RAT BRAIN ACETYLCHOLINESTERASE: REGIONAL ACTIVITY STUDIES FOLLOWING VARIOUS BEHAVIOURAL TREATMENTS.

by

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ABBREVIATIONS

Å	angstrom unit
ACh	acetylcholine
AChE	acetylcholinesterase
cyclic AMP	adenosine 3', 5' - monophosphate
DFP	di - isopropyl fluorophosphate
DTNB	5', 5' dithiobis 2 - nitrobenzoic acid
EEG	electroencephalograph
i.p.	intraperitoneal
K_m	Michaelis constant
mμ	wavelength in nanometres
O.D.	optical density
S	substrate
SEM	standard error of the mean
-SH	sulphydryl group
V	initial reaction velocity
Vmax	maximal reaction velocity
v/v	volume per volume
μ	micro -
w/v	weight per volume

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Section 3.

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ABSTRACT.

FACULTY OF SCIENCE

PHYSIOLOGY AND BIOCHEMISTRY

Doctor of Philosophy

RAT BRAIN ACETYLCHOLINESTERASE: REGIONAL ACTIVITY STUDIES FOLLOWING VARIOUS BEHAVIOURAL TREATMENTS

by Thomas Patrick Michael Durkin.

The activity of acetylcholinesterase, a stable enzyme and a relatively easily measurable component of the cholinergic transmission system, has been investigated in twelve regions of the adult rat brain following various behavioural treatments. The aim of the study has been to determine the nature and degree of changes of the enzyme activity following the treatments. For this purpose, treatments have been chosen which incorporate a wide spectrum of physiological stimulation.

Automated enzyme assay procedures were developed and used to assay the enzyme activity in females, only, of an inbred strain of Agus albino rat. The activity of acetylcholinesterase changes as a result of the behavioural treatments in five of the twelve brain regions. In four of these five the enzyme activity changes are directly related to the state of activation of the animals while, in the other region, the enzyme activity changes are of a different nature.

Enzyme - kinetic studies on the altered enzyme indicate that the activity changes are a result of an increased availability of active sites and are not due to changes in the affinity of the enzyme for its substrate. Time - course studies indicate that the enzyme activity changes persist for several days.

Protein synthesis inhibition studies are being conducted to investigate the mechanism of the observed enzyme activity changes. Other possible mechanisms are considered.

SECTION ONE

1 . 1. Introduction

The formal proposal of the chemical hypothesis of nerve transmission by Elliott (1904) and the discoveries, which followed, of Loewi (1921), Feldberg and Gaddum (1934), Brown et al (1936) and Eccles (1957) have all been major contributions to the advancement of our knowledge of chemical transmission and that of the cholinergic nervous system in particular.

The modification of neuronal activity is central to many theories of behaviour, learning and memory. The discovery that the electrical activity of neurones was mediated chemically at the synapse and the development of our knowledge of the detailed operation of the synapse were important in that they provided the possibility of a chemical mechanism for this modification.

Although Tanzi, as early as 1893, had suggested that learning and memory may be due to some form of change of synaptic conductance this statement came at a time when researchers had been attempting, but had failed, to demonstrate any gross physiological difference between the brains of trained and un-trained groups of animals. By the 1950's it was being suggested that, perhaps the re-organisation of behaviour consequent upon stimulation and experience might better be understood in terms of the dynamics of biochemical processes rather than in terms of differences in brain structure. Lashley (1950) accepted this view when, in summarising his 30 years in search of the neural engram for learning, he commented:

> "I sometimes feel, in reviewing the evidence on the localisation of the memory trace, that the necessary conclusion is that learning just is not possible".

This change in approach towards the dynamic aspects of behavioural adaptation, coupled with the growing knowledge of chemical transmission at the synapse and the development of sensitive and reliable investigative techniques, opened up new avenues of research into the neuro-chemistry of behaviour. The fact that the cholinergic system was the first chemical transmission system to be studied in detail and that more background data was available has led many groups, perhaps unwisely (Karczmar 1969), to study possible relations between the cholinergic system and behaviour. Two major branches of investigation into the nature of these relations have developed from the hypothesis that behaviour and neuro-chemical events are reciprocally related i.e. manipulation of neuro-chemical parameters may produce concomitant changes in behaviour and changes in behaviour may produce both concomitant and persisting alterations of neuro-chemical 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. The precision of any statement that a behavioural measure is some function of a neuro-chemical event is limited by the accuracy of the measuring techniques. Quantitative determinations of acetylcholine, whether by bio-assay or gas-chromatography, although sensitive, are not yet sufficiently reliable and do not, therefore, give the precision to test hypotheses about the ways in which this component of the cholinergic nervous system is related to behaviour (Russell 1969).

The availability of simple and reliable techniques for the assay of acetylcholinesterase has, therefore, focussed considerable attention on this component. Koelle (1969) has stated that the localisation of a high level of acetylcholinesterase activity in a group of neurones, in the absence of a more direct criterion, appears to be a reasonable index of cholinergic function and evidence already exists that changes in behaviour may be

reflected in persisting changes in enzyme activity and that experimental variation of such activity may induce specific changes in behaviour (Russell 1966). On the basis of this evidence and related theoretical considerations it has been argued that the development of new behavioural patterns is likely to occur at an enzyme level (Briggs and Kitto 1962, Smith 1962).

In the present study changes in the activity of acetylcholinesterase in various regions of the rat brain as a result of various behavioural treatments is investigated. Data on the nature, physical properties, distribution and function of the enzyme are, thus, essential to such a study.

* * * *

1.2. The physical properties of purified acetylcholinesterase.

Much information on the properties of purified acetylcholinesterase (acetylcholine acetyl hydrolase E.C. 3.1.1.7) has come from studies of the enzyme from the especially rich source of the electric tissue of the eel Electrophorus electricus (Rothenberg and Nachmansohn 1947, Lawler 1961, 1963). The enzyme was finally purified in a crystalline and electrophoretically homogeneous form by chromatographic procedures in 1967 (Leuzinger and Baker). The molecular weight was later found to be 260,000 daltons (Leuzinger et al 1969). Table 1 shows the results of several other studies for comparison.

The existence of the enzyme in several active forms with somewhat lower molecular weights has been reported (Dudai et al 1972, Massoulié et al 1970).

The enzyme is reported to be composed of four (Froede and Wilson 1970) or six sub-units (Millar and Grafius 1970) and contains four (Froede and Wilson 1970), or two active sites (Leuzinger 1971). The four sub-units (Froede and Wilson 1970) were obtained on disruption in 5 M Guanidine

and were inactive.

The techniques of affinity chromatography and gel-filtration have been applied successfully on a micro-gram scale (Berman and Young 1971).

TABLE 1. Studies on the nature and properties of acetylcholinesterase obtained from the eel (Electrophorus electricus) electric tissue.

Study	Molecular weight	Additional information
Lawler, H.C. (1961)	240,000	Not an homogeneous preparation.
Kremzner, L.T. Wilson, I.B. (1963)	230,000	Not an homogeneous preparation.
Leuzinger, W. Goldberg, M. Cauvin, E. (1969)	260,000	4 sub-units each of 64,000 daltons. Native enzyme is a dimeric hybrid. Protomers consist of non-identical a and b chains.
Millar, D.B. Grafius, M.A. (1970)	260,000	6 sub-units.
Pavlic, M. Wilson, I.B. (1970)	240,000	no active sub-units
Froede, H.C. Wilson, I.B. (1970)	224,000	4 sub-units with possible di-sulphide bonds between pairs of sub-units. 4 active sites proposed.

This technique has been modified and scaled - up so that relatively large amounts of enzyme are now available for study (Rosenberry et al 1972, Dudai et al 1972).

The acetylcholinesterase of mammalian brain is thought to be largely membrane bound (Whittaker et al 1964). This relatively firm association with membrane components has meant that solubilisation has been a major problem with attempts to purify the mammalian enzyme and numerous solubilisation procedures have been used. Early determinations of the molecular weight of the mammalian brain enzyme were conducted on crude, non-homogeneous solutions using the techniques of sucrose-gradient centrifugation or gel-filtration. Table 2 summarises some of the various solubilisation procedures that have been employed and the values for the molecular weight of the enzyme obtained.

Detergent-extracted enzymes (Kremzner et al 1967, Ho and Ellman 1969), as well as enzymes extracted in isotonic sucrose (Chan et al 1972 a,b) all show multiple active forms with increasing molecular weights. Hollunger and Niklasson (1967) reported that higher molecular weight forms of their solubilised enzyme preparation increased during storage at the expense of the low molecular weight form. They suggested that these high molecular weight forms of the enzyme merely represent different states of aggregation, a property of acetylcholinesterase reported by Changeux (1966) and Grafius and Millar (1967).

Chan et al (1972 b) extrapolate from the data on the purified eel enzyme to suggest that the three active forms reported by them represent dimers, tetramers and hexamers with the smaller molecular weight form predominating. They also point out that, although they are suggesting that these forms are multiples of a single active unit, their inter-conversion has not been established and the question of which form exists in the intact membrane remains unanswered. Grafius et al have stated (1971) that the aggregates of the eel enzyme may not be pure polymeric forms but may contain, at least, some "matrix".

TABLE 2. Studies on the properties of mammalian acetylcholinesterase

Study	Molecular weight	Additional information
Jackson, N. Aprison, M. (1966)	180,000	Butanol extraction. The single form resolved into 3 components on electrophoresis.
Hollunger, G. Niklasson, B. (1967)	85,000	Water extraction procedure.
Ho. I. Ellman, G. (1969)	100,000	Protease extraction procedure.
Chan, S. Shirachi, D. Trevor, A. (1972a)	291,000 493,000	Isotonic sucrose extraction. Small differences in substrate and pH optima and apparent Km values observed between the two forms.
Chan, S. Shirachi, D. Bhargarva, H. Gardner, E. Trevor, A. (1972b)	130,000 270,000 395,000	Isotonic sucrose extraction/ Affinity chromatography & gel-filtration. The three forms have different kinetic properties. Suggest these forms are dimers, tetramers & hexamers of a single unit.
Hollunger, G. Niklasson, B. (1973)	80,000	Ion-free solubilisation/Gel-filtration. Single form aggregated on storage to 3 forms of 250,000, 510,000 and 10^6 Suggest that the 2 lower mol. wt. forms are trimers and hexamers of a single unit of 80,000 daltons.

1.3. The nature and properties of cholinesterases.

Cholinesterases may be 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).

The cholinesterases may be divided into two groups with different properties:

Acetylcholinesterases: acetylcholine acetyl hydrolase (E.C. 3.1.1.7) and Pseudo-cholinesterases : acylcholine acyl hydrolase (E.C. 3.1.1.8). The enzymes may be differentiated via their substrate specificity, the presence of substrate inhibition and their reactivity towards the anti-cholinesterase agent DFP. There is, however, no rigid demarcation of properties and enzymes are found which have mixtures of these properties. Many tissues contain both acetyl- and pseudo-cholinesterase activity,but invariably, one is dominant. Thus, in nervous tissue, acetylcholinesterase activity is, normally, always greater than pseudocholinesterase activity (Augustinsson 1963).

Cholinesterase activity levels vary from species to species, from strain to strain within a species and with the age of the animal (Burgen and Chipman 1951, Elkes and Todrick 1955, Bennett et al 1958, 1961 and 1966 and Usdin 1970).

Iso-enzymes have been demonstrated for pseudocholinesterase on numerous occasions (Webb 1964) and their molecular properties have been reviewed by Svensmark (1965). In most vertebrate species the enzymes may be resolved into three groups of activity on the basis of their differential electrophoretic properties, tissue distribution, molecular weight and ontogeny (Holmes et al 1968, Kingsbury and Masters 1970).

Human serum pseudocholinesterase has been reported to contain four or five forms of activity (Harris et al 1962, Oki et al 1965 and Svensmark

(1965). These iso-enzymes are postulated as being molecular aggregates of different molecular weights : 82,000, 110,000, 170,000, 200,000 and 260,000 daltons (La Motta et al 1970). The molecular weight of the predominant iso-enzyme has been shown to be in excess of 300,000 daltons (Svensmark 1965, Kingsbury and Masters 1970 and Das and Liddell 1970), the enzymes are sialo-proteins but the sialic acid component may be removed without affecting their kinetic properties (Svensmark 1965).

The discovery that the serum cholinesterase, present in suxamethonium-sensitive individuals of a human population, is "atypical" in certain of its kinetic properties has also highlighted the fact that different phenotypes of the cholinesterase occur within a species (Kalow and Genest 1957, Kalow and Davies 1958 and Davies et al 1960). Studies using inhibitors (Kalow and Davies 1958, and Clark et al 1968) showed that the enzyme protein from suxamethonium-sensitive individuals must be qualitatively different in structure. The concentration, thermo-stability and electrophoretic mobility are all apparently the same as for the genetically "typical" enzyme, the altered kinetic properties of the enzyme being explained by an altered configuration of the anionic binding site.

Iso-enzymes have also been reported among the acetylcholinesterases Ecobichon and Israel (1967) reported that there were four iso-enzymes of acetylcholinesterase in the electric tissue of the eel Electrophorus electricus. Bauman et al (1972) report three from the same source.

Acetylcholinesterase has also been shown to exist as three electrophoretically distinct iso-enzymes in vertebrate brain and muscle tissue (Barron et al 1963, Maynard 1966, and Wilson et al 1969). The forms do not differ significantly in net surface charge but do have differing molecular weights of 220,000, 300,000 and 420,000 daltons (Wilson et al 1969), suggesting, once again, that the iso-enzymes are explicable in terms of aggregation phenomena.

Skangiel-Kramska and Niemierko (1971) demonstrated two bands of

acetylcholinesterase activity from the sciatic nerve of the rabbit on 5% polyacrylamide gels of molecular weights : 170,000 and 310,000 daltons. On 10% gels, a third band formed which was probably a polymer of the low molecular weight form.

Koelle et al (1970) report histochemical evidence from the cat and the mouse that the acetylcholinesterases of ganglia and muscle end - plates exist, predominantly, in different iso-enzymatic forms.

Vijayan et al (1972) have reported that administration of parathion (0,0 - diethyl - 0 - p nitro phenyl thiophosphate) to rats causes an alteration of the acetylcholinesterase iso-enzyme patterns in the brain.

Ontogenetic studies of acetylcholinesterase have shown that large changes in (a) activity (Nachmansohn 1939), (b) localisation (Mumenthaler and Engel 1961, Filogamo and Gebella 1967, and Wilson et al 1969) and (c) iso-enzyme patterns occur during tissue development (Maynard 1966, Barron et al 1968, Dabich et al 1968 and Wilson et al 1969). In chicken muscles the acetylcholinesterase activity decreases rapidly within two weeks of hatching, in normal individuals, but remains high in individuals homozygous for inherited muscular dystrophy (Wilson et al 1969, 1970). This decrease in activity suggests that changes in the extent of enzyme binding by neurones is a normal occurrence during development. Similar changes were observed in the iso-enzymes of the developing chick brain (Maynard 1966). It is, thus, apparent that the multiple forms of vertebrate acetylcholinesterase are probably polymeric iso-enzymes and that these undergo changes in the extent of their aggregation during ontogeny.

With regard to the functional significance of the occurrence of iso-enzymes, Baldwin and Hochachka (1970) observed that the acetylcholinesterase from the trout brain Salmo gardnerii occurs in two distinct forms. A single "warm" variant is present after acclimatisation, for four weeks, to 17°C; a single "cold" variant is present after acclimatisation to 2°C. Both

forms are present in fish acclimatised to an intermediate temperature. The Km values for the variants are different and temperature - dependent. They concluded that the Km - temperature relationship is adaptive and that the critical process, during acclimatisation, is the synthesis of a new enzyme variant that is better suited for catalysis and the control of catalysis at the different temperatures. This has great physiological significance for the trout as this species experiences annual temperature fluctuations of between 2°C. and 18°C. A suggestion that the rate of enzyme activity in cold - adapted organisms may be maintained via a lowering of activation energy (Vroman and Brown 1963) was investigated but the experiment yielded a non - linear Arrhenius plot. Non - linear Arrhenius plots, obtained with amino - acid oxidase, have been interpreted in terms of a temperature - dependent transition of the enzyme between two conformations (Koster and Veeger 1968, Massey et al 1966).

Evidence for similar temperature - dependent transitions between multiple forms of serum cholinesterase and acetylcholinesterase from erythrocytes has been reported by Main (1969) and similar relationships between habitat temperature and Km have been demonstrated for lactate dehydrogenases (Hochachka and Somero 1968), pyruvate kinases (Somero and Hochachka 1968), fructose diphosphatases (Behrisch 1969) and choline acetyltransferases (Hebb et al 1969) using material from several species of fish and crustaceans.

A number of other mechanisms that could conceivably act to raise the rate of hydrolysis in the cold - acclimatised state were explored by Baldwin and Hochachka (1970). Hickman et al (1964) observed rapid changes in brain sodium and potassium ion contents and longer term changes in brain chloride ion contents from cold - acclimatised trout. Baldwin and Hochachka (1970) studied the effect of ionic strength on

the activity of acetycholinesterase from 2°C. adapted trout and observed that increasing ionic strength leads to a marked increase in both the Km and the V max. of acetylcholine hydrolysis. At lower substrate concentrations, however, the rate of reaction was found to decrease as the ionic strength was increased. Reeves and Wilson (1969) observed an increase in intracellular and blood pH of 0.014 pH units/°C. in several poikilotherms when their temperature is lowered. With trout acetylcholinesterase a fall in temperature from 17°C to 2°C could result in a 12% increase in the rate of acetylcholine hydrolysis if the pH-activity relationship holds at physiological substrate concentrations.

Baslow and Nigrelli (1964) proposed an increase in the total amount of enzyme present in cold - acclimatised fish. Baldwin and Hochachka, however, observed no difference in the specific activity of the acetylcholinesterase from trout brain between the 2°C. and the 17°C. acclimatised fish. They concluded:

"The primary function of the acclimatisation process is the production of enzymes with Km values in a range likely to be optimum for the regulation of catalytic activity. ..... In evolutionary terms, it appears that there is a strong selection for enzymes permitting large changes in activity in response to physiological changes in substrate concentration. This is reflected in the patterns enzyme variants produced during acclimatisation and those selected during evolutionary adaptation".

* * * *

1 . 4. <u>The function of acetylcholinesterase</u>.

The primary role of acetylcholinesterase at synapses and the neuromuscular junction is to hydrolyse the acetylcholine released during transmission. There is still some dispute, however, as to whether acetylcholinesterase is an absolute requirement for transmission. Barnard and Wieckowski (1970) have reported that the progressive inhibition of acetylcholinesterase activity at the motor end - plate by DFP leaves some responses to low frequency stimulation unaffected. They conclude that the action of acetylcholinesterase in transmission is, therefore, only required for transmitter removal when rapid passage of impulses is involved; at low frequencies, removal of acetylcholine by diffusion must be adequate.

In support of this hypothesis is the estimate of the amount of acetylcholinesterase involved in transmitter removal (Barnard and Rogers 1967) and the lower concentration of acetylcholinesterase at tonic muscle junctions where the end-plate potential decays slowly (Hess and Pilar 1963).

Barnard and Wieckowski (1970) concede a requirement for the action of acetylcholinesterase, however, during rapid transmission of impulses and suggest that the arrangement, on the post - synaptic membrane, of the acetylcholinesterase and the cholinergic receptor sites must be a very efficient one. They, thus, infer a close spatial arrangement of the enzyme and receptors.

Another interesting observation, reported by Barnard and Wieckowski (1970), was that 60% of the DFP reactive sites in the motor end - plates do not have cholinesterase activity and are not receptor sites. They undergo the DFP reaction as rapidly as acetylcholinesterase and are, thus, by comparison with all other such cases, serine - dependent esterases. They conclude, since they are not receptors, that they could be enzymes required for turnover of lipid for membrane renewal in long - term

maintenance of synaptic function (e.g. vesicle formation) or that they could be some inactive precursor of acetylcholinesterase.

In assessing the functioning of acetylcholinesterase in transmission the rate of transmitter release and breakdown become very important. There has been considerable controversy about the synaptic concentration of acetylcholine. Ehrenpreis (1967) and Wilson and Harrison (1961) have estimated the local concentration <u>in vivo</u> as 5×10^{-5}M; this would be far below the optimal concentration of acetylcholine for its enzymic hydrolysis by acetylcholinesterase which would be at about 10^{-3}M. Nishi et al, (1967) however, have calculated, on the basis of the iontophoretic application of acetylcholine, that the synaptic concentrations of acetylcholine are very high - 10^{-3}M.

McIlwain (1966) performs the following calculation to demonstrate the role of acetylcholinesterase in transmission:

He points out that the synaptic vesicles from the mammalian cerebral cortex have been computed to contain 300 - 3,000 molecules of acetylcholine and that 3.8×10^{12} vesicles, not necessarily all from cholinergic endings, are present per gram of tissue (Whittaker and Sheridan 1965). Data from studies on the neuro - muscular junction indicates that acetylcholine exists at a concentration of 110mM. in the vesicles and, thus, on liberation, a large concentration gradient transitorily exists across the 300Å of the synaptic cleft; micro - seconds may suffice for its arrival at the post - synaptic membrane in adequate concentration and less than a milli - second for a large proportion of it to have been removed, by diffusion, to a distance of a few microns (Ogston 1955, Eccles 1964 and De Robertis 1964). Acetylcholinesterase, present at the synaptic region, acts during and after this time. The rate of breakdown of acetylcholine, using homogenates of acetylcholinesterase from brain tissue, has been noted to average 200 - 500 μMoles/ g. wet weight/ hour. Though this is approximately 300 times

its mean concentration in the brain "in vivo", the localisation of acetylcholine in the synapse means that suitably localised acetylcholinesterase could be working at maximal capacity shortly after liberation of the transmitter. If, in such a localised area, 1% of the cerebral acetylcholine were released (0.07 mμMoles/ g. tissue) its hydrolysis at a rate of 350 μMoles/ g./ hour would require 0.7 msec.

Thus, quantitative aspects of diffusion and breakdown of acetylcholine appear to be consistent with the highest rates of cell firing found in the brain of some 1000/sec. A similar calculation is performed and the same conclusion arrived at by McLennan (1970). A true acetylcholinesterase is found in red blood cells. Its function there is not clear. Nachmansohn (1959) has suggested that its localisation there is compatible with some role in ion movements, since the internal potassium ion concentration of red blood cells is high.

The function of pseudo - cholinesterase is, also, not clear. It has been suggested that it hydrolyses non - nervous acetylcholine and thus protects acetylcholinesterase from substrate inhibition (Jamieson 1963). McIlwain (1966) also points out that in the hypothalamus, where much of the pseudo - cholinesterase appears to be extra - neuronal, it may function by limiting the spread of acetylcholine by diffusion. Svensmark (1965) has reviewed several other possibilities.

* * * *

1 . 5. The general mechanism and kinetics of acetylcholinesterase.

Dixon and Webb (1964) have defined the active site as:

"...That part of the enzyme protein structure which combines with substrate and is responsible for the enzymic properties of the molecule".

Cohen and Oosterbaan (1963) have represented the active sites of acetylcholinesterase as shown in Figure 1, and Krupka (1966) has represented the functional groups at the active centre as shown in Figure 2.

Kitz (1964) has also stated that the active surface of acetylcholinesterase is best viewed as two sub - sites, an anionic and an esteratic site. The anionic site is responsible for binding and orienting substituted ammonium groups while the work of cleavage is done at the esteratic site. The details of the mechanism of the hydrolysis of acetylcholine by acetylcholinesterase are depicted in Figure 3.

Acetylcholinesterase exhibits substrate inhibition; a plot of substrate concentration against enzyme activity gives a bell - shaped curve with an assymptote at around $1 - 3 \times 10^{-3}$M. There has been some dispute as to the mechanism of substrate inhibition. Krupka and Laidler (1961) have suggested that the inhibition results from the action of substrate with the enzyme - substrate complex rather than with two equivalent anionic binding sites. Kato et al (1972) have proposed an allosteric mechanism for the inhibition.

The possibility that acetylcholinesterase can undergo conformational changes and allosteric interactions has been the foundation of several studies and is, obviously, of great relevance to the mechanism of any postulated role for acetylcholinesterase activity in the modification of synaptic activity.

* * * *

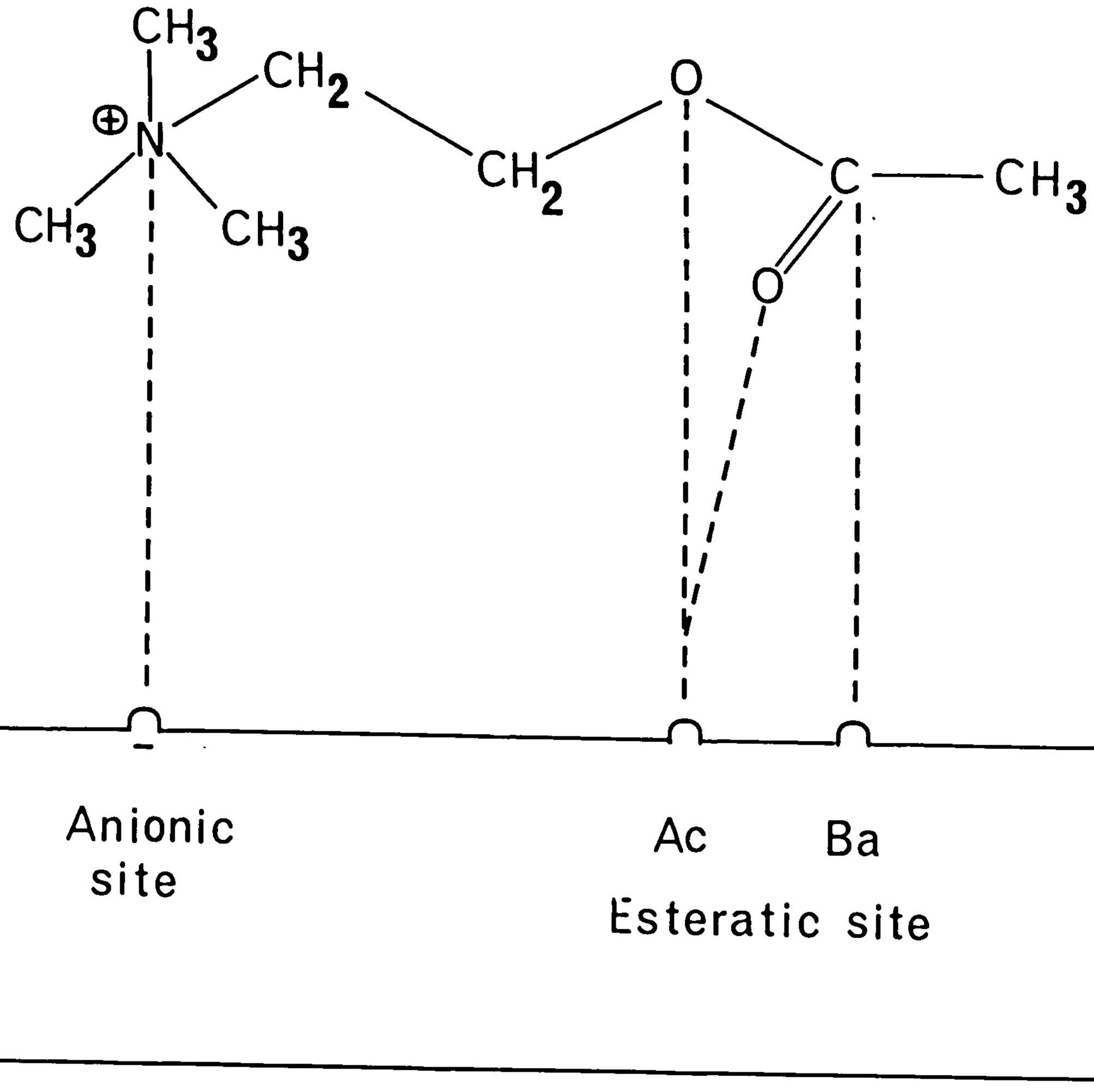
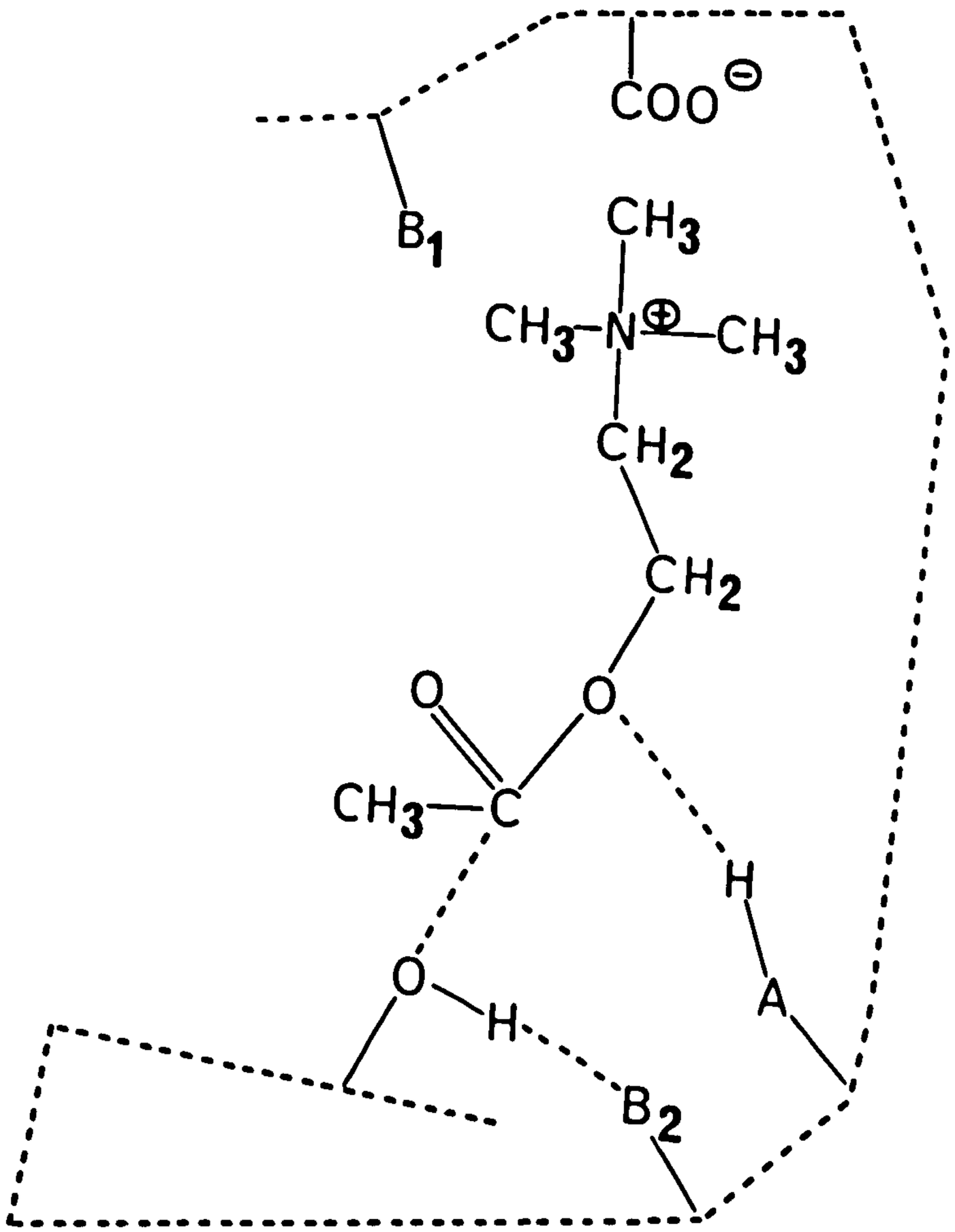

CH3
CH2
O
N
CH2
C
CH3
CH3
CH3
O
Anionic site
Ac
Ba
Esteratic site
Anionic site

FIGURE 1.

Schematic representation of the active site of acetylcholinesterase

(from Cohen and Oosterbahn 1963).

The active site is assumed to be composed of one or two anionic sites and the esteratic site, containing an acidic(Ac) and a basic (Ba) group.


COO⊖
B1
CH3
CH3–N⊕–CH3
CH2
CH2
O
O
CH3–C
O
H
A
O–H
B2

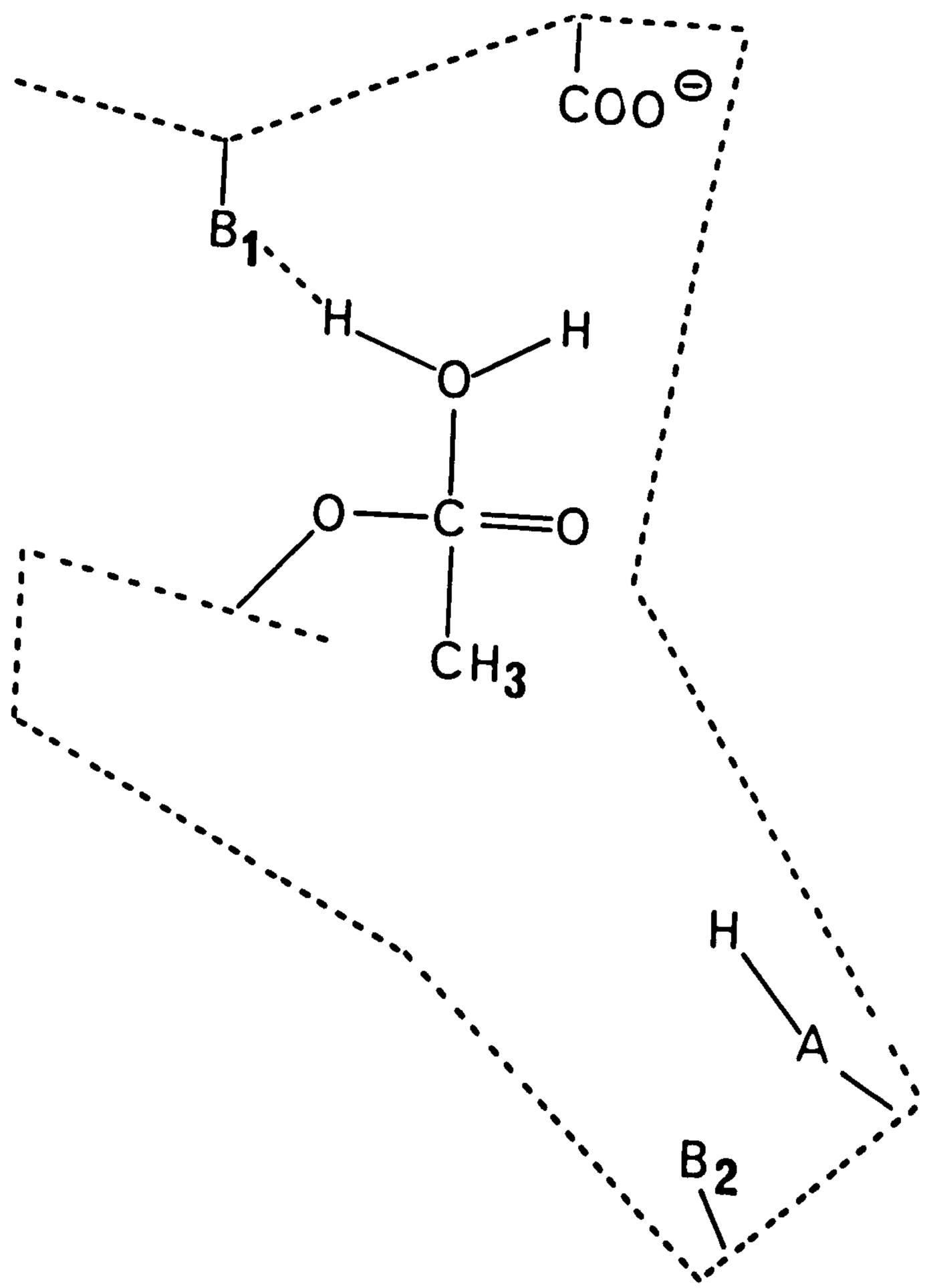

COO⊖
B1
H
H
O
O–C=O
CH3
H
A
B2

FIGURE 2.

Schematic representation of the active centre of acetylcholinesterase

(from Krupka 1966)

B_1 and B_2 are basic groups of pK 6.3 and 5.5 respectively. AH is an acidic group of pK 9.2. OH and COO^- are a serine - hydroxyl and the anionic site respectively.

The left - hand figure shows the enzyme - substrate complex for acetylcholine : while the substituted ammonium ion is held at the anionic site, B_2 and the acidic group catalyse transfer of the substrate acetyl group to the serine - hydroxyl (acetylation).

The right hand figure shows the product of this reaction, the acetyl - enzyme. As a result of a conformational change, the acetyl residue has been brought close to B_1, which catalyses the hydrolysis of the acetyl - enzyme (de - acetylation).

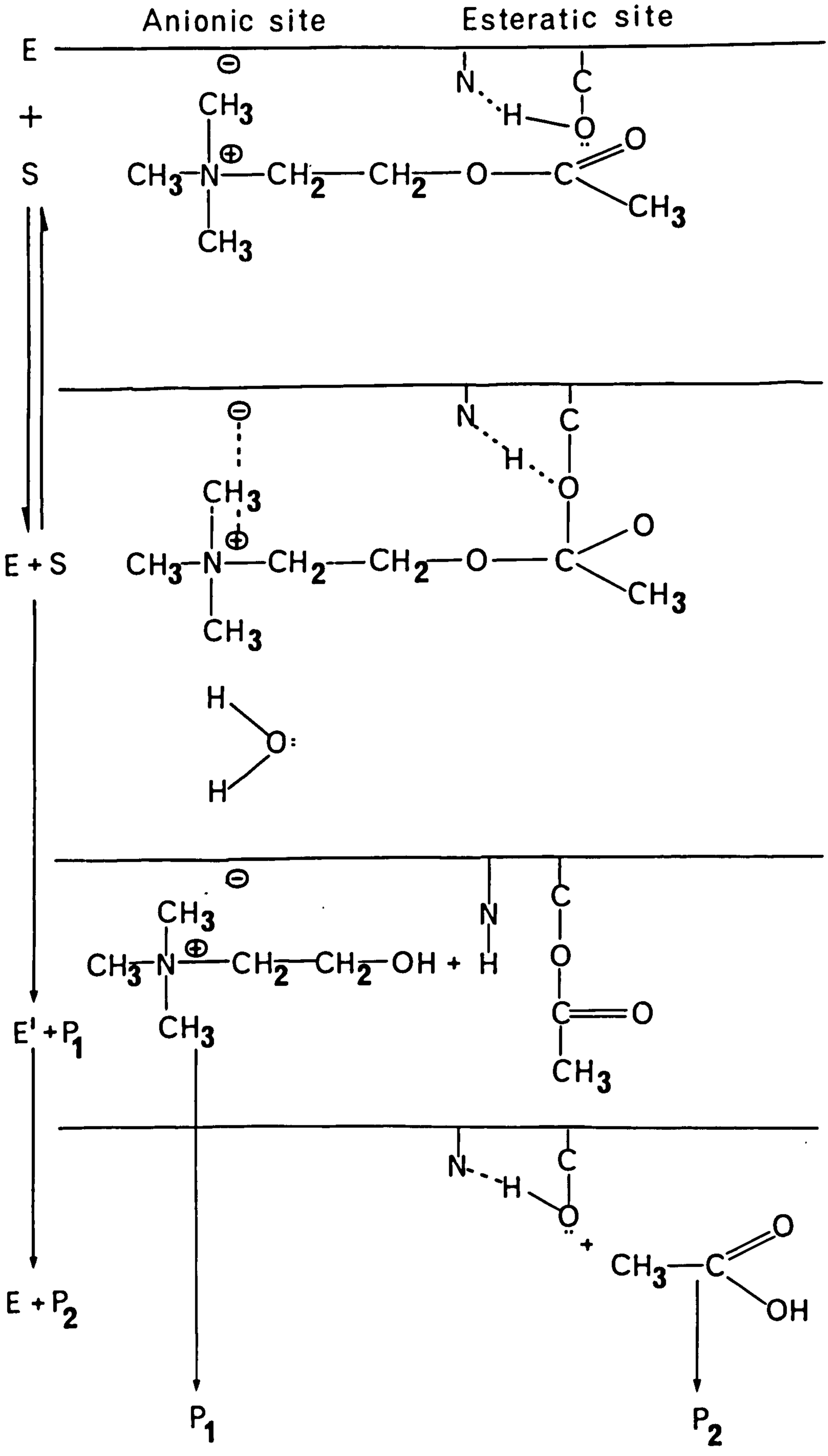

Anionic site
Esteratic site
E
+
S
E + S
E' + P1
E + P2
P1
P2

FIGURE 3.

