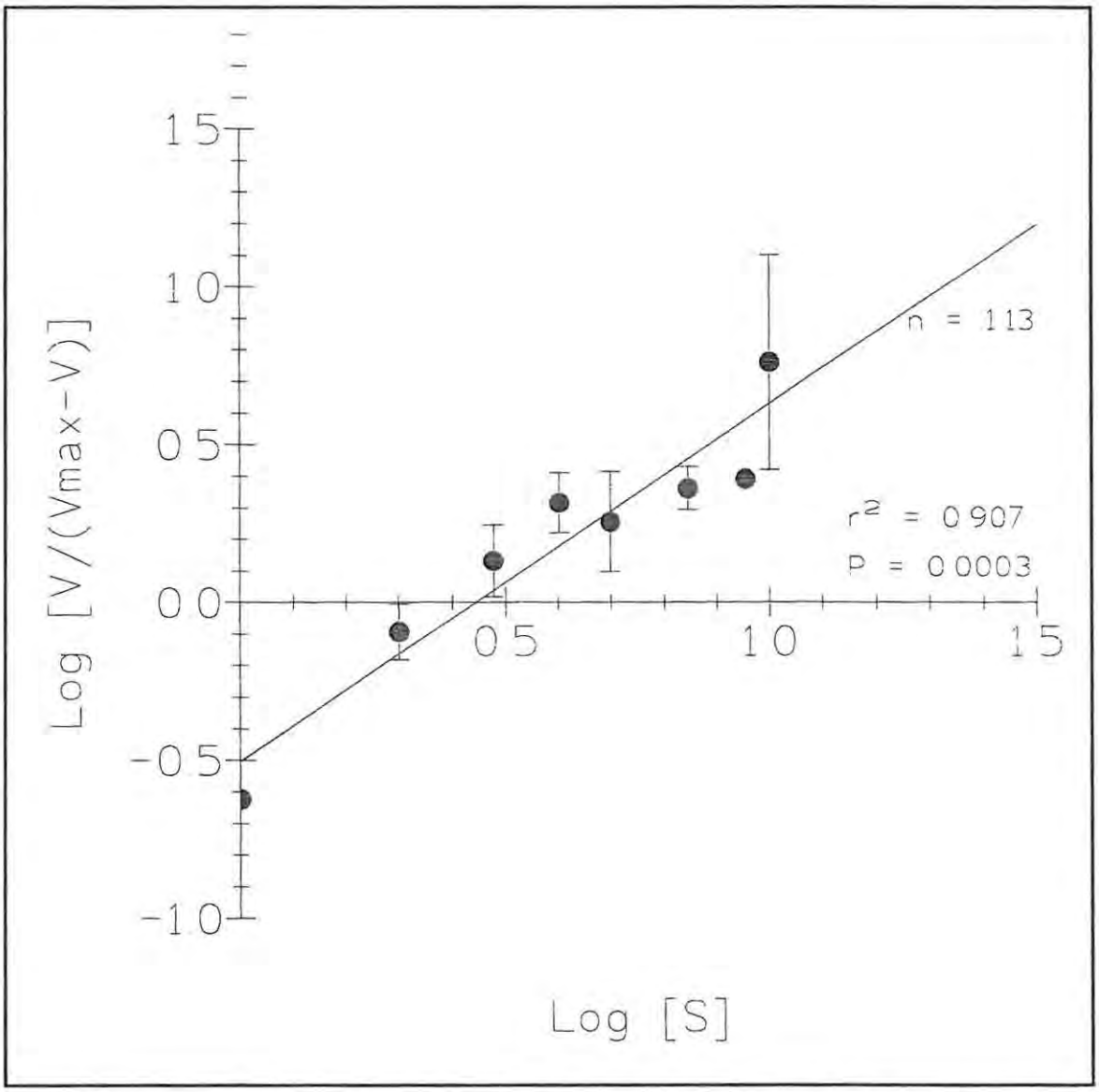

FIGURE 3.6. The Hill Plot of Log [$V_o/(V_0$ - Vmax)] versus Log [Substrate] for rat forebrain acetylcholinesterase activity (V_{max} = 15.79917 ± 0.3425). Acetylthiocholine concentration was varied from 0-10 mM and protein concentration was fixed at 0.4 mg/ml. The change in absorbance per min was calculated over a period of five minutes at a wavelength of 412 nm. The Rate was expressed as moles/min/mg according to the procedure described in section [3.2.2.4]. Each point represents the mean ± SEM (n=3). The experiment was carried out at 25 ^{0}C.

different to 1.

These results are an outcome of the substrate range used, and suggest that in this range, binding is occurring at only one substrate site. In other words, the substrate concentrations are not sufficient to fill more than one site on the enzyme. Thus, the number of binding sites indicated by these results is one.

3.5. EXPERIMENT: DETERMINATION OF THE EFFECTS OF INORGANIC TOXINS (ALUMINIUM AND MERCURY) ON CRUDE ACETYLCHOLINESTERASE (AChE) OF THE RAT FOREBRAIN.

3.5.1. INTRODUCTION

Aluminium in the brain has been implicated in various dementias. In AD for example, one characteristic feature is the generally diminished functioning of the cholinergic system. Another feature is the presence of aluminium in the brain of AD patients. It is not known whether aluminium is deposited in the brain as a result or a cause of AD, or whether it is involved in the etiology of AD (Graves *et al.*, 1990).

Mercury is an other known toxic element that is frequently used for dental treatment in the form of amalgam. Amalgam is the main source of exposure of the general population to inorganic mercury (Halbach, 1995). There is concern over the health consequences of humans exposed to mercury. A possible role of mercury in AD has been highlighted (Lorscheider and Vimy, 1993).

The effects of aluminium and mercury on AChE were examined in the present study.

3.5.2. MATERIALS AND METHODS

3.5.2.1. Chemicals, Drugs and Reagents

Aluminium chloride was purchased from Pal Chemicals, South Africa and mercuric chloride was purchased from Merck (Germany). The other materials used for the assay have been listed in [**3.2.2.1**].

3.5.2.2. Animals

The animals were maintained and sacrificed as described in section [**2.2.2.2.**]. The tissue was prepared as previously described in section [**3.2.2.2**].

3.5.2.3. The Acetylcholinesterase Assay

The assay was carried out as described in section [**3.2.2.4.**] with the inorganic toxin added *in vitro* to the sample in the following final concentrations: 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 10 µM, 1 mM and 0 (i.e. control). Acetylthiocholine was kept constant at a concentration of 75 mM. Statistical calculations and significance was determined using the *Unpaired Student's t test with a two-sided P-value.*

3.5.3. RESULTS

The activity rate of AChE from the rat forebrain, as determined *in vitro* in the presence of varying concentrations of aluminium chloride, is shown in **Table 3.2. Figure 3.7.** is a graphical representation of the results in **Table 3.2.** The rate is expressed as moles per min per mg. No significant inhibition by aluminium of AChE activity was detected within the concentration range of aluminium chloride from 1 nM - 1 mM.

The effect of mercuric chloride on crude AChE from the rat forebrain *in vitro* is shown in **Table 3.3.** A graphical representation of these results is illustrated in **Figure 3.8.** The rate is expressed as moles per min per mg. Inhibition of the enzyme was dose-dependent in the concentration range 100 nM - 1 mM. Significant inhibition was observed at 1 µM, 10 µM, 100 µM and 1 mM ($P < 0.05$). As the concentration of mercuric chloride increases, the inhibition increases.

TABLE 3.2. Rate of activity of acetylcholinesterase *in vitro* in the presence of varying concentrations of aluminium chloride. Each value represents the mean ± SEM (n=3).

ALUMIMIUM CHLORIDE CONCENTRATION	RATE (X 10^{-5}) moles/min/mg	SIGNIFICANCE
CONTROL	9.15 ± 0.43	-
1nM	9.23 ± 0.23	-
10nM	9.15 ± 0.12	-
100nM	9.25 ± 0.25	-
1µM	9.47 ± 0.24	-
10µM	9.12 ± 0.27	-
100µM	9.01 ± 0.18	-
1mM	9.33 ± 0.19	-

TABLE 3.3. Rate of activity of acetylcholinesterase *in vitro* in the presence of varying concentrations of mercuric chloride. Each value represents the mean ± SEM (n=3).

MERCURIC CHLORIDE CONCENTRATION	RATE (X 10^{-5}) moles/min/mg	SIGNIFICANCE
Control	1.21 ± 0.09	-
1nM	1.18 ± 0.06	-
10nM	1.19 ± 0.28	-
100nM	1.03 ± 0.07	-
1µM	0.83 ± 0.12	$P < 0.05$
10µM	0.70 ± 0.04	$P < 0.05$
100µM	0.12 ± 0.20	$P < 0.05$
1mM	0.00 ± 0	$P < 0.05$

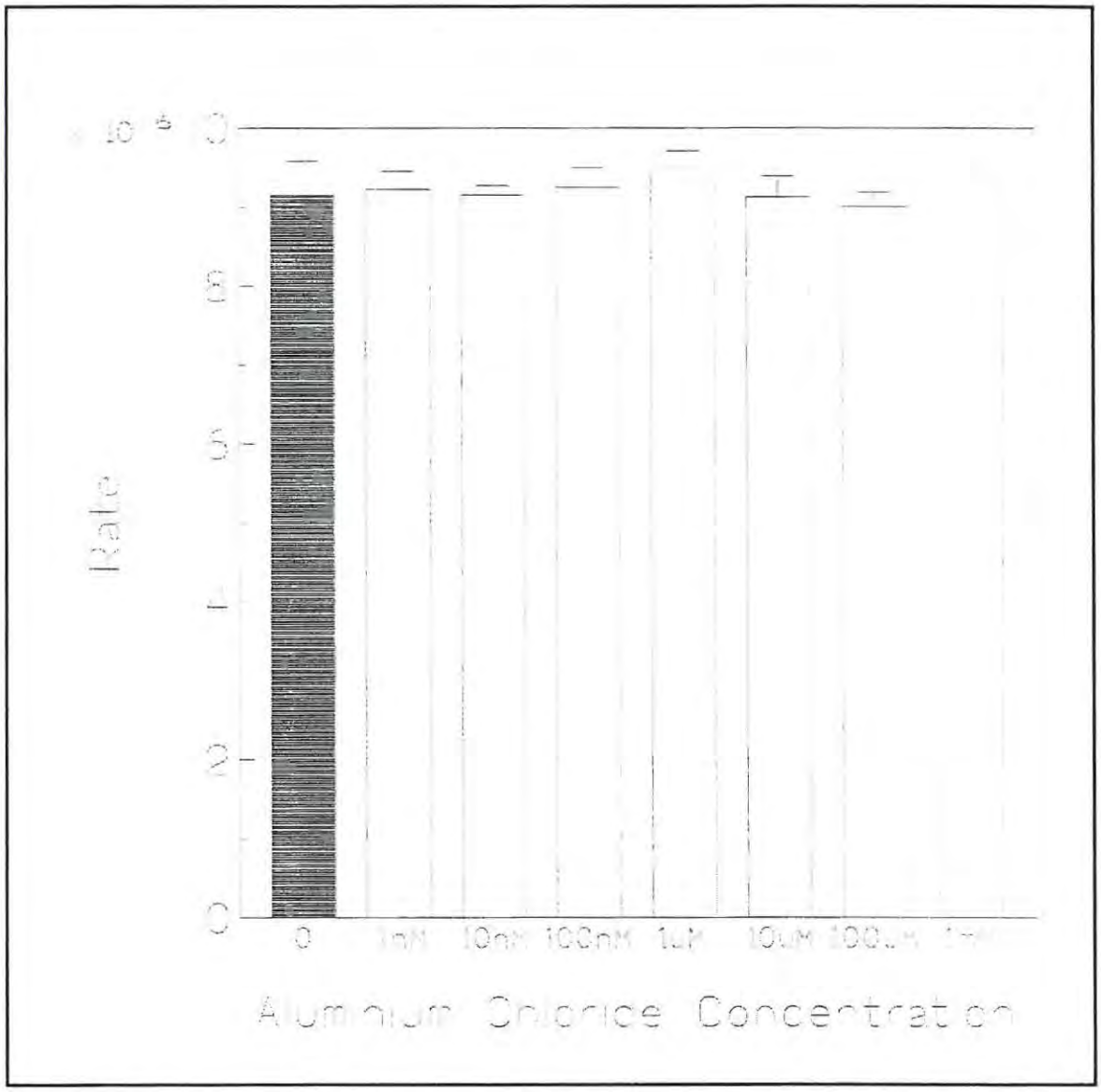

FIGURE 3.7. **The effect of different concentrations of aluminium chloride on the rate (moles/min/mg of protein) of activity of crude acetylcholinesterase from the rat forebrain according to the procedure described in section [3.5.2.3.]. The data represents the Mean ± SEM, n=3.**

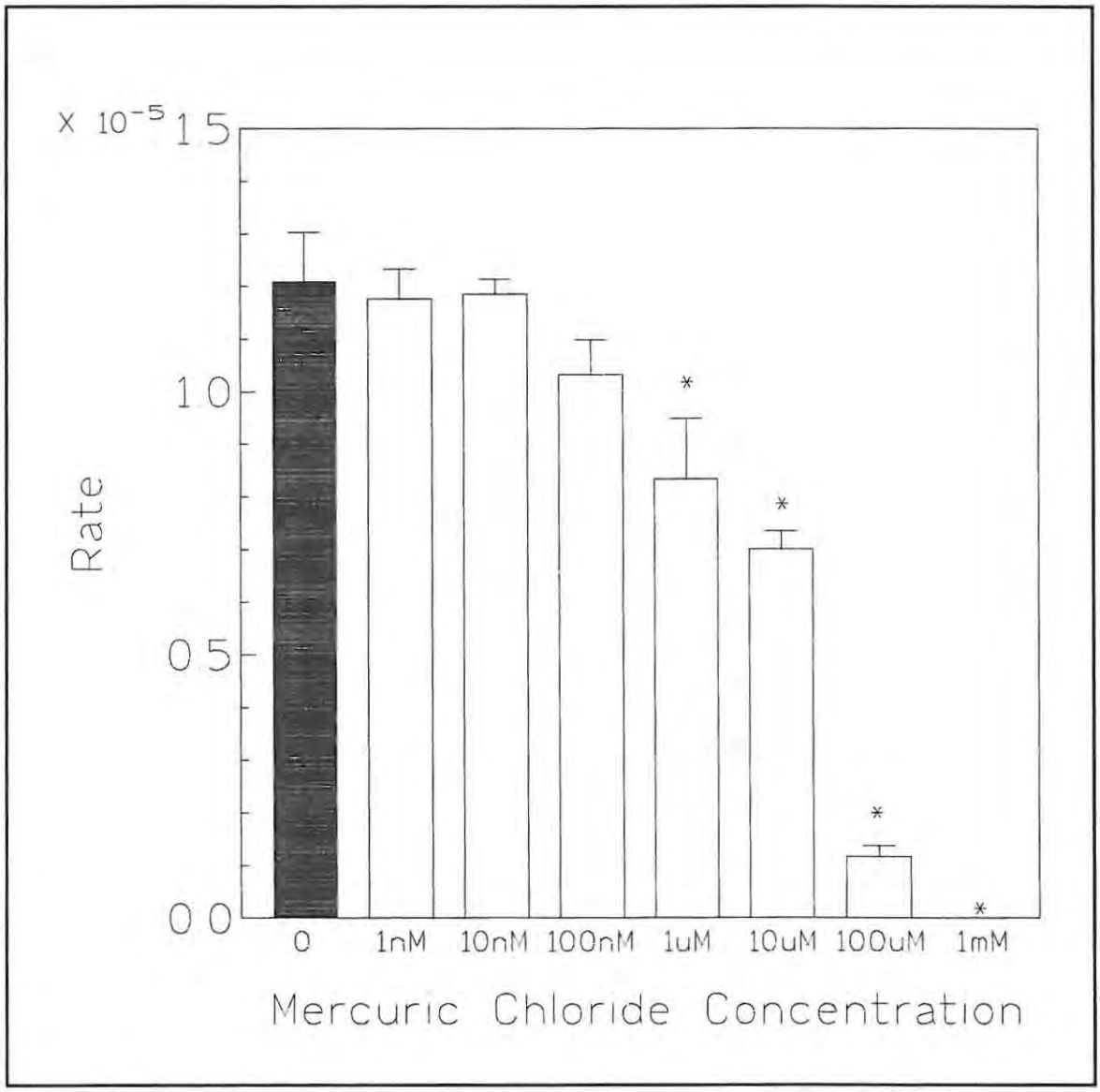

FIGURE 3.8. **The effect of different concentrations of mercuric chloride on the rate (moles/min/mg of protein) of activity of crude acetylcholinesterase from the rat forebrain according to the procedure described in section [3.5.2.3.]. The data represents the Mean ± SEM, n=3, * - P < 0.05 with respect to the control.**

