

UNIVERSITY OF SOUTHAMPTON

THE LABILITY OF BRAIN CHOLINESTERASES,
PROTEINS AND PROTEASES.

by

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CONTENTS.

	Page
<u>Section 1.</u> Introduction	1
<u>Section 2.</u> Changes in the activity of acetylcholinesterase and total proteins following behavioural treatments.	55
2.1. Introduction.	14
2.1.2. Nature and properties of AChE	15
2.1.3. Physical properties of purified cholinesterases	15
2.1.4. Mechanism and kinetics of AChE.	17
2.1.5. Evidence for allosterism in AChE	18
2.1.6. Localisation of AChE within the nervous system.	19
2.1.7. Synthesis and transport of AChE	24
2.1.8. Behavioural aspects of cholinergic transmission.	26
2.2. Methods	34
2.2.1. Quiet control procedure	34
2.2.2. Anaesthetisation procedure	34
2.2.3. Shock avoidance conditioning apparatus	35
2.2.4. Shock avoidance conditioning procedure	37
2.2.5. Preparation of samples	41
2.2.6. Acetylcholinesterase assay	43
2.2.7. Total protein assay.	50
2.3. Summary of results	83
2.4. Discussion	88

Section 2.3. Results. the activity of pseudoChE

2.3.1. The quiet control distribution of AChE	55
2.3.2. The effects of 45 minutes sodium-	
3.1. pentobarbitone anaesthesia on the	90
3.2. activity of AChE.	55
2.3.3. The effects of sodium-pentobarbitone	
anaesthesia on the activity of AChE	
measured 24 hours after the period of	100
3.2.2. anaesthesia of a specific assay for	60
2.3.4. The effects of pentobarbitone -sodium	106
3.2.3. anaesthesia on the activity of AChE	
in vitro.	63
2.3.5. Development of a specific assay for AChE	64
2.3.6. Modification of the technique for	
measuring the activity of AChE and	
total protein content and its use to	109
3.2.3. determine the effects of anaesthesia on	
these parameters. in regions of the rat	65
2.3.7. The effects of 45 min pentobarbitone-	112
3.2.6. sodium anaesthesia on the water content	114
3.3. of regions of the brain	74
<u>Section 2.3.8. The effects of shock avoidance conditioning:</u>	
conditioning and electrical stimulation	
4.1. on AChE activity and total protein content	117
4.2. of regions of the rat brain.	76
2.3.9 Summary of results the activity of proteinase	83
2.4. Discussion of the rat brain using casein	88

as the protein substrate	129
4.2.2. The automated analysis of trypsin-like	
and chymotrypsin-like enzymes in regions	
of the rat brain.	132

Section 3. Changes in the activity of pseudoChE
of rat brain following behavioural
treatments.

3.1. Introduction	90
3.2. Methods and Results.	
3.2.1. Distribution of cholinesterase activity in regions of the rat brain with different substrates.	100
3.2.2. Development of a specific assay for pseudoChE.	104
3.2.3. The effects of 45 minutes anaesthesia on the activity of pseudoChE	106
3.2.4. Changes in the activity of pseudoChE in regions of the rat brain following electrical stimulation and shock avoidance conditioning.	109
3.2.5. Comparison of changes in the activity of AChE and pseudoChE in regions of the rat brain following behavioural treatments	112
3.2.6. Summary	114
3.3. Discussion	116

Section 4. Determination of the activity of proteinases
following behavioural treatments

4.1. Introduction	117
4.2. Methods	
4.2.1. Determination of the activity of proteinases in regions of the rat brain using casein as the protein substrate	129
4.2.2. The automated analysis of trypsin-like and chymotrypsin-like enzymes in regions of the rat brain.	132

4.3.Results

- 4.3.1. Determination of proteinase activity with casein as the protein substrate. pH-activity curve for casein and of endogenous tissue proteins 137
- 4.3.2. The effect of anaesthesia on acidic and neutral proteinase activity in regions of the rat brain. 137
- 4.3.3. The effects of electrical stimulation and shock avoidance conditioning on the activity of acidic and neutral proteases 143
- 4.3.4. The pH dependence of the rate of hydrolysis of the tryptic (BAPA) and chymotryptic (GPNA) substrates. 149
- 4.3.5. The effects of 45 minutes anaesthesia on proteinase activity using the tryptic substrate (BAPA). 149
- 4.3.6. The effects of 45 min anaesthesia on proteinase activity using the chymotryptic substrate (GPNA). 154
- 4.3.7. The effects of electrical stimulation and shock avoidance conditioning on proteinase activity with a tryptic substrate. 157
- 4.3.8. The effects of electrical stimulation and shock avoidance conditioning on the rate of hydrolysis of the chymotryptic substrate (GPNA). 161
- 4.3.9. A comparison of the changes in activity of proteinases following behavioural treatments with changes in protein content 163

4.3.10. Summary	164
4.4. Discussion	166
<u>Section 5. Fractionation of brain proteins.</u>	
5.1. Introduction	168
5.2. Methods and results	
5.2.1. Polyacrylamide gel electrophoresis of soluble brain proteins.	169
5.2.2. Fractionation of insoluble brain proteins	177
5.2.3. Development of a microelectrophoretic technique for fractionating brain proteins	181
5.2.4. Gradient gel electrophoresis of brain proteins.	183
5.3. Discussion	189
Section 6. General discussion	192
Appendix A. Materials	200
Appendix B. Statistical Analysis of data	201
Bibliography	202

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ABSTRACT

FACULTY OF SCIENCE

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THE LABILITY OF BRAIN CHOLINESTERASES, PROTEINS AND PROTEASES

by Christine Ann Doberska.

The activity of acetylcholinesterase was determined in thirteen regions of the young adult rat brain following various behavioural treatments: anaesthesia, electrical stimulation, and shock avoidance conditioning. Initially, activity was measured in terms of protein content and changes in activity of this enzyme were observed in several regions of the brain following these treatments. Further experiments indicated that these treatments also produced changes in protein concentration in some regions of the brain. The technique for measuring acetylcholinesterase activity and total proteins was therefore modified and results were expressed in terms of wet weight of tissue.

The activity of pseudocholinesterase, an enzyme predominantly localised in glia cells, was also found to change in some regions following these behavioural treatments. Some of the changes observed correlated with changes in acetylcholinesterase activity while others were independent of changes in acetylcholinesterase. This could indicate a selective change in glia cell metabolism following these treatments.

Proteinase activity was also determined following behavioural treatments to investigate whether changes in protein content could be explained in terms of altered protein degradation.

An attempt was made to fractionate brain proteins to see if changes in protein concentration were general or specific.

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ABBREVIATIONS.

ADP	- adenosine 5' - diphosphate
ACh	- acetylcholine
AChE	- acetylcholinesterase, acetylcholine acetyl hydrolase (E.C. 3.1.1.7.)
ACTH	- adrenocorticotrophic hormone
AcThCh	- acetylthiocholine
cAMP	- cyclic adenosine 5'- monophosphate
ATP	- adenosine 5'- triphosphate
ATPase	- adenosine triphosphatase
BAA	- benzoylarginine anilide
BANA	- benzoylarginine - naphthylamide
DL-BAPA	- N-benzoyl-DL-arginine p-nitroanilide
BuChE	- butyrylcholinesterase
BW284C51	- 1:5-bis (4-allyldimethylammoniumphenyl) - pentan-3-one dibromide
ChAc	- choline acetyltransferase
ChE	- cholinesterase
CNS	- central nervous system
CSF	- cerebrospinal fluid
DFP	- diisopropylphosphorofluoridate
DNA	- deoxyribonucleic acid
DTNB	- 5,5' dithiobis-2-nitrobenzoic acid
EDTA	- ethylenediaminetetraacetic acid
EEG	- electroencephalograph
GABA	- γ - aminobutyric acid
L-GPNA	- N- glutaryl -L-phenylalanine p-nitroanilide
GTP	- guanosine triphosphate
GTPase	- guanosine triphosphatase
i.p.	- intraperitoneal
iso-OMPA	- tetramonoisopropylpyrophosphortetramide
K_m	- Michaelis constant
O.D.	- optical density

pseudoChE	- pseudocholinesterase, acylcholine acyl hydrolase (E.C.3.1.1.8.)
RNA	- ribonucleic acid
mRNA	- messenger ribonucleic acid
tRNA	- transfer ribonucleic acid
SDS	- sodium dodecyl sulphate
S.E.M.	- standard error of mean
-SH	- sulphhydryl group
TCA	- trichloroacetic acid
TEMED	- N,N,N,'N'- tetramethylethylenediamine
$V_{\max}$	- maximum reaction velocity
v/v	- volume per volume
w/v	- weight per volume

SECTION 1. INTRODUCTION.

Biochemical correlates of learning and memory have been suggested to occur in various systems of neuronal metabolism. The complex interactive processes of the brain which regulate the adaptation of the organism to the environment include the short term cellular responses such as neural habituation and the regionally more specific changes associated with gross changes in sensory stimulation (Blakemore & Cooper 1970), and are seen in the behavioural changes associated with learning and memory storage. Biochemical studies have aimed at determining which of the biochemical mechanisms support the co-ordinated responses of the organism, interacting with a diversity of environmental changes. This task is made more difficult by the sheer complexity of the brain, its regional differentiation and by the degree of cellular interaction: each of the millions of neurones may receive terminals from thousands of others (Cragg 1967; Hydén 1967).

There is no generally accepted definition of learning (Bateson 1970). Many types of learning have been described, these include: imprinting, one-trial learning, habituation, classical conditioning and operant conditioning. In a broader definition " Any functionally induced and persisting alteration of the functional properties of single neurones can be considered as a manifestation of neural plasticity and the elementary capacity of nerve tissue to store information." (Burřs & Buresova 1970).

It is also important to distinguish between the storage of information and performance on the basis of stored information. Glassman (1969) summarised the difficulties:

" Learning is inferred by a change in performance under specific circumstances. It is never observed directly. Thus

memory is assayed by the performance of the organism and not by direct measurements. Unfortunately, performance, is affected by factors other than memory. An unmotivated or tired animal may not perform, a frightened animal may freeze and a stimulated animal may perform better than it would otherwise. Such consideration makes the amount and kind of experimental information stored in the nervous system uncertain and extremely difficult to measure."

Thus, the study of the relationship between memory and biochemical events is made especially complex because of the experimental designs which are used at the behavioural level when attempts are made to dissect the components of memory encoding (learning), storage (long and short term) and retrieval from store (performance) (Norman 1973; Spear 1973).

Memory formation is considered to be a stepwise process having a characteristic time course. Consequently, memory is classified as ' short term memory ' which relates to processes responsible for memory storage during and shortly after training and ' long term memory ', that is, the processes responsible for maintaining the acquired information for later use. The experimental basis for this separation is that certain drugs (carbon dioxide, metrazol and inhibitors of protein synthesis) and certain treatments (electrical stimulation, hypothermia, and spreading depression) can affect memory only if applied at definite time intervals after learning has taken place. Electrophysiological and biochemical analyses also indicate that separate anatomical areas of the brain and different metabolic processes are involved in the different stages of formation and consolidation of memory.

Many investigations have been conducted to establish via

biochemical and behavioural means the physiological changes which lead to the altered performance of an animal measured at various times after a training experience. Results among reported studies, however are not usually comparable. Factors, such as species or even strain of animal used, the detailed construction of experimental apparatus, the sequence of training and the condition of animal housing appear to affect measured behaviour drastically.

The enormous complexity of the problem has led to a search for adequate models for the study of memory. One approach has been to use phylogenetically older animals whose nervous system is considered to be of a simpler structure than that of mammals. Summaries of this type of work are provided by Bullock (1967) and Eisenstein (1967).

Several theories have been suggested for the structural basis of the learning process. It has been suggested that structural changes occur in the connectivity of the CNS neurones. and the simplest theory proposes the facilitation of trans-synaptic propagation of nerve impulses (Hebb 1949). This has been suggested to occur in a number of ways: 1) formation and growth of new synapses 2) increase in the diameter of existing neurones 3) increase in production of transmitter substances in the pre-synaptic cell 4) modification of pre-synaptic membrane facilitating liberation of transmitter 5) modification of post-synaptic membrane making it more sensitive to neurotransmitters.

Whichever of these may be the case, it is likely that fundamental structural components such as proteins and lipids might be involved and that alterations in the synthesis of these or of RNA may be detected during the process of change. Much attention has, therefore, been focused on these components.

Changes in the structure or conformation of macromolecules has been implicated in the acquisition and storage of memory (Katz & Halstead 1950). Hydén (1967) proposed that afferent impulses were coded according to their frequency in changes in base composition of a messenger-type RNA. A similar hypothesis was proposed by Flexner & Flexner (1967). A linkage between release of neurotransmitter and altered rates of local protein synthesis resulting in a facilitation of conductivity in specific neuronal pathways was proposed by Baxter & Tewari (1970). Glial cells as well as neurones have been suggested to play a role in the learning process (Galambos 1961).

However realistic or otherwise these ideas may be there are certainly empirical grounds for implicating RNA and proteins. One approach to this problem has involved the use of inhibitors of RNA and protein synthesis. These agents are administered in association with training to test the hypothesis that one or other molecular process is critical in memory formation.

Agranoff and co-workers, using a shock-avoidance situation in goldfish found that injection of puromycin immediately after training resulted in a significant loss of retention during retesting four days later. This effect did not occur if injection was delayed for 60 min (Agranoff & Klinger 1964; Agranoff, Davis & Brink 1965; Davis & Agranoff 1966). Learning which occurs during this training period is also apparently resistant to this drug (Agranoff & Davis 1968; Agranoff 1970). Similar results were obtained with an alternative protein synthesis inhibitor acetoxycycloheximide (Agranoff et al 1967). However, acetoxycycloheximide did not impair recent or long term memory in mice (Flexner & Flexner 1966).

However, studies of this nature are complicated by the fact that metabolic inhibitors also produce gross effects unrelated

to the learning experience. For example, puromycin which resembles tRNA structurally and attaches to the growing polypeptide chain also blocks brain respiration (Jones & Banks 1969) and can produce hippocampal seizures (Cohen & Barondes 1967).

A second approach to this problem, has been to administer radioactively labelled amino acids and to measure the degree to which radioactivity is incorporated into brain proteins following training situations. This approach was adopted by Hydén and co-workers who used microtechniques such as hand dissection of neurons and glia and combustion gas radioactivity counting. Hungry rats were forced to balance along a steel wire at an angle of 45° in order to reach food. After four days of training RNA levels and RNA base ratios were examined in Deiter's nucleus (Hydén & Egyhazi 1962). An increase in RNA was found and in addition, there were changes in base ratios. However, in a passive stimulation experiment in which rats were subjected to rotation on an inclined disc, an increase in protein content (12%) and RNA content (4%) was found but base ratios did not change (Hydén 1960).

Neurons of the cerebral cortex also showed a change in total RNA content as well as in base ratios following a change in handedness experiment (Hydén & Egyhazi 1964). Using the same technique Hydén also detected increases in the rates of incorporation of labelled precursors into RNA and proteins of the hippocampus. (Hydén & Lange 1968). These studies were extended by protein fractionation on polyacrylamide gels and showed specific incorporation into three acidic proteins (Hydén 1969; Hydén & Lange 1970). Hydén & Lange (1972) have also reported decreases in incorporation into proteins of other areas of the brain (reticular formation, limbic system and cortical areas)

during the same learning experiment.

Similar correlates of learning were reported by Glassman and co-workers using a different experimental situation. Mice were trained to jump to a shelf from a electrified grid floor, in order to avoid shock, in response to a buzzer or flashing light. Incorporation of labelled precursor into RNA of animals receiving this training was compared with yoked controls (that is, animals which receive the electric shock simultaneously with experimental animals but were not trained to avoid the shock) and with quiet controls. Incorporation into RNA of trained animals was reported to be strikingly elevated (Zemp et al 1966; Zemp et al 1967); while rates of RNA synthesis in yoked animals were similar to those obtained for quiet control mice (Glassman & Wilson 1970a, b). The increase in RNA synthesis was apparently restricted to the nervous system since no similar alterations were observed in liver and kidney (Zemp et al 1966). It was suggested that it was unlikely that hormones of the adrenal gland played an important role in the increased incorporation into brain RNA (Adair et al 1968). Coleman et al (1971) reported similar findings using hypophysectomised and ovariectomised mice.

The increase in labelling of RNA was found not to be confined to a single species of RNA but was apparently distributed among many species (Glassman & Wilson 1970b). Attempts to regionally localise these effects indicated that enhancement of incorporation did not occur in whole brain but was confined to the diencephalic structures (Zemp et al 1967).

Levitan et al (1972) also studied amino acid incorporation into perikaryal and synaptosomal subcellular fractions of cortex and hippocampus in rats trained in the use of the non-preferred paw in the retrieval of food. They reported that after four

days, there was an increase in incorporation of ^3H -leucine in all subcellular fractions of the hippocampus except perikaryal mitochondria, where there was a decrease in incorporation. No differences were seen in cortex.

An alternative experimental situation which has been exploited involves the imprinting of day old chicks to a flashing light (Bateson et al 1968). It was found that 160 min after the start of imprinting and 90min after injection of ^3H -lysine, incorporation was significantly enhanced in the forebrain roof when compared with light controls and dark controls. An increase in the incorporation of labelled precursor into RNA has also been detected (Rose 1970).

A major assumption in this type of work is that the rate of incorporation of labelled amino acid administered directly reflects the rate of protein synthesis. This is true only if labelled amino acid has equilibrated with the precursor pool. Thus, if after the administration of a labelled amino acid an increase in incorporation into brain occurs following a specific treatment, two interpretations are available. Either the increase in amino acid incorporation into protein is due to an increase in ongoing brain protein synthesis or it is due to an increased access of labelled amino acid to the protein synthesising system which could be caused by, for example a change in local blood supply (Bondy & Morelos 1971). Therefore, to investigate the possible contribution of increased access it is necessary to measure both the amount of unlabelled amino acid and the amount of labelled amino acid over a time course. The specific activity of the precursor could then be expressed as radioactivity per mole amino acid in brain. This correction thus improves the estimate of the influence of the treatment on protein synthesis per se. However, measurements of this sort, if made are generally made

at the end of the incorporation period, whereas precursor pool fluctuations may occur throughout this period. Further ambiguities also arise if any part of the experimental treatment produces changes in precursors other than the labelled one. (Rose 1972).

While the incorporation of a labelled precursor is used as a measure of net synthesis, it is in fact, dependent on protein turnover. Degradation, as well as synthesis of macromolecules may be changed during treatment. Other complications may arise in tissue handling. An apparent depression of incorporation in a bulk protein extract may conceal an enhanced incorporation in a particular protein. Also, within a tissue sample there may be subregional variations in incorporation (Hydén & Lange 1972).

An alternative approach to this problem was adopted by Wenzel et al (1975). They investigated the ribosomes of CA1 and CA3 cells of the hippocampus by morphometric methods after acquisition of a shock-motivated brightness discrimination in rats. They reported an increase in the total number of ribosomes in CA3 cells of both active controls and trained animals and an increase in total number of ribosomes in the CA1 region of trained animals. A significant increase in membrane bound ribosomes was observed in CA3 and CA1 cells after training only. The results thus confirmed their earlier suggestion of an increased protein synthesis in hippocampal neurones during and after the acquisition of brightness discrimination, concluded from previous investigations on the incorporation of labelled amino acids under identical conditions. The authors also concluded that the protein synthesis in some neuronal cells may probably not only vary quantitatively but also qualitatively in trained and untrained animals.

Several investigators have looked at the synthesis of specific proteins during the learning process. Hydén & Lange (1970a) using a microelectrophoretic technique observed an increased

synthesis of two acidic protein fractions in the hippocampus of rats trained in the use of the non-preferred paw. The brain specific protein S-100 was observed to increase markedly during the reversal of handedness in hippocampal nerve cells. When the synthesis of S-100 in the hippocampal region was blocked with an anti-S100 protein antibody, further increases in the learned behaviour were prevented. Antibodies against S-100 protein were localised in the hippocampal structures by immunofluoresence (Hydén & Lange 1970 b).

Brain glycoproteins were suggested by Bogoch (1968 ; 1970) to play a role in learning. Two brain specific glycoproteins 10B (predominantly localised in glia) and 11A (predominantly localised in synaptosomes) have been extracted and purified (Bogoch 1970).

Transfer experiments have also been conducted. The essence of these experiments dealing with the possibility of a transfer of learned information is that the administration of extracts from trained animals (donor) can modify the behaviour of naive animals (recipients). Reinis (1965) reported an increase in the percentage of correct conditioned responses in rats which had been injected i.p. with brain homogenates from trained rats as compared with uninjected subjects or those injected with brain homogenate from untrained rats. Transfer training using RNA extracts has been reported by other workers (Babich et al 1965; Jacobson et al 1966; Albert 1966).

Other groups have provided evidence that peptides or low molecular weight proteins mediate transfer effects on conditioned escape or avoidance tasks (Rosenblatt et al 1966; Rosenblatt & Miller 1966). Ungar and co-workers have isolated substances mediating the transfer of dark avoidance in mice (Ungar et al 1968). This substance has been identified as a decapeptide

and its structure confirmed by synthesis (Ungar et al 1972).

Other workers have concentrated on detecting morphological and biochemical correlates of experience in non-training situations. This work has entailed sensory deprivation or stimulation in developing or mature animals. Cragg (1967) reported changes in number and density of synapses in the visual cortex of rats following three hours light exposure after dark rearing. Decrements in protein synthesis have also been reported after subjecting animals to forced exercise (Altman 1963; Jakoubek & Gutmann 1968); but in these cases stress has also been implicated as a contributory factor.

Richardson & Rose reported striking differences in the pattern of incorporation of lysine into soluble and insoluble proteins of the retina of dark reared rats on first exposure to light (Richardson & Rose 1973a). Using a double labelling technique, they reported significant differences in incorporation in 13 of the 41 protein fractions studied after one hour of light exposure. These 13 fractions were termed ' high differential activity' fractions and histochemical examination demonstrated that AChE was one of them.

Richardson & Rose (1973 b) reported similar differences in the visual cortex, where differential incorporation was reported in two fractions of the soluble and 7 fractions of the insoluble proteins. They suggested that exposure to a new experience involves a specific effect on protein synthesis rather than a general stimulation. In a further investigation (Rose et al 1973) an attempt was made to localise these effects at a cellular level. They reported that there were differences between the neuropil and neuronal fractions.

The results were taken to suggest that part of the protein synthesis

of the visual cortex is functionally controlled and that neuropil and neuronal fractions show a metabolic relationship which can be affected by environmental changes. Rose et al (1973) proposed the term ' state dependent ' to describe this relationship between neurochemical and environmental events.

A major uncertainty in the learning experiments is what it is that is happening in the experimental animal and not happening in the control animal, in addition to the learning process. Stress, heightened motor activity and the motivational state of the animal could produce the biochemical changes usually attributed to the learning process (Glassman 1969; Rose 1970; Bateson 1970). The use of the yoked control does not resolve all of these problems (Bateson 1970). For example, it has been shown that total brain catecholamine levels were higher in animals that could avoid or escape electrical stimulation than in those who could not (Weiss et al 1970). Thus it seems unlikely that animals learning an avoidance reaction are subjected to similar stresses as animals randomly shocked by painful stimulation.

It is extremely difficult to differentiate between those components of metabolism of macromolecules which change in response to excitation of neurones and those changes attributed to the more specific process called learning, since excitation always immediately precedes memory formation. There is evidence that excitation of neurones is itself able to produce profound changes in protein and RNA metabolism. The metabolism of macromolecules such as protein and RNA can be affected by motor activity (Jakoubek et al 1968); stress (Altman & Das 1965; Semiginovsky et al 1970) and by ACTH (Jakoubek et al 1972). Differences between experimental and control animal in any of these parameters are conceivable even when yoked controls are used.

The changes in macromolecular metabolism attributed to the learning process could be due to changes in hormonal balance of the animal which accompany any strong motivation. Many of the stimuli used in learning experiments are stressors, so that the hypothalamo-hypophyseal-adrenal system would also be activated. For example, stimulation of the reticular activating system at sites which gave sustained cortical arousal reaction also results in ACTH release occurring 5 min after stimulation and lasting for 30 - 40 min (Taylor 1969).

In a conditioning procedure, a conditioned hypoglycaemia was found to occur in the waiting period before stimulation (Gutmann & Jakoubek 1960). Bilateral adrenalectomy was found to abolish this conditioned hypoglycaemia. An activation of the adrenals was also implicated by increased levels of corticosteroids (Jakoubek et al 1970) and by depletion of fat in the adrenal glands of animals which had been subjected to long periods of anticipation stress (Jakoubek 1961).

The uptake and binding of corticosteroids in brain tissue has been described (McEwen et al 1972); and some effects of these hormones on nerve impulse conduction and on synaptic transmission have been described (Ojeman & Henkin 1967). Corticosteroids bind to the limbic system and midbrain reticular formation as well as the hypothalamus. Using radioactively labelled corticosteroid an intense labelling was also seen in the hippocampus (Gerlach & McEwen 1972).

There is also evidence that corticosteroids may be involved in 5HT synthesis (Azmitia & McEwen 1969). Corticosteroids were also found to change the metabolism of catecholamines under in vitro conditions (Maas & Mednick 1972).

The incorporation of labelled amino acids into cerebral

cortex of ACTH-treated mice was also found to be significantly stimulated (Semiginovsky & Jakoubek 1970).

However, hormonal mechanisms cannot be considered under all circumstances as the main cause of altered rates of macromolecular metabolism in brain. In experiments in which stimulation affects only some of the neuronal cells in a given anatomical structure, leaving other groups unaffected(Jarlstedt 1968; Lodin 1968) hormonal factors would not play a decisive role. However, neuroendocrine effects should always be considered when interpreting biochemical data on altered macromolecular metabolism following training procedures which induce stress in the experimental animal.

Thus, the experimental evidence presented in this section suggests an altered metabolism of brain macromolecules during the learning process. The features of this are difficult to discern from the conflicting data, which is due, at least in part to the considerable technical problems of biochemical interpretation surrounding the biochemical experiments.

SECTION 2. Changes in the activity of acetylcholinesterase
and total proteins following behavioural treatments.

2.1. Introduction.

The cholinergic nervous system was the first chemical transmission system to be studied in detail and this has led many groups to study the possible relationship between the cholinergic nervous system and behaviour. Two branches of investigation into the nature of these relations have developed from the hypothesis that behaviour and neurochemical events are reciprocally related i.e. manipulation of neurochemical parameters may produce changes in behaviour and changes in behaviour may produce both concomitant and persistent alterations of neurochemical events.

One of the most serious difficulties in the search for interactions between the cholinergic nervous system and behaviour is the requirement for the accurate measurement of both parameters. Thus, the precision of any statement that a behavioural measure is some function of a neurochemical event is limited by the accuracy of the measuring techniques. The availability of simple and reliable techniques for the assay of AChE has focused considerable attention on this component of the system. Koelle (1969) has stated that the localisation of a high level of AChE activity in a group of neurones in the absence of a more direct criterion, appears to be a reasonable index of cholinergic function. Although the presence of AChE in itself is insufficient evidence of the existence of a cholinergic pathway (Krnjević 1969), many areas tentatively designated cholinergic on the basis of their reaction for AChE have been found to fulfil the more rigorous criteria needed for their identification (Silver 1974).

2.1.2. Nature and properties of AChE.

Cholinesterases have been defined as " all hydrolases that cleave choline esters and are inhibited by physostigmine (eserine) at a concentration of $10^{-5}M$ " (Engelhard et al 1967). Two different groups of cholinesterase have been distinguished: acetylcholinesterase (E.C. 3.1.1.7.) and pseudocholinesterase (E.C. 3.1.1.8.). The two types of cholinesterase show different substrate specificities and kinetic constants and can be differentiated by inhibitors which are specific for acetylcholinesterase or pseudocholinesterase. The difference in behaviour of the two enzymes has been attributed to the structure of their active centres (Silver 1974).

Cholinesterase activity levels are found to vary from species to species, from strain to strain within a species and with the age of the animal (Burgen & Chipman 1951; Elkes & Todrick 1955; Bennett 1958; 1961; 1966).

2.1.3. Physical properties of purified cholinesterases.

Most of the information about the properties of purified AChE has been obtained from studies of the enzyme from the electric tissue of the eel Electrophorus electricus (Rothenberg & Nachmansohn 1947 ; Lawler 1961; 1963). The enzyme was finally purified in a crystalline and electrophoretically homogenous form by chromatographic procedures in 1967 by Leuzinger & Baker. The molecular weight was found to be 260,000 daltons (Leuzinger et al 1969). Several active forms of the enzyme have been reported with lower molecular weights (Massoulié et al 1970 ; Dudai et al 1972).

Determination of the molecular weight of AChE has indicated that the molecule can be broken down into subunits; but it is unclear what these subunits represent in terms of enzyme function. Leuzinger et al (1969) and Leuzinger (1969;

1971 a, b) suggested that AChE was a dimer formed from two protomers each of which was composed of two non-identical polypeptide chains. Leuzinger (1971a) also proposed that each molecule of AChE possesses only two active sites. The enzyme is reported to be composed of four subunits and contain four active sites (Froede & Wilson 1970); while Grafius & Millar (1967) suggested that AChE has six subunits and six active sites.

The AChE of mammalian brain is thought to be largely membrane bound (Whittaker et al 1964). This firm association with membrane components has meant that solubilisation has been a major problem with attempts to purify the mammalian enzyme. Detergent extracted enzymes (Kremzner et al 1967; Ho & Ellman 1969; Crone 1971) as well as enzymes extracted in isotonic sucrose (Chan et al 1972 a, b) show multiple forms with increasing molecular weights.

Higher molecular weight forms of the solubilised enzymes were reported to increase on keeping (Hollunger & Niklasson 1967) at the expense of lower molecular weight forms. They suggested that the higher molecular weight forms were different states of aggregation. This property of AChE was reported by Changeux (1966) and Grafius & Miller (1967). However it remains to be established whether these postulated multiples of a single active unit can interconvert and also which form exists in the intact membrane.

Isoenzymes of AChE have also been reported from studies on both electric tissue of the eel Electrophorus electricus (Echobichon & Israel 1967 ; Bauman et al 1972) and mammalian brain and muscle tissue (Barron et al 1963; Maynard 1966; Wilson et al 1969). Ontogenic studies (Maynard 1966; Barron et al 1963; Wilson et al 1969) have indicated that

changes in isoenzyme pattern occur during tissue development. The functional significance of the occurrence of isoenzymes of AChE from the trout brain Salmo gairdnerii was suggested by Baldwin & Hochachka (1970) to be associated with acclimatisation to different temperatures encountered by the trout.

2.1.4. Mechanism and kinetics of AChE.

The primary role of AChE at synapses and the neuromuscular junction is to hydrolyse the ACh released during transmission. The rate of hydrolysis of ACh by this enzyme is extremely fast which allows for a rapid removal of this transmitter substance (Nachmansohn 1972).

The enzyme contains an anionic site capable of binding the cationic portion of ACh and also an esteratic site characterised by an acidic and basic group upon which the enzyme activity is directly dependent (Wilson 1951). The anionic site is thus responsible for binding and orienting substituted ammonium groups while the work of cleavage is done at the esteratic site. Acetylcholine reacts with the enzyme at its active surface, forming a Michaelis-Menten complex. An internal electron shift then takes place and choline is released as the first product of the reaction. The acetate moiety remains covalently bound to the esteratic site. The acetyl-enzyme, so formed, hydrolyses regenerating the integrity of the active surface of the enzyme and releasing acetic acid as the second product of the reaction (Wilson 1950; Nachmansohn 1962). This scheme has been further elaborated by Rabin (1967) and Krupka (1966a, b).

AChE exhibits substrate inhibition; a plot of substrate concentration against enzyme activity gives a bell-shaped curve

with an asymptote at 3×10^{-3} M. Thus, AChE is inhibited by a large excess of substrate. There have been several suggestions to explain the mechanism of substrate inhibition shown by AChE.

Krupka (1963, 1964) suggests that at a high substrate concentration the excess substrate produces inhibition of the enzyme by binding to the anionic site when the enzyme is in the acylated form. The complex formed is deacylated slowly so that the active centre is unavailable for further hydrolysis and this then explains the inhibition seen at high substrate concentrations. It has also been proposed that substrate inhibition is the result of conformational changes produced by binding of excess substrate to an allosteric site (Changeux 1966; Brestkin et al 1965, 1966; Kato et al 1972; Kato 1972). It is suggested that this allosteric modification by excess substrate induces a conformational change which inhibits the catalytic process.

2.1.5. Evidence for allosterism in AChE.

The possibility that AChE can undergo conformational changes and allosteric interactions could be of great relevance to any postulated role of AChE activity in the modification of synaptic activity. Several investigators have suggested that AChE of electric eel possesses a regulatory site distinct from the active site (Changeux 1966; Kitz et al 1970).

These conclusions were derived from studies which indicated simultaneous binding of various drugs and substrates to the enzyme and suggested that AChE was an allosteric enzyme (Monod et al 1965). These investigations suggested an allosteric site capable of binding curare-like blockers and inducing conformational

changes at the active centre. Kitz & Kremzner (1968) have also reported that the highly purified enzyme undergoes conformational changes in response to heat, strong base, substrate and antiChEs.

There is some evidence that AChE from mammalian sources may also exhibit allosteric properties (Belleau et al 1970; Aldridge & Reiner 1969; Wombacher & Wolf 1971).

2.1.6. Localisation of AChE within the nervous system.

A Regional

The histochemical localisation and distribution of AChE in the central nervous system has been important in supplying supporting evidence for central cholinergic transmission. The identification of transmitters at the central synapse poses many problems. Werman (1966) proposed eight criteria which needed to be met in the identification of a substance as a natural transmitter. One of these criteria was the collection of the putative transmitter from the stimulated junction. Direct confirmation of this sort is difficult to obtain from reputed cholinergic synapses in CNS and evidence for suggesting that a pathway is cholinergic depends on the accumulation of indirect evidence. Thus the great difficulty in recovering and identifying the transmitter released following stimulation coupled with the fact that of the three characteristics of the cholinergic system (ACh, ChAc and AChE) only AChE could be localised histochemically, has meant that AChE distribution has been important in supplying evidence for cholinergic transmission.

Early studies of brain region homogenates indicated that the concentration of ACh, ChAc and AChE run fairly parallel for various regions of the brain (Burgen & Chipman 1951; Aprison et al 1964). With the use of ultramicrotechniques (Buckley et al 1967); improved systems for measuring ChAc

and axotomy(Lewis et al 1964) which causes both enzymes to accumulate on the neuronal side and to decrease on the distal side of the lesion, an even closer correlation between the concentration of the two enzymes was seen in most brain regions. An exception was found in the cerebellum, where the concentration of AChE was observed to be very high relative to that of ChAc (Silver 1967).

Shute & Lewis (1963, 1965, 1967) and Lewis & Shute (1967) have used the technique of histochemical localisation of AChE coupled with axotomy to map out several intensely staining tracts in the rat brain. They concluded that the fibres which stain for AChE come from two different areas of the brain. The dorsal tegmental pathway starts in the midbrain reticular formation and projects to the tectum, metathalamus and the thalamus. The second pathway, the ventral tegmental pathway, arises from neurones in the ventral tegmental area and the substantia nigra and sends fibres to the subthalamus, hypothalamus and basal forebrain areas. Fibres to the neocortex and striatum project from the reticular cells in various nuclei eg. globus pallidus. The cell groups projecting to the cortex are not only in continuity with each other and with neurones of the ventral tegmental pathway but are composed of large neurones with long dendrites. These features suggest that the cells may be activated from many different sources and is in agreement with the concept that the cholinergic system is likely to be involved in diffuse rather than specific activation as suggested by Krnjević (1969). Shute & Lewis suggest a relationship between the ascending reticular system they described and the reticular activating system described by Starzl et al (1951). This suggestion is supported by the EEG studies of Kanai & Szerb (1965 and Beani et al (1968). The overall picture of the distribution

of AChE activity is therefore, that it does not appear to be principally connected with the main afferent, efferent and commissural pathways but is involved in diffuse activation in the CNS.

Tebēcis (1974) reviewing the central action of ACh concluded that ACh was found to both excite and depress the firing of neurones and that sometimes both actions were apparent on the same neuron.. Characteristically, both the excitation and depression by ACh are usually of a slower time course than that shown by other transmitters eg. amino acid transmitters except in the case of the Renshaw cells and the many lateral geniculate neurons which are excited by ACh with a time course similar to that of the excitant amino acid transmitters.

In general ACh and other cholinomimetics stimulate the cerebral cortex and hippocampus and cholinolytic agents (atropine) depress it either by direct action on cortical neurons (Salmoiraghi & Stefanis 1967) or by activation of diffuse pathways which project to and innervate these neurons. Evidence has also been provided for an inhibitory action of ACh on a system of intracortical neurons (Phillis & York 1968) which had been previously described histochemically by Krnjević & Silver (1965).

Interpretation of the data for ACh is further complicated by the relative proportions of muscarinic and nicotinic properties of the neuron studied; these vary with the type of neuron and structures in which they are located. In addition some types of depression by ACh appear to be mediated by neither muscarinic or nicotinic receptors (Tebēcis 1974).

Although all cholinceptive cells are not necessarily sites of cholinergic transmission, the marked differential distribution of cholinceptive cells, together with other data, provide an indication of the possible sites where ACh may function as a transmitter. Shute & Lewis (1963) concluded

B. Subcellular localisation of AChE.

Subcellular fractionation studies have shown that AChE is associated with the particulate fraction (Ord & Thompson 1951; Nathan & Aprison 1955). The microsomal fraction of rabbit caudate nucleus was shown to contain the greatest AChE activity but activity was also seen in the mitochondrial fraction.(Nathan & Aprison 1955). AChE of rat brain has also been shown to be associated with the membrane component (Aldridge & Johnson 1959; Toschi 1959; Hanzon & Toschi 1959).

The finer details of the localisation of AChE are still controversial. One of the reasons for this is that AChE of the perikarya appears to be mainly associated with the reticulum but AChE of the nerve terminal is associated with the membrane fraction. Therefore, in any region the distribution of enzyme between the mitochondrial and microsomal fractions will depend on the relative distribution of nerve endings and nerve cells in the region (Silver 1974).

De Robertis et al (1962) fractionated the synaptosomes of whole rat brain and found the highest AChE activity associated with the coarse particulate fraction but the highest specific activity associated with the fraction he identified as containing synaptic vesicles and post-synaptic membranes. Rodríguez de Lores Arnaiz et al (1967) also provided evidence for the association of AChE with synaptic elements.

It is still uncertain whether AChE is present inside the nerve terminals as well as outside on the synaptic membrane. Nachmansohn (1950) compared the effects of lipid soluble anti-ChEs which could enter the axon with lipid insoluble anti-ChEs which could not, on an autonomic ganglia preparation. Koelle & Koelle (1959) have also used this approach and reported the presence of an external enzyme on the outside of the synaptic

membrane which was termed 'functional' and an internal termed 'reserve' enzyme.

The association of AChE with membrane structure has also been demonstrated by electron microscopy studies. Lewis & Shute (1969) found that in cholinergic cells the endoplasmic reticulum is always stained for AChE. The enzyme appears to be associated with the internal surface of the membrane rather than with the ribosomes themselves (Eränko et al 1967). The nuclear envelope has also stained for AChE in some cases (Lewis & Shute 1969). In some cases, the plasma membrane has been reported to be stained for AChE only where there is a synapse (Lewis & Shute 1966) whereas other studies have shown a more general staining of the plasma membrane (Kreutzberg et al 1973). The outer surface of the axolemmal membranes of cholinergic neurones also show staining for AChE (Lewis & Shute 1966).

Thus, evidence from subcellular fractionation studies and e.m. studies indicate that at the synapse the presynaptic enzyme is located on the external surface of the terminals and on the external surface of the cholinceptive post-synaptic cell.

2.1.7. Synthesis and transport of AChE.

Histochemical and biochemical studies have shown that if a nerve is ligated, AChE activity increases proximal to the lesion and decreases below the lesion and this has been interpreted as evidence that AChE is transported from the nerve cell body, where it is synthesised, along the axon to the nerve terminal (Fukuda & Koelle 1959; Lubińska et al 1961; Shute & Lewis 1961; Lewis et al 1964). The phenomena of axonal transport of proteins has also been demonstrated by injecting radioactively labelled amino

acids into nerve cells and following the fate of the radioactive label by autoradiography and subcellular fractionation (Grafstein 1969; Lubinska 1975).