Schematic representation of the hydrolysis of acetylcholine by acetylcholinesterase. (from Wilson and Harrison 1961)

E = enzyme

S = substrate

ES = Michaelis - Menten complex

E'= acetyl - enzyme

P_1= choline

P_2= acetic acid

Acetylcholine reacts with the enzyme at its active surface, initially forming a Michaelis - Menten complex. Under appropriate circumstances, an internal electron shift takes place and choline is split out as the first product of the reaction. The acetate moiety remains behind, covalently bound to the esteratic subsite. The acetyl - enzyme, so formed, hydrolyses in less than a tenth of a millisecond, thus regenerating the integrity of the active surface of the enzyme.

1 . 6. Evidence for conformational changes and allosterism in acetylcholinesterase.

Several reports have indicated that the acetylcholinesterase of the electric eel possesses a regulatory site distinct from the active site (Changeux 1966, Kitz et al 1970). This conclusion has been derived from studies which showed the simultaneous binding of various drugs and substrate to the enzyme and suggests that acetylcholinesterase is an allosteric enzyme (Monod et al 1965). Kitz and Kremzner (1968) have reported that the highly purified enzyme undergoes conformational changes in response to heat, strong base, substrate and anti - cholinesterase agents.

Acetylcholinesterase from mammalian sources has been less extensively studied but evidence is available that this enzyme has similar properties (Wombacher and Wolf 1971, Aldridge and Renier 1969 and Belleau et al 1970).

Crone (1973) has studied the modification of acetylcholinesterase activity by the interaction of ionic strength and quarternary nitrogen drugs to compare the mammalian brain with the data from the eel enzyme (Changeux 1966) and to compare the influence of these treatments on the re - activation of di - ethylphosphorylated enzyme (Kitz et al 1970). The phenomenon of the molecular aggregation of acetylcholinesterase (Changeux 1966, Grafius and Millar 1967) was also studied in order to determine whether this aggregation might form part of a regulatory mechanism. The results suggested that acetylcholinesterase exists in two extreme forms, the properties being observed, in practice, a resultant of the particular equilibrium established between them. In media of low ionic strength, or in hydrophobic areas of membranes, the enzyme is present in the form of complexes having apparent molecular weights in excess of 10^6 daltons. In this state the enzyme has high affinity for acetylcholine, low esteratic activity and is sensitive to

the presence of quarternary nitrogen drugs, e.g. gallamine, tubocurarine etc., and the rate of loss of phosphoryl groups from the active site is slow. The second form of the enzyme exists as the dis - aggregated molecule of molecular weight 260,000 daltons (Leuzinger et al 1969) when the ionic strength of the medium is high. The apparent affinity for acetylcholine is then lower but the catalytic activity is high. Gallamine or tubocurarine have little effect on this form of the enzyme and the phosphorylated enzyme is hydrolysed more rapidly to the active form. Rapid equilibrium occurs between the two states so that species of intermediate size may be observed on exclusion chromatography. In this situation possible control of enzyme activity could be achieved via several processes:

a) through a regulatory site distinct from the active site (Monod et al 1965).

b) through interaction between the two active sites on the enzyme (Leuzinger 1971, Koshland and Neet 1968).

c) by reversible adsorption to a surface (Masters et al 1969), or

d) by alteration of the rate of de - acetylation of the enzyme (Roufogalis and Thomas 1969).

Kitz and Braswell (1970), on the other hand, studied the de - activation by organophosphates, and the subsequent re - activation of acetylcholinesterase activity by sodium fluoride. They also concluded an allosteric site mechanism, capable of modulating activity at the active surface, presumably via conformational changes in the enzyme. They then, however, attempt to reconcile their findings with the hypothesis that acetylcholinesterase may be the "cholinergic receptor substance" (Zupancic 1963).

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1 . 7. Acetylcholinesterase and the cholinergic receptor.

The suggestion that the anionic binding site of acetylcholinesterase may be identical to the cholinergic receptor was, apparently, first put forward by Karassik (1946). More recently, several investigators have adopted the view that these macro - molecules may be similar (Belleau 1964), or, perhaps, even identical in some respects (Ehrenpreis 1967, Zupancic 1963, 1967).

Kitz and Braswell (1970) argue that acetylcholinesterase fulfils the classical and minimal requirements for an entity to be labelled as a cholinergic receptor viz.:

a) that it be principally protein or lipo-protein in nature.

b) that it react with the physiological transmitter, and

c) that it bind neuro - muscular blocking agents at the appropriate concentrations.

They then adopt a modification of the same two - state model as proposed by Monod et al (1963) and also suggest that the enzyme exists in equilibrium between two conformationally different states. They suggest, however, that the catalytic activity of the two states is now equal but that the affinity of the two states for acetylcholine is different. Receptor activators (depolarising neuro - muscular blocking agents) bind preferentially to the state having the highest affinity for acetylcholine. Receptor inhibitors (non - depolarising neuro - muscular blocking agents) are better bound to the low substrate affinity state. The allosteric effectors (muscle relaxants) shift the equilibrium between the two conformational states by stabilising the particular state to which they are bound. They suggest that, perhaps, one form is an aggregated form and the other a dis - aggregated form and associated, respectively, with an interference of sodium and potassium ion conductances and a facilitation of ion fluxes. In this way they implicate acetylcholinesterase

in a cholinergic receptor mechanism.

Belleau and DiTullio (1970) also conclude from their studies on quarternary ion binding to acetylcholinesterase that similarities exist between the anionic chain of the enzyme and the acetylcholine receptors of the myo - neural junction. They argue that:

> "...assuming that acetylcholinesterase is a primitive enzyme, the possibility definitely exists that, during the course of evolution, gene - duplications and mutations may have adapted the enzyme, or part of it, to the role of quarternary ion receptor in a variety of synaptic membranes".

The evidence that acetylcholinesterase is not the cholinergic receptor is, however, fairly convincing. Indirect evidence has been provided by Karlin (1967) and Albuquerque et al (1968). More recent and successful attempts at the isolation of the receptor proteo - lipid (DeRobertis and DePlazas 1970, DeRobertis et al 1970 and Miledi et al 1971) have shown that:

a) binding of ^{14}C acetylcholine was slightly reduced by - SH blocking agents, but was greatly inhibited by di - thio threitol followed by N - ethyl maleimide, a treatment known to inactivate the acetylcholine receptor without affecting acetylcholinesterase.

b) removal of acetylcholinesterase from the membrane did not affect the binding of cholinergic drugs.

c) Receptor proteo - lipid could be separated from normal and acetylcholinesterase - depleted membranes.

This and other evidence (Changeux et al 1971, Meunier et al 1971) demonstrates that acetylcholinesterase and the cholinergic receptor are two distinct macro - molecular entities.

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1 . 8. The localisation of cholinesterases in the central nervous system

The acetylthiocholine method, developed by Koelle and Friedenwald (1949) and Koelle (1950, 1951) and later modified by Koelle (1963) to improve the specificity and accuracy of localisation, remains the most specific histochemical procedure for the localisation of acetylcholinesterase and non - specific cholinesterases. It has been applied successfully to electron - microscopy (Lewis and Shute 1966).

Studies into the histochemical localisation and distribution of cholinesterases in the central nervous system have been important in supplying supporting evidence for central cholinergic transmission. The overwhelming obstacles to recovering and identifying the transmitter, released only at cholinergic terminals following stimulation, coupled with the fact that of the three characteristic constituents of cholinergic neurones, viz: acetylcholine, choline acetyltransferase and acetylcholinesterase, only acetylcholinesterase can be localised histochemically, at present, have been the reasons for the importance of this evidence.

Koelle (1969) has stated that the localisation of a high level of acetylcholinesterase activity in a group of neurones .....

> "is obviously not as direct a criterion of cholinergic function as would be the demonstration of the transmitter itself. Nevertheless, in the absence of a better index it appears to be a useful one".

Early studies on brain region homogenates showed that, in general, the concentration of acetylcholine, choline acetyltransferase and acetylcholinesterase run fairly parallel for various regions of the brain and spinal cord (Feldberg and Vogt 1948, Burgen and Chipman 1951, and Aprison et al 1964). Using ultra - microtechnique (Buckley et al 1967) better systems for the measurement of choline acetyltransferase (Hebb and

Silver 1956) and an axotomy (Hebb et al 1963, and 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 concentrations of the two enzymes has been observed in most brain regions and in certain central tracts. An exception was found in the cerebellum, where the concentration of acetylcholinesterase was observed to be very high, relative to that of choline acetyltranferase (Silver 1967).

Shute and Lewis (1967) and Lewis and Shute (1967) have used the technique of the histochemical localisation of acetylcholinesterase coupled with axotomy to map out several intensely staining tracts in the central nervous system of the rat. These various "cholinergic" projections ascend from the fore - brain and are predominantly related to the limbic system and striatum and may also form part of an ascending cholinergic system. Such an activating system may have an important role in the production and maintenance of cortical arousal.

Koelle (1963) points out several generalisations which can be made in regard to the distribution of acetylcholinesterase in the rat brain:

a) enzyme activity in the primary afferent neurones, as represented by the dorsal roots, their ganglion cells and their terminations in the spinal cord is consistently low.

b) enzyme activity in the primary motor neurones is consistently high.

c) little or no enzyme activity was noted in neurones synapsing directly with motor neurones, e.g. pyramidal cells and tracts and neurones of the red nucleus.

d) in the secondary, e.g. gracile and cuneate nuclei, and tertiary, e.g. lateral thalamic nucleus, sensory relay neurones a progressively increasing enzyme activity was observed.

These findings do not support the hypothesis, once offered by

Feldberg and Vogt (1948) that cholinergic and non - cholinergic neurones alternate in the central nervous system.

e) in certain correlation centres, particularly the basal ganglia, e.g. caudate nucleus, amygdaloid nuclei and the putamen, acetylcholinesterase activity is extremely high; whereas in others it is low, e.g. olivary nucleus, globus pallidus.

f) in the cerebellum more acetylcholinesterase activity was observed in the scattered, relatively large cells of the granular layer than in the few stained cells of the molecular layer. Stained fibres, however, were present in both layers.

The overall picture of the distribution of acetylcholinesterase activity in the central nervous system is that it does not appear to be principally connected with the main afferent, efferent or commisural pathways. With, at most, one or two exceptions, fibres rich in acetylcholinesterase do not form compact bundles. In most cases they occur as a relatively diffuse network of very fine fibres, in places intermingled with other pathways.

Krnjevic (1969) has pointed out that the acetylcholinesterase - containing fibres demonstrated by Shute and Lewis (1967) are associated, particularly, with phylogenetically ancient regions of the brain. Even the cortical projection from the striatal and septal regions appears to be distributed to the older, deeper layers of the cortex (Krnjevic and Silver (1965)).

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1 . 9. Aspects of the functional significance of the distribution of acetylcholinesterase in the central nervous system.

It has already been stated that studies into the distribution of acetylcholinesterase in the central nervous system have provided a source of important, but indirect, evidence for central cholinergic transmission. It is because of the lack of direct evidence that this evidence and, indeed, the whole subject is controversial.

In one of the earliest histochemical surveys of the rat central nervous system (Koelle 1954) a general finding was that relatively few neurones, or synaptic regions (at which the concentration of the enzyme is usually higher than along the fibre - tracts) appeared to be completely devoid of the enzyme. Neurones giving rise to known cholinergic peripheral fibres, e.g. those of the somatic motor nuclei and the neurones of origin of the pre - ganglionic autonomic fibres, consistently showed intense staining, as did those of certain basal ganglia, e.g. the caudate nucleus and putamen. However, the majority of other neuronal sites showed moderate or light concentrations of the enzyme. In the brain of the lower vertebrate, the frog, the distribution of intensely and moderately staining neurones is even more extensive (Shen et al 1955). From the many studies employing the iontophoretic application of acetylcholine onto central neurones it seems highly unlikely that cholinergic transmission obtains at all of these sites.

Similar considerations have led Krnjevic (1969) to state:

"All observers agree that the well - known pathways are not cholinergic, and that, with the sole exception of Renshaw cells, central neurones do not show the rapid and quickly reversible actions of acetylcholine expected of a conventional transmitter. Only two reasonable conclusions can, therefore, be drawn:

first, that in spite of the widespread distribution of acetylcholine and the enzymes required for its manufacture and destruction, acetylcholine plays no significant role in the central nervous system; alternatively, that the presence of acetylcholine is not entirely gratuitous but that it is connected with a type of function which differs appreciably from a conventional transmitter action. The latter seems a more useful way of looking at the problem".

He concludes, from neuro - pharmacological evidence of the slowly acting excitory effects of acetylcholine, that this action is not compatible with rapid "detonator" transmission and that a general facilitatory action on the cortex is more appropriate. This could have a significant function in the elevation of the general level of neuronal activity in the central nervous system, e.g. cortical arousal. Whether, also, the relatively weak depressant actions of acetylcholine could also contribute significantly to the inhibitory control of central activity remains unanswerable, at present.

Certain other features of the distribution of acetylcholinesterase appear inconsistent with the usual concept of cholinergic transmission. In the superior cervical and stellate ganglia of the cat essentially all the functional acetylcholinesterase is pre - synaptic (Koelle 1957). Koelle (1961) attempted an explanation of this observation by suggesting that the acetylcholine, released by the nerve action potential, acts initially at the pre - synaptic terminals to prolong, briefly, the depolarised state, resulting in the release of a greater number of quanta of acetylcholine to effect synaptic transmission. The duration of this self - amplifying feedback mechanism would then be limited by the pre - synaptically located acetylcholinesterase and, possibly also, by the desensitisation of the pre - synaptic cholino - receptors.

This pre - synaptic location of both acetylcholinesterase and the cholino - receptors has led Nachmansohn (1968) to postulate the pre - synaptic involvement of acetylcholine in a similar amplifier process.

The presence of acetylcholinesterase in the adrenergic neurones of most species (Eränko 1966) was explained, also, by Koelle (1961) by invoking a mechanism similar to that of Burn and Rand (1959) that acetylcholine participates in the release of nor - adrenaline from adrenergic fibres.

The wide - spread presence of acetylcholinesterase at synapses in the guinea - pig cerebellar cortex led Kasa and Csillik (1965, 1966) to conclude that acetylcholine is the universal transmitter in this structure. This is difficult to reconcile with the very low levels of choline acetyltransferase found in the cerebellar cortex of this species (Goldberg and McCaman 1967). There is, moreover, no apparent correlation between the levels of choline acetyltransferase and acetylcholinesterase in the various strata of the cerebellar cortices of different species (Goldberg and McCaman 1967).

* * * *

1 : 10. Behavioural aspects of cholinergic transmission.

The search for inter - relations between the cholinergic nervous system and behaviour has had to rely, largely, on essentially indirect experimental approaches. Russell (1969) has stated:

> "One could argue that the ultimate criterion for establishing a relation between the cholinergic system and a particular behaviour pattern would be the demonstration that arousal of, or changes in the behaviour occur following the release of acetylcholine. This would require the collection of acetylcholine and its

quantitative assessment during the same period when the behaviour was being measured, a technological nicety which now lies beyond our capability".

In general terms these indirect approaches fall into two main categories:

a) determination of the degree to which individual differences in some measure of behaviour are correlated with individual differences in some neuro - chemical parameter.

b) the experimental manipulation of the neuro - chemical parameter and the measurement of consequent changes in the measure of behaviour, or, the experimental manipulation of behaviour and the measurement of consequent changes in the neuro - chemical parameter.

An example of the first approach is given by the early studies of Rosenzweig et al (1960). They proposed:

"..... that variation in brain chemistry is a major determinant of variation in adaptive behaviour among normal individuals".

Using rats, they attempted a correlation between a measure of adaptive behaviour, in a variety of maze - learning situations and the activity of acetylcholinesterase in the cortex and sub - cortical areas. Selective breeding techniques were also used to maximise the range of individual variation in acetylcholinesterase activities. They reported a positive and significant correlation between higher acetylcholinesterase activity and the degree of adaptive behaviour exhibited under their experimental conditions. The differences in acetylcholinesterase activity in the cortical regions between "high" adapting and "low" adapting animals were quite small - 5%. The significance of such small differences has been questioned by many researchers, who usually argue that cholinesterases are generally regarded as enzymes working with a large safety margin. This type of correlation approach provided no concrete basisfor the interpretation of the results and led to the statement (Rosenzweig et al 1960):

"Obviously it is hazardous to argue from correlations to the existence of a causal relation.....the traditional procedure in seeking (such relations)....would be to manipulate experimentally the biochemical variable and observe whether.....performance changed....in a manner appropriate to the hypothesis being tested".

Many groups have adopted this second type of approach. Of these, some have chosen to determine the behavioural consequences of the progressive inhibition of central and peripheral acetylcholinesterase activity. Russell (1954, 1958) studied the effect of the chronic reduction of acetylcholinesterase activity to around 40% of normal by intra - peritoneal injection of the organo-phosphorus agent O, O-diethyl S-ethylmercaptoethanol thiophosphate. 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 later confirmed by Russell et al (1961).

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

These results have been confirmed in an extension of this study by Glow and Rose (1965) and Glow et al (1966). Using acute as well as chronic reduction of acetylcholinesterase activity by intraperitoneal 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. Evidence was provided that the effect on extinction could not be explained in terms of motivational or performance factors. They also observed that there occur "out of control" increases

in the concentration of acetylcholine following the acute reduction of acetylcholinesterase activity to below 40% of normal. Aprison (1962) has reported that it is at about this level that the enzyme loses control of its substrate and Wilson and Cohen (1953) have also reported that it is at about this level that a sharp drop in nerve conductance begins to occur.

The results of these two studies, therefore, suggest a relation between the cholinergic nervous system and the behavioural process of extinction.

The anti - cholinesterase DFP has been used by Aprison et al (1954) to examine relations between acetylcholinesterase activity and behaviour in rabbits. They reported that the injection of the same dose of DFP (0.1 mg./Kg.) into the right common carotid artery of rabbits produced three different behavioural patterns:

a) forced circling to the right.

b) forced circling to the left.

c) no circling in either direction.

Assay of the acetylcholinesterase activity in five brain areas, each on the left and right sides of the brain revealed that forced circling was associated with an asymmetric reduction of enzyme activity on the two sides of the brain. They explained this by assuming a biochemical lesion, the inactivation of acetylcholinesterase, which results in the abnormal accumulation of acetylcholine which then stimulates the affected brain areas. In animals that circle to the left, the right frontal cortex and the right caudate nucleus were primarily involved: whereas, in animals that circled right the frontal cortex on both sides of the brain as well as the right caudate nucleus were implicated. They state that it is noteworthy that a 36% reduction of acetylcholinesterase activity in the left frontal cortex is associated with a change in behaviour; a reversal in the direction of circling.

Another example of the research into the relations between the central cholinergic nervous system and behaviour is provided by the studies into the genetically pre - coded behavioural pattern of drinking. It is known that in the rat the regulation of the drinking response is mediated by a functional neural circuit which involves several limbic and diencephalic structures (Morgane 1964). That this circuit transmits quantitative information has been suggested by studies which measure the amount of water consumed by normal animals after varying periods of water deprivation. Grossman (1962) has shown that direct cholinergic stimulation of the circuit will elicit the drinking response in satiated animals and that the circuit is selectively cholinoceptive. The circuit is, in fact, so selective that it has been traced by cholinergic stimulation (Fisher and Coury 1962).

Levitt and Fisher (1966) have reported that the intra - peritoneal injection of atropine eliminates the drinking response and that the application of atropine to different parts of the functional circuit prevented drinking in response to the stimulation of other parts of the circuit. Khavari and Russell (1966) have shown that direct cholinergic stimulation of the circuit was equally as effective as water - deprivation in supporting the acquisition and re - learning of a maze response and that they had similar effects on extinction. They also demonstrated that a response learned under water - deprivation was readily elicited by direct central stimulation of the circuit.

Deutsch and his collaborators have been investigating the cholinergic synapse in relation to its possible role in memory mechanisms. Using evidence from human retrograde amnesia studies they hypothesised that if the substrate of memory is synaptic and if it can slowly change then there is the possibility of following such synaptic changes using pharmacological methods. Deutsch (1971) has stated:

"If the same dose of a synaptically acting drug has different effects on remembering that depend on the age of the memory (and this can be shown for a number of synaptically acting drugs) then we may assume that there has been a synaptic alteration as a function of time after learning, and we may infer that such a synaptic change underlies memory".

In a series of experiments (Deutsch and Liebowitz 1966, Deutsch et al 1966) this group have shown that cholinergic drugs could alter recall. Using intra - hippocampal injections of DFP into rats they showed that animals trained to run to an illuminated arm of a Y - maze showed amnesia when injected with the anti-cholinesterase agent 30 mins. after training. No amnesia is produced if the injection takes place 3 days after training. Complete amnesia, however, is seen if the injection is given 14 days after training. Using intra - cerebral injection of DFP in rats they showed good re - call 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 partly learned. On the basis of these and other results Deutsch argued that the anti - cholinesterase was facilitating low conductance synapses and inhibiting 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 anti-cholinesterase would have no effect soon after training but would cause a block as soon as conductance of the synapse improved.

To test this hypothesis parallel experiments were performed with scopolamine (Deutsch and Rocklin 1967), an anticholinergic which decreases the acetylcholine 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 and Rocklin (1967) reported that this

was indeed the case. Deutsch (1971) combines the results of these and other studies to conclude:

a) that cholinergic synapses are modified as a result of learning and that it is probably the post - synaptic membrane that becomes increasingly more sensitive to acetylcholine, up to a certain point.

b) after this point the sensitivity declines leading to the phenomenon of forgetting.

c) there is good evidence that there is an initial phase of declining sensitivity to anti - cholinesterases or increasing sensitivity to anti - cholinergics. This could reflect the existence of a parallel set of synapses with a fast decay characteristic that serve as a short - term store.

d) increasing the amount of learning leads to an increase in conduction in each of a set of synapses without an increase in their number.

e) both original learning and extinction are subserved by cholinergic synapses.

Single - dose studies, of this kind, using anti - cholinesterases have been criticised (Weiss and Heller 1969).

Other major approaches to this study have been to vary behaviour and examine neuro - chemical parameters which may alter as a consequence. It has been conceived that behavioural patterns are a series of processes, the first of which is sensory stimulation. Relations between the cholinergic nervous system and behaviour might, therefore, be observable as changes resulting from sensory stimulation.

Liberman (1962) took two groups of age-matched rats; he reared one group in normal light - dark conditions, and the other he reared in total darkness for 17 weeks. He reported that the acetylcholinesterase activity in the retinas of the dark - raised rats was very significantly lower than

in the control group. This could be explained by assuming that the lower level of stimulation led to less acetylcholine being released; if now the synthesis and maintenance of acetylcholinesterase activity were dependent on the level of acetylcholine, then this too would be lower, as observed. Since the retina is an embryological outgrowth of the diencephalon and is independently capable of neural integration, it is plausible that central acetylcholine concentrations and acetylcholinesterase activity might also be affected, in this manner, by sensory input and, thus, have behavioural implications.

De Robertis and 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 electron - microscopic 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. (Perri et. al. 1971, Csillik and Bense 1971 and Feher et al 1972).

Feher et al (1972) performed acoustic stimulation on cats, both in the waking state and following anaesthesia by either pentobarbital or chloralose. After only 45 mins. of stimulation they reported changes in the vesicular density of the 4th and 5th cortical layers. Categorising axons by cross - sectional area, they found differential effects in the waking state, with both increases in density in some categories and decreases in others. With pentobarbital anaesthesia, significant decreases in vesicular density were observed in all categories but one, where no effect was observed. It was suggested that the reduction of vesicular density, resulting from the combined procedure of stimulation under pentobarbital anaesthesia was possibly due to the 70% inhibition of choline transport across the blood - brain barrier by pentobarbital

reported by Diamond (1970), thus, hindering the supply of precursor for transmitter synthesis.

As well as this data on vesicles, there is also histochemical evidence that the concentration of acetylcholinesterase in central neurones may be correlated, directly, with their level of activation by afferent input, over varying periods. Pepler and Pearse (1957) reported that procedures that promoted an increase in secretory activity of the anterior or posterior pituitary, e.g. high salt intake, lactation, castration etc., resulted in an increased staining for acetylcholinesterase in certain hypothalamic neurones of the rat.

Hess (1960) studied the effect of eye - removal at different stages of maturity on the histochemical localisation of acetylcholinesterase in the superior colliculus of mice. Removal of the eye at birth resulted, consistently, in decreased staining for the enzyme in the contralateral stratum griseum superficiale. This decrease was age - dependent and no effect was seen in mature animals. Hess (1960) concluded that afferent activation might be essential for acetylcholinesterase formation only during developmental stages.

This possible link between sensory input and central acetylcholinesterase activity and other neuro - anatomical measures has been the basis of an extensive series of studies conducted by Bennett and Rosenzweig. Bennett et al (1964), Rosenzweig et al (1971) reviewed these studies.

In their early studies (Rosenzweig et al 1960) they found evidence that the ratio of cerebral acetylcholine concentration to acetylcholinesterase activity was positively related to problem solving ability in rats. On the basis of these results they hypothesised that differential experience in rats would lead to quantitative changes in the cholinergic synaptic transmitter system. Specifically they predicted that enhanced stimulation and training would lead to an increased rate of liberation of acetylcholine,

which would lead, in turn, to an increased rate of synthesis of acetylcholinesterase (Krech et al 1960).

Their standard approach has been to assign same sex littermate rats to two groups. From weaning, one group, the "experience - enriched" condition is housed in large social groups in a large cage with adequate food and water and a variety of objects that they can play with; the other group, the "experience - impoverished" condition are housed singly in small cages and receive minimal environmental stimulation. At the end of a pre - determined experimental period (0 - 80 days) the animals were killed, their brains removed and a series of anatomical and neuro - chemical tests were performed. It was observed (Bennett et al 1964) that rats that had 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 acetylcholinesterase. However, the amount of enzyme activity per unit of tissue weight decreased. They also reported that the brain samples exhibited a greater pseudo - cholinesterase activity and a greater glial - neuronal ratio. Altman and Das (1964) confirmed the effect of environmental - enrichment on glial proliferation finding increases of up to 140% in 3 months.

The differences in acetylcholinesterase activity reported by Bennett et al (1964) were not large - 3% but they are reported as significant and repeatable. In the subcortical areas acetylcholinesterase activity increased but no effect on pseudo - cholinesterase activity was observed. The group has produced control evidence to show that the effects are not due to differences in handling, stress or perhaps altered rates of maturation. Later experiments, (Rosenzweig et al 1968) showed that the same brain measures could be obtained by exposing rats to the "experience - enriched" condition for only 2 hours a day for 30 days.

Bennett et al (1964) assert that they wish to make it clear that

finding these changes in the brain consequent upon experience does not prove that they have anything to do with the storage of memory, merely that the brain is responsive to environmental change, a fact central to physiological theories of learning and memory. However, they then consider whether their findings are compatible with the suggestion of Ramon y Cajal (1904) that long term memory storage involves the growth of new synaptic connections among neurones and conclude that the observed effects are, indeed, compatible with a substantial increase in the number of synaptic connections.

Quantitative electron microscopy has been used to determine the effects of differential environments on synaptic size and number. Cragg (1967, 1968, and 1969) has measured the axon - terminals of rats reared in the dark or removed for various periods of light exposure. He reports (1969) that terminals in the lateral geniculate nucleus are 15% greater in diameter but 34% less numerous, in dark reared rats.

Fifkova (1970) has measured axo - dendritic synapses in layers 2 - 4 of the occipital cortex of rats after 6 weeks of unilateral lid suture. She reports 20% fewer synapses in the hemisphere contralateral to the sutured eye but that they were 7.5% greater in cross - sectional area than controls.

Mollgaard et al (1971) showed significant changes in the size and number of synapses between experience "enriched" or "impoverished" groups after 30 days. They found that rats from the experience "enriched" condition had synapses 52% greater in length but that were only 67% as numerous. The experience "enriched" rats had more large synapses as well as fewer small synapses than did the "impoverished" condition rats, so the distribution could not be explained in terms of the loss of small synapses from the "impoverished" condition distribution. The total area of synapses in the experience - "enriched" condition was, taking both size and number of contacts into account, 40% greater than in the

"impoverished" group. They point out that this could explain the increased acetylcholinesterase activity found in their earlier studies, but that although there are 40% differences in synaptic area this would not be expected to produce a similar increase in the enzyme activity. They also suggest that the increased synaptic area could indicate a facilitation of those synapses and from this consideration they suggest the hypothesis that the "impoverished" condition brain represents a more immature condition with many small synapses that are not committed to functional circuits. Learning and experience in the "enriched" condition could modify this distribution, increasing the effectiveness of functional pathways and suppressing connections that yield undesirable results.

More recently, Rosenzweig et al (1972) have demonstrated that the cerebral effects of differential experience (Bennett et al 1964) occur also in hypophysectomised rats. They conclude that these effects do not require mediation by hormones of the pituitary or of those regulated by the pituitary.

Many groups have found that the activity of central acetylcholinesterase changes in response to a variety of physical and chemical treatments which alter the behavioural state of the animal. It cannot be stressed too highly that many of these treatments also affect the levels of acetylcholine in the brain, a fact which has great relevance to any postulated role for the enzyme activity changes. The tables (3 - 7) summarise the variety of treatments chosen and their effects on acetylcholine concentrations and acetylcholinesterase activity. For ease of presentation important additional information on time courses of the effects and the % changes have been omitted.

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1 . 11. Summary and plan of experiments.

It is apparent from the foregoing reviews that there are a wide variety of treatments which alter the behavioural state of the organism and also alter their acetylcholinesterase activity and the concentrations of acetylcholine in the brain. Many of these studies have been done on whole brain homogenates or, at best, on a very few regions of the brain. The assay of such large portions of the brain can fail to detect changes occurring at a local level and this can lead to misinterpretation.

From the few studies that have employed regional analyses it is clear that the effects of drugs on the concentration of acetylcholine in the brain differ from area to area. Giarman and Pepeu (1962) have shown that at a dose of 0.63 mg./Kg. the effects of scopolamine hydrobromide on acetylcholine concentrations are restricted to the cerebral hemispheres and that the sub - cortical areas are not affected. However, Beani et al (1964) have shown that at doses of 5 and 25 mg./Kg. decreases in the acetylcholine concentration occur in the caudate nucleus, parietal cortex and olfactory lobes, while the thalamus and cerebellar regions are unaffected. Crossland and Merrick (1954) reported that barbiturate anaesthesia raised acetylcholine levels in the cerebral hemispheres, medulla and pons, cerebellum and upper brainstem but that the increases were unequal in these various regions. Schmidt (1966) has confirmed these regional effects on acetylcholine concentration increases using halothane anaesthesia. He also found that the increases also varied with the depth of the anaesthesia. Attempts to use micro - methods and regional studies are, therefore, most important in the search for relations between neuro - chemical and biochemical variables.

TABLE 3. Summary of treatments found to alter acetylcholine concentrations and acetylcholinesterase activity in the central nervous system.

Study	Animal	Treatment	AChE	ACh
Richter, D.	Rat	Barbiturate anaesthesia		+
Crossland, J. (1949)		Sleep		+
		Emotional states		-
		Electrical stimulation		-
		Convulsions		-
Richter, D. (1952)	Rat	Hypoxia		-
Crossland, J.		Barbiturate anaesthesia		+
Merrick, A. (1954)	Rat	Seizures		-
Crossland, J. et al (1955)	Rat	Insulin hypoglycemia		-
Murali Mohan, P. Murali K-Dass, P. (1969)	Snail	Aestivation	-	
Naik, S. et al (1970)	Rat	Stress	+	-
		Fasting	-	+
Schmidt, K. (1966)	Rat	Halothane anaesthesia		+
Freedman, D. et al (1966)	Rat	Sleep - deprivation		-

TABLE 4. Summary of treatments found to alter acetylcholine concentrations and acetylcholinesterase activity in the central nervous system.

Study	Animal	Treatment	AChE	ACh
Kling, A. et al (1965)	Rat	Handling	-(cortex) +(caudate)	
		Light stimulation	-(cortex) +(caudate)	
Bennett, E. et al (1964)	Rat	Differential experience	+	
Toru, M. et al (1966)	Rat	Avoidance responding :		
		increased		-
		decreased		+
Mitchell, J. (1963)	Cat	Electrical stimulation		-
Kanai, T. Szerb, J. (1965)	Cat	Electrical stimulation		-
Phillis, J. (1968)	Cat	Electrical stimulation		-
Krivanek, J. Bures, J. (1968)	Rat	Electrical stimulation	-	
		Cortical spreading depression	+	
Westermann, K. et al (1971)	Mouse	Barbital narcosis		+

TABLE 5. Summary of treatments found to alter acetylcholine concentrations and acetylcholinesterase activity in the central nervous system.

Study	Animal	Treatment	AChE	ACh
Kalchev, L. Ivanov, R. (1969)	Cat	Urethane anaesthesia		+
Kerkut, G. et al (1970)	Cockroach	Shock - avoidance conditioning	-	
Cymborowski, B. et al (1970)	House cricket	Circadian rhythm	-	
Hanin, I. et al (1970)	Rat	Circadian rhythm		+
Kerkut, G. et al (1972)	Snail	Shock - avoidance conditioning	+	
Ling, A. (1970)	Rat	Thyroidectomy	-	
		Hypothyroid	-	
		Hypermetabolic	+	
		+ T_4	+	
McKinney, T. (1970)	Rat	Adrenal de-medullation singly - caged	-	
		group - caged	+	
Oba, T. et al (1971)	Rat	Suckling (2 mins.)	-	

TABLE 6. Summary of treatments found to alter acetylcholine concentrations and acetylcholinesterase activity in the central nervous system.

Study	Animal	Treatment	AChE	ACh
Alexsidze, N.		Training to use the		
Balavadze, M.		non - preferred paw	+	
(1971)	Rat			
Vernadakis, A.		Pentobarbital anaesthesia	-	
Rutledge, C.		Ether anaesthesia	-	
(1973)	Rat			
Waser, P.		Morphine	-	
Schaub, E.		LSD	-	
(1970)	Guinea-pig	Amphetamine	-	
		Psilocybin	-	
Beani, I.		Drugs which increase		
Bianchi, C.		brain Dopamine		-
(1970)	Guinea-pig			
Kabes, J. et al (1973)	Rat	LSD (0.2µg./ Kg.)	-	+
Pepeu, G. et al (1973)	Rat	Septal lesion		-
Kling, A. et al (1970)	Monkey	Frontal cortex lesion	+	
		Alternation training	+	

TABLE 7. Summary of treatments found to alter acetylcholine concentrations and acetylcholinesterase activity in the central nervous system.

Study	Animal	Treatment	AChE	ACh
Naik, S.		Reserpine	-	+
Sheth, U.		Chlorpromazine	-	+
(1970)	Rat	Pentobarbital	no effect	+
		Codeine	no effect	-
		Pethidine	-	-
		Morphine	no effect	+
		Norcamphane	+	-
		Amphetamine	-	-
		Atropine	no effect	-
Brown, C.		visual stimulation		
King, M.		Formal and informal		
(1971)	Rat	training	+	
Beani, I.				
et al (1964)	Rat	Scopolamine (5 and 25 mg./Kg.)		-
Giarman, N.				
Pepeu, G.				
(1962)	Rat	Scopolamine (0.63mg./Kg.)		-
Quay, W.				
et al (1971)	Rat	Circadian rhythm	none	

Note: + and - refer to increases or decreases, respectively of either acetylcholine concentration or acetylcholinesterase activity.

The aim of the present study has been to examine, more closely, the changes in activity of acetylcholinesterase which result from behavioural manipulations.

In order to do this, automated semi - micro - methods for the assay of acetylcholinesterase from small sample of brain tissue, 2 - 5 mg. wet weight, were developed. Twelve regions of the rat brain were chosen for study and a range of behavioural procedures were adopted, which incorporated a wide range of physiological stimulation.

Since there are numerous models which could provide a mechanism for such enzyme activity changes in a relatively short time period, e.g. allosterism and conformational changes, rapid protein synthesis and naturally occurring inhibitors and activators etc., and since any of these models may apply to acetylcholinesterase, kinetic investigations, time - course studies and protein synthesis inhibition studies were carried out in order to elucidate the mechanism of the enzyme activity changes.

* * * *

SECTION TWO.

2 . 1. MATERIALS

a) Animals. Several breeding pairs of an inbred strain of Agus albino rat were obtained from the M.R.C. unit at Carshalton, Surrey, U.K. A stock of rats was bred from these pairs in this department and maintained on an unlimited supply of the Porton rat diet. From weaning, female rats were separated and housed in littermate groups of up to six. Female littermates, only, were used in this study, aged between 100 - 120 days and weighing between 190 - 220 g.: this was done in order to eliminate possible sex differences and to use only animals with a stable adult concentration of acetylcholinesterase (Bennett et al 1958, 1966).

b) Chemicals and reagents. All chemicals and reagents were purchased from BDH biochemicals, Poole, Dorset, with the following exceptions: Pentobarbitone sodium : standard Nembutal solution (60 mg./ml.) supplied by Abbott Laboratories, Queenborough, Kent.

Lever IV and Brij - 35 (30% solution), detergents for the auto - analyser, were obtained from Technicon Instruments Co. Ltd., Basingstoke, Hants.

Lubrol - WX : gift from C. Fewtrell.

2,2' - dithiodi - pyridine (aldrithiol - 2) was obtained from Ralph Emmanuel Ltd., Wembley, Middx.

N-Acetylthiocholine iodide: was obtained from the Sigma Chemical Company, St.Louis, Missouri, U.S.A.

Halothane (Fluothane) was obtained from I.C.I. Chemicals Ltd.

* * * *

2 . 2. METHODS.

2 . 2. 1. Quiet control procedure.

A pair of female littermates was taken from the stock cage, weighed and transferred to a second cage supplied with food and water. The animals were taken to a quiet room with even lighting and temperature and left for one hour before sacrificing. An average of eight animals, chosen at random, was used to make up the experimental and control groups.

2 . 2. 2. Anaesthetisation procedure.

A pair of female littermates was taken from the stock cage, marked on the tail for identification, weighed and placed into a second cage. They were then taken to the quiet room and one was injected, intra-peritoneally, (i.p.), via a 25 gauge 5/8" needle using a standard anaesthetic dose (60 mg./Kg.) of Nembutal (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 then tested for the abolition of reflexes using the standard testing procedures of:

a) loss of righting reflex.

b) loss of paw - withdrawal reflex.

c) loss of response to pinching the base of the tail.

d) loss of corneal reflex (eye - blink response).

Having established the abolition of reflexes by these procedures the anaesthetised animals were left for a further 45 mins. with their body temperature maintained by a 60 watt light bulb placed at a distance of 24 inches (approx.).

The other rat of the pair was used as a control and injected at the same time with an equivalent volume of pyrogen - free physiological saline (0.9% w/v.) pH corrected to 7.2. The control rats were then placed back into the cage and left in the quiet room, away from the anaesthetised

animal, for the same period, before sacrifice.

2 . 2. 3. Anaesthetisation coupled with non - specific electric stimulation procedure.

Animals were anaesthetised and tested for the abolition of reflexes as for animals in group 2.2.2. Body temperature was maintained also, for the animals in this group, by a 60 watt light bulb at approximately 24 inches.

A pair of stimulating leads were then attached, via spring pins, one to the rear left paw and the other to the front right paw. A stimulus pulse was then applied from a Palmer stimulator at 5 second intervals with a pulse width of 100msec. and at a voltage just sufficient to elicit a twitch response in the stimulated paws (25 - 28 volts.). This treatment was maintained for 30 mins. during the normal anaesthetised period.

The other rat of the pair in this group was injected, at the same time, with an equivalent volume of pyrogen free physiological saline (0.9% w/v.) pH corrected to 7.2, and left for the same time period as the experimental animal, in a cage in the quiet room before sacrifice.

2 . 2. 4. Two - way shock-avoidance conditioning apparatus.

To obtain data from rats subjected to a broad spectrum of physiological stimulation, it was necessary to electrically stimulate conscious animals. In order to do this, an automatic rat reflex conditioning apparatus (Ugo Basile, Milan cat. no. 7502), or shuttle - box, was used. This apparatus is fully automated, and is designed to study conditioned reflexes (avoidance reaction) in rats.