3.5.4. DISCUSSION

These *in vitro* experiments were carried out using crude AChE from the rat forebrain. Aluminium chloride had no effect on the activity of AChE (**Figure 3.7.**). In 1982, Marquis and Lerrick found that concentrations of aluminium chlorohydrate in the range of 0.1 mM to 0.5 mM have a non-competitive inhibitory effect on the enzyme. There was no change in the K_m value but an altered V_{max} for the substrate Ach was observed. Later, Marquis and Black (1984) found that aluminium elevates AChE activity in the bovine caudate at concentrations in the range of 1-10 µM. *In vivo* experiments by Bilkei-Gorzo (1993) showed increased AChE activity following administration of aluminium. Bilkei-Gorzo performed the experiment on 5 groups of rats. The increases reported were found to be significant in two of the five groups of rats (Bilkei-Gorzo, 1993). Hetnarski *et al.* (1980) found no change in rabbit AChE activity five days after being treated with aluminium. Other workers have recently reported a significant decrease in the activity of AChE in the serum of rats exposed to a subacute dose of aluminium for a period of four weeks. These authors also found a reduction in AChE activity in the cerebral cortex, hippocampus and corpus striatum of these rats (Julka and Gill, 1995). The reports are conflicting. This may be attributed to the fact that different species of animals were used for the experiments. Also, the conditions and areas of the brain examined, varied.

In the present study, mercury was found to have no significant effect on crude AChE activity at concentrations of 1 nM to 100 nM. At concentrations higher than this, significant inhibition was observed. The inhibition also appeared to be dose-dependent from 1 µM to 1 mM. At 1 mM, no activity was detected. Mercury released from amalgam has been of concern with regard to its toxic potential (Lorshceider and Vimy, 1993).

The average absorbed dose of mercury for human subjects with eight fillings, is estimated to be 10 µg per day (Halbach, 1995). The results of the present study show that very low doses of mercury have no significant effect on the activity of AChE.

Increased exposure and increased absorbance of mercury will, however, cause a substantial inhibition of AChE activity. Therefore, a person with a large number of amalgam fillings could have a high risk of the mercury leaching, and one manifestation would be decreased activity of brain AChE.

3.6. EXPERIMENT: DETERMINATION OF THE EFFECTS OF THE EXCITATORY AMINO ACIDS, KYNURENIC ACID AND QUINOLINIC ACID, ON CRUDE ACETYLCHOLINESTERASE FROM THE RAT FOREBRAIN

3.6.1. INTRODUCTION

Kynurenic acid is a product of tryptophan metabolism. For some time now, tryptophan metabolism has interested researchers, as it leads to the production of melatonin in the pineal gland and the production of KA and QA. Accumulations of QA have been hypothesized to cause a broad spectrum of human neurological diseases (Blight *et al.*, 1995). Increases in quinolinic acid among other tryptophan metabolites in the CNS, could alter neuronal function (Basile *et al.*, 1995). Therefore, the present experiment investigates the effect of KA and QA on AChE.

3.6.2. MATERIALS AND METHODS

3.6.2.1. Chemicals, Drugs and Reagents

Quinolinic acid and kynurenic acid were purchased from Sigma Chemical Co., USA. The other chemicals used in this assay have been listed in section [**3.2.2.1**].

3.6.2.2. Animals

The animals were maintained and sacrificed as described in [**2.2.2.2.**]. The tissue was prepared as previously described in [**3.2.2.2**].

3.6.2.3. The Acetylcholinesterase Assay

The assay was carried out as described in [**3.2.2.4.**] with the excitatory amino acid

added *in vitro* to the sample in the following final concentrations: 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 100 µM, 1 mM and 0 (i.e control). Acetylthiocholine was kept constant at a concentration of 75 mM. Statistical calculations and significance were determined using the *Unpaired Student's t test with a two-sided P-value.*

3.6.3. RESULTS

In **Table 3.4.** the activity of crude AChE from the rat forebrain, as determined *in vitro* in the presence of KA, is represented. **Figure 3.9.** is a graphical representation of these results. No significant change in the activity of AChE was observed at any concentration of KA tested.

The activity of crude AChE from the rat forebrain was determined *in vitro* at different concentrations of QA (1 nM to 1 mM), and the results obtained are represented in **Table 3.5.** These results are graphically represented in **Figure 3.10.** No significant change in the activity of the enzyme was detected at any of the QA concentrations tested.

3.6.4. DISCUSSION

KA acid has been shown to exist in the brain (Schwarcz *et al.*, 1992). The role of KA in neurobiology is complex and not clearly defined because of a lack of pharmacological tools that can selectively influence KA. The compound is said to steadily increase with age. The possibility that KA may have a role in neurological disease's (such as Huntington's) as a neuroprotective agent, has been suggested (Schwarcz *et al.*, 1992). Therefore, its effect on AChE was investigated. The activity of the enzyme did not change in the presence of the tryptophan metabolite at the different concentrations (**Table 3.4.** and **Figure 3.9.**).

TABLE 3.4. Rate of activity of acetylcholinesterase *in vitro* in the presence of varying concentrations of kynurenic acid. Each value represents the mean ± SEM (n=3).

KYNURENIC ACID CONCENTRATION	RATE (X 10^{-6}) moles/min/mg	SIGNIFICANCE
CONTROL	7.95 ± 0.19	-
1nM	8.27 ± 0.43	-
10nM	8.05 ± 0.02	-
100nM	7.74 ± 0.61	-
1µM	7.96 ± 0.00	-
10µM	7.77 ± 0.04	-
100µM	7.79 ± 0.28	-
1mM	7.88 ± 0.33	-

TABLE 3.5. Rate of activity of acetylcholinesterase *in vitro* in the presence of varying concentrations of quinolinic acid. Each value represents the mean ± SEM (n=3).

QUINOLINIC ACID CONCENTRATION	RATE (X 10^{-5}) moles/min/mg	SIGNIFICANCE
Control	1.09 ± 0.03	-
1nM	1.07 ± 0.03	-
10nM	1.09 ± 0.01	-
100nM	1.01 ± 0.02	-
1µM	1.07 ± 0.00	-
10µM	1.06 ± 0.07	-
100µM	1.02 ± 0.03	-
1mM	1.05 ± 0.02	-

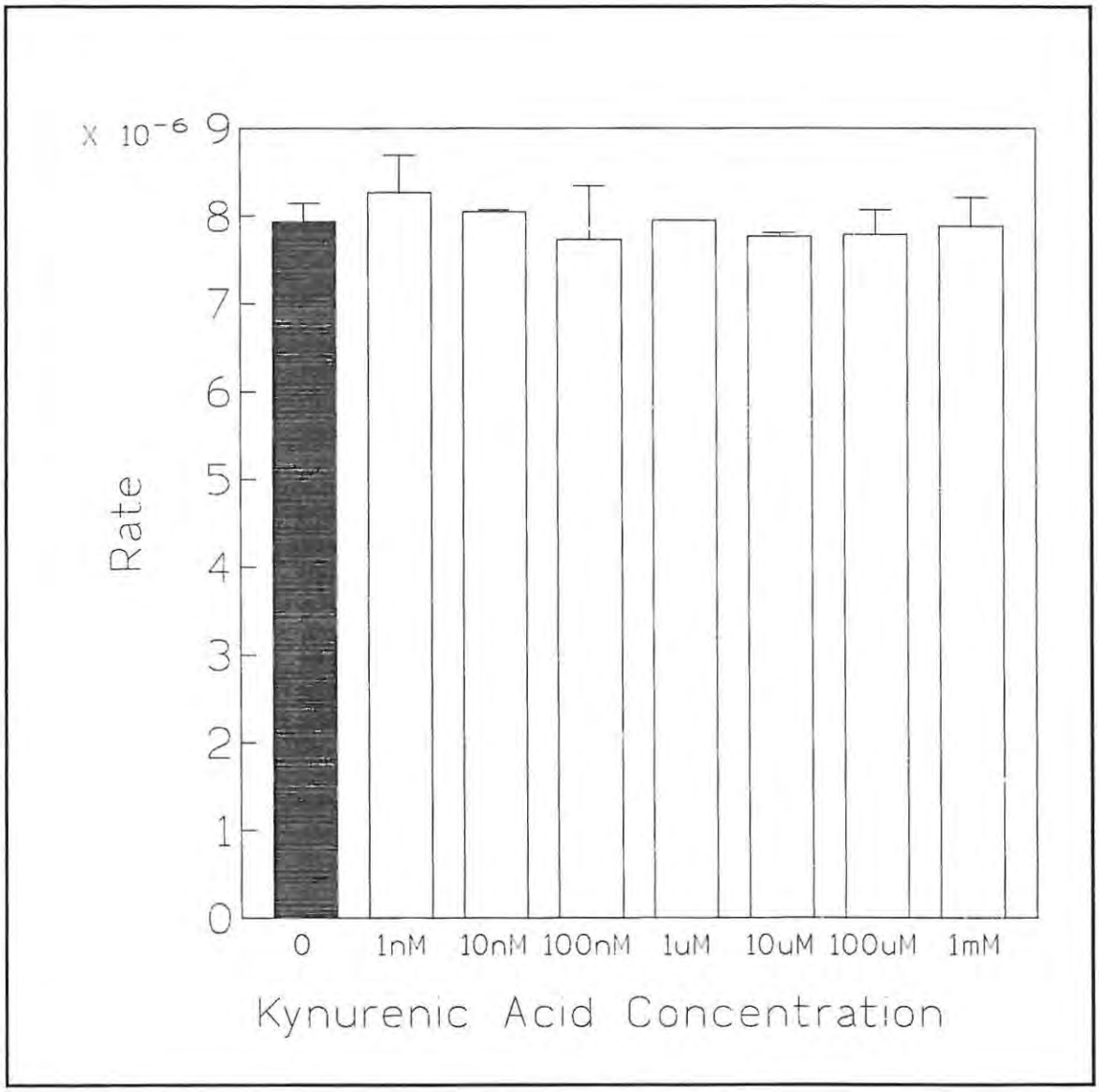

FIGURE 3.9. The effect of different concentrations of kynurenic acid on the rate (moles/min/mg of protein) of activity of crude acetylcholinesterase from the rat forebrain according to the procedure described in section [2.6.2.3.]. The data represents the Mean ± SEM, n=3.

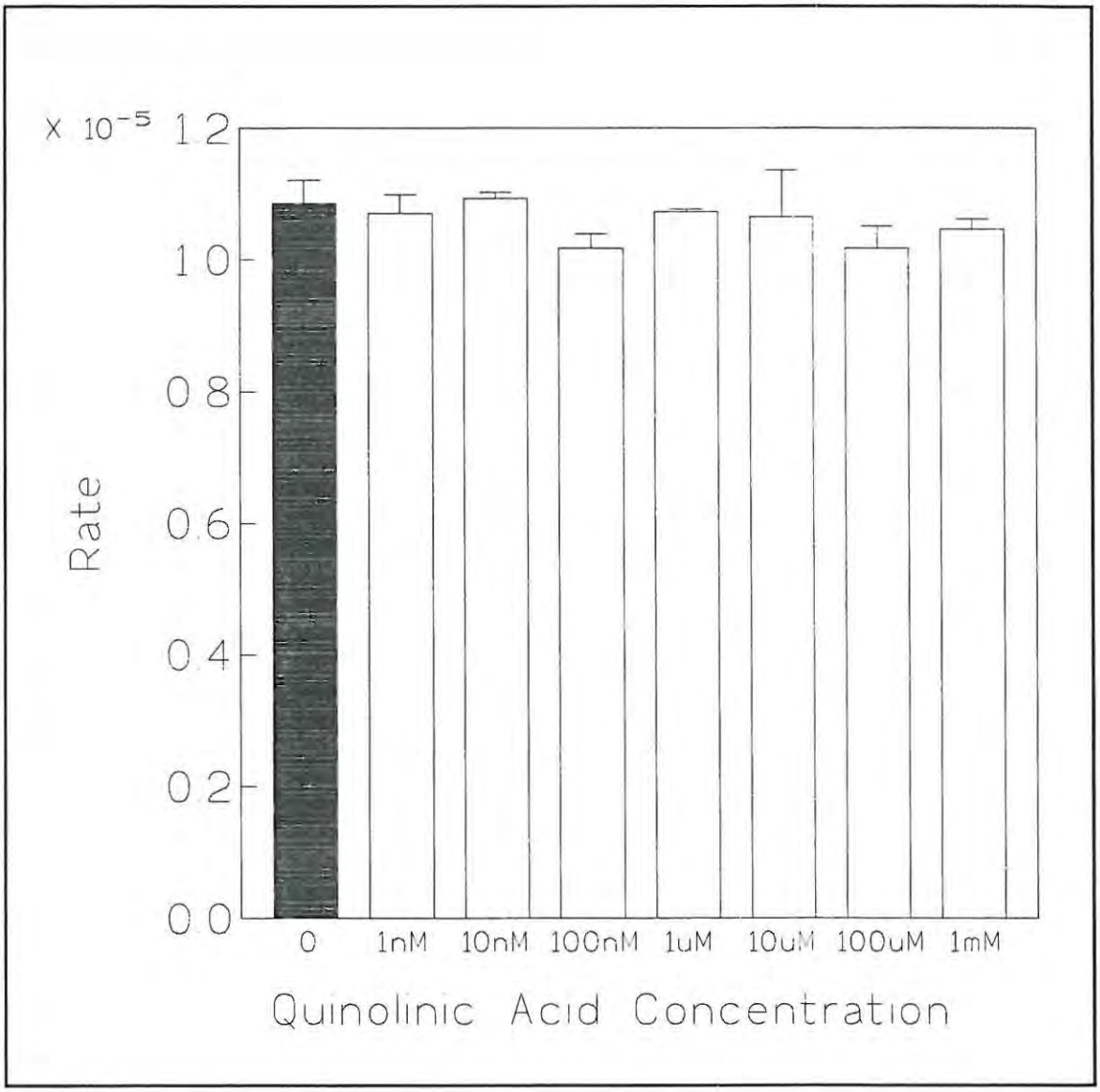

FIGURE 3.10. **The effect of different concentrations of quinolinic acid on the rate (moles/min/mg of protein) of activity of crude acetylcholinesterase from the rat forebrain according to the procedure described in section [2.6.2.3.]. The rate represents the mean ± SEM, n=3.**

QA is known to be present in the rat brain and the human brain. The QA concentration has been shown to increase with age. The increase in QA concentrations has been of some concern as it is not known why this occurs (Moroni *et al.*, 1984a). In this experiment (See **Table 3.5.** and **Figure 3.10.**), it's effect on AChE was determined.