There is no general agreement about the mechanism of transport of proteins along axons. McEwen & Grafstein (1968) suggest that there are two components, a fast moving component (40 mm / day) and a slow moving component (0.4mm / day). The suggestion that there are two components with different rates of transport is also supported by the work of Cuénod & Schonbach (1971) who followed the movement of ^3H - leucine injected into the optic system of the pigeon. They reported a slower component moving at 100-200 mm / day and a faster component moving at 400 mm / day.

Subcellular fractionation studies have led to the idea that the fast component is associated with the particulate fraction and the slow component is associated with the soluble protein (McEwen & Grafstein 1968 ; Karlsson & Sjostrand 1971 ; Schonbach et al 1971). However, other workers have suggested that transport occurs over a range of velocities and not as two distinct components. (Bradley et al 1971; Cuénod & Schonbach 1971).

There are several reports of specific transport rates for AChE. Using peroneal nerve preparation of dog, Lubińska (1964) found that the rate of flow of AChE was 30 - 60 mm / day. Lubińska & Niemierko (1971) measured the rate of accumulation of AChE in ligated nerve and found that 15% of the AChE travelled at 260 mm / day, that is within the fast range described from amino acid transport studies. Ranish & Ochs (1972) also reported that a small proportion of AChE was transported by a fast mechanism (431 mm / day), while the remainder moved by

a slower process. Results from studies with ligated nerves are however difficult to interpret and may not be comparable to the transport of enzyme in intact nerve (Lubińska & Niemierko 1971).

Kása et al (1973) compared the rate of transport of AChE with choline acetyltransferase in the sciatic nerve of rat and found that AChE was transported by a fast process, while ChAc was associated with a slower mechanism. Histochemical studies revealed the presence of AChE within the vesicles and associated with neurotubules while ChAc appeared free in the cytoplasm bound to the neurofilaments and on the outer surface of vesicles and tubules. Schmitt (1968) also suggested that neurotubules were involved in rapid transport of proteins and suggested that proteins were within vesicles which moved along the neurotubules utilising the energy produced by hydrolysing ATP or GTP by vesicular ATPase or GTPase.

Fast axoplasmic transport depends upon oxidative phosphorylation with ATP supplying the energy to the transport mechanism locally in the axons and also requires calcium ions. On the basis of this Ochs (1971; 1972) proposed a transport filament model for fast axoplasmic transport. Microtubules are suggested as stationary members of the sliding filament pair and transport filaments, which are synthesised in the cell body are the moving members of the pair of sliding filaments. Proteins which are synthesised in the perikarya are suggested to be bound to this transport filament.

In addition to protein synthesis in the perikarya there is also evidence that local synthesis of proteins occurs in axons and nerve terminals (Koenig & Koelle 1960;

1961; Clouet & Waelisch 1961). RNA has been reported to be present in Mauthner cells of the goldfish (Edström 1964) and there have also been reports that RNA is present in mammalian nerve cells (Bondy 1972; Jarlstedt & Karlsson 1973). Protein synthesis has also been demonstrated in isolated axons and in synaptosomes (Koenig 1967; Morgan & Austin 1968; Goldberg 1971; 1972).

The idea that protein synthesis can occur in isolated axons has been disputed by several workers (Lubińska et al 1964; Austin & James 1970) who have failed to demonstrate any local production of AChE in axons.

Many questions, therefore remain to be answered about what controls the synthesis of AChE in the perikarya and the mechanism of its subsequent transport along the axon. It also remains to be established whether synthesis within the axon has any significance in the function of the cholinergic neuron.

2.1.8. Behavioural aspects of cholinergic transmission.

There is much evidence in the literature to support the contention that there is an association between central cholinergic mechanisms and behaviour. For example, many workers have provided evidence that the activity of central AChE changes in response to a number of chemical and physical treatments (Krinavec & Bureš 1968; Naik & Sheth 1970;

Haywood et al 1975; Kling et al 1965). However, conclusive identification of cholinergic mechanisms responsible for specific behaviours has been difficult to achieve mainly because of extensive anatomical and functional overlap of central pathways. Variations in experimental data caused by species differences, housing conditions of experimental animals, diurnal rhythms, maturation and nutrition have also contributed to this problem.

When investigating the inter-relationship between the cholinergic system and behaviour three approaches can be taken:

- i) Determination of the degree to which individual differences in some measure of behaviour are correlated with individual differences in some neurochemical parameter.
- ii) Experimental manipulation of a neurochemical parameter and the measurement of consequent changes in a measure of behaviour.
- iii) Experimental manipulation of behaviour and the measurement of consequent changes in the neurochemical parameter.

Many workers have attempted correlations between measures of adaptive behaviour in a variety of learning situations and variations in the activity of AChE. This approach was used in the early studies of Rosenzweig(1960). Using rats his group attempted a correlation between a measure of adaptive behaviour, in a variety of maze-learning situations and the activity of AChE in the cortex and in the subcortical areas. Selective breeding techniques were used to maximise the range of individual variation in AChE activities. They reported a positive and significant correlation between higher AChE activity and the degree of adaptive behaviour exhibited under their experimental conditions. The differences in AChE activities in the cortical regions between the high adapting and low adapting animals were

quite small (approximately 5%). The significance of such small differences is questionable since cholinesterases are generally regarded as enzymes working with a large safety margin. This type of correlation does not provide a concrete basis for the interpretation of results.

Many groups have therefore adopted the second approach, that is to determine the behavioural consequences of the progressive inhibition of central and peripheral AChE activity. Russell (1954, 1958) studied the effect of chronic reduction of AChE activity to around 40% of normal by i.p. injection of the organophosphorus reagent O,O-diethyl S-ethylmercaptoethanol thiosulphate. Using a wide variety of standardised behavioural testing procedures he reported that his results indicated no effect on the acquisition of new responses but that extinction was slower for experimental as compared to control animals. This effect was further studied by Russell et al (1961).

Dose response studies showed that the relation between AChE activity and extinction was not linear and there was a critical level between 40-50% of normal enzyme activity below which speed of extinction is inversely related to AChE activity.

These results have been confirmed in an extension of this study by Glow & Rose (1965) and Glow et al (1966). Using acute as well as chronic reduction of AChE activity by peripheral injection of DFP they found that extinction was slower for the experimental group and that this effect was observed below 40-45% of normal enzyme activity. It was reported by Aprison (1962) that at about this concentration that the enzyme loses control of its substrate and Wilson & Cohen (1953) have also reported at about this level there is a sharp drop in membrane conductance.

The results of these two studies suggested a relation between the cholinergic nervous system and the behavioural

process of extinction.

In a series of experiments Deutsch & Liebowitz (1966), Deutsch et al (1966) have shown that cholinergic drugs can alter recall. They suggest that if the substrate of memory is synaptic and if it can slowly change then there is a possibility of following such synaptic changes using pharmacological methods. Using intrahippocampal injections of DFP into rats they showed that animals trained to run to an illuminated arm of a Y-Y-maze showed amnesia when injected with the anticholinesterase reagent 30 min after training. No amnesia takes place if the injection takes place 3 days after training. Complete amnesia is however seen if the injection is given 14 days after training. Using intra-cerebral injections of DFP in rats they showed good recall of an otherwise almost forgotten habit learned 28 days previously. The same injection produced temporary amnesia for the same habit otherwise well remembered, learned 14 days previously but had no effect at this time, if the habit was only partially learned. On the basis of these and other studies Deutsch argued that the anticholinesterase facilitated low conductance synapses and inhibited high conductance synapses. He concluded that, with the exception of a short time after training, the conductance of a synapse increased with time after training. In this way the anticholinesterase would have no effect soon after training but would cause a block as soon as the conductance of the synapse improved.

To test this hypothesis, parallel experiments were performed with scopolamine (Deutsch & Rocklin 1967) an anticholinergic which decreases the ACh content of the brain. It was expected, therefore, that the effects observed with scopolamine would be the mirror-image of the effects observed with DFP. Deutsch & Rocklin (1967) reported that this was indeed the case.

Burěšova et al (1964) reported that atropine administered 20 min prior to training caused impairment of acquisition of an avoidance response. Downs et al (1972) demonstrated that impairment of a classically conditioned eyelid reflex in rabbits was produced by centrally acting atropine sulphate; atropine methyl nitrate, which does not pass the blood-brain barrier, had no effect. It thus appears that the deficits were of acquisition and resulted from the central rather than peripheral effects of the antimuscarinic drug.

Thus evidence from several species and different learning tasks demonstrates that cholinergic inhibitors influence both learning and memory. However, all of these studies on the effects of antiChEs on central neurons are at most only speculative, since it is well known that these agents also inhibit cellular respiration , and enzymes other than AChE (Brooks et al 1949; Michaelis et al 1949). These data also do not rule out the possibility of a deficit in performance possibly due to the central effects on motor mechanisms. ACh is thought to be an important transmitter in the extrapyramidal system, blockage could cause deficits in the initiation of behaviour and possibly also in the temporal integration of behaviour (Prado-Alcala et al 1972).

A further criticism of this work has been that in many of the studies performed cholinomimetics and cholinolytic agents have been administered systemically and that this has led to many problems in both interpreting and reproducing the observed behavioral effects. Some of the difficulties encountered when using systemic injections were summarised by De Feudis (1975) as being :

- 1) lack of or slow entry of injected substances into the brain due to blood-brain barrier mechanisms.

- ii) actions of injected substances at peripheral sites which can modify cerebral activity by secondary or reflex actions.
- iii) metabolism or inactivation of injected substances.
- iv) local effects of agents on vascular mechanisms which can lead to alterations in cellular metabolism (Hebb & Krnjević 1962).

Cholinergic mechanisms have also been implicated in cortical arousal (Rinaldi & Himwich 1955a,b). Injections of ACh or cholinomimetics (carbachol) directly into the mesencephalic or thalamic reticular formation of the rat produced reversible behavioural changes in both appetitive and aversive test situations (Grossman 1966 ; Grossman & Grossman 1966; Grossman 1968). These workers concluded that cholinergic stimulation markedly increased the animals responsiveness to sensory stimulation. These results indicated that the cholinergic reticular mechanisms might be involved in behavioural arousal which is necessary for the learning process.

Krnjević (1965) suggested that the level of cortical arousal might be related to the amount of ACh released from cholinergic fibres which ascend from subcortical structures to innervate deep pyramidal cells of the cerebral cortex.

Several workers have also suggested the involvement of the cholinergic system in predatory aggression (eg. mouse-killing in rats; rat-killing by cats). This type of behaviour is stereotyped and stimulus specific (Wasman & Flynn 1962). Direct injections of carbachol, ACh and eserine or physostigmine into sites of lateral hypothalamus have been found to facilitate this predatory aggression behaviour (Bandler 1969, 1970).

Other major approaches to this study have been to vary behaviour and to examine the neurochemical parameters which may alter as a consequence. It has been suggested that behavioural patterns are a series of processes, the first of which is sensory

stimulationA relation between the cholinergic nervous system and behaviour might, therefore, be observable as changes resulting from sensory stimulation.

Lieberman (1962) took groups of age-matched rats and reared one group in normal light-dark conditions and the other he reared in total darkness for 17 weeks. He reported that the AChE activity in the retinae of the dark reared rats was very significantly lower than in the control group. This could be explained by assuming that the lower levels of stimulation led to a less ACh being released; if now the synthesis and maintenance of AChE activity were independent on the level of ACh then this too would be lowered as observed.

De Robertis & Franchi (1956) conducted a similar experiment and observed using electron-microscopy, a sharp decrease in the size of synaptic vesicles in the retina after 9 days in complete darkness. Similar studies using e.m. observations of the size, number and distribution of vesicles in various nerve endings, following various behavioural treatments such as electrical stimulation and anaesthesia, have been valuable in supplying supporting information on this relation between substrate concentration and sensory stimulation (Csillik & Bengé 1971; Fehér et al 1972).

The possible link between sensory input and central AChE activity and other neurochemical parameters has been the basis of an extensive series conducted by Bennett & Rosenzweig(reviewed by Bennett et al 1964; Rosenzweig et al 1971), in which they compared animals reared in an experience enriched environment with those reared in an experience impoverished environment. They found that rats that spent from 4-10 weeks in the enriched condition had, compared to the impoverished condition group, a greater weight of cerebral cortex, a greater thickness of cortex

and a greater total activity of cortical AChE. However, the amount of enzyme activity per unit of tissue weight decreased. They also reported that the brain samples exhibited a greater pseudoChE activity and a greater glial-neuronal ratio.

Rosenzweig et al (1972) demonstrated that the cerebral effects of differential experience (Bennett et al 1964) occur also in hypophysectomised rats. They concluded that these effects do not require mediation by hormones.

Thus many workers have reported that the activity of central AChE changes in response to a variety of physical and chemical treatments which alter the behavioural state of the animal. Many of the previous studies were done on whole brain or very few regions of the brain. The assay of such large regions of the brain can fail to detect changes occurring at a local level and this could lead to misinterpretation. The aim of this present study was thus to examine more closely the changes in activity of AChE which result from behavioural manipulation. Durkin (1973) developed a semi-micro method for the assay of AChE from small regions of the brain. This technique has been used to examine the effect of a range of behavioural procedures which incorporated a range of physiological stimulation; these include the effects of anaesthesia, electrical stimulation and shock avoidance conditioning on the activity of AChE from selected regions of the rat brain.

Section 2.2. Methods.

2.2.1. Quiet Control Procedure.

Female littermates of an inbred strain of Agus Albino rats were used in all experiments when they were 90 - 100 days old. It has been reported that acetylcholinesterase activity increases until 100 days and thereafter steadily declines (Bennett et al 1966) so that animals were used when the activity of AChE was maximal. Animals were killed with the minimum of trauma by stunning and immediate exsanguination from the carotid artery. The brains were rapidly dissected and immediately frozen on petri dishes embedded on crushed dry ice. Throughout the project, rats were routinely killed between 10.30 a.m. and 11.30 a.m. to eliminate any diurnal changes in the activity of the enzyme. .

2.2.2. Anaesthetisation Procedure.

A pair of female littermates were taken from the stock cage, marked on the tail for identification and weighed. One of the pair was then injected intraperitoneally by a 25G 5/8" needle using a standard anaesthetic dose of 60 mg/kg pentobarbitone sodium. The injections were performed as quickly as possible in order to minimise handling and injection stress to the animals. The injected animals were tested for the abolition of reflexes using the standard procedure of:-

- a) loss of righting reflex
- b) loss of paw withdrawal reflex
- c) loss of response to pressure to the tail
- d) loss of corneal reflex

When the animals were fully anaesthetised they were left for a further 45 mins with their body temperature maintained by a 60 watt light bulb. The other rat of the pair was used as a control and was injected with a volume of pyrogen-free dextrose saline equivalent to the volume of anaesthetic .

The control rat was then replaced

in its cage and was killed at the same time as the experimental animal.

2.2.3. Shock Avoidance Conditioning Apparatus.

The apparatus consists of a separate programming/recorder unit (cat. no. 7501, Ugo Basile, Milan) connected via 4m of cable to the cage. The separation of the two units at this distance ensures that there is no noise interference at the cage. The cage is built of opaque Perspex and is divided into two sections by a communicating door at floor level (see Fig.1.).

Acoustic and visual stimulators are mounted into the transparent roof of the cage and supply the conditioning stimuli. The floor of the cage is hinged centrally so that the floor tilts through a few degrees. Reinforcement consists of an electrical stimulus applied to the metal floor bars of the cage by a 'static scrambler circuit'. This device ensures that the rat receives the stimulus irrespective of the relative position of its paws. The apparatus operates the conditioning sequence as follows:-

- a) $t = 0$ secs : conditioning stimuli on (lights and buzzer)
- b) $t = 3$ secs : conditioning stimuli remain on; reinforcement stimulus applied.
- c) $t = 6$ secs : both conditioning and reinforcement stimuli switched off.
- d) $t = 30$ secs : conditioning sequence repeats.

A rat placed in this apparatus can cancel the conditioning sequence by moving from the compartment it is in to the other compartment. The movement of the animal from one compartment to the other causes the floor to tilt. This tilting operates a magnetic micro switch which cancels the conditioning sequence. A hydraulic damping mechanism ensures that any violent movement by the rat eg. jumping will not cause an accidental operation

Conditioning Sequence

$t = -3$ secs;
lights and buzzer on

$t = 0$ secs;
lights, buzzer and
electrical stimulus on

$t = +3$ secs;
lights, buzzer and
electrical stimulus off

$t = +27$ secs;
cycle repeats

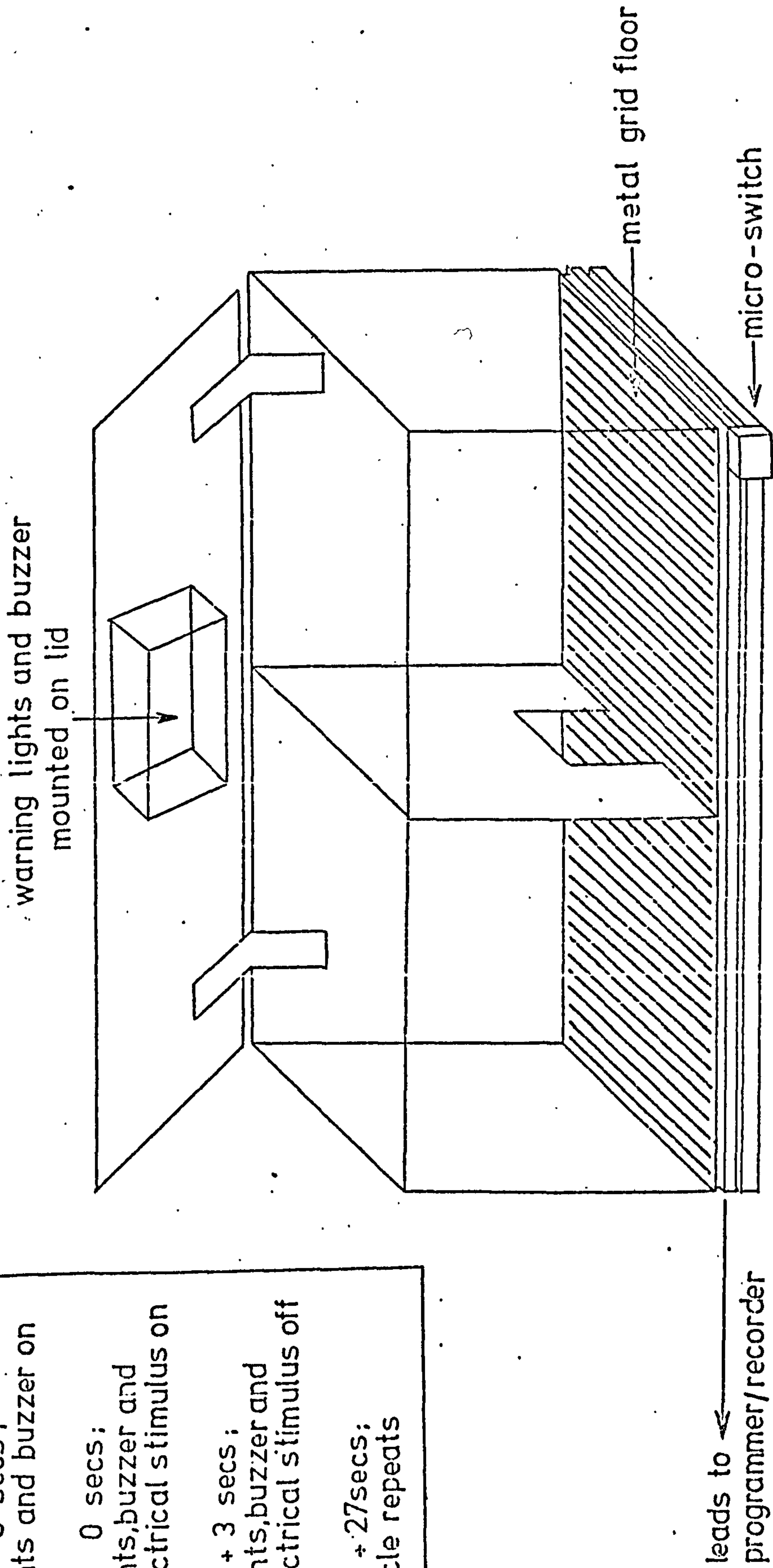


Fig.1 The UGO BASILE rat reflex conditioning apparatus

of this switch.

The programmer/recorder unit supplies a pre-set number of stimuli. The intensity of the acoustic stimulus and the reinforcement stimulus can be varied. The recording unit records the responses of the animal via a writing mechanism which allows the investigator to discriminate responses to the conditioning stimuli from those requiring the reinforcement stimulus. A timer mounted into the recording unit displays waiting time (waiting time = time from onset to cancellation of an individual conditioning sequence) for each response. Waiting times are a measurement of an animal's response to the conditioning stimulus and they become progressively shorter as the animal becomes conditioned. Example traces during the various stages of training are shown in Figure 2. By plotting the waiting times against stimulus number a training curve can be produced (Fig.3).

Regarding the use of this apparatus in avoidance reaction behavior studies, it should be noted that it evokes two way shock avoidance conditioning i.e. both active and passive responses compete for expression. Since what the animal does is instrumental in avoiding shock, the behaviour pattern is classified into the more general category of instrumental conditioning, a term introduced to separate it from classical conditioning (Hilgard & Marquis 1940).

2.2.4. Shock Avoidance Conditioning Procedure

In order to differentiate between the effects of stress following electrical stimulation and responses more exclusively related to learning, a yoked control procedure was adopted. Two cages were connected to the same programmer/recorder unit. The micro switch of the control animal's cage was inactivated so that this animal received the same electrical stimulation as the experimental animal but could not by its own movement

Figure 2. Example traces obtained from automatic reflex conditioning apparatus during a training session.

1 - rat not conditioned

2 - rat partially conditioned

3 - rat fully conditioned.

Figure 3. An example training curve.

The total waiting times (the time from the onset to the cancellation of a conditioning sequence) are plotted against the stimulus number. As the rat becomes conditioned it can be seen that the waiting times become progressively smaller. The horizontal line through the graph represents the arrival of the reinforcing stimulus. Responses which represent true avoidances are those which are plotted beneath this line. The animals were trained until they reached the criterion of 8 successive avoidances. This particular rat learnt in a comparatively short time.

cancel the electric shock sequence. The floor micro switch of the experimental animal's cage was activated so that this animal could by its movement cancel the sequence and thus learn to avoid the shock.

Animals were taken from their stock cage and placed into the apparatus. They were left for 10 min to become accustomed to the environment. Care was taken after each experiment to clean the cage in order to remove traces of the previous animal.

Experience from earlier pilot experiments indicated the correct intensity of the stimuli for efficient conditioning: if the reinforcing stimulus was too low the animals did not respond at all, if too high then the animals 'froze' after receiving only a few stimuli. The correct setting of the stimuli was thus at an intermediate intensity of 30 volts. The intensity of the acoustic conditioning stimulus was set at the arbitrary scale of 2.0.

After allowing time for the rats to become accustomed to the environment of the cage, the programmer was set to deliver a train of stimuli at 30 sec intervals and switched on. The responses of the experimental animal were recorded automatically and the general behaviour of both animals was observed.

Animals behaved characteristically by moving to an alert posture throughout the conditioning stimuli and at the onset of the reinforcement began moving their paws rapidly about the grid finally running to the opposite compartment and remaining there until the end of the sequence. A few animals exhibited rearing behaviour randomly during the first few conditioning sequences but their behaviour rapidly stabilised to conform with the pattern outlined. A few animals (two) apparently learned to avoid the shock as a result of this rearing behaviour. They responded on the arrival of the

reinforcement by placing their front paws onto the side walls of the cage and standing on their rear paws. These animals were discarded from the study. Another characteristic feature of the behaviour of the rats in this apparatus was that inter-trial crossing behaviour (moving from one compartment to the other during the period in between the conditioning sequence) was almost entirely absent. Inter-trial crossing has been used as a measure of spontaneous activity levels of rats in this apparatus.

Pilot experiments using rats in the acquisition of the shock avoidance response were conducted to determine the optimal conditions for acquisition. On the basis of these studies it was decided that an arbitrary criterion of learning could be set at 8 successive avoidances; and that this criterion ought to be achieved after about 45 mins of training (Fig.3). Figure 4 shows the retention of the avoidance response after both 24 hours and 6 days.

On two occasions the rats did not learn at all, responding only on the arrival of the reinforcing stimulus. These animals were excluded from the group.

Animals were left for one hour after the training session before they were killed. The experimental group was compared with both the yoked controls and a group of quiet controls; the yoked control group was also compared with the quiet controls to detect any changes due to stress.

2.2.5. Preparation of samples.

The brains were partially thawed to allow dissection of the samples which were hand dissected with a scalpel blade (Swann Morton 24). The following 12 regions were initially used and the hippocampus was also used in later studies:

1) frontal dorsal cortex 2) medial dorsal cortex 3) posterior

Figure 4. Training curves obtained after retesting animals
24 hours and 6 days following the original training.

There is a significant degree of retention of the conditioned response both after 24 hours and 6 days.

dorsal cortex 4) cerebellum 5) ventral cortex 6) medulla oblongata 7) pons 8) hypothalamus 9) thalamus 10) superior colliculus 11) olfactory tubercle 12) caudate nucleus. Figure 5 shows the location of the areas dissected. The approximate wet weight of the samples was 2-4 mg.

The dissected samples were then refrozen until homogenates were made by homogenising at 2,000 r.p.m. for one minute in a Voss vertical homogeniser fitted with a teflon pestle in a glass homogenising tube containing 2.0 ml of 0°C pH 8.25 sodium barbital buffer. Duplicate aliquots (0.5 ml) of the homogenate were then added to a further 0.5 ml barbital buffer and the resulting solution was assayed for acetylcholinesterase.

The regions were chosen on the basis of two main considerations: 1) accurate dissection : all regions chosen were relatively large and anatomically well defined 2) within the limitations of 1,, regions were chosen on the basis of either interesting cholinergic inputs or properties: several of the regions selected being situated on the ascending cholinergic reticular activating system (Shute & Lewis 1967).

2.2.6. Acetylcholinesterase Assay

Samples were assayed for acetylcholinesterase by the method of Ellman et al (1961). This method has been adapted for the auto-analyser by Durkin (1974). Details of the preparation of reagents and the pump tube sizes are given in Table 1. Figure 6 gives a flow diagram of the assay procedure.

The principle of the method is the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolysed. This is accomplished by the continuous reaction of the thiol with 5,5'-dithiobis-2-nitrobenzoate which yields the highly coloured 5-thio-2-nitrobenzoate ion. The rate of colour formation at maximum absorption (420 nm) was measured.

Figure 5. Diagrams to illustrate the localisation of the tissue samples taken from the brain regions used in the study

The diagrams show the dorsal, ventral and medial sagittal views of the brain. The stippled areas indicate the location of the tissue samples used in the study.

Table 1. Details of the preparation of reagents and buffers for the assay of acetylcholinesterase.

1) Sodium barbital buffer (pH 8.25)

Barbitone sodium	1.237g
Potassium dihydrogen phosphate	0.136g
Sodium chloride	17.535g
Water (distilled)	to 1 litre.

2) NAcetylthiocholine iodide (made fresh daily)

76.68mg acetylthiocholine iodide in 100 ml sodium barbital buffer.

3) D.T.N.B.

5,5' dithiobis - 2 - nitrobenzoic acid (396 mg) and Sodium bicarbonate (150 mg) were dissolved in 100 mls of 0.1M Sodium phosphate buffer (pH 7.0).

4) Sodium phosphate buffer (pH 7.0)

Sodium dihydrogen phosphate (0.1M) 31.0 mls were added to Disodium hydrogen phosphate (0.1M) 69.0 mls.

5) Saline

9g Sodium chloride dissolved in 1 litre distilled water and adjusted to pH 8.5.

Pump tube sizes for the auto - analyser.

N acetylthiocholine iodide	0.23 ml / min
D.T.N.B.	0.10 ml / min
Sodium barbital buffer pH 8.25 (plus 2% v / v Brij 35)	0.23 ml / min
Saline (plus 2% v / v Brij 35)	1.00 ml / min
Enzyme sample	0.42 ml / min
Distilled water (plus 2% v / v Brij 35)	2.00 ml / min

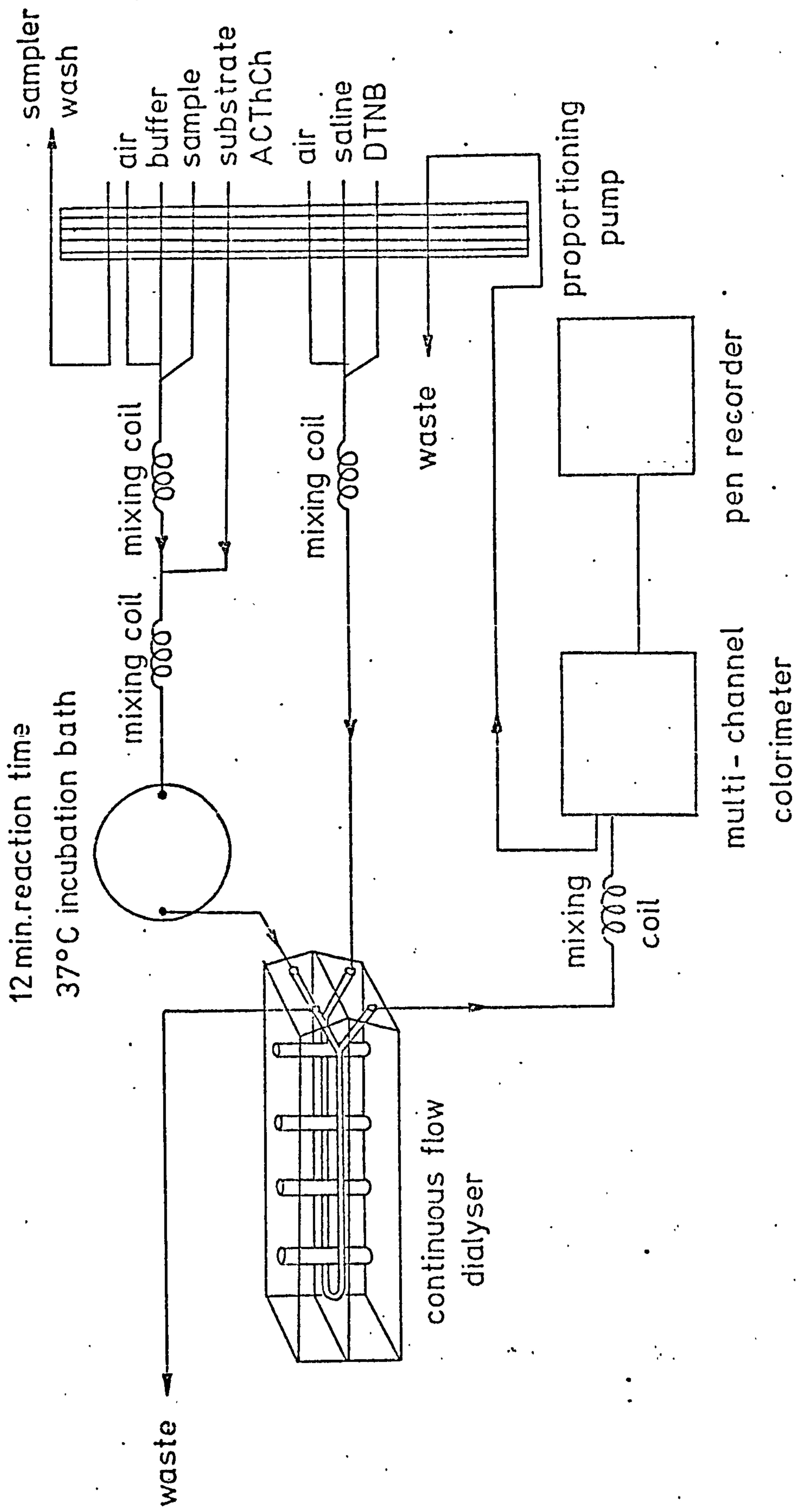


Fig.6. FLOW DIAGRAM FOR ACETYLCHOLINESTERASE ASSAY

using Technicon Auto-analyser 2

A peristaltic pump draws the various components from their storage bottles. Since the speed of the pump is fixed, the rate of pumping is dictated entirely by the internal diameter of the pump tubes. The enzyme sample is injected via a probe from the automatic sampler at a rate of one per minute interspersed with a 10 second wash injection from a reservoir of detergent. The enzyme sample and buffer are mixed thoroughly via a system of coils and are pushed along a tube system. Air bubbles are injected at two second intervals to prevent laminar flow phenomenon mixing adjacent samples in the flow system. This decreases the wash time and ensures that intersample washings effectively decontaminate the tubes. The substrate is then added and mixed. The enzyme incubation mixture is then pumped into a 10 min delay coil which is surrounded by an oil filled heating bath kept at 37°C . The mixture then passes into a continuous flow dialysis system. This is a Perspex block with a 12" U-shaped groove cut into the upper and lower halves of the block. The two blocks fit together with a screw tightened by a 25lb torsion wrench and are separated from each other by a cellophane dialysis membrane. This arrangement means that the two streams, the acceptor and the donor flow over each other and are only separated by the membrane. The dialysis is only 15% efficient in the transfer of products to the acceptor stream and this is a serious limitation of the method since a large reduction in sensitivity is inherent.

From the top stream of the dialyser the products of the reaction (thiocholine) dialyse through the membrane into the lower stream which contains the colour reagent D.T.N.B. The colour so produced in the acceptor stream is mixed once again and led to a continuous flow colorimeter where the extinction at 420 nm is measured. The wavelength is selected

Figure 7. Graph to show the standard curve, using reduced glutathione standard solutions (0 - 200 μ g / ml) for the automated assay of acetylcholinesterase activity.

The standard curve is from 8 independent determinations and the standard errors are less than 1% and are thus too small to be clearly shown.

Figure 8. A copy of a trace obtained from the automated assay of acetylcholinesterase activity.

The traces show the reproducibility between the duplicate samples (within 2%). The peaks do not return to the zero base line; this is the result of the short wash time between samples.

by using an interference filter. The optical density is recorded as a series of peaks which are read off against a calibrating set of glutathione standards.

The use of a detergent is an essential feature also of the operation of the machine, since surface tension can affect the hydrodynamic properties of the flow system. The detergent however must not affect the ongoing reaction. It was found that the use of an 0.2% v/v solution of Brij 35 (30% solution) was compatible with both operations.

2.2.7. Total Protein Assay

The total protein of the samples was estimated by the method of Lowry et al (1951) which has been adapted for the auto-analyser. 0.1ml aliquots of the remaining homogenate were taken in duplicate and made up to a final volume of 1.0 ml with protein solubilising fluid (Table 2). A standard curve was constructed using solutions of Bovine Serum Albumin Cohn Fraction V containing 0 - 100 µg/ml. The solubilised sample is mixed thoroughly with Lowry reagent D and pumped into a 9 min delay coil. The diluted Folin's reagent is then added and mixed. The colour is allowed to develop for 18 min and is then read in a continuous flow colorimeter at 750 nm. This shortening of the development period from 30 to 18 mins is a feature of the auto-analyser and such measurements are routinely conducted in steady state. The optical density of the resulting solution is recorded as a series of peaks which can be read off as concentrations against a standard curve.

A compatible non-phosphate detergent (Levor IV) was used in a 0.2% v/v solution. The reproducibility of the duplicate samples using this method was found to be within 2.0%.

Figure 9 shows a flow diagram of the assay procedure and Table 2 give details of the preparation of reagents.

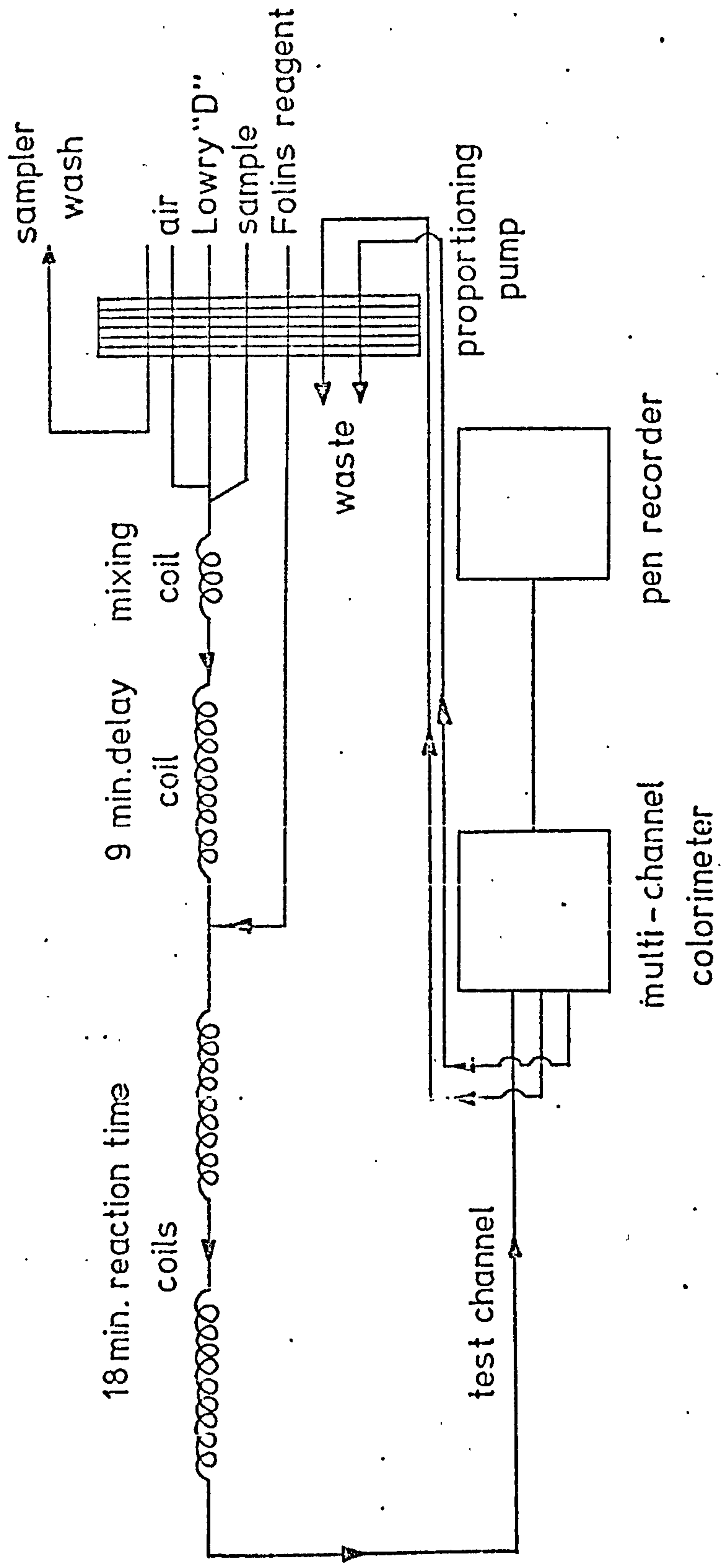


Fig.9 FLOW DIAGRAM FOR TOTAL PROTEIN ASSAY

using Technicon Auto - Analyser 2

Table 2. Details of the preparation of reagents for the assay of total proteins.

1) Solubilising fluid

Sodium chloride (0.9% w / v) containing 5.0 g / litre sodium hydroxide.

2) Lowry reagent

Sodium carbonate (anhydrous) 20 g and Sodium hydroxide 4.0 g were dissolved in distilled water and made up to 1 litre. To 500 mls of this reagent were added 8.0 mls cupric sulphate (1.0% w / v) and 8.0 mls Sodium D - tartrate (2.0 % w / v) and 1.0 ml Levor IV detergent. This mixture was pumped with the protein sample into the auto - analyser. To this mixture was added Folin and Ciocalteus phenol reagent diluted x 2.5 with mixing.

Pump tube sizes.for the assay of total proteins

Lowry D	1.0 ml / min
Protein sample	0.16 ml / min
Folin and Ciocalteus phenol reagent	0.10 ml / min
Distilled water (plus 2% v / v Levor IV)	2.00 ml / min

Figure 10. Graph to show the standard curve, using Bovine serum albumin standard solutions (0 - 100 μ g / ml) from the automated assay of total proteins.

The standard curve is from 8 separate determinations carried out in duplicate. The standard errors are less than 2% and are thus too small to be clearly shown.

Figure 11. A copy of part of a trace obtained from the automated assay of total proteins.

The trace shows the reproducibility between the duplicate samples (within 3%). The peaks do not return to the zero base - line because of the short wash time between samples.

Section 2.3. Results.

2.3.1. The distribution of acetylcholinesterase activity in regions of the rat brain.

The quiet control distribution of acetylcholinesterase activity is shown in Table 3 and Figure 12. The lowest activity was found in the posterior cortex and the highest in the caudate nucleus; the difference in activity between the regions was 20 fold. The standard errors of the measurement of AChE activity of the quiet control group for fairly accessible brain regions eg. cerebellum is low indicating the consistency of the sampling technique and the reliability of the assay.