The apparatus consists of a separate programming/recorder unit (cat. no. 7501) connected, via 4 metres 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 central partition, into which, is cut an inter - communicating door at floor level (see Figure 4). 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 in a seesaw fashion.

The "reinforcement" consists of an electrical stimulus applied to the metal floor bars of the cage by a special "static - scrambler" circuit. This device ensures that the rat receives the stimulus irrespective of the relative positions of its paws.

The apparatus operates the conditioning sequence, automatically, as follows:

a) time = 0 seconds : conditioning stimuli (buzzers and lights in the roof) switched on.

b) time = 3 seconds : conditioning stimuli remain on: reinforcement stimulus applied.

c) time = 6 seconds : both conditioning and reinforcement stimuli switched off.

d) time = 30 seconds : conditioning sequence repeats.

A rat, placed in the 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, e.g. jumping, will not cause an accidental operation of this switch.

The programming / recording unit supplies a pre - set number of stimuli. The intensity of the acoustic stimulus and the reinforcement stimulus can be varied.

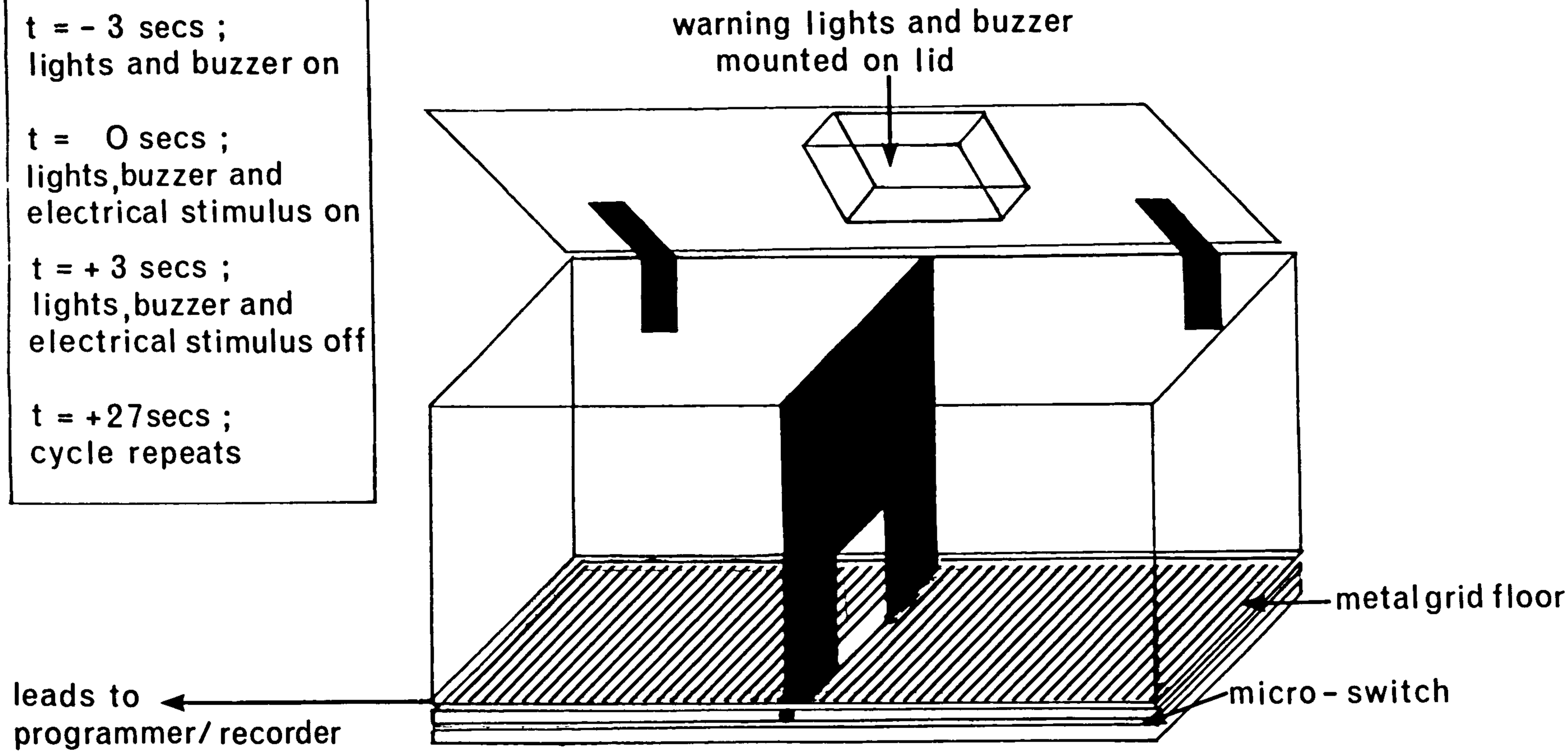
conditioning sequence

t = - 3 secs ;
lights and buzzer on

t = O secs ;
lights, buzzer and
electrical stimulus on

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

t = +27secs ;
cycle repeats



The UGO BASILE rat reflex conditioning apparatus

FIGURE 4. Diagram of the construction of the two - way shock - avoidance conditioning apparatus.

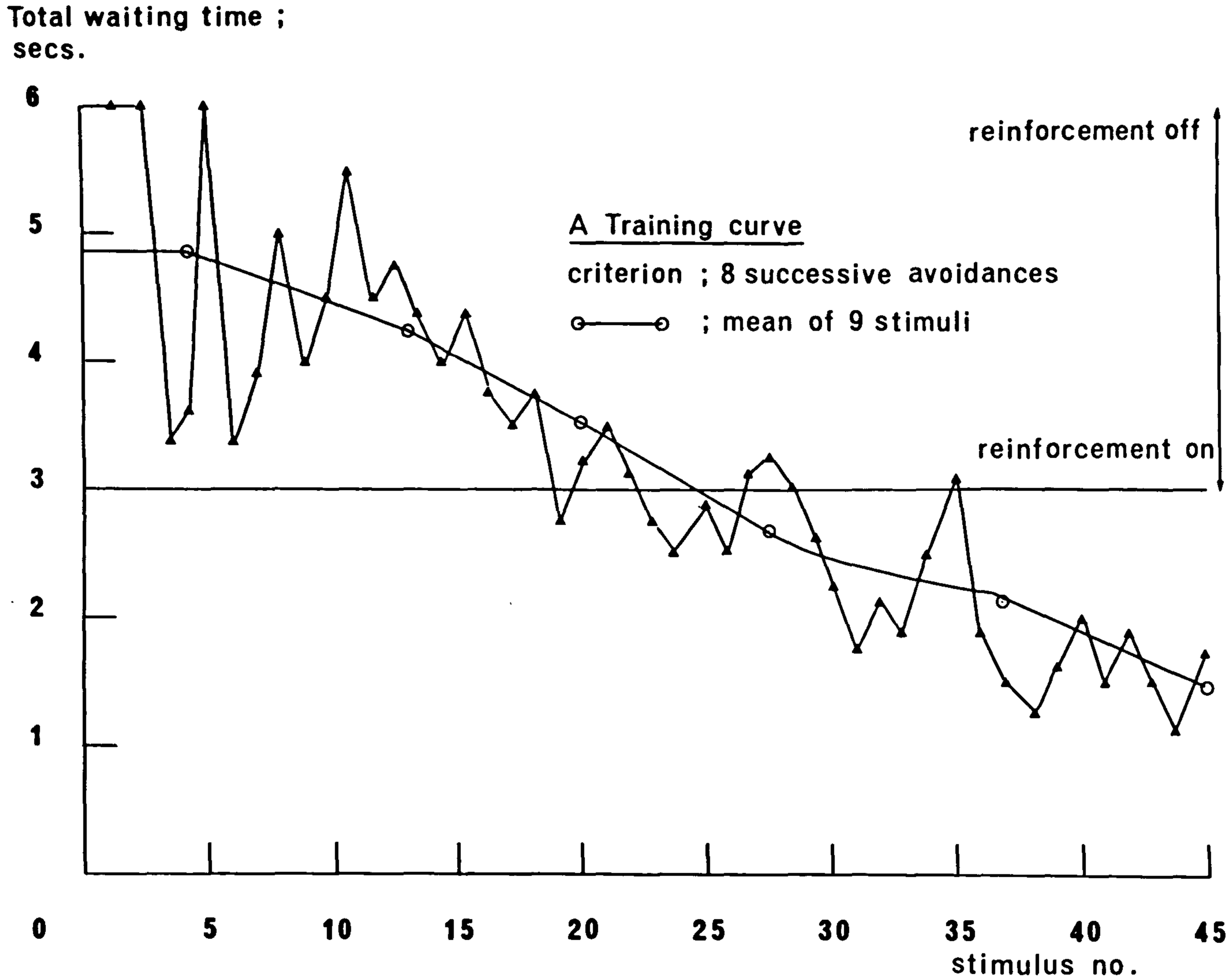

Total waiting time ;
secs.
6
5
4
3
2
1
0
5
10
15
20
25
30
35
40
45
stimulus no.
reinforcement off
reinforcement on
A Training curve
criterion ; 8 successive avoidances
; mean of 9 stimuli

FIGURE 5. A typical training curve.

The total waiting times (the time from onset to cancellation of an individual conditioning sequence) are plotted against the stimulus, or sequence number. It can be seen that the waiting times become progressively smaller as the animal becomes conditioned. The horizontal line, drawn through the graph represents the arrival of the reinforcing stimulus. Responses which represent true avoidances are those which are plotted beneath this line. The arbitrary criterion of learning of 8 successive avoidances was normally achieved at 45 ± 5 stimuli.

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 reinforcing stimulus. A timer, mounted into the recording unit displays the waiting time (waiting time = time from onset to cancellation of an individual conditioning sequence) for each response in 1 / 12.5 seconds.

The waiting times are a measurement of the rats response to the conditioning stimulus and they become progressively shorter as the animal becomes conditioned. By plotting the waiting times against stimulus number a training - curve can be produced (see Figure 5).

The successful use of this apparatus for conditioning rats requires great care to be taken with regard to the housing of the equipment. The apparatus was placed in a quiet, dimly lit, temperature and humidity controlled room. The temperature was maintained between 22 - 25°C., and the humidity at 70%. Diffuse and indirect illumination was used to light the room.

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

2 . 2. 5. Electrical stimulation : procedure.

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

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

After becoming accustomed to the environment of the cage the programmer was set to deliver a train of 40 stimuli at 30 second intervals and switched on. The floor micro - switch was inactivated in this group of experiments so that these animals could neither cancel the conditioning sequence nor, hence, become conditioned; they were merely stimulated electrically.

The animals in this group behaved, characteristically, by moving to an alert posture throughout the conditioning stimulus, 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 cancellation 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 very few animals (2) apparently learnt to avoid the shock as a result of this rearing behaviour. They responded on arrival of the reinforcement by placing the 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 conditioning sequences) was almost entirely absent. Inter - trial crossing has been used as a measure of the sponteneous activity level of rats in this apparatus.

A group of quiet control animals was used to compare the two groups. The animals were sacrificed within 15 mins. of the completion of the stimulation.

2 . 2. 6. <u>Two - way shock - avoidance conditioning procedure</u>.

The operation of the apparatus has been described in 2.2.4. Unlike the treatment used in 2.2.5., the floor micro - switch was activated so that the animals could, by their movements, cancel the sequence and, thus, learn to avoid the shock.

Pilot experiments using rats in the acquisition of the shock - avoidance response were conducted to determine the optimal conditions for acquisition, the time - course of the acquisition and the behavioural homogeneity of the responses exhibited using this treatment. On the basis of these studies it was decided that an arbitrary criterion of learning could be set at 8 successive avoidance and that this criterion ought, normally, to be achieved after about 30 mins. of training (see Figure 5).

Rats in this group were also allowed 10 mins. in the cage prior to the start of the training, to accustom themselves to the environment. The programmer was then set to deliver 60 stimuli and switched on. The responses of the animal were recorded automatically and the general behaviour continuously monitored by observation. The pattern observed in the pilot experiments was observed also for the animals in this group (see Figure 5).

On two occasions animals did not learn at all, responding only on arrival of the reinforcing stimulus. These animals were excluded from the group. It was again noted that there was a consistent absence of inter - trial crossing, although this behaviour was eventually observed in the two animals that did not learn after receiving 50 stimuli.

Trained animals were tested for their degree of the retention of the avoidance response at 4 and 10 days following initial training. The re-test curves are shown in Figure 6. The response is retained very well, for at least, 10 days.

The animals in this experimental group were left in quiet for one hour after the completion of the training. A group of quiet control animals was again used to compare this group.

This experimental procedure was also conducted for other groups of animals and they were sacrificed on day 2 (24 hours) and day 4 after training. This was done in order to determine the time - course of any possible changes observed as a result of training.

2 . 2. 7. <u>Yoked control procedure</u>

In groups 2.2.5. and 2.2.6., both procedures involved the electrical stimulation of rats, although only in the shock - avoidance conditioning group did learning occur. However, a direct and meaningful comparison of the two groups is not possible since the pattern of stimulation received by the two groups was entirely different. A direct comparison of two groups where both were identically stimulated under the same conditions yet where only one group achieved shock - avoidance conditioning is essential to any study that aims to examine the cerebral effects of the conditioning. A yoked control procedure was conducted to fulfil this aim.

Two cages were connected to the same programmer / recorder unit.

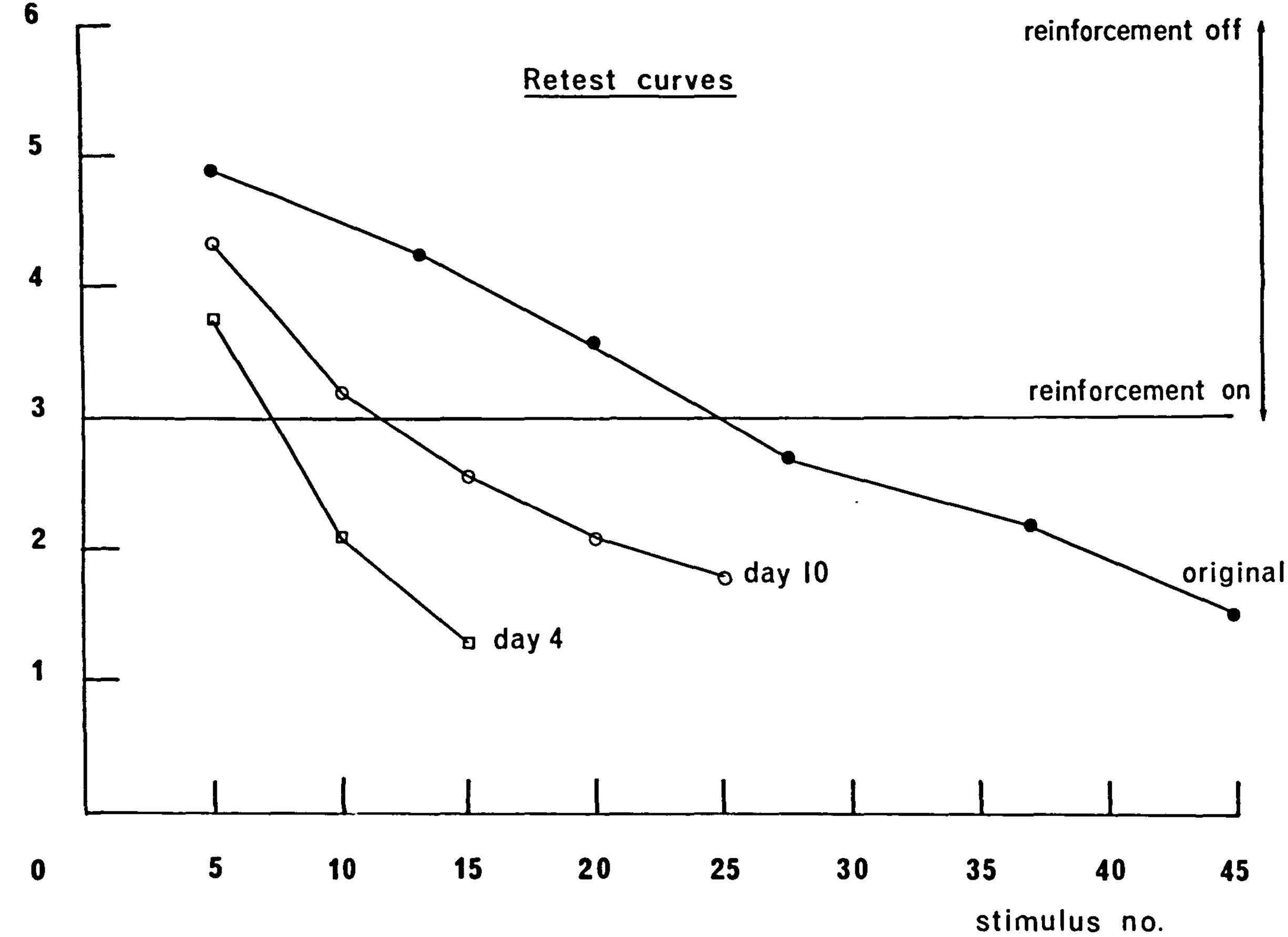

Total waiting time ;
secs.
6
5
4
3
2
1
0
Retest curves
reinforcement off
reinforcement on
day 10
day 4
original
5
10
15
20
25
30
35
40
45
stimulus no.

FIGURE 6. <u>Training curves obtained by re - testing animals 4 days and 10 days following initial training</u>.

There is a significant degree of retention of the conditioned response both after 4 and 10 days.

The micro - switch of one cage was inactivated so that the resulting arrangement was that of master - slave. The rat in the master cage was in an executive capacity i.e. its movement only could cancel the conditioning sequence, the rat in the slave cage received an identical pattern of stimulation, simultaneously but could not, by its movements, cancel the sequence.

Other details of the procedure were as for group 2.2.6. The behaviour of the yoked group was very similar to that described for 2.2.5. The animals were again left for one hour, in quiet, before sacrifice. Other groups were left for 24 hours and 4 days after training to provide control groups for the trained animals.

2 . 2. 8. Acetylcholinesterase assay.

a) Preparation of the samples.

Twelve regions of the rat brain were chosen for study. They were: 1) frontal dorsal cortex, 2) medial dorsal cortex, 3) posterior dorsal cortex, 4) cerebellum, 5) ventral cortex, 6) hypothalamus, 7) thalamus, 8) medulla, 9) pons, 10) superior colliculus, 11) olfactory tubercle and 12) caudate nucleus. Figure 7 shows the location of the various brain regions chosen. The regions were chosen on the basis of two main considerations:

1) accurate dissection: all the regions chosen are relatively large, anatomically well defined and many are situated peripherally on the surface of the brain so that they can be dissected easily and accurately. The regions chosen were defined using a stereotaxic atlas of the rat brain (Koenig and Klippel 1963). Electron micro-graphs of the morphology of the brain region samples and electron micro-graphs showing the histochemical localisation of acetylcholinesterase in the samples are presented in appendix A.

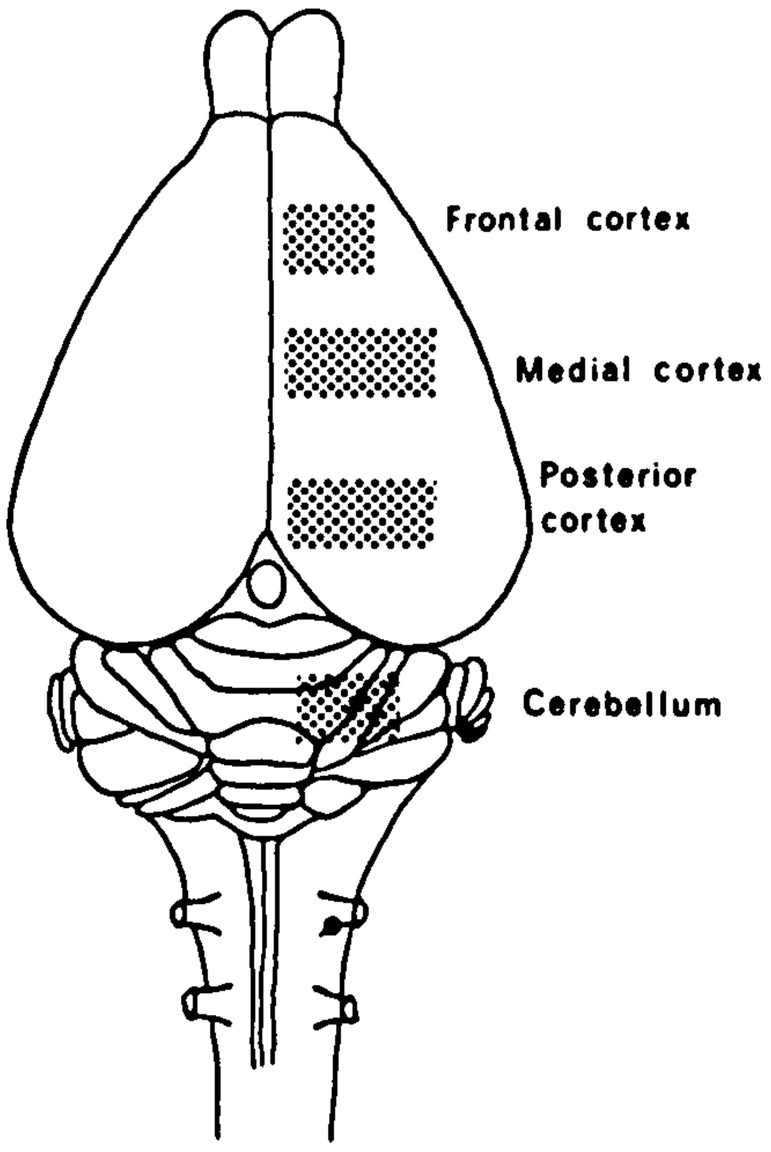
2) interesting cholinergic properties : with the limitation of the considerations given in 1) the regions were chosen on the basis of either interesting cholinergic input or properties, several of the regions being situated on the ascending cholinergic reticular activating system (Shute and Lewis 1967).

All the animals were killed, with a minimum of trauma, by stunning and immediate exsanguination from the carotid arteries. The brains were rapidly dissected (1 min.) and immediately frozen by placing them into petri - dishes embedded in powdered dry - ice. The brains were then partially thawed to allow dissection of the tissue samples; approximately 2 - 5 mg. wet weight was taken from each of the twelve regions (see Figure 6) by hand dissection using a Swann - Morton no. 11 scalpel blade. The dissected samples were then re - frozen on dry - ice until homogenates were made; 2,000 r.p.m. for 1 minute using a Voss vertical homogeniser fitted with a teflon pestle in a glass homogenising tube containing 2.0 ml. $0^{o}C$. pH 8.2 sodium barbital buffer. These samples were then stored at $+4^{o}C$. for approximately 2 hours until coding, randomisation and assay for acetylcholinesterase activity.

b) automated assay procedure for acetylcholinesterase activity using the Technicon Auto - analyser 2.

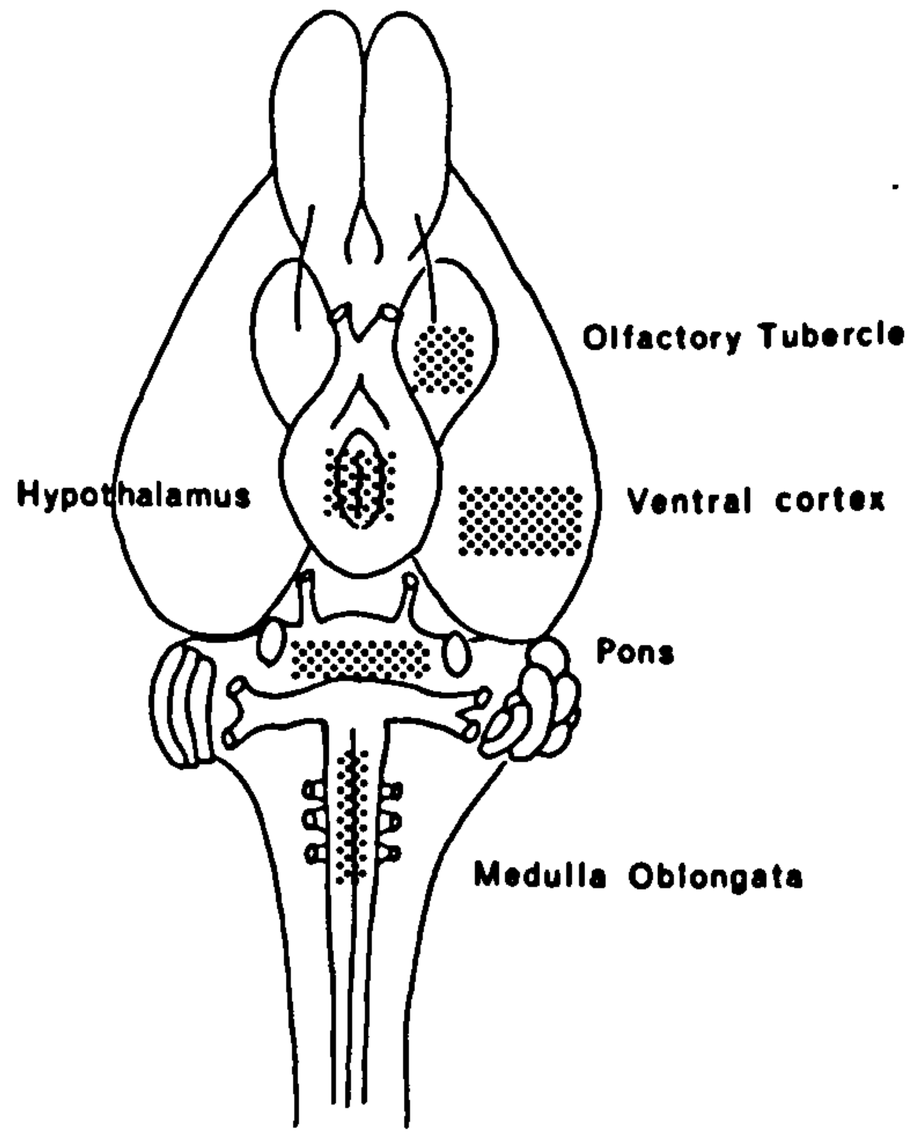
It was decided to seek an automated assay procedure for the enzyme assay in view of the time - factor inherent in the large number of measurements involved and, also, to reduce experimental errors to a minimum.

An automated procedure for the assay of acetylcholinesterase, using a Technicon Auto - analyser 1 was available (Gage and Litchfield 1966): but experience with this method, which uses the physiological substrate, acetylcholine, and measures the decolourisation of a phenol - red solution due to the liberation of acetic acid by the reaction, indicated that the method was relatively insensitive and unreliable.



RAT BRAIN ; DORSAL VIEW

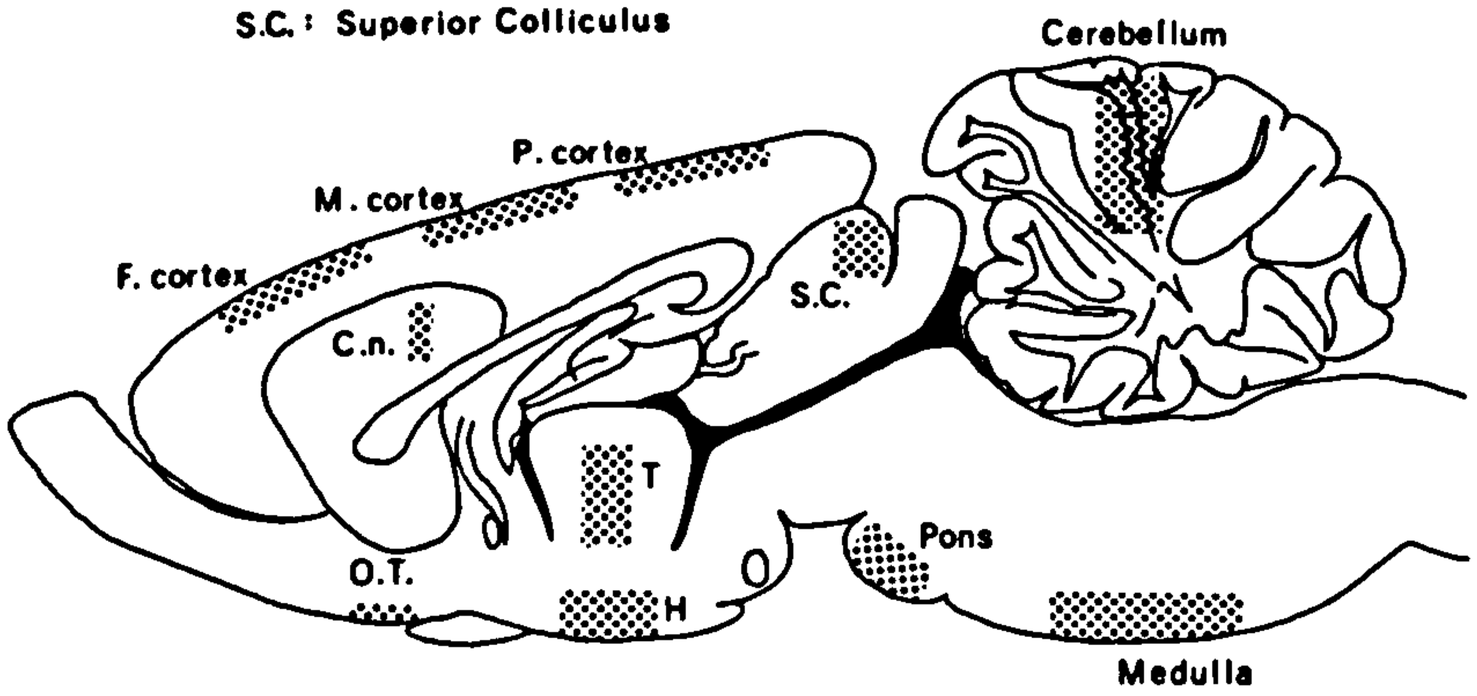
to illustrate positions of samples



RAT BRAIN ; VENTRAL VIEW

to illustrate positions of samples

Key to abbreviations ; H : Hypothalamus
O.T. : Olfactory Tubercle
C.n. : Caudate nucleus
T : Thalamus
S.C. : Superior Colliculus



RAT BRAIN ; MEDIAL SAGITTAL SECTION

to illustrate positions of samples

FIGURE 7. Diagrams to indicate the localisation of the tissue samples taken from the brain regions used in this study.

The diagrams show the dorsal, ventral and medial saggital views of the rat brain. The stipled areas indicate the localisation of the tissue samples used in this study. Further evidence of the localisation of the tissue samples is available from histological sections and from the electron micrographs presented in appendix A.

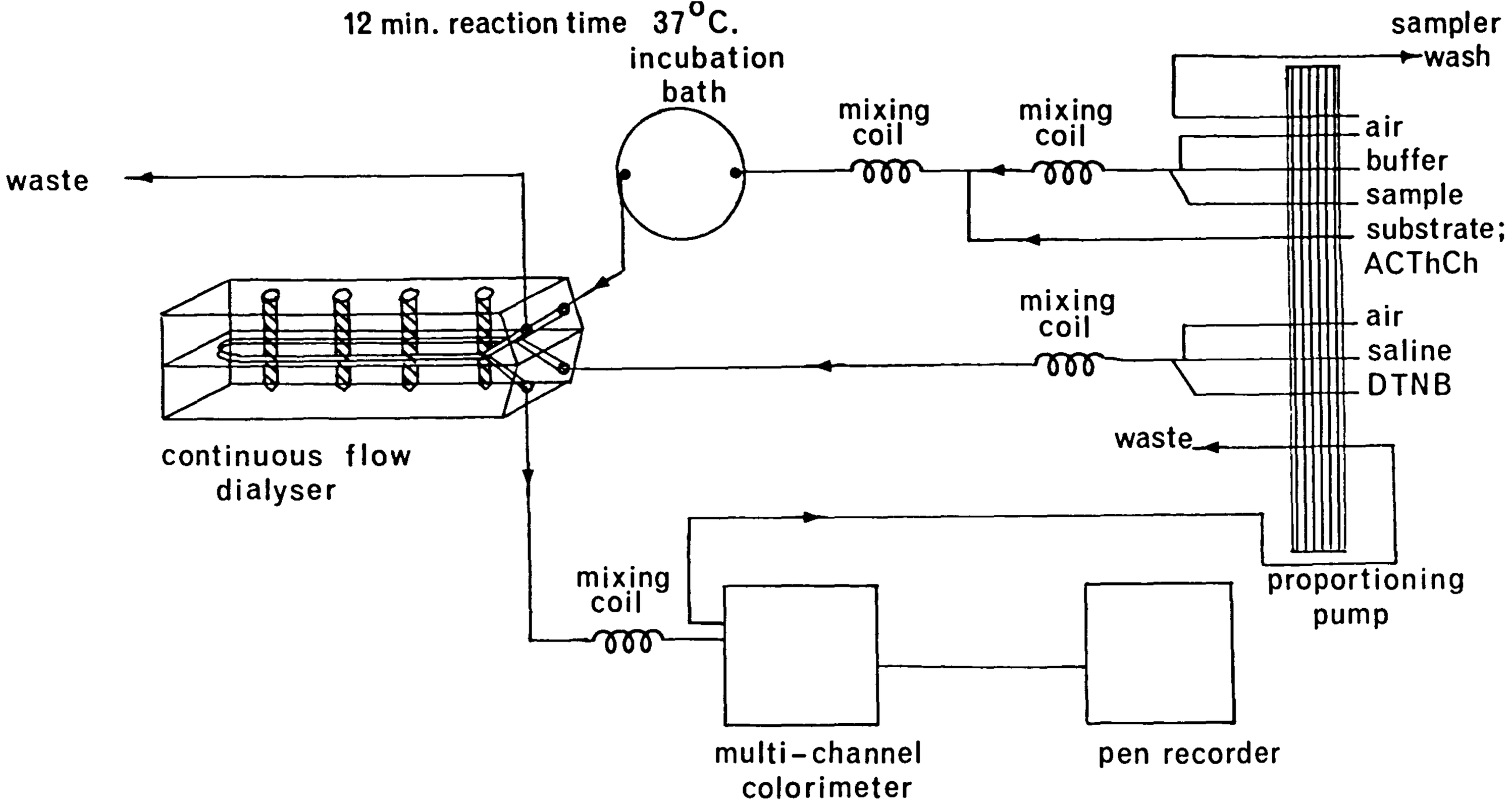
A very sensitive assay procedure for acetylcholinesterase was published by Ellman et al (1961). It was decided to attempt the modification of this assay procedure to the auto - analyser. The standard assay procedure (Ellman et al 1961) was conducted as follows:

5, 5' dithiobis - 2 nitrobenzoic acid ..... 0.10 ml.
Enzyme homogenate ..... 0.10 ml.
Buffer (sodium pH 8.2) ..... to 3.0 ml. volume.
Substrate (acetylthiocholine) ..... 0.25 ml.

For the details of the reagents and buffers please see appendix B.

The solutions listed, with the exception of the substrate, were pipetted into cells of 3.0 ml. capacity and 10 mm. ligh path and allowed to equilibrate to 22°C. for 4 to 5 minutes. The reaction was started by the addition of the substrate. The hydrolysis of the substrate leads to the production of thiocholine and this is estimated by disulphide exchange with 5, 5' dithiobis- (2 nitrobenzoic acid) which yields the highly coloured 5-thio -2 nitrobenzoate ion. The increase in extinction at 412 nm. was monitored, continuously, using a Pye Unicam SP 1800 spectrophotometer coupled with a Pye Unicam AR 25 linear recorder against a reagent blank from which the substrate was omitted. The initial reaction velocity was taken as the slope of the line over the first 2 minutes of reaction. The rate was linear for at least 12 minutes following the addition of the optimal concentration of the substrate (1.0mM.).

The modification of this method to the auto - analyser required its conversion into a continuous - flow system. A peristaltic - type pump draws the various assay components from their storage bottles. The correct proportioning of the assay mixture is achieved by varying the internal diameter of the pump tubing selected. The enzyme sample is injected, via a probe from an automatic sampler at the rate of one sample per minute, interspersed with a 10 second wash injection from a



FLOW DIAGRAM FOR ACETYLCHOLINESTERASE ASSAY

using Technicon Auto-Analyser 2

FIGURE 8. Diagram of the flow - system used for the assay of acetylcholinesterase activity, using the Technicon Auto - analyser 2.

Details of the reagent compositions and pump tube sizes are given in appendix B. To facilitate presentation only the test channel has been shown. The blank channel, which has been omitted, is identical with the test channel except that, following the injection of the enzyme sample, the flow is led through a heating bath at 95°C. to heat - destroy the enzyme and then through a cooling system to return the temperature to that of the test channel. The time - delay of this operation is matched to that of the test channel so that the substrate is injected into both channels at the same time.

reservoir. The enzyme sample and buffer are mixed intimately via a system of coils and are pushed along a tube system (see Figure 8). Air is injected as a small bubble at 2 second intervals to prevent laminar flow phenomena mixing adjacent samples in the flow system and to ensure that inter - sample washings effectively de - contaminate the tubes. The substrate is then added and mixed intimately to initiate the reaction. The enzyme incubation mixture is then pumped into a 10 minute delay - coil, which is surrounded by an oil - filled heating bath, kept at 37°C. The mixture emerges from the heating bath and is led into a continuous - flow dialysis system. This is a Perspex block with a 12" U - shaped groove cut into upper and lower halves of the block. The two blocks fit together with screws, tightened by a 25 lb. torsion wrench and are separated from each other by a cellophane dialysing membrane. This arrangement means that two streams, the donor and the acceptor, flow over each other and are only separated by the membrane. From the top stream (the enzyme incubation) the products of the reaction (thiocholine) dialyse into the bottom stream which contains the colour - reagent (DTNB). The dialysis system is only 15% effective in the transfer of product to the acceptor stream and this is a serious limitation of this automated method since a large reduction in sensitivity is inherent. The colour, so produced, in the acceptor stream is mixed once again and led to a continuous - flow colourimeter where the extinction at 420 nm. is measured. The wavelength is selected by use of an interference filter. The optical density is recorded as a series of peaks, which can be read off against a calibrating set of reduced glutathione standard solutions (0 - 200µg./ml.). The standard curve is shown in Figure 8.

The flow - system (Figure 8) was the result of testing with numerous permutations of reagent volumes and concentrations to achieve good hydro - dynamic properties, this being an important factor in the reliable

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

The system was tested with reduced glutathione standards (0 -200µg./ml.) and a standard curve obtained (see Figure 9). The reproducibility of the method was found to be within 2% (see Figure 12).

2 . 2. 9. Total proteins assay.

a) preparation of the samples.

The remainder of the homogenate from the acetylcholinesterase assay (2.2.8.) was stored at -5°C. for approximately 18 hours before they were thawed and re - homogenised. 0.1 ml. aliquots of the homogenate were taken, in duplicate, and made up to a final volume of 1.0 ml. with protein solubilising solution (see appendix B). These samples were then coded, randomised and taken for assay of total proteins.

b) automated assay procedure for total proteins using the Technicon Auto - analyser 2.

A sensitive colorimetric assay procedure for total proteins was published by Lowry et al (1951). This assay procedure was adapted to the auto-analyser.