The results obtained show that increases in QA concentration up to 1 mM have no effect on AChE activity under the given assay conditions *in vitro*.

CHAPTER 4

MUSCARINIC RECEPTOR

[^{3}H] QNB BINDING STUDIES

4.1. INTRODUCTION

Neuroscientists have for a long time researched the properties of neurotransmitter receptors. The techniques employed while indirectly confirming the existence of receptors could not disclose how the receptors and the ligands interacted. Only in the sixties, did the existence of the transmitter and receptors become a directly demonstrated fact, rather than a deduction from pharmacological responses of whole tissues (Birdsall and Hume, 1976). The advent of the radioreceptor assay proved to be a solution, and the interaction of the receptor with the ligand could then be observed.

In 1965 the first radioligand binding studies of the muscarinic receptor were performed. Paton and Rang (1965) demonstrated that the binding of tritiated atropine can be used to determine muscarinic receptors in guinea pig smooth muscle. However, detailed biochemical experimentation was not possible as the specific activity was very low and intact pieces of tissue were used. Almost ten years later, the feasibility of using ligands of higher specific radioactivity to identify and characterize muscarinic receptors was demonstrated (Nathanson, 1987).

The development of these radioligands with high affinity and specificity for muscarinic receptors has played a key role in increasing the available biochemical information, such as direct measurement of muscarinic sites, providing data regarding quantitation,

distribution, pharmacological specificity and modulation of muscarinic receptors (Nathanson, 1987; Watson *et al.*, 1986b). One of the antagonists that has been derived is [^{3}H] quinuclidinyl benzilate (QNB). QNB is an alkylating agent derived from the muscarinic antagonist benzilylcholine, and binds irreversibly to muscarinic receptors (Yamamura and Snyder, 1974a). The muscarinic antagonist has been shown to antagonize the acetylcholine-induced contractile response of the guinea pig ileum (Yamamura and Snyder, 1974b).

A few criteria have to be satisfied to establish specific binding by a radiolabelled drug rather than non-specific binding. These are:
(1) Saturability. A component of binding should saturate with increasing concentrations of the radioactive ligand.
(2) Specificity. Pharmacologically effective concentrations of drugs which act at the given receptor should displace the saturable component of binding, whilst pharmacologically effective concentrations of drugs with different receptor specificities should be ineffective.
(3) Localisation. The saturable component of binding should be localized to tissues, and regions of tissues, known from pharmacological experiments to contain the receptor. These criteria are fulfilled by QNB. (Birdsall and Hulme, 1976; Hulme *et al*, 1978)

4.2. MUSCARINIC RECEPTOR [^{3}H] QNB BINDING DETERMINATION TECHNIQUE

4.2.1. Theory of the Assay

Specific [^{3}H] QNB binding is experimentally determined from the difference between counts of [^{3}H] QNB bound in the absence and presence of 1 μM atropine sulphate (Roeske and Yamamura, 1980). Displacement of [^{3}H] QNB binding is greatest with muscarinic antagonists, and the relative affinity of muscarinic cholinergic agonists tends

to parallel their pharmacological potency. Atropine interacts with the specific choline uptake system of cholinergic neurons in the brain, but only at concentrations several orders of magnitude greater than its affinity for QNB-binding sites (Yamamura and Snyder, 1974b).

4.2.2. Materials and Methods

4.2.2.1. Chemicals, Drugs and Reagents

Tritium labelled L-quinuclidinyl [phenyl 4-^{3}H]-benzilate([^{3}H] QNB) (specific activity 50 Ci/mmol) was purchased from Amersham International, England. Atropine was obtained from Sigma Chemical Co., USA. All other drugs and chemicals used were of the purest grade available and all solutions were prepared fresh.

4.2.2.2. Animals

The rats used in this study were inbred albino adult male rats of the Wistar strain, and they were maintained and sacrificed as previously described in section [**2.2.2.2.**].

4.2.2.3. Preparation of Tissue Extraction

Rat forebrain tissue that had been previously stored at -70^{0}C was allowed to thaw. The forebrain tissue was then homogenized in a glass homogenizer with 20 volumes of 10 mM Tris-HCl Buffer (pH 7.4), containing 1 mM EDTA. The homogenates were then centrifuged at 400 rpm for 10 minutes in a Mixtasel Table top Centrifuge that was housed in a cold room with a temperature of 5 ^{0}C. The supernatant was discarded and the pellet was resuspended in 20 volumes of 10 mM Tris-HCl Buffer (pH 7.4) containing 1 mM EDTA and centrifuged at 20 000 rpm for 60 minutes at 4 ^{0}C in a refrigerated centrifuge (Beckman L40 Ultracentrifuge). The supernatant was discarded and the pellet was resuspended in 10 mM Tris-HCl Buffer (pH 7.4) containing 0.9% NaCl. The suspension was then rehomogenized to a smooth suspension. The crude membrane

preparation was used within the following 2 hours.

A protein assay was carried out on the preparation to determine the protein concentration. Using the results obtained, a 1 mg protein/ml solution of the membrane preparation was obtained to carry out the receptor binding assay. The protein concentration was determined by the Folin Lowry Protein Assay as outlined in section [**2.2.4.**]. See **Figure 2.1.** for a typical protein standard curve.

4.2.2.4. Radioligand Binding Assay

A scheme for the binding assay is shown in **Table 4.1.** Radioligand receptor binding assays were carried out by the addition of 400 µl aliquots of membrane suspension (1 mg protein/ml) into glass tubes that contained 60 µl Tris buffer or appropriate drug atropine (10^{-5} M) in buffer. Dilutions of the [H] QNB were made to give ten concentrations ranging from 0.5 nM - 10.0 nM. The reactions were initiated by the addition of [^{3}H] QNB to give the required dilutions in the set concentration range. The incubations were performed at 25 °C for 100 minutes. After this time the reactions were terminated by the addition of 3 ml of 10 mM Tris-HCl (pH 7.5), and were then filtered through Schleicher and Schnell glass fibre No. 6 filters under vacuum. Subsequently each filter was rapidly washed twice with 3 ml ice cold 10 mM Tris-HCl Buffer (pH 7.5). The receptor bound radioactivity which was trapped on the filters was then measured by liquid scintillation solution spectrometry in 3 ml Emulsifier Scintillator Plus scintillation cocktail in a Beckman LS-2500 Scintillation counter.

The non-specific ligand binding was assessed in the presence of excess "cold" drug.

4.3. RESULTS

The saturability of specific [^{3}H] QNB binding in rat forebrain homogenate was measured

SCHEME FOR THE BINDING ASSAY

TABLE 4.1. TOTAL BINDING

[^{3}H]QNB	0.5	1.0	1.5	2.0	3.0	4.0	5.0	7.0	8.0	10.0
PROTEIN (µl)	400	400	400	400	400	400	400	400	400	400
[^{3}H]QNB (µl)	2	4	6	8	12	16	20	28	32	40
BUFFER (µl)	98	96	94	92	88	84	80	72	68	60
TOTAL (µl)	500	500	500	500	500	500	500	500	500	500

TABLE 4.2. NON-SPECIFIC BINDING

[^{3}H]QNB	0.5	1.0	1.5	2.0	3.0	4.0	5.0	7.0	8.0	10.0
PROTEIN (µl)	400	400	400	400	400	400	400	400	400	400
[^{3}H]QNB (µl)	2	4	6	8	12	16	20	28	32	40
ATROPIN E (10^{-5}) (µL)	20	20	20	20	20	20	20	20	20	20
BUFFER (µl)	78	76	74	72	68	64	60	52	48	40
TOTAL (µl)	500	500	500	500	500	500	500	500	500	500

***** REACTION IS STARTED BY ADDING 20 µl [^{3}H]QNB ******

Perform the assay for 100 minutes at 25 °C.

Terminate by filtering the incubation media through Whatman GF-C filter paper under vacuum.

Wash the filter three times with Ice Cold 10 mM Tris Buffer (pH 7.5) containing 0.9% NaCl.

Place filters into vials containing 3 ml Scintillation Cocktail.

as a function of [^{3}H] QNB concentration ranging from 0 - 10 nM (**Figure 4.1.**). Specific binding was calculated as the difference between non-specific binding which is in the presence of [^{3}H] QNB and 10 μM atropine, and total binding, which is in the presence of [^{3}H] QNB in the concentration range from 0.5 nM - 10 nM. Specific binding was expressed as specific [^{3}H] QNB bound (pmoles/mg of protein). The line of best fit was ascertained using the GraphPad InPlot computer program developed by Graph Pad Software, Inc, USA. The correlation coefficient (r^2) was equal to 0.952. The data represents the mean ± SEM of 3 experiments.

The data was then replotted as a single straight line according to the method of Scatchard (1949) (**Figure 4.2.**). The Scatchard plot is a graph of [^{3}H] QNB Bound/Free (pmoles/mg of protein/nM versus [^{3}H] QNB Bound (pmoles/mg of protein). The nonspecific equilibrium dissociation constant (K_D) and the receptor density (B_{max}) were determined from **Figure 4.2.** The K_D (i.e. the radioactive drug concentration at which fifty percent of the receptors are saturated) value was determined to be 0.31 ± 0.80 nM, and B_{max} (i.e. the receptor density) was 0.806 pmoles/mg. **Figure 4.1.** represents the saturation curve and shows the concentration range in which this study was carried out. The plateau indicates the saturation zone where receptor sites reach saturation.

4.4. DISCUSSION

In 1974, Yamamura and Snyder successfully isolated muscarinic receptor sites in the rat brain and found that an alkylating agent derived from the muscarinic antagonist benzilylcholine, known as 3-Quinuclidinyl benzilate (QNB), was able to bind specifically to homogenates of rat brain. The specific binding displayed many of the characteristics which might be expected of interactions with muscarinic cholinergic receptors in the rat brain. Since then, QNB has been used to isolate muscarinic receptor sites in the brain, the pineal gland, the heart and the lung of various animals (Fields *et al.*, 1978; Luthin and Wolfe, 1984; Govitrapong *et al.*, 1989; Watson *et al.*, 1986; Bloom *et al.*, 1987; Goodwin *et al.*, 1995;).

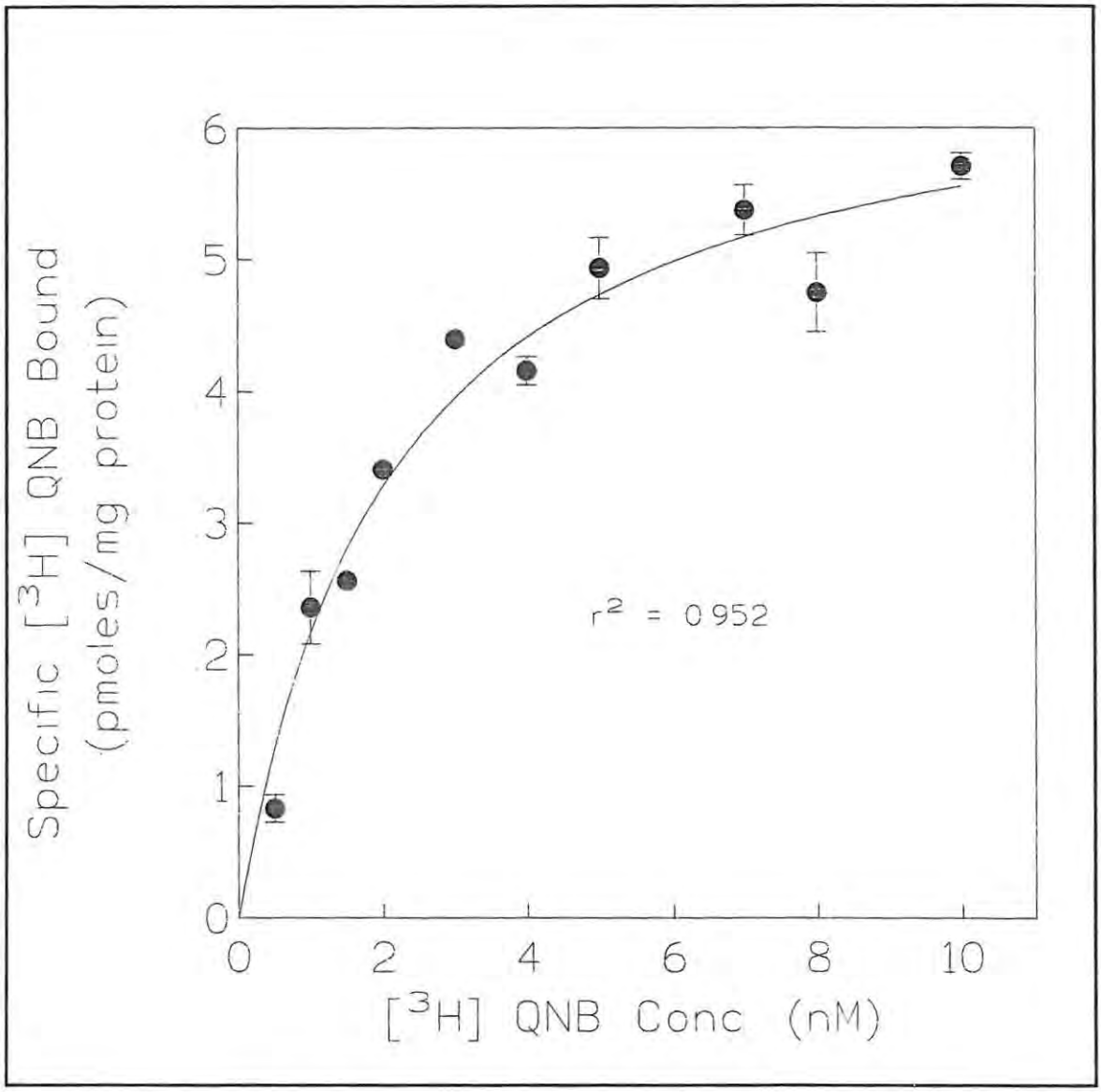

FIGURE 4.1. A saturation curve of [³H] QNB binding to rat forebrain membranes. The experiment was carried out using 10 concentrations of [³H] QNB, ranging from 0.5 - 10.0 nM. Each point represents the mean of triplicate determinations ± SEM.