2.3.2. The effects of 45 minutes sodium-pentobarbitone anaesthesia on the activity of acetylcholinesterase.

The effects of anaesthesia on the activity of AChE is shown in Table 4 and Figure 13.

- 1) No significant difference between the saline injected control group and the anaesthetised group was noted in the cerebellum, ventral cortex, hypothalamus, pons, superior colliculus and olfactory tubercle.
- 2) In all three dorsal cortical regions there was a slight activation of the enzyme activity as compared with the saline injected control group; only in the medial cortex is this significant.
- 3) A significant decrease in enzyme activity following anaesthesia was noted in the caudate nucleus (34%), thalamus (29%) and medulla (23%).

Thus the activity of AChE has been found to change significantly in some regions of the brain following 45 minutes of sodium-pentobarbitone anaesthesia.

TABLE 3. The quiet control distribution of acetylcholinesterase activity.

Region	Mean activity	S.E.M.	n
Frontal cortex	2.531	± 0.185	8
Medial cortex	1.641	± 0.164	7
Posterior cortex	1.629	± 0.074	8
Cerebellum	1.651	± 0.076	8
Ventral cortex	3.122	± 0.272	6
Medulla	5.350	± 0.203	6
Pons	7.622	± 0.337	6
Hypothalamus	3.953	± 0.264	6
Thalamus	5.200	± 0.409	5
Superior colliculus	9.016	± 0.208	5
Olfactory tubercle	31.421	± 0.480	5
Caudate nucleus	31.716	± 1.933	6

Enzyme activity is expressed as μ moles acetylthiocholine hydrolysed / hr / mg proteins.

Figure 12. Histogram of the quiet control distribution of the activity of acetylcholinesterase in regions of the rat brain.

The brain regions have been divided into 3 groups of activity and thus the scales of the axes are different. The columns represent the means of the number of determinations shown in Table 3. The S.E.M. is represented by the vertical bars.

Key:

C		cerebellum
PC		posterior cortex
MC		medial cortex
FC		frontal cortex
VC		Ventral cortex
H		Hypothalamus
T		Thalamus
M		Medulla
P		Pons
SC		Superior colliculus
OT		Olfactory tubercle
CN		Caudate nucleus

Enzyme activity is expressed as μ moles acetylthiocholine hydrolysed / hr / mg proteins.

TABLE 4. The effects of 45 mins anaesthesia on the activity of acetylcholinesterase in regions of the rat brain.

Region	SALINE CONTROL Mean $\pm$ S.E.M.	ANAESTHESIA Mean $\pm$ S.E.M.	n	p
Frontal cortex	1.574 $\pm$ 0.137	1.786 $\pm$ 0.100	5	ns
Medial cortex	1.393 $\pm$ 0.113	1.598 $\pm$ 0.103	5	0.01
Posterior cortex	1.466 $\pm$ 0.180	1.741 $\pm$ 0.254	6	ns
Cerebellum	1.491 $\pm$ 0.158	1.782 $\pm$ 0.615	6	ns
Ventral cortex	4.037 $\pm$ 0.453	3.251 $\pm$ 0.303	7	ns
Medulla	2.633 $\pm$ 0.248	2.024 $\pm$ 0.270	7	0.05
Pons	9.245 $\pm$ 0.376	9.087 $\pm$ 0.399	5	ns
Hypothalamus	2.912 $\pm$ 0.569	2.649 $\pm$ 0.349	6	ns
Thalamus	6.047 $\pm$ 0.912	4.185 $\pm$ 0.845	4	0.01
Superior colliculus	5.210 $\pm$ 0.409	5.944 $\pm$ 0.733	5	ns
Olfactory tubercle	31.762 $\pm$ 2.888	32.051 $\pm$ 1.488	5	ns
Caudate nucleus	31.738 $\pm$ 1.559	21.034 $\pm$ 0.737	6	0.001

Enzyme activity is expressed as μ moles acetylthiocholine hydrolysed / hr / mg proteins.

Figure 13. Histogram to show the effects of 45 mins anaesthesia on the activity of acetylcholinesterase in regions of the rat brain.

The rat brain regions have been divided into 3 groups of activity and thus the scales on the axes are different. The columns represent the mean of the number of observations shown in Table 4. The S.E.M. is represented by the vertical bars. The groups that are statistically significant are indicated by shading.

Key: FC Frontal cortex
MC Medial cortex
PC Posterior cortex
C Cerebellum
VC Ventral cortex
H Hypothalamus
T Thalamus
M Medulla
SC superior colliculus
P pons
OT Olfactory tubercle
CN Caudate nucleus

Enzyme activity is expressed as μ moles acetylthiocholine hydrolysed / hr / mg proteins.

c = control animal

a = anaesthetised animal

2.3.3. The effects of sodium-pentobarbitone anaesthesia on the activity of AChE measured 24 hours after the period of anaesthesia.

It was reported by Vernadakis & Rutledge (1973) that when rats were killed during sodium-pentobarbitone anaesthesia there were no changes in the activity of AChE in either of the brain regions they looked at (cerebral cortex and corpus striatum). It is significant to note that male rats only were used in their study and that similar experiments in this laboratory indicated that changes in activity found in the female rat were not paralleled by changes in a comparable group of male rats.

Other rats were allowed to recover and were killed one and three weeks after the period of general anaesthesia. There was a significant decrease in AChE activity after one week and after three weeks in the corpus striatum (Vernadakis & Rutledge 1973).

Thus it was decided to investigate whether the changes in AChE activity obtained were reversible and if so to determine the time course of the reversal. The experimental rats in this group were injected with the standard anaesthetic dose of sodium-pentobarbitone (60mg/kg) and allowed to recover. They were then kept for a further 24 hours before they were killed and their brains assayed for AChE. Complete recovery from this dose of anaesthetic was found to take on average $5\frac{1}{2}$ hours. A control group were injected with an equivalent volume of dextrose-saline at the same time as the experimental group and killed 24 hours later. The results are shown in Table 5 and Figure 14.

- 1) As in the case when animals were killed after 45 min of anaesthesia, there was no difference between the enzyme activity of the saline injected control group and the anaesthetised group in the cerebellum , ventral cortex,

TABLE 5. The effects of anaesthesia on the activity of acetylcholinesterase measured 24 hours after the period of anaesthesia.

Region	CONTROL Mean $\pm$ S.E.M.	ANAESTHESIA Mean $\pm$ S.E.M.	n	p
Frontal cortex	2.060 $\pm$ 0.09	2.392 $\pm$ 0.15	4	0.05
Medial cortex	2.522 $\pm$ 0.31	1.792 $\pm$ 0.16	4	ns
Posterior cortex	2.098 $\pm$ 0.17	1.774 $\pm$ 0.22	4	ns
Cerebellum	1.695 $\pm$ 0.28	1.595 $\pm$ 0.14	4	ns
Ventral cortex	4.383 $\pm$ 0.50	3.769 $\pm$ 0.41	4	ns
Medulla	2.855 $\pm$ 0.79	2.479 $\pm$ 0.79	4	ns
Pons	8.885 $\pm$ 0.39	8.659 $\pm$ 0.42	5	ns
Hypothalamus	2.946 $\pm$ 0.21	2.909 $\pm$ 0.19	4	ns
Thalamus	4.781 $\pm$ 0.84	4.932 $\pm$ 0.38	5	ns
Superior colliculus	4.133 $\pm$ 0.29	3.477 $\pm$ 0.48	4	ns
Olfactory tubercle	33.463 $\pm$ 2.11	31.823 $\pm$ 2.14	4	ns
Caudate nucleus	33.101 $\pm$ 2.05	30.834 $\pm$ 2.03	4	ns

Enzyme activity is expressed as μ moles acetylthiocholine hydrolysed / hr / mg proteins.

Figure 14. The effects of anaesthesia on the activity of acetylcholinesterase measured 24 hours after the period of anaesthesia.

The brain regions have been divided into 3 groups of activity and thus the scale of the axes is different. The columns represent the means of the number of determinations shown in Table 5. The S.E.M. is represented by the vertical bars. The group that is significant is indicated by shading.

Key: C Cerebellum
 PC Posterior cortex
 MC Medial cortex
 FC Frontal cortex
 VC Ventral cortex
 H Hypothalamus
 T Thalamus
 M Medulla
 SC Superior colliculus
 P Pons
 OT Olfactory tubercle
 CN Caudate nucleus

Enzyme activity is expressed as μ moles acetylthiocholine hydrolysed / hr / mg proteins.

c = control animal

a = anaesthetised animal

pons, hypothalamus, superior colliculus and olfactory tubercle.

- 2) The significant activation of enzyme activity seen in the medial cortex after 45 mins of anaesthesia was followed by a non significant decrease in activity after 24 hours. In the frontal cortex there was a significant activation of enzyme activity after 24 hours (16%). The enzyme activity of the posterior cortex of the anaesthetised animal was not significantly different from the saline control.
- 3) In the caudate nucleus, there was again a decrease in the enzyme activity but this was not significant. The significant decrease in AChE activity seen in the thalamus and medulla after 45 min of anaesthesia was not observed after 24 hours.

Thus the significant changes in enzyme activity observed after 45 minutes of anaesthesia were not seen 24 hours later, indicating that the activity changes are transient.

2.3.4. The effects of pentobarbitone-sodium anaesthesia on the activity of AChE in vitro.

It has been shown that the activity of AChE is affected significantly in some regions of the brain following anaesthesia. This can be explained in two ways:

- 1) The anaesthetic is eliciting these effects by some intermediate mechanism in specific and localised areas only.
- 2) These regional effects could be due to the direct action of the anaesthetic on the enzyme activity. In this case a factor such as the heterogenous nature of cerebral blood flow could be causing differential regional concentrations of the anaesthetic, which would account for the observed effects.

In order to test the second hypothesis, an experiment was performed to determine whether pentobarbitone-sodium could effect, in vitro, the activity of the enzyme. It was calculated on the basis of data presented by Sharpless (1965) that 10% of the

i.p. injected dose of anaesthetic would be present in the brain 10 minutes following the injection. The caudate nucleus was chosen for this study since this was the region which had shown the greatest change in activity after 45 minutes of anaesthesia. Three concentrations of anaesthetic were prepared: 1.0 mg / ml; 0.1 mg / ml and 0.01 mg / ml of homogenate by diluting the standard sodium pentobarbitone. The homogenates were incubated for 45 minutes at 37°C before assaying for AChE. The results are presented in Table 6.

Table 6. The effects of sodium - pentobarbitone anaesthesia on the activity of AChE of the caudate nucleus in vitro.

CONTROL	HOMOGENATE + 1.0 mg / ml anaesthetic	HOMOGENATE + 0.1 mg / ml anaesthetic	HOMOGENATE + 0.01 mg / ml anaesthetic
29.78	29.25	30.08	29.13
± 0.53	± 0.83	± 0.74	± 0.36
n = 4	n = 4	n = 4	n = 4

Enzyme activity is expressed in terms of μ moles acetylthiocholine hydrolysed / hr / mg proteins.

The results indicate that sodium pentobarbitone does not exert a direct effect on the activity of AChE.

2.3.5. The development of a specific assay for AChE.

Pseudocholinesterase will also hydrolyse acetylthiocholine, although it is not its optimal substrate so that the activity of this enzyme will also contribute to the enzyme activity observed. In order to differentiate between the effects of behavioural treatments on the activity of AChE as distinct from any changes due to pseudocholinesterase, it was necessary to develop specific assays for the two enzymes. The specific inhibition of pseudoChE by tetramonoisopropylpyrophosphortetramide (iso - OMPA) was investigated in order to develop a specific

assay for AChE.

The whole caudate nucleus was dissected out and homogenised in 12 ml of buffer. Duplicate aliquots of 0.5 ml of homogenate were added to a further 0.4 ml of buffer and 0.1 ml of iso-OMPA of the appropriate concentration. The range of concentrations used was 0.1 μ M to 100 μ M final volume.

The above experiment was repeated using 1mM butyrylthiocholine as substrate. The results are shown in Figure 15.

In the presence of iso-OMPA at a concentration of 10 μ M the activity of cholinesterase with acetylthiocholine as substrate is inhibited by 12% of its original value and thereafter does not vary significantly. At this concentration with butyrylthiocholine as substrate the activity of cholinesterases is inhibited by 85%. Thus by conducting the assay for AChE in the presence of this concentration of inhibitor, a relatively specific assay for AChE can be obtained.

2.3.6. Modification of the technique for measuring the activity of AChE and total protein concentration and its use to determine the effect of anaesthesia on these parameters.

In the previously reported experiments determining the effects of anaesthesia on AChE activity, the results were expressed in terms of mg of proteins. If anaesthesia alters the metabolism of other proteins besides AChE, the results will be affected. Thus a change in the activity of AChE expressed in terms of mg total proteins may reflect a change in protein content and not in the activity of AChE itself. To investigate this possibility a series of experiments were performed to determine whether the total protein content of the brain regions alters during anaesthesia.

The dissected samples were thawed and the surface water removed by partially drying the samples on Whatman filter

Figure 15. The specific inhibition of pseudocholinesterase by tetramonoisopropylpyrophosphortetramide the caudate nucleus.

- A. Inhibition of pseudocholinesterase with acetylthiocholine as substrate. The values given are a mean of 3 independent determinations and the standard errors of the points are less than 3.01%.
- B. Inhibition of pseudocholinesterase with butyrylthiocholine as substrate. The values given are the means of 3 independent determinations.

paper no 54. The samples were weighed in small vials on an Oertling five figure balance. The standard errors of the measurements were quite high: this was largely due to the difficulty in transferring the samples from weighing vial to homogenising tube with such a small volume of buffer available for washing out the sample. However these preliminary experiments did indicate that protein concentration did change in some regions of the brain following anaesthesia. These findings indicated that to determine the effect of any behavioural treatment on AChE activity it is necessary to express the activity in terms independent of other proteins; thus in future experiments enzyme activity is expressed in terms of wet weight of brain tissue.

The following set of experiments were conducted to establish the effects of anaesthesia on AChE activity expressed in terms of wet weight of tissue and also to establish the effect of anaesthesia on total protein content. The experimental technique was improved by making a Teflon homogeniser which fitted a standard set of small glass tubes and weighing the brain samples in the tubes before homogenising. This eliminates the error in transferring tissue from weighing vessel to homogenising tube. PseudoChE was inhibited by the addition of 10 μ M iso-OMPA.

The AChE activity and the protein content of a quiet control group was also determined and the results compared with the saline injected control group and the anaesthetised group. The results are presented in Tables 7 & 8 and Figures 16 & 17.

1) Changes in the activity of AChE / g wet weight.

1) The results indicate that enzyme activity can be expressed in terms of wet weight with a reasonable degree of accuracy. This is indicated by the values of the standard errors in the measurement of AChE activity in the caudate nucleus of the

Table 7. The effects of 45 mins anaesthesia on the activity of acetylcholinesterase in regions of the rat brain.

Region	Quiet Control	Saline Control	Anaesthesia
Frontal cortex	3.95 $\pm$ 0.16	5.12 $\pm$ 0.21 ⁺⁺	5.08 $\pm$ 0.36 ⁺
Medial cortex	4.76 $\pm$ 0.03	4.56 $\pm$ 0.18	3.99 $\pm$ 0.20 ⁺⁺⁺
Posterior cortex	4.41 $\pm$ 0.15	4.89 $\pm$ 0.31	5.06 $\pm$ 0.33
Cerebellum	3.84 $\pm$ 0.44	3.52 $\pm$ 0.17	4.45 $\pm$ 0.22 ^{**}
Ventral cortex	8.91 $\pm$ 0.65	7.79 $\pm$ 0.83	8.40 $\pm$ 0.77
Hippocampus	11.87 $\pm$ 1.16	11.83 $\pm$ 1.06	8.73 $\pm$ 0.21 ⁺
Hypothalamus	9.90 $\pm$ 0.59	7.75 $\pm$ 1.10	9.81 $\pm$ 2.15
Thalamus	10.61 $\pm$ 0.80	12.58 $\pm$ 0.41	8.32 $\pm$ 0.31 ⁺⁺⁺
Medulla	6.38 $\pm$ 0.59	5.51 $\pm$ 0.30	8.03 $\pm$ 0.42 ^{****}
Pons	27.33 $\pm$ 1.48	25.37 $\pm$ 1.30	27.59 $\pm$ 2.31
Superior colliculus	22.42 $\pm$ 2.14	19.84 $\pm$ 4.84	18.48 $\pm$ 2.87
Olfactory tubercle	103.88 $\pm$ 7.47	95.01 $\pm$ 1.78	83.99 $\pm$ 4.95
Caudate nucleus	98.11 $\pm$ 3.50	101.30 $\pm$ 6.25	76.97 $\pm$ 4.80 ⁺⁺⁺

Enzyme activity is expressed as μ moles acetylthiocholine hydrolysed / min / g wet weight. The values given are the means of at least 6 determinations.

+ = significantly different from quiet controls (+ p < 0.02;

++ p < 0.01; +++ p < 0.001). * = significantly different from saline controls (* p < 0.02; ** p < 0.01; *** p < 0.001).

Figure 16. The effects of 45 mins anaesthesia on the activity of acetylcholinesterase in regions of the rat brain.

The columns represent the mean of the number of determinations shown in Table 7. The white columns represent the quiet controls; the black columns represent the saline injected controls and the stippled columns the anaesthetised group. The S.E.M. is represented by the vertical bars.

Key: FC Frontal cortex
 MC Medial cortex
 PC Posterior cortex
 C Cerebellum
 VC Ventral cortex
 HP Hippocampus
 H Hypothalamus
 T Thalamus
 M Medulla
 P Pons
 SC Superior colliculus
 OT Olfactory tubercle
 CN Caudate nucleus

□ = significantly different from quiet controls

△ = significantly different from saline injected controls.

Results are expressed in terms of μ moles acetylthiocholine hydrolysed / min / g wet weight.

control rats relative to protein content and wet weight i.e.

X 32.61 $\pm$ 1.32 μ Moles acetylthiocholine hydrolysed / h / mg proteins
and 98.11 $\pm$ 3.5 μ moles acetylthiocholine hydrolysed / min / g wet
weight.

ii) The rank order of the distribution of AChE / g wet weight is slightly different from the order of activity / mg proteins. The activity expressed in terms of wet weight varied from 3.84 units in the cerebellum to 103.88 units in the olfactory tubercle , a 27 fold difference in activity.

iii) There was no change in enzyme activity following anaesthesia compared with either the quiet controls or the saline injected controls in the posterior cortex, ventral cortex, hypothalamus, pons, superior colliculus and olfactory tubercle.

iv) Only in the frontal cortex is there a significant change in the enzyme activity between the quiet control and saline control animals (an increase of 29%). A similar difference in activity in this region was observed in the anaesthetised group and must be attributed to stress during handling and injection. In all other regions of the brain investigated stress during handling and injection had no effect on enzyme activity.

v) There is a significant decrease in enzyme activity compared with both quiet controls and saline controls in the medial cortex 16.6% (quiet) and 12.5% (saline control); hippocampus (26.5% (quiet) and 26.2% (saline control); thalamus 21.6% (quiet) and 33.8% (saline control); caudate nucleus 21.6% (quiet) and 24.0% (saline control.).

vi) There is a significant increase in enzyme activity in the medulla when compared with the quiet controls (25%) and with the saline controls (46%). In the cerebellum, there is a significant increase in enzyme activity compared with the saline

controls (26.4%)³.

2) Changes in protein content of brain regions.

i) The protein content does not differ very much between regions: the highest value being found in the caudate nucleus (172.33 mg / g wet weight) and the lowest in the hypothalamus (142.04 mg / g wet weight).

ii) The protein content of the saline injected group was not significantly different from the quiet control group in any measured region of the brain.

iii) There is a significant increase in protein content in the posterior cortex compared with the quiet controls (8.02%) and with the saline injected group (18.3%). In the caudate nucleus, there is a significant increase in protein content compared with the saline controls (18.9%).

iv) In the ventral cortex, there is a significant decrease in enzyme activity compared to the quiet control group (8.8%).

Thus, the activity of AChE and the total protein content of some regions of the brain change following sodium pentobarbitone anaesthesia.

Table 8. The effects of 45 mins anaesthesia on the total protein content of regions of the rat brain.

Region	Quiet Control	Saline Control	Anaesthesia
Frontal cortex	159.99 $\pm$ 5.26	146.71 $\pm$ 10.39	163.32 $\pm$ 10.86
Medial cortex	154.19 $\pm$ 6.50	140.95 $\pm$ 6.04	156.23 $\pm$ 6.48
Posterior cortex	150.81 $\pm$ 4.26	137.71 $\pm$ 5.96	162.91 $\pm$ 4.48 ⁺⁺
Cerebellum	160.82 $\pm$ 7.15	137.61 $\pm$ 9.48	144.41 $\pm$ 4.47
Ventral cortex	164.76 $\pm$ 3.60	154.72 $\pm$ 18.36	150.34 $\pm$ 3.00 ⁺
Hippocampus	146.69 $\pm$ 5.04	152.33 $\pm$ 8.55	162.45 $\pm$ 13.45
Hypothalamus	142.04 $\pm$ 7.78	160.41 $\pm$ 3.82	180.80 $\pm$ 22.46
Thalamus	155.48 $\pm$ 24.60	177.37 $\pm$ 26.94	166.95 $\pm$ 2.10
Medulla	146.92 $\pm$ 4.49	152.08 $\pm$ 14.89	164.04 $\pm$ 24.02
Pons	157.40 $\pm$ 8.26	145.07 $\pm$ 14.49	145.85 $\pm$ 12.77
Superior colliculus	143.19 $\pm$ 6.30	152.30 $\pm$ 4.40	166.87 $\pm$ 22.60
Olfactory tubercle	169.91 $\pm$ 5.60	159.61 $\pm$ 24.14	168.02 $\pm$ 21.93
Caudate nucleus	172.33 $\pm$ 7.62	160.77 $\pm$ 3.58	204.96 $\pm$ 13.86 [*]

Results are expressed in terms of mg total proteins / g wet weight.

+ = significantly different from quiet control (p < 0.05)

* = significantly different from saline control (p < 0.05).

The values given are the means of at least 6 determinations.

Figure 17. The effects of 45 mins anaesthesia on the total protein content of regions of the rat brain.

The columns represent the mean of the number of determinations shown in Table 8. The white columns represent the quiet controls; the black columns represent the saline injected controls and the stippled columns represent the anaesthetised group. The S.E.M. is represented by the vertical bars.

Key: FC Frontal cortex
 MC Medial cortex
 PC Posterior cortex
 C Cerebellum
 VC Ventral cortex
 HP Hippocampus
 H Hypothalamus
 T Thalamus
 M Medulla
 P Pons
 SC Superior colliculus
 OT Olfactory tubercle
 CN Caudate nucleus

□ = significantly different from quiet controls

△ = significantly different from saline injected controls

Results are expressed in terms of mg total proteins / g wet weight.

2.3.7. The effects of 45 minutes pentobarbitone - sodium anaesthesia on the water content of regions of the brain.

If anaesthesia alters the water content of the brain regions investigated, there would be a difference in the wet weight determinations between the two groups. The water content of the regions of the brain of anaesthetised and control rats were therefore compared to see whether anaesthesia changes water content and hence influences total protein estimations.

A series of experiments were performed comparing wet and dry weights of brain samples from anaesthetised and control rats. The anaesthetisation procedure and wet weight determinations were conducted as previously described. After wet weight determination the beakers containing the brain samples were heated to constant weight in an oven at 105°C. Drying of samples was found to take a minimum of six hours at this temperature. The effects of anaesthesia on water content of the brain regions is shown in Table 9.

The water content of the regions varied from 71.13% in the hypothalamus of the saline injected group to 85.3% in the olfactory tubercle of the experimental group. The values obtained agree well with those reported by Donaldson & Hatai (1931) who found that the percentage of water in the total brain of female rats of the same age to be 79% and the water content of the cerebellum to be 79%.

The ventral cortex is the only region of the brain to show a significant difference in water content between the saline injected and the anaesthetised group. There was found to be a increase of 3% in this region (i.e. from 80.4% to 83.8%). Changes in protein content and AChE activity cannot be reliably determined in this region by expressing them in terms of wet weight. In all other regions investigated changes in water content

TABLE 9. The effects of anaesthesia on water content of regions of the rat brain.

Region	CONTROL Mean $\pm$ S.E.M.	ANAESTHESIA Mean $\pm$ S.E.M.	n	p
Frontal cortex	80.72 $\pm$ 0.85	82.22 $\pm$ 1.20	4	ns
Medial cortex	82.21 $\pm$ 0.88	83.54 $\pm$ 1.10	6	ns
Posterior cortex	83.64 $\pm$ 1.59	80.92 $\pm$ 0.83	6	ns
Cerebellum	79.70 $\pm$ 0.65	79.73 $\pm$ 0.49	6	ns
Ventral cortex	80.44 $\pm$ 0.79	83.82 $\pm$ 1.19	5	0.05
Hippocampus	81.60 $\pm$ 0.87	81.34 $\pm$ 0.46	5	ns
Medulla	72.20 $\pm$ 2.35	73.79 $\pm$ 2.94	5	ns
Pons	73.79 $\pm$ 3.70	81.77 $\pm$ 1.36	4	ns
Hypothalamus	71.13 $\pm$ 3.65	81.94 $\pm$ 3.58	5	ns
Thalamus	77.64 $\pm$ 1.19	79.28 $\pm$ 0.99	5	ns
Superior colliculus	75.76 $\pm$ 2.21	76.82 $\pm$ 3.65	6	ns
Olfactory tubercle	78.03 $\pm$ 2.22	85.26 $\pm$ 3.76	5	ns
Caudate nucleus	77.83 $\pm$ 0.90	77.91 $\pm$ 0.59	5	ns

Results are expressed in terms of % water content.

are not significant and so would not affect the measurement of AChE activity in terms of wet weight.

2.3.8. The effects of shock avoidance conditioning and electrical stimulation on the AChE activity and total protein content of regions of the rat brain.

The effects of shock avoidance conditioning and electrical stimulation via foot shock on AChE activity and total protein content was determined. The results are shown in Tables 10 & 11 and Figures 18 & 19.

1) The effects of behavioural treatments on AChE activity.

- a) There was no significant change in enzyme activity in the medial cortex, cerebellum, pons and superior colliculus between 1) quiet controls and yoked controls ii) quiet controls and conditioned animals, or iii) yoked controls and conditioned animals.
- b) Electrical stimulation caused a significant increase in enzyme activity in the frontal cortex (23.3%) and medulla (52.4%).
- c) A decrease in AChE activity was observed following electrical stimulation in the hypothalamus (40%); thalamus (28.7%); olfactory tubercle (29.3%) and caudate nucleus (24.0%).
- d) There was no significant difference between the yoked controls and quiet controls in the posterior cortex, ventral cortex and hippocampus.
- e) There was a significant increase in the enzyme activity of the conditioned group compared with the yoked control group (17.1%) and compared with the quiet control group (22.9%) in the posterior cortex, indicating that this change was specifically related to the conditioning experience.
- f) In the hippocampus, there was a significant increase in the enzyme activity between the yoked control and conditioned group (17.0%) ; but no significant difference between the

Table 10. The effects of electrical stimulation and shock avoidance conditioning on the activity of acetylcholinesterase in regions of the rat brain.

Region	Quiet Control	Yoked Control	Conditioned
Frontal cortex	3.95 $\pm$ 0.16	4.88 $\pm$ 0.13 ⁺⁺⁺	4.40 $\pm$ 0.43
Medial cortex	4.55 $\pm$ 0.15	4.36 $\pm$ 0.16	4.87 $\pm$ 0.25
Posterior cortex	4.46 $\pm$ 0.10	4.68 $\pm$ 0.21	5.48 $\pm$ 0.17 ⁺⁺⁺⁺
Cerebellum	3.84 $\pm$ 0.44	4.13 $\pm$ 0.25	3.97 $\pm$ 0.32
Ventral cortex	8.91 $\pm$ 0.65	7.64 $\pm$ 0.75	6.91 $\pm$ 0.67 ⁺
Hippocampus	11.35 $\pm$ 1.36	9.91 $\pm$ 0.15	11.60 $\pm$ 0.32 ^{**}
Hypothalamus	9.90 $\pm$ 0.59	6.94 $\pm$ 1.25 ⁺	9.33 $\pm$ 0.65 [*]
Thalamus	9.97 $\pm$ 0.95	7.11 $\pm$ 0.56 ⁺	10.25 $\pm$ 0.65 ^{**}
Medulla	6.78 $\pm$ 0.58	10.33 $\pm$ 2.37 ⁺	9.10 $\pm$ 0.91
Pons	27.33 $\pm$ 1.48	27.56 $\pm$ 1.28	28.32 $\pm$ 1.02
Superior colliculus	22.42 $\pm$ 2.14	20.03 $\pm$ 2.50	24.69 $\pm$ 1.20
Olfactory tubercle	103.88 $\pm$ 7.47	73.51 $\pm$ 5.21 ⁺	73.13 $\pm$ 6.00 ⁺
Caudate nucleus	98.11 $\pm$ 3.50	74.55 $\pm$ 4.18 ⁺⁺	81.93 $\pm$ 5.50 ⁺

Enzyme activity is expressed in terms of μ moles acetylthiocholine hydrolysed / min / g wet weight. The values given are the means of at least 6 determinations.

+ = significantly different from quiet controls (+p < 0.05;

++ p < 0.01; +++ p < 0.001). * = significantly different from yoked controls (* p < 0.05; ** p < 0.01; *** p < 0.001).

Figure 18. The effects of shock avoidance conditioning and electrical stimulation on the activity of acetylcholinesterase in regions of the rat brain.

The columns represent the mean of the number of observations shown in Table 10. The white columns represent the quiet controls; the black columns represent the the yoked controls and the stippled columns represent the shock avoidance conditioned group. The S.E.M. is represented by the vertical bars.

Key: FC Frontal cortex
 MC Medial cortex
 PC Posterior cortex
 C Cerebellum
 VC Ventral cortex
 HP Hippocampus
 H Hypothalamus
 T Thalamus
 M Medulla
 P Pons
 SC Superior colliculus
 OT Olfactory tubercle
 CN Caudate nucleus

□ = significantly different from quiet controls

△ = significantly different from yoked controls.

The results are expressed in terms of μ moles acetylthiocholine hydrolysed / min / g wet weight.

Table 11. The effects of electrical stimulation and shock avoidance conditioning on the total protein content of regions of the rat brain.

Region	Quiet Control	Yoked Control	Conditioned
Frontal cortex	159.99 $\pm$ 5.26	133.45 $\pm$ 6.40 ⁺⁺	132.41 $\pm$ 10.70 ⁺
Medial cortex	154.19 $\pm$ 6.51	145.36 $\pm$ 9.00	127.14 $\pm$ 8.07 ⁺⁺
Posterior cortex	150.81 $\pm$ 4.26	163.13 $\pm$ 11.79	134.99 $\pm$ 5.02 ⁺
Cerebellum	160.82 $\pm$ 7.15	145.90 $\pm$ 4.52	134.61 $\pm$ 2.45 ⁺⁺
Ventral cortex	164.76 $\pm$ 3.60	143.33 $\pm$ 4.40 ⁺	123.76 $\pm$ 3.41 ⁺⁺⁺⁺
Hippocampus	146.69 $\pm$ 5.04	130.30 $\pm$ 1.81 ⁺	131.52 $\pm$ 7.66 ⁺
Hypothalamus	142.04 $\pm$ 7.78	168.03 $\pm$ 27.20	122.44 $\pm$ 9.20
Thalamus	155.48 $\pm$ 24.61	136.58 $\pm$ 7.46	168.58 $\pm$ 3.23 ^{**}
Medulla	146.92 $\pm$ 4.49	135.09 $\pm$ 4.20	163.45 $\pm$ 13.21
Pons	157.40 $\pm$ 8.26	155.48 $\pm$ 14.40	158.73 $\pm$ 15.81
Superior colliculus	143.19 $\pm$ 6.30	154.74 $\pm$ 13.10	147.53 $\pm$ 8.81
Olfactory tubercle	169.91 $\pm$ 9.70	151.09 $\pm$ 24.30	130.13 $\pm$ 13.16
Caudate nucleus	172.33 $\pm$ 7.63	133.73 $\pm$ 14.11 ⁺	141.20 $\pm$ 10.90 ⁺

Results are expressed as mg total proteins / g wet weight. The values given are the means of at least 6 determinations.

+ = significantly different from quiet controls (+ p < 0.05

++ p < 0.01; +++ p < 0.001). * = significantly different from

yoked controls (* p < 0.05; ** p < 0.01; *** p < 0.001).

Figure 19. The effects of shock avoidance conditioning and electrical stimulation on the total protein content of regions of the rat brain.

The columns represent the mean of the number of determinations shown in Table 11. The white columns represent the quiet controls; the black columns represent the yoked controls and the stippled columns represent the shock avoidance conditioned group. The S.E.M. is represented by the vertical bars.

Key: FC Frontal cortex
 MC Medial cortex
 PC Posterior cortex
 C Cerebellum
 VC Ventral cortex
 HP Hippocampus
 H Hypothalamus
 T Thalamus
 M Medulla
 P Pons
 SC Superior colliculus
 OT Olfactory tubercle
 CN Caudate nucleus

□ = significantly different from quiet controls

△ = significantly different from yoked controls

Results are expressed in terms of mg total proteins / g wet weight.

quiet control and conditioned groups.

- g) In the ventral cortex, the activity of the conditioned group was significantly lower than the quiet control group (22.3%); the activity of the conditioned group was not significantly lower than the yoked control group.
- h) In the caudate nucleus, there was a significant decrease in the activity of AChE_{of the conditioned group} compared with the quiet controls (16.5%); there was a similar decrease in activity in the yoked control group compared with the quiet controls, which indicates that the effect is probably a result of stress following electrical stimulation. Similarly , in the olfactory tubercle there is a decrease in enzyme activity compared with the quiet control group (29.6%) which is comparable to the decrease in enzyme activity between the quiet control group and the yoked control group; indicating that in this region too the decrease in enzyme activity is probably a result of stress following electrical stimulation.
- i) In the thalamus, the enzyme activity of the trained group is significantly higher than the yoked controls (44.2%) but not different from the quiet controls. However, the yoked controls are significantly lower than the quiet controls indicating that in this region the effect of stress is to lower the enzyme activity while the effect of the conditioning is to increase the enzyme activity. Similarly in the hypothalamus the activity of the conditioned group is significantly different from the yoked controls but not from the quiet controls.
- j) There is no significant difference between the quiet control group and the conditioned group or between the yoked control group and the conditioned group in the frontal cortex and medulla.

Thus the activity of AChE in some regions of the brain was found to alter significantly following either electrical stimulation or shock avoidance conditioning.

2) The effects of behavioural treatments on the total protein content

- a) There is no change in the total protein content between
 - i) the quiet controls and the yoked controls, ii) the quiet control group and the conditioned group or iii) the yoked control group and the conditioned group in the medulla pons, hypothalamus, olfactory tubercle and superior colliculus.
- b) There was a decrease in protein content in the yoked controls compared with the quiet controls in the frontal cortex (16.6 %) , ventral cortex (13.0%) , hippocampus (11.2%) and caudate nucleus (22.4%).
- c) There was no significant difference between the yoked controls and quiet controls in the medial cortex, posterior cortex, cerebellum and thalamus.
- d) There was a significant decrease in total protein content of the conditioned group compared with the quiet control group (24.9%) and compared with the yoked control group (13.7%) in the ventral cortex, indicating that this change may be specifically related to the training experience.
- e) In the thalamus there was a significant increase in total protein of the conditioned group compared to the yoked control group (23.4%); there was no similar difference between the quiet control group and the conditioned group.
- f) There were significant decreases in protein content between the quiet control groups and conditioned groups in the medial cortex (17.5%) , posterior cortex (10.5%) and cerebellum (16.3%). In all of these regions there is a slight but non significant decrease between the yoked controls and conditioned groups.

g) Total protein content of the conditioned group is significantly lower than the protein content of the quiet control group in the frontal cortex (17.2%), hippocampus (10.4%) and in the caudate nucleus (18.1%). However, in all of these areas there is a similar decrease in the protein content of the yoked controls compared with the quiet controls, which indicates that the decrease is probably a result of stress following electrical stimulation in the shuttle box.

Changes in total proteins are, therefore, seen in some regions of the brain following either electrical stimulation via foot shock or shock avoidance conditioning. Thus it is necessary to estimate AChE activity in terms of wet weight to obtain any meaningful relationship between behavioural treatments and enzyme activity.

2.3.9. Summary of results.

- 1) The quiet control distribution of AChE activity was determined in 12 regions of the rat brain and the effects of anaesthesia on its activity was determined. It was found that anaesthesia produced an increase in enzyme activity in the medial cortex and an inhibition of activity in the medulla, thalamus and caudate nucleus.
- 2) When the effects of anaesthesia was determined 24 hours following the period of general anaesthesia, the significant decrease in activity in the medulla, thalamus and caudate nucleus and the significant increase in the medial cortex were no longer seen, indicating that these are short term changes. However, there was a significant increase in enzyme activity in the frontal cortex 24 hours after the period of anaesthesia.
- 3) It was shown that the changes in enzyme activity were not due to a direct effect of the anaesthetic on the enzyme.
- 4) A specific assay for AChE was developed: it was found that

iso-OMPA at a concentration of 10 μ M selectively inhibited pseudocholinesterase activity.

- 5) Preliminary experiments indicated that anaesthesia produced changes in the protein concentration of some regions of the brain. This indicated that it was necessary to measure the activity of AChE in terms independent of other proteins. The technique for determining AChE activity and protein content was modified and used to determine the effects of anaesthesia electrical stimulation and shock avoidance conditioning on these parameters. The results are summarised in Tables 12 and 13. It must be noted here that expressing results in terms of percentage values tends to exaggerate differences especially where rates are low and the numbers small.
- 6) Pentobarbitone sodium anaesthesia was found to produce an increase in protein content in some regions of the rat brain i.e. posterior cortex, caudate nucleus and a decrease in protein content in the ventral cortex. This indicated that in order to investigate changes in activity of AChE following specified treatments it was necessary to measure the activity of the enzyme in terms independent of other proteins.
- 7) AChE activity expressed in terms of wet weight was found to increase in the cerebellum, medulla and to decrease in the medial cortex, hippocampus, thalamus and caudate nucleus.
- 8) The water content of the regions of the regions of the brain investigated was unaltered by anaesthesia except in the ventral cortex where there was a small increase. This indicated that in all other regions the protein content and AChE activity could be reliably determined by expressing results in terms of wet weight.
- 9) A decrease in protein content of the rat brain was observed following electrical stimulation via foot shock in the

frontal cortex, ventral cortex, hippocampus and caudate nucleus. Following shock avoidance conditioning, decreases in protein content were observed in the medial cortex, posterior cortex, cerebellum and ventral cortex; in the thalamus an increase in protein content was observed.

10) Since there are changes in brain protein content following these treatments it was necessary to measure AChE activity in terms of wet weight to study the relationship between behavioural treatments and levels of AChE activity. Changes in AChE activity were observed following electrical stimulation via foot shock in the frontal cortex and medulla where there was an increase in activity and in the hypothalamus, thalamus, olfactory tubercle and caudate nucleus where there was a decrease in activity. Following shock avoidance conditioning the activity of AChE increased in the posterior cortex, hippocampus, hypothalamus and thalamus ; and the activity of the enzyme decreased in the ventral cortex.

Table 12. The effects of behavioural treatments on the activity of acetylcholinesterase in regions of the rat brain.

Region	Saline Control	Anaesthesia	Yoked Control	Avoidance Conditioned
Frontal cortex	+29.6% [□]	+28.6% [□]	+23.5% [□]	-
Medial cortex	-	-16.6% [□] -12.5% [△]	-	-
Posterior cortex	-	-	-	+22.9% [□] +17.1% [△]
Cerebellum	-	+26.4% [△]	-	-
Ventral cortex	-	-	-	-22.3% [□]
Hippocampus	-	-26.5% [□] -26.2% [△]	-	+17.0% [△]
Hypothalamus	-	-	-40.0% [□]	+57.1% [△]
Thalamus	-	-21.6% [□] -33.8% [△]	-28.7% [□]	+44.2% [△]
Medulla	-	+ 25.9% [□] + 45.7% [△]	+52.4% [□]	-
Pons	-	-	-	-
Superior colliculus	-	-	-	-
Olfactory tubercle	-	-	-29.3% [□]	-29.6% [□]
Caudate nucleus	-	-21.6% [□] -24.0% [△]	-24.0% [□]	-16.5% [□]

□ = significantly different from quiet control.