The standard assay procedure was conducted as follows:

The diluted sample, containing 0 - 100µg./ml. proteins, was pipetted into a thoroughly cleaned test - tube. To this was added 5.0 ml. of freshly made sodium - potassium tartrate/copper sulphate reagent (Lowry, D, see appendix B) and the mixture allowed to stand for 10 minutes. Then 0.5 ml. of Folin and Ciocalteaus' phenol reagent (diluted $x\frac{1}{2}$ from the

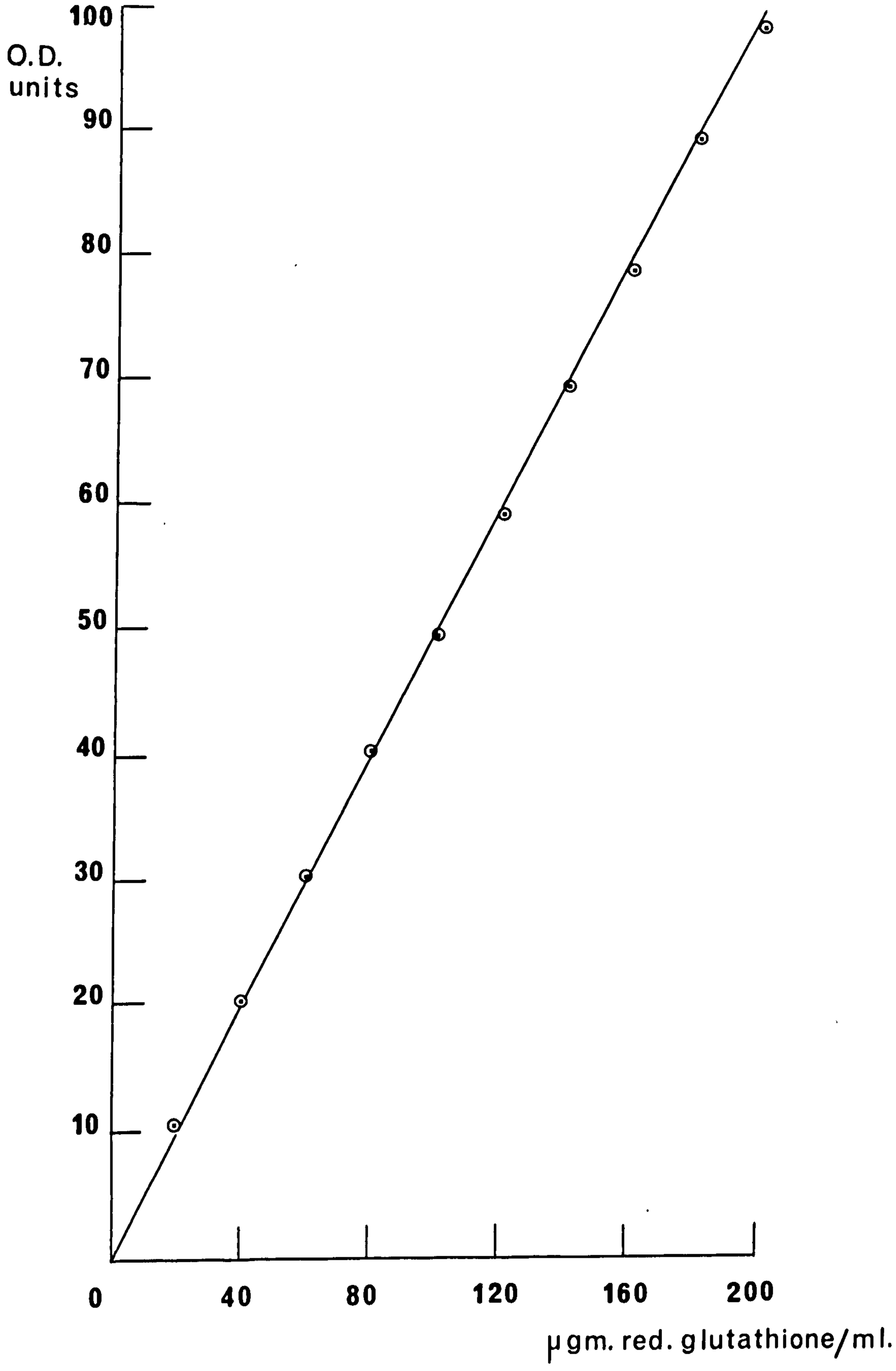
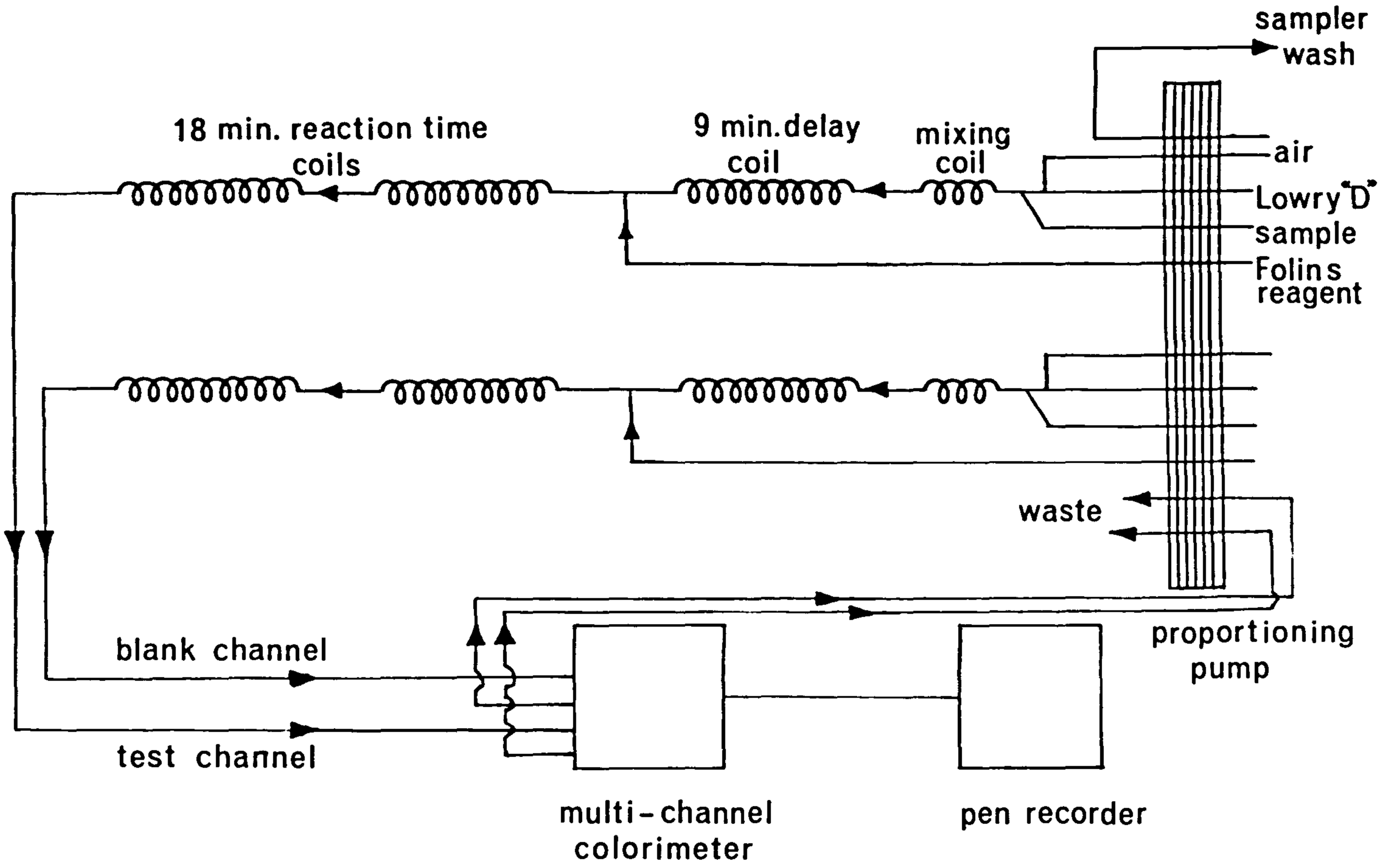

100
O.D.
units
90
80
70
60
50
40
30
20
10
0
40
80
120
160
200
μgm. red. glutathione/ml.

FIGURE 9. Graph to show the standard curve, using reduced glutathione standard solutions (0 - 200 µg./ml.) from the automated assay of acetylcholinesterase activity.

The standard curve is a composite of 15 determinations carried out in duplicate.



FLOW DIAGRAM FOR TOTAL PROTEIN ASSAY

using Technicon Auto- Analyser 2

FIGURE 10. Diagram of the flow - system used for the assay of total proteins, using the Technicon Auto - analyser 2.

Details of the reagent compositions and pump tube sizes are given in appendix B.

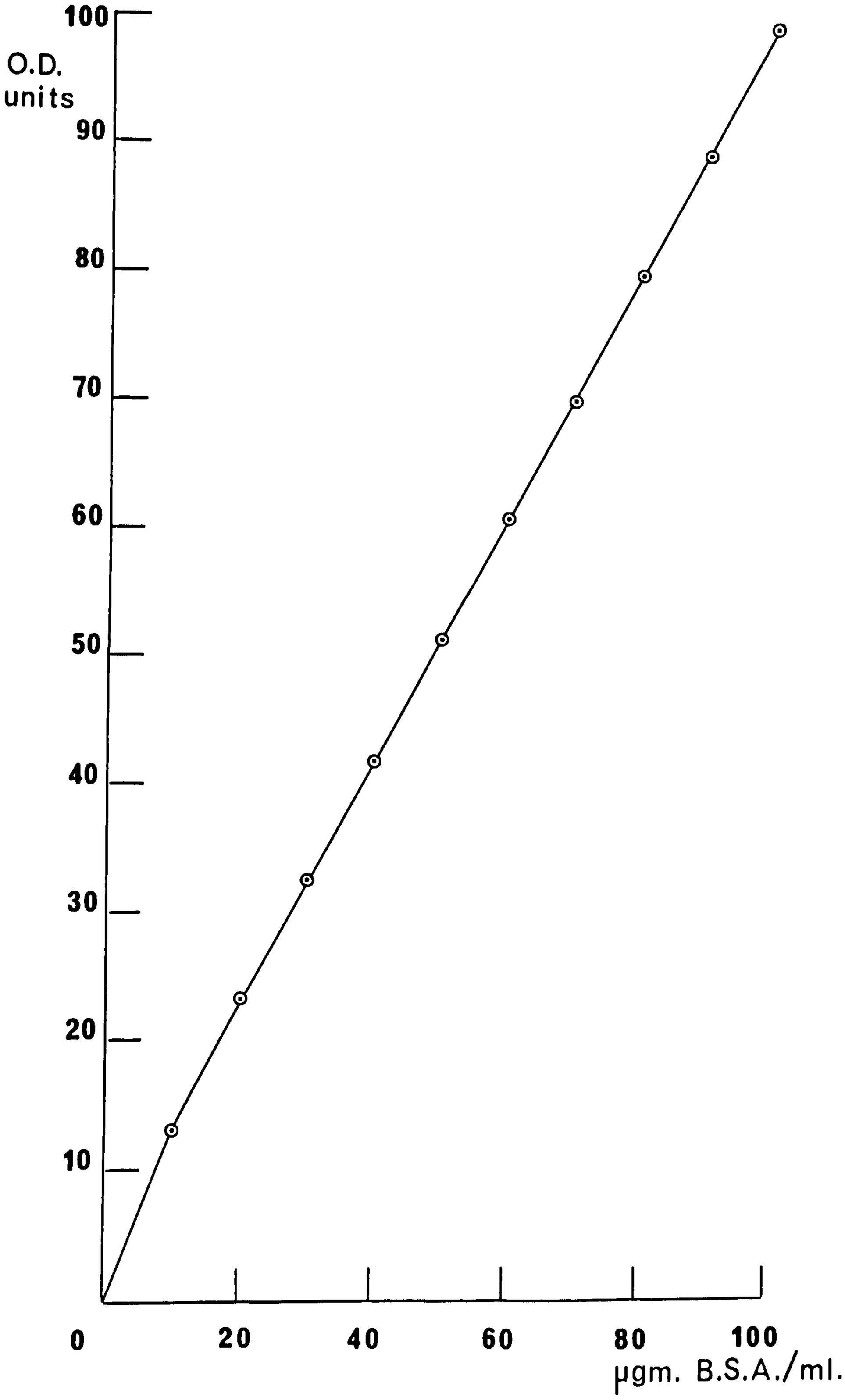

100
O.D.
units
90
80
70
60
50
40
30
20
10
0
20
40
60
80
100
µgm. B.S.A./ml.

FIGURE 11. Graph to show the standard curve, using Bovine serum albumin standard solutions (0 - 100 µg./ml.) from the automated assay of total proteins.

The standard curve is a composite of 10 determinations carried out in duplicate. The marked curvature of the line at low protein concentrations was observed in all determinations.

Absorbance units

90

80

70

60

50

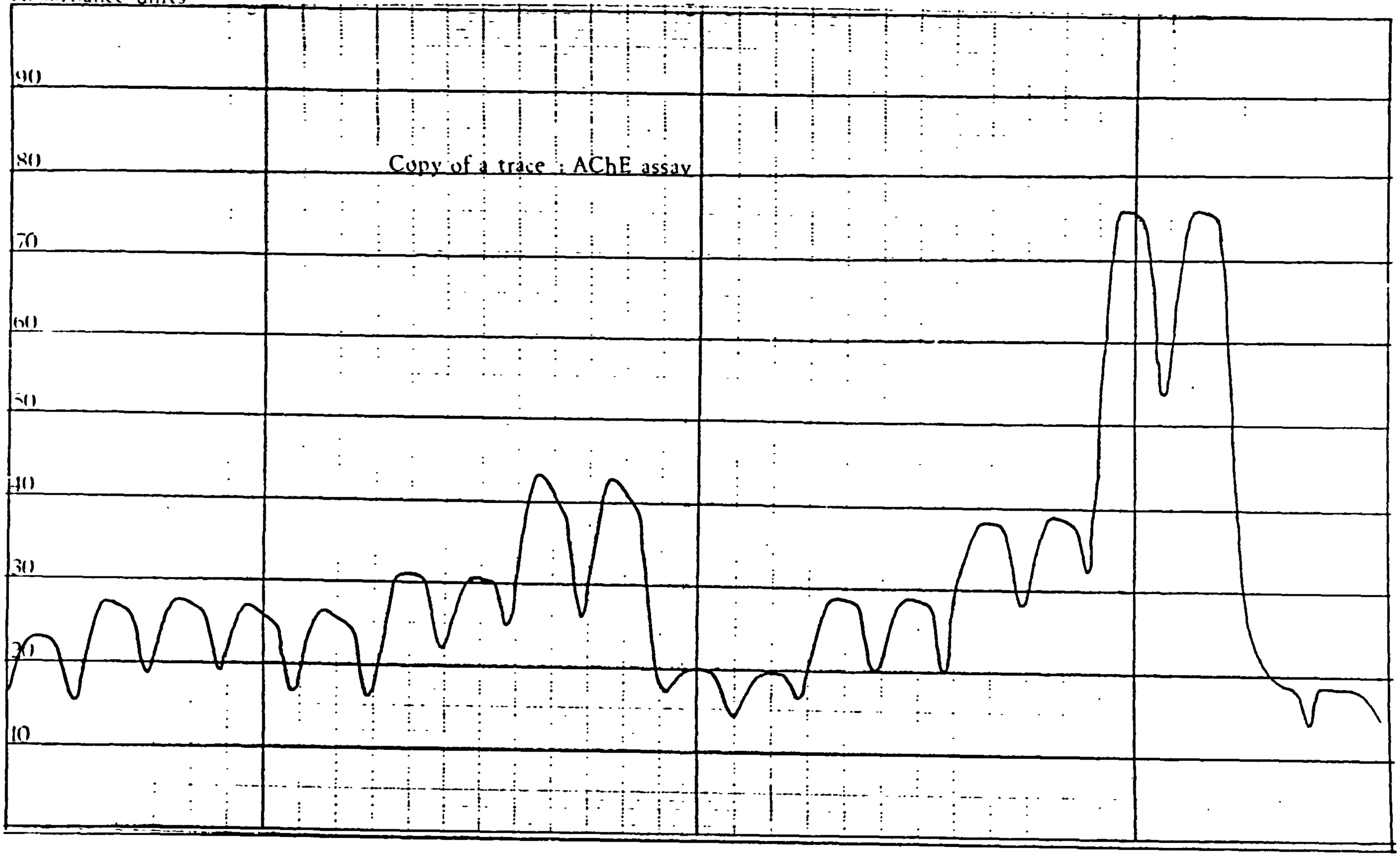
40

30

20

10

Copy of a trace : AChE assay



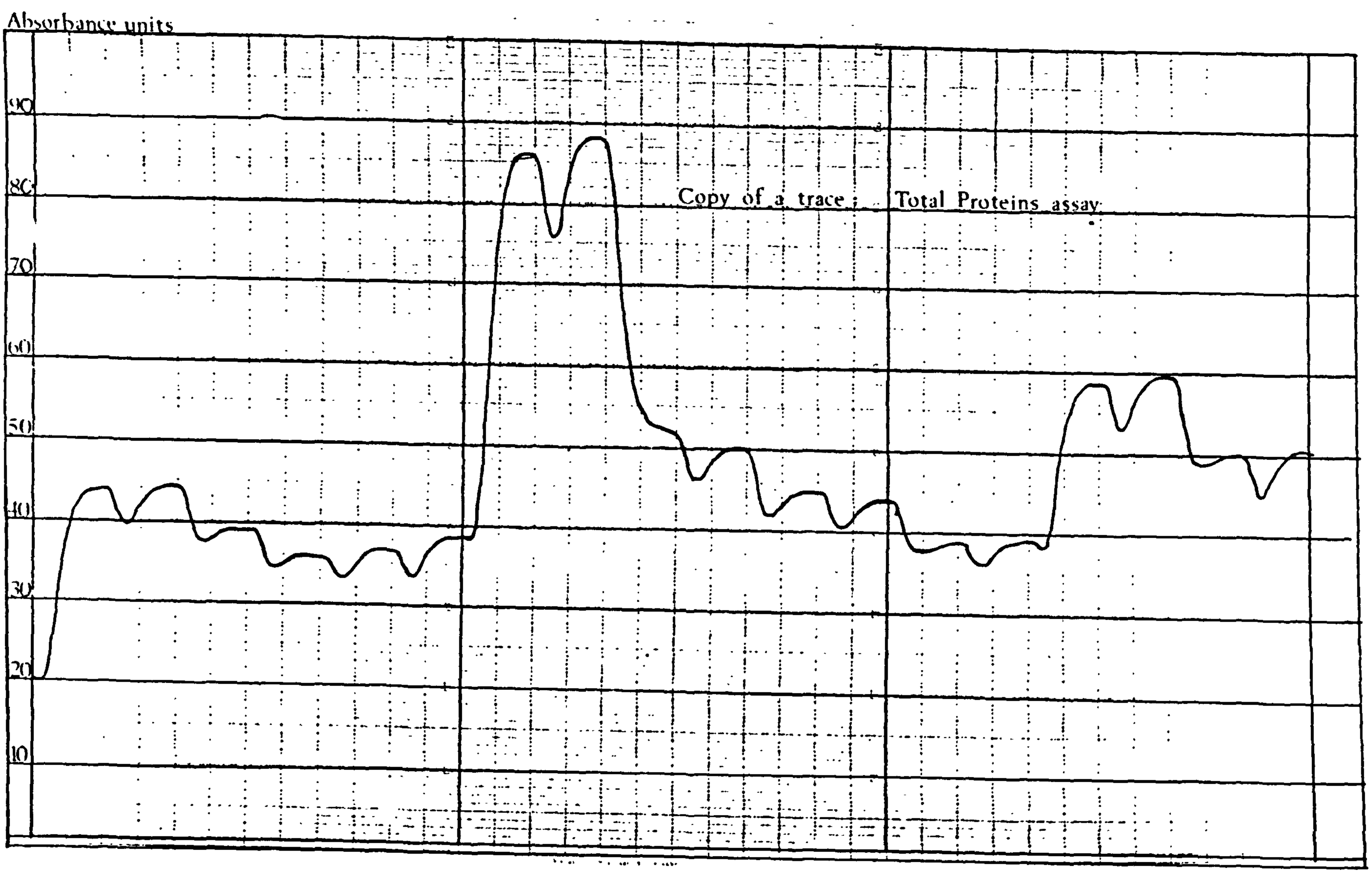

Absorbance units
90
80
70
60
50
40
30
20
10
Copy of a trace ; Total Proteins assay

FIGURE 12. Copies of traces obtained from the automated assay of a) acetylcholinesterase activity and b) total protein concentration.

The traces show the reproducibility between the duplicate samples. The peaks do not return to a zero base - line. This is as a result of the short wash time programmed into the operation of this machine.

commercial stock solution) was added with immediate and thorough mixing using a vortex mixer. After 30 minutes the extinction at 660 nm. was determined. A standard curve was prepared using solutions of Bovine serum albumin Cohn fraction 5 containing 0 - 100 μg./ml.

The modification of this assay to the auto - analyser was more straightforward. The solubilised sample is mixed intimately with the sodium - tartrate/ copper sulphate reagent (modified Lowry D, see appendix B) and pumped into a 10 minute delay - coil (see Figure 10). The diluted Folin and Ciocalteaus' phenol reagent, (see appendix B), is then added and mixed intimately. The colour is allowed to develop for 18 minutes only. This shortening of the colour development period from 30 to 18 minutes is a feature of the operation of the auto - analyser and such measurements are routinely conducted in "steady - state". The colour, developed in the coil system, is read in a continuous - flow colourimeter at 660 nm. The optical density of the solution is recorded as a series of peaks, (see Figure 12), which can be read off as concentrations against a calibrating set of Bovine serum albumin Cohn fraction 5 standard solution (0 - 100μg. /ml. see Figure 11).

A compatible non - phosphate detergent (Levor 4) was used in an 0.2% v/v. solution. The reproducibility between duplicate samples, using this method was usually within 3% (see Figure 12).

2 . 2. 10. Statistical analysis of the data.

Whenever possible, the mean and the standard error of the mean (S.E.M.) were calculated for each group of data. The significance of the difference between the means of control and experimental groups was estimated by Students-t test. Students-t test tested the null hypothesis that an observed difference in a measured parameter had arisen, by chance, due to the random variation inherent in the individual measurements. All the calculations were carried out on a Diehl Combitron

S from the following formulae:

$$S.E.M. = \frac{1}{\sqrt{n}}\sqrt{\frac{\Sigma x^2 - \Sigma x/n}{n-1}}$$

where x = individual data in a group

and n = number of observations.

$$S.E.\ diff = \sqrt{\frac{(n_a\,(n_a-1)\,S.E._a{}^2 - n_b\,(n_b-1)\,S.E._b{}^2\,(n_a-n_b)}{n_a \cdot n_b\,(n_a+n_b-2)}}$$

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.

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

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 the tables of Fisher and Yates (1953).

* * * *

SECTION THREE

3 . 1. THE PROPERTIES OF RAT BRAIN ACETYLCHOLINESTERASE.

A number of experiments investigating the properties of rat brain acetylcholinesterase were conducted in order to assess the optimal conditions for and the reliability of the assay procedures.

3 . 1. 1. pH optimum.

The pH optimum was determined using the colourimetric method of Ellman et al (1961) (see 2.2.8.). The rate of hydrolysis of 1.0 mM. acetylthiocholine was determined, in duplicate, in a range of 0.05M sodium barbital buffers from pH 6.8 - 9.0, spaced at approximately 0.4 pH unit intervals. The assays were conducted at an equilibrated temperature of 22°C. The increase in extinction at 412 n.m. was monitored every 30 seconds for 2.5 minutes in a Pye Unicam S.P. 600 spectrophotometer.

Figure 13 shows the rate of hydrolysis plotted as a function of the pH. The pH optimum was found to be at 8.2. The rate of hydrolysis at pH 7.0 is approximately 50% of the rate at the pH optimum. Above pH 8.5 the rate of hydrolysis begins to fall off and at pH 9.0 the rate is approximately 66% of the rate at pH 8.2. Above pH 8.5, also, there is a marked increase in the blank, indicating an increased non - enzymic hydrolysis of the substrate.

3 . 1. 2. Substrate optimum.

The substrate optimum was determined using the same experimental system as for 3.1.1. but using pH 8.2 sodium barbital buffer (see appendix B). A range of substrate concentrations from 0.5 - 3.0 mM. was used to determine the rates of hydrolysis in duplicate at each substrate concentration. Figure 14 shows the rate of hydrolysis of acetylthiocholine plotted as a function of the substrate concentration.

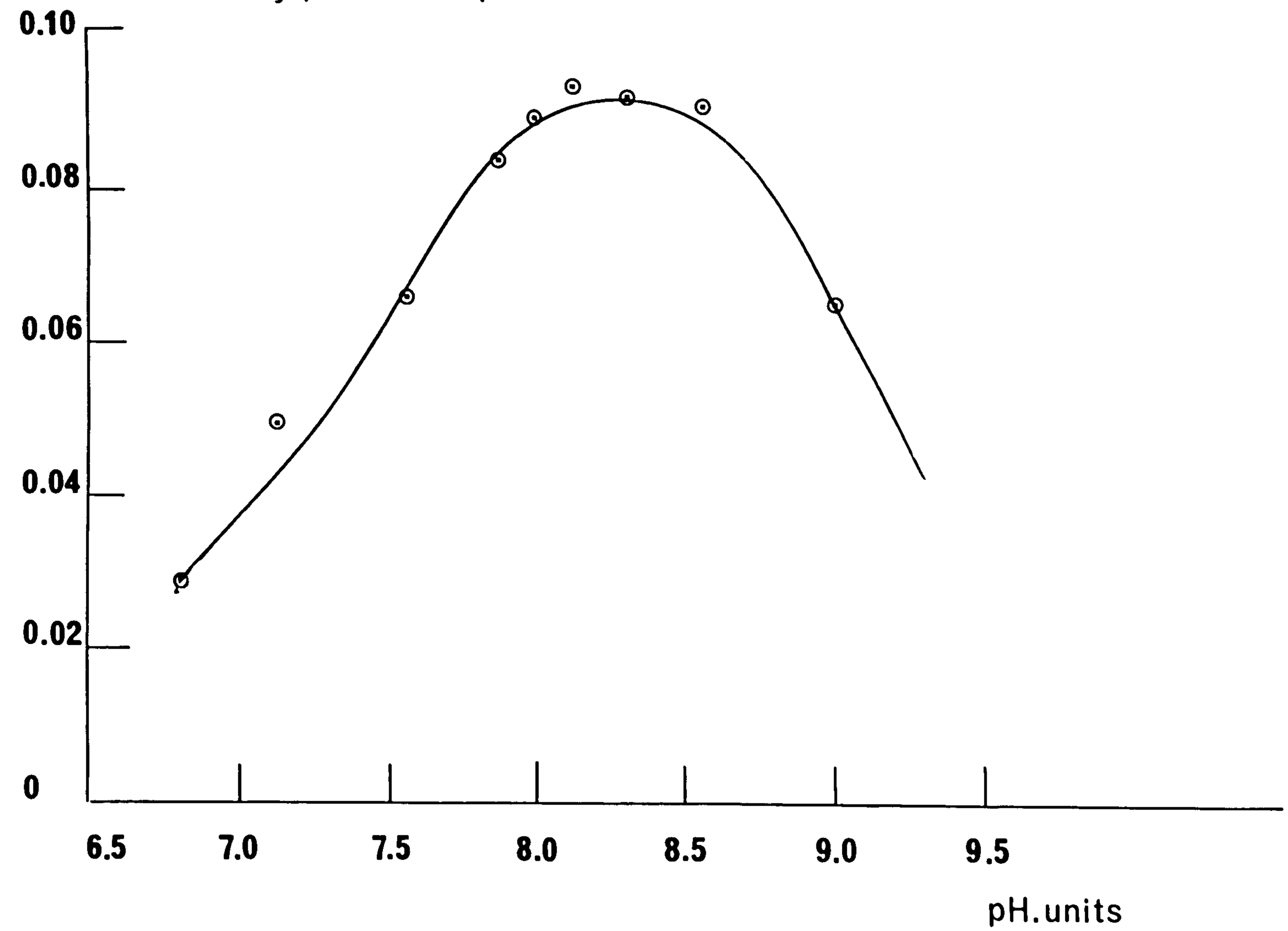

Reaction velocity ; O.D. units/min.
0.10
0.08
0.06
0.04
0.02
0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
pH. units

FIGURE 13. A graph showing the rate of hydrolysis of 1.0 mM. acetylthiocholine substrate plotted as a function of pH over the range 6.8 - 9.0.

The curve is bell-shaped with a peak (pH optimum) at pH 8.2.

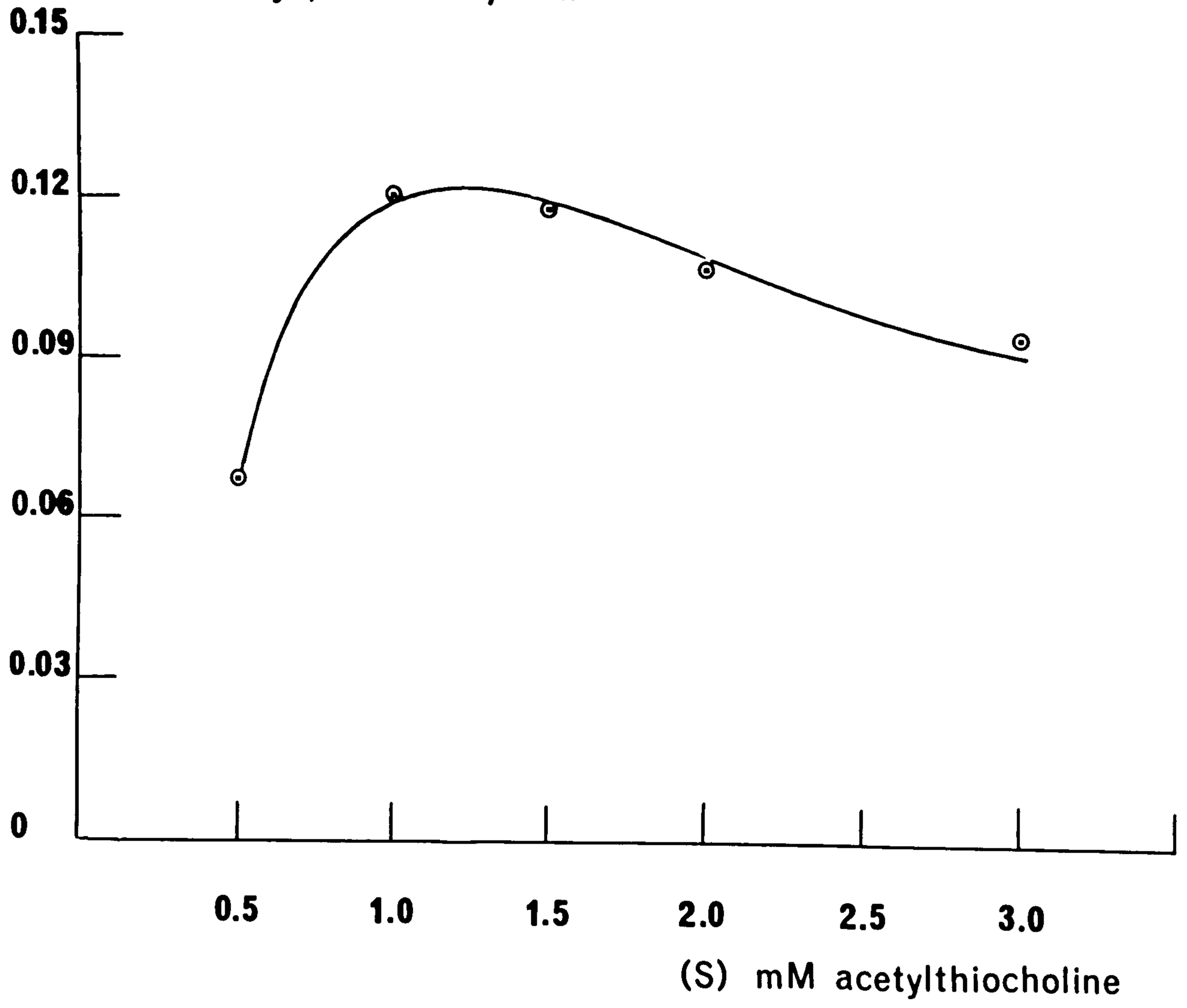

Reaction velocity ; O.D. units/min.
0.15
0.12
0.09
0.06
0.03
0
0.5
1.0
1.5
2.0
2.5
3.0
(S) mM acetylthiocholine

FIGURE 14. <u>A graph showing the rate of hydrolysis of acetylthiocholine plotted as a function of its concentration over the range 0.5 - 3.0mM</u>.

The substrate optimum can be seen to be at about 1.0 mM. The decrease in the rate of hydrolysis above 1.0 mM. is due to substrate inhibition of acetylcholinesterase activity.

The substrate optimum was found to be at about 1.0 mM. Above 1.0mM. there was a decrease in the rate of hydrolysis due to substrate inhibition of enzyme activity. The phenomenon of end - product inhibition of acetylcholinesterase activity was not studied.

3 . 1. 3. <u>The stability of enzyme activity in homogenates</u>.

The activity of some enzymes is known to decay rapidly in homogenates or when treated by other solubilisation procedures. This may be due to sub - unit disruption or aggregation phenomena etc. An experiment was conducted to determine the stability of acetylcholinesterase activity in homogenates.

An homogenate of approximately 3 mg. wet weight rat brain caudate nucleus tissue was made up in 2.0 ml. 0°C. pH 8.2 sodium barbital buffer using a Voss vertical homogeniser at 2,000 r.p.m. for 1 minute. The rate of hydrolysis of 1.0 mM acetylthiocholine was determined, in triplicate, at 10 minutes, 2 hours, 6 hours and 24 hours of storage at +4°C. Figure 15 shows the time - decay relationship for the enzyme activity in the homogenates.

It can be seen that the enzyme activity is quite stable, losing less than 4% of its activity in 6 hours and only 20% in 24 hours. Chan et al (1972a) and Phillis (1970) have both reported that acetylcholinesterase is a stable enzyme.

3 . 1. 4. <u>Determination of the efficiency of the homogenisation procedure in the solubilisation of enzyme activity from brain region samples</u>.

Since it was possible that different amounts of enzyme activity might be released by the homogenisation of the brain region samples, it was necessary to determine the efficiency of the solubilisation of the enzyme activity.

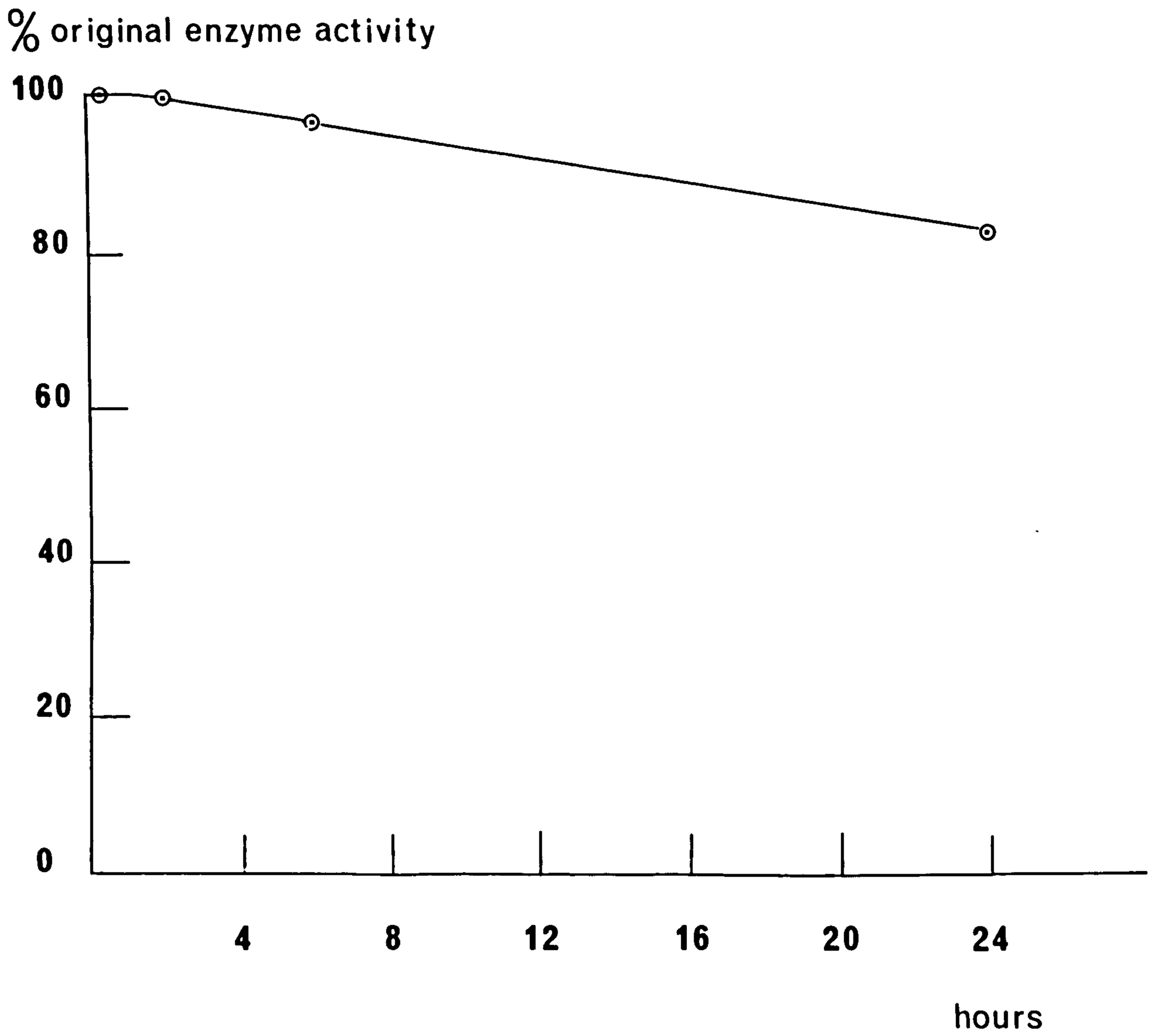

% original enzyme activity
100
80
60
40
20
0
4
8
12
16
20
24
hours

FIGURE 15. A graph to show the decay of enzyme activity in an homogenate of rat brain caudate nucleus tissue plotted as a function of time.

The activity of acetylcholinesterase in homogenates is quite stable, losing less than 4% of its activity in 6 hours of storage at $+4^{o}C$.

In order to test this, homogenates of approximately 3 mg. wet weight rat.brain caudate nucleus were made up in 2.0 ml. 0°C. pH 8.2 sodium barbital buffer using a Voss vertical homogeniser at 2,000 r.p.m. for 1 minute. Aliquots were then taken to determine the rate of hydrolysis of 1.0 mM acetylthiocholine substrate.

0.1% Lubrol X detergent was then added to the homogenate and the mixture re - homogenised at 2,000 r.p.m. for a further minute. It was found that the standard homogenisation procedure was releasing a constant fraction (85%) of the available enzyme activity in the fresh tissue.

3 . 1. 5. Investigations into the contribution to the observed enzyme activity by pseudo - cholinesterase.

Since pseudo-cholinesterase will also hydrolyse acetylthiocholine, although it is not its maximal substrate, the activity of this enzyme will also contribute to the enzyme activity observed using the method of Ellman et al (1961). It was, thus, important to determine the activity of this enzyme in the brain region homogenates.

In order to test this, homogenates of various brain regions (dorsal cortex, medulla, thalamus and caudate nucleus) were made up. Aliquots (0.1 ml.) of the homogenates were incubated with 1.0 mM. acetylthiocholine as substrate and the rate of hydrolysis, in duplicate, was determined. Aliquots (0.1 ml.) were also incubated with 1.0mM. butyrylthiocholine as substrate and the rate of hydrolysis again tested in duplicate. In all the brain regions tested the pseudo - cholinesterase activity was between 4% - 6% of the activity of acetylcholinesterase in terms of mμMoles of substrate hydrolysed per minute. This value agrees with those reported by Bennett et al (1964), Blume et al (1970), Vernadakis and Rutledge (1973) and Hiller and Simon (1973).

It is, thus, apparent that any change in enzyme activity observed by this method must reflect, predominantly, changes in the activity of acetylcholinesterase.

3 . 1. 6. Investigations into the linearity of the initial rate.

Since the auto - analyser method uses an 11 minute 20 second incubation period it was necessary to determine that the initial rate remained linear throughout this period for all samples. It was possible that, with high activity samples, substrate exhaustion might cause non - linearities in its hydrolysis.

In order to test this an homogenate of approximately 5.0 mg. wet weight of caudate nucleus tissue was made up in 2.0 ml. pH 8.2 sodium barbital buffer. The activity of the homogenate being, at least, twice the maximum value for any sample in auto - analysis. Using 1.0 mM acetylthiocholine as substrate the linearity of the initial rate was found to be maintained for, at least, 12 minutes.

3 . 1. 7. Investigations into protein stability during storage.

The total protein assay is an integral part of the determination of acetylcholinesterase activity. It was decided to conduct, as a corollary to the experiments described in 3.1.3., an investigation to determine whether storage of an homogenate at -5°C. for 18 hours could cause any alteration in the measured total protein concentration. A standard total protein assay using the method of Lowry et al (1951) and as described in 2.2.9. was conducted on the enzyme homogenates from the rat brain caudate nucleus at 1 hour and 24 hours of storage. No differences in the total protein concentration were observed. It was concluded that the protein concentration of the homogenates was quite stable during this period of storage.