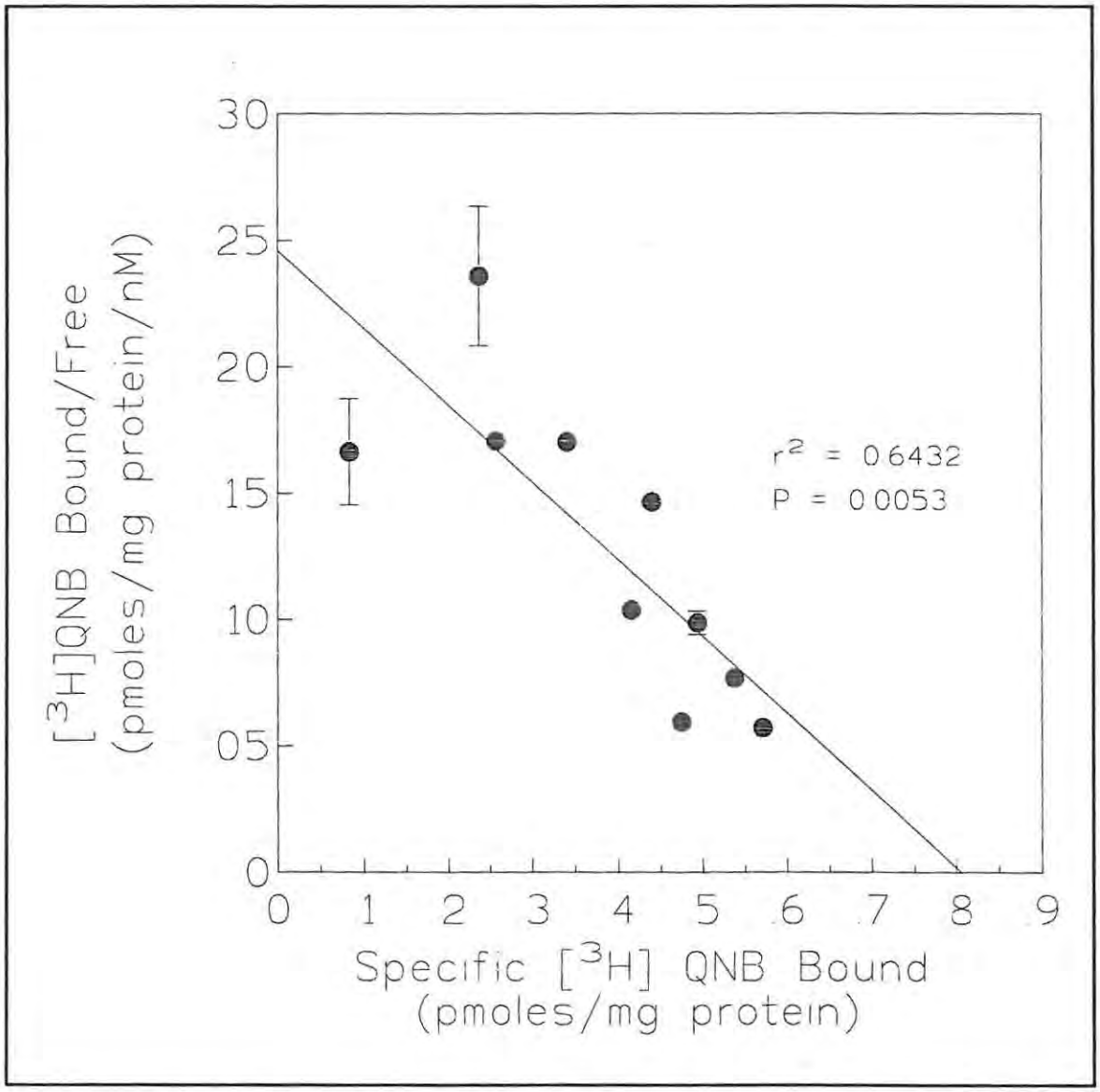

FIGURE 4.2. A Scatchard analysis plot of [^{3}H] QNB binding to the rat forebrain membranes. This was carried out using ten concentrations of [^{3}H] QNB, ranging from 5 - 10.0 nM. Each point represents the mean of triplicate determinations ± SEM.

In this study, the K_D value determined for muscarinic cholinergic receptors of the rat forebrain is agreeable with the value of 0.4 nM that was determined by Yamamura and Snyder in 1974. The K_D for bovine (Govitrapong *et al.*, 1989) and for developing chick heart (Galper *et al.*, 1977) were similar to the K_D reported in this study. However the K_D reported was approximately twenty times that reported by Luthin and Wolfe (1984) for rat brain muscarinic cholinergic receptors, about thirty-six times the K_D for rabbit peripheral receptors (Bloom *et al.*, 1987), and five times the K_D reported by Goodwin *et al.* (1995) for rat nucleus tractus solitarius and rat heart muscarinic receptors.

4.5. EXPERIMENT: DETERMINATION OF THE EFFECTS OF INORGANIC TOXINS (ALUMINIUM AND MERCURY) ON CRUDE CHOLINERGIC MUSCARINIC RECEPTORS FROM THE RAT FOREBRAIN.

4.5.1. INTRODUCTION

In the past few years, there has been an increasing interest in the effects of toxic elements on biochemical processes. Most toxic elements affect multiple organ systems, with specific biochemical processes and (or) organelles as targets (Bush *et al.*, 1995). Toxic effects are often pronounced at the neurological level (Felton, 1972; Silbergeld and Goldberg, 1974; Rustam *et al.*, 1975). An example of this is mercury, which is toxic to many enzymes, but has particularly severe effects on the central nervous system which are pronounced during cerebral maturation (Bondy *et al.*, 1979). Poor coordination, tremors and other movement disturbances caused by compounds with toxic elements, suggest that the elements may in part exert their effects by interfering with nerve function (Bondy and Agrawal, 1980).

In the present study, the effects of aluminium and mercury on the cholinergic muscarinic receptors of rat forebrain are investigated. The effects of different concentrations of the inorganic metals on the receptors is determined.

4.5.2. MATERIALS AND METHODS

4.5.2.1. Chemicals, Drugs and Reagents

Aluminium chloride was obtained from Pal Chemicals, South Africa, and mercuric chloride was obtained from Merck (Germany). All the other chemicals and reagents used are outlined in section [**4.2.2.1.**].

4.5.2.2. Method

To determine the effect of inorganic toxins, aluminium and mercury, on cholinergic muscarinic receptors from the rat forebrain, the radioligand binding assay described in section [**4.2.2.4.**] was used with the following changes. [^{3}H] QNB concentration was kept constant at 0.31 nM, which is the K_D calculated in section [**4.3.**]. The inorganic toxins in question, were added to give final concentrations of 1 nM, 10 nM, 1 μM, 10 μM, 100 µM and 1 mM to the incubation mixtures.

4.5.3. RESULTS

The effect of varying concentrations of aluminium chloride, on the affinity of muscarinic receptors for [^{3}H] QNB is presented in **Table 4.3.**, and graphically in **Figure 4.3.** The results are expressed as specific [^{3}H] QNB bound (pmoles/mg protein). Each point represents the mean ± SEM (n=3). Significance is computed using a One-Way Analysis of Variance followed by Student-Newman-Keuls Multiple Range test. Aluminium chloride in the range from 1 nM - 100 µM did not have any significant effect on the binding of [H^3] QNB to acetylcholine muscarinic receptors of the rat forebrain. At a concentration of 1 mM of aluminium chloride, the affinity of the receptors for [^{3}H] QNB appears to have increased significantly ($P < 0.01$), resulting in increased specific [^{3}H] bound QNB (See **Table 4.3.** and **Figure 4.3.**).

Table 4.4. and **Figure 4.4.** show the effect of varying concentrations of mercuric chloride on the affinity of muscarinic receptors for [^{3}H] QNB. The results are expressed as specific [^{3}H] QNB bound (pmoles/mg protein). Each point represents the mean ± SEM (n=3). Significance is computed using a One-Way Analysis of Variance followed by Student-Newman-Keuls Multiple Range test.

The results in **Table 4.4** and **Figure 4.4.** show that mercuric chloride, from a concentration of 1 nM to 100 µM, does not significantly affect the specific binding

TABLE 4.3. The effects of varying concentrations of aluminium chloride on specific muscarinic receptor binding in the presence of 0.31 nM [^{3}H] QNB. Each value represents the mean ± SEM (n=3).

ALUMINIUM CHLORIDE CONCENTRATION	SPECIFIC BINDING (specific [^{3}H] QNB bound) pmoles/mg protein	SIGNIFICANCE
Control	0.15 ± 0.02	-
1 nM	0.16 ± 0.02	-
10 nM	0.15 ± 0.16	-
100 nM	0.14 ± 0.02	-
1 µM	0.17 ± 0.02	-
10 µM	0.15 ± 0.01	-
100 µM	0.16 ± 0.02	-
1 mM	0.30 ± 0.03	$P < 0.01$

TABLE 4.4. The effects of varying concentrations of mercuric chloride on specific muscarinic receptor binding in the presence of 0.31 nM [^{3}H] QNB. Each value represents the mean ± SEM (n=3).

MERCURIC CHLORIDE CONCENTRATION	SPECIFIC BINDING (specific [^{3}H] QNB bound) pmoles/mg protein	SIGNIFICANCE
Control	0.15 ± 0.02	-
1 nM	0.14 ± 0.002	-
10 nM	0.13 ± 0.02	-
100 nM	0.14 ± 0.01	-
1 µM	0.14 ± 0.02	-
10 µM	0.13 ± 0.01	-
100 µM	0.13 ± 0.02	-
1 mM	0.06 ± 0.01	$P < 0.01$

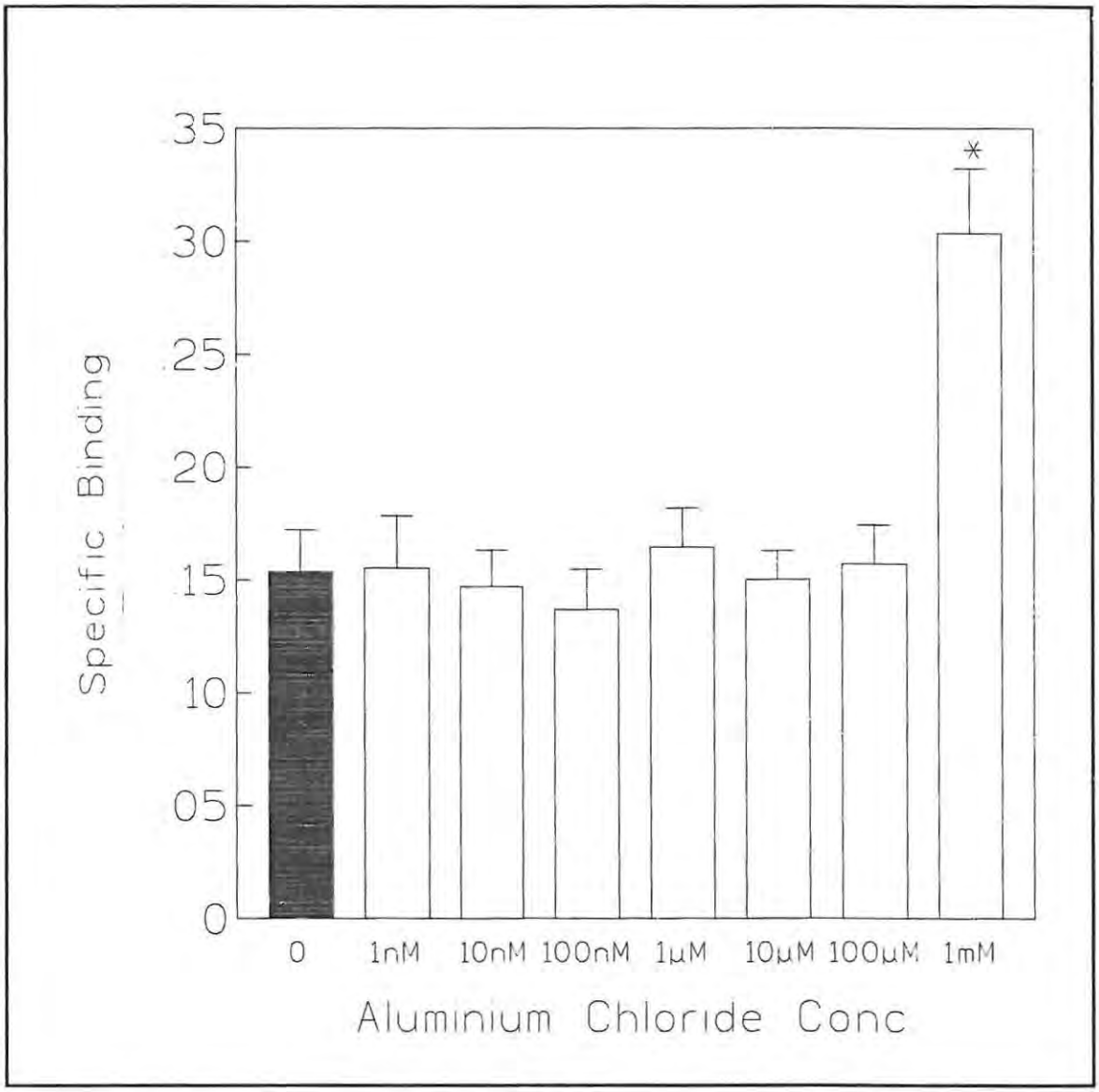

FIGURE 4.3. **The effect of different concentrations of aluminium chloride on the specific binding of rat forebrain acetylcholine muscarinic receptors. The Specific binding is expressed as specific [^{3}H] QNB bound (pmoles/mg protein). Each value represents the Mean ± SEM (n=3). Significance is computed using a One-Way Analysis of Variance followed by Student-Newman-Keuls Multiple Range test.**