△ = significantly different from saline injected control
(in case of anaesthesia); or significantly different
from yoked control (in case of avoidance conditioning).

Table 13. The effects of behavioural treatments on the total protein content of regions of the rat brain.

Region	Saline Control	Anaesthesia	Yoked Control	Avoidance Conditioned
Frontal cortex	-	-	-16.5% [□]	-17.2% [□]
Medial cortex	-	-	-	-17.5% [□]
Posterior cortex	-	+8.02% [□] +18.3% [△]	-	-10.5% [□]
Cerebellum	-	-	-	-16.3% [□]
Ventral cortex	-	-8.8% [□]	-13.0% [□]	-24.9% [□] -13.6% [△]
Hippocampus	-	-	-11.2% [□]	-10.4% [□]
Hypothalamus	-	-	-	-
Thalamus	-	-	-	+23.4% [△]
Medulla	-	-	-	-
Pons	-	-	-	-
Superior colliculus	-	-	-	-
Olfactory tubercle	-	-	-	-
Caudate nucleus	-	+18.9% [△]	-22.4% [□]	-18.1% [□]

□ = significantly different from quiet control

△ = significantly different from saline injected control

(in case of anaesthesia); or significantly different from the yoked control (in case of avoidance conditioning)

Section 2.4. Discussion.

The rank order of distribution of and the absolute values of the activity of AChE in the various regions studied are closely similar to those reported for the rat brain by Bennett et al (1966), Bajgar & Urban (1971) and Durkin (1974).

Results presented in the present study indicate that AChE is capable of undergoing relatively rapid changes in activity in response to various behavioural treatments. A criticism of the method used to determine AChE activity in this study is that the natural substrate is not used and that changes detected could be due to changes in sulphhydryl groups following treatment. However, using an alternative titrimetric technique with ACh as substrate similar changes in activity have been obtained (Anand 1977). It must also be re emphasised that changes in enzyme activity and protein content following shock avoidance conditioning cannot be unequivocally attributed to the training experience since the yoked control animal is not subjected to exactly the same stress as the trained animal as discussed in Section 1.

Kinetic studies have suggested that changes observed are probably due to a change in V_{max} of the enzyme (i.e. change in the number of sites available for hydrolysis) rather than in an alteration of the affinity of the enzyme for its substrate (Durkin 1974; Anand 1977).

Several previous studies have indicated that the activity of AChE is capable of undergoing rapid changes in response to behavioural manipulation. Oba et al (1971) found that the activity of AChE decreased significantly in the hypothalamus of lactating rats within 2 min of receiving the suckling stimulus. and that this effect was reversible in 15 min.

A study by Harris et al (1971) lends support

to the argument that there can^{also} be a rapid alteration in the rate of synthesis of AChE. The possible contribution of de novo synthesis of AChE to the return of enzyme activity in retinae of guinea-pigs following administration of an anticholinesterase was determined. The results indicated that AChE activity of the retinae recovered significantly between 30 min and 6h after poisoning. Yaksh (1975) reported $\frac{1}{2}$ times of recovery for cholinesterase in brain tissue to range from 0.9 - 3.8 days. An iso-enzyme of AChE has been reported by Davis & Agranoff (1968) which has a half life of only 3 hours.

The time course study on the effect of anaesthesia on AChE activity measured 24 hours following the period of anaesthesia indicates that the changes in activity of AChE are transient. This has also been reported by Haywood et al (1975) who were investigating the effects of imprinting on day-old chicks. The activity of AChE was found to increase by 11% in the fore-brain of the exposed birds one hour after exposure; in the base by 13% and in the mid-brain by 8% after 6 hours of exposure. At 12 hours a lowered mid-brain AChE was reported.

The protein content was found to increase in three regions of the brain investigated following anaesthesia. Glazer & Sartorelli (1973) reported that phenobarbital caused a stimulation of protein synthesis of 2.6 times in 3 to 14 hours of both free and bound polyribosomes of the liver. It is possible that a similar increase in protein synthesis occurs in some regions of the brain following pentobarbitone anaesthesia.

Changes in protein levels were also seen in some regions of the brain following electrical stimulation and shock avoidance conditioning. The observed changes in protein concentration could be the result of either increased synthesis or degradation. Alexandrovskaya (1972) reported that administration of electric

shocks resulted in a decrease of protein synthesis in the hippocampus which compares well with our findings of a decrease in protein content in this area. Stewart & Urban (1972) concluded from their study of S-100 of mouse brain that the accumulation of protein during development is the result of modulation of the rates of both synthesis and degradation. There is also evidence in the literature that function dependent alterations in the activities of proteinases occurs (Glushchenko 1971; Poberai et al 1972; Bass & Moore 1971).

Thus the present investigation provides a basis for selecting specific regions of the brain for study of the lability of AChE and proteins. It should be emphasised at this point, that the AChE component of the central cholinergic transmission system does not function in isolation but as an integral part of a total transmission system which includes transmitter synthesis, storage, release, receptor-interaction, uptake and hydrolysis. All of these processes have their rate limiting steps and some, if not all, are intimately connected with each other. It can, thus, be seen that further speculation as to the behavioural significance of these enzyme activity changes must await further studies into the functioning of the total transmission system. It must be established, whether and to what extent other components of the transmission system also alter in response to such behavioural treatments.

These investigations were continued by studying regional protease activity to see if protein degradation is a possible mechanism for the observed changes in protein content. This study was also followed up by fractionation of proteins of brains of trained and control animals to see if the changes observed were general or specific to particular protein species. The following section, however, deals with pseudocholinesterase.

SECTION 3. Changes in the activity of pseudocholinesterase
of rat brain following behavioural treatments.

Section 3.1. Introduction.

Cholinesterases can be divided into two groups with different properties: 1) acetylcholinesterase (E.C. 3.1.1.7.) and 2) pseudocholinesterase (E.C. 3.1.1.8.). The enzymes can be differentiated via their substrate specificity, the presence of substrate inhibition and their reactivity towards various anticholinesterases. The characteristic of pseudoChE is that it hydrolyses higher esters of choline such as butyrylcholine and propionylcholine more rapidly than it hydrolyses ACh. Another feature which differentiates pseudoChE from AChE is the ability of the former to hydrolyse aliphatic and aromatic esters such as tributyrin at an appreciable rate (Augustinsson 1948, 1959 a,b,c).

Cholinesterase type can also be determined by the reaction of the enzymes towards various antiChEs which are specific for either AChE (Wellcome anti-ChE BW 284 C51) or pseudoChE (iso-OMPA). AChE may also be distinguished from pseudoChE by the phenomenon of substrate inhibition. AChE is inhibited at higher concentrations of ACh but pseudoChE does not show this inhibition.

Purification of pseudoChE has indicated that it is a glycoprotein and in both horse and man the enzyme appears to be associated with sialic acid. This can be split off from the enzyme by neuraminidase without loss of hydrolytic activity (Augustinsson 1971). PseudoChE is more soluble and easier to extract from tissues than AChE (Das & Liddell 1970).

The essential differences in the behaviour of AChE and pseudoChE are thought to be due to the configuration of the active centres of the two enzymes. Several hypotheses

have been advanced to explain these differences. Adams & Whittaker (1950) concluded from their studies of erythrocyte AChE and plasma cholinesterase that the behaviour of the two enzymes was consistent with the existence of a negatively charged atom in the active centre of erythrocyte cholinesterase which is absent from plasma cholinesterase. Zupančič (1964a,b), however, suggested that pseudoChE also possessed an anionic site while Bergmann (1955, 1958) suggested that the difference in behaviour of the two enzymes was in the number of the anionic sites.

Augustinsson (1966) proposed that pseudoChE contained a second 'non-esteratic' site which was different from the anionic site of AChE and that this was the main feature which led to the difference in behaviour of the enzymes. He also presented evidence which suggested that the esteratic sites of the two cholinesterases differed.

The hypothesis has also been put forward that it is not the nature of the esteratic and anionic sites of the enzymes which account for the difference in the behaviour but differences in the surrounding groups (Kabachnik et al 1970).

Silver (1974) suggests that since certain enzymes may possess characteristics typical of both AChE and pseudoChE that it was likely that the differences in behaviour of the enzymes may be due to the specific structural arrangements of the active centres rather than to differences in the chemical nature of the active groups.

In the nervous system, pseudoChE has been found to be concentrated in white matter (Ord & Thompson 1952; Koelle 1952; Burgen & Chipman 1951). High levels of pseudoChE have however been reported in some nuclei of gray matter (Abrahams et al 1957)

where pseudoChE activity does not appear to be concentrated in the perikarya with the AChE activity but occurs diffusely between the cells.

It is now generally accepted that pseudoChE activity is associated with glia rather than with axons (Hebb et al 1953). There is some controversy about the type of glial cell that pseudoChE is associated with. Koelle (1954) reported that the majority of cells which stained for pseudoChE were fibrous astrocytes. In contrast, Friede (1967) identified the glial cells which stained for pseudoChE as oligodendroglia; he also reported that in certain tracts pseudoChE staining was also seen in axons rather than in glial cells. The histochemical technique he used has been criticised by Silver (1974).

There is further evidence to suggest that pseudoChE is not confined to the astroglia. It has been reported (Büllbring et al 1953; Wolleman & Zoltan 1962) that high levels of pseudoChE can be detected biochemically both in oligodendrogliomas and astrocytomas but not in non-glial tumours. Roessmann & Friede (1966) also provide histochemical evidence of pseudoChE activity associated with oligodendroglia in the cat and reported only minimal staining of the astrocytes.

Ord & Thompson (1952) provided biochemical evidence for the presence of pseudoChE in both white and gray matter of rat cerebrum; but the histochemical studies of Roessmann & Friede (1966) failed to detect pseudoChE activity in the glial cells of the cortex of man or cat. The discrepancy between the histochemical and biochemical results could be explained by the insensitivity of the histochemical technique caused perhaps by a diffusion barrier imposed by the glial cell membrane. Alternatively, since it is known that the endothelial

cells of blood vessels of rat CNS are rich in pseudoChE (Navaratnam & Lewis 1970; Flumerfelt 1973) it is possible that the activity measured biochemically could be due to endothelial cells rather than to glial cells.

The distribution pattern of pseudoChE appears to be distinct from that of AChE and of non-specific esterases and this suggests that pseudoChE has a specific role in brain metabolism. This is reinforced by the scheme proposed by Augustinsson (1968) which suggests that pseudoChE is not a less efficient precursor of AChE.

Various suggestions have been put forward to explain the function of pseudoChE both outside and within the central nervous system. Considering its role outside the nervous system it has been implicated in:- i) membrane permeability, particularly in blood vessels (Fourman 1966, 1967; Flumerfelt et al 1973; Joó & Csillik 1966). ii) fatty acid metabolism (Clitherow 1963) iii) choline metabolism (Zeller & Bissegger 1943) iv) regulation of choline plasma levels (Funnell & Oliver 1965) v) lipid metabolism (Ballantyne 1967; Fourman & Ballantyne 1967) vi) tissue growth (Ballantyne & Burwell 1965) vii) hydrolysis of non-nervous ACh (Jamieson 1963).

The function of pseudoChE in the CNS has not been firmly established. The predominant localisation of pseudoChE in white matter, where it is mainly associated with glia together with experimental evidence that organophosphorus anti-ChEs could produce demyelination led to the suggestion that the enzyme was involved in the maintenance of myelin (Earl & Thompson 1952a,b). Davison (1953) showed that demyelination did not inevitably follow BuChE inhibition.

Desmedt & La Grutta (1955, 1957) injected pseudoChE

inhibitors into the carotid of the encéphale isolé preparation of cat and reported an alteration of spontaneous electrical activity in the cerebral cortex. They suggested that the presence of pseudoChE in the nervous system was of functional rather than structural significance.

McIlwain (1966) puts forward the suggestion that in the hypothalamus where pseudoChE is mainly extraneuronal, it may function by restricting the spread of ACh by diffusion.

It was reported by Vernadakis & Rutledge (1973) that sodium pentobarbitone and ether anaesthesia inhibit the activity of BuChE in rat brain. BuChE activity markedly decreased in both the corpus striatum and cerebral cortex at one week and three weeks after the period of anaesthesia. The activity of AChE did not change in the cerebral cortex and was much less affected in the corpus striatum than BuChE. If BuChE is primarily localised in glial cells, this data suggests that glia cell metabolism are selectively altered by general anaesthesia and that this alteration persists as long as three weeks after anaesthesia. The altered activity of neurones during anaesthesia may lead to prolonged changes in the metabolism of glia cells as reflected by a decrease in BuChE activity.

It was decided to investigate whether there were any changes in the activity of BuChE following anaesthesia, electrical stimulation and shock avoidance conditioning to give an indication of any changes in glia cell metabolism following these treatments; and also to compare these changes with changes observed in the activity of AChE.

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Section 3.2. Methods and Results.

3.2.1. Distribution of cholinesterase activity in regions of the rat brain with different substrates.

The distribution of cholinesterases using different substrates was first investigated. The method was as described in Section 2.2.6.; and the substrates used were acetylthiocholine (0.67 mM), butyrylthiocholine (1.0mM) and propionylthiocholine (1.0mM). The distribution of activity with these substrates is shown in Tables 3, 14, and 15 and in Figure 20.

- 1) The activity of cholinesterases using butyrylthiocholine as substrate is much lower than the activity with acetylthiocholine as substrate in all of the regions investigated. The percentage activity obtained with this substrate compared with acetylthiocholine varies regionally from 1.5% in the caudate nucleus to 12.6% in the cerebellum. The distribution of activity of cholinesterases with this substrate varies much less between regions than the activity with acetylthiocholine as substrate. The activity is very low in the dorsal cortical regions , the ventral cortex and the cerebellum but varies very little between other regions.
- 2) The activity of cholinesterases obtained using propionylthiocholine as substrate was lower in all regions than the activity with acetylthiocholine; the percentage activity compared with acetylthiocholine varied from 26.4% in the medulla to 97.5% in the ventral cortex. The activity of cholinesterases with propionylthiocholine as substrate was always higher than with butyrylthiocholine as substrate.

Table 14. The distribution of cholinesterases in regions of the rat brain using butyrylthiocholine as substrate.

Region	Mean	S.E.M.	n	% of activity using AcThCh.
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Frontal cortex	0.194	0.010	5	7.6%
Medial cortex	0.171	0.010	3	10.4%
Posterior cortex	0.187	0.014	4	11.4%
Cerebellum	0.208	0.018	3	12.6%
Ventral cortex	0.252	0.019	5	8.0%
Hippocampus	0.466	0.014	3	12.1%
Hypothalamus	0.454	0.067	5	11.5%
Thalamus	0.417	0.009	4	8.0%
Medulla	0.464	0.084	5	8.7%
Pons	0.467	0.058	5	6.1%
Superior colliculus	0.617	0.142	3	6.8%
Olfactory tubercle	0.500	0.046	5	1.6%
Caudate nucleus	0.459	0.045	3	1.5%

Enzyme activity is expressed as μ moles butyrylthiocholine hydrolysed / hr / mg proteins.

Table 15. The distribution of cholinesterases in the rat brain using propionylthiocholine as substrate.

Region	Mean	S.E.M.	n	% of activity using AcThCh
Frontal cortex	1.658	0.08	4	65.5%
Medial cortex	1.469	0.22	4	90.3%
Posterior cortex	1.491	0.22	4	91.5%
Cerebellum	0.822	0.48	3	46.8%
Ventral cortex	3.074	0.19	4	97.5%
Hippocampus	3.033	0.45	3	78.9%
Hypothalamus	2.647	0.57	4	66.9%
Thalamus	5.031	0.88	3	96.7%
Medulla	1.501	0.30	4	26.4%
Pons	5.337	0.40	4	70.5%
Superior colliculus	5.759	1.31	3	63.8%
Olfactory tubercle	21.781	3.51	3	69.3%
Caudate nucleus	15.433	3.88	3	48.6%

Enzyme activity is expressed as μ moles propionylthiocholine hydrolysed / hr / mg proteins.

Figure 20. The distribution of cholinesterases in regions of the rat brain using acetylthiocholine, propionylthiocholine and butyrylthiocholine as substrates.

The columns represent the means of the number of determinations shown in Tables 3, 14 & 15. The white columns represent the activity obtained with acetylthiocholine as substrate; the black columns represent the activity obtained with propionylthiocholine as substrate and the stippled columns represent the activity obtained with butyrylthiocholine as substrate. The S.E.M. is represented by the vertical bars.

Key: FC Frontal cortex
MC Medial cortex
PC Posterior cortex
C Cerebellum
VC Ventral cortex

H Hypothalamus
T Thalamus
M Medulla
P Pons
SC Superior colliculus
OT Olfactory tubercle
CN Caudate nucleus

Enzyme activity is expressed as μ moles substrate hydrolysed / hr / mg proteins.

3.2.2. Development of a specific assay for pseudocholinesterase.

The specific inhibition of AChE using the specific Wellcome inhibitor BW 284 C51 (dibromide of 1:5 bis (p-allyl-N-methylaminophenyl) pentan-3-one) was investigated using both acetylthiocholine and butyrylthiocholine as substrates. The range of concentrations of inhibitor used was 10^{-7} M to 10^{-4} M. Since the activity using butyrylthiocholine as substrate is much lower than the activity with acetylthiocholine the assay requires larger brain samples (10mg) to obtain readings which fall on the most sensitive part of the recorder trace. The results are presented in Figure 21.

It can be seen that at an antiChE concentration of 3×10^{-5} M the activity of cholinesterases using acetylthiocholine as substrate in the caudate nucleus has decreased to 94% of its original value and thereafter does not decrease significantly. With butyrylthiocholine as substrate the degree of inhibition at this concentration varies from 9% in the medulla to 45% in the cerebellum. The degree of inhibition will vary depending on the ratio of the activities of the enzymes in the regions.

The pI_{50} for acetylthiocholine (i.e. the negative logarithm of the molar concentration causing 50% inhibition) in the caudate nucleus is 6.95; this agrees well with the value quoted by Wellcome of 6.7 for whole rat brain.

Thus acetylcholinesterase can be selectively inhibited in the assay of pseudocholinesterase by the addition of 3×10^{-5} M BW 284 C51.

Figure 21. The specific inhibition of acetylcholinesterase
by BW 284 C51 in the rat brain.

- A. Inhibition of acetylcholinesterase in the caudate nucleus with acetylthiocholine as substrate. The values given are the mean of 4 independent determinations and the standard error of each point is less than 1.1%.
- B. Inhibition of acetylcholinesterase with butyrylthiocholine as substrate. The values given are the mean of 3 independent determinations. The standard error of each point is less than 5.3%.

3.2.3. The effects of 45 minutes anaesthesia on the activity of pseudoChE in regions of the rat brain.

The effects of 45 minutes anaesthesia on the activity of pseudoChE is shown in Table 16 and Figure 22.

- 1) The quiet control distribution of pseudoChE activity does not vary very much between regions: the activity in the cortical regions, hippocampus, and cerebellum is low and there is very little variation in activity between the other regions.
- 2) The pseudoChE activity of the saline injected group was not significantly different from the quiet control group in any measured region of the brain.
- 3) There was a decrease in the activity of pseudoChE in the anaesthetised group compared with the quiet control group and saline control group in the medial cortex (18.8% quiet; 14.4% saline), cerebellum (26.5% quiet; 30.1% saline) and caudate nucleus (25.6% quiet; 24.9% saline).
- 4) There was an increase in activity of pseudoChE in the anaesthetised group compared with both the saline and quiet controls in the ventral cortex (14.0% quiet; 19.7% saline). In the medulla, there was an increase in the activity of pseudoChE in the anaesthetised group compared with the saline controls (14.0%); there was no significant difference compared with the quiet controls.
- 5) There was no significant change in the activity of pseudoChE of the anaesthetised group compared with either the quiet controls or saline controls in the frontal cortex, posterior cortex, hippocampus, hypothalamus, thalamus, pons, superior colliculus and olfactory tubercle.

Table 16. The effects of 45 mins anaesthesia on the activity of pseudocholinesterase in regions of the rat brain.

Region	Quiet Control	Saline Control	Anaesthesia
Frontal cortex	0.408 $\pm$ 0.021	0.548 $\pm$ 0.077	0.512 $\pm$ 0.044
Medial cortex	0.521 $\pm$ 0.034	0.494 $\pm$ 0.012	0.423 $\pm$ 0.012 ⁺⁺⁺
Posterior cortex	0.458 $\pm$ 0.021	0.509 $\pm$ 0.031	0.447 $\pm$ 0.050
Cerebellum	0.444 $\pm$ 0.007	0.473 $\pm$ 0.019	0.327 $\pm$ 0.023 ⁺⁺⁺
Ventral cortex	0.437 $\pm$ 0.014	0.416 $\pm$ 0.006	0.498 $\pm$ 0.018 ⁺⁺
Hippocampus	0.441 $\pm$ 0.025	0.517 $\pm$ 0.040	0.516 $\pm$ 0.048
Hypothalamus	1.358 $\pm$ 0.305	1.337 $\pm$ 0.158	1.561 $\pm$ 0.284
Thalamus	1.072 $\pm$ 0.075	0.961 $\pm$ 0.238	1.413 $\pm$ 0.194
Medulla	1.123 $\pm$ 0.058	1.026 $\pm$ 0.030	1.170 $\pm$ 0.026 ^{**}
Pons	1.207 $\pm$ 0.109	0.981 $\pm$ 0.160	1.082 $\pm$ 0.083
Superior colliculus	1.862 $\pm$ 0.075	2.317 $\pm$ 0.239	2.109 $\pm$ 0.349
Olfactory tubercle	1.381 $\pm$ 0.226	1.381 $\pm$ 0.334	1.449 $\pm$ 0.241
Caudate nucleus	0.835 $\pm$ 0.037	0.827 $\pm$ 0.050	0.621 $\pm$ 0.054 ⁺⁺

Enzyme activity is expressed as μ moles butyrylthiocholine hydrolysed / min / g wet weight. The values given are the means of at least 4 determinations.

+ = significantly different from quiet control (+p < 0.05; ++ p < 0.01; +++ p < 0.001). * = significantly different from saline control (* p < 0.05; ** p < 0.01; *** p < 0.001).

Figure 22. Changes in the activity of pseudocholinesterase
in regions of the rat brain following 45 mins anaesthesia.

The columns represent the mean of the number of observations shown in Table 16. The white columns represent the quiet controls; the black columns represent the saline controls; and the stippled columns represent the anaesthetised group. The S.E.M. is represented by the vertical bars.

Key: FC Frontal cortex
MC Medial cortex
PC Posterior cortex
C Cerebellum
V/C Ventral cortex
HP Hippocampus
H Hypothalamus
T Thalamus
M Medulla
P Pons
SC Superior colliculus
OT Olfactory tubercle
CN Caudate nucleus

▲ = significantly different from quiet controls

□ = significantly different from saline controls

Enzyme activity is expressed as μ moles butyrylthiocholine
hydrolysed / min / g wet weight.

3.2.4. Changes in the activity of pseudoChE in regions of the rat brain following electrical stimulation and shock avoidance conditioning.

The effects of electrical stimulation and shock avoidance conditioning on the activity of pseudoChE are shown in Table 17 and Figure 23.

- 1) There was no change in the activity of pseudoChE between,
 - i) quiet controls and yoked controls, ii) quiet controls and conditioned animals, iii) yoked control group and conditioned group in the hippocampus, hypothalamus, thalamus, pons, superior colliculus and olfactory tubercle.
- 2) There was an increase in activity of pseudoChE in the yoked control group compared with the quiet control group in the frontal cortex (44.8%), medial cortex (26.9%), posterior cortex (40.3%), cerebellum (25.4%), ventral cortex (35.5%) and medulla (47.7%).
- 3) In the caudate nucleus, there was a decrease in activity of pseudoChE in the yoked control group compared with the quiet control group (27.7%).
- 4) There was an increase in activity of pseudoChE compared with the quiet control in the medial cortex (30.7%) and cerebellum (17.8%). However, in both cases there was a similar increase in pseudoChE activity of the yoked controls compared with the quiet controls, which indicates that the increase is probably due to stress following electrical stimulation in the shuttle box. Similarly, in the caudate nucleus the decrease in enzyme activity seen in the conditioned group compared to the quiet control group (32.8%) corresponds to the decrease in activity of the electrically stimulated group.
- 5) In the frontal cortex, the enzyme activity in the trained

Table 17. The effects of electrical stimulation and shock avoidance conditioning on the activity of pseudocholinesterase.

Region	Quiet Control	Yoked Control	Conditioned
Frontal cortex	0.408 $\pm$ 0.021	0.591 $\pm$ 0.025 ⁺⁺	0.484 $\pm$ 0.033 ^{**}
Medial cortex	0.521 $\pm$ 0.034	0.661 $\pm$ 0.025 ⁺	0.681 $\pm$ 0.019 ⁺⁺
Posterior cortex	0.458 $\pm$ 0.021	0.643 $\pm$ 0.009 ⁺⁺⁺	0.578 $\pm$ 0.025 ⁺⁺
Cerebellum	0.444 $\pm$ 0.007	0.557 $\pm$ 0.016 ⁺⁺⁺	0.523 $\pm$ 0.014 ⁺⁺
Ventral cortex	0.437 $\pm$ 0.014	0.592 $\pm$ 0.023 ⁺⁺	0.539 $\pm$ 0.088
Hippocampus	0.441 $\pm$ 0.025	0.511 $\pm$ 0.044	0.548 $\pm$ 0.057
Hypothalamus	1.358 $\pm$ 0.305	1.809 $\pm$ 0.140	1.977 $\pm$ 0.110
Thalamus	1.072 $\pm$ 0.075	1.208 $\pm$ 0.080	1.222 $\pm$ 0.050
Medulla	1.123 $\pm$ 0.058	1.659 $\pm$ 0.054 ⁺⁺⁺	1.194 $\pm$ 0.084
Pons	1.207 $\pm$ 0.109	1.300 $\pm$ 0.108	1.298 $\pm$ 0.130
Superior colliculus	1.862 $\pm$ 0.075	2.076 $\pm$ 0.357	1.945 $\pm$ 0.241
Olfactory tubercle	1.381 $\pm$ 0.226	1.487 $\pm$ 0.349	1.471 $\pm$ 0.227
Caudate nucleus	0.835 $\pm$ 0.037	0.604 $\pm$ 0.060 ⁺	0.561 $\pm$ 0.035 ⁺⁺

Enzyme activity is expressed as μ moles butyrylthiocholine hydrolysed / min / g wet weight. The values given are the means of at least 4 determinations.

+ = significantly different from quiet controls (+ p < 0.05; ++ p < 0.01; +++ p < 0.001). * = significantly different from yoked controls (* p < 0.05; ** p < 0.01; *** p < 0.001).

Figure 23. Changes in the activity of pseudocholinesterase
in regions of the rat brain following electrical
stimulation and shock avoidance conditioning.

The columns represent the mean of the number of observations shown in Table 17. The white columns represent the quiet controls; the black columns represent the yoked controls and the stippled columns represent the shock avoidance conditioned group. The S.E.M. is represented by the vertical bars.

Key: FC Frontal cortex
MC Medial cortex
PC Posterior cortex
C Cerebellum
VC Ventral cortex
HP Hippocampus
H Hypothalamus
T Thalamus
M Medulla
P Pons
SC Superior colliculus
OT Olfactory tubercle
CN Caudate nucleus

▲ = significantly different from quiet controls

□ = significantly different from yoked controls

Enzyme activity is expressed as μ moles butyrylthiocholine
hydrolysed / min / g wet weight.



group is significantly lower than the yoked control group (18.1%), but not significantly different from the quiet controls. However, the enzyme activity of the yoked control group is significantly higher than the activity of the quiet control group, indicating that in this region the effect of stress could be to increase the activity of the enzyme while the effect of conditioning could be to decrease the enzyme activity.

- 6) In the posterior cortex, the enzyme activity of the trained group is significantly lower than the yoked control group (10.1%); and significantly higher than the quiet control group (26.2%). Therefore, in this region the effect of stress could be to increase the enzyme activity, while the effect of conditioning is to lower the activity of the enzyme.

3.2.5. A comparison of changes in activity of AChE and pseudoChE in regions of the rat brain following behavioural treatments.

A comparison of the changes in activity of these two enzymes is shown in Table 18.

- 1) Some of the changes observed in AChE activity following anaesthesia correlate with changes in pseudoChE activity (decrease in activity of enzymes in the medial cortex and caudate nucleus; increase in activity of the enzymes in the medulla).

- 2) In other regions of the brain, the changes in activity of the enzymes following anaesthesia do not correlate.

There is a decrease in activity of AChE in the hippocampus and thalamus but no corresponding change in pseudoChE activity.

In the ventral cortex, there is an increase in activity of pseudoChE but no corresponding change in the activity of AChE. In the cerebellum, the activity of AChE increases, while the activity of pseudoChE decreases following

Table 18. A Comparison of changes in the activity of AChE and pseudoChE following behavioural treatments.

Region	Anaesthesia		Electrical Stimulation		Conditioning	
	AChE	BuChE	AChE	BuChE	AChE	BuChE
Frontal cortex	-	-	+24%	+44%	-	-18%
Medial cortex	-13%	-14%	-	+27%	-	-
Posterior cortex	-	-	-	+40%	+17%	-10%
Cerebellum	+26%	-30%	-	+25%	-	-
Ventral cortex	-	+20%	-	+35%	-	-
Hippocampus	-26%	-	-	-	+17%	-
Hypothalamus	-	-	-40%	-	+34%	-
Thalamus	-22%	-	-29%	-	+44%	-
Medulla	+25%	+14%	+52%	+47%	-	-
Pons	-	-	-	-	-	-
Superior colliculus	-	-	-	-	-	-
Olfactory tubercle	-	-	-29%	-	-	-
Caudate nucleus	-24%	-25%	-24%	-28%	-	-

anaesthesia.

- 3) Similarly, some of the changes observed in AChE activity following electrical stimulation correspond with changes in pseudoChE activity (the increase in activity of the enzymes in the frontal cortex and medulla; and the decrease in activities of the enzymes in the caudate nucleus).
- 4) There was no change in the activity of AChE but an increase in the activity of pseudoChE following electrical stimulation in the medial cortex, posterior cortex, cerebellum and ventral cortex. There was a decrease in activity of AChE in the hypothalamus, thalamus and olfactory tubercle; but no corresponding change in the activity of pseudoChE in these regions.
- 5) None of the changes in activities of AChE and pseudoChE following conditioning correlated. The increase in activity of AChE following conditioning in the hypothalamus, thalamus and hippocampus were not paralleled by changes in pseudoChE activity. There was no change in AChE activity in the frontal cortex following conditioning but there was a decrease in pseudoChE activity. In the posterior cortex, the effect of conditioning was to increase the activity of AChE but to decrease the activity of pseudoChE.

3.2.6. Summary.

- 1) The rates of hydrolysis of acetylthiocholine, butyrylthiocholine and propionylthiocholine were determined. It was found that the rate of hydrolysis of acetylthiocholine was greater than the hydrolysis of propionylthiocholine in all regions of the brain investigated; the rate of hydrolysis of propionylthiocholine was always greater than the rate of hydrolysis of butyrylthiocholine.
- 2) A specific assay for pseudoChE was developed; it was found that at a concentration of $3 \times 10^{-5} \text{ M}$ BW 284 C51, the AChE activity

was selectively inhibited.

- 3) The quiet control distribution of pseudoChE was determined and it was found to differ completely from AChE.
- 4) Pentobarbitone sodium anaesthesia was found to produce a decrease in activity of pseudoChE in the medial cortex, cerebellum and caudate nucleus, while in the ventral cortex and medulla there was an increase in activity.
- 5) Electrical stimulation was found to produce an increase in activity of pseudoChE in the frontal cortex, medial cortex posterior cortex, cerebellum, ventral cortex and medulla; while a decrease in activity was observed in the caudate nucleus.
- 6) A decrease in activity of pseudoChE was observed in the frontal cortex and posterior cortex following shock avoidance conditioning.
- 7) Some of the changes produced by anaesthesia and electrical stimulation, but not by conditioning correlated with changes in activity of AChE (eg. medulla and caudate nucleus); while others were independent of changes in AChE activity (cerebellum and thalamus). This could indicate a selective change in glia cell metabolism following these treatments.

Section 3.3. Discussion

The total activity of pseudoChE in rat brain was found to be much less than the activity of AChE by Ord & Thompson (1952). A comparison with our results is difficult since we measured regional activity. Ord & Thompson reported that the activity of pseudoChE was 6.% of AChE for total brain. The ratio of the enzymes will vary regionally because the regional pattern of the enzymes differs greatly. Burgen & Chipman (1951) reported that in the dog there was a high level of enzyme in the thalamus and this was also found in the present study.

The data presented in this section suggests that the activity of pseudoChE can change rapidly in response to behavioural treatments. Some of the responses correlated with changes observed in AChE activity; while others were independent of changes in AChE activity. This suggests that glial cell metabolism might be selectively altered by the treatments.

Although there was no reported change in water content following the treatments, the possibility of a change in extracellular volume was not discounted in this study. If, however, the activity of AChE and pseudoChE change in opposing directions in the same region following some treatment, it is difficult to explain this on the basis of a change in extracellular volume caused by cell shrinkage or swelling. Such a selectivity of enzyme activity changes was indeed obtained eg. in cerebellum, thus providing further support for the suggestion that there are specific changes in enzyme activity.

The possible mechanisms which might explain these changes in enzyme activity are discussed in Section 6.

SECTION 4. Determination of the activity of proteinases following behavioural treatments.

Section 4.1. Introduction.

The continuous turnover of proteins in brain as in other tissues is an extensive but markedly heterogenous process (Lajtha & Marks 1971). Protein turnover includes not only synthesis, but also the breakdown of proteins according to the physiological and metabolic requirements of the tissue. While knowledge of the events of protein synthesis and their control is well advanced (Lipmann 1969), knowledge concerning protein breakdown, which is an integral part of the regulatory mechanism affecting protein composition, is still rudimentary. A full understanding of degradative processes is needed to interpret the many factors which influence protein turnover; these include the effects of hormones and drugs (Lajtha 1964), pathological states (Weinstock & Iodice 1964) and states of excitation and exhaustion (Ungar 1963).

Brain contains a variety of proteins, with variable half-lives, the average is 15 days but minor components show a range extending from less than an hour to several months (Lajtha 1964). The major pathway for the breakdown of proteins is considered to involve the initial cleavage of proteins by peptidyl-peptide hydrolases (E.C.3.4.4.) followed by sequential attack by the many peptide hydrolases (E.C.3.4.) present in all tissues (Marks & Lajtha 1970). Exact schemes for protein breakdown can be formulated only when knowledge is available for isolating individual enzymes and their native substrates together with knowledge of their localisation, mechanism of inactivation and peptide bond specificity. As yet, none of these criterion has been fully met. Despite the relative abundance of degradative enzymes, a number of questions remain

to be answered about the factors which determine when and why a particular protein or structural element is broken down. These questions are part of the problem of total regulation of all enzyme activities within cells.

The role of proteinases in the physiology of the nervous system was first demonstrated by Soula (1912) and then Faure & Soula (1913) who showed that excitation of nerve centres resulted in increased aminogenesis. Since then there have been many reports of functional roles for proteinases in the nervous system. Excitation has been found to result in increased catabolism of proteins (Waelsch 1957, Ungar 1957, Hydén 1955; Geiger et al 1956; Ungar 1963).

Hooper (1962, 1964, 1966) suggested that proteinases might be involved in the catabolism of physiologically active polypeptides such as oxytocin, in the hypothalamus. There is also evidence that proteinases may be involved in demyelination, particularly in relation to pathological states such as Wallerian degeneration (Porcellati & Curti 1960; Adams & Glenner 1962). Gabrielescu (1970; 1975) has also suggested the involvement of proteolytic enzymes in stress conditions.

Biochemical studies of Takasaka (1927) and Schurr et al (1950) demonstrated the presence of proteinases in the nervous system. The presence of two types of proteinase, an acid proteinase with a pH optima of 3.8 and a neutral proteinase with a pH optima of pH 7.4, was shown by Ansell & Richter (1954a, b). Subsequent work (Lajtha 1964, Marks and Lajtha 1965, 1971) has revealed a widespread distribution of both endopeptidases and exopeptidases within the nervous system.

Regional distribution studies of proteinases within the

nervous system have shown that acid proteinase activity is predominantly associated with gray matter; while neutral proteinase activity is generally associated with white matter and in particular with myelin (Ansell & Richter 1954). A myelin-bound proteolytic enzyme system has also been described by Adams (1965); Adams et al (1963, 1968).

Subcellular fractionation studies of nervous tissue have revealed a complex organisation of proteinases at the subcellular level. Acid proteinase has been shown to be particulate bound (Beaufay et al 1957) and later Palladin (1961) and Palladin et al (1963) demonstrated that over 80% of the acid proteinase activity was associated with the crude mitochondrial fraction. Neutral proteinases and peptidases as well as acid proteinases were reported to be associated with the light mitochondrial fraction (Waelsch & Lajtha 1961). The presence of acid and neutral proteinase activity in the mitochondrial fraction was also shown by D'Monte et al (1970).

DeDuve et al (1955) isolated lysosomes from the mitochondrial fraction and demonstrated the presence of cathepsins B, C and D in this fraction. The association of acid proteinases with lysosomes has also been reported by Marks et al (1970) and Marks & Lajtha (1970).

Classification.

Insufficeint data are available to assign intracellular proteinases to specific categories. The methods used for classification are based on the pH optima observed during autolysis of tissue, or on properties of the partially purified enzyme in relation to substrate specificity and on the effects of added cofactors. During autolysis, brain extracts are found to display two pH optima, one in the acidic region (pH 2 - 6)

and the other in neutral or alkaline range (Lajtha 1970).

The term cathepsin was proposed by Willstätter & Baumann (1929) for the enzyme active at acidic pH. This term was adopted by Bergmann (1942) and Fruton (1960) to describe a series of proteolytic enzymes active at acidic pH. These studies were facilitated by the synthetic substrates introduced by Bergmann (1942) for the characterisation of trypsin and similar enzymes and led to the reported presence in tissues of enzymes homospecific with pepsin (Cathepsin A), with trypsin (Cathepsin B) and with chymotrypsin (Cathepsin C). The use of synthetic substrates as the only criterion for characterising these enzymes has been misleading, since these studies failed to recognise the significance of an acid proteinase described earlier by Anson (1939 a, b) and present in all tissues, which does not hydrolyse these simpler substrates. Press et al (1960), suggested the term Cathepsin D for this important intracellular proteinase that accounted for over 2 / 3 of the haemoglobin-splitting activity of tissues. Later, the existence of an unstable form of cathepsin was reported; this was named Cathepsin E (Leprescle & Webb 1962).

Cathepsin A.

Cathepsin A is a pepsin-like sulfhydryl dependent hydrolase (Fruton 1960; Lichtenstein & Fruton 1960). The activity of Cathepsin A is difficult to detect in brain (Marks & Lajtha 1965) and its presence in tissues has been disputed by other workers (Press et al 1960).

Cathepsin B.

Cathepsins of this group hydrolyse substrates which are homospecific with trypsin but can be distinguished from trypsin by lack of inhibition by DFP, by their acid pH optima

and by the fact that they are sulfhydryl dependent enzymes. Two distinct enzymes of Cathepsin B group are differentiated by their substrate specificity: Cathepsin B which hydrolyses benzoylarginine β -naphthylamide (BANA) and Cathepsin B' which hydrolyses benzoylarginine anilide (BAA).

Low levels of CathepsinB-like activity can be demonstrated in brain (Marks & Lajtha 1965). The detection of such low levels of activity might be accounted for by their great lability and by the interference of tissue inhibitors released during their determination (Marks & Lajtha 1971).

Cathepsin C.

Cathepsin C is a sulfhydryl- and chloride-dependent enzyme (Gutmann 1948; Tallan et al 1952) which hydrolyses dipeptidyl amides at pH 5-6. In addition, this enzyme polymerises some peptidyl amides at neutral pH. (Metrione et al 1966). The activity of Cathepsin C in brain is very low in comparison with other tissues and may be attributable to the presence of endogenous inhibitors (Marks & Lajtha 1965; McDonald et al 1969; Marks et al 1968).

Cathepsin D.

This proteinase is defined by its capacity to hydrolyse denatured protein substrates at acidic pH and its inability to hydrolyse synthetic substrates (Fruton 1960). It is not a sulfhydryl-dependent cathepsin and does not require the presence of metal ions. It has been found to be responsible for 2 / 3 of the haemoglobin splitting activity in many tissues (Press et al 1960; Marks & Lajtha 1963).

Neutral proteinases.