* * * *

3 . 2. <u>The use of cortical spreading depression to provide a functional unilateral decortication in rats</u>.

A major technical difficulty in the search for inter - relations between the central cholinergic system and behaviour has been the necessity of using groups of animals to compare the effects of differential behavioural treatments. It would be obviously advantageous if a method could be used where each experimental animal also acted as its own control. This could be achieved via surgical methods which both lateralise the sensory input and section the corpus callosum and the commissural pathways so that one hemisphere, only, receives sensory information. However, a large amount of damage is caused to the nervous system by such procedures and the process is, of course, irreversible, rendering the technique of limited value for behavioural studies.

The discovery that this state could be achieved, reversibly, and without gross surgical procedure thus offered exciting possibilities for behaviour research. In a series of papers (Bures, 1959, Bures and Buresova 1960a, 1960b and Travis and Sparks 1963) it was reported that the local application, to the exposed cortical surface of rats, of a saturated (25%) solution of potassium chloride elicited a spreading depression of neuronal activity in the cortex of the treated hemisphere, so producing a functional unilateral decortication. The acquisition of different types of avoidance responding were not affected in the untreated hemisphere and ~~that~~ there was no transfer of the response to the treated hemisphere. This was confirmed by Russell and Ochs (1963) using an operant conditioning paradigm (bar pressing by rats for a food reward). In a later paper, however, (Bures et al 1964) it was reported that some inter - hemispheric transfer of a passive avoidance response occurred, raising the question of the neuro - physiological basis of this difference.

Krivanek and Bures (1968) reported that the cortical spreading

depression procedure, itself, produced changes in acetylcholinesterase activity. Increases in enzyme activity (referred to protein content) of 16% and 30% were reported after 4 and 6 hours respectively after maintaining the depression at maximum. They also reported changes in the protein content (referred to sample volume) which were even more pronounced - 24% in 4 hours. In a further investigation (Krivanek 1969) the changes in protein content were confirmed. It was also reported that the incorporation of ^{14}C leucine into cerebral cortex protein was inhibited by 33% after 6 hours and that the potassium and water content of the treated hemisphere increased. Similar changes in the ipsilateral sub - cortex were also reported but ~~that~~ no changes were observed in the untreated cortex. The changes in potassium and water content were reversible; potassium levels recovering in 24 hours while water content recovered in 13 days.

Buresova and Bures (1969) studied learning and memory impairment evoked by prolonged cortical spreading depression in rats. They reported impairment in the acquisition of an avoidance response for 3 - 5 days following the spreading depression, normal functioning being observed after 6 - 7 days. Since the spontaneous E.E.G. and evoked responses were found to be normal after 24 hours they concluded that the spreading depression was interfering with the metabolic phase of engram formation.

An experiment was conducted to assess the value of using cortical spreading depression to provide a functional decortication of one hemisphere of the rat. Providing that this could be achieved in a relatively short time period (30 mins. - 1 hour) rats could be trained in the acquisition of an avoidance response and then the activity of acetylcholinesterase in symmetrical regions of opposite hemispheres compared. In this way it might be possible to detect differences in enzyme activity as a result.

A pilot study was conducted to determine:

a) the degree of variability in the resting levels of acetylcholinesterase activity in symmetrically opposite regions of both hemispheres, and b) whether the cortical spreading depression, maintained for a short time - period (approximately 1 hr.), would alter the enzyme activity in the treated hemisphere.

Using the auto - analyser methods for the enzyme activity assay it was found that there were no significant differences in the resting levels of the enzyme activity between the right and left frontal cortex samples and the right and left caudate nucleus samples (see Table 8).

The cortical spreading depression was produced, using the modified method of Bures et al (1964). 110 day old female Wistar albino rats were anaesthetised by administration of 3% concentration of halothane in a 25% oxygen/ 75% nitrous oxide carrier stream, delivered at a rate of 3 litres per minute from a mini - Boyle apparatus. A trephine opening 5 m.m. in diameter was made over the right frontal cortex using a dentist drill. Care was taken not to rupture the dura. Animals with ruptured dura or with signs of brain oedema were discarded. For technical reasons the cortical spreading depression was elicited while the animals were still under anaesthesia, by placing a filter paper (3 x 3 mm.) soaked in 25% potassium chloride onto the exposed cortical surface. After 30 minutes of spreading depression the filter paper was removed and the animals killed. The brains were dissected and cut into half longitudinally to divide right and left halves. Right and left frontal cortex and caudate nucleus samples were then dissected and analysed, in duplicate, using the automated methods for the assay of acetylcholinesterase activity.

In order to interpret the results a control sham procedure was used where the animals were treated in exactly the same manner but filter paper soaked in pyrogen - free pH 7.2 physiological saline was used in place of the potassium chloride.

The results (Table 8) indicated that the spreading depression

treatment caused a slight, but significant, activation of acetylcholinesterase activity, a finding consistent with that reported by Krivanek and Bures (1968). It can be seen, also, that the sham procedure also caused a slight activation of the enzyme activity. These experiments were conducted on a small number of animals and none of the right / left differences observed in any one experimental treatment were significant at the 95% level. It was felt, however, in view of the results obtained, that any results obtained after behavioural treatment might be very difficult to interpret and require the use of control procedures. Since this defeated the object of the experiment, further use of the technique was abandoned.

3 . 3. <u>The effects of the various behavioural treatments on the activity of acetylcholinesterase from regions of the rat brain</u>.

3 . 3. 1. <u>The quiet control distribution of acetylcholinesterase activity</u>.

The quiet control distribution of acetylcholinesterase activity in the 12 brain regions studied is shown in Table 9 and Figure 16. The regions have been listed in order of increasing enzyme activity to indicate the rank order of distribution of activity. The lowest activity was found in the cerebellum; the highest in the caudate nucleus sample. The difference in activity between these two regions was 18 - fold.

As can be seen from Table 9 the standard error of the measurement of acetylcholinesterase activity of the quiet control group for easily accessible brain regions e.g. cerebellum, cortex, is very low, indicating: a) the consistency of the sampling techniques and b) the reliability of the assay procedures.

In the case of tissue samples from regions such as the hypothalmus, thalamus or superior colliculus there is greater opportunity for dissection error since the boundaries are not so well defined. In these cases the magnitude of the standard error is normally between 2 - 3%.

TABLE 8. The effect of cortical spreading depression on the activity of acetylcholinesterase from the cortex and caudate nucleus.

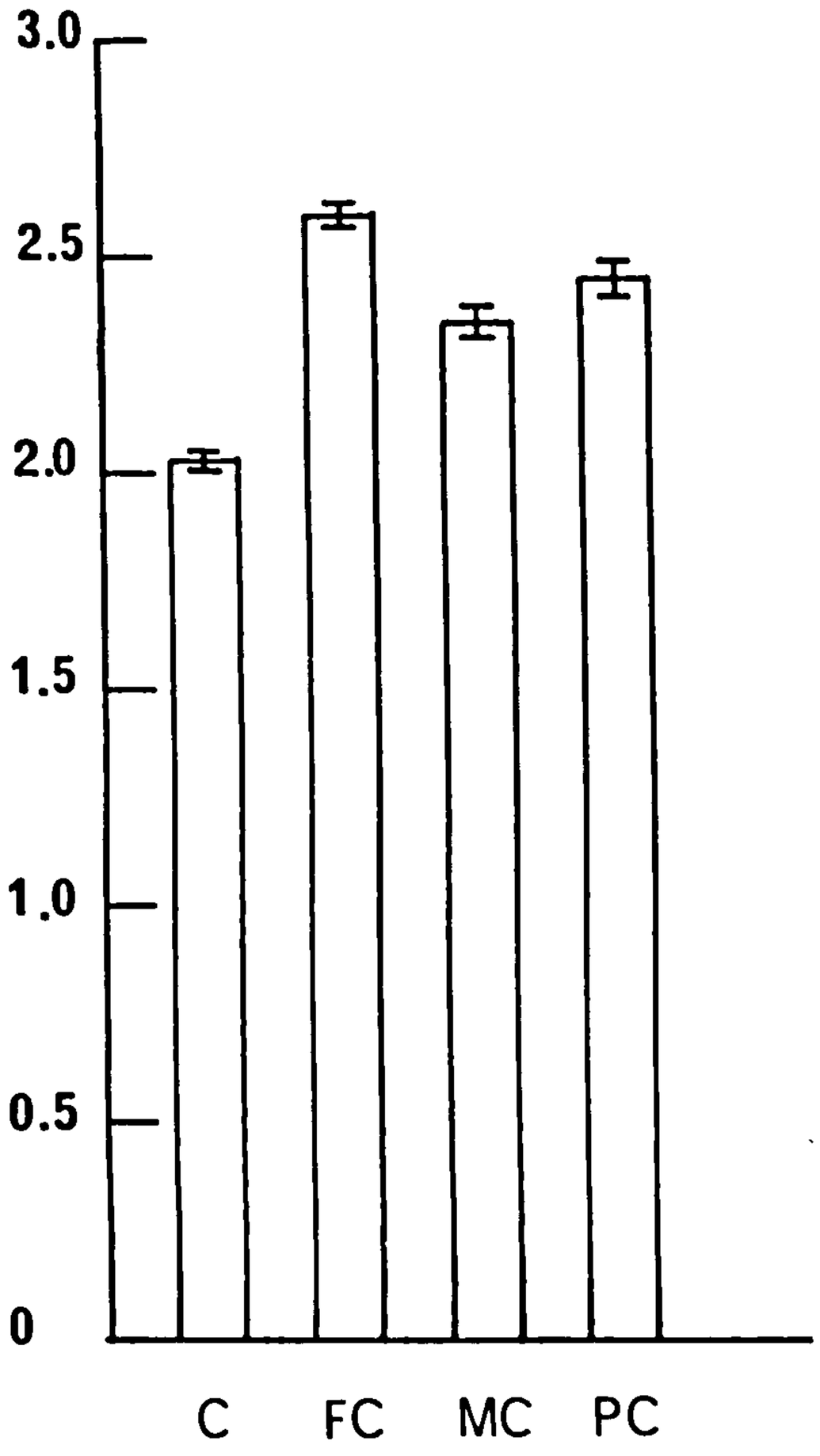
Region	Control	Spreading depression	Sham
Left cortex	2.565 ±0.032	2.659 ±0.047	2.645 ±0.052
n	3	3	3
Right cortex	2.582 ±0.040	2.749 ±0.061	2.669 ±0.058
n	3	3	3
Left caudate	35.232 ±0.691	36.819 ±0.072	36.664 ±0.080
n	3	3	3
Right caudate	36,348 ±0.670	37.727 ±0.083	37,011 ±0.075
n	3	3	3

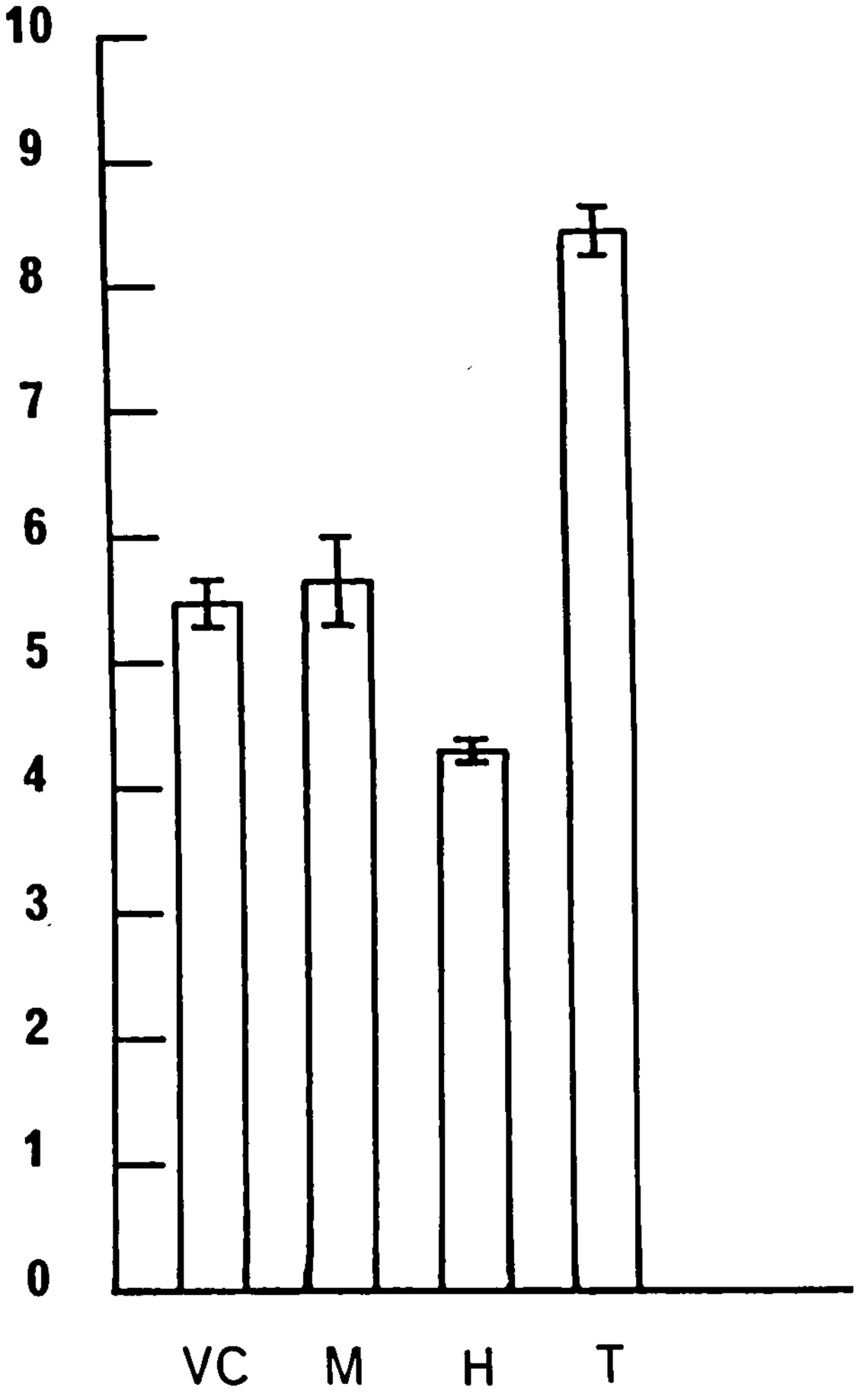
The results are expressed in μMoles acetylthiocholine hydrolysed/ hr./mg.Proteins ± S.E.M. No significant differences in enzyme activity were observed between right and left control samples from either the dorsal frontal cortex or the caudate nucleus. None of the right / left differences in any one experimental treatment were significant at the 95% level. Both the spreading depression and the sham operation caused an activation of the enzyme activity.

TABLE 9. The quiet control distribution of acetylcholinesterase activity.

Region	Mean activity	S.E.M.	n
Cerebellum	2.028	0.021	6
Medial cortex	2.346	0.036	7
Posterior cortex	2.445	0.042	7
Frontal cortex	2.594	0.026	7
Hypothalamus	4.294	0.079	6
Ventral cortex	5.467	0.188	6
Medulla	5.642	0.348	7
Thalamus	8.431	0.197	6
Superior colliculus	12.716	0.362	6
Pons	13.424	0.314	7
Olfactory tubercle	33.553	0.521	8
Caudate nucleus	36.161	0.553	9

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


3.0
2.5
2.0
1.5
1.0
0.5
0
C
FC
MC
PC


10
9
8
7
6
5
4
3
2
1
0
VC
M
H
T

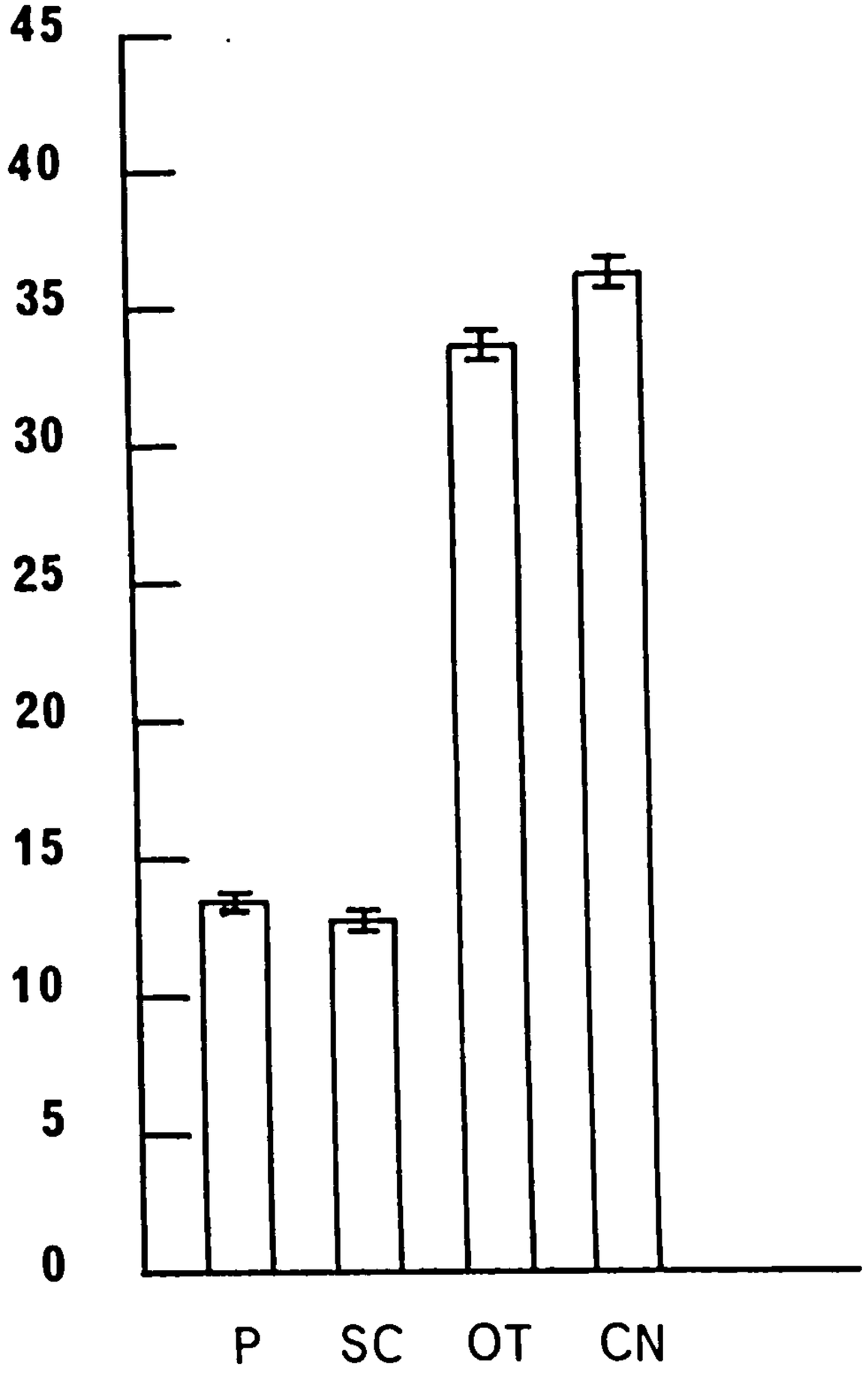

45
40
35
30
25
20
15
10
5
0
P
SC
OT
CN

FIGURE 16. Bar diagram of the quiet control distribution of the activity of acetylcholinesterase in the 12 brain regions.

The brain regions have been divided into 3 groups of activity and thus the scales of the axes are different. The diagrams have been drawn by a Hewlett Packard statistical plotter. The columns represent the means of the number of determinations shown in Table 9. The S.E.M. is represented by the vertical bars.

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

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

3 . 3. 2. <u>The effects of pentobarbitone - sodium anaesthesia on the activity of acetylcholinesterase in 12 regions of the rat brain</u>.

The effects of pentobarbitone - sodium anaesthesia on the activity of acetylcholinesterase in the 12 brain regions is shown in Table 10 and Figure 17.

a) In all 3 dorsal cortical regions, there is a slight, but non - significant, activation of the enzyme activity as compared with the saline - injected control group. An increase in the standard error of the measurement possibly indicates that some differential effect of the anaesthetic is operating in the individual rats comprising the experimental group.

b) No significant differences in enzyme activity were found, as a result of the anaesthesia, in the samples from the olfactory tubercle, hypothalamus, pons or superior colliculus. In all these regions the standard error of the measurement again increased.

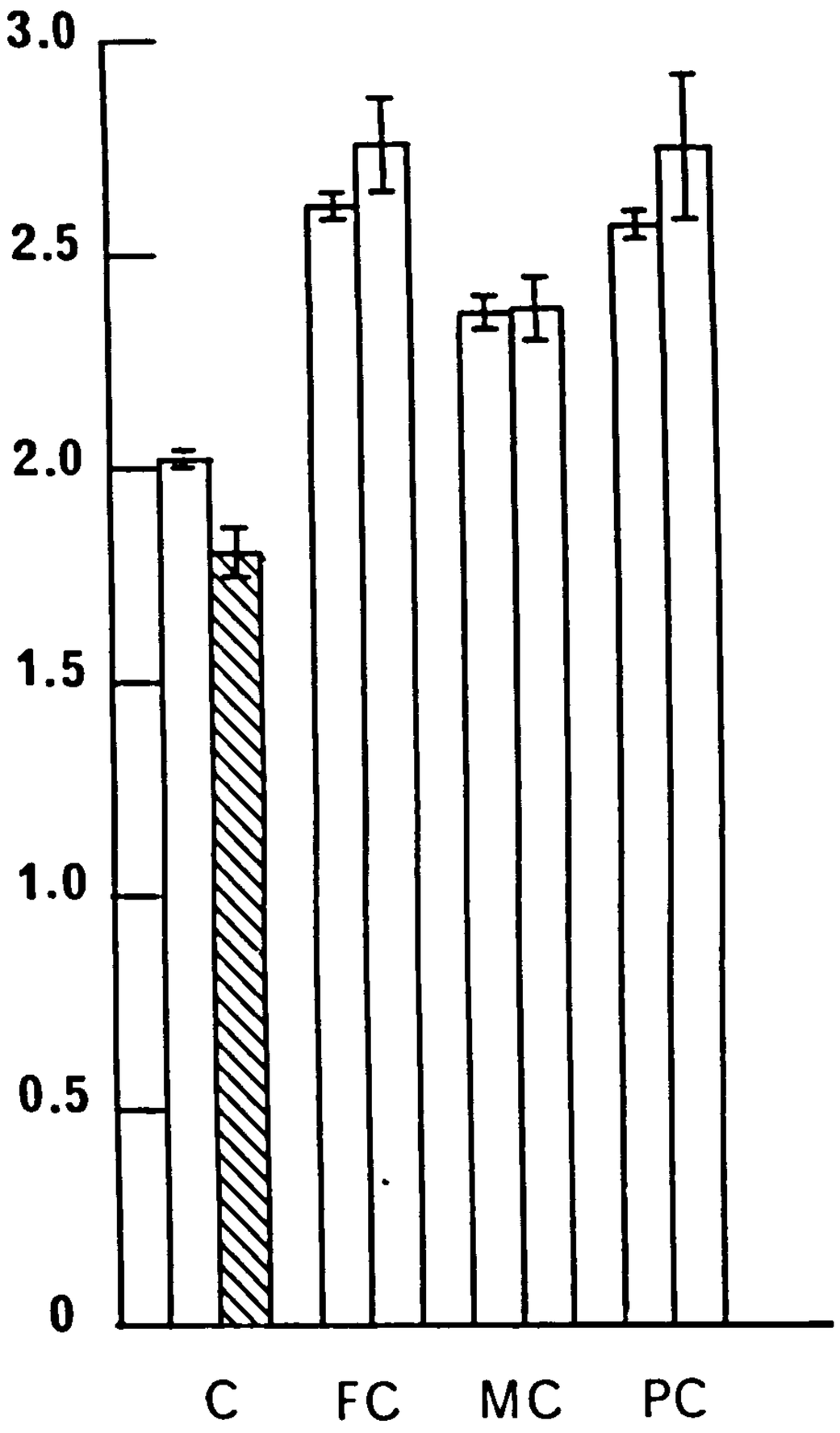
c) Significant decreases in enzyme activity were observed, following anaesthesia, in the cerebellum (10%), ventral cortex (26%), thalamus (39%) and the caudate nucleus (24%) as compared with the saline - injected control group. In the remaining region, the medulla, it can be seen that there was an increase in enzyme activity (38%) following anaesthesia.

d) No significant effect on acetylcholinesterase activity was found, as a result of the saline injection, in any of the brain regions as compared with the quiet control group (Table 9).

TABLE 10. The effects of anaesthesia on acetylcholinesterase activity.

	ANAESTHESIA		CONTROL		
Region	Mean ± S.E.M.	n	Mean ± S.E.M.	n	p
Cerebellum	1.801 0.058	(11)	2.021 0.019	(8)	0.02
Medial cortex	2.365 0.075	(9)	2.357 0.039	(8)	ns
Posterior cortex	2.740 0.168	(8)	2.558 0.033	(8)	ns
Frontal cortex	2.754 0.108	(12)	2.611 0.030	(8)	ns
Hypothalamus	4.038 0.374	(6)	4.312 0.085	(6)	ns
Ventral cortex	4.029 0.286	(6)	5.502 0.158	(6)	0.001
Medulla	7.795 0.156	(6)	5.667 0.199	(7)	0.001
Thalamus	5.153 0.743	(6)	8.306 0.172	(6)	0.001
Sup. colliculus	12.072 0.531	(8)	11.998 0.301	(8)	ns
Pons	12.125 0.672	(8)	12.961 0.330	(8)	ns
Olf. tubercle	33.864 0.728	(6)	33.611 0.585	(6)	ns
Caudate nucleus	27.261 0.854	(13)	36.687 0.512	(8)	0.001

Enzyme activity is expressed as μMoles acetylthiocholine hydrolysed /hr./mg. proteins.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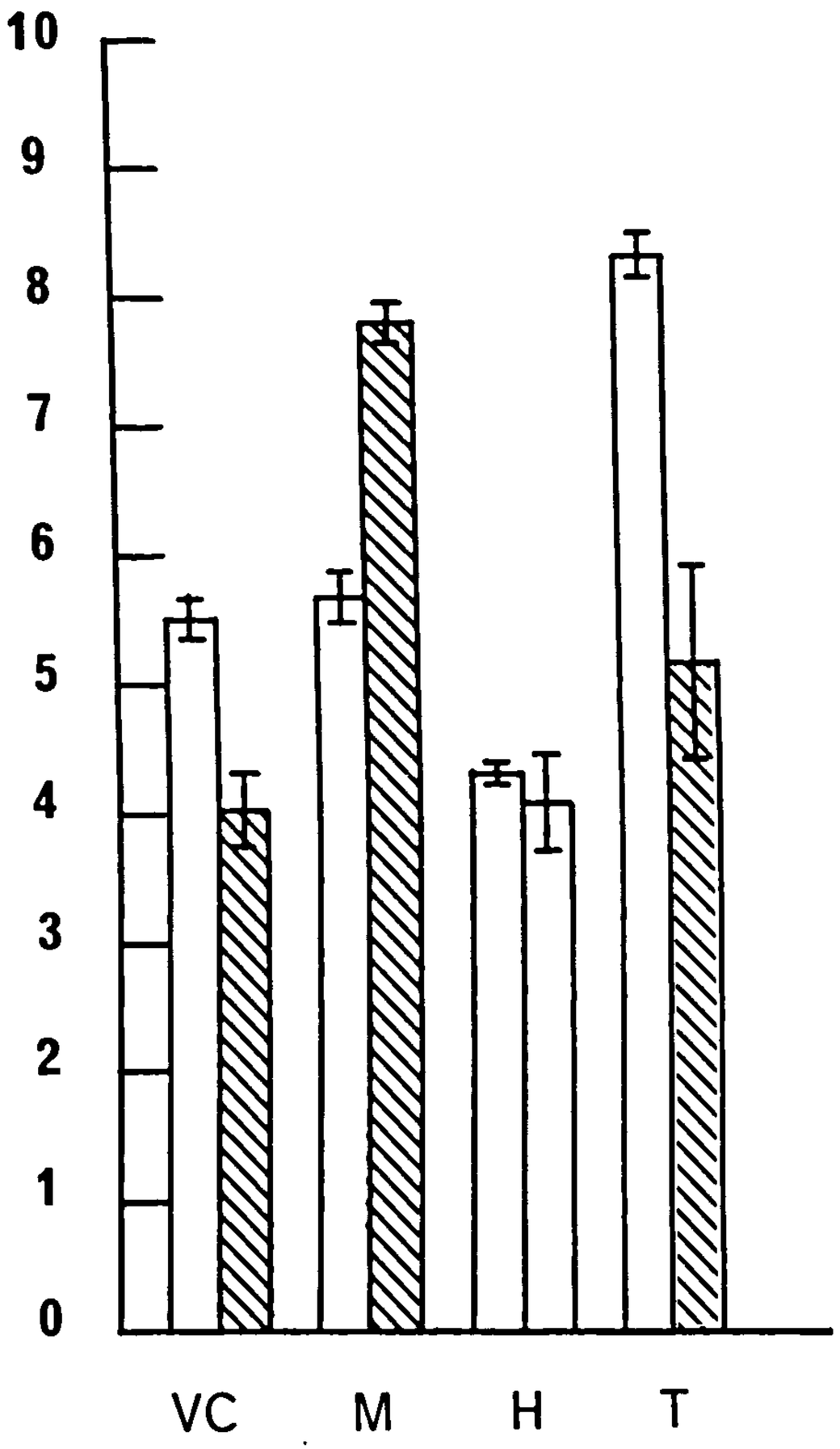
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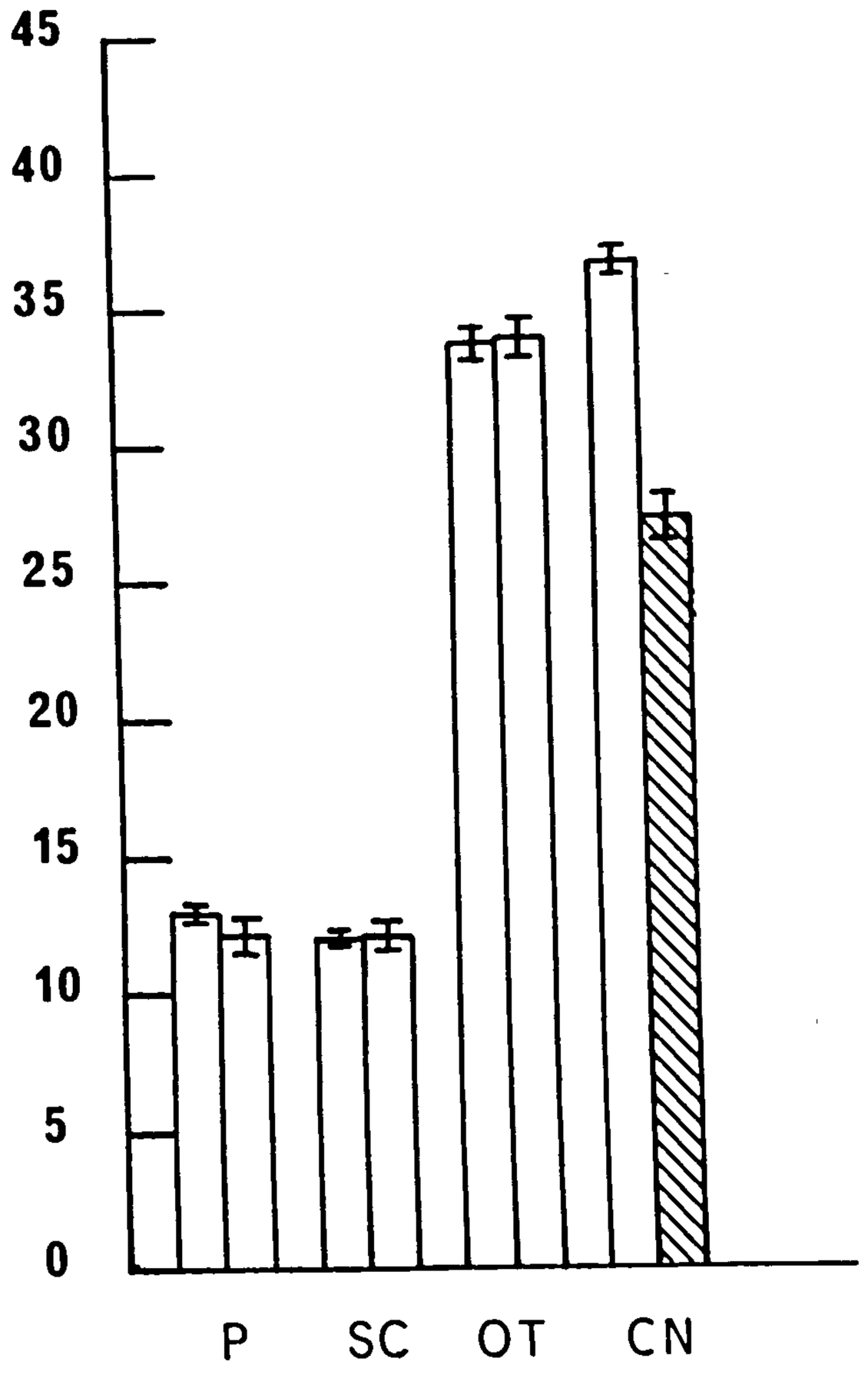
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FIGURE 17. Bar diagram to show the effects of anaesthesia on the activity of acetylcholinesterase in the 12 brain regions.

The brain regions have been divided into 3 groups of activity and thus the scales of the axes are different. The diagrams have been drawn by a Hewlett Packard statistical plotter. The columns represent the means of the number of determinations shown in Table 10. The S.E.M. is represented by the vertical bars. The groups that are significantly different are indicated by shading.

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

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

3 . 3. 3. The effects of pentobarbitone - sodium anaesthesia coupled with non - specific electrical stimulation on the activity of acetylcholinesterase in 12 regions of the rat brain.

The effects of pentobarbitone - sodium anaesthesia coupled with non-specific electrical stimulation on the activity of acetylcholinesterase in the 12 brain regions are shown in Table 11 and Figure 18.

a) In the 3 dorsal cortical regions the treatment produced no significant differences in enzyme activity as compared with the control group. The slight activation of enzyme activity observed following anaesthesia only is, however, not observed in the experimental group following this treatment.

b) No significant differences were found as a result of the treatment in the samples from the thalamus, hypothalamus or cerebellum. In the cerebellum, however, the treatment produced a slight, though non - significant, activation of enzyme activity. This compares with the significant decrease of enzyme activity observed following anaesthesia.

c) The samples from the superior colliculus and the olfactory tubercle were excluded from this study and, hence, no data is available on the effects of this treatment on the enzyme activity of these regions.

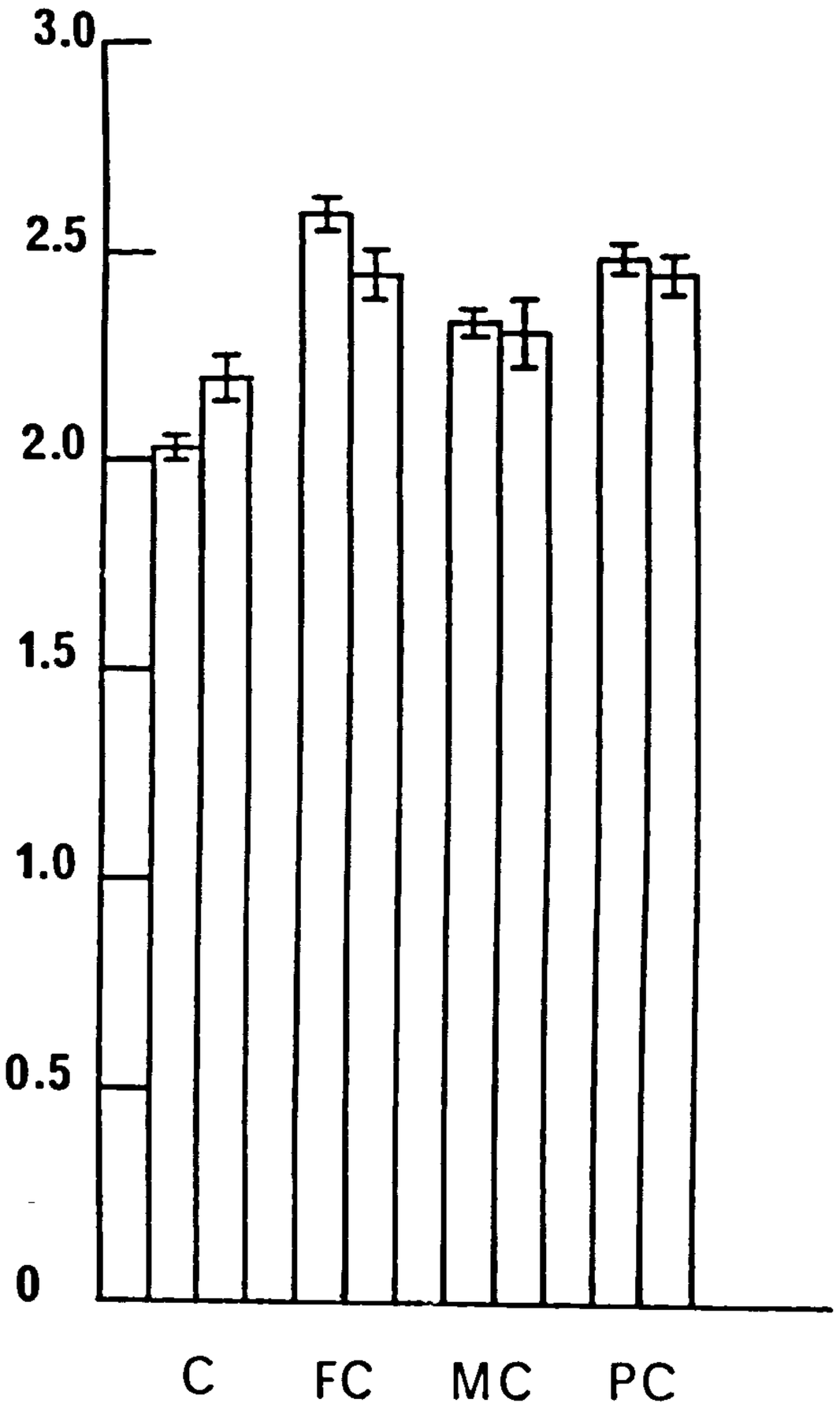
d) Significant decreases in enzyme activity were observed as a result of this treatment in the pons (9%), ventral cortex (20%), medulla (37%) and caudate nucleus (19%). In the ventral cortex and the caudate nucleus the decreases in enzyme activity were not so great as those observed as a result of anaesthesia only.

e) With the exception of the sample from the pons, no significant differences in enzyme activity in any region of the brain was observed as a result of the saline injection, by comparison with the quiet control group.

TABLE 11. The effects of anaesthesia/stimulation on AChE activity.