*** - P < 0.01.**

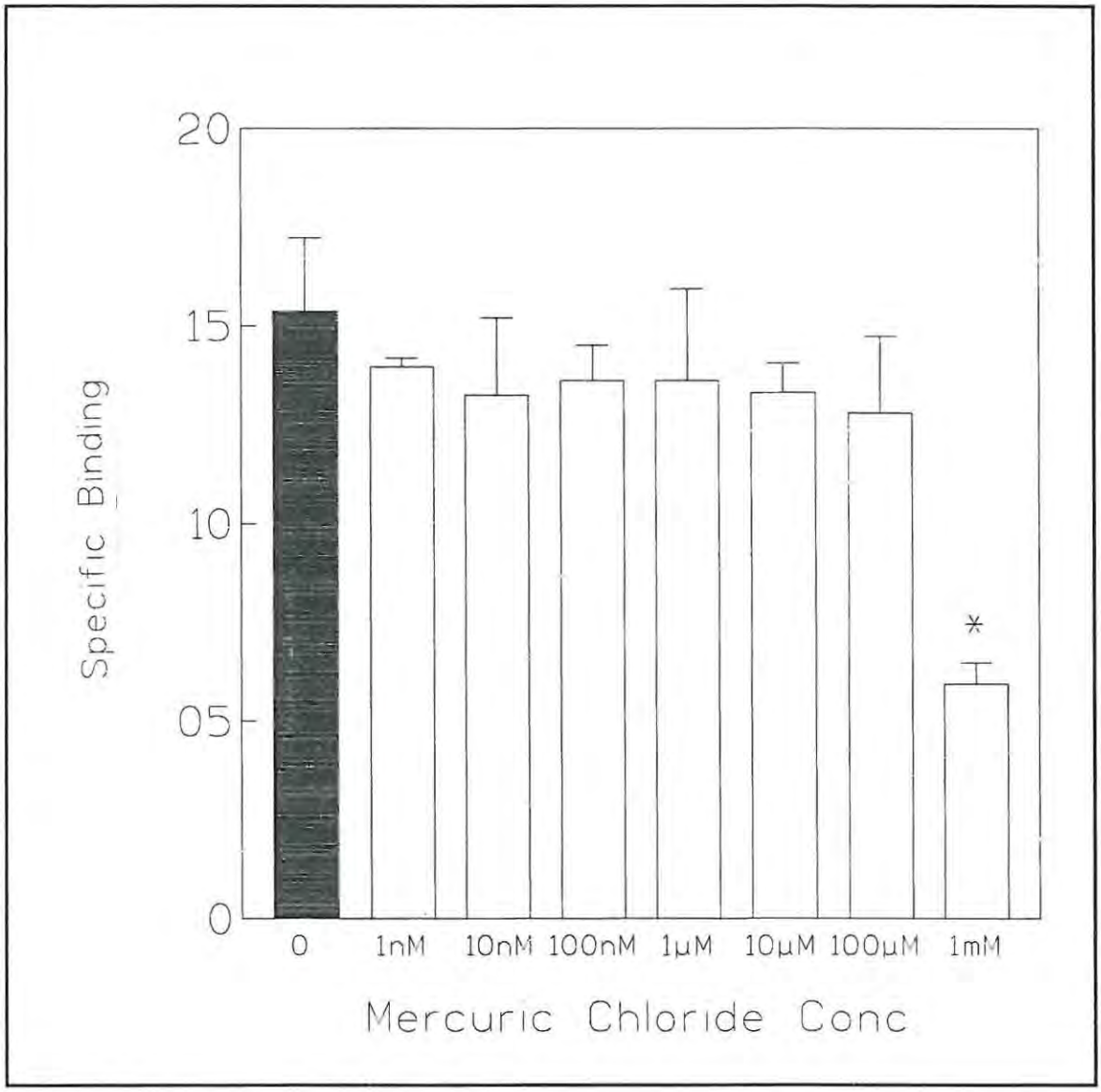

FIGURE 4.4. The effect of different concentrations of mercuric chloride on the specific binding of rat forebrain acetylcholine muscarinic receptors. The Specific binding is expressed as specific [^{3}H] QNB bound (pmoles/mg protein). Each value represents the Mean ± SEM (n=3). Significance is computed using a One-Way Analysis of Variance followed by Student-Newman-Keuls Multiple Range test.
*** - P < 0.01.**

of [^{3}H] QNB to acetylcholine muscarinic receptors. At a concentration of 1 mM there is a significant decrease in the ability of [^{3}H] QNB to bind to the receptors ($P < 0.01$).

4.5.4. DISCUSSION

Muscarinic receptors are altered in several neurological disorders associated with cognitive deficits. The receptors are also highly sensitive to neurotoxins (Schulte *et al.*, 1994). Therefore, this study investigated the *in vitro* effects of varying concentrations of aluminium chloride and mercuric chloride on muscarinic receptors from the rat forebrain.

Muscarinic receptors seem to be rather insensitive to aluminium chloride in the concentration range from 1 nM to 100 µM (See **Table 4.3.** and **Figure 4.3.**), and there was no statistically significant change in the receptors ability to bind to [^{3}H] QNB *in vitro*. At a concentration of 1 mM, however, there appeared to be increased binding of the receptors to [^{3}H] QNB ($P < 0.01$).

Mercuric chloride was found to have no statistically significant effect on *in vitro* muscarinic receptor binding from a concentration of 1 nM to 100 µM. The ionic mercuric chloride at 1 mM appeared to strongly block binding ($P < 0.01$). In 1980, Bondy and Agrawal reported strong inhibition of muscarinic receptors of the cortical region at a mercuric chloride concentration of 5 µM. Other authors have also reported on the effects of heavy metals on muscarinic receptors. These authors have shown the presence of sulfhydryl groups in rat brain muscarinic receptors, and attribute the inhibition of mercury to its ability to interact with these sites (Aronstam *et al.*, 1978; Aronstam and Eldefrawi, 1979). Hurko (1978) also demonstrated the critical nature of sulfhydryl groups to the muscarinic receptor binding site. Since inhibition of binding was statistically significant only at 1 mM, this suggests that there must be an excess of mercury present to facilitate its interaction with the sulfhydryl sites in the receptors.

4.6. EXPERIMENT: DETERMINATION OF THE EFFECTS OF EXCITATORY AMINO ACIDS (KYNURENIC ACID AND QUINOLINIC ACID) ON CRUDE CHOLINERGIC MUSCARINIC RECEPTORS FROM THE RAT FOREBRAIN.

4.6.1. INTRODUCTION

In 1981, Stone and Perkins reported that QA was an excitant of mammalian central neurons. QA is able to serve as a ligand of excitatory amino acid (EAA) receptors. The concentration of QA in the brain is several orders of magnitude lower than that of other EAA's. However, it is the most potent endogenous excitotoxin able to reproduce the neuropathological characteristics of several human neurological diseases. QA is an agonist of forebrain N-methyl-D-aspartate (NMDA) receptors, and may play a role in the memory and cognitive impairment that may be linked to NMDA receptor dysfunction. Neurodegeneration by EAA receptor agonists, especially that of QA can be prevented by KA. KA is an antagonist and acts competitively at the NMDA receptor complex. Thereby, KA may be able to exert control over NMDA receptor activity (Schwarcz *et al.*,1992).

Quinolinic acid (QA) has been observed to increase with age in rats (Stone and Connnick, 1985). In experiments conducted by Moroni *et al.* (1984b) on the brains of rabbit, guinea pig and rat, the cortex was shown to contain the highest concentration of QA. Moroni *et al.*(1984a) found that QA concentration increases during the development and ageing process of the animals. Kynurenic acid (KA) has also been noted to increase with age (Martin and Beal, 1992).

The cholinergic system in the central nervous system is important for learning, memory and cognition. A decline in cholinergic innervation of the human brain is a feature of Alzheimer's disease (AD) (Bogdanovic *et al.*, 1993).

In the present study, keeping in mind that QA and KA have been suggested to have

possible roles in neurological diseases and are an agonist and antagonist respectively of NMDA receptors, the effects of QA and KA on cholinergic muscarinic receptors of the rat forebrain are investigated.

4.6.2. MATERIALS AND METHODS

4.6.2.1. Chemicals, Drugs and Reagents

QA and KA were purchased from Sigma Chemical Co., USA. All the other chemicals and reagents were obtained as outlined in section [**4.2.2.1.**].

4.6.2.2. Method

To determine the effect of excitatory amino acids on cholinergic muscarinic receptors from the rat forebrain, the radioligand binding assay described in section [**4.2.2.4.**] was used with the following changes. [^{3}H] QNB concentration was kept constant at 0.31 nM, which is the K_D calculated in section [**4.3.**]. The amino acid in question was added to give final concentrations of 1 nM, 10 nM, 1 μM, 10 μM, 100 µM and 1 mM to the incubation mixtures.

2.6.3. RESULTS

Table 4.5. and **Figure 4.5.** show the effect of varying concentrations of kynurenic acid, in the presence of 0.31 nM [^{3}H] QNB, on specific muscarinic receptor binding. The results are expressed as specific [^{3}H] QNB bound (pmoles/mg protein). Each point represents the mean ± SEM (n=3). Significance is computed using a One-Way Analysis of Variance followed by Student-Newman-Keuls Multiple Range test.

KA does not appear to affect the specific binding of [^{3}H] QNB to muscarinic receptors

significantly. However, an increase in specific binding is observed at a KA concentration of 1 mM (See **Table 4.5.** and **Figure 4.5.**). This increase was not statistically significant.

The effects of varying concentrations of QA in the presence of 0.31 nM [^{3}H] QNB on specific muscarinic receptor binding are set forth in **Table 4.6.**, and presented graphically in **Figure 4.6.** The results are expressed as specific [^{3}H] QNB bound (pmoles/mg protein). Each point represents the mean ± SEM (n=3). Significance is computed using a One-Way Analysis of Variance followed by Student-Newman-Keuls Multiple Range test.

QA appears to significantly increase the specific binding of the muscarinic receptors to [^{3}H] QNB from 10 nM to 1 mM. There is no significant change in the binding at 1 nM (See **Table 4.6.** and **Figure 4.6.**). The change in binding from 1 nM to 10 nM of QA is statistically significant. From a QA concentration of 10 nM to 1 mM the increased ability of the receptors to bind to [^{3}H] QNB appear to be fairly consistent.

4.6.4. DISCUSSION

QA is the most potent endogenous excitotoxin that is capable of reproducing the neuropathologic features of several human neurologic diseases. QA may also play a role in the memory and cognitive impairment that has been linked to N-methyl-D-aspartate (NMDA) receptors dysfunction (Schwarcz *et al.*, 1992). QA is weakly active at NMDA binding sites (Fagg and Baud, 1988). QA is an NMDA receptor agonist. QA in the liver is rapidly transformed to nicotinic acid mononucleotide, but its metabolism in the nervous system is unclear (McLennan, 1984). It is, however, known that QA in the cortical region increases with age (Moroni, 1984a). KA has also been found to increase with age (Schwarcz *et al.* 1992). KA is a broad spectrum excitatory amino acid antagonist of NMDA receptors (Davies and Stanley, 1988). KA can act competitively at the glycine site associated with the NMDA receptor comlex. KA can

TABLE 4.5. The effects of varying concentrations of kynurenic acid on specific muscarinic receptor binding in the presence of 0.31 nM [^{3}H] QNB. Each value represents the mean ± SEM (n=3).

KYNURENIC ACID CONCENTRATION	Specific Binding (specific [^{3}H] QNB bound) pmoles/mg protein	SIGNIFICANCE
Control	0.15 ± 0.02	-
1 nM	0.16 ± 0.01	-
10 nM	0.15 ± 0.04	-
100 nM	0.16 ± 0.01	-
1 µM	0.16 ± 0.00	-
10 µM	0.17 ± 0.01	-
100 µM	0.16 ± 0.01	-
1 mM	0.21 ± 0.01	-

TABLE 4.6. The effects of varying concentrations of quinolinic acid on specific muscarinic receptor binding in the presence of 0.31 nM [^{3}H] QNB. Each value represents the mean ± SEM (n=3).

QUINOLINIC ACID CONCENTRATION	Specific Binding (specific [^{3}H] QNB bound) pmoles/mg protein	SIGNIFICANCE
Control	0.15 ± 0.02	-
1 nM	0.18 ± 0.01	-
10 nM	0.22 ± 0.001	$P < 0.001$
100 nM	0.21 ± 0.008	$P < 0.01$
1 µM	0.24 ± 0.005	$P < 0.001$
10 µM	0.24 ± 0.01	$P < 0.001$
100 µM	0.23 ± 0.0	$P < 0.001$
1 mM	0.23 ± 0.003	$P < 0.001$

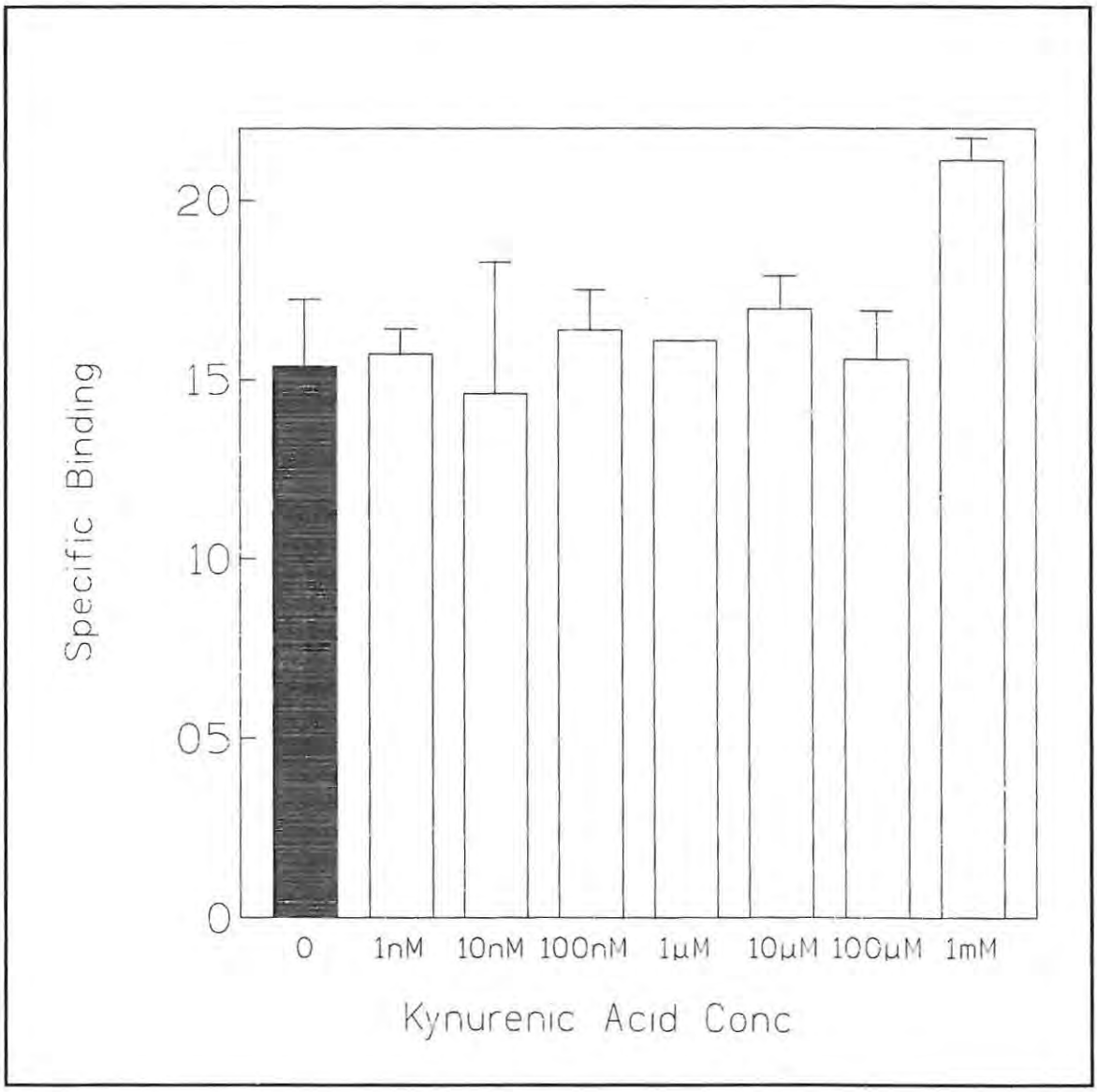

FIGURE 4.5. **The effect of different concentrations of kynurenic acid on the specific binding of rat forebrain acetylcholine muscarinic receptors. The Specific binding is expressed as specific [^{3}H] QNB bound (pmoles/mg protein). Each value represents the Mean ± SEM (n=3). Significance is computed using a One-Way Analysis of Variance followed by Student-Newman-Keuls Multiple Range test.**