Studies of proteinases active in the physiological pH range are hampered by the fact that these proteinases are

very labile. The presence of neutral proteinases in brain has been reported by several workers (Ansell & Richter 1954; Lajtha 1961; Marks & Lajtha 1965). A neutral proteinase was isolated from brain by Marks & Lajtha (1965) and shown to be activated by calcium and to have a sulfhydryl group at its active centre. This partially purified proteinase could degrade haemoglobin, casein and albumin at neutral pH.

There have been several suggestions for the physiological role of proteinases. Marks & Lajtha (1971) suggested a role for neutral proteinases in the turnover of proteins particularly in synaptic function and in releasing newly synthesised proteins from ribosomes. They have also been implicated in the mechanism of demyelination both in the peripheral and central nervous systems (Porcellati & Curti 1960; Riekkiniin & Rinne 1968).

Distribution.

Ansell & Richter observed autolysis in aqueous extracts of brains from different species higher in white matter compared to gray matter.

Most studies indicate that acid proteinase is particulate bound in tissues, with the highest concentration in the crude mitochondrial-lysosomal fractions. Lajtha (1961) in early studies reported high endogenous acid proteinase in the crude mitochondrial-synaptosomal fractions of rat cortex with levels of about 10% hydrolysed per hour in descending order by crude nuclear (5%), debris (3%), microsome (2%) and finally supernatant fractions (1.4%). Cathepsins A,B and D were identified in the lysosomes of liver by DeDuve et al (1962) and Beaufay et al (1964). Shibko & Tappel (1964) demonstrated lysosomal integration of Cathepsin C. In nervous tissue, Cathepsins B and D are connected with neurolysosomes (Marks & Lajtha 1965).

In nervous tissue, neutral proteinase activity is also particulate - bound. On fractionation of the mitochondrial fraction, neutral proteinase activity has been found mainly in nerve endings and myelin with some activity in the lysosomes (Marks et al 1968).

After subfractionation of the mitochondrial fraction, the activity of acid proteinases was found to be highest in the lysosomal fraction with some activity also present in the nerve ending fraction and slight activity in the fraction identified as containing myelin. The activity of neutral proteinases appeared to be concentrated in the nerve ending subfraction, with some activity also detectable in the lysosomal fraction (Marks & Lajtha 1965; Lajtha 1964).

The microsomal fraction has also been found to show proteinase activity (Lajtha 1964; Palladin 1961); but Datta et al(1967) failed to detect either acid or neutral proteinase activity in this fraction. Marks & Lajtha (1965) suggest that this activity is involved in releasing newly synthesised proteins from ribosomes.

Both acid and neutral proteinase activity have also been detected in the nuclear fraction (Marks 1968; Marks & Lajtha 1971). Their function in the nucleus has been suggested to be in releasing histones from the nucleoprotein complex (Marks & Lajtha 1965).

These subcellular fractionation studies, therefore, indicate that proteinases are not homogenously distributed in subcellular structures but that acid and neutral proteinases appear to be associated with different structures: acid proteinase being found predominantly in the lysosomal

fractions; while maximal neutral proteinase activity is seen in the nerve ending subfraction. The intracellular separation of proteinases is suggested to be of considerable significance to the functional activities of these enzymes within the cell (Gabrielescu 1975). The relationship of proteinases to the structures they are associated with thus influences the activity and the site of action of proteolytic enzymes. It is of relevance, therefore, to consider in more detail the nature of the relationship between the enzymes and the morphological structures they are associated with.

Several hypotheses have been formulated concerning the integration of proteinases into lysosomes. The first of these was proposed by DeDuve (1959, 1963) who proposed that the enzymic molecules of the lysosomes are released within the organelle, and that the activity of the enzymes was therefore controlled by the lysosomal membrane. Subsequent experimental data has shown however that the behaviour of various lysosomal hydrolases differ in that the rate of release of enzymes varies in terms of the activation stimulus and also the time course. This individual behaviour has been suggested to reflect different kinds of bonds within the lysosomes (Gabrielescu 1975). Therefore, the idea arose that there was a labile physico-chemical binding of the enzymes either to the lysosomal membrane or to the matrix.

The second hypothesis was put forward by Desai et al (1964)

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and postulates an ionic bond between the enzyme molecules and lysosomal membrane. According to Tappel et al (1963), the lysosomal membrane is not just a semipermeable barrier but access to enzyme substrate is due to translocation through the lysosomal membrane that depends upon the electric charge on the surface of the membrane and electrovalent bonds with the polarised states.

The third hypothesis was described by Koenig et al (1962, 1963, 1965, 1966, 1969). which postulates supramolecular integration of hydrolases in the lysosomal matrix by electrovalent bonds.

Very little is known about the binding of proteinases in other cellular structures. Integration of proteolytic enzymes in the myelin membrane system is far from clear; the enzymes appear to participate as soluble protein components integrated by electrovalent bonds into the lipoprotein structure (Adams & Bayliss 1968).

The response of proteinases to functional stimulation and stress.

There are many reports in the literature that function dependent alterations in the activities of proteinases occur in both the central and peripheral nervous systems. Ungar et al (1957) found that electrical stimulation increased chymotrypsin like activity of peripheral nerve. To test whether similar changes could be observed after increased muscular activity, the activity of neutral proteinases in rat sciatic nerves was investigated (Jakoubek et al 1963 ; Jakoubek & Gutmann 1968; Jakoubek et al 1968). Sharp increases in the proteolytic activity of rat sciatic nerve measured at pH 7.2 were found during increased motor activity. Poberai et al (1972) also reported that following supramaximal stimulation of rat motor end plates there occurs an intense proteolytic activity,

Glushchenko (1971) found that prolonged excitation in rat leads to an increase in the activity of both acidic and neutral proteinases in the brain.

Benatato & Gabrielescu (1967) looked at the significance of proteinase activity in the course of excitation in the dog by analysing kinetically the histochemical reaction of these enzymes in the peripheral nerve, cortex and hypothalamus under the influence of electrical stimulation in vivo. These experiments provided evidence of the activation of acid and neutral proteinases in nervous tissue. The enzyme reaction appeared to be correlated with the functional state of the structures and was found to be proportional to the intensity, frequency and duration of the stimuli. Intense stimulation (20V) together with long duration (20 min) produced activation of proteinases with a positive reaction in the CSF indicating the release of neural enzymes.

Subsequent observations by Gabrielescu and co-workers used various experimental models of stress; 1) stress caused by swimming to the point of fatigue (Gabrielescu & Bordeianu 1968) 2) stress caused by adaptation to the environment under high temperature conditions (Benetato & Gabrielescu 1968) 3) gravitational overstrain caused by acceleration (Gabrielescu 1970) 4) emotional stress produced in rats by the presence of a cat (Gabrielescu & Bordeianu 1971). Histochemical analysis of neuroprotease activity under these varied conditions showed similar common alterations irrespective of the nature of the stimulus. Again the intensity of the increased proteolysis was found to be proportional to the stimulus and affected both white and gray matter. The histochemical method used in this investigation is unselective for endopeptidases and an attempt was made to try to identify proteinase type on the basis of

substrate specificity and of their specific activators and inhibitors. The activity of Cathepsin D, as well as trypsin-like tryptase and aminopeptidase was found to increase.

Gabrielescu (1975) also reported the action of certain biologically active mediators of stress and found that noradrenaline and oxytocin had no apparent reaction; while vasopressin and bradykinin exercise a moderate effect; whereas adrenaline and histamine in very small doses produce a violent reaction of brain proteases, suggesting a possible correlation between chemical mediators and intracellular proteinase activity in stress conditions.

Bass & Moore (1971) have implicated proteolytic enzymes at the synapse as part of a memory model.

Thus, it was decided to investigate further the changes in protein content following behavioural treatments reported in Section 2, to see if they could be explained by an altered rate of protein degradation. Three assay systems were used in this study: in the first, the rate of hydrolysis of the protein substrate casein was determined; an attempt was made to further characterise the proteases involved by looking at the hydrolysis of a specific tryptic and a specific chymotryptic substrate following the behavioural treatments.

Section 4.2. Methods.

4.2.1. Determination of the activity of proteinases in regions of the rat brain using casein as the protein substrate.

The assay is based on that described by Bergmeyer (1963), in which casein is hydrolysed with the formation of hydrolysis products whose tyrosine and tryptophan content can be measured spectrophotometrically after the precipitation of residual substrate. Details of the preparation of reagents and buffers for this assay are given in Table 19.

The regions used in this study were those which showed changes in either protein content or cholinesterase activity following our behavioural treatments and included:- frontal cortex, medial cortex, posterior cortex, cerebellum, hippocampus, thalamus, medulla and caudate nucleus. Tissue samples were dried and then weighed on an Oertling five figure balance. Homogenates of these regions were made by homogenising approximately 15mg of tissue in 1 ml ice-cold 0.02M potassium phosphate buffer pH 7.6 for 5 min at 2,000 r.p.m. in a George/Griffiths teflon / glass homogeniser.

Determination of enzyme activity was performed at 37°C in stoppered tubes with shaking. The buffer solutions employed were as follows:- pH 1.5-3.0 glycine - HCl; pH 3.0-8.0 sodium phosphate or sodium acetate; pH 8.0-10.5 Tris-HCl. The pH values were checked at 37°C in the incubation mixtures at the beginning and end of the incubation under all experimental conditions. The ionic strength of the buffer used in the incubation prevented any change greater than 0.1 pH unit following the addition of tissue homogenate or during incubation. The incubation time was 30 min and during this time the reaction rate was constant. The substrate and tissue homogenate concentrations were also chosen from the range in which

Table 19. Details of the preparation of reagents and buffers
for the assay of proteinases.

1. Homogenisation buffer - 0.02 M potassium phosphate (pH 7.6).

6.74 ml 0.02 M potassium dihydrogen phosphate were added to 0.02 M disodium hydrogen phosphate (31 ml) and the mixture was adjusted to the correct pH.

2. 0.1 M potassium phosphate buffer for dissolving casein (pH 7.6).

0.157 g potassium dihydrogen phosphate (KH_2PO_4) and 1.575g disodium hydrogen phosphate($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) were dissolved in 90ml distilled water the solution was adjusted to pH 7.6 and diluted to 100 ml with distilled water.

3. Casein substrate solution. (prepared as described by Bergmeyer 1962)

4 g casein (Hammersten) were suspended in 100 ml 0.1M phosphate buffer pH 7.6 and the mixture was heated for 15 min.

4. Incubation buffers.

a) glycine - hydrochloride buffer

25 ml 1.0 M glycine were added to x ml 1.0 M HCl.

pH	x ml 1.0 M HCl
2.0	25.9
2.5	14.2
3.0	5.7

b) sodium acetate

x ml 1.0 M sodium acetate were added to y ml 1.0M acetic acid

pH	x	y
4.0	18.0	82.0
5.0	70.0	30.0

c) sodium phosphate

x ml 1.0 M disodium hydrogen phosphate were added to y ml 1.0M sodium dihydrogen phosphate.

pH	x	y
6.0	6.15	43.85
7.0	30.50	19.50

Table 19 continued.

4 d) tris - hydrochloride buffer

50 ml 1.0 M tris (hydroxymethyl) aminomethane were added
to x ml 1.0 M HCl.

pH	x
8.0	29.2
9.0	5.8

linearity was obtained with varying enzyme activity of homogenates. The incubation medium consisted of:- 1 M buffer 0.2 ml; homogenate - 0.1 ml; substrate - 0.2 ml; distilled water 0.5 ml. In experiments without an exogenous substrate distilled water was used instead of substrate.

The reaction was terminated by the addition of 0.2 ml 25% TCA solution. For controls the TCA was first added and then the homogenate. The difference between test and control samples was taken as a measure of enzyme activity. The precipitated protein was then centrifuged out in a bench centrifuge at maximum speed for 20 min.

The volume of supernatant was measured and a 0.5 ml aliquot of this was taken out for determination of Folin-Ciocalteus positive material according to Lowry et al (1951). The 0.5 ml of supernatant was added to 1 ml of 0.5N sodium hydroxide and with continuous shaking (Rotamixer) 0.3 ml of Folin & Ciocalteus phenol reagent (diluted 1x3) was added.

After waiting 30 min for the colour to develop, the colour was read at 750 nm on a Unicam SP 600 Series 2 Spectrophotometer. A standard curve was constructed with tyrosine and the results expressed as tyrosine equivalents liberated during incubation.

4.2.2. The automated analysis of trypsin-like and chymotrypsin-like enzymes in regions of the rat brain.

The chromogenic substrates N-benzoyl-DL-arginine p-nitroanilide (DL - BAPA) which is specific for trypsin and N-glutaryl-L-phenylalanine p-nitroanilide (L-GPNA) which is specific for chymotrypsin were developed by Erlanger and his co-workers (1961, 1966). Their hydrolysis can be followed by direct colorimetric analysis of released p-nitroaniline. The automation of these methods was described

by Stewart (1973).

A flow diagram of the assay system is shown in Figure 24 and details of the preparation of reagents and buffers is given in Table 20.

The regions of the brain described in 4.2.1. were also used in this study. The dissected samples were thawed and the surface water removed by partially drying the samples. The samples were then weighed on an Oertling five figure balance. Homogenates of the brain regions were prepared by homogenising approximately 15 mg of tissue in 2.0 ml Tris-HCl buffer pH 8.0 (0.05M) for 5 min. The homogenate is first mixed with buffer through a system of coils in the auto analyser. The substrate was then added and the resulting solution was incubated in a water bath at 37°C for 10 min. The reaction was terminated by the addition of 10% acetic acid. The colour was then read in a colorimeter at 420 nm and read off against a trypsin or chymotrypsin standard curve.

Table 20. Details of the preparation of reagents and buffers for the assay of trypsin and chymotrypsin.

1) Substrate solutions.

- a) The tryptic substrate N - benzoyl - DL - arginine p - nitroanilide (BAPA) was prepared fresh daily by dissolving BAPA in 3 ml dimethyl sulphoxide (50 mg BAPA / ml) with gentle heating and then diluting the solution with 0.5 M tris - hydrochloride buffer (pH 8.0) or 0.5 M sodium acetate buffer (pH 5.0) which contained 0.02 M calcium chloride to a final concentration of 1.5 mg BAPA / ml.
- b) The chymotryptic substrate N - glutaryl - L - phenylalanine p - nitroanilide (GPNA) was prepared fresh daily by dissolving GPNA in 7 ml methanol (20 mg GPNA / ml) with mild heating and then diluting the solution with 0.05 M tris - hydrochloride buffer (pH 8.0) or sodium acetate buffer (pH 5.0) containing 0.02 M calcium chloride to a final concentration of 0.5 mg GPNA / ml.

2) Buffers.

- a) tris - hydrochloride buffer 0.5 M (pH 8.0) was prepared by adding 50 ml 0.5 M tris to 29.2 ml 0.5 M HCl and adjusting the solution to pH 8.0.
- b) sodium acetate buffer 0.5 M (pH 5.0) was prepared by adding 0.5 M sodium acetate (70 ml) to 0.5 M acetic acid (30 ml) and adjusting to pH 5.0.

3) Standard enzymes

- a) Trypsin from beef pancreas (peptidyl peptide hydrolase 3.4.4.4.) was prepared in the appropriate buffer (1.1 mg trypsin / 100 ml). The activity of the enzyme was 10, 000 BEAA units / mg solid. One BEAA unit is defined as causing an increase in absorbance at 253 nm of 0.001 per minute with benzoyl - L-arginine ethyl ester (BEAA) as substrate at pH 7.6 at 25[°]C.

Table 20 .

3) b) A standard solution of chymotrypsin was made in the appropriate buffer (1 mg chymotrypsin / 100 ml). The activity of the enzyme was 45 units of activity / mg solid. One unit of activity is defined as that which will hydrolyse 1.0 μ mole of N - benzoyl - L - tyrosine ethyl ester (BTEE) per minute at pH 7.8 at 25°C.

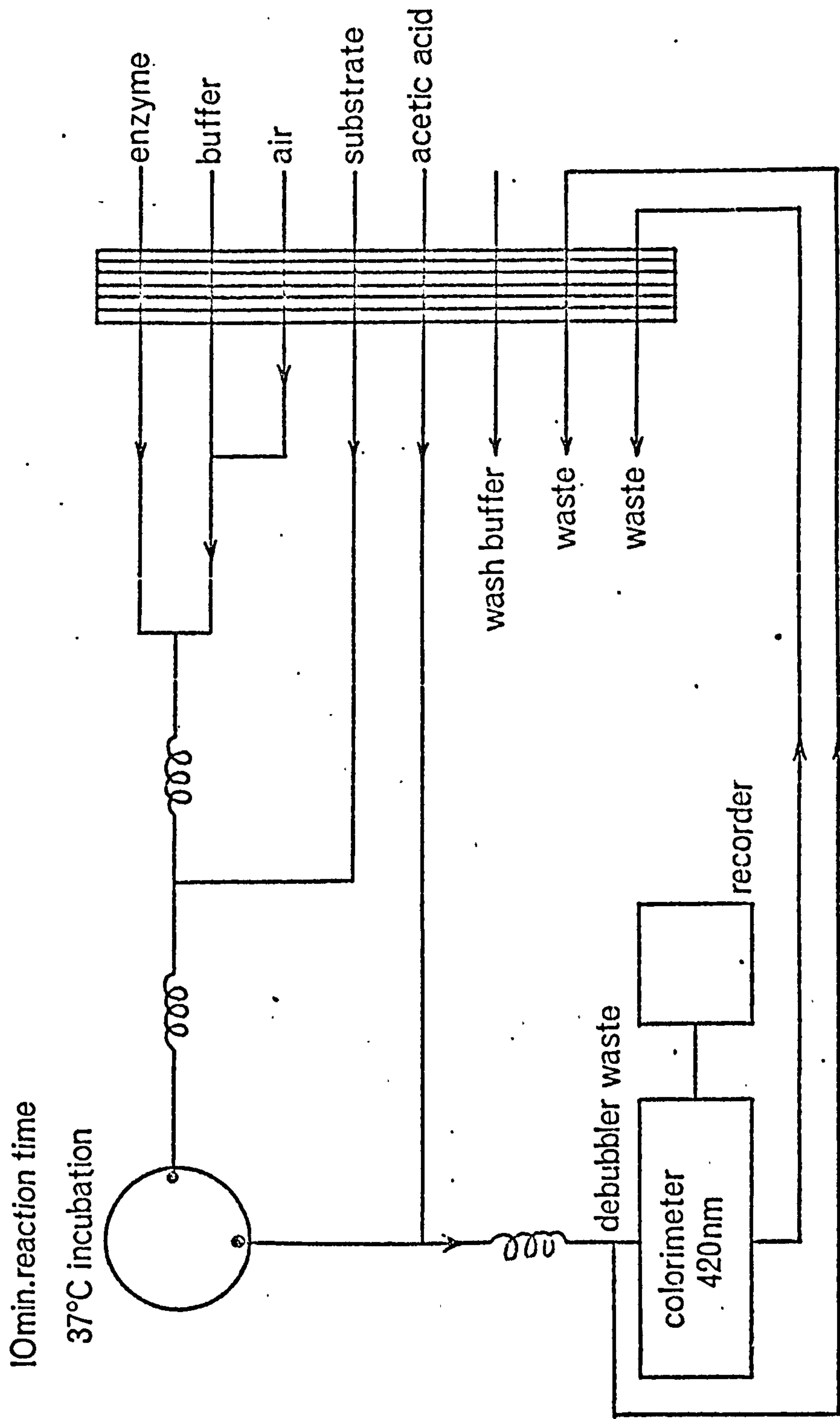


Fig.24 FLOW DIAGRAM FOR ANALYSIS OF TRYPSIN OR CHYMOTRYPSIN

Section 4.3. Results.

4.3.1. Determination of proteinase activity with casein as the protein substrate. pH-activity curve for casein and of endogenous tissue proteins.

The pH dependence of enzyme activity of brain homogenates was first determined. The pH-activity curve was constructed using homogenates of dorsal cortex of rat brain. The buffers used at the various pH regions are given in Table 19. The results of this study are shown in Figure 25.

It can be seen that the activity of proteinases with casein as substrate as well as the rate of hydrolysis of tissue proteins was highest in the acidic pH region. Maximum activity was found at pH 3.0; another distinct peak of activity was seen at pH 7.0. The hydrolysis of tissue proteins proceeded very slowly at neutral and at alkaline pH regions.

It was therefore decided to look at the activity of proteinases in regions of the brain at these two peak activities (pH 3.0 and pH 7.0) following behavioural treatments.

4.3.2. The effects of anaesthesia on acidic and neutral proteinase activity in regions of the rat brain.

The effects of anaesthesia on acidic and neutral proteinases is shown in Tables 21 & 22 and Figures 26 & 27.

- 1) The highest activity in the acidic region was seen in the caudate nucleus (266.4 ± 26.3) and the lowest activity was seen in the cortical regions (eg. frontal cortex 118.1 ± 6.9). A similar distribution was seen for neutral proteinases.
- 2) At pH 3.0 there was no change in the activities of proteinases following anaesthesia in any measured region of the brain.
- 3) At neutral pH decreases in enzyme activity were observed following anaesthesia in the posterior cortex (21% compared to quiet controls and 12% compared to saline controls); in

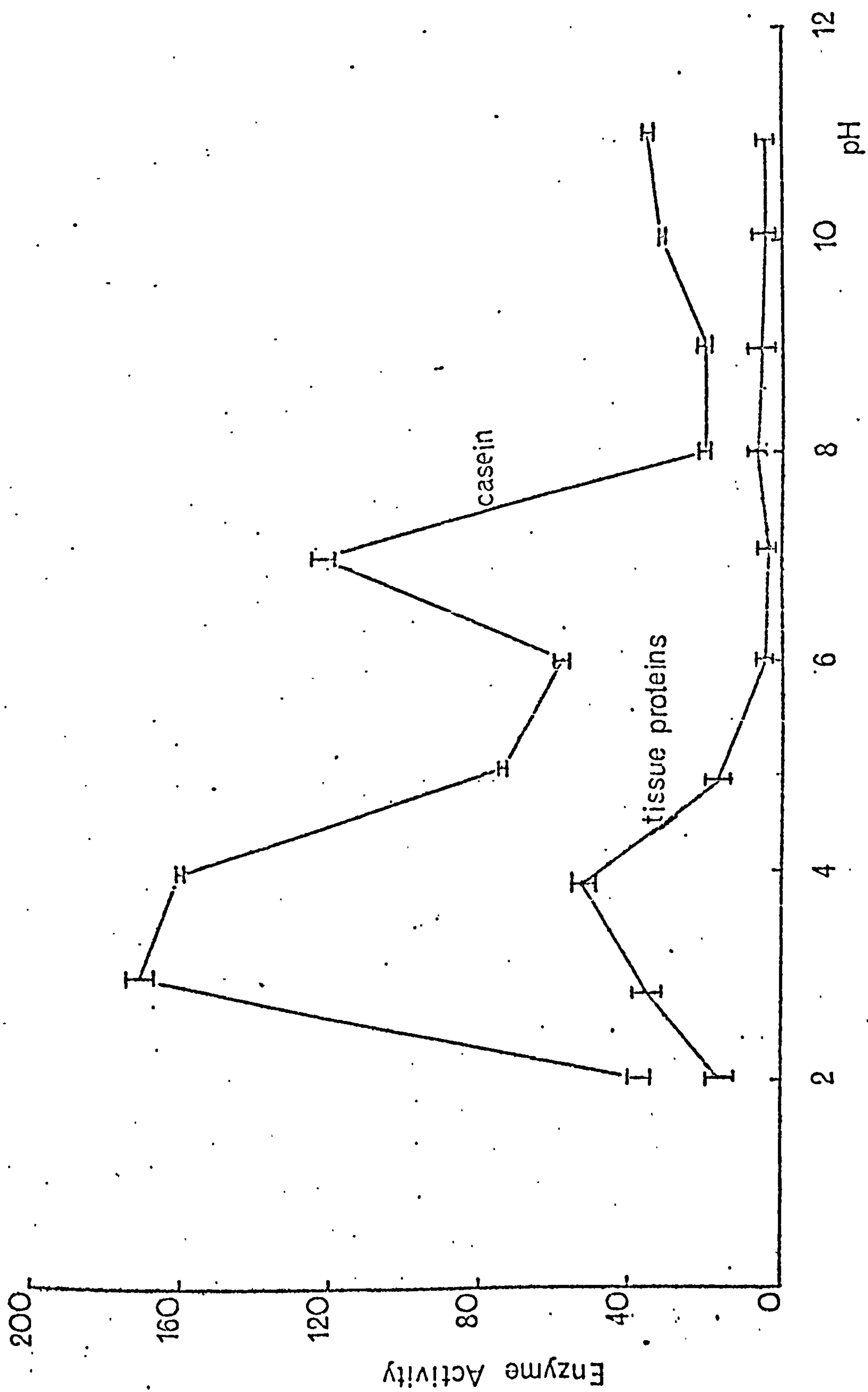


Fig. 25 pH-activity curve for casein and of endogenous proteins

Table 21. The effects of 45 min anaesthesia on the activity of acidic proteinases.

Region	Quiet Control	Saline Control	Anaesthesia
Frontal cortex	118.07 ± 6.9	122.26 ± 9.9	122.36 ± 10.9
Medial cortex	138.11 ± 4.3	160.00 ± 13.6	150.23 ± 13.9
Posterior cortex	156.12 ± 3.6	150.17 ± 8.5	142.90 ± 4.6
Cerebellum	165.42 ± 6.5	177.81 ± 15.7	180.14 ± 5.7
Hippocampus	176.04 ± 6.4	172.68 ± 4.7	167.41 ± 7.7
Thalamus	199.25 ± 16.0	237.99 ± 17.5	211.40 ± 22.7
Medulla	212.95 ± 11.8	197.14 ± 14.4	212.00 ± 20.8
Caudate nucleus	266.44 ± 26.3	246.83 ± 19.6	246.25 ± 17.5

Values given are the mean $\pm$ S.E.M. of at least 4 determinations.

Enzyme activity is expressed as μ moles tyrosine equivalents liberated / hr / g wet weight.

Figure 26. The effect of anaesthesia on the activity of acidic proteinases.

The columns represent the means of the number of observations shown in Table 21. The white columns represent the activity of the quiet controls ; the black columns represent the activity of the saline controls and the stippled columns represent the activity of the anaesthetised group.

key: FC Frontal cortex
 MC Medial cortex
 PC Posterior cortex
 C Cerebellum
 HP Hippocampus
 T Thalamus
 M Medulla
 CN Caudate nucleus

The enzyme activity is expressed in terms of μ moles tyrosine equivalents liberated / hr / g wet weight.

Table 22. The effects of 45 min anaesthesia on the activity of neutral proteinases.

Region	Quiet Control	Saline Control	Anaesthesia
Frontal cortex	84.88 ± 8.1	85.70 ± 14.6	99.54 ± 9.1
Medial cortex	87.12 ± 5.6	79.61 ± 4.7	94.12 ± 12.2
Posterior cortex	91.21 ± 4.3	81.32 ± 5.7	71.85 ⁺⁺ ± 3.1
Cerebellum	104.40 ± 3.1	97.65 ± 7.4	72.52 ± 24.8
Hippocampus	100.19 ± 3.3	92.85 ± 5.3	61.47 ⁺⁺⁺⁺ ± 1.8
Thalamus	127.58 ± 5.2	124.90 ± 14.9	97.13 ⁺⁺ ± 3.7
Medulla	180.38 ± 3.1	193.71 ± 11.9	173.15 ± 10.1
Caudate nucleus	173.30 ± 5.1	174.90 ± 9.6	195.60 ± 10.4

Values given are the mean ± S.E.M. of at least 4 determinations.

Enzyme activity is expressed as μ moles tyrosine equivalents liberated / hr / g wet weight.

+ = significantly different from quiet controls (+ p < 0.05 ;

++ p < 0.01 ; +++ p < 0.001).

* = significantly different from saline controls (* p < 0.05 ;

** p < 0.01 ; *** p < 0.001).

Figure 27. The effects of anaesthesia on the activity of neutral proteinases.

The columns represent the means of the number of observations shown in Table 22. The white columns represent the activity of the quiet control group ; the black columns represent the activity of the saline group and the stippled columns represent the activity of the anaesthetised group.

key : FC Frontal cortex
MC Medial cortex
PC Posterior cortex
C Cerebellum
HP Hippocampus
T Thalamus
M Medulla
CN Caudate nucleus

■ = significantly different from quiet controls

△ = significantly different from saline controls

Enzyme activity is expressed in terms of μ moles tyrosine equivalents liberated / hr / g wet weight.

the hippocampus (38.6% compared with quiet controls; 33.8% compared with saline controls) and in the thalamus (24% compared with quiet controls).

Thus the activity of neutral proteinases decreases in some regions of the brain following anaesthesia.

4.3.3. The effects of electrical stimulation and shock avoidance conditioning on the activity of acidic and neutral proteinases.

The effects of electrical stimulation and shock avoidance conditioning on proteinase activity is shown in Tables 23 & 24 and Figures 28 & 29.

- 1) No changes in the activities of either acidic or neutral proteinases were seen following either electrical stimulation or shock avoidance conditioning in the medulla.
- 2) Electrical stimulation caused a significant increase in activity of both acidic and neutral proteinases in the frontal cortex (26% acidic proteinases; 42% neutral proteinases) and in the hippocampus (39% acidic proteinases; 22% neutral proteinases)
- 3) There was no significant change in activity of yoked control group compared with quiet control group in the medial cortex, posterior cortex, cerebellum, thalamus and caudate nucleus.
- 4) There was a significant increase in activity of both acidic and neutral proteinases in the frontal cortex of conditioned group compared with quiet control group (33% acidic; 40% neutral) but no significant difference in activity between the yoked controls and the conditioned group. Since there was a similar increase in activity of the yoked control group this indicates that this effect is probably due to stress following electrical stimulation. Similarly, in the hippocampus there was an increase in activity of neutral proteinases of the conditioned group compared with the

Table 23. The effects of electrical stimulation and shock avoidance conditioning on the activity of acidic proteinases.

Region	Quiet Control	Yoked Control	Conditioned
Frontal cortex	118.07 ± 6.9	148.97 ⁺ ± 5.9	156.89 ⁺⁺ ± 5.2
Medial cortex	138.11 ± 4.3	152.00 ± 7.9	172.06 ⁺⁺ ± 7.6
Posterior cortex	156.12 ± 3.6	142.57 ± 8.8	184.66 ⁺ ± 9.9
Cerebellum	165.42 ± 6.5	164.96 ± 3.1	190.75 ⁺⁺⁺ ± 6.4
Hippocampus	176.04 ± 6.4	246.08 ⁺⁺⁺ ± 6.3	204.23 ⁺⁺ ± 6.1
Thalamus	199.25 ± 16.0	216.90 ± 20.7	146.76 ⁺ ± 3.0
Medulla	212.95 ± 11.8	220.43 ± 19.0	222.11 ± 10.8
Caudate nucleus	266.44 ± 26.3	220.04 ± 16.3	297.30 ^{**} ± 16.5

Values given are the mean ± S.E.M. of at least 4 determinations.

Enzyme activity is expressed as μ moles tyrosine equivalents liberated / hr / g wet weight.

+ = significantly different from quiet controls (+ p < 0.05 ;

++ p < 0.01 ; +++ p < 0.001).

* = significantly different from yoked controls (* p < 0.05 ;

** p < 0.01 ; *** p < 0.001).

Figure 28. The effects of electrical stimulation and shock avoidance conditioning on the activity of acidic proteinases.

The columns represent the means of the number of observations shown in Table 23. The white columns represent the quiet controls ; the black columns represent the activity of the yoked controls and the stippled columns represent the activity of the shock avoidance conditioned group.

key : FC Frontal cortex
MC Medial cortex
PC Posterior cortex
C Cerebellum
HP Hippocampus
T Thalamus
M Medulla
CN Caudate nucleus

□ = significantly different from quiet controls

Δ = significantly different from yoked controls

Enzyme activity is expressed as μmoles tyrosine equivalents liberated / hr / g wet weight.

Table 24. The effects of electrical stimulation and shock avoidance conditioning on the activity of neutral proteinases.

Region	Quiet Control	Yoked Control	Conditioned
Frontal cortex	84.88 ± 8.1	128.95 ⁺⁺ ± 4.7	127.64 ⁺⁺ ± 5.9
Medial cortex	87.12 ± 5.6	108.64 ± 10.3	120.87 ⁺⁺ ± 9.1
Posterior cortex	91.21 ± 4.3	87.70 ± 2.5	119.40 ⁺⁺⁺ ± 2.6
Cerebellum	104.40 ± 3.1	114.81 ± 4.1	138.11 ⁺⁺⁺ ± 3.5
Hippocampus	100.19 ± 3.3	121.95 ⁺⁺ ± 3.9	126.63 ⁺⁺ ± 6.0
Thalamus	127.58 ± 5.2	136.85 ± 4.2	111.09 ± 6.0
Medulla	180.38 ± 3.1	207.83 ± 20.3	204.43 ± 14.6
Caudate nucleus	173.30 ± 5.1	177.04 ± 15.8	188.82 ± 6.6

Values given are the mean and S.E.M. of at least 4 determinations.

Enzyme activity is expressed as μ moles tyrosine equivalents liberated / hr / g wet weight.

+ = significantly different from quiet controls (+ p < 0.05 ;

++ p < 0.01 ; +++ p < 0.001).

* = significantly different from yoked control (* p < 0.05 ;

** p < 0.01 ; *** p < 0.001).

Figure 29. The effects of electrical stimulation and shock avoidance conditioning on the activity of neutral proteinases.

The columns represent the means of the number of determinations shown in Table 24. The white columns represent the quiet controls ; the black columns represent the yoked controls and the stippled columns represent the shock avoidance conditioned group.

key : FC Frontal cortex
MC Medial cortex
PC Posterior cortex
C Cerebellum
HP Hippocampus
T Thalamus
M Medulla
CN Caudate nucleus

◻ = significantly different from quiet controls

Δ = significantly different from yoked controls

Enzyme activity is expressed as μmoles tyrosine equivalents liberated / hr / g wet weight.

quiet control group (26%); but no change compared with the yoked controls. However, the activity of acidic proteinases in the hippocampus did show a significant change compared to the activity of the yoked controls (17%); indicating that the effect of stress was to increase activity, while the effect of training was to decrease it.

- 5) The activity of acidic proteinases of the conditioned group was found to increase significantly compared with the quiet control group in the medial cortex (25%); and posterior cortex (18%). In these two regions there was no significant increase in activity of the conditioned group compared with the yoked control group.

In the caudate nucleus, the activity of the conditioned group increased significantly (35%) compared to the yoked control group. However, in the cerebellum the activity of acidic proteinases of the conditioned group increased significantly compared to both the quiet control group (15%) and to the yoked control group (15%), indicating that this change was specifically related to the training experience.

- 6) The activity of neutral proteinases showed significant increases following conditioning compared with both quiet controls and yoked controls in the medial cortex (39% quiet controls; 11% yoked controls); posterior cortex (31% quiet controls; 36% yoked controls) and cerebellum (32% quiet controls; 20% yoked controls).

- 7) There was no significant change in enzyme activity of the conditioned group in the caudate nucleus and thalamus compared either with quiet controls or yoked controls at neutral pH.

4.3.4. The pH dependence of the rate of hydrolysis of the tryptic (BAPA) and chymotryptic (GPNA) substrates.

The pH-activity curve was constructed using homogenates of dorsal cortex of rat brain. The buffers used for the various pH regions were those described in Section 4.2.1. The pH dependence of hydrolysis of the tryptic and chymotryptic substrates are shown in Figures 30 & 31 respectively.

From Figure 30, it can be seen that the highest activity with the tryptic substrate is seen at pH 8.0, with a second peak of activity at pH 5.0. The activity with the chymotryptic substrate reaches a maximum at pH 6.0 and there is a second peak of activity at pH 8.0.

It was therefore decided to investigate the activity of these two enzymes at pH 5.0 and pH 8.0 to see if there was any change in these proteolytic enzymes following behavioural treatments.

4.3.5. The effects of 45 min anaesthesia on proteinase activity using the tryptic substrate (BAPA).

The rates of hydrolysis of BAPA for the quiet control group, saline group and anaesthetised group at pH 5.0 and pH 8.0 are shown in Tables 25 & 26 and Figures 32 & 33 respectively.

- 1) The rates of hydrolysis of BAPA was greatest in the medulla at both pH values; the variation in activity between the other regions was not very great. In all regions of the brain investigated the activity with this tryptic substrate was greater at pH 8.0 than at pH 5.0.
- 2) There was no significant change in activity with this substrate following anaesthesia at either pH value investigated in any region of the brain examined.

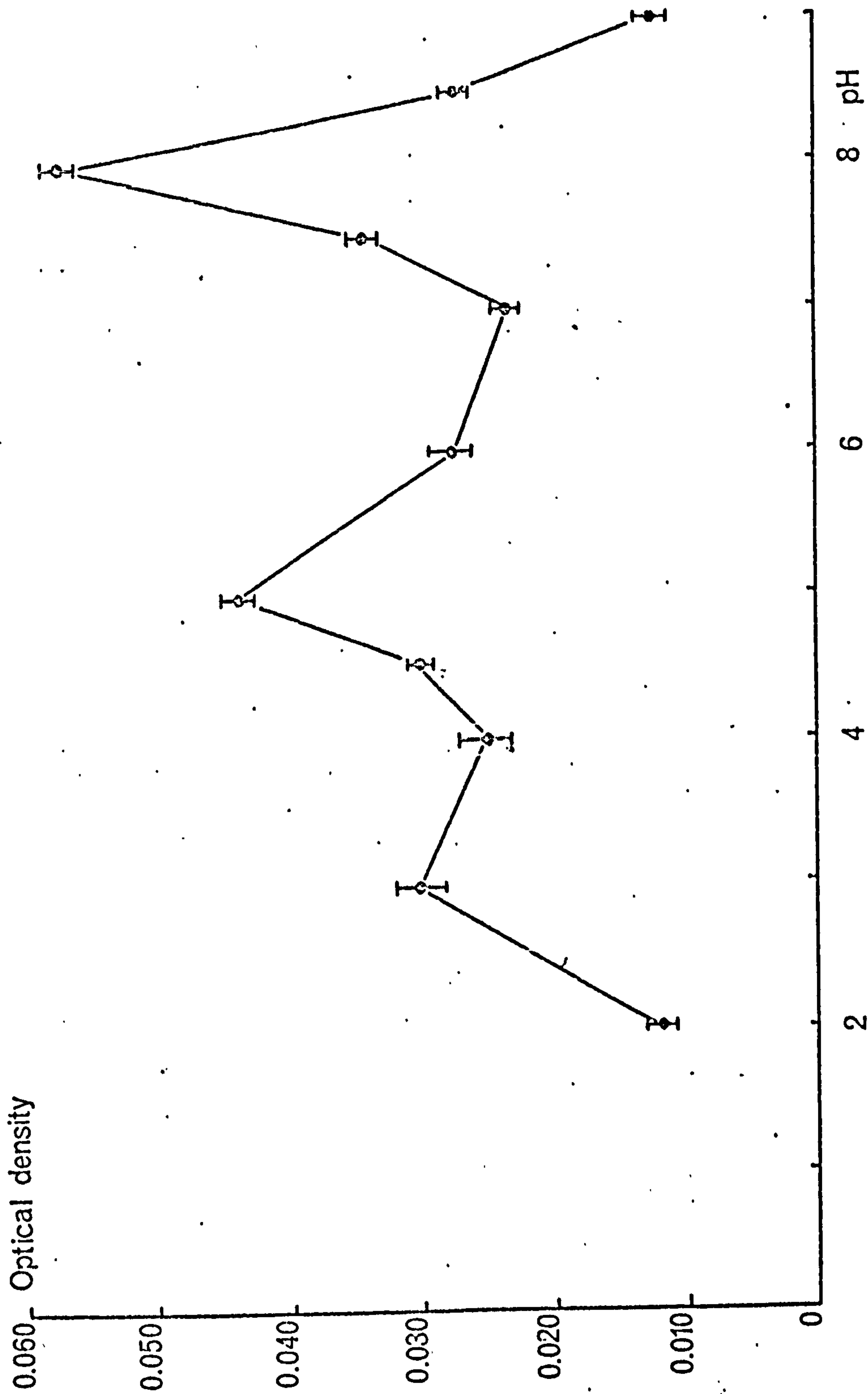


Fig.30 pH dependence of the rate of hydrolysis of the tryptic substrate BAPA

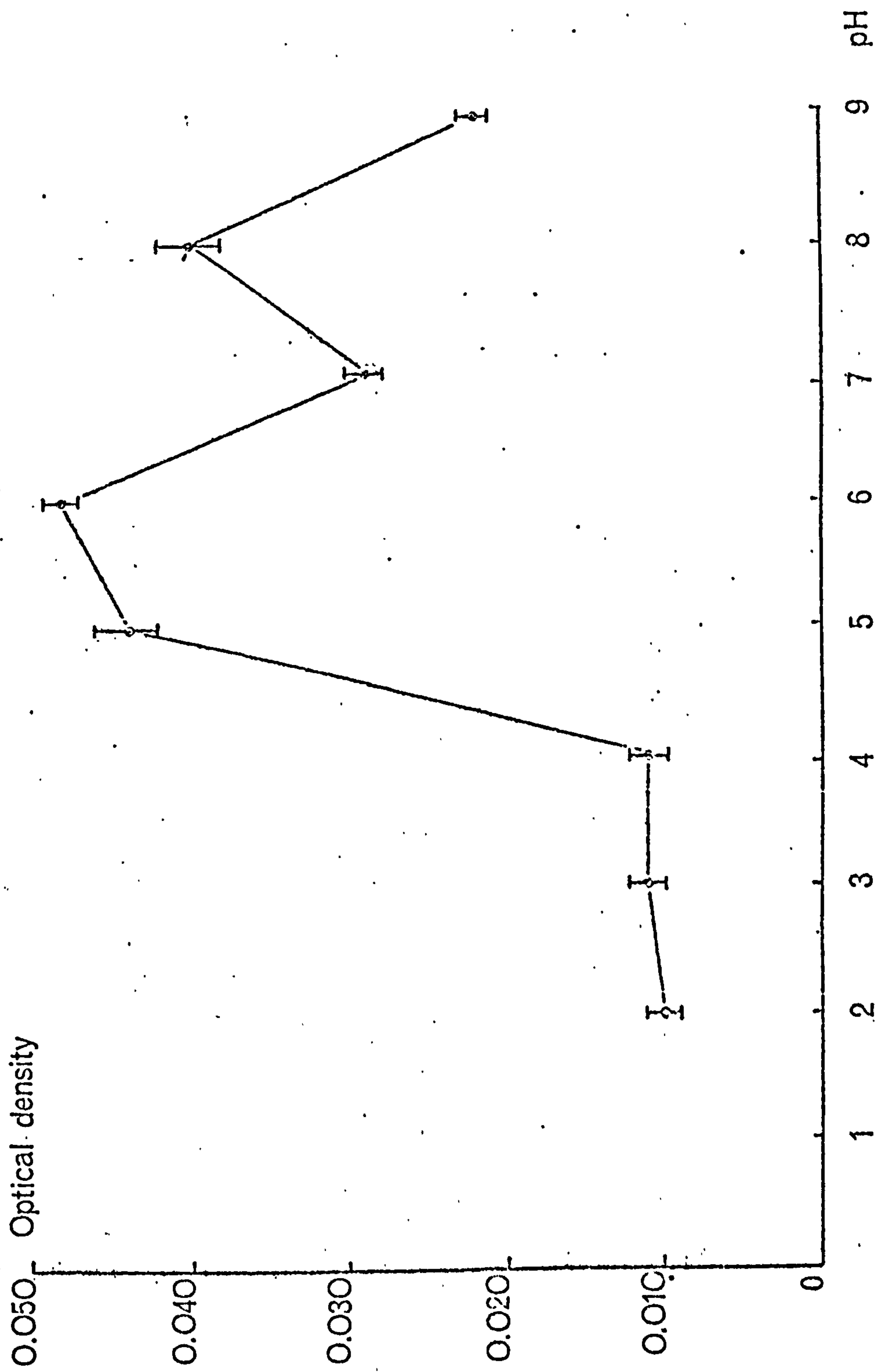


Fig.31 pH dependence of rate of hydrolysis of the chymotryptic substrate GPNA

Table 25. The effects of 45 min anaesthesia on the rate of hydrolysis of the tryptic substrate BAPA at acidic pH.