	ANAESTHESIA/STIM.			CONTROL			
Region	Mean ± S.E.M.		n	Mean ± S.E.M.		n	p
Cerebellum	2.197	0.055	(13)	2.030	0.030	(8)	ns
Medial cortex	2.309	0.082	(6)	2.335	0.032	(6)	ns
Posterior cortex	2.448	0.046	(6)	2.488	0.035	(6)	ns
Frontal cortex	2.449	0.058	(6)	2.593	0.039	(6)	ns
Hypothalamus	4.059	0.087	(8)	4.184	0.063	(7)	ns
Ventral cortex	4.391	0.174	(6)	5.278	0.092	(6)	0.001
Medulla	3.860	0.290	(7)	6.184	0.221	(7)	0.001
Thalamus	8.132	0.155	(6)	8.427	0.285	(6)	ns
Sup. colliculus	no data						
Pons	11.079	0.173	(6)	12.148	0.357	(6)	0.05
Olf. tubercle	no data						
Caudate nucleus	28.467	0.854	(13)	35.107	0.783	(13)	0.001

Enzyme activity is expressed as μMoles acetylthiocholine hydrolysed /hr./mg. proteins.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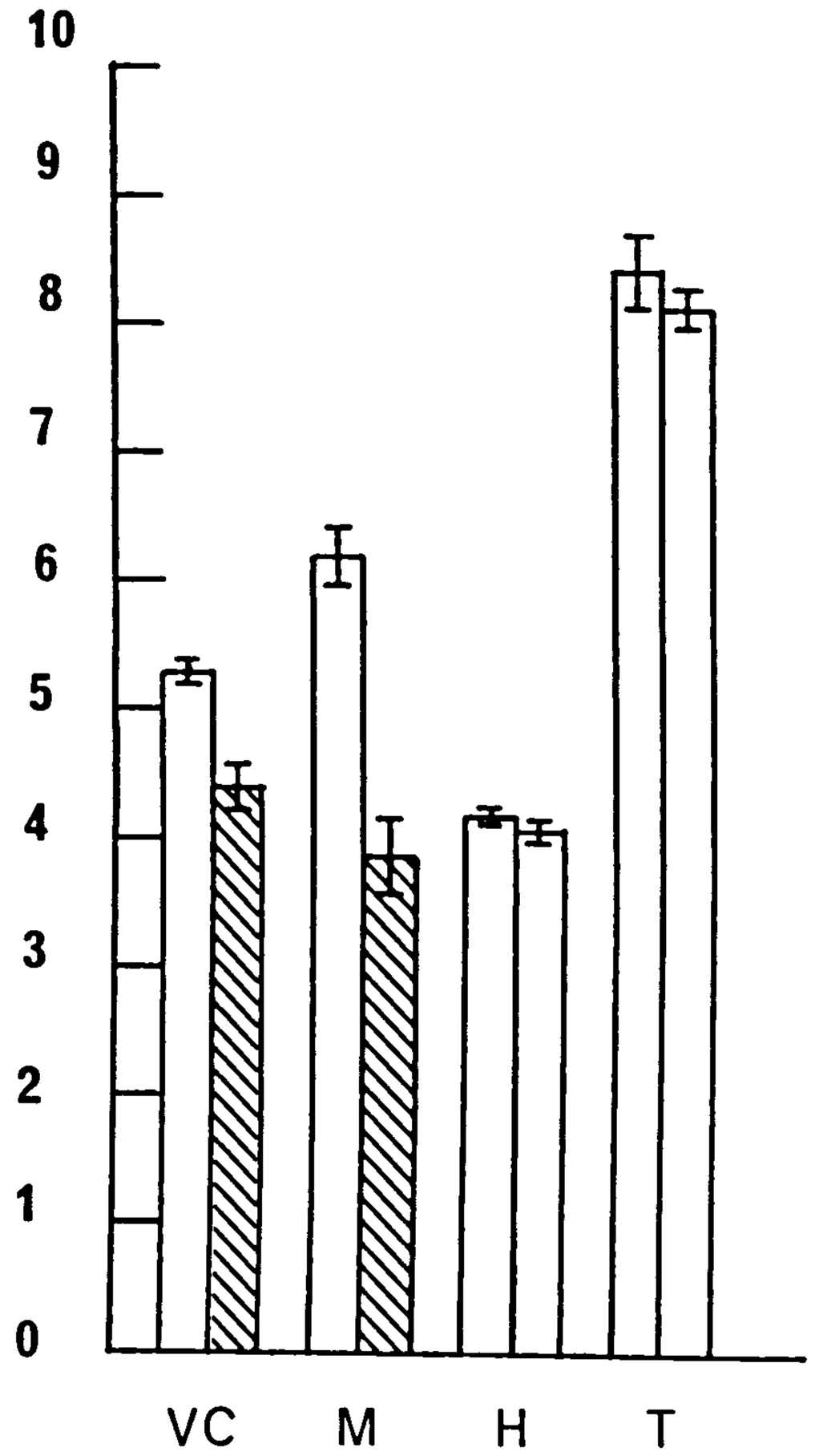
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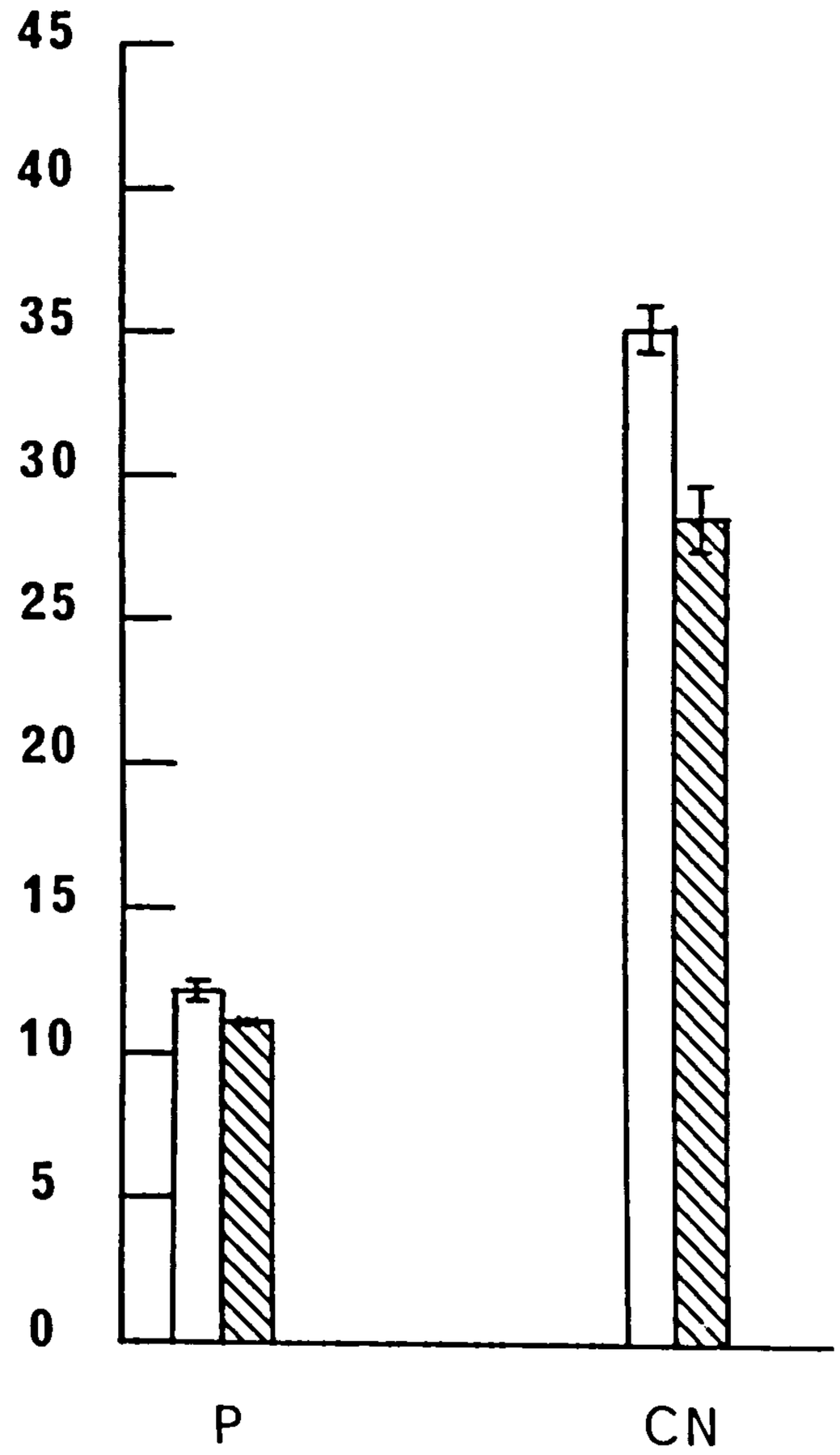
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FIGURE 18. Bar diagram to show the effects of anaesthesia coupled with electrical stimulation on the AChE activity of the 12 regions.

The brain regions have been divided into 3 groups of activity and thus the scales of the axes are different. The diagrams have been drawn by a Hewlett Packard statistical plotter. The columns represent the means of the number of determinations shown in Table 11. The S.E.M. is represented by the vertical bars. The groups that are significantly different are indicated by shading.

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

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

3 . 3. 4. <u>The effects of electrical stimulation in a "shuttle - box" on the activity of AChE in 12 regions of the rat brain</u>.

The effects of electrical stimulation in a "shuttle - box" on the activity of acetylcholinesterase in the 12 regions of the rat brain are shown in Table 12 and Figure 19.

a) In the 3 dorsal cortical regions the treatment produced no significant differences in enzyme activity. All the regions exhibited a slight, but non - significant activation of enzyme activity as compared with quiet control group animals.

b) A similar pattern of slight, but non - significant, activation of enzyme activity was also observed in the ventral cortex, hypothalamus (where the quiet control value for the enzyme activity was significantly increased over the previous value), the olfactory tubercle and the caudate nucleus. In the thalamus and pons no significant differences in enzyme activity were observed, although, in the pons, the quiet control value for the enzyme activity was lower than the previous value.

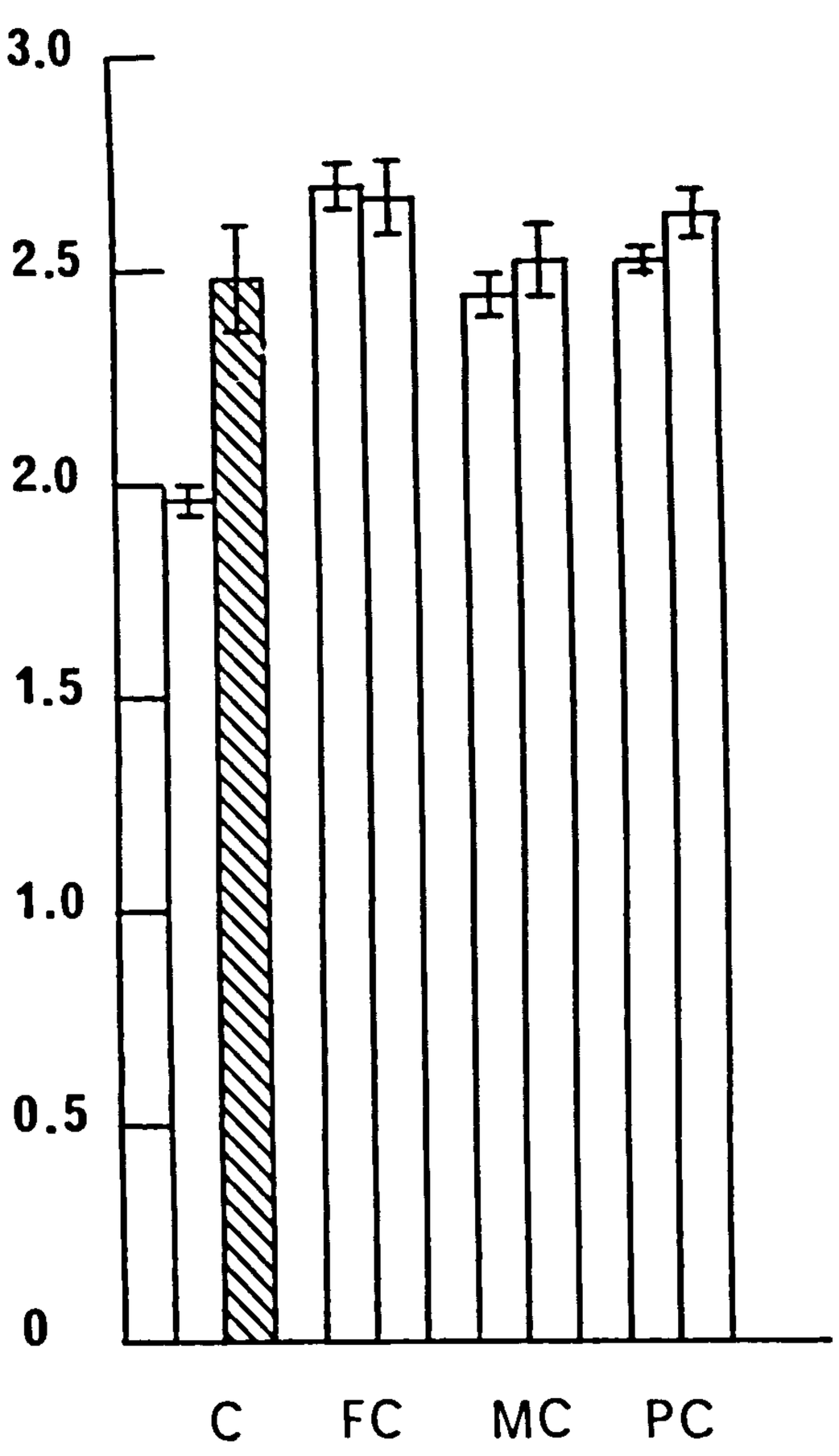
c) A highly significant increase in enzyme activity was observed as a result of the stimulation in the cerebellum (28%). In the superior colliculus an increase in enzyme activity was also observed (11%), although, here too, the quiet control value was lower than the previous value.

d) In the remaining region, the medulla, a highly significant decrease (24%) in enzyme activity was observed as a result of stimulation.

TABLE 12. The effects of electrical stimulation on AChE activity.

	STIMULATION			CONTROL			
Region	Mean ± S.E.M.		n	Mean ± S.E.M.		n	p
Cerebellum	2.479	0.125	(9)	1.961	0.035	(7)	0.01
Medial cortex	2.515	0.084	(8)	2.435	0.049	(7)	ns
Posterior cortex	2.623	0.055	(8)	2.516	0.030	(7)	ns
Frontal cortex	2.666	0.085	(8)	2.693	0.053	(7)	ns
Hypothalamus	5.251	0.167	(8)	5.017	0.058	(7)	ns
Ventral cortex	5.812	0.293	(8)	5.271	0.145	(7)	ns
Medulla	4.553	0.342	(12)	5.667	0.076	(7)	0.05
Thalamus	8.093	0.385	(8)	8.110	0.253	(8)	ns
Sup. colliculus	12.146	0.290	(8)	10.936	0.128	(7)	0.01
Pons	11.670	0.384	(8)	12.841	0.381	(7)	ns
Olf. tubercle	35.579	0.302	(8)	34.488	0.769	(7)	ns
Caudate nucleus	37.704	0.702	(8)	36.147	0.891	(7)	ns

Enzyme activity is expressed as μMoles acetylthiocholine hydrolysed /hr./mg. proteins.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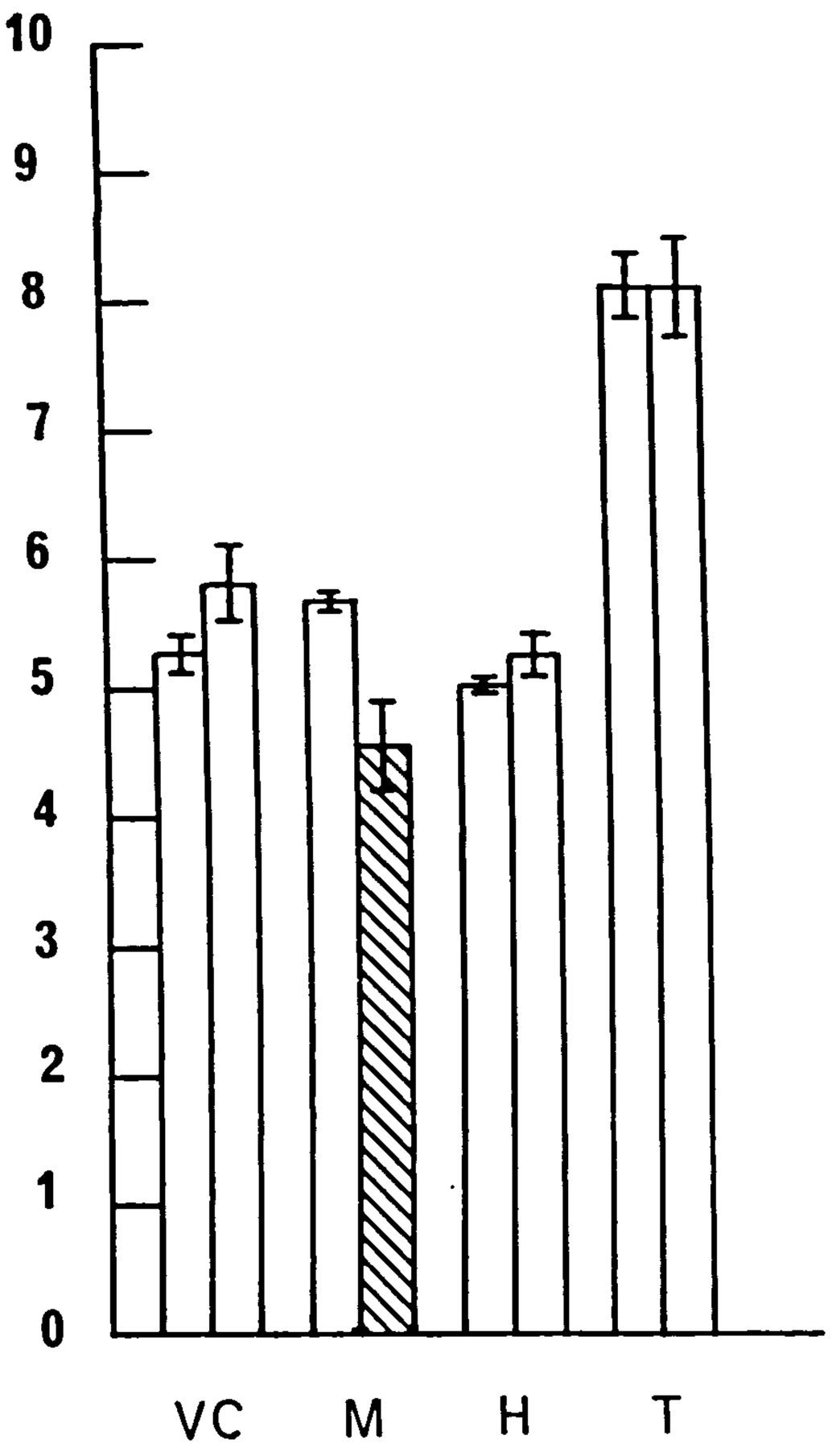
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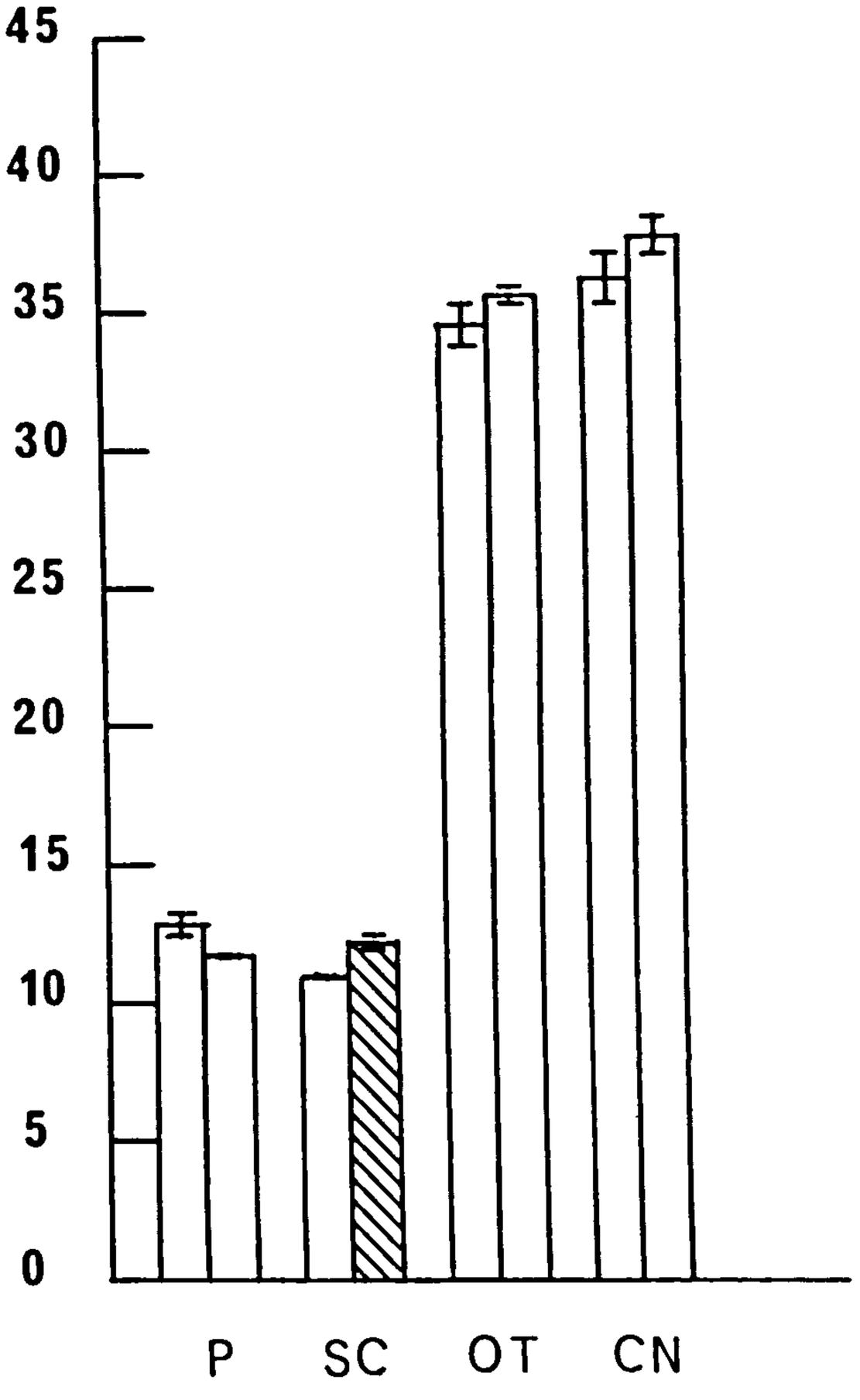
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FIGURE 19. Bar diagram to show the effects of electrical stimulation in a "shuttle - box" on the AChE activity in the 12 brain regions.

The brain regions have been divided into 3 groups of activity and thus the scales of the axes are different. The diagrams have been drawn by a Hewlett Packard statistical plotter. The columns represent the means of the number of determinations shown in Table 12. The S.E.M. is represented by the vertical bars. The groups that are significantly different are indicated by shading.

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

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

3 . 3. 5. <u>The effects of 2 - way shock - avoidance conditioning on the activity of AChE in 12 regions of the rat brain</u>.

The effects of 2 - way shock - avoidance conditioning on the activity of acetylcholinesterase in the 12 regions of the rat brain are shown in Table 13 and Figure 20. These results were obtained by assaying the enzyme activity 1 hour after completion of the training and are compared here with quiet control group animals.

a) In the 3 dorsal cortical regions significant increases in enzyme activity were observed in all 3 regions. None of the changes was greater than 10%.

b) Significant increases in enzyme activity were also observed in the cerebellum (8%), thalamus (10%) and the caudate nucleus (8%); while in the superior colliculus a significant decrease (12%) in enzyme activity was observed.

c) In the remaining regions, the ventral cortex, hypothalamus, pons medulla and olfactory tubercles, no significant differences in enzyme activity were observed.

d) The quiet control value for the pons was again found to be lower than that measured in the original quiet control distribution of enzyme activity experiment, although the values for the superior colliculus and hypothalamus were now found to be the same as those measured previously. The quiet control values for the enzyme activity in all other regions remained unchanged in 3 replications.

TABLE 13. The effects of avoidance - conditioning on AChE activity.

	CONDITIONING			CONTROL			
Region	Mean ± S.E.M.		n	Mean ± S.E.M.		n	p
Cerebellum	2.174	0.022	(8)	2.004	0.022	(8)	0.001
Medial cortex	2.642	0.046	(8)	2.486	0.039	(8)	0.01
Posterior cortex	2.764	0.036	(8)	2.551	0.020	(8)	0.001
Frontal cortex	2.864	0.030	(8)	2.635	0.014	(8)	0.001
Hypothalamus	4.664	0.133	(8)	4.555	0.127	(8)	ns
Ventral cortex	5.440	0.088	(8)	5.372	0.124	(8)	ns
Medulla	5.935	0.471	(8)	5.651	0.094	(8)	ns
Thalamus	9.192	0.310	(8)	8.295	0.166	(8)	0.02
Sup. colliculus	11.037	0.326	(8)	12.421	0.132	(8)	0.01
Pons	11.141	0.126	(8)	11.180	0.143	(8)	ns
Olf. tubercle	35.054	0.642	(8)	35.196	0.729	(8)	ns
Caudate nucleus	40.282	0.369	(8)	36.909	0.285	(8)	0.001

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

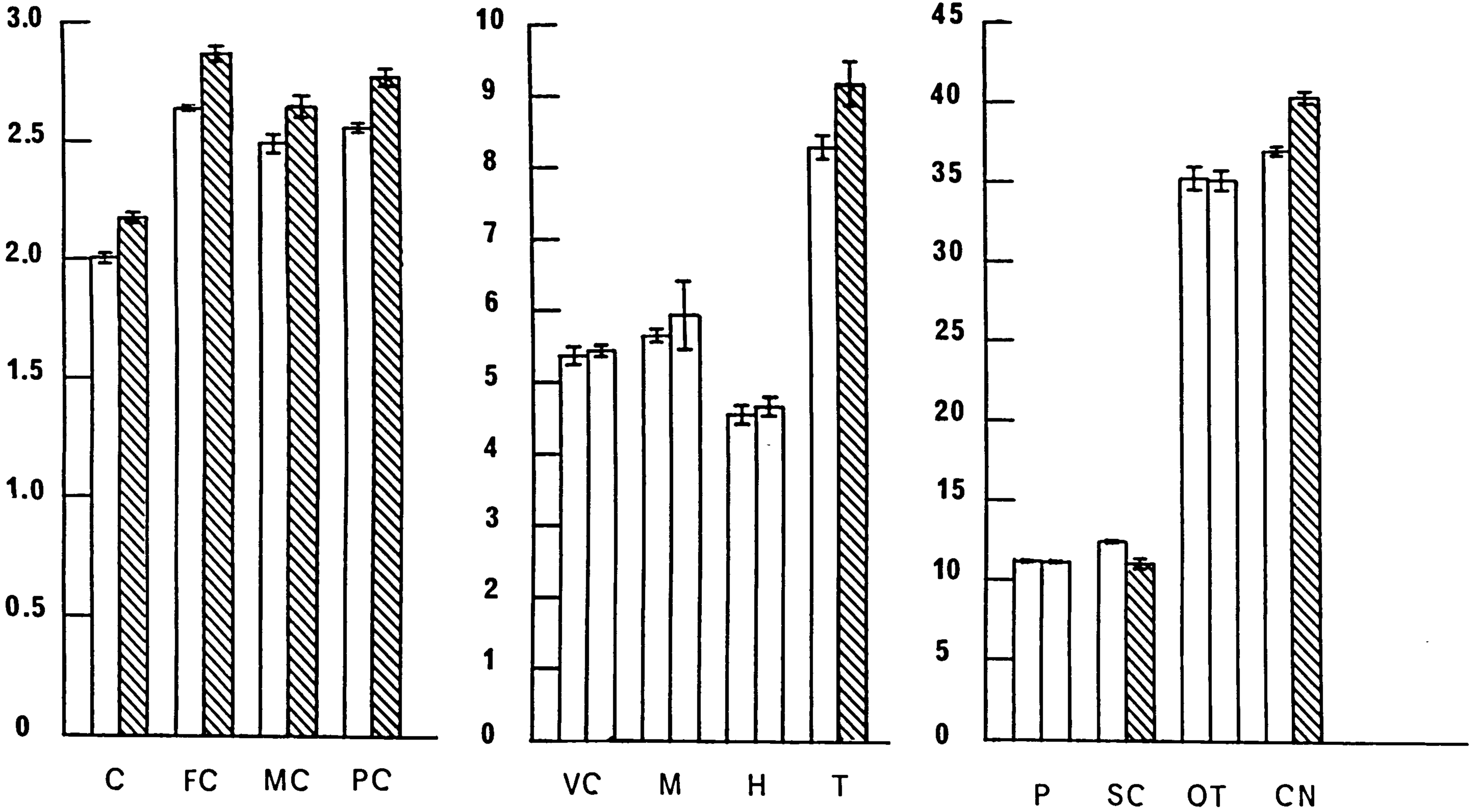
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FIGURE 20. Bar diagram to show the effects of 2 - way shock - avoidance conditioning on the AChE activity in the 12 brain regions.

The regions have been divided into 3 groups of activity and thus the scales of the axes are different. The diagrams have been drawn by a Hewlett Packard statistical plotter. The columns represent the means of the number of determinations shown in Table 13. The S.E.M. is represented by the vertical bars. The groups that are significantly different are indicated by shading.

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

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

3 . 3. 6. <u>The effects of 2 - way shock - avoidance conditioning on the activity of AChE in 12 regions of the rat brain, (b). A repeat experiment carried out with a yoked control group</u>.

The effects of 2 - way shock - avoidance conditioning on the activity of acetylcholinesterase in the 12 regions of the rat brain are shown in Table 14 and Figure 21. These results were obtained by assaying the enzyme activity 1 hour after completion of the training and are compared here with yoked - control group animals.

a) In the 3 dorsal cortical regions the enzyme activity is increased to a comparable extent as in 3.3.5. The enzyme activity in the yoked - control group is, however, also increased, although not to such a great extent. Thus, in no cortical region was a statistically significant difference in enzyme activity observed.

b) In the cerebellum and caudate nucleus, although the same pattern of elevated enzyme activity was observed in both trained and yoked groups, the differences in enzyme activity achieved statistical significance.

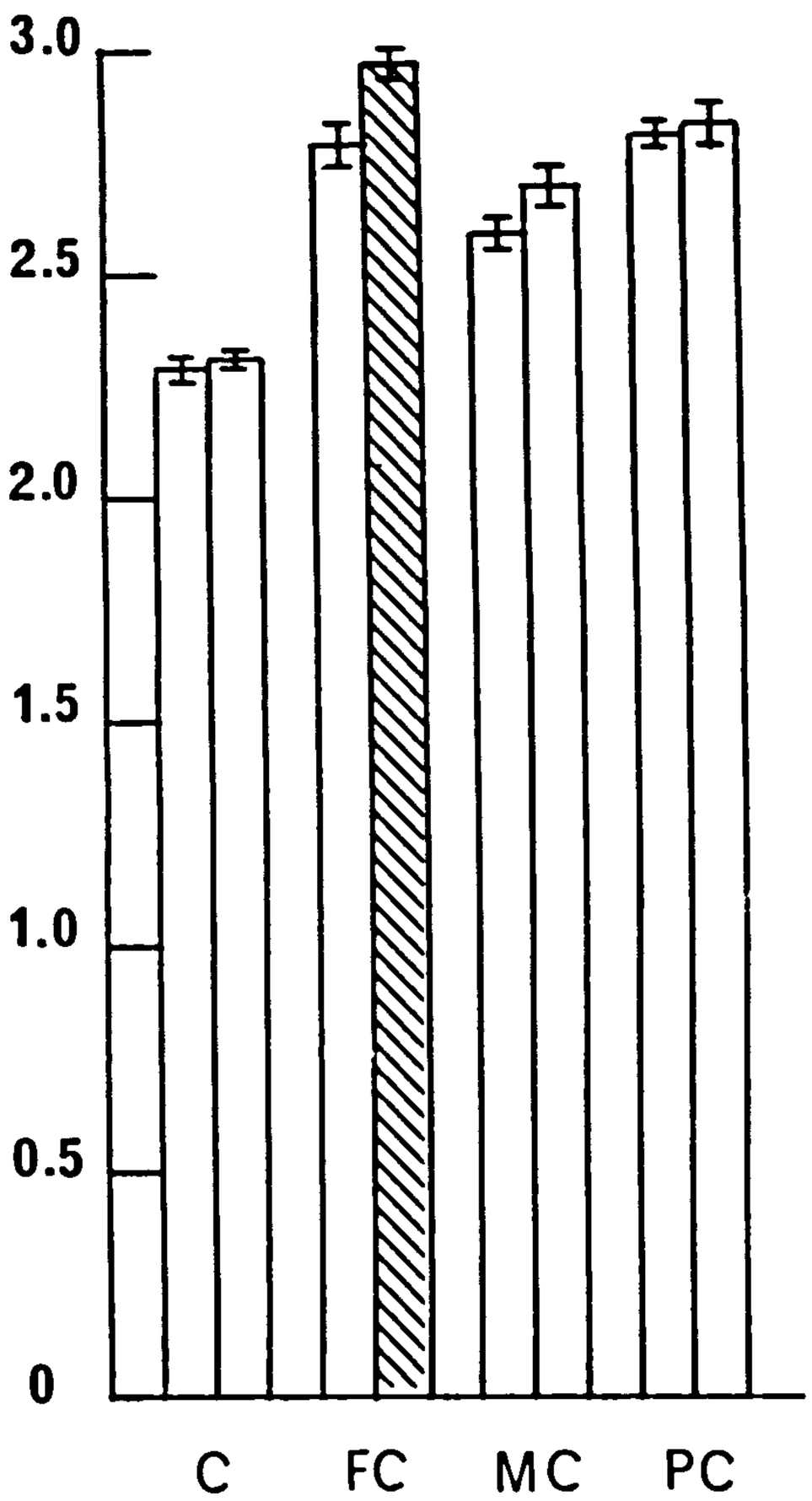
c) In the thalamus, ventral cortex and medulla the same pattern of elevated enzyme activity was observed in both groups but the difference in enzyme activity did not achieve statistical significance.

d) In all the regions the elevation of enzyme activity, as a result of shock - avoidance conditioning are comparable to the values reported for the previous experiment.

TABLE 14. The effects of avoidance - conditioning on AChE activity (b).

	CONDITIONING		YOKED CONTROL		
Region	Mean ± S.E.M.	n	Mean ± S.E.M.	n	p
Cerebellum	2.298 0.029	(8)	2.198 0.035	(8)	0.05
Medial cortex	2.599 0.038	(8)	2.526 0.051	(8)	ns
Posterior cortex	2.797 0.042	(8)	2.723 0.039	(8)	ns
Frontal cortex	2.881 0.029	(8)	2.750 0.057	(8)	ns
Hypothalamus	no data				
Ventral cortex	5.699 0.092	(8)	5.500 0.144	(8)	ns
Medulla	5.896 0.361	(8)	5.477 0.521	(8)	ns
Thalamus	9.207 0.257	(8)	8.489 0.396	(8)	ns
Sup. colliculus	no data				
Pons	no data				
Olf. tubercle	no data				
Caudate nucleus	39.986 0.384	(8)	38.021 0.462	(8)	0.01

Enzyme activity is expressed as μMoles acetylthiocholine hydrolysed/hr./mg. proteins.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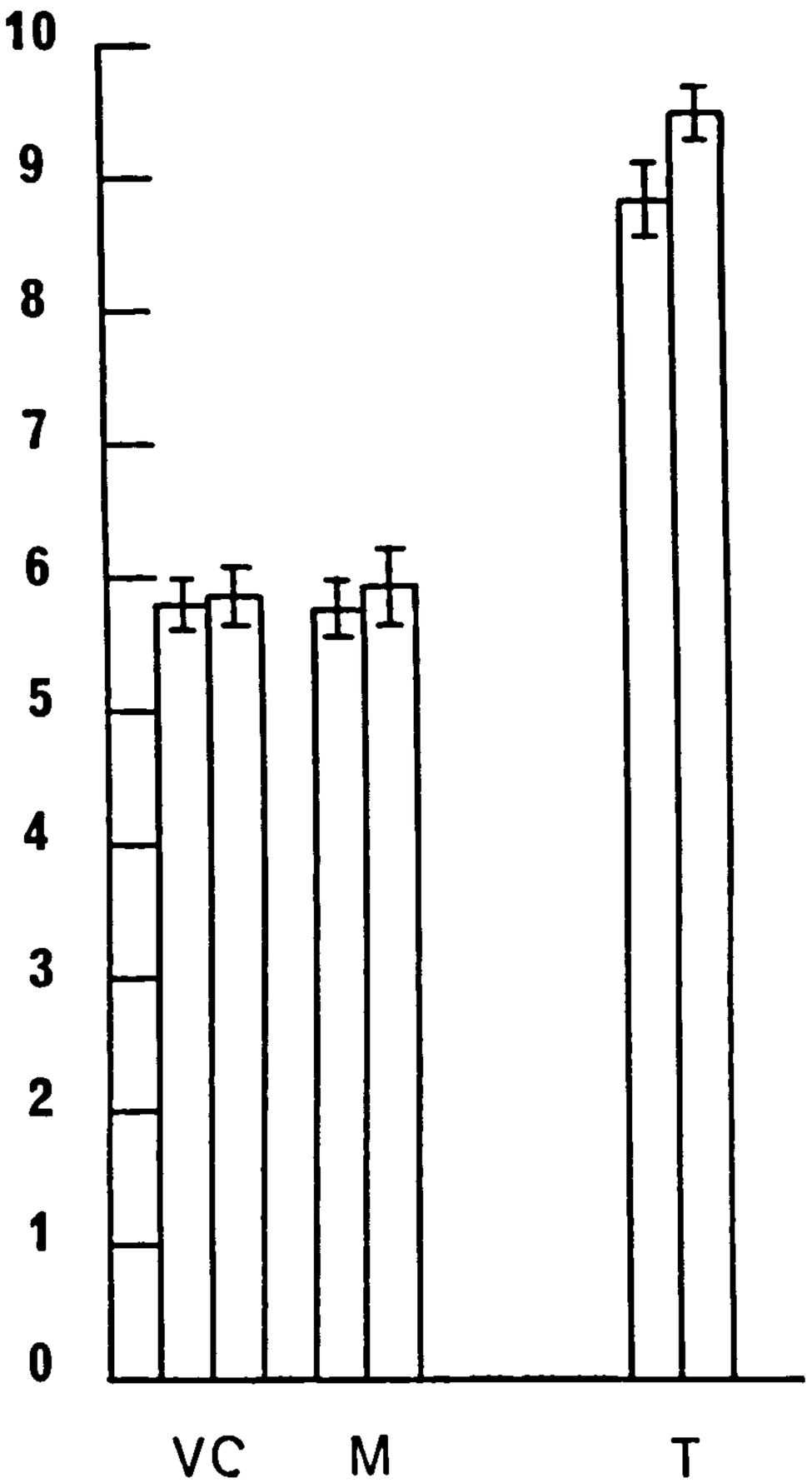
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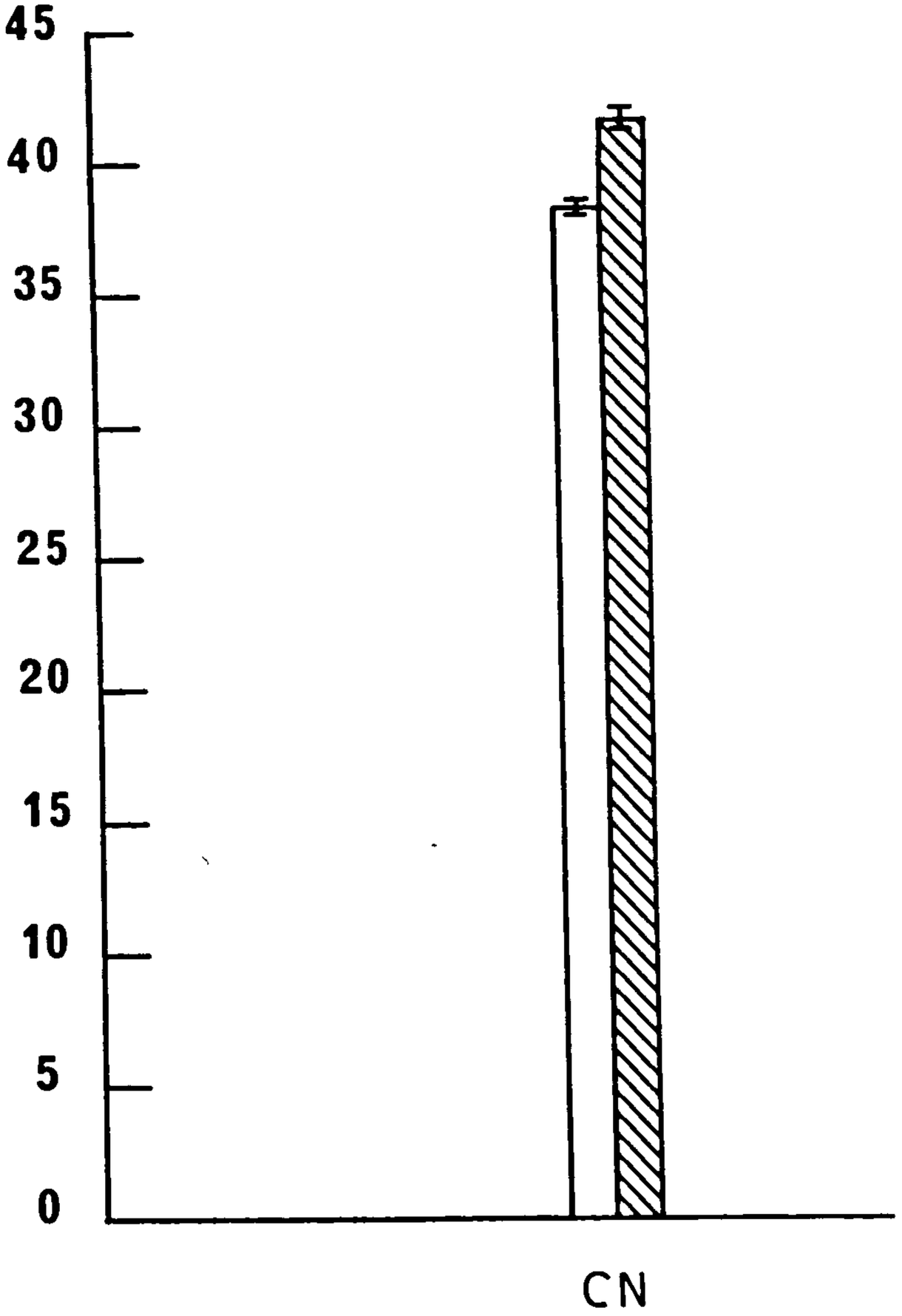
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FIGURE 21. Bar diagram to show the effects of 2 - way shock - avoidance conditioning on the AChE activity of the 12 regions. (b). A repeat experiment carried out with a yoked control group.