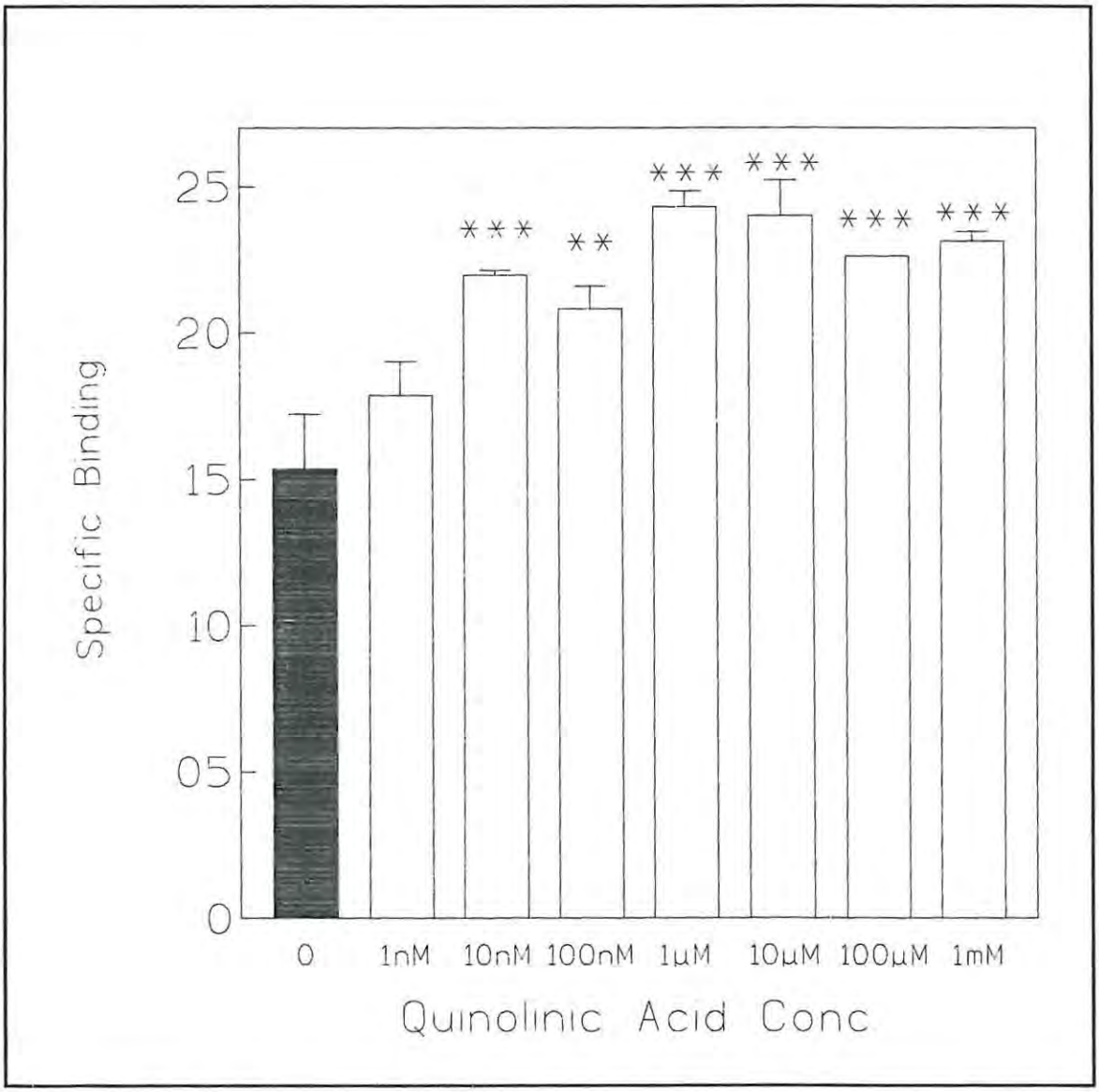

FIGURE 4.6. **The effect of different concentrations of quinolinic acid on the specific binding of rat forebrain acetylcholine muscarinic receptors. The Specific binding is expressed as specific [³H] QNB bound (pmoles/mg protein). Each value represents the Mean ± SEM (n=3). Significance is computed using a One-Way Analysis of Variance followed by Student-Newman-Keuls Multiple Range test.**

**** - P < 0.01, *** - P < 0.001.**

therefore exert control over NMDA receptor activity and thereby prevent neurodegeneration induced by EAA receptor agonists (Schwarcz *et al.*, 1992).

These are the effects that KA and QA have on NMDA receptors. In this study, the effects that KA and QA have on acetylcholine muscarinic receptors, were investigated. KA did not alter the sensitivity of the muscarinic receptors to [^{3}H] QNB. QA facilitated the binding of the receptors to the ligand significantly from a concentration of 10 nM to 1 mM. QA may possibly interfere with the protein configuration of the receptor, such that the binding affinity of the ligand is increased.

CHAPTER 5

ORGAN CULTURE STUDIES

5.1. INTRODUCTION

In the last few decades, the pineal gland has finally been recognized as being a functional organ (Reiter, 1989). Present knowledge of the pineal gland function is not absolutely satisfying in detail. It does, however, show that the gland is not just an insignificant phylogenic relic, but an active organ that synthesises a number of compounds. The secretory products that the pineal produces are released into the systemic circulation, and will exert an effect on several endocrine organs (Kappers, 1976). The pineal gland is an integral and important component of the neuroendocrine system (Reiter, 1989). In recent years, the pineal gland has been the centre of considerable attention. It has been connected with affecting, and being influenced by, a number of physiological and endocrine processes.

In order to quantify the metabolism of indoles in the pineal gland, a technique is needed which will permit sensitive monitoring of all metabolites that are undergoing biochemical or pharmacological manipulations. It must mimic, as closely as possible, normal physiological processes.

Therefore, a technique was sought in which indole metabolism could be monitored with sensitivity and reproducibility, using individual rat pineal glands. The pineal gland of certain species (for example, the rat) is small and readily accessible, making it ideal for intact organ culture. A number of methods have been developed and used with some

success. However most of the developed techniques have disadvantages in that they either do not monitor all metabolites, or are not sensitive enough to allow the use of a single gland (Morton, 1990). The technique of pineal gland organ culture is suitable by virtue of its favourable size and accessibility. It is a useful technique as it eliminates the complexities of organ interaction, and allows for direct pharmacological manipulation. The pineal gland in organ culture is able to utilise exogenous radioactive serotonin to produce various indoles including the neurohormone, melatonin (Daya *et al.,* 1989).

5.2. ORGAN CULTURE TECHNIQUE

5.2.1. Theory of Assay

The pineal gland in culture is able to maintain its metabolic function, thereby synthesizing the various indole metabolites that are associated with the pineal metabolism. This is achieved by utilizing an exogenous radioactive precursor (tryptophan (TRP), 5-hydroxytryptophan or serotonin). It has been shown that 95% of the synthesized radioactive indoles are secreted into the culture medium during the incubation period. The radioactive indoles are then isolated from the culture medium and quantified.

Previously, isolation of pineal indoles was achieved through preparative organic extractions, and then chromatographic separations using combinations of thin layer adsorbents, paper and different solvents. Presently, the method employed to separate radioactive indoles is a bi-dimensional thin layer chromatography system, and the radioactivity is quantitated by radiospectrometry (Klein and Notides, 1969). Two organic solvents are used in this technique. The first solvent separates melatonin (aMT) from N-acetylserotonin (aHT), and the 5-hydroxyindoles from the 5-methoxyindoles. The second solvent improves the separation of 5-methoxyindole acetic acid (MA) and 5-methoxytryptophol (ML) from aMT, and the separation of 5-hydroxyindole acetic acid (HA)

and 5-hydroxytryptophol (HL) from aHT. TRP, serotonin (HT), 5-hydroxytryptophan and 5-methoxytryptamine (MT) are not affected by either of the solvents, and therefore remain at the origin. This thin layer chromatographic technique is quick and simple, and effectively separates trace quantities of the indoles.

5.2.2. Materials and Methods

5.2.2.1. Chemicals, Drugs and Reagents

5-Hydroxy (side-chain-2-^{14}C) tryptamine creatine sulphate (specific activity 55 mCi/mmol) was obtained from Amersham International Pla, England. The aluminium TLC plates coated with silica gel 60, Type F_{254} (0.25 mm) were purchased from Merck, Germany. BGJ_b culture medium (Fitton-Jackson modification) was obtained from Gibco, Europe. The composition of the medium is presented in **Table 5.1.** Beckman Ready-Sol multipurpose premixed liquid scintillation solution was obtained from Beckman RIIC Ltd, Scotland. The antibiotics benzyl penicillin and streptomycin were purchased from Hoechst, South Africa. The standard unlabelled indoleamines, 5-methoxytrytamine (MT), 5-hydroxindole acetic acid (HA), 5-hydroxytryptophol (HL), 5-methoxyindole acetic acid (MA) and 5-methoxytryptophol (ML), melatonin (aMT) and N-acetyl serotonin (aHT) were obtained from Sigma Chemical Co, USA. All other chemicals and reagents were obtained from local commercial sources and were of the highest purity available.

5.2.2.2. Animals

Adult male Wistar rats, weighing between 200 - 250 grams, were used. The animals were maintained as detailed in section [**2.5.1.2**]. The animals were sacrificed by cervical dislocation and decapitation. Using a pair of scissors, an incision was made through the bone from the foramen magnum to the area near the orbit of the eye. A clean sterile

dissecting forceps was inserted into the incision, and the skull and adhering brain meninges was lifted and folded back. The brain was gently peeled away from the dura and the skull, exposing the pineal gland. The pineal gland was removed and the stalk, and any tissue adhering to the gland was removed.

5.2.2.3. Organ Culture of the Pineal Gland

The pineal glands were placed individually into sterile (borosilicate 75 X 10 mm) culture tubes containing 52 µl BGJb culture medium. The components of the culture medium and their respective concentrations are listed in **Table 5.1**. To each of the tubes, 8 µl of (^{14}C) serotonin (specific activity 55 mCi/mmol) was added. The tubes were then saturated with carbogen (95% O_2 / 5% CO_2) and sealed. These tubes were then incubated at 37 °C in the dark for 24 hours in a Forma Scientific model 3028 incubator. After the 24 hour incubation period, the reaction was stopped by the removal of the pineal glands from the culture medium.

5.2.2.4. Separation of the Indoles by Thin Layer Chromatography

To separate the radiolabelled indoles, the technique used a modification of the method of Klein and Notides (1969). To measure the amount of (^{14}C) indoles present, 10 µl of the culture medium was spotted on a 10 cm X 10 cm TLC plate (Silica gel 60, Type F_{254} (0.25 mm), aluminium plates). 10 µl of the standard solution containing unlabelled pineal indoles was spotted onto the spotted culture medium. The standard solution of the indoles was prepared as follows: 1 mg of each standard indoleamine was dissolved together in a test tube containing 2.5 ml of 95 % ethanol. 2.5 ml of 1% ascorbic acid in 0.1 N HCl is added and serves as an antioxidant. The solution was stored in darkness at -20 °C. The plates were spotted in subdued light to prevent photo-oxidation of the indole metabolites. A gentle stream of nitrogen was used to dry the spots, which were no larger than 0.5 cm.

TABLE 5.1. Constituents of BGJb Culture Medium (Fitton Jackson Modification).

CONTENTS	*CONCENTRATIONS (mg/l)*
AMINO ACIDS	
L-alanine	250.00
L-Arginine	175.00
L-Aspartic acid	150.00
L-Cysteine HCl	90.00
L-Glutamine	200.00
Glycine	800.00
L-Histidine	150.00
L-Isoleucine	30.00
L-Leucine	50.00
L-Lysine HCl	240.00
L-Methionine	50.00
L-Phenylalanine	50.00
L-Proline	400.00
L-Serine	200.00
L-Threonine	75.00
L-Tryptophan	40.00
L-Tyrosine	40.00
DL-Valine	65.00
INORGANIC SALTS	
Dihydrogen sodium orthophosphate	90.00
Magnesium sulphate $7H_2O$	200.00
Potassium chloride	400.00
Potassium dihydrogen phosphate	160.00
Sodium bicarbonate	3 500.00
Sodium chloride	5 300.00

CONTENTS	CONCENTRATIONS (mg/l)
VITAMINS	
α-Tocopherol phosphate	1.00
Ascorbic acid	50.00
Biotin	0.20
Calcium pantothenate	0.20
Choline chloride	50.00
Folic acid	0.20
Inositol	0.20
Nicotinamide	20.00
Para aminobenzoic acid	2.00
Pyridoxal phosphate	0.20
Riboflavin	0.20
Thiamine HCl	4.00
Vitamin B_{12}	0.04
OTHER COMPONENTS	
Calcium lactate	555.00
Glucose	10 000.00
Phenol red	20.00
Sodium acetate	50.00

The spotted plates were then placed in a TLC tank containing chloroform : methanol : glacial acetic acid (93:7:1), and developed twice in the same direction. The total front movement allowed during each development was 9 cm (the position of the solvent front was marked), and after each development the plates were dried under a gentle stream of nitrogen. Following this, the plate was developed once in ethyl acetate at right angles to the first direction of development, with a front movement of 7 cm. The plate was then dried under nitrogen and sprayed with Van Urks reagent (1 g of 4-dimethylamino benzaldehyde dissolved in 50 ml of 25 % HCl, followed by the addition of 50 ml of 95 % ethanol), and dried in an oven at 60 °C for 10-20 min to allow for the colour development of the spots.

The spots were then cut and placed into plastic scintillation vials. Absolute ethanol (1 ml) was added to the vials, together with 3 ml scintillation cocktail fluid (Beckman Read-Solv HP-B), and shaken on a Vortex Rotor-mixer for 30 seconds. The radioactivity was then measured in a Beckman LS 2800 scintillation counter.