Region	Quiet Control	Saline Control	Anaesthesia
Frontal cortex	23.27 $\pm$ 1.1	24.51 $\pm$ 0.4	21.96 $\pm$ 0.8
Medial cortex	27.09 $\pm$ 0.5	26.47 $\pm$ 0.6	26.56 $\pm$ 0.8
Posterior cortex	26.52 $\pm$ 0.5	26.97 $\pm$ 0.4	26.14 $\pm$ 0.4
Cerebellum	23.44 $\pm$ 0.4	23.68 $\pm$ 0.5	23.96 $\pm$ 0.6
Hippocampus	28.61 $\pm$ 0.6	29.29 $\pm$ 0.4	29.85 $\pm$ 0.5
Thalamus	24.17 $\pm$ 0.7	23.54 $\pm$ 0.4	23.73 $\pm$ 0.6
Medulla	70.44 $\pm$ 0.6	70.19 $\pm$ 0.4	69.69 $\pm$ 0.3
Caudate nucleus	23.66 $\pm$ 0.6	23.77 $\pm$ 0.4	24.46 $\pm$ 0.5

Table 26. The effects of 45 min anaesthesia on the rate of hydrolysis of the tryptic substrate BAPA at alkaline pH.

Region	Quiet Control	Saline Control	Anaesthesia
Frontal cortex	29.84 $\pm$ 0.8	27.47 $\pm$ 1.2	29.08 $\pm$ 2.1
Medial cortex	33.43 $\pm$ 2.0	28.36 $\pm$ 2.4	28.97 $\pm$ 3.8
Posterior cortex	32.26 $\pm$ 1.1	28.47 $\pm$ 1.7	29.37 $\pm$ 2.0
Cerebellum	26.16 $\pm$ 1.5	25.88 $\pm$ 1.5	27.64 $\pm$ 1.9
Hippocampus	32.81 $\pm$ 1.1	35.55 $\pm$ 5.1	31.32 $\pm$ 1.4
Thalamus	29.72 $\pm$ 1.0	31.04 $\pm$ 0.8	32.77 $\pm$ 1.3
Medulla	77.64 $\pm$ 3.1	79.41 $\pm$ 3.5	83.38 $\pm$ 2.7
Caudate nucleus	27.18 $\pm$ 1.9	29.55 $\pm$ 1.5	29.72 $\pm$ 1.1

Values given are the mean $\pm$ S.E.M. of at least 4 determinations. Enzyme activity is expressed as trypsin equivalents / min / mg wet weight.

Fig.32

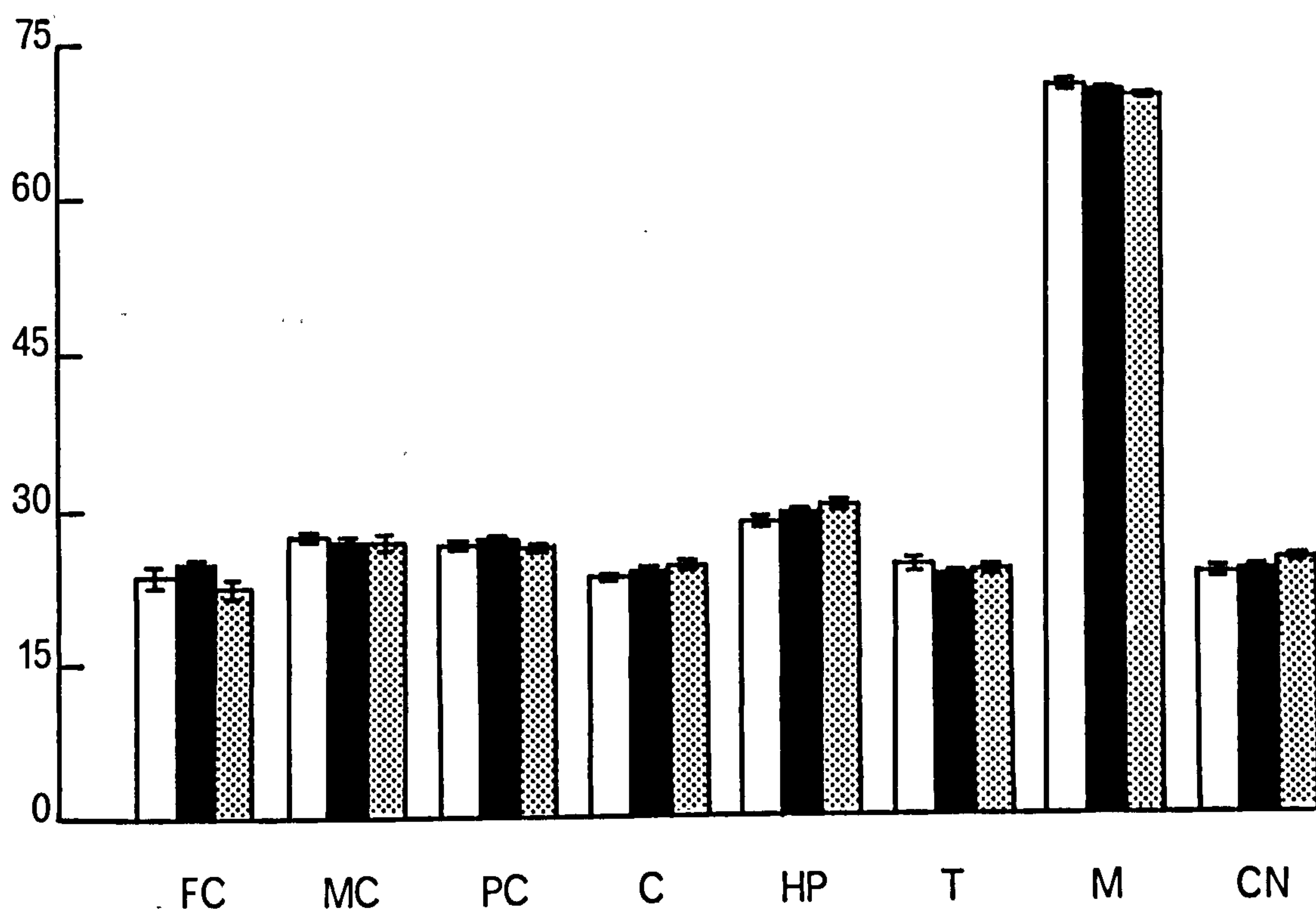
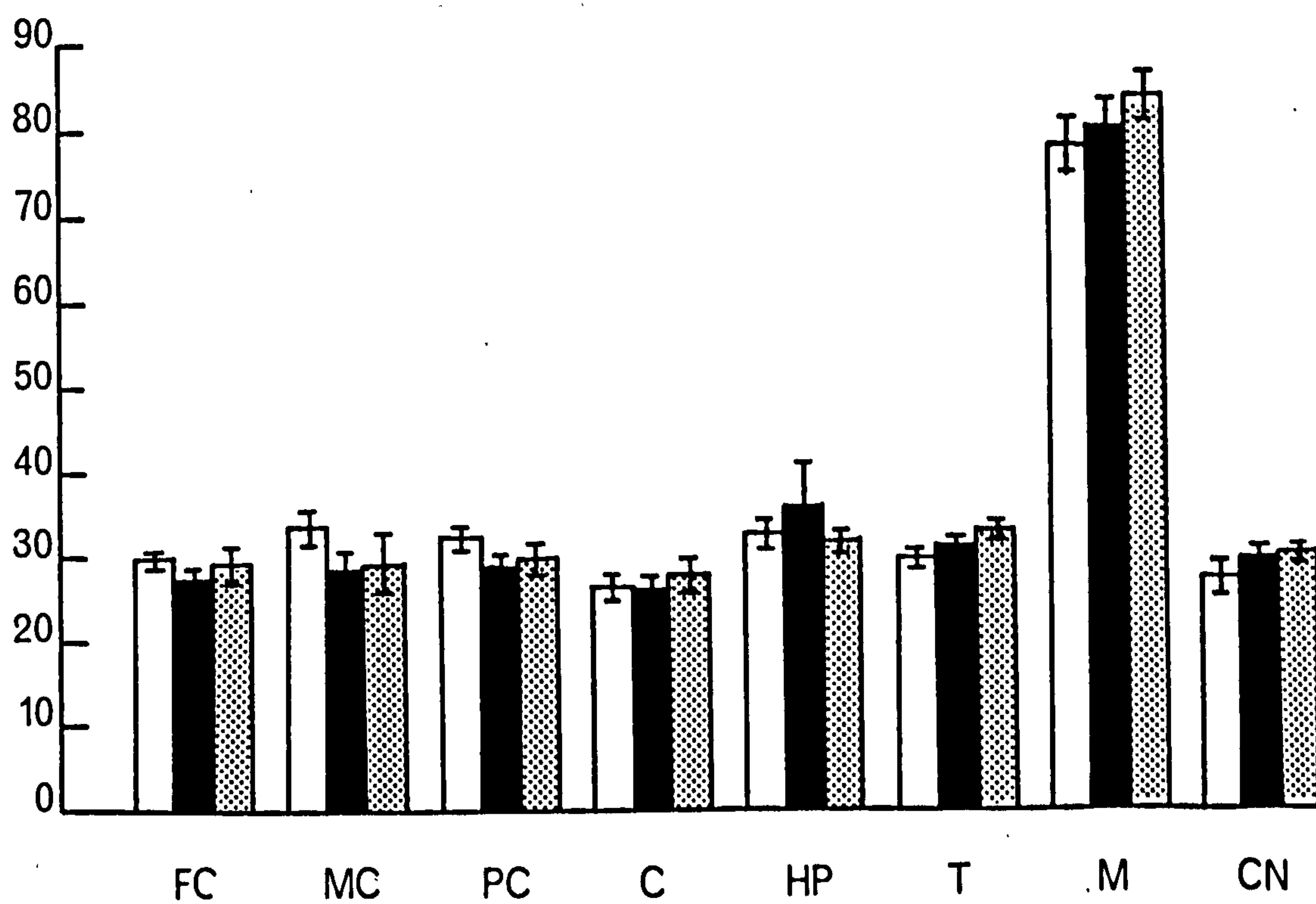


Fig.33



The effects of anaesthesia on the rate of hydrolysis of a tryptic substrate at acid and alkaline pH.

Figure 32 shows the effects of anaesthesia on the rate of hydrolysis of the tryptic substrate at acidic pH ; while Figure 33 shows the effect of anaesthesia on the rate of hydrolysis of the tryptic substrate at alkaline pH.

The columns represent the means of the number of observations shown in Tables 25 & 26. The white columns represent the quiet controls ; the black columns represent the saline controls and the stippled columns represent the anaesthetised group.

key : FC Frontal cortex
MC Medial cortex
PC Posterior cortex
C Cerebellum
HP Hippocampus
T Thalamus
M Medulla
CN Caudate nucleus

Enzyme activity is expressed in terms of trypsin equivalents / min / mg wet weight.

4.3.6. The effects of 45 min anaesthesia on proteinase activity using the chymotryptic substrate (GPNA).

The rates of hydrolysis of GPNA for quiet controls, saline controls and anaesthetised animals at pH 5.0 and pH 8.0 are shown in Table 27 & 28 and Figures 34 & 35. respectively.

- 1) The rate of hydrolysis of GPNA was much greater in the medulla at both pH regions than in any other region examined; there was little variation in activity between the other regions investigated. In all regions of the brain investigated the activity at pH 5.0 was greater than the activity at pH 8.0.
- 2) As with the tryptic substrate, there was no change in the activity of the anaesthetised group with this substrate compared with either quiet controls or saline controls in any region of the brain investigated at either pH values.

Table 27. The effects of 45 min anaesthesia on the rate of hydrolysis of the chymotryptic substrate GPNA at acidic pH.

Region	Quiet Control	Saline Control	Anaesthesia
Frontal cortex	10.72 $\pm$ 0.3	10.64 $\pm$ 0.2	10.60 $\pm$ 0.3
Medial cortex	9.62 $\pm$ 0.1	9.58 $\pm$ 0.2	9.62 $\pm$ 0.1
Posterior cortex	9.69 $\pm$ 0.1	9.73 $\pm$ 0.1	9.74 $\pm$ 0.1
Cerebellum	10.15 $\pm$ 0.3	10.23 $\pm$ 0.3	10.45 $\pm$ 0.4
Hippocampus	12.81 $\pm$ 0.2	12.65 $\pm$ 0.2	12.96 $\pm$ 0.2
Thalamus	14.05 $\pm$ 0.4	14.09 $\pm$ 0.5	14.38 $\pm$ 0.3
Medulla	29.62 $\pm$ 0.3	29.16 $\pm$ 0.5	29.50 $\pm$ 0.3
Caudate nucleus	12.66 $\pm$ 0.4	13.14 $\pm$ 0.2	12.58 $\pm$ 0.4

Table 28. The effects of 45 min anaesthesia on the rate of hydrolysis of the chymotryptic substrate GPNA at alkaline pH.

Region	Quiet Control	Saline Control	Anaesthesia
Frontal cortex	10.02 $\pm$ 0.7	9.27 $\pm$ 0.5	9.89 $\pm$ 0.6
Medial cortex	9.26 $\pm$ 0.5	9.26 $\pm$ 0.5	9.36 $\pm$ 0.6
Posterior cortex	9.59 $\pm$ 0.3	9.51 $\pm$ 0.5	9.86 $\pm$ 0.2
Cerebellum	10.09 $\pm$ 0.5	9.85 $\pm$ 0.4	11.54 $\pm$ 0.6
Hippocampus	11.71 $\pm$ 0.2	11.46 $\pm$ 0.4	10.95 $\pm$ 0.3
Thalamus	14.18 $\pm$ 0.8	15.93 $\pm$ 1.1	14.52 $\pm$ 1.2
Medulla	27.31 $\pm$ 0.8	27.48 $\pm$ 1.9	25.73 $\pm$ 1.2
Caudate nucleus	11.43 $\pm$ 0.6	13.23 $\pm$ 1.1	12.05 $\pm$ 1.2

Values given are the mean $\pm$ S.E.M. of at least 4 determinations.

Enzyme activity is expressed as chymotrypsin equivalents / min / g wet weight.

Fig.34

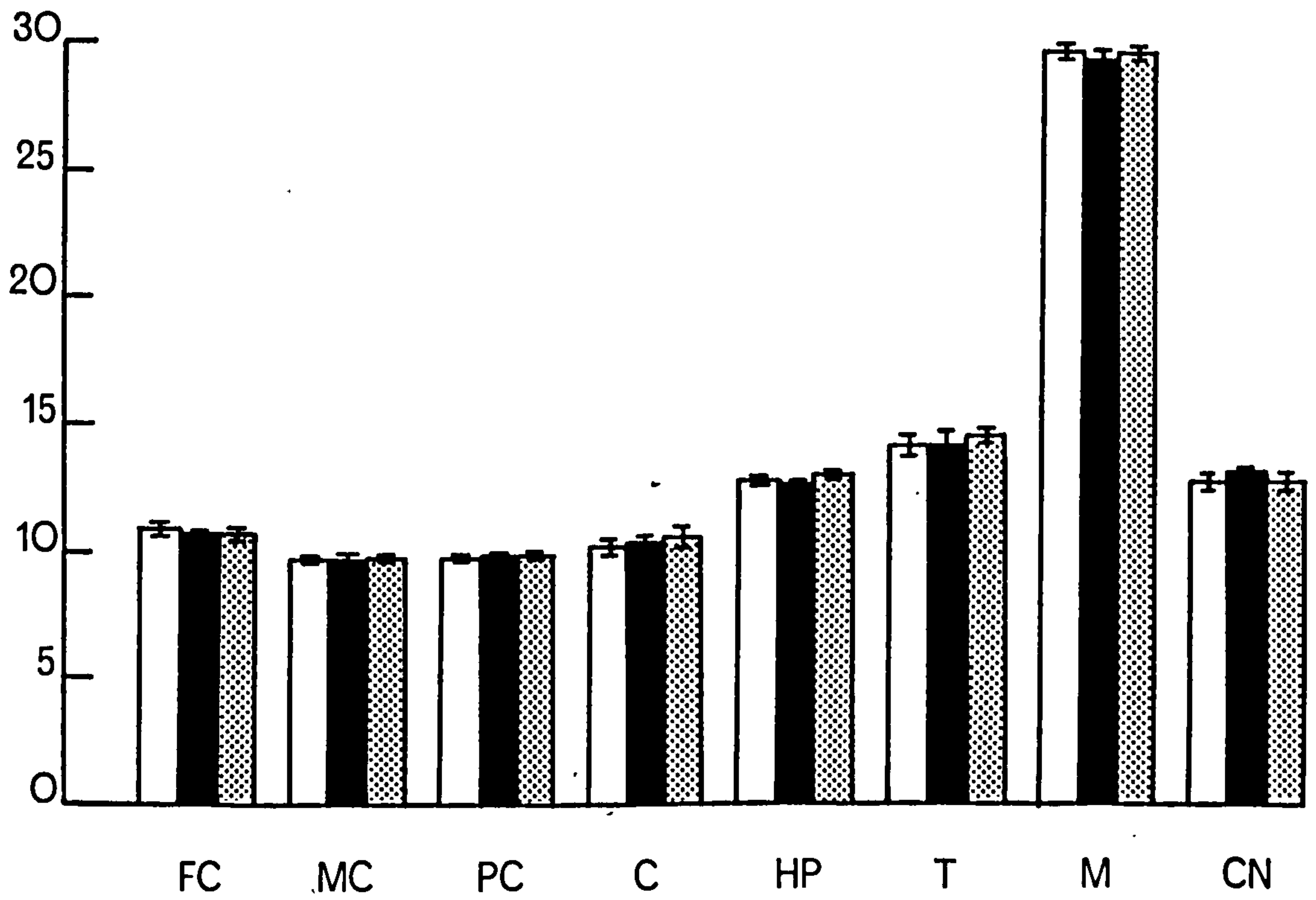
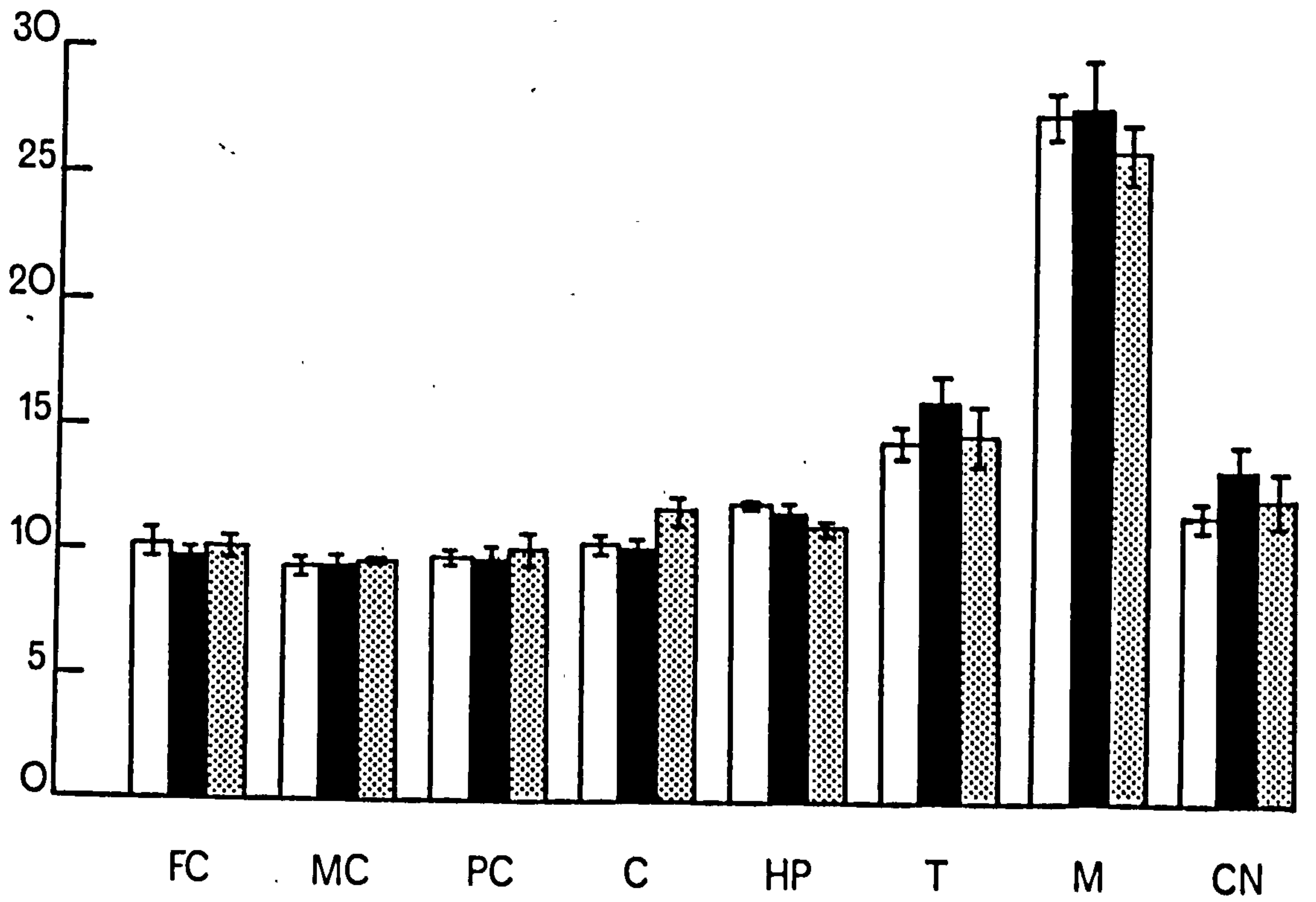


Fig.35



The effects of anaesthesia on the rate of hydrolysis of a chymotryptic substrate at acidic and alkaline pH.

Figure 34 shows the effects of anaesthesia on the rate of hydrolysis of the chymotryptic substrate GPNA at acidic pH; while Figure 35 shows the effects of anaesthesia on the rate of hydrolysis of this substrate at alkaline pH.

The columns represent the means of the number of observations shown in Tables 27 & 28. The white columns represent the quiet control group ; the black columns represent the saline control group and the stippled columns represent the anaesthetised group.

,

key : FC Frontal cortex
MC Medial cortex
PC Posterior cortex
C Cerebellum
HP Hippocampus
T Thalamus
M Medulla
CN Caudate nucleus

Enzyme activity is expressed in terms of chymotrypsin equivalents / min / g wet weight.

4.3.7. The effects of electrical stimulation and shock avoidance conditioning on proteinase activity with a tryptic substrate.

The effects of electrical stimulation and shock avoidance conditioning on the rate of hydrolysis of BAPA at pH 5.0 and 8.0 are shown in Tables 29 & 30 and Figures 36 & 37 respectively.

- 1) There was no change in the rate of hydrolysis of BAPA following either electrical stimulation or shock avoidance conditioning at either pH value in the medial cortex, thalamus, medulla, and caudate nucleus.
- 2) There was a significant increase in enzyme activity of the yoked control group compared with the quiet control group in the hippocampus at pH 5.0 and pH 8.0 (12% pH 5.0; 19% pH 8.0).
- 3) Electrical stimulation produced a significant increase in enzyme activity (25%) at pH 8.0 in the frontal cortex but no change in activity at pH 5.0
- 4) There was no change in enzyme activity following electrical stimulation in the posterior cortex or cerebellum at pH 8.0 or pH 5.0
- 5) There was no significant change in enzyme activity of the conditioned group at pH 5.0 in any region of the brain examined compared with either yoked control or quiet control group.
- 6) The increase in enzyme activity of the frontal cortex of the conditioned group at pH 8.0 compared with the quiet control group (32%) is comparable with the increase in activity of the yoked control group compared with the quiet control group; and is, therefore, probably due to stress following foot shock.
- 7) Shock avoidance conditioning produced an increase in hydrolysis of BAPA at pH 8.0 in the cerebellum (16%) compared to the yoked control group.

Table 29. The effects of electrical stimulation and conditioning
on the rate of hydrolysis of the tryptic substrate BAPA
at pH 5.0.

Region	Quiet Control	Yoked Control	Conditioned
Frontal cortex	23.27 $\pm$ 1.1	22.23 $\pm$ 1.0	26.62 $\pm$ 0.8
Medial cortex	27.09 $\pm$ 0.7	25.75 $\pm$ 1.0	27.02 $\pm$ 0.7
Posterior cortex	26.52 $\pm$ 0.6	26.58 $\pm$ 0.4	24.99 $\pm$ 0.9
Cerebellum	23.44 $\pm$ 0.4	23.82 $\pm$ 0.5	23.15 $\pm$ 0.4
Hippocampus	28.61 $\pm$ 0.6	31.99 $\pm$ 0.4 ⁺⁺	29.85 $\pm$ 1.0
Thalamus	24.17 $\pm$ 0.7	23.82 $\pm$ 0.5	24.63 $\pm$ 0.3
Medulla	77.44 $\pm$ 0.6	70.72 $\pm$ 0.8	69.86 $\pm$ 0.5
Caudate nucleus	23.66 $\pm$ 0.6	23.90 $\pm$ 0.6	23.63 $\pm$ 0.6

Table 30. The effects of electrical stimulation and conditioning
on the rate of hydrolysis of the tryptic substrate BAPA
at pH 8.0.

Region	Quiet Control	Yoked Control	Conditioned
Frontal cortex	29.84 $\pm$ 0.8	37.27 $\pm$ 1.1 ⁺⁺	39.24 $\pm$ 1.1 ⁺⁺⁺
Medial cortex	33.43 $\pm$ 2.0	31.19 $\pm$ 1.3	32.00 $\pm$ 0.9
Posterior cortex	32.26 $\pm$ 1.1	30.62 $\pm$ 0.3	36.79 $\pm$ 0.6 ^{***}
Cerebellum	26.16 $\pm$ 1.4	25.60 $\pm$ 1.3	29.76 $\pm$ 1.2 [*]
Hippocampus	32.81 $\pm$ 1.1	38.97 $\pm$ 0.5 ⁺⁺	33.63 $\pm$ 1.3 [*]
Thalamus	29.72 $\pm$ 1.0	29.35 $\pm$ 0.5	29.61 $\pm$ 0.4
Medulla	77.64 $\pm$ 3.1	76.91 $\pm$ 2.5	77.15 $\pm$ 1.3
Caudate nucleus	27.18 $\pm$ 1.9	27.88 $\pm$ 0.7	25.70 $\pm$ 2.2

Values given are the mean $\pm$ S.E.M. of at least 4 determinations.

Enzyme activity is expressed as trypsin equivalents / min / mg wet

weight. + = significantly different from quiet controls (+ p < 0.05 ;

++ p < 0.01 ; +++ p < 0.001). * = significantly different from yoked

controls (* p < 0.05 ; ** p < 0.01).

Fig.36

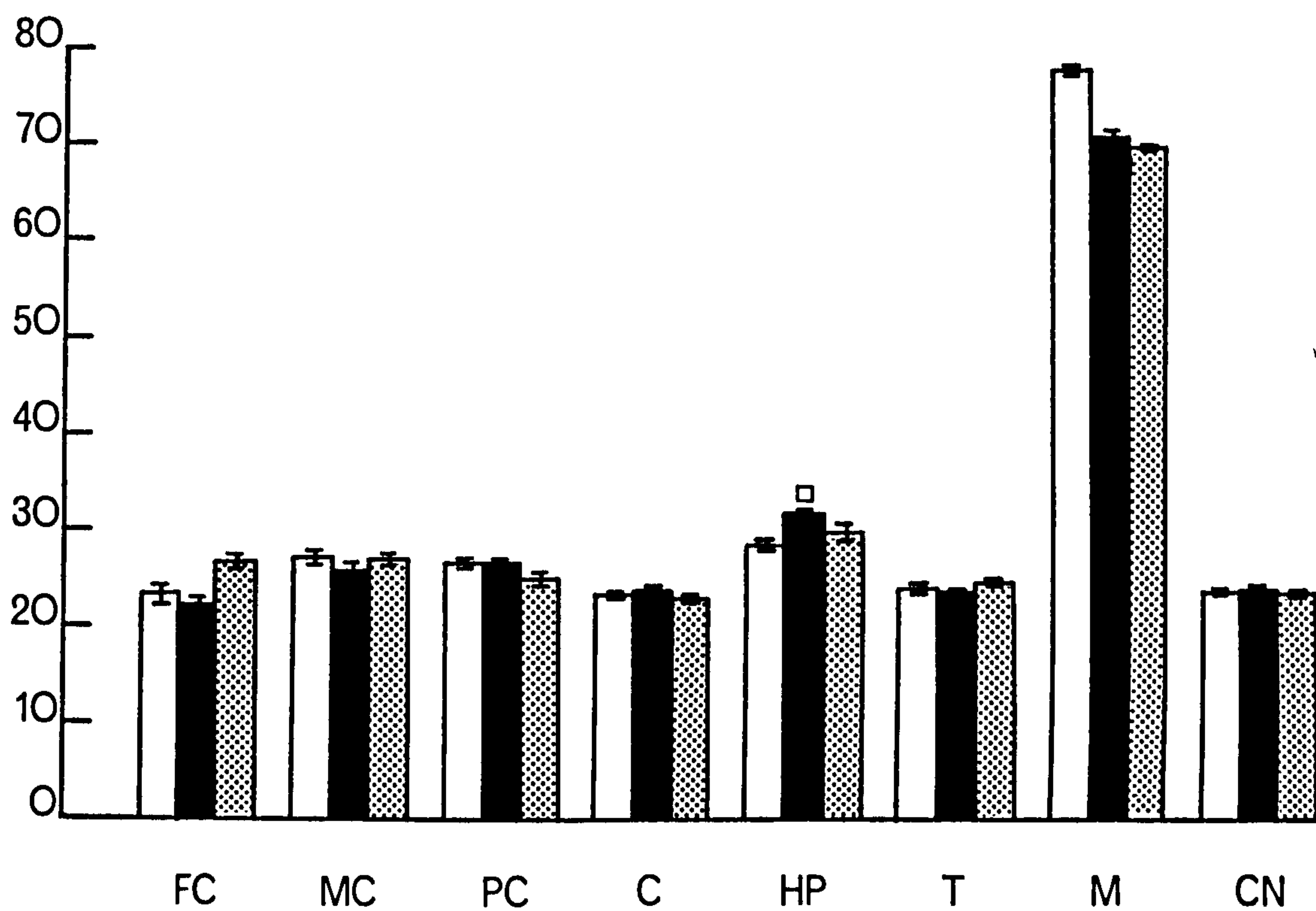
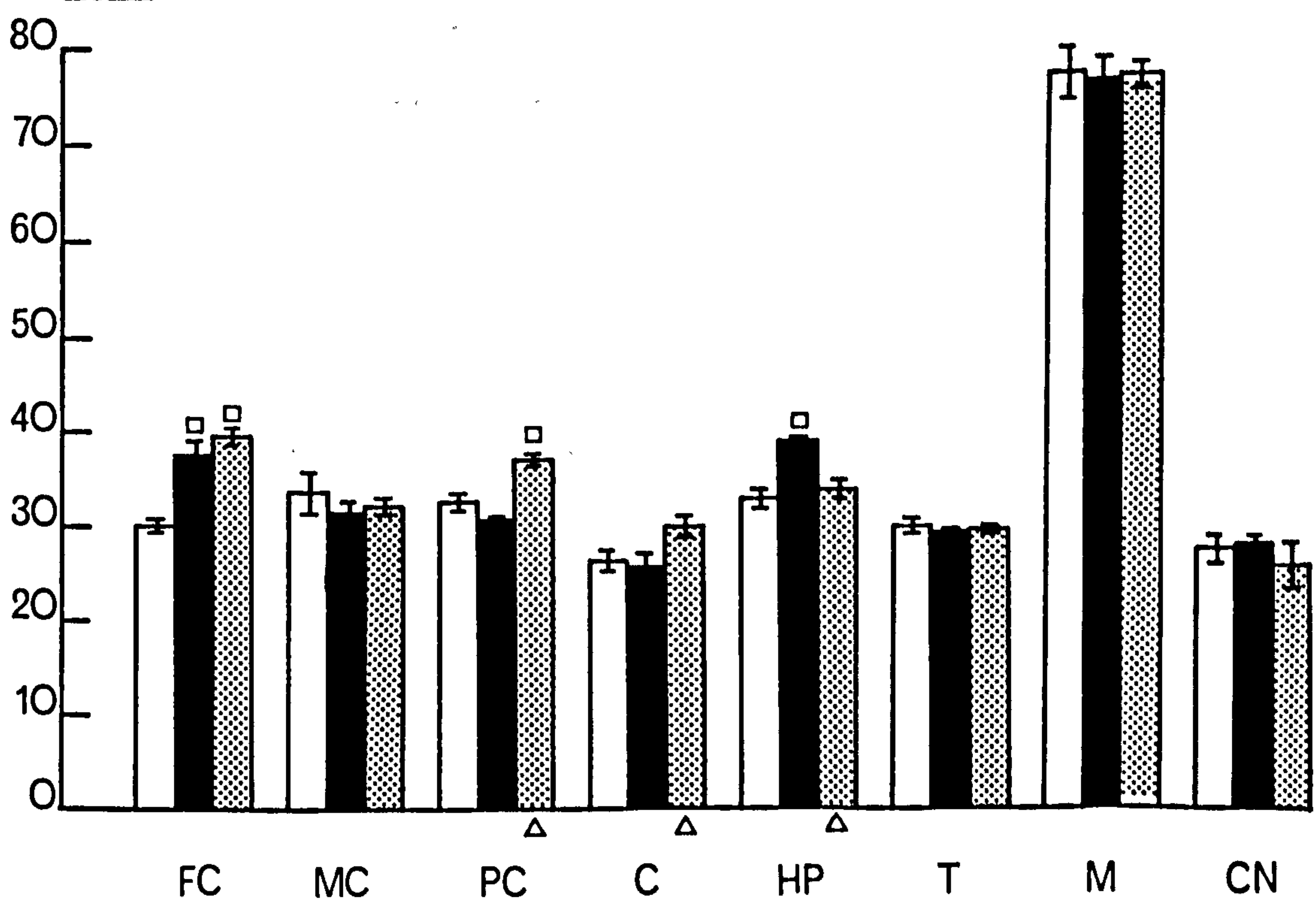


Fig.37



The effects of electrical stimulation and shock avoidance conditioning on the rate of hydrolysis of a tryptic substrate at acidic and alkaline pH.

Figure 36 shows the effects of electrical stimulation and shock avoidance conditioning on the rate of hydrolysis of the tryptic substrate BAPA at acidic pH ; while Figure 37 shows the effects of electrical stimulation and conditioning on the rate of hydrolysis of this substrate at alkaline pH.

The columns represent the means of the number of observations shown in Tables 29 & 30. The white columns represent the quiet controls ; the black columns represent the yoked controls and the stippled columns represent the conditioned group.

key : FC Frontal cortex
MC Medial cortex
PC Posterior cortex
C Cerebellum
HP Hippocampus
T Thalamus
M Medulla
CN Caudate nucleus

□ = significantly different from quiet controls

△ = significantly different from yoked controls

Enzyme activity is expressed in terms of trypsin equivalents / min / mg wet weight.

- 8) In the hippocampus, there was a decrease in activity of the shock avoidance conditioned group at pH 8.0 compared to the yoked control group (14%) but no change in activity compared to the quiet control group. Thus stress following foot shock had caused an increase in activity; while conditioning had decreased the enzyme activity.
- 9) In the posterior cortex, at pH 8.0 there was an increase in the rate of hydrolysis of BAPA of the conditioned group compared with both the quiet control group and the yoked control group (14% quiet; 20% yoked). Thus, the change in activity could be specific to the conditioning experience.
- 10) There was no change in activity of medial cortex at pH 8.0 following conditioning.

4.3.8. The effects of electrical stimulation and shock avoidance conditioning on the rate of hydrolysis of the chymotryptic substrate GPNA.

- 1) There was no change in the rate of hydrolysis of GPNA following electrical stimulation or shock avoidance conditioning at either pH values in the frontal cortex, medial cortex, cerebellum, thalamus, medulla and caudate nucleus.
- 2) Electrical stimulation produced an increase in the rate of hydrolysis of GPNA in the posterior cortex and hippocampus (11% posterior cortex; 18% hippocampus) at pH 8.0 but no change in enzyme activity was observed at pH 5.0.
- 3) In the posterior cortex and hippocampus the increase in enzyme activity of the conditioned group at pH 8.0 compared to the quiet control group, was comparable to the increase in activity of the yoked control group in these regions and is probably due to stress following foot shock.

Table 31. The effects of electrical stimulation and conditioning on the activity of chymotrypsin at acidic pH.