The regions have been divided into 3 groups of activity and thus the scales of the axes are different. The diagrams have been drawn by a Hewlett Packard statistical plotter. The columns represent the means of the number of determinations shown in Table 14. The S.E.M. is represented by the vertical bars. The groups that are significantly different are indicated by shading.

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

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

3 . 3. 7. <u>The effects of 2 - way shock - avoidance conditioning on the activity of AChE in the 12 regions of the rat brain (c). A repeat experiment carried out 24 hours following conditioning</u>.

The effects of 2 - way shock - avoidance conditioning on the activity of acetylcholinesterase in the 12 regions of the rat brain are shown in Table 15 and Figure 22. The results were obtained by assaying the enzyme activity 24 hours after the completion of the training and are compared here with yoked control group animals.

a) In the 3 dorsal cortical regions the enzyme activity level in both trained and yoked - control animals is again elevated. Comparing the increases to those seen in 3.3.6., in all cases, the activity levels are further increased. Only in the frontal cortex is a statistically significant difference in enzyme activity observed compared to the yoked control group.

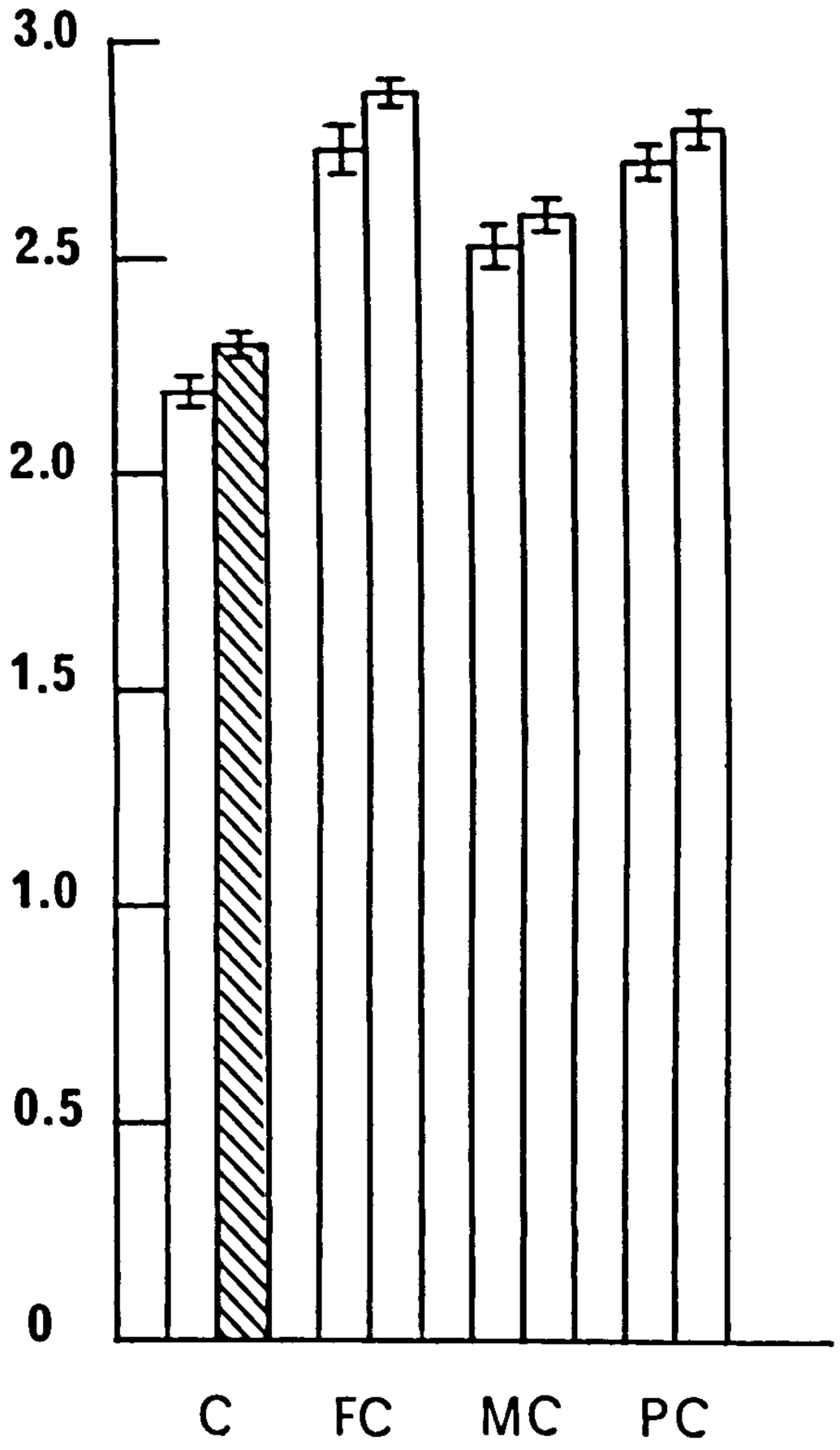
b) In the caudate nucleus the pattern is similar to a), with both trained and yoked groups exhibiting further increased enzyme activity levels. The difference in enzyme activity between trained and yoked control groups was highly significant.

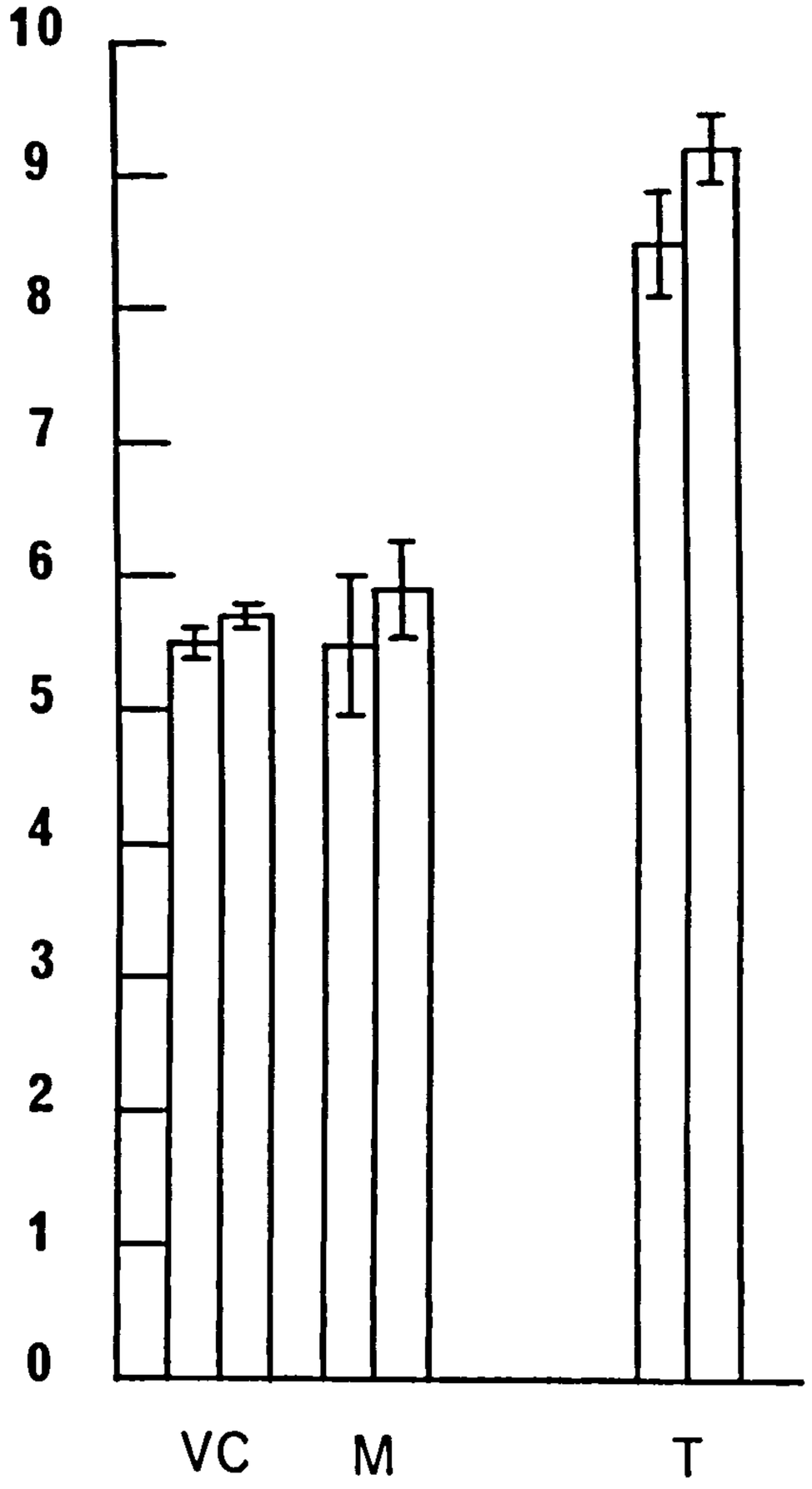
c) The same pattern was observed for the samples from the cerebellum, ventral cortex, thalamus and medulla. In these regions, however, none of the difference in enzyme activity achieved statistical significance.

TABLE 15. The effects of avoidance - conditioning on AChE activity (c).

	CONDITIONING		YOKED CONTROL		
Region	Mean ± S.E.M.	n	Mean ± S.E.M.	n	p
Cerebellum	2.309 0.021	(8)	2.287 0.027	(8)	ns
Medial cortex	2.693 0.044	(8)	2.588 0.037	(8)	ns
Posterior cortex	2.834 0.046	(8)	2.810 0.029	(8)	ns
Frontal cortex	2.970 0.035	(8)	2.788 0.049	(8)	0.02
Hypothalamus	no data				
Ventral cortex	5.858 0.221	(8)	5.801 0.190	(8)	ns
Medulla	5.922 0.286	(8)	5.761 0.212	(8)	ns
Thalamus	9.468 0.199	(8)	8.822 0.277	(8)	ns
Sup. collicul us	no data				
Pons	no data				
Olf. tubercle	no data				
Caudate nucleus	41,609 0.408	(8)	38.226 0.299	(8)	0.001

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


3.0
2.5
2.0
1.5
1.0
0.5
0
C
FC
MC
PC


10
9
8
7
6
5
4
3
2
1
0
VC
M
T

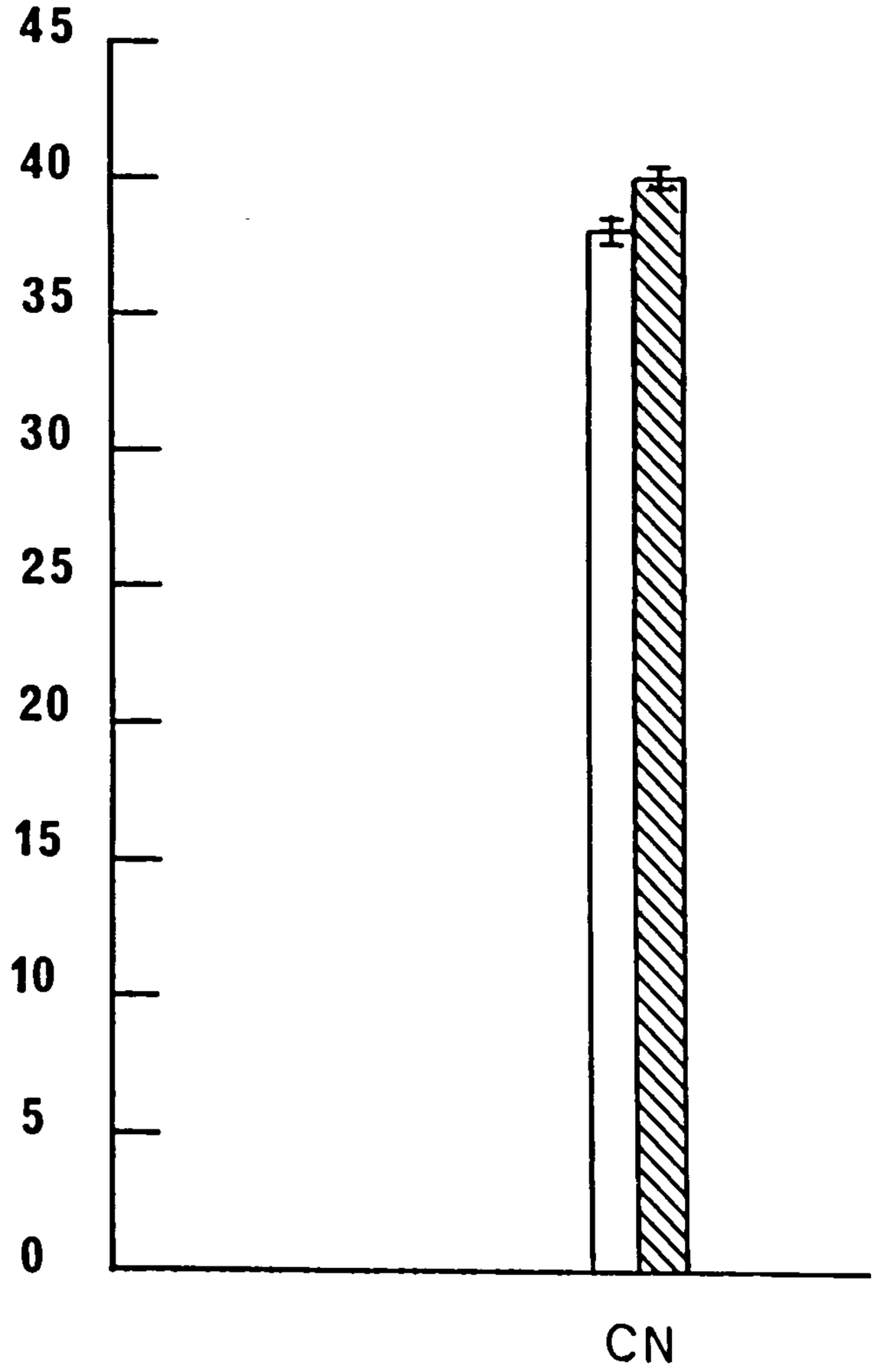

45
40
35
30
25
20
15
10
5
0
CN

FIGURE 22. Bar diagram to show the effects of 2 - way shock - avoidance conditioning on the AChE activity of the 12 regions (c). A repeat experiment carried out 24 hours following conditioning.

The regions have been divided into 3 groups of activity and thus the scales of the axes are different. The diagrams have been drawn by a Hewlett Packard statistical plotter. The columns represent the means of the number of determinations shown in Table 15. The S.E.M. is represented by the vertical bars. The groups that are significantly different are indicated by shading.

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

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

3 . 4. Summary.

The general properties of rat brain acetylcholinesterase were investigated. The results are summarised in Table 16.

An attempt to use potassium chloride - induced cortical spreading depression as a means of providing a unilateral functional decortication, in order to use each experimental animal as its own control in behavioural studies was abandoned. Experimental evidence indicated that the treatment, itself, induced rapid changes in the acetylcholinesterase activity.

The results of the behavioural treatments on the activity of acetylcholinesterase from the various regions of the rat brain are summarised in Table 17. There are definite changes in the response of the enzyme activity following the various behavioural treatments:

a) The activity of acetylcholinesterase appears to vary directly with the state of activation of the animal in the cerebellum, ventral cortex, thalamus and the caudate nucleus, i.e. decreased enzyme activities are observed following anaesthesia, decreased enzyme activities, but to a lesser extent, are observed following anaesthesia coupled with electrical stimulation; increased enzyme activities are observed following electrical stimulation, as shown both by this group (2.2.5) and by the yoked control groups (2.2.7) and in shock - avoidance conditioning.

b) The increases in enzyme activity, exhibited in the various brain regions from both the trained and yoked control groups, appear to be further increased when the assays are conducted 24 hours following the treatment.

c) In the 3 dorsal cortical regions no significant changes in enzyme activity were observed, as a result of the behavioural treatments, except following shock - avoidance conditioning when all 3 regions exhibited increased enzyme activities.

d) The medulla appears to be an exception to this pattern, exhibiting

TABLE 16. The properties of rat brain acetylcholinesterase.

1) pH optimum	8.2
2) Substrate optimum . . .	1.0 mM acetylthiocholine.
3) Substrate inhibition . .	Above 1 mM acetylthiocholine.
4) Stability	< 4% loss of activity in 6 hrs.
5) Pseudo - cholinesterase . .	4 - 6% of the activity of AChE.
6) Multiple forms . . .	at least 2 (see Appendix C).

TABLE 17. The effects of the behavioural treatments on AChE activity.

	Anaes. 3.3.2.	Anaes.Stim. 3.3.3.	Stim. 3.3.4.	Cond.(a) 3.3.5.	Cond.(b) 3.3.6.	Cond.(c) 3.3.7.
Cerebellum	- 10%	*	+28%	+8%	+5%	*
Medial cortex	*	*	*	+6%	*	*
Posterior cortex	*	*	*	+10%	*	*
Frontal cortex	*	*	*	+8%	*	+9%
Hypothalamus	*	*	*	*	*	*
Ventral cortex	-26%	-20%	*	*	*	*
Medulla	+38%	-37%	-24%	*	*	*
Thalamus	-39%	*	*	+10%	*	*
Sup. colliculus	*	*	+11%	-12%	*	*
Pons	*	-9%	*	*	*	*
Olf. tubercle	*	*	*	*	*	*
Caudate nucleus	-24%	-19%	*	+8%	+6%	+8%

an increased enzyme activity following anaesthesia; a decreased enzyme activity following anaesthesia coupled with electrical stimulation and a decreased activity following stimulation in a shuttle - box. Thus, in general terms and with the exception of the results observed following shock - avoidance conditioning, where no significant enzyme activity changes were observed, the response of the enzyme from the medulla appears to be a "mirror - image" of the pattern in a), page 98.

e) In the superior colliculus, pons and hypothalamus the enzyme activity changes, where they occur, do not appear to obey any pattern and, hence, may possibly reflect seasonal variations in baseline activity levels.

f) No enzyme activity change were observed following any of the treatments in either the olfactory tubercle or hypothalamus.

* * * *

3 . 5. Discussion.

The rank order of the quiet control distribution and the absolute values of the acetylcholinesterase activity (Table 9 and Figure 16) in the various regions studied are closely similar to those reported for the rat brain by Bennett et al (1966) and Bajgar and Urban (1971).

Bennett et al (1966) report the values for acetylcholinesterase activity in units of mμMoles acetylcholine hydrolysed/min./mg. fresh weight. In order to compare the two sets of results an experiment was conducted to determine the average protein content per mg. wet weight of various brain region tissue samples. The results of this experiment indicated an 8.6 $\pm$0.1% mean protein content of the brain samples. Using this figure to convert those results to units of μMoles acetylthiocholine hydrolysed/hr./mg. proteins, the results agree closely with only one exception. Bennett et al (1966) report the highest enzyme activity in the brain regions they

measured, as being in the olfactory tubercle (33.5 μMoles acetylthiocholine hydrolysed /hr./mg. proteins), with the caudate nucleus and putamen sample in second position in the rank order (26.0 μMoles acetylthiocholine hydrolysed/ hr./mg. proteins). In this study the caudate nucleus sample had the higher enzyme activity (36.1 μMoles acetylthiocholine hydrolysed/hr./mg. proteins) and that the enzyme activity in the olfactory tubercle was slightly lower (33.5 μMoles acetylthiocholine hydrolysed/hr./mg.proteins). The reason for this difference may be that Bennett et al (1966) sampled all of the caudate nucleus and putamen and in this study only a part of the caudate nucleus was sampled. The absolute values for the enzyme activity in the olfactory tubercle in the two studies are the same. Bennett et al (1966) also reported that they consistently found that the anterior region of the dorsal cortex had a higher enzyme activity than the posterior region of the cortex. The present results support this finding, though a true antero - posterior gradient was not found.

These results are also comparable, to some extent, to those reported for the acetylcholinesterase activity in several regions of the dog - brain (Burgen and Chipman 1951). They report the highest activity of the enzyme in the caudate nucleus. In both dog and rat the superior colliculus has a relatively high enzyme activity, whilst the activity in the cortices of both is low. They also report that the anterior region of the cortex has a higher enzyme activity than the posterior region (visual cortex). There are, however, several differences reported for the distribution of the enzyme activity between the two studies:

a) the cerebellum has the next to the highest acetylcholinesterase activity in the dog but the lowest activity in the rat.

b) in the dog the hypothalamus has a higher enzyme activity than the thalamus, whereas, in the rat the reverse is found. This difference may, again, be explicable in terms of sampling differences for the thalamus.

In a recent study by Vernadakis and Rutledge (1973) the effects of

both ether and pentobarbital anaesthesia on the activities of pseudo - cholinesterase and acetylcholinesterase was investigated in two regions of the brains of young adult rats. They reported that the activity of acetylcholinesterase decreases in the corpus striatum and cerebral cortex by 20% after one hour of pentobarbital anaesthesia. These values were measured one week following treatment. Decreases of a slightly greater magnitude, in acetylcholinesterase activity were observed, at this time, following anaesthesia for one hour with ether. Significant decreases in the activity of pseudo - cholinesterase were also observed following either pentobarbital or ether anaesthesia. These results confirm that:

a) decreases in the activity of acetylcholinesterase occur following anaesthesia with pentobarbitone - sodium for a short time period.

b) changes in the activity of acetylcholinesterase activity may be observable for, at least, up to one week following acute treatments of this nature.

Many investigators (Tobias et al 1946, Richter and Crossland 1949, Elliot et al 1950 and Takahashi and Aprison 1964), have reported that the concentration of acetylcholine in the brain seems to vary inversely with the general state of activation of the animal e.g. during sleep or following the administration of anaesthetics or convulsants etc. Studies investigating the release of acetylcholine from regions of the brain (Mitchell 1963, Kanai and Szerb 1965, Celesia and Jasper 1966 and Phillis 1968) reported that the rate of spontaneous release of acetylcholine could be augmented by stimulation. Phillis (1968) found that peripheral stimulation caused comparable increases in acetylcholine output from all of the cortical areas tested, regardless of the type of stimulation employed. Similar studies have demonstrated similar effects on the rate of release of acetylcholine from the cerebellar cortex, thalamus and caudate nucleus. The pattern of the increases in the rate of release of acetylcholine from the cerebral cortex during stimulation has been taken to indicate

(Phillis 1970) that the cholinergic fibres form part of a diffuse projection system, rather than being connected with the main afferent pathways. This has also been stated by Krnjevic (1969).

An examination of the data presented in Tables 3 - 7 and of the results of this study indicate that it is possible that there could be a relationship between the factors that cause an increase in brain acetylcholine concentrations and a decrease in acetylcholinesterase activity. This hypothesis was suggested by Krech et al (1960).

* * * *

SECTION FOUR

4 . 1. Kinetic investigations into the observed enzyme activity changes using the method of Ellman et al (1961).

In order to elucidate the mechanism of the observed enzyme activity changes following the various behavioural treatments (see section 3.3.) standard kinetic studies were employed. In this manner it was hoped to determine whether the enzyme activity had altered via a change in the affinity of the enzyme for its substrate, as shown by a change in the Km value or whether, by some other mechanism, there was some change in the number of active sites available for acetylcholine hydrolysis, as shown by an altered Vmax. characteristic of the enzyme.

In order to maximise the procedures, samples from brain regions which showed particularly large changes in enzyme activity following a particular behavioural treatment were selected. These were compared with the enzyme from the same brain regions from control animals. In all cases the samples were coded and randomised before assay in order to conduct the assays "blind".

The enzyme activity was determined at pH 8,2 and 22°C. by the method of Ellman et al (1961), as outlined in 2.2.8., using a wide range of substrate concentrations (0.01 - 1.0 mM acetylthiocholine). The assays were performed in duplicate using three animals per group.

The results, shown in Figures 23, 24 and 25, are presented using the Lineweaver - Burk plot (1934). For the Lineweaver - Burk plots the lines of best fit to the data were compared by using a Diehl combitron S calculator with a program for linear regression analysis placing equal emphasis on all points.

Figure 23 shows that in the medulla, where in the previous

Lineweaver-Burk plots obtained from Medulla homogenates following ;

a) Anaesthesia

b) Resting control

$Km_a = 0 \cdot 8 \times 10^{-4}$ M.

$Km_b = 0 \cdot 8 \times 10^{-4}$ M.

difference in activity at Vmax.= 28%

$\frac{1}{V}$ O.D. units/hr./mgm. Protein

·08 ·07 ·06 ·05 ·04 ·03 ·02 ·01

-30 -20 -10 0 10 20 30 40 50 60 70 80 90 100

$\frac{1}{S}$ mM. ACThCh

FIGURE 23. Double reciprocal plot of the hydrolysis of acetylthiocholine by acetylcholinesterase from the medulla.

(b) Incubations with the enzyme from the medulla of quiet - control animals

(a) Incubations with the enzyme from the medulla of anaesthetised animals

There is no change in the Km but only in the V. max. characteristic.

Lineweaver-Burk plots obtained from Thalamus homogenates following ;

a) Anaesthesia

b) Resting control

$Km_a = 1{\cdot}2 \times 10^{-4}$ M.

$Km_b = 1{\cdot}2 \times 10^{-4}$ M.

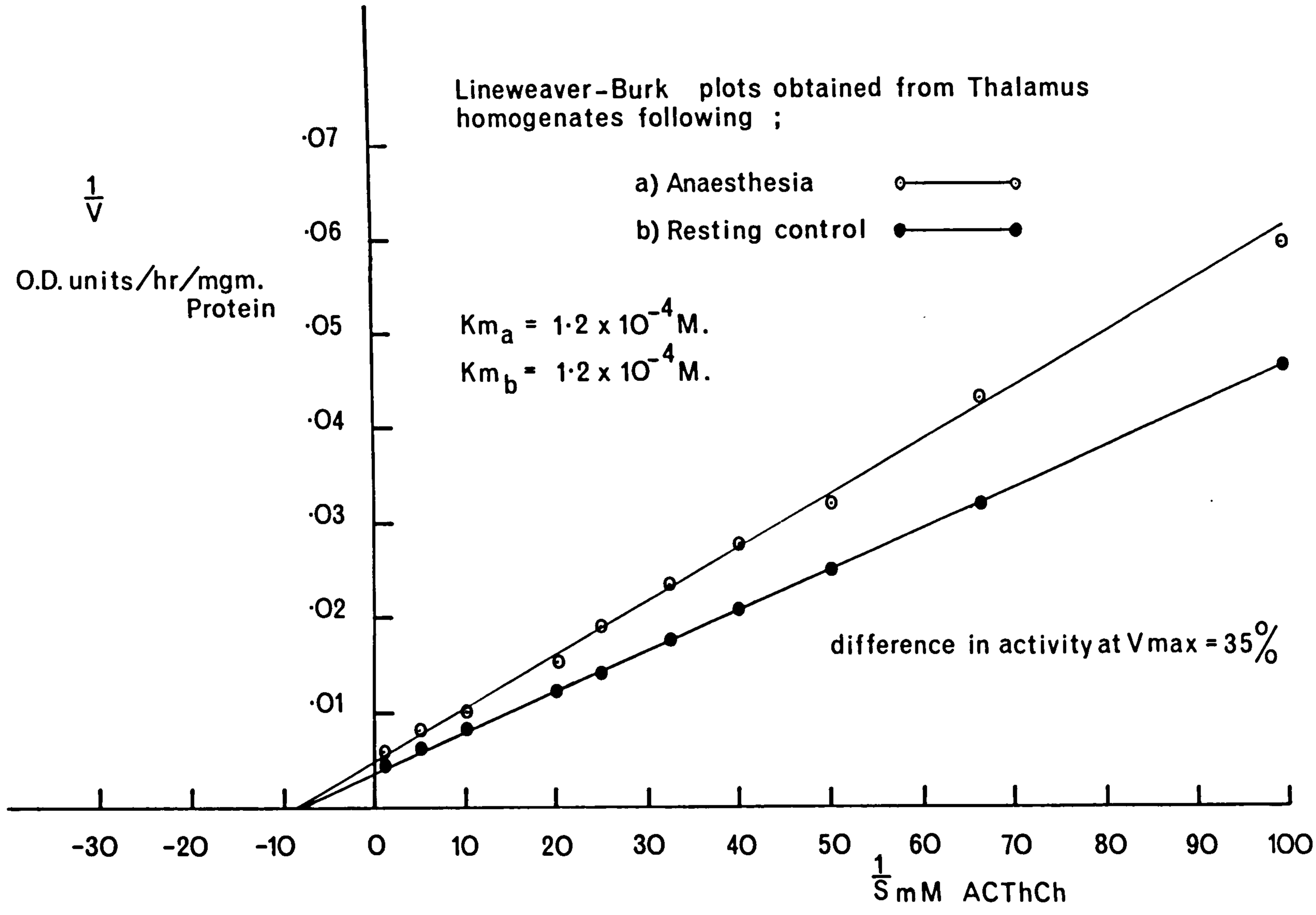


FIGURE 24. <u>Double reciprocal plot of the hydrolysis of acetylthiocholine by acetylcholinesterase from the thalamus</u>.

(b) Incubations with the enzyme from the thalamus of quiet - control animals.

(a) Incubations with the enzyme from the thalamus of anaesthetised animals.

There is no change in the Km value but only in the V max. characteristic.

Lineweaver-Burk plots obtained from Caudate nucleus homogenates following ;

a) Anaesthesia

b) Shock-avoidance conditioning

$Km_a = 1{\cdot}0 \times 10^{-4}$ M.

$Km_b = 1{\cdot}2 \times 10^{-4}$ M.

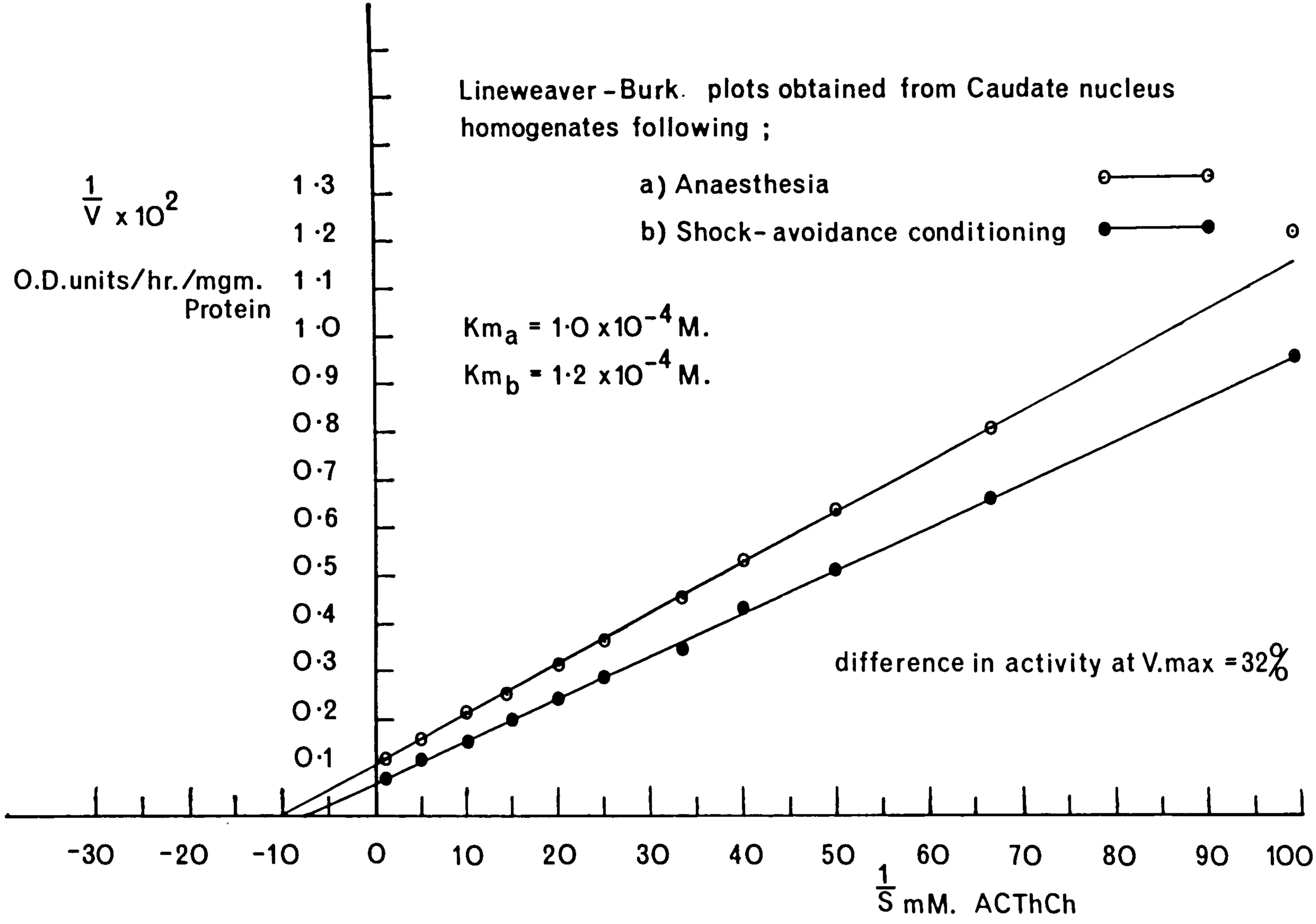


FIGURE 25. Double reciprocal plot of the hydrolysis of acetylthiocholine by acetylcholinesterase from the caudate nucleus.

(a) Incubations with the enzyme from the caudate nucleus of anaesthetised animals.

(b) Incubations with the enzyme from the caudate nucleus of trained animals.

The small change in the Km value is not large enough to account for the observed enzyme activity change. The change in the V max. is great enough to account for this.

experiment, the activity of acetylcholinesterase following anaesthesia was 38% greater than the saline - injected control group, the activity of the enzyme at V max. was 28% greater than for the quiet control animals. No difference in the Km values (0.8×10^{-4}M.) was observed between the two samples.

Figure 24 shows that in the thalamus, where the activity of acetylcholinesterase following anaesthesia was observed to be 39% less than the saline - injected control group, (page 81) the activity of the enzyme at V max. was 35% less than for the quiet control animals. In this region, also, no difference in the Km value (1.2×10^{-4}M.) was again observed.

Figure 25 shows that in the caudate nucleus there is a difference in enzyme activity at V max. of 32% between the enzyme samples obtained following anaesthesia and shock - avoidance conditioning. In the previous experiment a 24% decrease in enzyme activity was observed following anaesthesia and an 8% increase in enzyme activity following two - way shock - avoidance conditioning. The Km values of the two caudate nucleus samples were observed to be 1.0×10^{-4} M. and 1.2×10^{-4}M. This small difference is not large enough to account for the observed enzyme activity change. This change can be explained by the observed change in the V max.

* * * *

4 . 2. Kinetic investigations into the observed enzyme activity changes using the method of Brownson and Watts (1973).

The experiments outlined in section 4.1 had been completed when Brownson and Watts (1973) published results indicating that the colour reagent, 5, 5' dithiobis 2 - nitrobenzoic acid (DTNB) in the method of Ellman et al (1961) caused, itself, a modification of cholinesterase activities. They reported that the presence of DTNB in the incubation medium at a concentration of 0.35 mM caused a 30% activation of human serum acetylcholinesterase at 30°C. pH 7.4. The effect was, predominantly, related to a change in the Km value from 0.55×10^{-4}M. in the presence of DTNB to 0.83×10^{-4}M. in the presence of 2, 2' dithiodi - pyridine (aldrithiol - 2). This latter substance was reported to be free of any such kinetic interactions. The measurement of acetylcholinesterase activity, they report, is further complicated by the fact that the 5' thio 2 - nitrobenzoate ion also appears to interact with the enzyme, resulting in slightly lowered absorbance values. They also reported that the acetylthiocholine substrate competes for the DTNB - binding site so that activation is essentially eliminated by saturating concentrations of substrate.

They explained this by suggesting that DTNB binds to the catalytically active sub - unit and either directly influences the catalytic process or causes a limited conformational change that does not affect sub - unit interactions. The DTNB would, thus, be displaced from this site by high substrate concentrations. This suggestion of a non - catalytic substrate binding site on acetylcholinesterase is compatible with the finding that eel acetylcholinesterase also binds acetylcholine at sites on the enzyme other than the catalytic site (Changeux 1966).

Brownson and Watts (1973) concluded that reliable kinetic studies could only be carried out with aldrithiol - 2 but that DTNB was acceptable for routine studies.

They stated that:

".....although the coupled assay with NbS_2 (DTNB) is both sensitive and easy to use the nature of the interactions with both human acetylcholinesterase and pseudo - cholinesterase, and probably cholinesterases from other sources as well, make it quite unsuitable for reliable kinetic investigations. Aldrithiol - 2, on the other hand, appears to be free from any of these difficulties.

.....the disadvantage of aldrithiol - 2 ..... is that its molar extinction coefficient is only about half that of NbS_2 (DTNB)."

For these reasons the kinetic investigations conducted previously (see section 4.1.) were re - investigated using the method of Brownson and Watts (1973).

The first investigation was to confirm the activation of acetylcholinesterase activity by DTNB on the enzyme from the rat brain. Enzyme homogenates, containing 2 mg. (approx.) wet weight caudate nucleus tissue, were made up, as outlined in section 2.2.8., from quiet control animals. The assays, using the method of Brownson and Watts, were routinely conducted as follows:

Sodium barbital buffer	2.55 ml.
Acetylthiocholine	0.25 ml.
Enzyme homogenate	0.10 ml.
Aldrithiol - 2	0.10 ml.

All measurements were made with a Pye Unicam SP 1800 spectro - photometer coupled with a Pye - Unicam AR 25 linear recorder. Silica cells of 3.0 ml. capacity and 10 mm. path length were used. The cell contents were equilibrated for 4 mins. to 22°C. and the enzyme added to initiate the reaction. The cell contents were mixed intimately and the increase in absorbance at 343 nm. was continuously monitored for 4 mins. This procedure was conducted, in duplicate for each enzyme sample.

The progress curve was linear for at least 8 minutes. Initial velocities were measured from the slope of the recorder trace and are expressed as O. D. units /min./ mg. proteins.

The results, shown in Figures 25, 26, 27 and 28 are presented using a Lineweaver - Burk (1934) plot and the lines of best fit to the data were compared by using a computerised linear regression analysis which places equal emphasis on all points.

Figure 26 shows that the results of Brownson and Watts are supported by the data from the rat brain enzyme. A 25% activation of acetylcholinesterase activity was observed with DTNB present in the incubation medium as compared with the rates of hydrolysis of acetylthiocholine measured with aldrithiol - 2. This effect is predominantly related to a change in the Km value of the enzyme from 0.95×10^{-4} M. (in the presence of DTNB) to 1.55×10^{-4} M. (in the presence of aldrithiol - 2), see Fig. 25. It is confirmed that there is little or no activation of enzyme activity at saturating concentrations of the substrate as shown by the intercepts on the abscissa, indicating an identical V max.