5.3. RESULTS

A typical bi-dimensional thin layer chromatogram, showing the separation of the indole metabolites, can be seen in **Figure 5.1.** Very good separations of the indoles were achieved. A list of the different indoles are also present in **Figure 5.1.**

5.4. DISCUSSION

In this present group of studies, female rats were not used, because of the variation in the (^{14}C) serotonin metabolism noted in female rats in different stages of the estrus cycle. Hydroxyindole O-methyltransferase sensitivity to oestradiol has been reported to vary during the estrus cycle of female rats (Daya and Potgieter, 1982).

The separation of N-acetylserotonin, melatonin, 5-hyroxyindole acetic acid, 5-hyroxytryptophol, 5-methoxyindole acetic acid and 5-methoxytryptophol were achieved by TLC. In this method, acetic acid was included in solvent A, since it was shown to be essential for good separation of the 5-methoxyindole acetic acid and 5-methoxytryptophol indoles.

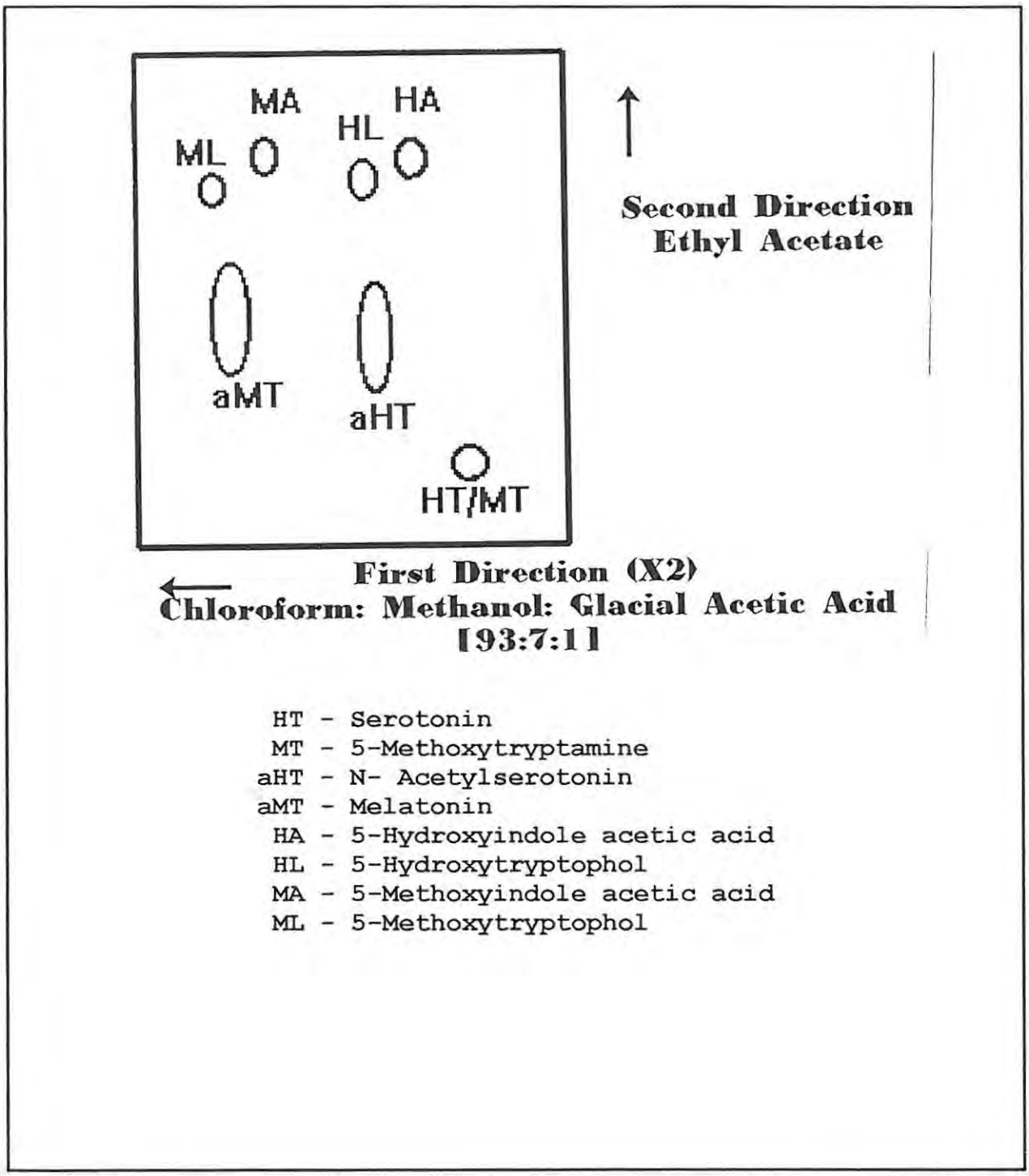

FIGURE 5.1. A trace of TLC plate showing the direction in which the solvents were run and the subsequent location of the pineal indole metabolites.

5.5. EXPERIMENT: DETERMINATION OF THE EFFECTS OF EXCITATORY AMINO ACIDS (KYNURENIC ACID AND QUINOLINIC ACID) ON INDOLE METABOLISM OF THE RAT PINEAL GLAND

5.5.1. INTRODUCTION

The biosynthesis of the indole derivative melatonin in the pineal gland, is initiated by the uptake of the tryptophan from the blood stream. Once in the pineal gland, some of the tryptophan is utilized for protein synthesis, while a large fraction is metabolised via the indole pathway. However, the major route for tryptophan metabolism in the pineal gland, appears to be the oxidative cleavage of the indole pyrrole ring to kynurenine by the enzyme indoleamine 2,3 dioxygenase (tryptophan pyrrolase) (Cardinali, 1981).

A possible role for the pineal gland and its principal hormone melatonin has been proposed in the process of ageing and age-related diseases. These theories arise from the fact that melatonin is important in a number of biological functions and the fact that melatonin production is gradually lost throughout life, so that in the very old of any species the circadian rhythm is barely noticeable (Reiter, 1995b).

Apart from melatonin, KA and QA are also tryptophan metabolites. These amino acids are synthesised via the kynurenine pathway. In the pinealocytes, the major route via which tryptophan is metabolised appears to be by the kynurenine pathway (**Figure 1.12.**). The amino acid is converted to L-kynurenine by oxidative cleavage of the pyrrole ring by the enzyme indoleamine 2,3-dioxygenase (Cardinali, 1981). L-Kynurenine accounts for 32% of all tryptophan metabolites in the pineal gland (Stone and Connick, 1985). Kynurenine can then be converted to KA and also to QA. Alternatively, tryptophan is also metabolized via the indole pathway to melatonin (Cardinali, 1981).

Kynurenine, the precursor to KA and QA, is known to reduce serotonin levels and its synthesis. This may occur by diverting the TRP into the kynurenine pathway (Gould and Handley, (1978) cite Green and Curzon, 1970). However, Gould and Handley (1978) report that administration of kynurenine does not decrease serotonin levels and hydroxyindole acetic acid. The administered kynurenine acid was distributed into the brain, and was signified by an increase in brain levels of the TRP metabolite.

The aim of these experiments was to establish the effect of kynurenic acid (KA) and quinolinic acid (QA) on rat pineal gland biosynthesis of indoles during the light phase.

5.5.2. METHODS AND MATERIALS

5.5.2.1. Chemicals, Drugs and Reagents

KA and QA were purchased from Sigma Chemical Co., USA. All the other chemicals and reagents were obtained as listed previously in section [**5.2.2.1.**].

5.5.2.2. Animals

The rats used were adult males of the Wistar strain. These were housed in groups of 5 per cage as described in [**2.5.1.2**]. The rats were killed by cervical dislocation and decapitation, and their pineal glands were removed as previously described in section [**5.2.2.2.**].

5.5.2.3. Method

The pineal gland organ culture and the separation of the labelled indoles were performed

as outlined in sections [**5.2.2.3.**] and [**5.2.2.4.**]. Five pineal glands were cultured individually in the absence of any excitatory amino acid, and served as the control (group 1). In group 2, five pineal glands were cultured individually in BGJb culture medium containing 0.4 µCi of (^{14}C) Serotonin in the presence of KA (10^{-3} M), and group 3 also had five glands which were cultured individually in the presence of QA (10^{-3} M). The glands were incubated at 37 °C for 24 hours. The (^{14}C)indoles in the culture medium were then maintained as described in section [**5.2.2.3.**].

5.5.2.4. Data Analysis Section

Data is expressed as DPM values/10 µl medium/pineal calculated as the mean ± SEM (n= 5). Comparisons between the results was performed using a two-tailed unpaired Student t-test.

5.5.3. RESULTS

Good separation of the indoles was achieved, enabling the precise isolation of each metabolite. The results illustrating the effects of KA (1 mM) on indole metabolism are presented in **Table 5.2.** The results are expressed as DPM/10 µl medium/pineal and represent the mean ± SEM (n = 5). Each experiment was repeated 5 times. **Figure 5.2.** is a graphical representation of these results. The results show that there is a significant decrease in the levels of aHT ($P < 0.001$) and aMT ($P < 0.003$) in the presence of 1 mM KA, as compared to the control values. HA increased in the presence of KA but this was not significant. MA did not appear to change. HL showed an insignificant decrease in its level, while the level of ML showed no change.

Table 5.3. represents the results of the effects of 1 mM QA on indole metabolism. The

results are expressed as DPM/10 µl medium/pineal and represent the mean ± SEM (n = 5). Each experiment was repeated 5 times. These results are presented graphically in **Figure 5.3.** In the presence of 1 mM QA, there is a significant decrease in the levels of the indoles aHT ($P < 0.001$) and aMT ($P < 0.015$). ML ($P < 0.0005$) showed a significant increase in its level. No significant changes were observed with regard to the other indoles. However, there were increases in the levels of HA and HL.

5.5.5. DISCUSSION

Ageing has been defined as a general decline in organic functions, as well as a decrease in adaptiveness to change and to restore disrupted homeostasis. The pineal gland which is reported to be a circadian ageing clock, undergoes several functional and morphological alterations. In ageing rodents, a decrease of the secretory processes and reduction in the synthesis of nocturnal melatonin has been noted (Humbert and Pevét, 1994). Humbert and Pevét also cite a report by Skene (1990) which states that a disappearance of the day-night rhythm of melatonin in old and Alzheimer's patients occurs. In old rats, a decrease in pineal activity, a reduction of adrenergic innervation and a reduction of the number of pinealocytes has been shown, but the cause of this melatonin decrease is not clear (Humbert and Pevét, 1994).

Both KA and QA appear to cause a decrease in melatonin production in the pineal gland (See **Tables 5.2** and **5.3.**, and **Figures 5.2.** and **5.3.**) Serotonin is converted to melatonin by two enzymatic steps. The first step yields aHT and the enzyme involved is serotonin *N*-acetyltransferase (SNAT). A methyl group is then transferred from S-adenosylmethionine to the 5-hydroxy group of aHT to produce melatonin. This reaction is catalysed by the enzyme hydroxindole-*O*-methyl transferase (HIOMT). The tryptophan metabolites KA and QA possibly interfere with the conversion of serotonin to aHT, thereby resulting in the decrease in aHT and aMT levels.

TABLE 5.2. The effects of kynurenic acid on (^{14}C) serotonin metabolism in the rat pineal gland.

METABOLITES	CONTROL	KYNURENIC ACID (1 mM)	SIGNIFICANCE
HT/MT	14798.77 ± 6578.91	21464.85 ± 4888.38	NS
aHT	1352.13 ± 97.85	747.02 ± 60.11	P < 0.001
aMT	599.36 ± 66.33	137.55 ± 22.58	P < 0.003
HA	9796.64 ± 2915.73	12695.48 ± 1947.48	NS
HL	8212.03 ± 852.87	5957.88 ± 1307.64	NS
MA	275.62 ± 35.15	272.34 ± 49.14	NS
ML	557.48 ± 52.34	566.18 ± 62.75	NS

10 µl aliquots of the medium were chromatographed by TLC. The results were expressed as DPM/ 10 µl medium/pineal (mean +/- SEM; n=5 individually cultured pineal glands per group.

NS - Not significant, Significance is computed using a two- tailed unpaired Student t-test.)

TABLE 5.3. The effects of quinolinic acid on (^{14}C) serotonin metabolism in the rat pineal gland.

METABOLITES	CONTROL	QUINOLINIC ACID (1 mM)	SIGNIFICANCE
HT/MT	14798.77 ± 6578.91	25351.89 ± 1530.71	NS
aHT	1352.13 ± 97.85	702.78 ± 33.54	P < 0.001
aMT	599.36 ± 66.33	315.77 ± 46.15	P < 0.015
HA	9796.64 ± 2915.73	12161.62 ± 1184.16	NS
HL	8212.03 ± 852.87	10897.72 ± 981.94	NS
MA	275.62 ± 35.15	285.38 ± 41.5	NS
ML	557.48 ± 52.34	952.39 ± 46.09	P < 0.0005

10 µl aliquots of the medium were chromatographed by TLC. The results were expressed as DPM/ 10 µl medium/pineal (mean ± SEM; n=5 individually cultured pineal glands per group.

NS - Not significant, Significance is computed using a two-tailed unpaired Student t-test.)

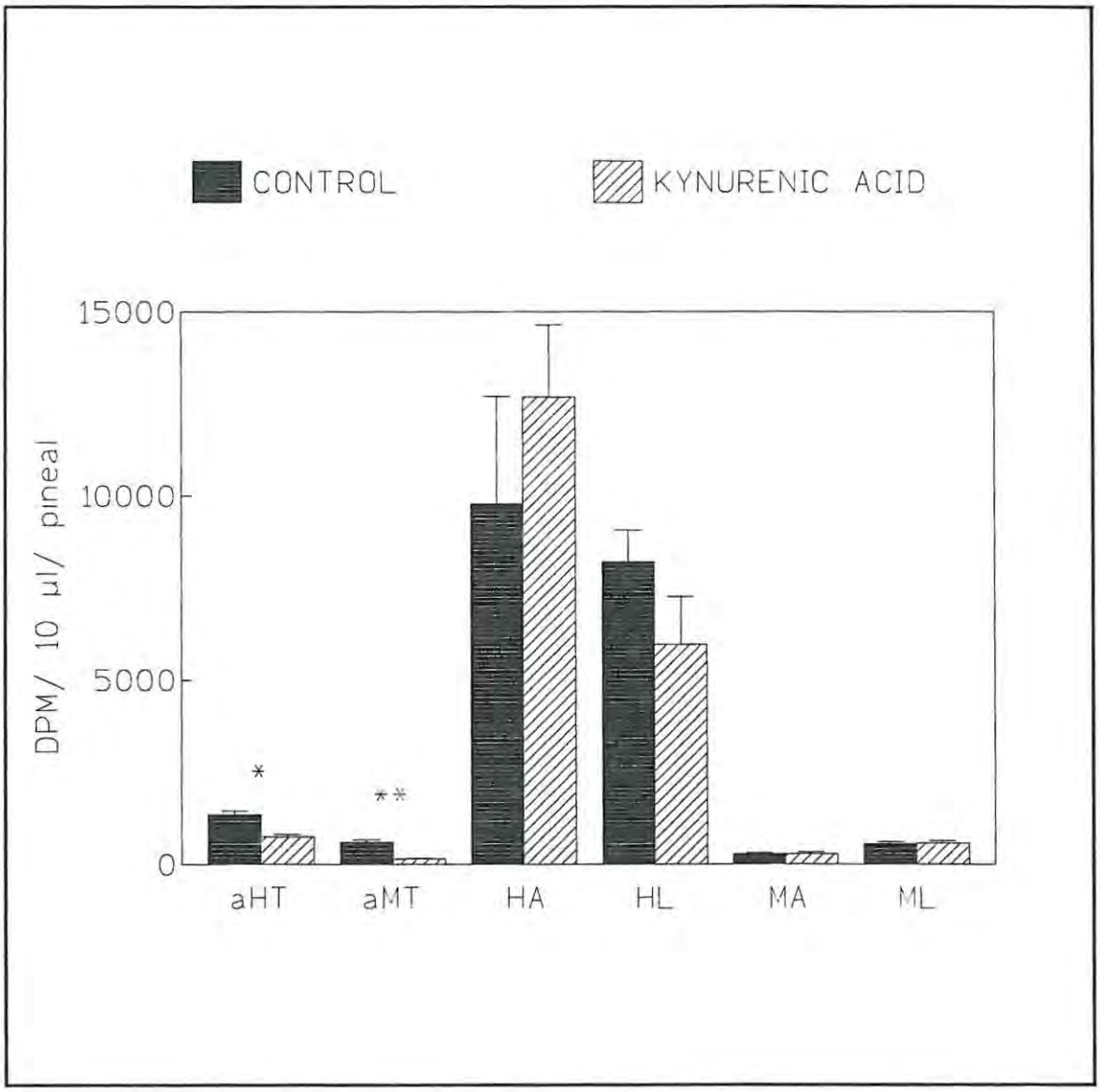

FIGURE 5.2. **The effects of kynurenic acid on [^{14}C] serotonin metabolism by the rat pineal glands in organ culture. Values represent the mean ± SEM (n = 5).**

*** P < 0.001, ** P < 0.003**

Significance is computed using a two-tailed unpaired Student t-test.