Region	Quiet Control	Yoked Control	Conditioned
Frontal cortex	10.72 $\pm$ 0.3	10.62 $\pm$ 0.23	10.52 $\pm$ 0.2
Medial cortex	9.62 $\pm$ 0.1	9.64 $\pm$ 0.1	9.61 $\pm$ 0.1
Posterior cortex	9.70 $\pm$ 0.1	9.83 $\pm$ 0.1	9.82 $\pm$ 0.1
Cerebellum	10.15 $\pm$ 0.3	10.49 $\pm$ 0.3	9.84 $\pm$ 0.3
Hippocampus	12.81 $\pm$ 0.2	13.53 $\pm$ 0.3	12.57 $\pm$ 0.3
Thalamus	14.25 $\pm$ 0.43	14.23 $\pm$ 0.5	14.46 $\pm$ 0.3
Medulla	29.62 $\pm$ 0.3	29.40 $\pm$ 0.5	29.59 $\pm$ 0.3
Caudate nucleus	12.66 $\pm$ 0.5	12.79 $\pm$ 0.5	12.59 $\pm$ 0.3

Table 32. The effects of electrical stimulation and shock avoidance conditioning on the activity of chymotrypsin at pH 8.0.

Region	Quiet Control	Yoked Control	Conditioned
Frontal cortex	10.02 $\pm$ 0.7	10.42 $\pm$ 0.5	9.82 $\pm$ 0.6
Medial cortex	9.26 $\pm$ 0.5	9.76 $\pm$ 0.7	9.56 $\pm$ 0.5
Posterior cortex	9.89 $\pm$ 0.3	10.95 $\pm$ 0.1 ⁺⁺	11.75 $\pm$ 0.3 ⁺⁺
Cerebellum	10.89 $\pm$ 0.5	11.47 $\pm$ 0.3	11.77 $\pm$ 0.5
Hippocampus	11.70 $\pm$ 0.2	13.76 $\pm$ 0.3 ⁺⁺	13.43 $\pm$ 0.5 ⁺
Thalamus	14.18 $\pm$ 0.8	13.15 $\pm$ 0.3	14.76 $\pm$ 0.6
Medulla	27.31 $\pm$ 0.8	25.30 $\pm$ 0.5	25.43 $\pm$ 1.3
Caudate nucleus	11.43 $\pm$ 0.6	13.85 $\pm$ 0.3	12.47 $\pm$ 0.9

Values given are the mean $\pm$ S.E.M. of at least 4 determinations.

Enzyme activity is expressed as chymotrypsin equivalents / min / g wet weight. + = significantly different from quiet controls (+ p < 0.05; ++ p < 0.01 ; +++ p < 0.001).

Fig.38

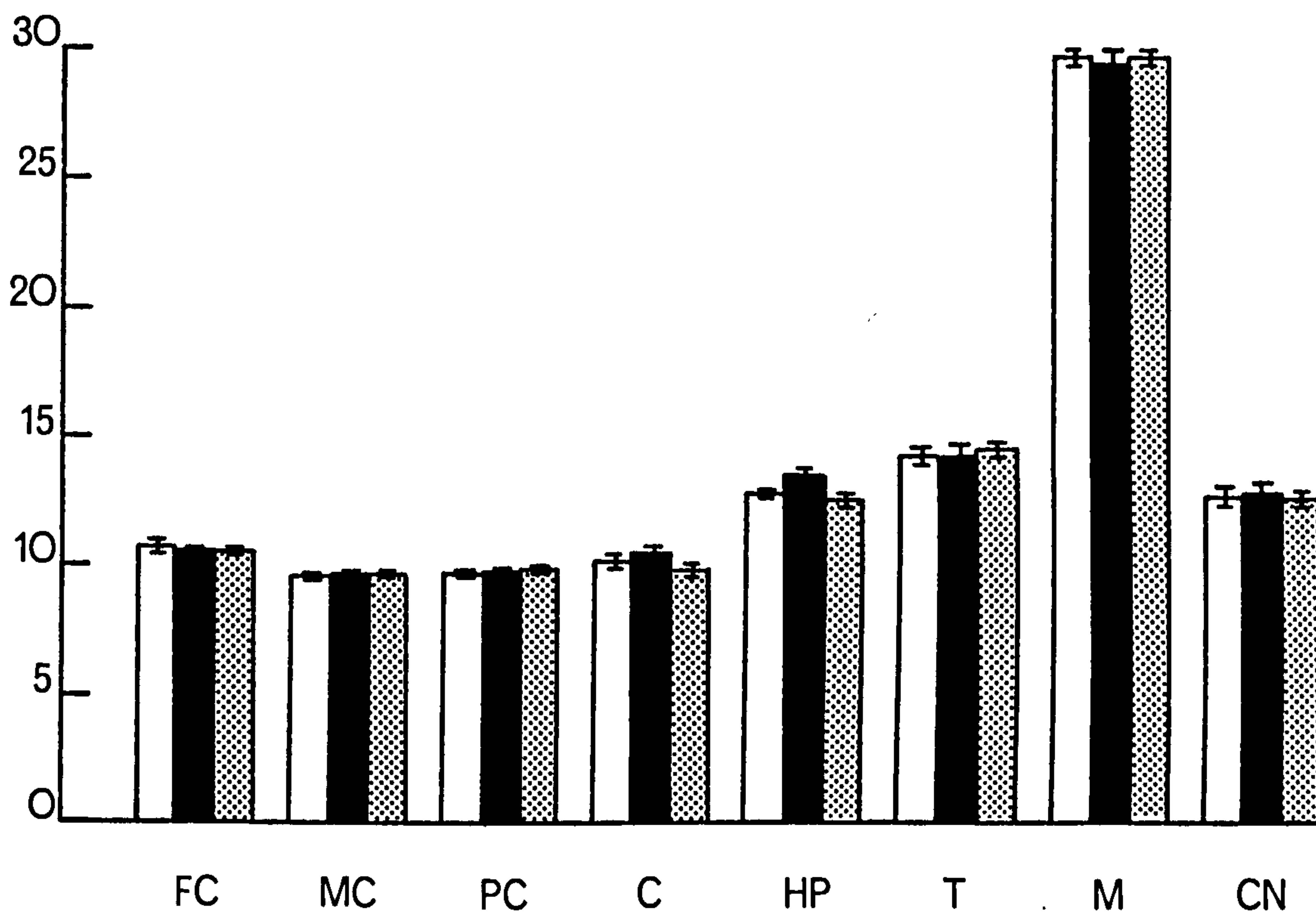
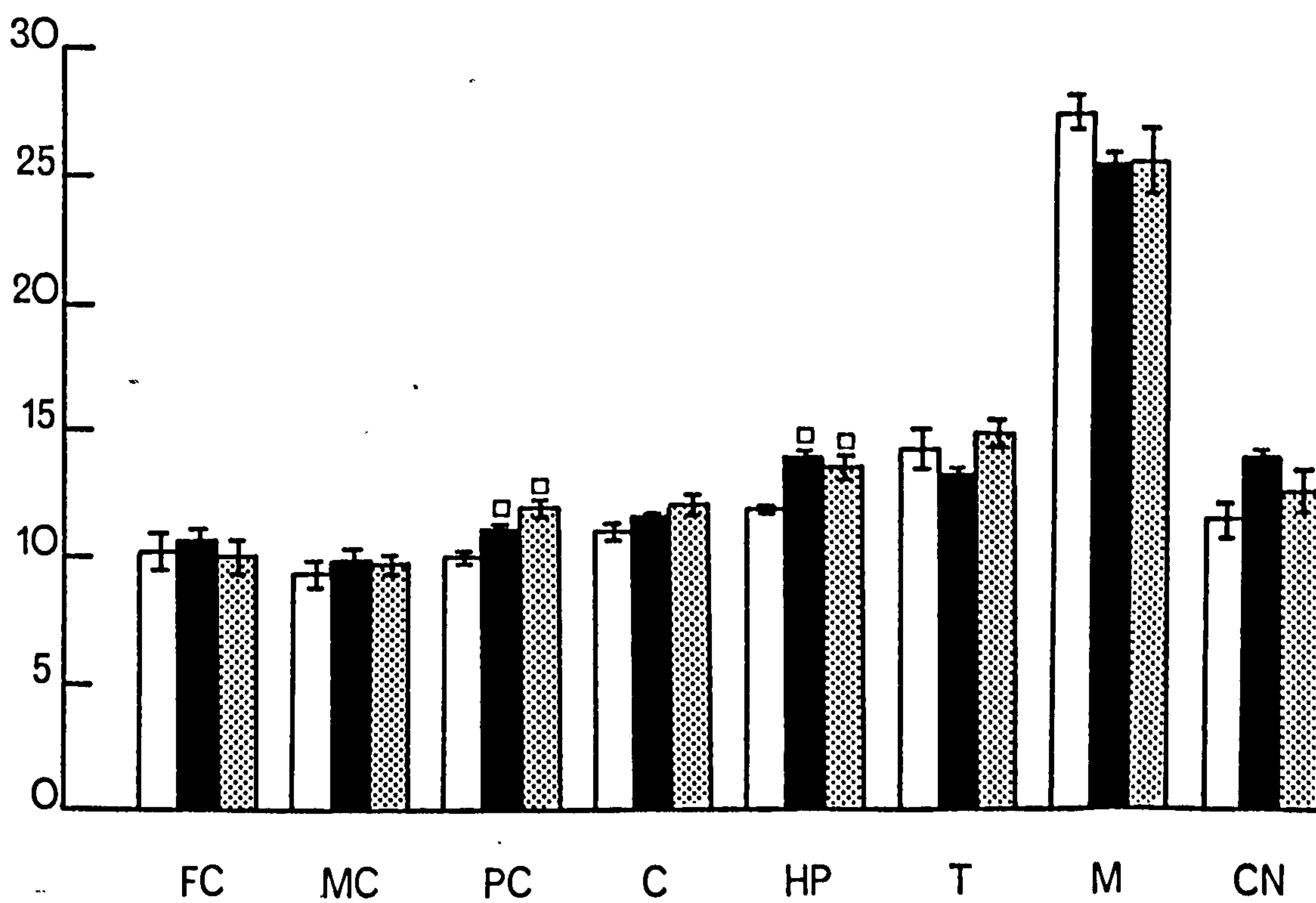


Fig.39



The effects of electrical stimulation and shock avoidance conditioning on the rate of hydrolysis of a chymotryptic substrate at acidic and alkaline pH.

Figure 38 shows the effects of electrical stimulation and shock avoidance conditioning on the rate of hydrolysis of the chymotryptic substrate GPNA at acidic pH ; while Figure 39 shows the effects of these treatments on the hydrolysis of this substrate at alkaline pH.

The columns represent the means of the number of observations shown in Tables 31 & 32. The white columns represent the quiet controls ; the black columns represent the yoked controls and the stippled columns represent the conditioned group.

key : FC Frontal cortex
MC Medial cortex
PC Posterior cortex
C Cerebellum
HP Hippocampus
T Thalamus
M Medulla
CN Caudate nucleus

□= significantly different from quiet controls

Enzyme activity is expressed in terms of chymotrypsin equivalents / min / g wet weight.

4.3.9. A comparison of changes in the activity of proteinases following behavioural treatments with changes in protein content.

It is clear that there are some changes in proteinase activity following behavioural treatments and it is useful here to compare these with changes observed in protein content following the same treatments.

Anaesthesia was found to produce increases in protein concentration in two regions of the brain: the posterior cortex and the caudate nucleus. Following anaesthesia, there was a decrease in activity of proteinases when assayed with the protein substrate casein, in the posterior cortex, hippocampus, and thalamus at neutral pH; no change in the rate of hydrolysis of the tryptic or chymotryptic substrate was observed in any measured region of the brain. Thus, the change in protein content of the posterior cortex could be due to altered protein degradation.

Changes in the activity of proteinases following electrical stimulation correlate well with changes in protein content: electrical stimulation produced decreases in protein content in the frontal cortex, hippocampus and caudate nucleus and an increase in the activity of proteinases in the frontal cortex and hippocampus at both acidic and neutral pH. The rate of hydrolysis of the tryptic substrate was also found to increase at both acidic and alkaline pH in hippocampus and to increase at alkaline pH in the frontal cortex. There was also an increase in the rate of hydrolysis of the chymotryptic substrate at alkaline pH in the hippocampus. This suggests that changes in the rate of protein degradation may account for protein content changes observed in some regions following electrical stimulation eg. frontal cortex and hippocampus. It also suggests that another mechanism

may be responsible for the changes in other regions eg. caudate nucleus.

There was a similar correlation in some regions between protein content changes and changes in proteinase activity following conditioning. Conditioning produced decreases in protein content in medial cortex, posterior cortex and cerebellum and an increase in protein content in the thalamus. Proteinase activity was found to increase in the medial cortex, posterior cortex and cerebellum at both acidic and alkaline pH using casein as the substrate; while activity of proteinases decreased in the thalamus at acidic pH. Training also resulted in an increase in the rate of hydrolysis of the tryptic substrate at alkaline pH in the posterior cortex and cerebellum.

Thus, some of the changes in protein concentration observed following behavioural treatments are possibly suggested by these preliminary studies, to be explicable in terms of altered proteolysis.

4.3.10. Summary

- 1) Methods for determining the activity of proteinases using a protein substrate, a tryptic substrate and a chymotryptic substrate were described.
- 2) With casein as the protein substrate, two distinct peaks of activity were seen at pH 3.0 and pH 7.0. With the tryptic substrate a peak of activity was seen at pH 8.0, with a second peak of activity at pH 5.0; while with the chymotryptic substrate, maximal activity was seen at pH 6.0, a second peak was seen at pH 8.0.
- 3) Following anaesthesia, the activity of neutral proteinases using casein as the protein substrate was found to decrease in the posterior cortex, hippocampus and thalamus. No change in acidic proteinase activity was detected following

anaesthesia and no change was observed in the rate of hydrolysis of either the tryptic or chymotryptic substrate at either pH value investigated.

- 4) Proteinase activity increased in the frontal cortex and hippocampus following electrical stimulation at both pH 3.0 and pH 7.0^{with casein as substrate}. There was also an increase in the rate of hydrolysis of the tryptic substrate in the hippocampus at acidic and alkaline pH. and in the frontal cortex at alkaline pH. The rate of hydrolysis of the chymotryptic substrate was also found to increase at pH 8.0 in the posterior cortex and hippocampus.
- 5) Shock avoidance conditioning led to an increase in proteinase^{with casein as substrate} activity in the medial cortex, posterior cortex and cerebellum at both acid and neutral pH; there was a decrease in activity in thalamus at pH 3.0. Conditioning also led to an increased rate of hydrolysis of the tryptic substrate in the posterior cortex and cerebellum at pH 8.0.
- 6) Changes in protein content following treatments were compared with changes in proteinase activity following the same treatments.

It was found that some of the changes in protein concentration could possibly be explained in terms of altered protein degradation (eg. decrease in protein content of frontal cortex and hippocampus following electrical stimulation); while other changes in protein content could not be explained in terms of altered protein degradation (eg. the increase in protein concentration in caudate nucleus following anaesthesia).

Section 4.4. Discussion.

Brain tissue contains many proteinases. The present study however, gives information only about their combined activity at two pH regions. The significance of proteolytic activity at a very low pH far from the physiological pH is also open to question. In spite of their low pH optima, these acid proteinases may also participate in the breakdown of tissue proteins in vivo, since locally eg. in lysosomes the intracellular pH may be relatively low.

The present study, indicates that proteolysis may be a mechanism to explain the observed protein changes following behavioural manipulations. Much work needs to be done before any definite conclusion on the role of proteinases can be clarified. Only a thorough separation and purification of brain proteinases would enable definite conclusions to be drawn about changes in individual proteinases following the behavioural treatments investigated. It is also necessary to carry out more extensive investigations to see how proteinases affect specific proteins in the brain.

Gabrielescu (1975) suggests that the structural compartmentation of proteinases is essential to the function and metabolism of these enzymes in the nervous system. The catabolism of proteins thus stimulates the turnover and rate of renewal of proteins within the nervous system and provides a mechanism of regulation of protein levels (Bohley 1968).

Many reports have suggested that excitation in the nervous system results in an increase in proteinase activity (Ungar 1963; Jakoubek & Gutmann 1968; Gabrielescu 1966). It is not clear how this increase in proteinase activity is related to neuronal function. Bass & McIlroy (1968) have suggested that a tryptase in the membrane may be involved

in the reversible proteolysis of a ' gate protein ' of the ionic pump. Other workers have suggested that lysosomes at the synapse may create the amino acid resource for protein synthesis at the presynaptic ending (Gordon et al 1968) or that proteinases may have a role in axonal flow mechanism (Marks et al 1970; Orrego 1969).

SECTION 5. Fractionation of brain proteins.

Section 5.1. Introduction.

One of the major problems in neurochemistry is that of isolating and identifying specific proteins in the nervous system and determining their function. Nervous tissue is very rich in proteins and their high turnover rate indicates their importance (Roberts et al 1970; D'Monte et al 1970). The specific neuronal components such as myelin, synaptosomes neurofilaments and neurotubules have all been shown to contain certain characteristic proteins. In particular the two acidic proteins S-100 and 14-3-2 have attracted much interest, mainly because they appear to be specific to the nervous system, their concentration in brain being at least a thousand fold higher than in other organs (Moore & Perez 1968). In behavioural experiments (e.g. in transfer of handedness in rats) it was found that the amount of S-100 in the CA3 region of hippocampus of rats increased in trained animals and that S-100 was specifically correlated with learning (Hydén & Lange 1970). The possibility that proteins are concerned with information processing or storage has subsequently become an accepted idea (Agranoff 1970; Ungar 1970).

In the present Section, an attempt was made to fractionate the proteins of regions of the brain which had been found to change in total protein content following behavioural treatments; and to determine whether protein changes observed are general or whether they could be related to particular protein species.

5.2.1. Polyacrylamide gel electrophoresis of soluble brain proteins.

One of the best procedures for analysing proteins is polyacrylamide gel electrophoresis. This is partly because the gels can be prepared easily from analytically pure starting materials, and by varying the proportions of the starting materials, gels of different density and pore diameter can be prepared. Also, the gels are mechanically stable, can be used over a wide range of pH, are insoluble in most of the solvents used for electrophoresis and other substances can be copolymerised into the gel. Polyacrylamide gel electrophoresis exploits differences both in molecular size and charge for the purpose of fractionation.

The gel is formed by polymerisation of acrylamide in a tube or slab mould and the tube or mould is positioned between two buffer chambers. The sample is then placed on the gel and an electric field applied across them. Most of the components then migrate as individual bands each with a characteristic electrophoretic mobility depending on its size and net charge (Maurer 1971). Fixation and staining subsequent to electrophoresis reveals characteristic band patterns.

The synthetic polymer, polyacrylamide, is produced by polymerising acrylamide with N,N'-methylene-bisacrylamide as the cross linking component. Usually, certain catalyst redox systems which yield free radicals are used to initiate polymerisation e.g. ammonium persulphate and N,N,N',N'- tetramethylethylenediamine (TEMED). By varying the concentration of reagents in the polymerisation reaction, various pore sizes can be achieved.

Electrophoresis on polyacrylamide gels can be either continuous (Raymond & Weintraub 1959) or discontinuous (Ornstein 1964; Davis 1964). With continuous electrophoresis, homogenous

buffers and a fixed pH are used; while with discontinuous electrophoresis different buffers and different pHs are employed.

The electrophoresis of soluble proteins was performed by the method of Davis (1964).

Preparation of gels.

Details of the preparation of reagents and buffers is given in Table 33. Acrylamide and N,N'-methylene-bisacrylamide were recrystallized from chloroform and acetone respectively. Solutions were stored in dark bottles at 4°C and were kept up to two months except ammonium persulphate which was renewed weekly. All solutions were filtered before use to remove insoluble material which would inhibit polymerisation.

Perspex tubes (0.6 x 6.0 cm) were used for gel preparation since acrylamide does not stick to the surface of this material to the extent to which it sticks to glass. They were sealed with parafilm at one end and placed in a small test tube rack. The small pore gel was prepared as shown in Table 33; 7.5% acrylamide concentration was found to give the best resolution. The solution was deaerated on a water pump for 2 min and then added to the tubes to a depth of 4.5cm. A thin layer of water (distilled) was then placed on top of this with a Pasteur pipette with care being taken not to disturb the surface of the acrylamide solution. The solution was allowed to polymerise which took approximately 30 min, after which time the water was pipetted off.

Solutions were then mixed for the large pore gel. After deaeration this solution was added to the top of the small pore gel to a depth of 0.5-0.7 cm and again overlaid with water. The gel was exposed to light from a 200 watt light bulb placed directly above it and photopolymerisation of the acrylamide gel took place in 30 min. The gel was then left for a further 30 min

Table 33. Details of the preparation of solutions for polyacrylamide gel electrophoresis of soluble brain proteins.

<u>Small pore gel</u>		<u>Large pore gel</u>	
1a)		1b)	
Tris	36.3g	Tris	5.7g
N HCl	48.0ml	N H ₃ PO ₄	25.6ml
TEMED	0.92ml	H ₂ O to	100.0ml
H ₂ O to	100.0ml		
2a)		2b)	
Acrylamide	30g	Acrylamide	10g
Bis	0.8g	Bis	2.5g
Potassium ferricyanide	15mg	H ₂ O to	100ml
H ₂ O to	100ml		
3a)		3b)	
Ammonium persulphate	0.28g	Riboflavin	4mg
H ₂ O to	100ml	H ₂ O to	100ml

Preparation of gels : To obtain a given concentration of acrylamide in the gel the solutions were mixed in the following proportions:

	<u>% acrylamide</u>							
	4%	5%	6%	7%	7.5%	8%	9%	10%
1a)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
2a)	1.3	1.6	2.0	2.3	2.5	2.7	3.0	3.3
3a)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
H ₂ O	3.7	3.4	3.0	2.7	2.5	2.3	2.0	1.7

The large pore gel was always prepared by mixing solutions in the following proportions:

1b)	1.0	2b)	2.0	3b)	1.0	H ₂ O	4.0
-----	-----	-----	-----	-----	-----	------------------	-----

Reservoir buffer contains: 6g Tris; 28.8g glycine per litre pH 8.6.

Bis = N,N' - methylenebisacrylamide

after it had turned opaque to ensure complete polymerisation after which time the parafilm was removed. Gels were always used within 4 hours of being cast.

Sample preparation:

Some difficulty was experienced in trying to obtain enough protein from the brain regions to load onto the gels. The samples were homogenised in 1.0 ml phosphate buffer pH 8.0 and then centrifuged at 100,000g at 4°C for an hour in an MSE 65 centrifuge. The protein of the sample added to the gel should be of the order of 50 µg for maximal resolution and this can be contained in up to 200µl. Even with 10 mg of starting material after homogenisation and centrifugation only 30% of the protein was in the supernatant and the protein content fell far short of the optimal.

Electrophoresis.

Reservoir buffer was added to the upper and lower compartments of a Shandon Analytical Polyacrylamide Gel Electrophoresis Apparatus and the tubes were placed in position. The sample was made 10% in sucrose to make it sufficiently dense to layer on top of the gel displacing the upper reservoir buffer. 10µg bromophenol blue dye (20mg/ml) was added to the sample and this moved at the solvent front and acted as a marker. A Shandon Disc Power pack was used for electrophoresis and was set to deliver a constant current. In all cases the apparatus was set with the lower reservoir connected as the anode. Electrophoresis was carried out at room temperature.

A current of 2 mamps per gel was supplied for the first 5 min to allow the protein to migrate into the large pore gel and the current was then increased to 5mamps per gel for the remainder of the electrophoresis period. This was continued

Fig.40 Schematic diagram of gels obtained after electrophoresis of soluble brain proteins.

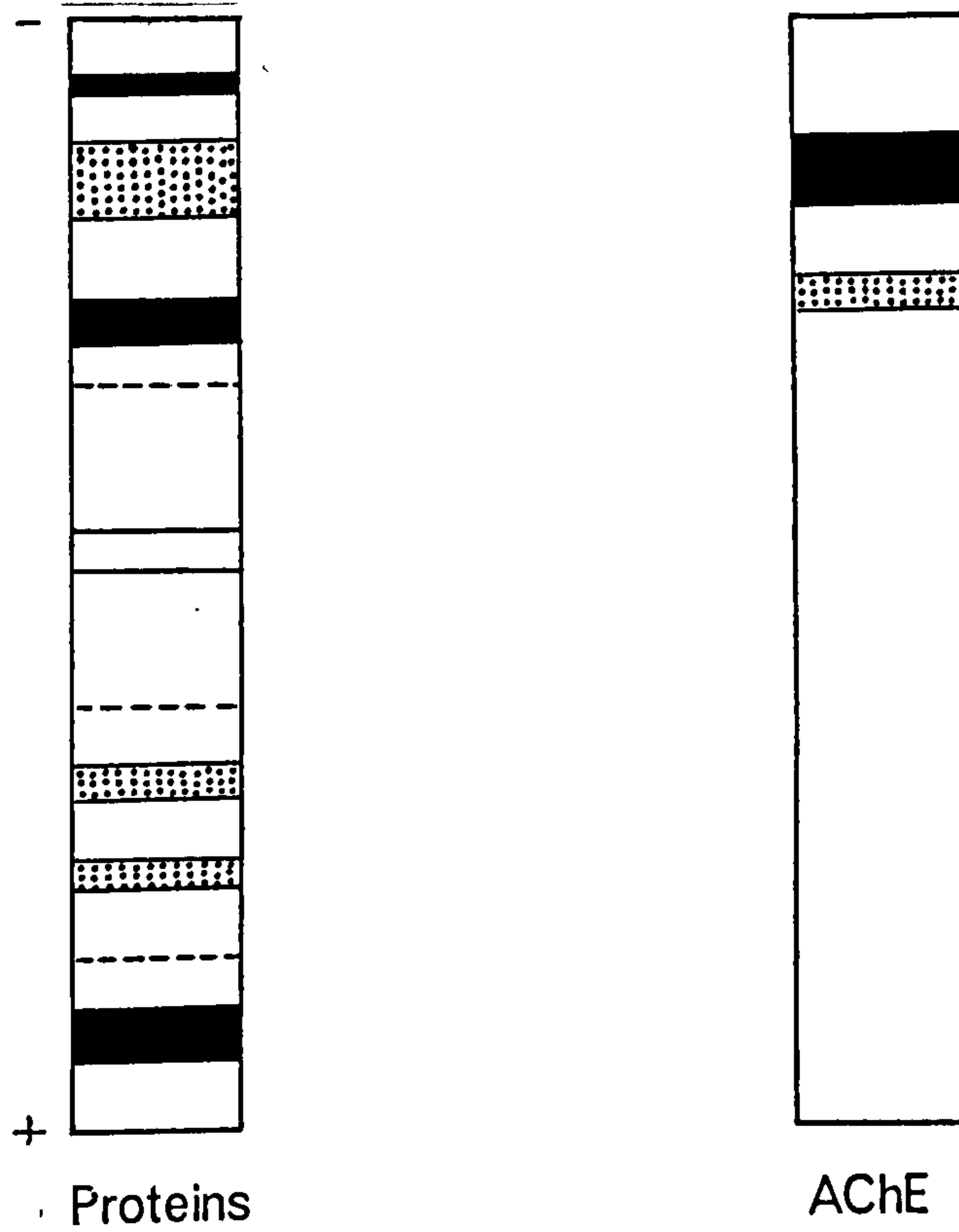
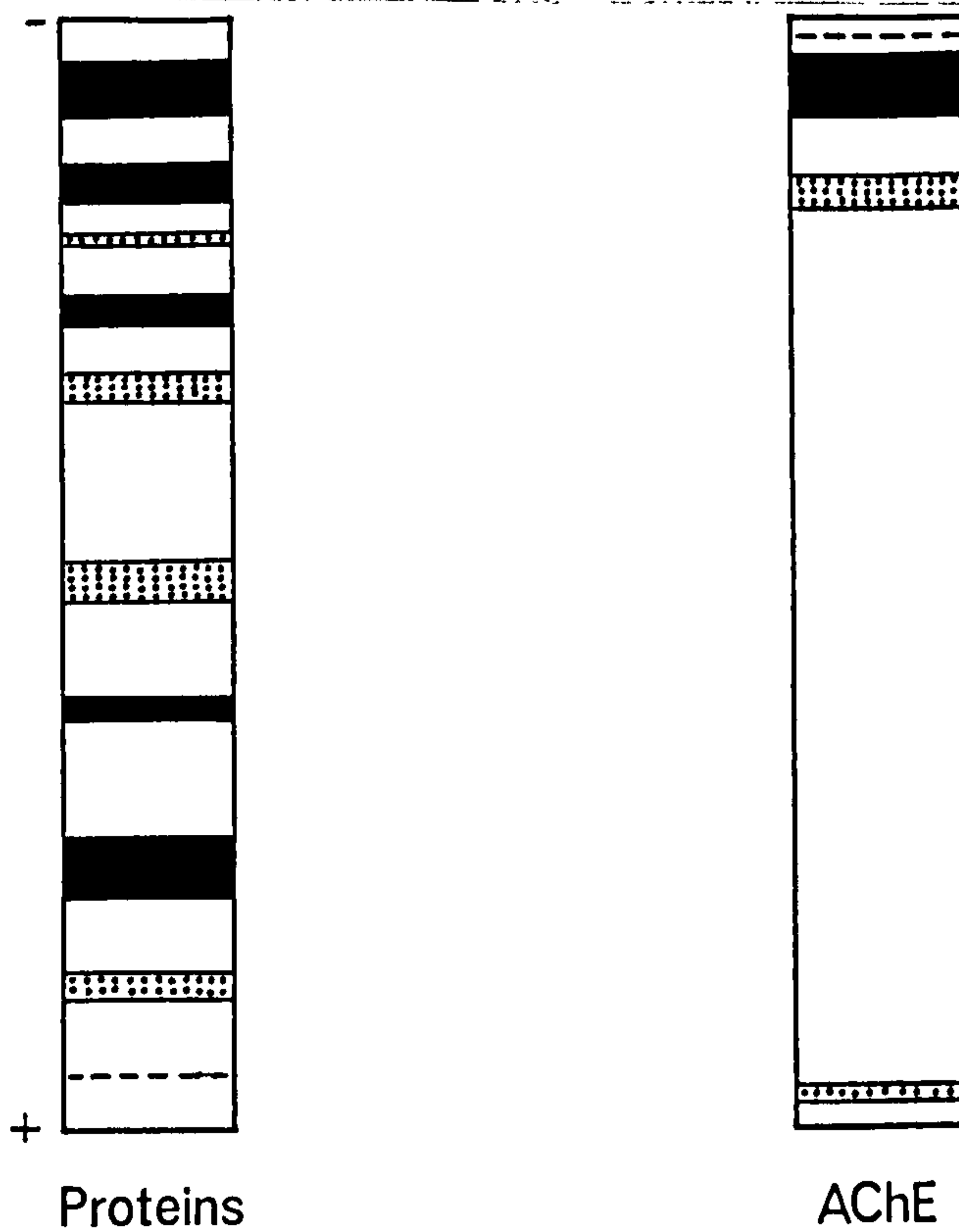


Fig.44 Schematic diagram of gels obtained after electrophoresis of insoluble brain proteins.



until the bromophenol blue band had reached about 0.3 cm from the end of the tube. The tubes were then removed and the gels extruded into 7% acetate. After allowing one hour for the fixing of the protein bands, the gels were stained in Coomassie brilliant blue (0.01% coomassie in 50% methanol; 7% acetate). Staining was continued overnight and the gels were then destained in 25% methanol 7% acetate until the background was clear. Schematic diagrams of the gels obtained are shown in Figure 40.

Scanning of gels.

Gels were initially scanned in a Joyce Loebel u.v. polytrace. The optical density was measured and plotted on a pen recorder. Example traces obtained are shown in Figure 42A. It was intended to measure the area under the curve of these traces to determine intensity of staining of each fraction. In this scanner the gel is scanned at 1.0 mm intervals so that bands narrower than this are not distinguished very clearly and the pen does not return to the baseline between bands. A Zeiss microscope photometer was adapted for measuring the intensity of staining to see if it gave better resolution and peaks whose area could be accurately measured. The features of this photometer are illustrated in Figure 41. A trace obtained from a logarithmic Vitatron recorder connected to this instrument is shown in Figure 42B. The regions of the brain investigated produced on average 10 bands of activity following electrophoresis.

Specific staining for AChE.

Gels were also specifically stained for AChE, so that if there were any change in protein bands following behavioural treatments it would be possible to see if these changes effect AChE bands and confirm the findings of Section 2. This technique also permits changes in isoenzyme patterns to be observed.

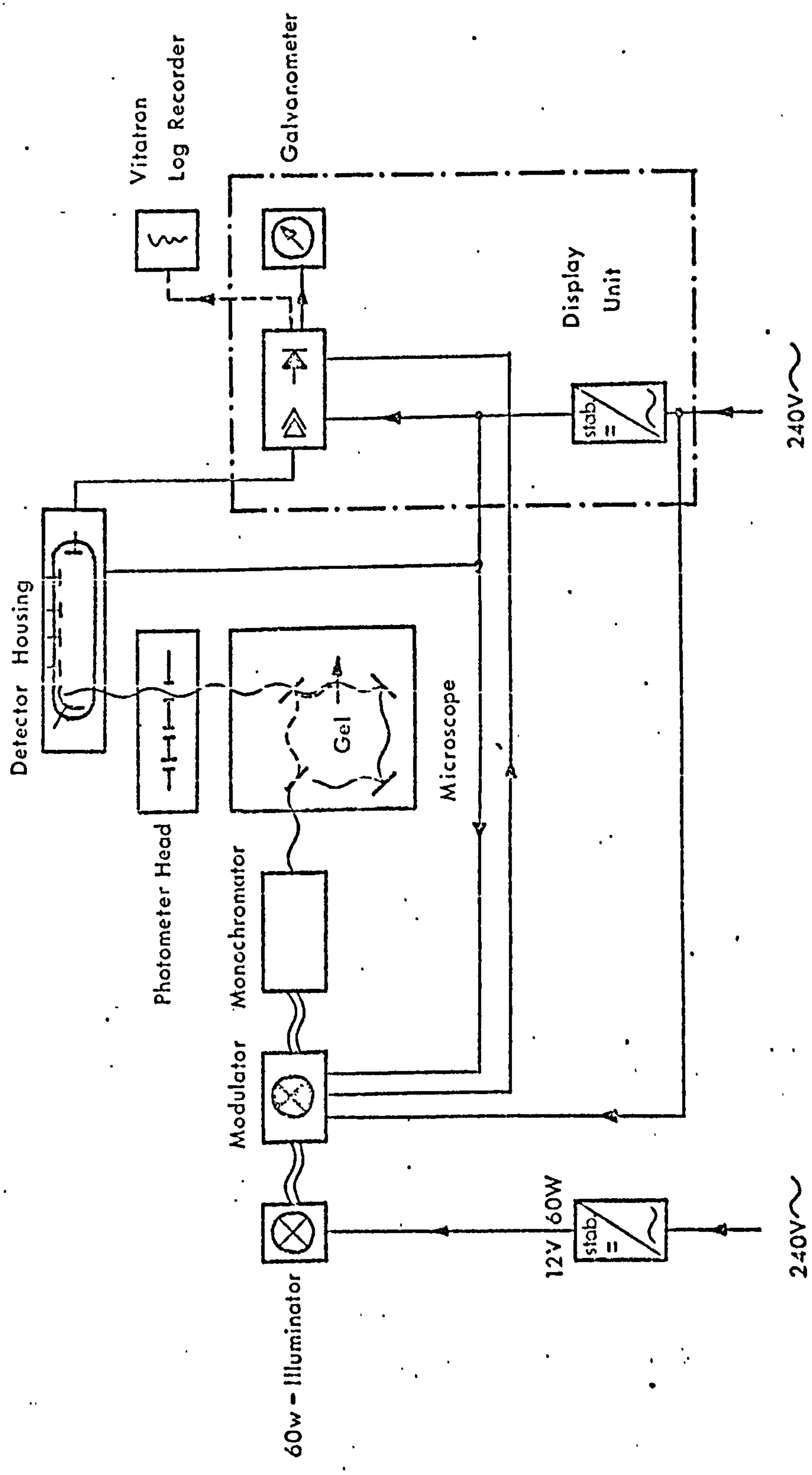


Fig.41 SCHEMATIC DIAGRAM OF MPM MICROSCOPE PHOTOMETER

Figure 42. A copy of densitometric traces of soluble proteins
of dorsal cortex of control rats.

A - trace obtained using Joyce Loebl Chromoscan.

B - trace obtained using Zeiss Microscope Photometer.

Methods available for AChE staining include: 1) production of copper thiocholine sulphate 2) conversion of white copper thiocholine sulphate to copper sulphide 3) conversion of copper thiocholine sulphate to copper dithiooxamide. These three methods were tried and it was found that method 3 gave the best results. The method used was a modification of the method of Koelle (1951) by Lim, Davies & Agranoff (1971).

The gels were incubated at 25°C in 3 x 10 min changes of preincubation medium: 0.499 g copper sulphate; 9.867 g magnesium sulphate; 0.75 g glycine and 10⁻⁶M iso-OMPA. The gels were then incubated for one hour at 25°C in preincubation medium plus 3mg/ ml acetylthiocholine iodide. Following incubation the gels were left overnight at 4°C in ammonium sulphate (3M) - 100 ml per gel. The gels were then transferred to a saturated solution of dithio oxamide (10 mg in 0.2 M acetate) and stained for 8 hours after which time they were put into 7% acetate solution and scanned 24 hours later.

Traces of AChE activity are shown in Figure 43. It can be seen that there are 3 distinct bands of activity: the peak with the highest activity being closest to the origin; this is followed by a smaller peak and then a very faint band of activity.

5.2.2. Fractionation of insoluble brain proteins.

Only 30%-40% of the brain proteins can be extracted with classical aqueous solvents. The other proteins are membrane bound and exist as aggregates within a lipid matrix. The term insoluble is used here to refer to those proteins present in the homogenate residue after extraction with sodium phosphate buffer and which react positively with protein specific stains after solubilisation and electrophoresis. Solubilisation requires dissaggregation of these proteins from their lipid matrix with the aid of solvents.

Figure 43. A copy of densitometric traces of the AChE isoenzymes of the caudate nucleus of rat brain.

A - trace obtained using the Joeyce Loeb1 Chromoscan

B - trace obtained using Zeiss Microscope photometer.

The types of solvent most commonly used are urea, sodium dodecyl sulphate and Triton X100, sometimes with the addition of a reducing agent such as 2-mercaptoethanol to break the -SH bridges.

Inevitably, this rather drastic procedure entails considerable structural and presumably conformational changes which limits the procedure as a step for isolating and identifying species specifically associated with environmental stimulation. However, it does not negate its facility with respect to assessing changes in protein concentration following behavioural treatments.

Insoluble proteins were fractionated by the method of Neville (1971); this method involves SDS polyacrylamide electrophoresis of proteins using a discontinuous buffer system.

The pellet obtained after centrifugation was solubilised in 50 mM sodium carbonate containing 2% sodium dodecyl sulphate (specially pure BDH) and 10% 2-mercaptoethanol. The pellet was homogenised in this mixture and then incubated overnight in a shaking water bath at 37°C. After incubation the solution was centrifuged at 100,000g for one hour at 4°C. The supernatant was then used for electrophoresis.

The details of the preparation of reagents for this method is given in Table 34. The solutions for the small pore (lower) gel were mixed in the desired proportions (11%) and deaerated for two minutes in a vacuum water pump. The TEMED was added to initiate polymerisation and the gel was cast in perspex tubes to a depth of 4.5 cm. The polymerisation of the lower gel was usually complete in 30 min after which time the water was pipetted off and the upper gel polymerised by chemical polymerisation on top of the lower gel to a depth of 0.5 cm. After a further 30 min the gels were ready for use.

The gels were then placed in the electrophoresis apparatus

Table 34. Details of the preparation of solutions for polyacrylamide gel electrophoresis of insoluble brain proteins.

A. Lower gel.

	1		2		3
Acrylamide	27.5g	Tris	12.85g	Ammonium persulphate	0.3g
Bis	0.25g	HCl	77mM	H ₂ O	to 100 ml
H ₂ O	to 100 ml	total volume 100ml			

B. Upper gel.

	1	2	3
Acrylamide	7.5g	Tris	1.64g as above
Bis	0.6g	H ₂ SO ₄	66.8mM
H ₂ O to 100ml	total volume 100ml		

These solutions were mixed in the following proportions

		<u>% acrylamide</u>		
	11	7.5	5.5	Upper gel
H ₂ O	-	2ml	3ml	-
1	6ml	4ml	3ml	2ml
2	6ml	6ml	6ml	2ml
3	2ml	2ml	2ml	1ml
Temed	22μl	22μl	22μl	22μl

Reservoir buffers.

1. Upper reservoir

Tris 4.97g 41mM
Boric acid to pH 8.64
SDS 1.0g
H₂O to 1 litre

Lower reservoir

Tris 51.39g 0.42M
HCl to pH 9.5
H₂O to 1 litre.

and the upper and lower reservoir filled with the appropriate buffers. The sample which can be applied is up to 200ul and up to 10 µg can be run on each gel. The sample was made 10% in sucrose and 10ul of bromophenol blue was added as tracker dye. The sample was then carefully applied to the top of the gel displacing the upper reservoir buffer. Electrophoresis was carried out at room temperature. A constant current of 1 mamp per gel was then applied with the anode in the lower compartment until the protein has entered the upper gel. The current was then increased to 2 mamps per gel until the dye front is 0.3 cm from the bottom of the tube; this was usually after two hours.

Gels were then placed in 7% acetate for one hour to fix the proteins and then stained overnight in a solution of 10% acetic acid; 45% methanol and 0.01% Coomassie blue. Gels were destained in 7% acetate and 10% methanol; and then stored in 7% acetate until scanning. Insoluble proteins were also stained for AChE. Diagrams of gels obtained following electrophoresis are shown in Figure 44.

5.2.3. Development of a microelectrophoretic technique for fractionating brain proteins.

The possibility of applying polyacrylamide gel electrophoresis to separate very small amounts of material was developed by Pun & Lombrozo (1964) to fractionate proteins from regions of the brain. The microprocedure consisted of gel moulds of vertical glass channels (microcells of 1.8x1.0x70 mm) cut into a glass plate. Thus the cross section of the gel strips is smaller than 1/10 of the standard gel cylinder (5-7 mm diameter). This made it possible to analyse from 0.8 to 2mg of nervous tissue and the concentrating effect of the system allowed relatively large volumes of extracts to be applied.

An alternative method used by some workers is to use Drummond capillaries; this idea was first developed by Grossbach (1965).

Since the resulting gels from experiments were required for quantitative densitometry, it was decided to use a microslab technique since this has the advantage over cylindrical gels in that the gel can be conveniently evaluated by densitometry without risk of optical artifacts. Also, the technique permits the use of different stains on samples run in the same gel. Maurer & Dati (1972) have also described a polyacrylamide gel system on microslabs in which gels are formed between two glass plates separated by perspex strips; and the individual gels are formed by a comb inserted into the top of the apparatus. This technique was combined with the gel size described by Pun & Lombrozo (1964) and a microslab electrophoresis apparatus was constructed.

The gel was cast between two glass plates (75 x 56 mm) separated by two perspex strips (thickness 1.0mm). The glass plates were first cleaned by rinsing in Kodak photoflo (1 : 200 v/v in distilled water). Vaseline was applied to the perspex strips to form a good seal with the glass plates which were then held together with bulldog clips. The glass plates were then placed vertically on a strip of plasticine which was sealed about the base of the plates.

Small pore gel was prepared as described in Section 5.2.1. was then added between the plates by means of a Pasteur pipette. The gel was then overlaid with water and allowed to polymerise which took on average 15 min. The water was then removed and 3 cm of large pore gel (see Section 5.2.1.) was carefully added on top of the small pore gel. A perspex comb was then inserted between the glass plates so that wells were left in the gel for sample application. The teeth of the comb were 1.8mm x 0.5mm long and 1.0mm gaps were left between them.

When the large pore gel had polymerised, the glass plates

were removed from the plasticine and secured between the buffer compartments of the electrophoresis apparatus. 100 μ l of supernatant (prepared as in 5.2.1.) was added to each well; the sample was first made 10% in sucrose and 10 μ l of bromophenol were added. The power pack was set to deliver a constant current of 0.5 mamps per gel until the dye front was 0.3 cm from the bottom of the tube.

It was found that Amido black was a more preferable stain than Coomassie blue for this method. The gels were first placed in 7% acetate for 30 min to fix the proteins and they were then stained in 1% Amido black in 7% acetic acid for 40 min. Destaining was in 7% acetic acid. Examples of protein bands of soluble proteins obtained with this method are given in Figure 45.

It can be seen that this method offers greater resolution of protein bands; sixteen protein bands were distinguishable in this fraction.

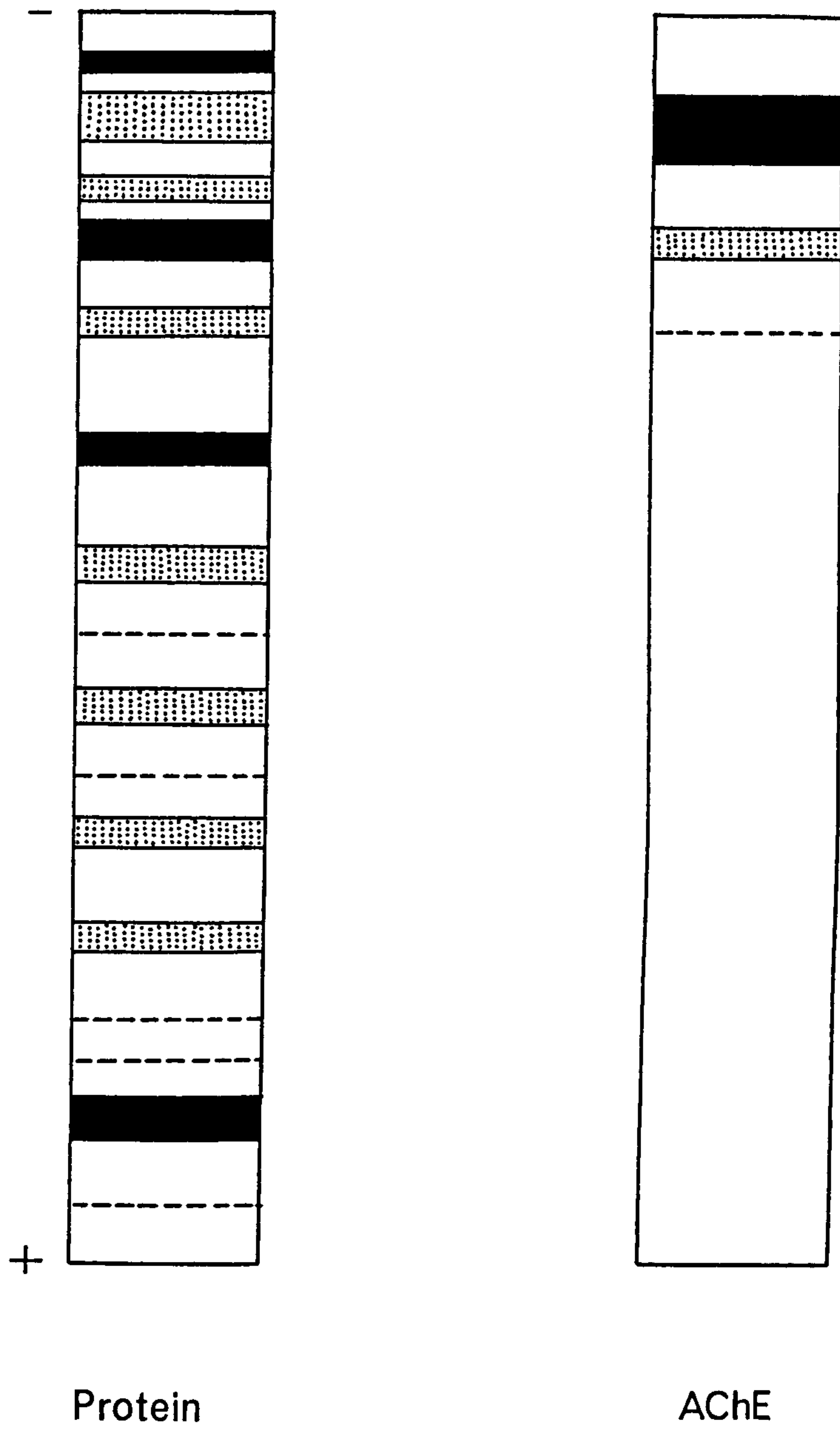
5.2.4. Gradient gel electrophoresis of brain proteins.

Gradient gel electrophoresis was used in the fractionation of brain proteins to see if better resolution of bands could be obtained. In gradient gel electrophoresis, the pore sizes of the separating gels are not homogenous but range from small (at the high concentration part of the gel) to large (at the low concentration part of the gel). Such a gel allows for a greater separation of different size of proteins since molecules are basically separated by molecular sieving, with the result that more information can be obtained on the protein composition of a sample than when it is fractionated on a system where a homogenous pore size separating gel is used.

Apparatus.

A Uniscil Gradient Gel Electrophoresis system was used (Figure 46). The upper anode electrode and the lower cathode electrode

Fig.45 Schematic diagram of gels obtained after electrophoresis
of soluble brain proteins.



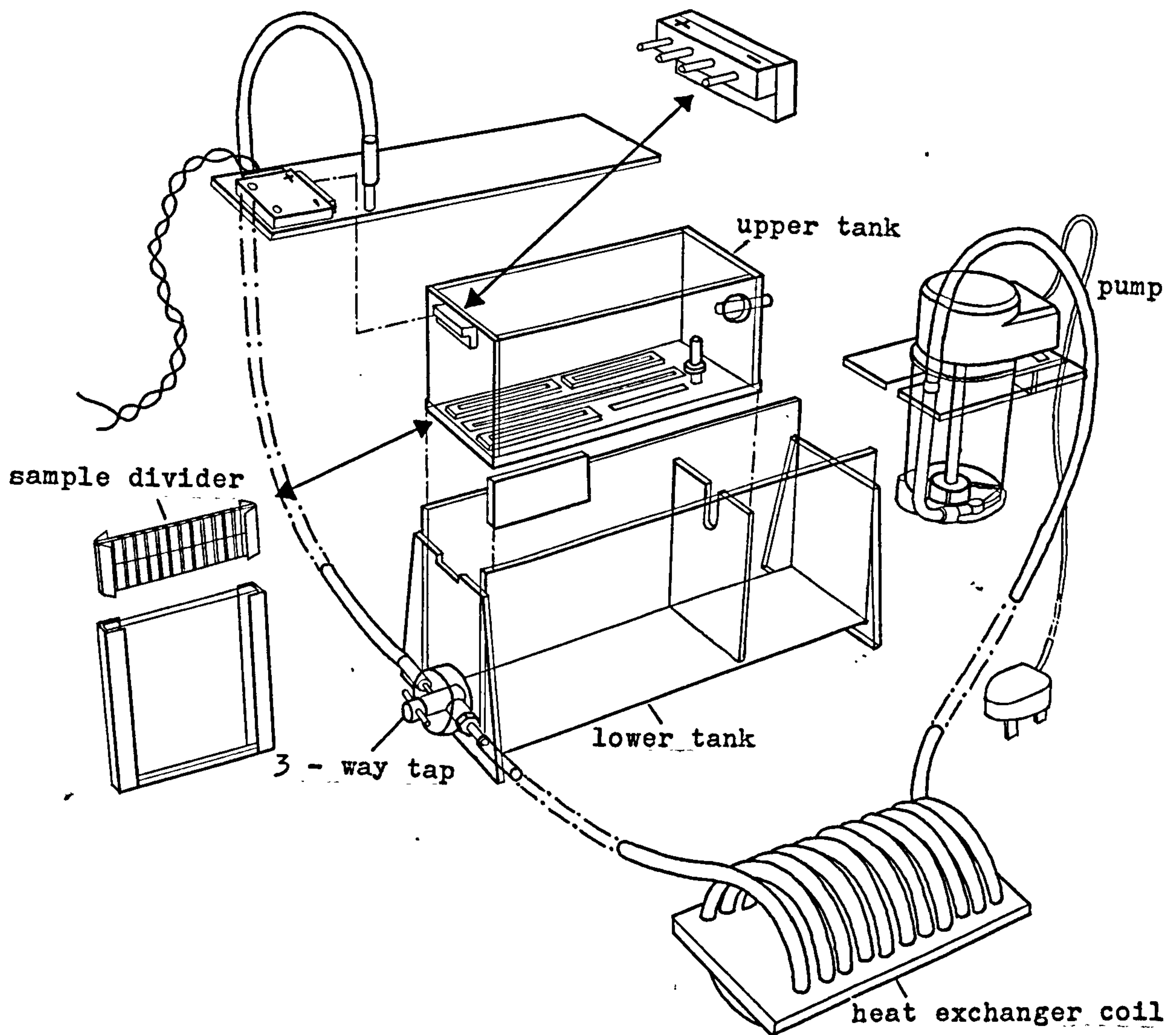


Fig.46 Diagram of gradient gel electrophoresis

apparatus

are both mounted in the upper tank and terminate in the plug at the top left hand side. The tap system on the outer tank is used to control the direction of cooled buffer flow. In the case of a continuous buffer system, buffer is passed into the upper and lower tank and in the case of a discontinuous buffer system, the entire flow is passed into the lower tank.

A vertical divider in the outer tank separates the gels from the pump and maintains a constant level of buffer in the electrophoresis tank. Buffer is pumped out of the constant level reservoir, through the heat exchanger coil into the lower cathodic, buffer tank via the three way tap system. The cooled buffer flows across the gels, displaces warmer buffer over the constant level divider to be recirculated. A small tube connects from the three way tap into the upper (anode) chamber and buffer passes through this to maintain a supply of cooled buffer to this chamber. The displaced buffer then flows through a small tube into the pump chamber. The heat exchanger was routinely kept in ice to ensure adequate cooling.

Preparation of gels.

Prepared Gradipore gradient gels were used; these are supplied with a constant gradient from 4% to 26%. The gels have a separation range from molecular weight range 8×10^6 down to 30,000. They are cast in buffer (1g sodium EDTA; 1g boric acid; 2.5g ammonium sulphate in distilled water to 1 litre).

The gel buffer is first equilibrated with the running buffer (Tris-glycine pH 8.6) by electrophoresing for 30 min. After pre- electrophoresing the running buffer was drained away and the gels were removed.

Sample preparation.

Approximately 10 mg of brain tissue was dissected out,

blotted and weighed and then homogenised for two minutes at 2,000 r.p.m. in 1.0 ml of Tris-chloride buffer pH 7.4. The sample was then homogenised at 100,000g in an MSE 65 centrifuge for one hour. 10 µl of bromophenol blue (20 mg/ml) were then added to 100 µl of the resulting supernatant and the sample was made 10% in sucrose. The resulting solution was used for electrophoresis.

Electrophoresis.

A sample divider was used for multiple sample application. This was constructed of thin acrylic strips separated by a number of white plastic rods sealed between the strips to form discrete channels. The separator was inserted into the free space above the gels and then firmly pushed down to penetrate the gel surface by about 1.0 cm. The soft gel was then squeezed up into the bottom of the separator to form discrete wells. The gel was supported vertically by inverting the upper chamber and inserting the dense end of the slab gel into the gaskets by 1.0 cm and the sample is then carefully applied by means of a Pasteur pipette. After loading the gel, it is removed from the gasket and inserted into the upper chamber so that it protrudes about 1.0 cm into the upper chamber; and the upper chamber is then replaced in the apparatus. The upper chamber is then carefully filled with buffer to avoid the sample being washed out of the wells. Electrophoresis continued for 20-30 min; the current is then switched off and the sample spacers removed. Electrophoresis is then continued for the desired period. Separation on gradient gels tends to be self limiting. It is usual, therefore, to electrophorese under conditions of constant voltage for 12-24 hours. Generally higher voltages do not improve resolution but long time periods do. It was found that optimal conditions

for electrophoresing were obtained with a voltage of 30 volts for 12 hours.

After electrophoresis gels were removed from the cassettes and stained for AChE, pseudoChE, non-specific esterases and proteins.

Staining techniques.

- 1) Proteins. Gels were placed in 7% acetate for one hour to fix the proteins and were then stained in Amido black (0.01% Amido black in 7% acetate) for 12 hours. Gels were destained by diffusion in a Uniscil electrophoretic destainer. This method was found to give more satisfactory results than electrophoretic destaining. The destainer consists of two compartments, one of which contains the pump which circulates the destaining solution (5% acetic acid). Destaining solution is passed over the gels and the excess dye is adsorbed onto woollen pads in the pump compartment.
- 2) Cholinesterases. Gels were stained for AChE in the presence of the pseudoChE inhibitor iso-OMPA as described in Section 5.2.1. Gels were stained for pseudoChE by the same method with butyrylthiocholine as substrate and in the presence of the AChE inhibitor BW 284 C51.
- 3) Non specific esterases. Since the AChE technique previously used did not give very satisfactory bands, an attempt was made to use a non specific esterase stain to see if the AChE bands were more clearly defined and could be distinguished from the other esterase bands. The technique employed was that of Gomori (1958). 1.0 ml of stock reagent (1% α -naphthyl acetate in 50% acetic acid) was added to 50 ml of water and to this was added 2.0 ml 0.2M disodium phosphate and 30 mg of Diazo fast blue BB. The mixture was stirred and filtered. The

gels were then incubated in this mixture at 4°C for 30 min. They were then rinsed in distilled water and stored in 7% acetate.

Fractionation of insoluble brain proteins.

The pellet from the first centrifugation was suspended in 1.0 ml of 0.01 M sodium phosphate buffer pH 7.0 containing 1% sodium dodecyl sulphate and 1% mercaptoethanol. The resulting mixture was incubated overnight with shaking at 37°C. The sample was then centrifuged at 100,000g for one hour and the supernatant was used for electrophoresis. Electrophoresis conditions were the same for soluble proteins except that the reservoir buffer was 0.01M sodium phosphate buffer pH 7.0 containing 0.1% SDS.

Schematic diagrams of gels obtained from soluble and insoluble fractions are shown in Figure 47.

Section 5.3. Discussion.

Using the technique of gradient gel electrophoresis much clearer bands were obtained. Twenty five distinct bands of activity were obtained from the soluble protein fraction and twenty three bands of activity were obtained from the insoluble fraction.

Three bands of activity of AChE were detected in the soluble fraction by this method; with the insoluble fraction there were also 3 bands of activity and in addition there was a faint staining at the gel front. In spite of the variation in individual and total activities in isoenzyme regions between brain regions, it was observed that the relative percentage values of the zones were constant in all areas examined with iso-enzyme 3 accounting for at least 50% of the enzyme activity. With butyrylthiocholine as substrate, a faint staining was observed at zone 3. With α -naphthyl acetate as substrate a variety of esterase bands were observed including bands 1 - 3; however, this staining technique did not offer any greater resolution of the AChE bands.

Fig. 47 Schematic diagram of gels obtained after gradient gel electrophoresis of soluble brain proteins.

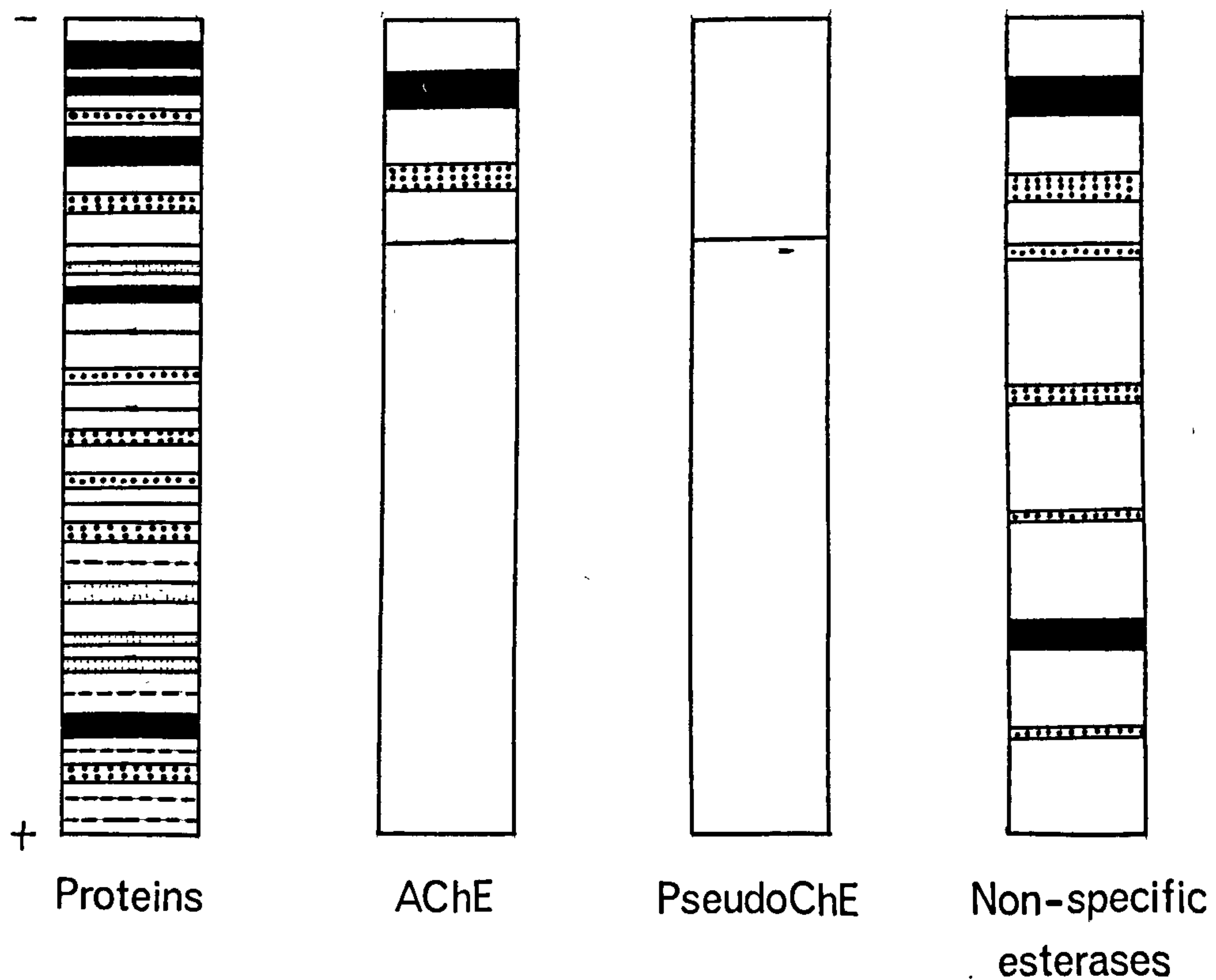
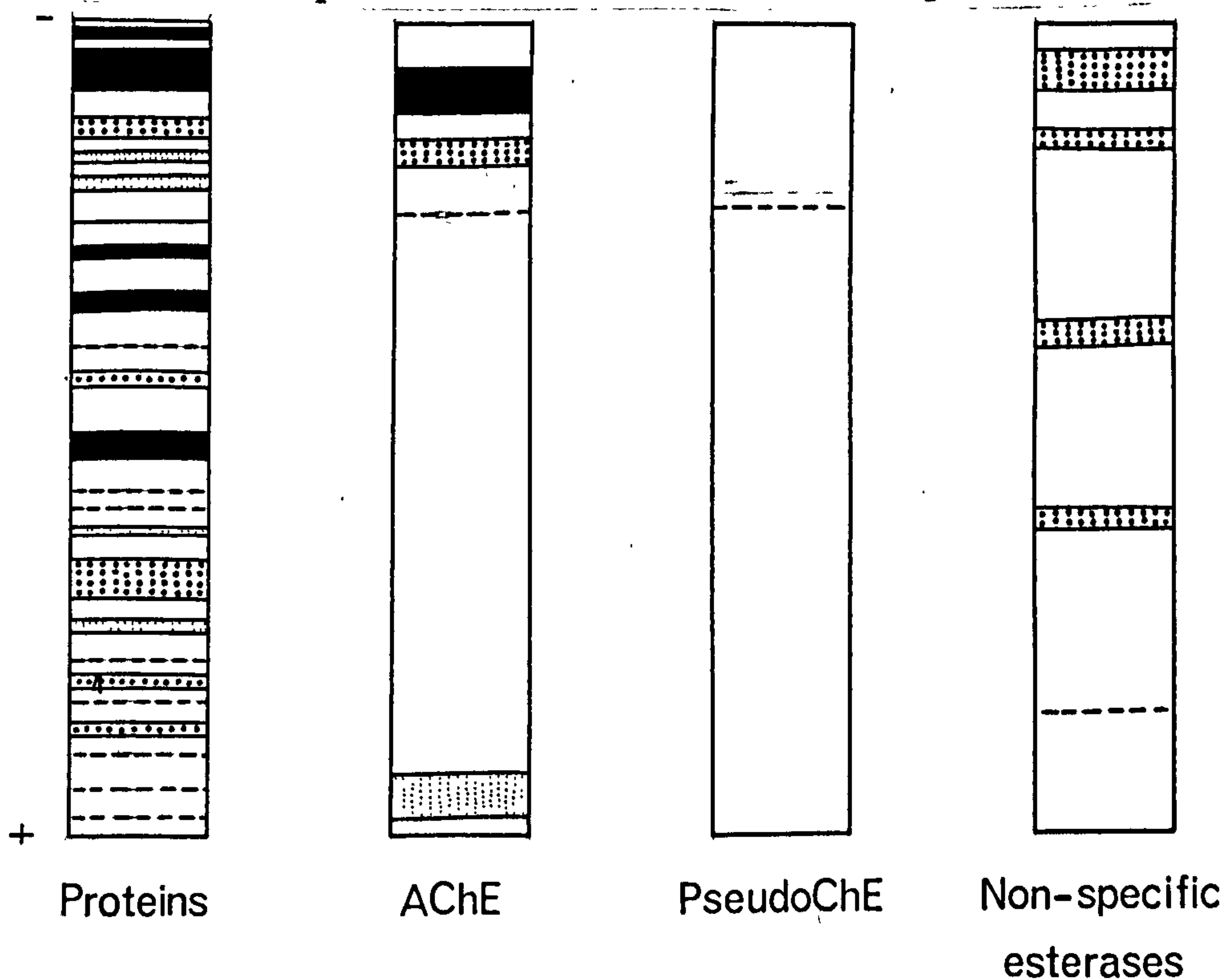


Fig. 48 Schematic diagram of gels obtained after gradient gel electrophoresis of insoluble brain proteins.



Vijayan & Brownson (1974) reported a similar staining pattern for AChE with three anodally migrating isoenzyme zones with low relative mobilities. The identical electrophoretic mobilities of pseudoChE and AChE isoenzyme compare well with similar findings by other workers (Barron & Bernsohn 1968; Davis & Agranoff 1968).

As the technique of electrophoresis was developed, the resolution of protein bands was improved. However, even with the gradient gel system, it was found difficult to determine the intensity of staining of the protein bands. It was thus impossible to complete the original aim of this section, that is to measure the intensity of staining of bands to establish whether the protein changes observed following behavioural treatments were general or specific to particular protein species.

An alternative method to resolve this problem would have been to inject animals with radioactively labelled amino acids, and to measure the rate of incorporation of the amino acids into the fractionated brain proteins. However, in this case the problems discussed in Section 1 of eg. precursor pool availability would still remain to be resolved before any correlation could be made between incorporation of labelled amino acids and rate of protein synthesis.

SECTION 6. General Discussion

The experiments described in this study have indicated that the activity of AChE, pseudoChE and the concentration of total proteins from various regions of the young adult rat brain are responsive to stimulation. Further studies have implicated altered proteolysis as a possible mechanism for the observed changes.

A major question which arises from these studies is what are the mechanisms involved in controlling these enzyme levels. Various techniques have demonstrated the importance of both synthesis and degradation in altering specific enzyme levels (Schmike & Doyle 1970). Changes in levels of enzymes in mammalian tissues may result from i) alteration in rate of synthesis of enzyme or ii) stabilisation or labilisation of existing enzyme in the presence of continuing enzyme synthesis. In order to investigate these possible mechanisms, it is necessary to purify the enzyme to establish whether or not a change in enzyme activity is the result of an altered level of the enzyme protein.

Regulation of functional levels may occur in the following ways:

- 1) synthesis of mRNA
 - 2) activation of existing mRNA
 - 3) conformational or allosteric changes
 - 4) addition of other groups eg. phosphates
 - 5) altered degradation of protein
- > altered protein synthesis
- > altered protein structure

Two forms of regulation are thus based on specific increases in the number of enzyme molecules. In the first, the amount of mRNA is limiting and synthesis of additional copies of mRNA

results in increased synthesis of the protein for which it codes. This is the classical mechanism of enzyme induction whereby, an inducer-- often the substrate of the enzyme in question - leads to the synthesis of more mRNA which directs the synthesis of more enzyme. There is also evidence that regulation occurs beyond the synthesis of mRNA; in this case existent mRNA is activated, thereby directing the synthesis of a greater number of protein molecules for which it codes. Studies on the synthesis of noradrenaline by the adrenal medulla which may be taken as a simple model for nervous tissue, have shown that the amount of tyrosine hydroxylase the limiting enzyme of noradrenaline synthesis, may be enhanced by prolonged nerve stimulation. There is evidence that this process is mediated by synthesis of new mRNA (Weiner 1970).

Other major mechanisms for regulating protein function depend on changing the conformation of proteins or on covalent addition of molecules to protein. Conformational changes are the basis of allosteric regulation, in which the end product of a sequence of enzymic reactions interacts with and inhibits an enzyme early in the sequence, thereby decreasing the activity of the total pathway. For example noradrenaline can interact with and inhibit tyrosine hydroxylase (Weiner 1970).

Addition of molecules to proteins after they have been released from polysomes is another mechanism of regulation. In brain, proteins may be altered structurally in many ways. Phosphates, sugars and lipids can be added to proteins to form phosphoproteins (Walsh et al 1970) glycoproteins (Brunngraber et al 1969) and lipoproteins respectively. Phosphorylation of proteins may also alter their enzymic properties. This is seen in the phosphorylation of phosphorylase responsible for liver glycogenolysis by a protein kinase which is activated by

cAMP (Walsh et al 1970).

The synthesis of brain proteins is reported to occur at high rates comparable with liver (Hydén 1960 ; Richter 1965). However, it was pointed out by Ford & Rhines (1967) that there was a considerable masking of protein synthesis in neurones by less active surrounding tissue. It was reported (Ford & Rhines 1969 a,b) that uptake of radioactively labelled amino acids by neurones was higher than that of liver and any other tissue. Satake & Abe (1966) also reported very high rates of incorporation of labelled amino acids in isolated nerve cell perikarya from rat cerebral cortex. It is now widely accepted that neurones and glia cells show different rates of protein synthesis (Droz & Leblond 1963; Blomstrand & Hamberger 1969). The biosynthetic abilities of neurones are found to be substantially higher than those of glial cells.

The turnover rates of brain proteins is very wide varying from minutes to several weeks (Lajtha 1964). Large differences between subcellular fractions have also been reported (Rodríguez et al 1971).

Proteins are synthesised on polysomes in all cells and in addition there is also a protein synthesising system in mitochondria which is directed by intramitochondrial DNA and mediated by mitochondrial mRNA. These two processes, polysomal and mitochondrial protein synthesis exist in the nervous system (Campbell et al 1966). A unique characteristic of nerve cells is that they have extremely long axonal processes far removed from the DNA of the cell nucleus. It was originally thought that the polysomes of the neurones are clustered in the nerve cell body and that no morphologically identified protein synthesising system occurred in the axon or

nerve ending except for the intra-axonal mitochondrial protein system (Palay & Palade 1955).

A large body of evidence indicates that proteins are transported from the nerve cell body to the axon and nerve terminals (Ochs 1970; McEwen & Grafstein 1968). Evidence has also been presented that supports the existence of a local protein synthesising system in the axons and nerve terminals (Edstrom 1969; Austin & Morgan 1967).

Active local metabolism of macromolecules in nerve endings is also suggested by in vivo studies which show that glucosamine and its carbohydrate derivatives are incorporated into polypeptides at the nerve ending after these polypeptides have been transported from their site of synthesis in the nerve cell body (Barondes 1969). The addition of carbohydrates to proteins in this way is generally the prelude to the secretion of glycoproteins onto the cell surface or into the extracellular space.

All these studies indicate that protein metabolism is active at the nerve ending in brain and could play an important role in the regulation of nerve ending function. Regulation can occur in four major ways:-

- i) via rapid transport from the nerve cell body
- ii) by local protein synthesis in mitochondria and ribosomes
- iii) by modification of protein structure by addition of other groups eg. sugars
- iv) by local allosteric regulation of key enzymes

Changes in protein synthesis observed following behavioural manipulations using techniques of incorporation of radioactively labelled amino acids were discussed in Section 1. Brain polysomes appear to be very unstable in comparison to those of the liver in that they dissociate more rapidly during incubation in amino

acid incorporating systems (Zomzely et al 1968). It is conceivable that some of the factors controlling polysome stability in neural tissue may represent part of a highly responsive mechanism coupling the synthesis of specific proteins with the coding, storage and utilisation of biological information in the CNS. Vesco & Giuditta (1968) reported that application of electric shocks to the cerebral cortex of rats leads to a marked decrease in polysome/ribosome ratio. Similar results were obtained by MacInnes et al (1970).

Changes in the rate of mitochondrial protein synthesis have also been reported following increased functional activity (Hamberger et al 1969). The possible role of RNA polymerases in response of neural tissue to light stimulation has also been examined (Rose 1970).

Results from Section 3 suggested that glial cell metabolism might also be involved in behavioural adaptation. Morphological (Friede 1962), electrophysiological (Kuffler 1967; Trachtenberg & Pollen 1970) and immunological (Mihailovic & Hydén 1969) data indicate very close functional connections between glia and neuronal cells under resting conditions and especially during increased stimulation of nerve cells.

A metabolic coupling between glia and neuronal cells can also be inferred from observations of simultaneous reciprocal changes in RNA concentration and in energy and respiratory metabolism of these two cell types (Hydén 1960; 1967a; Hydén & Pigon 1960; Hydén & Lange 1962).

Many groups have tried to identify naturally occurring chemical compounds which could alter the rate of central protein synthesis. Goldberg (1972) measured the effects of putative transmitter substances on the incorporation of ^{14}C - amino acids into protein in synaptosomal preparations. He reported

a 50% inhibition of incorporation by dopamine, 5 - hydroxytryptamine and DOPA; and a 30% inhibition of incorporation by adrenaline and noradrenaline. No effect on incorporation was reported for either ACh or GABA. These effects were not reversed by ATP, ADP or cAMP. Granitsas (1970) measured the incorporation of ^{14}C - alanine and ^{14}C - leucine into proteins in the white blood cells of the rat. He reported that the presence of ACh in the medium caused an increase in the incorporation.

Guth et al (1967) studied the role of nerve impulses and ACh release on the regulation of ChE activity in adult rat soleus muscle. They reported that miniature end plate potentials and the quantal release of ACh were not important in the neural regulation of ChE. Tenotomy produced no decrease in ChE activity and so it was concluded that the nerve impulses themselves were not essential for the neurotrophic process. Studies using electrotherapy on denervated muscle failed to retard the rate of loss of ChE activity. Since neither the quantal release of ACh or the impulse directed release of ACh or muscular contractions were important in the neural regulation of ChE activity an alternative neurotrophic mechanism was proposed i.e. that the synthesis of this protein was regulated by the transmission of specific messenger substances or de-repressor agents from nerve to muscle. A possible explanation was that the nerve liberates either messenger nucleic acids or de-repressor agents at nerve terminals and thereby helps to direct the synthesis of specific proteins by muscle.

Rose & Glow (1967) studied the effects of denervation on the de novo synthesis of muscle ChE and the effects of ACh availability on the AChE activity of the retina. They reported that ACh availability had no effect on muscle ChE

but that it did increase the rate of retinal AChE synthesis. They suggested two explanations to account for their observations ACh availability regulates a) AChE synthesis or b) transfer of specific substances from the axon.

Donnelly et al (1973) have reported the existence of a heat stable protein from the lobster tail muscle which modulates the activity of cyclic nucleotide dependent protein kinase. A similar modulator was found in rat and rabbit muscle. The protein is reported to be capable of existing as aggregates and different aggregates can have either activating or inhibitory effects on the kinase.

Changes in enzyme activity observed could be due to the synthesis of an effector or inhibitor. This explanation would assume the release of a molecule during synaptic stimulation which combines with the enzyme to effect an alteration in enzyme activity. In the case of AChE, the effect must be non-competitive since no effect on the K_m was observed. Evidence for the occurrence of a naturally occurring antiChE by the cockroach has been reported by Menn & McBain (1968). They reported that it was of a phospholipid nature and was produced within four hours of stressing nymph cockroaches.

A simple hypothesis of a rapidly increased AChE activity as a result of stimulation would be the activation, by some mechanism related to the degree of usage of a neuronal pathway, of an inactive precursor of the enzyme. The inactive component could be stored as an integral part of the post-synaptic membrane and be activated in proportion to the amount of 'traffic' in the system. This type of activation could be brought about by a molecule co-released with ACh from the synaptic vesicles or by ACh itself. Conformational changes taking place in the post-synaptic membrane as a result of interaction of ACh and its receptor and the subsequent depolarisation could thus provide

a model for the activation of the transmitter removal system. Evidence in support of this hypothesis was provided by Barnard & Wieckowski (1970).

Many reports have indicated that the activity of AChE is affected by inorganic ions (Cohen & Oosterbaan 1963 ; Changeux 1966; Kitz et al 1970; Crone 1973; Crone & Dawson 1973). The raising or lowering of the ionic concentration in the synaptic cleft, possibly via ionic pump mechanisms, would alter the properties of AChE on the post-synaptic membrane.

It is also possible that the observed enzyme activity changes might be, at least in part, explicable in terms of redistribution of multiple forms of the enzyme activity eg. isoenzymes. Many studies have shown that changes in isoenzyme pattern and aggregation states of AChE occur during development (Maynard 1966; Barron et al 1968; Dabich et al 1968; Wilson 1969). Vijayan et al (1972) have also reported that parathion treatment causes an alteration of the distribution of the isoenzyme patterns of AChE of brain.

The results of this project, together with many others in the literature indicate a highly sensitive and interactive biochemical process which responds to changes in the environment and in the behavioural state of the animal. Brain biochemistry appears to be labile with respect to cholinesterases, proteins and proteases in a regionally specific manner.

APPENDIX A

MATERIALS

1) Animals .

Several breeding pairs of an inbred strain of Agus albino rats were obtained from the M.R.C. unit at Carshalton, Surrey U.K. A stock of rats were bred from these pairs in the Department and were maintained on an unlimited supply of Porton rat diet. From weaning, female rats were separated and housed in littermate groups of up to six.

2) Chemicals.

All chemicals and reagents were obtained from BDH Chemicals, Poole, Dorset with the following exceptions:

1. Pentobarbitone sodium: standard Nembutal solution (60 mg/ml) supplied by Abbott Laboratories, Queensborough, Kent.
2. Levor IV and Brij 35 (30%) were obtained from Technicon Instruments Co. Ltd, Basingstoke, Hants.
3. Anticholinesterase BW 284 C51 dibromide was obtained from Wellcome Reagents Limited, Beckenham, England.
4. Butyrylthiocholine iodide, propionylthiocholine iodide, iso - OMPA and acrylamide were obtained from Koch-Light Laboratories, Colnbrook, Bucks, England.
5. Glutathione reduced was supplied by Boehringer Corporation, Mannheim, Germany.
- 6) Coomassie Brilliant Blue R was obtained from Baird & Tatlock Romford, Essex.
- 7) α - naphthyl acetate was obtained from Eastman Laboratories .
(no 2380)
- 8) Dextrose-saline - 4.3% dextrose; 0.18% sodium chloride - was obtained from Polyfusor- Boots, Nottingham.
- 9) Naphthol Blue Black no N3005, N' acetylthiocholine iodide and chymotrypsin (from bovine pancreas) were obtained from Sigma Chemical Co, St. Louis, Missouri, U.S.A.

Appendix B

Statistical Analysis of Data

All calculations were carried out on a Hewlett Packard 9810A calculator. Students t-test tested the null hypothesis that an observed difference in a measured parameter had arisen by chance, due to random variation inherent in the individual measurements.

Values of t were calculated from the following formulae:-

$$S.E.M. = \frac{1}{\sqrt{n}} \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n-1}}$$

Where x = individual data in a group

n = number of observations

$$S.E.\text{difference} = \sqrt{\frac{(n_a(n_a-1)S.E.a^2 + n_b(n_b-1)S.E.b^2 + (n_a-n_b))}{n_a \cdot n_b (n_a + n_b - 2)}}$$

Where n_a = number of observations in group a

n_b = number of observations in group b

S.E.a = standard error of the mean of group a

S.E.b = standard error of the mean of group b

$$t = \frac{\bar{x}_a - \bar{x}_b}{S.E.\text{diff}}$$

Where $\bar{x}_a$ = mean of group a

$\bar{x}_b$ = mean of group b

S.E.diff. = standard error of the difference between the means

The values of t obtained by this method were expressed as p, the probability that an observed difference had arisen by chance. The values of p were obtained from tables stored in the calculator programme.

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