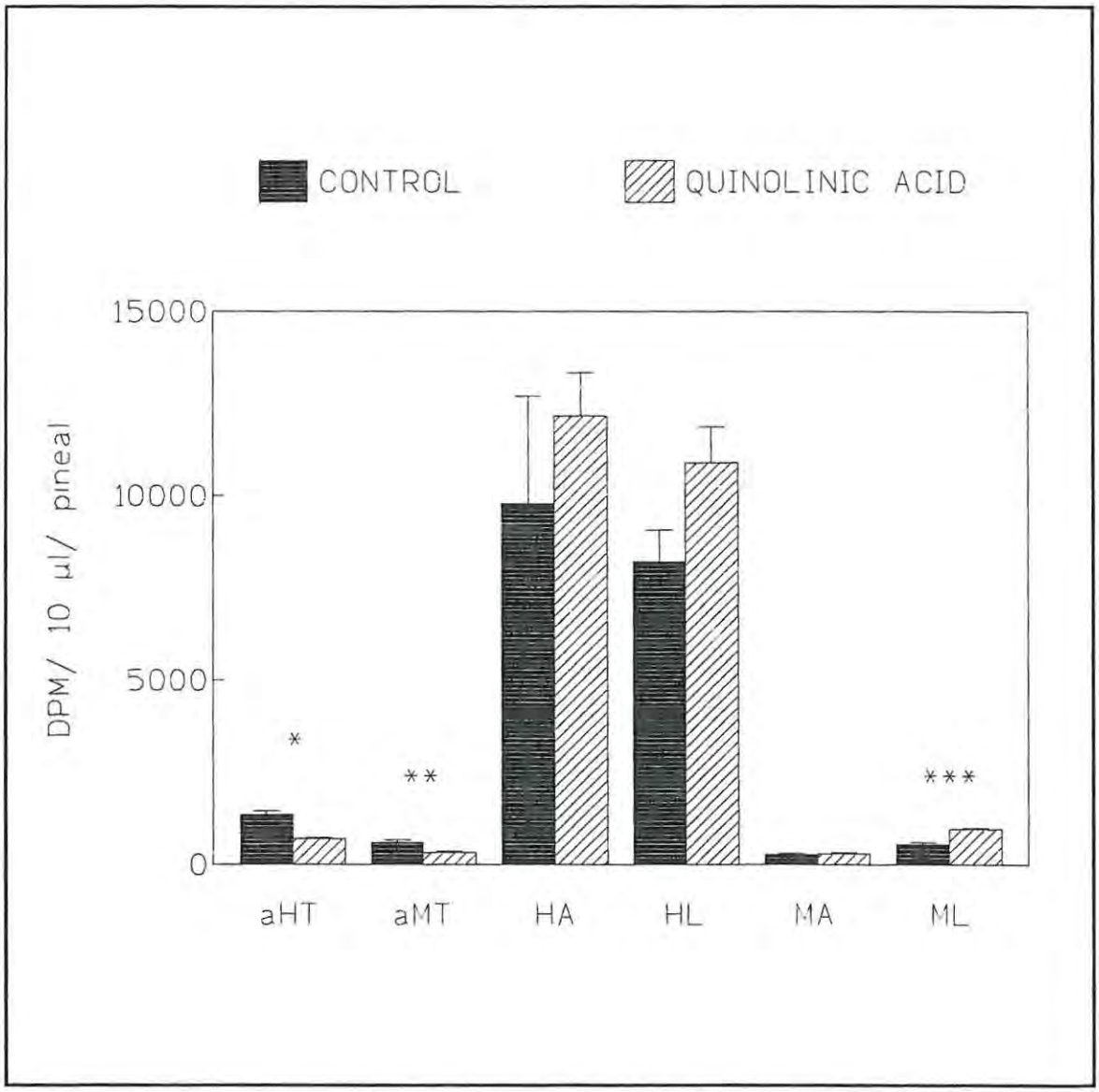

FIGURE 5.3. **The effects of quinolinic acid on [^{14}C] Serotonin metabolism by the rat pineal glands in organ culture. Values represent the mean ± SEM (n = 5).**

*** P < 0.001, ** P < 0.015, *** P < 0.0005**

Significance is computed using a two-tailed unpaired student t-test.

Serotonin is also converted to 5-hydroxyindole acetaldehyde by monoamine oxidase (MAO). This is an unstable intermediate and is oxidized to 5-hydroxyindole acetic acid (HA) which is then converted to 5-methoxyindole acetic acid (MA). 5-Hydroxyindole acetaldehyde is also reduced to 5-hydroxytryptophol (HL), which is then converted to 5-methoxytryptophol (ML). QA (**Table 5.3.** and **Figure 5.3.**) caused a significant increase in ML. An increase in HL was also noted, but it was not significant.

Melatonin has recently been considered to have a neuroprotective function against neurological insults (Daya, 1994). Melatonin also appears to have a role as an anticancer hormone, as it is thought to have an effect on major intracellular, biochemical pathways that stimulate cell growth (Blask *et al.*, 1989). Experiments by Reiter (1995a) have shown that melatonin is a free radical scavenger in the nuclei of cells, thereby protecting DNA from damage. It is also possible that the indole may stimulate DNA repair mechanisms. The decline in melatonin production that is associated with age may be related to the onset of age-related diseases (Reiter, 1995a). There also appears to be a link between the production of melatonin and the immune system. A decrease in indole production may result in a variety of diseases (Maestroni, 1993).

KA and QA have both been shown to increase during the ageing process. Rat brain KA increases steadily with age, not during ontogeny, but continuously up to old age. Therefore the brain cortex of a two-year old rat contains approximately ten times the amount of KA found in the brain of a two-month old rat (Schwarcz *et al.*, 1992). Experimental evidence from rats indicates that a large variability exists in the QA increase. The possibility that QA may be a contributing factor in pathological age related diseases has been inferred (Moroni *et al.* 1984a).

The results in this study (**Tables 5.2.** and **5.3.**, and **Figures 5.2.** and **5.3.**) show that KA and QA reduce melatonin production. This means that if KA and QA have this effect *in vivo*, then all the protection that melatonin offers that has been described above falls away, leaving the individual exposed to a vast variety of diseases.

CHAPTER 6

SUMMARY OF RESULTS

CHAPTER 1

All behaviour is thought to be a reflection of brain function. The action of the brain underlies simple behaviour such as walking, and also more elaborate functions like feeling, learning and thought. This means that any dysfunction in brain function will result in neurotic and psychotic illness (Kandel and Schwartz, 1986).

The nervous system is a vast electrochemical conducting network that extends from the brain through the rest of the body. The pineal gland, which is located between the cerebral cortex and the cerebellum, has been of great interest in the past few decades. The gland produces melatonin which is regulated by a circadian rhythm. The hormone is known to be very important to the endocrine system (Wilson *et al.*, 1989).

CHAPTER 2

The choline acetyltransferase (ChAT) assay developed by Fonnum (1969, 1975) was employed to determine ChAT activity in the rat forebrain. The assay is sensitive enough to determine the minute quantities of Acetylcholine (ACh) produced, and is also able to isolate ACh from acetyl carnitine (ACar), which is also produced in cell homogenates. ACh must be isolated from ACar and extracted so that an artificially high activity is not acquired for ChAT activity (Hamprecht and Amano, 1974).

Aluminium chloride, kynurenic acid (KA) and quinolinic acid (QA), in this *in vitro* study, did not have any affect on ChAT activity. *In vivo* experiments cited by Cherroret *et al.* (1994) report that aluminium affects ChAT activity. Cherroret *et al.* (1994) found that

there is no inhibition of ChAT in adult rats by aluminium chloride at any concentration. These authors found that aluminium chloride (1 mM) inhibits the enzyme in 15 day old rats.

In this study, mercuric chloride is shown to decrease ChAT activity *in vitro* from a concentration of 10 µM to 1 mM (See **Table 2.5.** and **Figure 2.9.**). At lower concentrations, mercuric chloride does not decrease the enzymes activity.

CHAPTER 3

The acetyl cholinesterase (AChE) assay employed in this study of the rat forebrain was reported by Ellman *et al.* (1961). It is a simple and efficient assay. The assay is applicable to small amounts of tissue and low concentrations of enzyme.

The present study found that aluminium chloride, KA and QA did not affect the activity of AChE. Marquis and Lerrick (1982) have shown that AChE is inhibited in the presence of aluminium chlorohydrate (0.1 mM - 0.5 mM). In 1984, Marquis and Black reported an increase in AChE activity from bovine caudate in the presence of aluminium (1 - 10 µM). Similar increases in rat brain AChE activity were shown by Bilkei-Gorzo (1993). There are a number of reports on the effect of aluminium on AChE, but these are conflicting. The differences may be a result of the conditions under which the experiments were carried out, or the different species of animals.

Mercuric chloride, in this study, was shown to inhibit AChE activity at high concentrations (1 µM - 1 mM). The inhibition in this range appears to be dose-dependent (See **Table 3.3.** and **Figure 3.8.**).

CHAPTER 4

Studies examining muscarinic receptors in the rat forebrain were carried out with the

use of radioligand binding assay that utilise an antagonist [^{3}H] quinuclidinyl benzilate (QNB) which binds to muscarinic receptors. Aluminium chloride (1 nM - 100 µM) had no affect on muscarinic receptor binding. At a concentration of 1 mM, there was a statistically significant increase in the receptors binding affinity for [^{3}H] QNB ($P < 0.01$) (See **Table 4.3.** and **Figure 4.3.)**. Mercuric chloride appeared to interfere significantly with the binding of [^{3}H] QNB to the receptor at a concentration of 1 mM ($P < 0.01$). Mercury is thought to interact with sulfhydryl groups in muscarinic receptors, thereby blocking binding of the receptor and ligand (**Table 4.4**. and **Figure 4.4.**).

KA had no statistically significant effect on muscarinic receptor binding. At a KA concentration of 1 mM, receptor affinity for the ligand increases, but not significantly. However, a significant increase in binding affinity of muscarinic receptor for [^{3}H] QNB was determined in the presence of QA from a concentration of 10 nM - 1 mM (**Table 4.6.** and **Figure 4.6.**).

CHAPTER 5

The organ culture studies showed that KA and QA at a concentration of 1 mM reduces N-acetylserotonin (aHT) and melatonin levels in the pineal gland (See **Tables 5.2. and 5.3.** and **Figures 5.2 and 5.3.**). KA and QA may possibly have an inhibitory effect on the enzyme serotonin *N*-acetyltransferase responsible for converting serotonin to aHT.

CHAPTER 7

CONCLUSIONS AND FUTURE STUDIES

This study examined the effects of varying concentrations of aluminium chloride, mercuric chloride, kynurenic acid (KA) and quinolinic acid (QA) on the cholinergic enzymes, choline acetyltransferase (ChAT) and acetyl cholinesterase (AChE), and on acetylcholine muscarinic receptors (m-AChR). This study also examined the effect of KA and QA, which are tryptophan metabolites, on melatonin synthesis in the pineal gland. All the investigations undertaken in the present exploration of the cholinergic system and the pineal gland were *in vitro* studies.

Aluminium chloride, KA and QA had no significant effect on the cholinergic system enzymes and m-AChR in an *in vitro* environment. At high concentrations, mercuric chloride appears to have significant inhibitory effects on ChAT and AChE enzymes.

Future investigations are required into the effects of the above mentioned substances on the cholinergic system to give a clearer understanding of the interactions of the cholinergic system with inorganic toxins and tryptophan metabolites.
Possible areas of future study would be to :

(1) Determine of the effects of aluminium, mercury, KA and QA on ChAT and AChE activities in an *in vivo* situation.
(2) Establish of the effects of long term exposure to the inorganic toxins and tryptophan metabolites examined in this study.
(3) Determination of the type of inhibition effected by mercuric chloride on ChAT and

AChE.

(4) Establish the levels of mercuric chloride, KA and QA present in the brains of patients that have died as a result of Alzheimer's Disease and Huntington's Disease.

KA and QA concentrations are known to increase in the brain with age (Moroni *et al.*, 1984a). Another possible area of study would be to determine the role, if any, of these tryptophan metabolites in binding to synaptosomal membranes. The amino acids are present in the brain and the means for its synthesis are present. Therefore KA and QA may be considered as possible neurotransmitters. A stumbling block to this is the fact that there does not appear to be any system for its removal. Further investigation into this is necessary to satisfactorily answer the question.

The pineal gland studies carried out in this investigation show that KA and QA do affect the synthesis of melatonin production. Further studies are necessary. Enzyme assay studies need to be conducted to determine the effect of KA and QA on serotonin N-acetyltransferase, which is the enzyme responsible for the conversion of serotonin to N-acetylserotonin, and on the enzyme, hydroxyindole-O-methyl-transferase, which converts N-acetylserotonin to melatonin.

The kynurenine pathway of tryptophan metabolism that results in the production of KA and QA is present in the pineal gland (Cardinali, 1981). It is also known that melatonin production in the pineal gland is under the control of the circadian rhythm. Keeping all this in mind, the possibility that KA and QA production in the pineal gland may also be controlled by this mechanism needs to be explored.

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