Having confirmed the activation of rat brain acetylcholinesterase by DTNB the effects described in section 4.1 were re - investigated. The same brain region samples as for 4.1 were investigated following the same behavioural treatments. The results are shown in Figures 27, 28 and 29.

Figure 27 shows in the medulla the activity of the enzyme at V max. from the anaesthetised animals was 24% greater than for the quiet control animals. No difference in the Km values for the two enzyme samples was observed (1.58×10^{-4} M.). These observations confirm the data from the earlier experiments, (section 3.3.2.) where a 38% difference in enzyme activity was observed between the enzymes from anaesthetised and saline - injected control groups (section 4.1.) where a 28% difference in enzyme activity at V max. was observed with enzyme samples from anaesthetised and quiet control group animals.

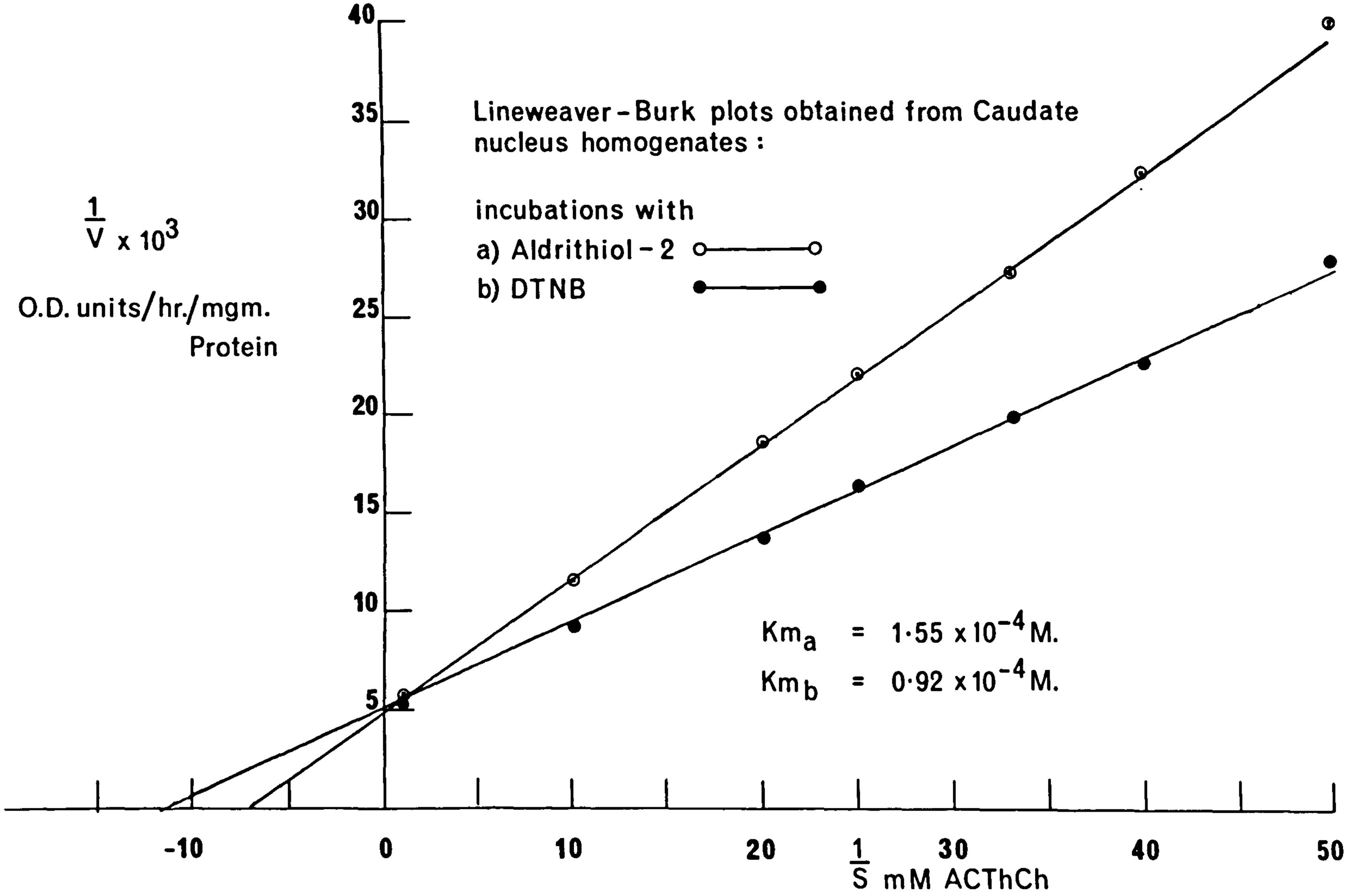

Lineweaver-Burk plots obtained from Caudate nucleus homogenates :
incubations with
a) Aldrithiol-2
b) DTNB
$\frac{1}{V} \times 10^3$
O.D. units/hr./mgm. Protein
40
35
30
25
20
15
10
5
$Km_a = 1{\cdot}55 \times 10^{-4}$ M.
$Km_b = 0{\cdot}92 \times 10^{-4}$ M.
-10
0
10
20
30
40
50
$\frac{1}{S}$ mM ACThCh

FIGURE 26. Double reciprocal plot of the hydrolysis of acetylthiocholine by acetylcholinesterase from the caudate nucleus.

(b) Incubations of the enzyme with DTNB present.

(a) Incubation of the enzyme with aldrithiol - 2 present.

Note that the activating effect of DTNB is predominantly related to a change in the Km value. The identical intercepts on the abscissa indicate an identical V max. The activation is not seen at saturating concentrations of the substrate.

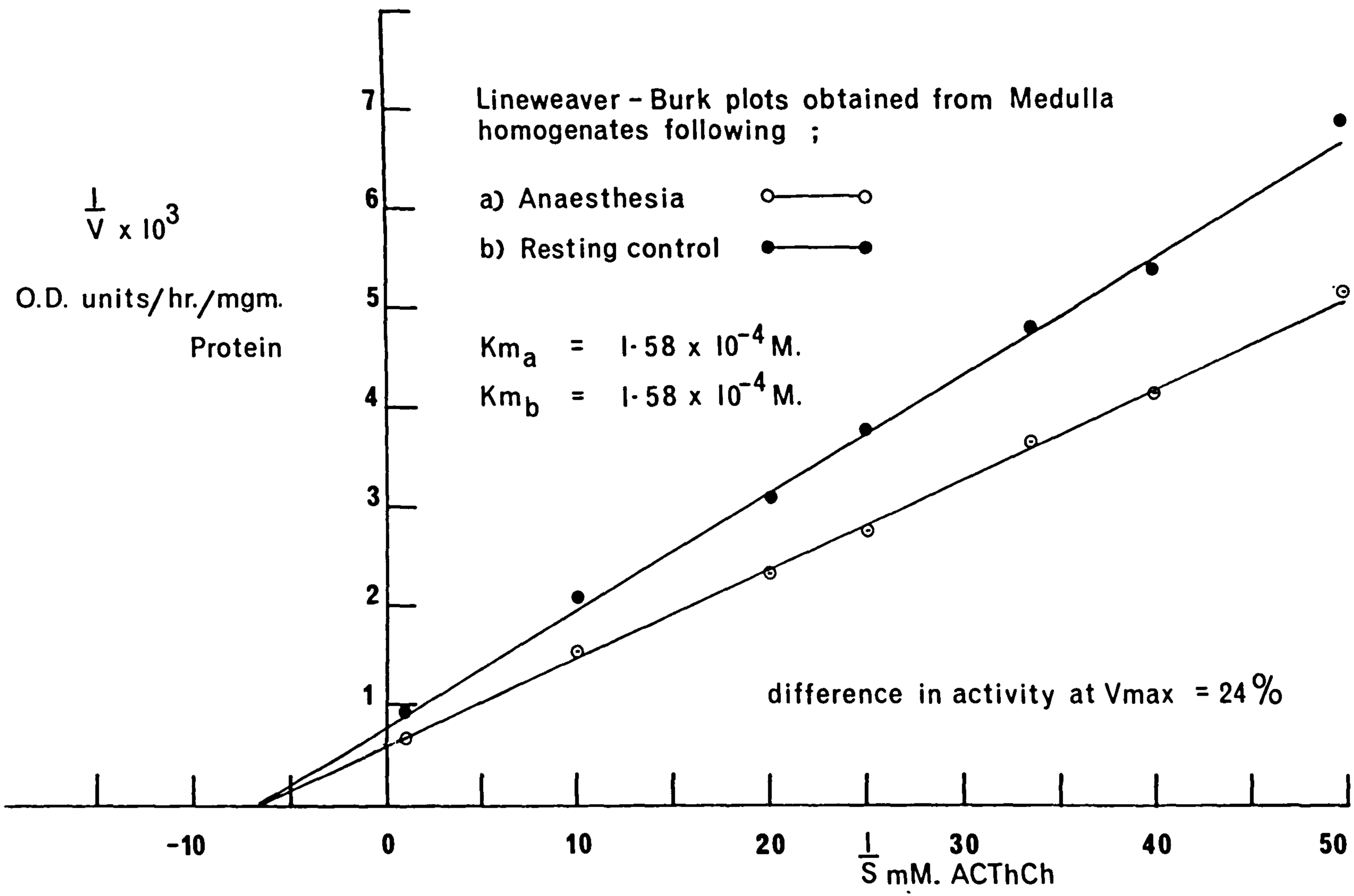

Lineweaver - Burk plots obtained from Medulla homogenates following ;
a) Anaesthesia
b) Resting control
Km_a = 1·58 x 10^{-4} M.
Km_b = 1·58 x 10^{-4} M.
difference in activity at Vmax = 24 %
$\frac{1}{V} \times 10^3$
O.D. units/hr./mgm. Protein
7
6
5
4
3
2
1
-10
0
10
20
30
40
50
$\frac{1}{S}$ mM. ACThCh

FIGURE 27. Double reciprocal plot of the hydrolysis of acetylthiocholine by acetylcholinesterase from the medulla.

(b) Incubations with the enzyme from quiet control animals.

(a) Incubations with the enzyme from anaesthetised animals.

There is no change in the Km value but only in the V max.

Lineweaver - Burk plots obtained from Thalamus homogenates following ;

a) Anaesthesia

b) Resting control

$Km_a = 1{\cdot}6 \times 10^{-4}$ M.

$Km_b = 1{\cdot}6 \times 10^{-4}$ M.

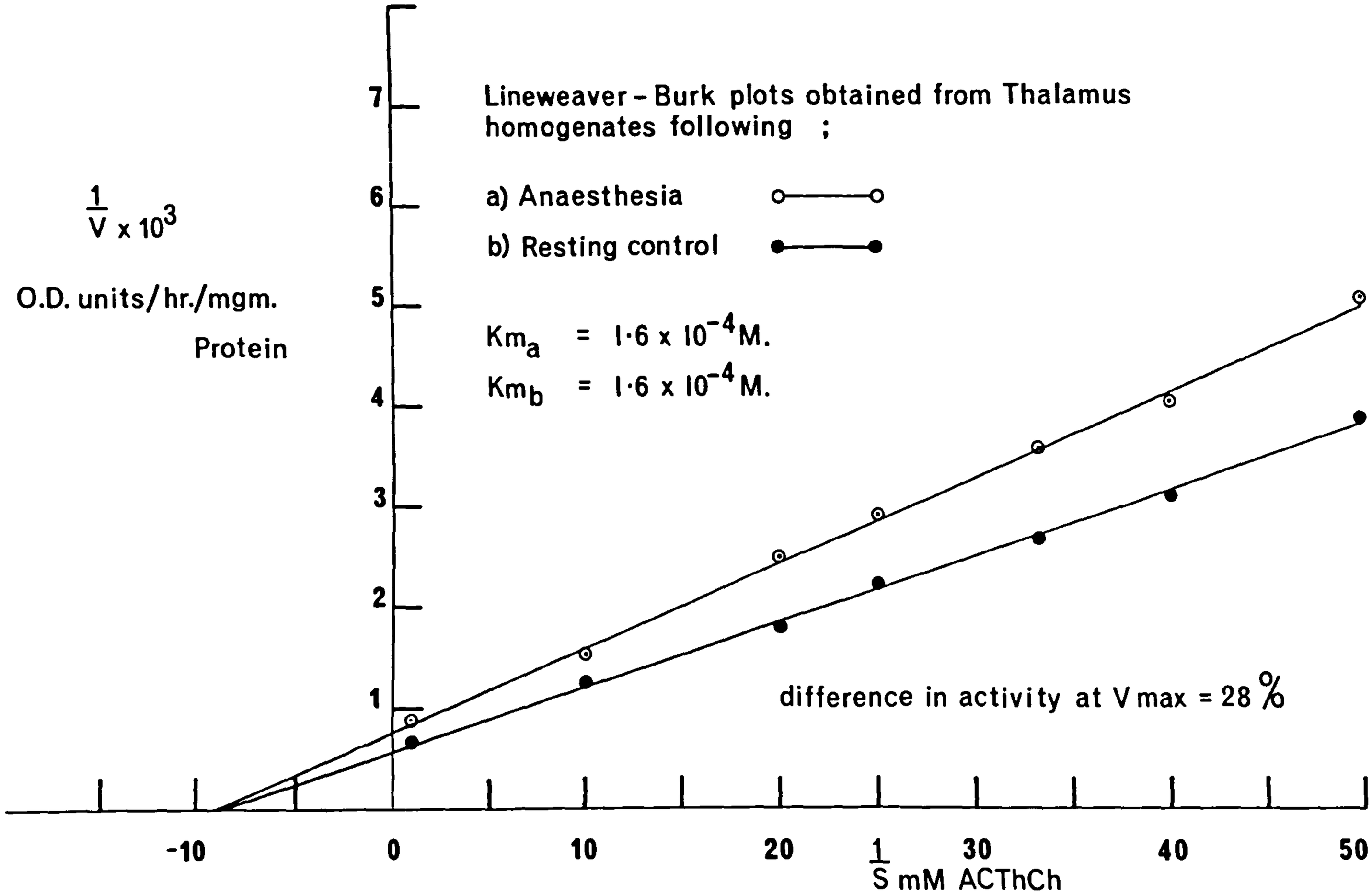


FIGURE 28. Double reciprocal plot of the hydrolysis of acetylthiocholine by acetylcholinesterase from the thalamus.

(b) Incubations with the enzyme from quiet control animals.

(a) Incubations with the enzyme from anaesthetised animals.

There is no change in the Km value but only in the V max.

Lineweaver-Burk plots obtained from Caudate nucleus homogenates following ;

a) Anaesthesia

b) Resting control

$Km_a = 1{\cdot}54 \times 10^{-4}$M.

$Km_b = 1{\cdot}54 \times 10^{-4}$M.

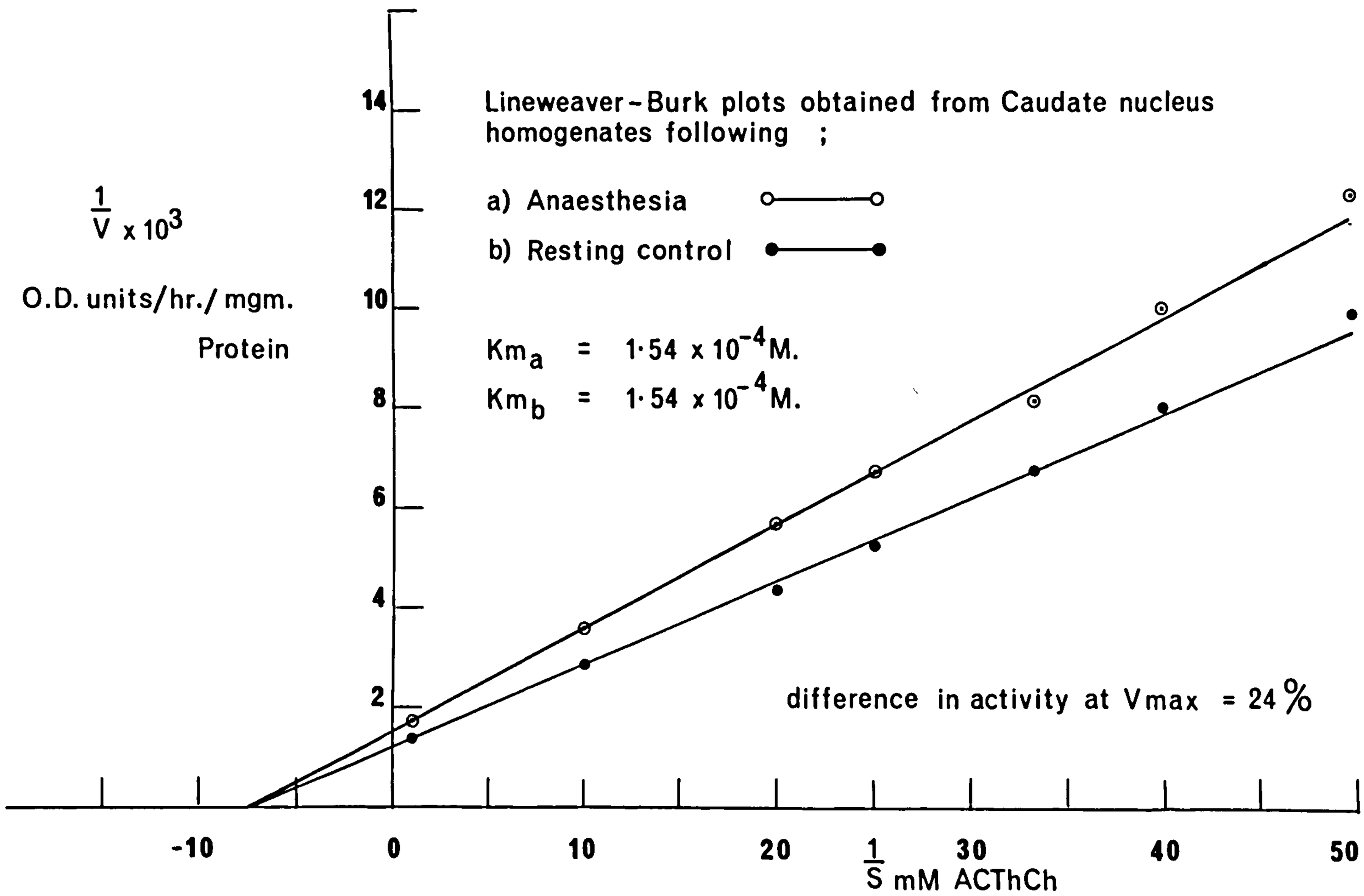


FIGURE 29. Double reciprocal plot of the hydrolysis of acetylthiocholine by acetylcholinesterase from the caudate nucleus.

(b) Incubations with the enzyme from quiet control animals.

(a) Incubations with the enzyme from anaesthetised animals.

There is no change in the Km value but a change in the V max.

In the thalamus (Figure 28) the activity of the enzyme from the quiet control group animals was observed to be 28% greater than that from the anaesthetised animals. Again, no change in the Km value was observed (1.6×10^{-4}M). This data compares with the 39% decrease in enzyme activity following anaesthesia reported in section 3.3.2. and the 35% decrease reported in section 4.1.

In the caudate nucleus (Figure 29) a similar picture is seen. Comparing, this time, anaesthetised and quiet control animals a 24% decrease in enzyme activity is observed at V max. following anaesthesia and, again, no difference in the Km values ($1,54 \times 10^{-4}$M.). This data compares with the 24% decrease in enzyme activity following anaesthesia reported in 3.3.2. and the 32% difference in enzyme activities reported in 4.1. comparing the enzymes obtained from anaesthetised and trained animals.

It can be seen from the foregoing results that the activity of acetylcholinesterase is affected quite markedly, in some regions of the brain, following anaesthesia yet not in others. It is, therefore, conceivable that:

a) the pentobarbitone - sodium is eliciting these effects by some mechanism in specific and localised areas only (see 1.11).

b) these regional effects were due to a direct action of the anaesthetic on enzyme activity but that, due for example, to the heterogeneous nature of the cerebral blood flow, differential regional concentrations of the anaesthetic could possibly occur, thus accounting for the observed effects.

In order to test hypothesis (b) an experiment was conducted to determine whether pentobarbitone - sodium could affect, <u>in vitro</u>, the activity of acetylcholinesterase. It was calculated, on the basis of data presented by Sharpless (1965) that approximately 10% of an intra - peritoneally injected dose of pentobarbitone - sodium would be present in the brain 10 mins. following the injection. Using this evidence, an

homogenate of rat brain caudate nucleus tissue was prepared (as described in section 2.2.8.). Three concentrations of pentobarbitone - sodium were prepared by diluting the standard Nembutal solution (60 mg./ml.). with sodium barbital buffer pH 8.2. The dilutions were a) 6.0 mg./ml. b) 0.60 mg./ml. and c) 0.060 mg./ml. The enzyme activities at 0.1, 0.5 and 1.0 mM acetylthiocholine substrate concentrations were performed, in triplicate, at 22°C in sodium barbital buffer pH 8.2. The assays were conducted as outlined in 4.2.

The results, shown in Table 18, indicate that there is no direct effect on the activity of acetylcholinesterase by pentobarbitone - sodium.

* * * *

4 . 3. Summary.

The results of the kinetic investigations are summarised in Table 19.

a)The unifying feature of both studies, using the methods of Ellman et al (1961) and Brownson and Watts (1973) is that in all cases the observed enzyme activity changes were explicable in terms of an altered V max. value and that no change of the Km value was involved.

b) The activation effect of DTNB on acetylcholinesterase (Brownson and Watts 1973) was investigated using rat brain enzyme and the effect confirmed.

c) Subsequent investigations carried out using aldrithiol - 2, while changing the Km value of the enzyme as compared with the investigation using DTNB, all gave similar results to those found with DTNB.

d) An experiment, conducted to test the hypothesis that the activity of acetylcholinesterase could be affected directly by pentobarbitone - sodium, showed that no effect was observed in vitro as a result of enzyme incubations carried out in the presence of varying concentrations of the anaesthetic.

* * * *

TABLE 18. The effect of pentobarbitone - sodium on AChE activity in vitro.

Dose	0.1 mM.		0.5 mM.		1.0 mM.	
None	0.0337	0.0004	0.0600	0.0005	0.0872	0.0004
+ 0.06 mg./ml.	0.0330	0.0005	0.0595	0.0003	0.0880	0.0006
+ 0.60 mg./ml.	0.0320	0.0003	0.0589	0.0004	0.0875	0.0006
+ 6.0 mg./ml.	0.0322	0.0003	0.0592	0.0005	0.0871	0.0005

Enzyme activities are expressed in Optical density units/min. $\pm$ S.E.M.

N = 3 in each group.

None of the differences were significant.

The incubations are as described in section 4.2.

TABLE 19. Summary of the results of the Kinetic investigations.

Behavioural treatment	DTNB		ALDRITHIOL - 2	
	Km	% diff.Vmax.	Km	% diff. Vmax.
Quiet control	0.92×10^{-4}M.		1.55×10^{-4}M.	
Quiet control (medulla)	0.8×10^{-4}M.	28%	1.58×10^{-4}M.	24%
Anaesthesia (medulla)	0.8×10^{-4}M.		1.58×10^{-4}M.	
Quiet control(thalamus)	1.2×10^{-4}M.	35%	1.6×10^{-4}M.	28%
Anaesthesia (thalamus)	1.2×10^{-4}M.		1.6×10^{-4}M.	
Trained (caudate nucleus)	1.2×10^{-4}M.	32%	-	
Anaesthesia (caudate)	1.0×10^{-4}M.			
Quiet control(caudate)	-		1.54×10^{-4}M.	24%
Anaesthesia (caudate)	-		1.54×10^{-4}M.	

In each case the change in enzyme activity observed following the behavioural treatments is accountable for by a change in V max.

In only one case was a change in Km observed.

4 . 4. Discussion.

The properties and kinetic constants for rat brain acetylcholinesterase, found in this study, are listed in Tables 18 and 19. Chan et al (1972 a) report two forms (A and B) of the enzyme from bovine brain:

	A	B
Km acetylthiocholine	0.87×10^{-4}M.	1.4×10^{-4}M.
Optimal substrate concn.	1.25 - 1.5 mM.	1.0 - 1.25 mM.
pH optimum.	8.2	7.8

These values were obtained using the method of Ellman et al (1961) at 25°C. pH 8.0. They also report that the partly purified enzyme was very stable and that even at a very high dilution of the enzyme (15 - 60 µg./ml.) the enzyme activity was completely stable for one week.

Bajgar and Zizkovsky (1971) have reported two iso - enzymatic forms of acetylcholinesterase from the rat brain. They report their kinetic constants as:

	A	B
Km acetylthiocholine	$0.7 - 2.0 \times 10^{-4}$M	$0.7 - 2.0 \times 10^{-4}$M.
pH optimum.	7.8 - 8.2	7.8 - 8.2

These measurements were conducted at 25°C. and pH 8.0.

Kaplay and Jagannathan (1970) report the kinetic constants for the ox - brain as:

Km acetylthiocholine	0.7×10^{-4}M.
Optimal substrate concn.	3.3 mM.
pH optimum.	7.5 - 8.5

These measurements were conducted at 30°C pH 7.5.

These literature results on the properties of mammalian acetylcholinesterase agree well with those reported in this study.

Morero et al (1972) conducted kinetic studies into the allosteric properties of erythrocyte acetylcholinesterase, by fluoride inhibition, from fat - deficient rats. They reported that a transition from abnormal to normal allosteric - type kinetics was observed within 8 days of return to a normal fat diet. This was explained by suggesting that the structure of the membrane could be responsible for the phenomenon of phenotypic allosteric desensitisation in membrane - bound enzymes.

Evidence from a study into the inter - relations between behaviour and the central cholinergic nervous system (Smissaert and Hitman 1971) indicated that the acetylcholinesterase from the brains of Tryons' rat strains, which have been reported to differ genotypically in maze - learning ability and acetylcholinesterase activity, was identical. The results of heat - denaturation studies indicated that a structural difference was improbable and that the observed enzyme activity differences should be due to differences in the amount of enzyme per unit weight of brain tissue. This is evidence for acetylcholinesterase activity differences in the rat brain being explicable in terms of an altered Vmax. value of the enzyme.

Waser and Schaub (1972), using homogenates of the synaptosome fraction of Guinea-pig cortex, have reported that phenobarbital has no direct effect on the activity of acetylcholinesterase.

* * * *

SECTION FIVE

5 . 1. General discussion.

The hypothesis that acetylcholine is a transmitter in the mammalian central nervous system has not, as yet, been completely established (Krnjevic 1969). However, acetylcholine is known to have both excitatory and inhibitory actions on central neurones in many regions of the brain and in certain structures there is growing and convincing evidence for cholinergic synaptic transmission. These include the cerebral and cerebellar cortices, thalamus, lateral and medial geniculate nuclei and the caudate nucleus (Phillis 1970, McLennan 1970).

Acetylcholinesterase, a stable and relatively easily measurable component of the cholinergic nervous system, has been singled out for studies into the possible inter - relations between the central cholinergic nervous system and behaviour. Its function in transmitter breakdown and predominant localisation at the post - synaptic membrane places it in a key position to effect alterations in synaptic security and, hence, neuronal activity via changes in enzyme activity.

The results, presented in this study, indicate that acetylcholinesterase is capable of undergoing relatively rapid changes in its activity in response to various behavioural treatments and that this is possibly due to an alteration in the number of active sites available for hydrolysis of the substrate rather than in an alteration of the affinity of the enzyme for its substrate. The direction of the enzyme activity changes seem to bear a direct relation to the state of activation of the animal.

Several studies (see Tables 3 - 7) have shown that acetylcholinesterase is capable of undergoing changes in its activity in response to a variety of behavioural treatments. These studies also indicate that,

in general, the enzyme activity varies directly with the state of activation of the animal. The results of Oba et al (1972) amply demonstrate that the enzyme is capable of undergoing rapid and significant changes in its activity. They reported that the activity of acetylcholinesterase decreased significantly in the hypothalamus of lactating rats within 2 minutes of receiving a suckling stimulus and that this effect was reversible within 15 minutes. They concluded that a cholinergic mechanism might function in the release of oxytocin.

The mechanism of the enzyme activity changes remains to be established. It remains, also, to be established whether these changes in acetylcholinesterase activity could cause alterations in synaptic security and neuronal activity and whether these would have any behavioural significance e.g. in learning and memory.

With regard to the mechanism of the enzyme activity changes there are many possibilities which can be invoked. However, taking into account the data presented in this study which indicates a pure V max. change the following explanations should be considered:

a) <u>Synthesis and release of an effector or inhibitor</u>.

This explanation would assume the release of a molecule during synaptic stimulation which combines with the enzyme to effect an alteration in enzyme activity. The effect must be a non - competitive one since no effect on the Km is observed and, hence, the combination with the enzyme must be at a second site. In this case the binding of the substrate would not be affected but the rate of its hydrolysis would be altered. This molecule must be quite firmly attached, perhaps even irreversibly, to account for the persistence of the enzyme activity changes.

Evidence for the production of a naturally occurring anti-cholinesterase by the cockroach has been reported by Menn and McBain (1968).

They reported that it was of a phospho-lipid nature and was produced within four hours of stressing nymph cockroaches. Anti-cholinesterases of the organo-phosphate type are generally regarded as irreversible inhibitors.

b) Rapid alteration of the rate of enzyme protein synthesis.

If the turnover of acetylcholinesterase were of a sufficiently high rate then the rapid alteration of the rate of enzyme protein synthesis would lead to a change in enzyme activity at the synapse. Dingman and Sporn (1964) stated:

> "Since protein synthesis is one of the most fundamental of all cellular processes and since the proteins of a cell are largely responsible for its behaviour one could expect that the process of memory storage in a neuron might well involve some participation of the protein synthesising mechanism. It is not surprising to find that this mechanism may undergo some change of state during cellular activity, or that interference with this mechanism may cause changes in the overall behaviour of a cell".

In support of the hypothesis of the rapid alteration of the rate of acetylcholinesterase synthesis is a study by Harris et al (1971). They measured the possible contribution of de novo synthesis of acetylcholinesterase to the return of enzyme activity in the retinae of guinea-pigs poisoned with the anti-cholinesterase agents soman (pinacolyl methyl phosphonofluoridate) or sarin (isopropyl methyl phosphonofluoridate). Their results indicated that the acetylcholinesterase activity of the retinae recovered significantly, in surviving animals, between 30 minutes and 6 hours after poisoning with either compound. Treatment of poisoned guinea-pigs with cycloheximide, an inhibitor of protein synthesis, markedly reduced both survival and the recovery of acetylcholinesterase activity. While de-phosphorylation of the poisoned enzyme was a factor

in the recovery of sarin-poisoned animals, they concluded that:

> "....in soman-poisoned guinea-pigs the return of acetylcholinesterase activity and survival appears strongly related to the synthesis of new enzyme protein".

In support of the hypothesis of the rapid inductions of acetylcholinesterase synthesis are the studies of Blume et al (1970) and Hiller and Simon (1973). Blume et al (1970) reported that the specific activity of acetylcholinesterase in a mouse neuroblastoma cell line increased greatly during restricted cell-division, i.e. during the stationary phase or when serum was removed from the culture medium. They presented preliminary evidence that protein synthesis may be involved in this induction.

Hiller and Simon (1973) investigated this phenomenon of the induction of acetylcholinesterase in cell-cultures incubated in the presence of levorphanol, a congener of morphine. They reported that levorphanol inhibited this increase in the concentration of acetylcholinesterase at concentrations of the drug which did not affect overall protein synthesis nor were the base levels of the enzyme affected by the presence of the drug. They concluded that this effect of levorphanol had a certain specificity for the synthesis of acetylcholinesterase. It was also reported that the enzyme, as in other systems, appeared to be largely membrane-bound and that levorphanol did not affect the distribution of the enzyme between membrane and supernatant fractions.

Much research has been directed towards the identification of 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-hydroxy tryptamine and DOPA and a 30% inhibition of incorporation by adrenaline and nor - adrenaline. No effect on incorporation was reported for either ACh or GABA. It was also reported

that these effects on the incorporation were not reversed by ATP, ADP or cyclic AMP. Although these studies were carried out at concentrations of these compounds of 10^{-3}M. it was concluded that synaptic protein synthesis could be affected by putative transmitters. Granitsas (1970) measured the incorporation of ^{14}C-alanine and ^{14}C-leucine into protein in the white blood cells of the rat. He reported that the presence of acetylcholine in the medium caused an increase in the incorporation. Whether this effect was caused by an increase in the rate of amino acid transport or whether it was caused by a stimulation of protein synthesis was unknown.

Guth et al (1967) studied the role of nerve impulses and acetylcholine release on the regulation of cholinesterase activity of adult rat soleus muscle. They reported that miniature end-plate potentials and the random quantal release of acetylcholine were not important in the neural regulation of cholinesterase. Tenotomy produced no decrease in cholinesterase activity and so it was concluded that the nerve impulses themselves were not essential to this neurotrophic process. Studies using electrotherapy on denervated muscle failed to retard the rate of loss of cholinesterase activity. Since neither random quantal release of acetylcholine, impulse-directed release of acetylcholine nor muscular contractions were important in the neural regulation of cholinesterase activity an alternative neurotrophic mechanism was proposed:

> "....perhaps the synthesis of this protein is regulated by the transmission of specific messenger substances or de-repressor agents from nerve to muscle.
>
> .....an attractive hypothesis is that the nerve liberates either messenger nucleic acids or de-repressor agents at the nerve terminals and thereby help to direct the synthesis of specific proteins by the muscle".

Rose and Glow (1967) studied the effects of denervation on the <u>de novo</u>

synthesis of muscle cholinesterase and the effects of acetylcholine availability on the acetylcholinesterase activity of the retina. They reported that acetylcholine availability had no effect on muscle cholinesterase but that it did increase the rate of retinal acetylcholinesterase synthesis. They put forward two explanations of this observation:

a) acetylcholine availability regulates acetylcholinesterase synthesis

b) transfer of specific substances from the axon

Donnelly et al (1973) have reported the existence of a heat-stable protein from lobster tail muscle (M. Wt. 34,000 daltons) which modulates the activity of cyclic nucleotide dependent protein kinases. This modulator is similar to the inhibitory factor reported in rat and rabbit skeletal muscle. The protein is reported to be capable of existing as aggregates and that different aggregates can have either activating or inhibitory effects upon the kinase. They suggest that a possible function, in vivo, is to modulate the substrate specificity of the protein kinase.

Kelly and Luttges (1972), however, reported that drugs known to produce a facilitation of learning and memory did not affect protein synthesis in vitro. They suggested that, since there was no direct effect on amino acid uptake or protein synthesis, some secondary effect upon protein synthesis might be possible.

Other groups have studied the effects of behavioural treatments on the modification of the rate of central protein synthesis. Dunn (1971) reported that the amnesia caused by electroshock is the result of protein synthesis inhibition caused by the electroshock. He suggested that the protein synthesis component of consolidation of the memory trace was erased and that a possible site for this effect was at the nerve ending. Aleksandrovskaya et al (1972) reported that the administration of 6 electric shocks spaced at 5 minute intervals resulted in the inhibition of protein synthesis in the hippocampus and that this was followed by an activation of

synthesis after 2 hours. Glazer and Sartorelli (1973) have reported that phenobarbital caused a stimulation of nascent protein synthesis of 2.6^{x} in 3 - 14 hours on free and membrane-bound polyribosomes of normal and regenerating liver. It is, therefore, possible that phenobarbital might have similar effects on the rate of central protein synthesis in general and on that of acetylcholinesterase in particular.

In a recent series of papers Richardson and Rose (1973 a) reported striking differences in the pattern of incorporation of lysine into soluble and insoluble proteins of the retina occur in dark-reared rats on first exposure to light. Using a double-labelling technique they reported significant differences in incorporation into 13 of 41 protein fractions studied after 1 hour of light exposure. These 13 fractions were termed high differential activity fractions and histochemical examination demonstrated that acetylcholinesterase was among these.

Richardson and Rose (1973 b) report similar effects in the visual cortex where differential incorporation of lysine was reported in 2 fractions of the soluble proteins and 7 fractions of the insoluble proteins. They suggest 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 the 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. The results of these studies are combined to conclude that:

a) The protein synthesis changes, as shown by the enhanced incorporation of lysine into the high differential activity fractions, may be directly associated with synaptic modification.

b) That, taken in combination with other observations, the rates of

a number of biochemical processes are maintained in a delicate equilibrium responding to changes in the behavioural states of the animal and to changes in the environment in measurable ways.

Rose et al (1973) propose the general term "state-dependence" to describe this relationship of neurochemical and environmental events. Changes in the synthesis of specific electrophoretic fractions have been reported in other stimulus-linked situations. Hyden and Lange (1970) reported increases in the amounts of the S-100 protein in the hippocampus of rats during learning to use the non-preferred paw. This same learning task is also accompanied by increases in the specific activity of protein fractions containing the 14-3-2 protein.

The S-100 protein was the subject of a study conducted by Stewart and Urban (1972). They measured changes in the rate of the appearance of the protein during the development of the central nervous system of the mouse. They concluded that the control of the accumulation of the protein during development is as a result of the modulation of the rates both of synthesis and degradation. The degradation of enzyme protein and rapid alterations in the rate of degradation might also be a possible mechanism to explain the observed changes in acetylcholinesterase activity. There is, also, a certain amount of evidence in the literature that function - dependent alterations in the activity of proteinases occur. Glushchenko (1971) has reported thatprolonged motor excitation in the rat leads to an increase in the activity both of acid and neutral proteinase activity in the brain. Poberai et al (1972) reported that following supra-maximal stimulation of rat motor end-plates there occurs an intense proteolytic activity. It was suggested that the degradation products of this function-dependent axo-terminal proteolysis (oligopeptides, amino acids etc.) may take part in the process of synaptic impulse transmission by a retrograde axoplasmic flow. In this manner they are implicated as part of a metabolic feedback mechanism.

Bass and Moore (1971) have implicated the proteolytic enzymes at the synapse as part of a memory model. They suggest that the proteolytic enzymes selectively attack a gate protein when the axolemma is depolarised. In this manner the neuron could act as a memory element by switching from one stationary state to another with a corresponding change in the integration time.

The mechanisms proposed in this section depend to a large extent on the rapid alteration on the rate of production and transport of enzyme protein. Evidence in support of the rapid production and transport of acetylcholinesterase is given by the work of Ranish and Ochs (1972). They reported that the rate of axoplasmic transport of acetylcholinesterase was 431 mm./ day or about 2 cm. / hour. The rate of retrograde transport of acetylcholinesterase was found to be 220 mm/ day. They also reported that the freely moving fraction of the enzyme was estimated to be 15% of the total enzyme present in the nerve (10% forward, 5% backward). This evidence shows that the turnover time for the acetylcholinesterase at the synapse may be very fast - only a few hours may suffice to completely turn over the synaptic enzyme.

Studies currently being carried out in this laboratory to investigate whether the enzyme activity changes, observed following behavioural treatments (see section 3.3.) are also observable in cycloheximide-injected animals, are giving preliminary indications that the enzyme activity changes are not as great. It appears that a contribution of <u>de novo</u> enzyme protein synthesis is a possible explanation of the observed enzyme activity changes.

Continuing time-course studies on the activity of acetylcholinesterase in seven regions of the rat brain following 2-way shock-avoidance conditioning give preliminary indications that, when measured 4 days following the treatment, the activity changes are at least as great and possibly greater than the levels measured both after 1 hour and 24 hours.

This may be taken to indicate a possible short-term and a longer term metabolic component.

> In spite of this evidence Glassmans' statement (1969) is still true: "Many groups have shown that changes in protein synthesis occur in the brain during learning. In this respect the nervous system seems to be able to respond faster than any other tissue. The functional significance of such changes is, however, still in doubt and no cause-effect relationships can yet be postulated".

c) <u>The activation of an inactive precursor of acetylcholinesterase</u>

and d) <u>The modification of membrane structure</u>

A simple hypothesis of a rapidly increased acetylcholinesterase 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 precursor can be envisaged as being stored as an integral component of the post - synaptic membrane and activated in proportion to the amount of "traffic" in the system. This type of activation could possibly be brought about by a molecule co - released with the acetylcholine from the synaptic vesicles e.g. cyclic AMP, or by acetylcholine itself. Conformational changes taking place in the post - synaptic membrane as a result of the interaction of acetylcholine and its receptor and the subsequent depolarisation may, thus, provide a model for the activation of the transmitter removal system.

Evidence in support of this hypothesis is given by the study of Barnard and Wieckowski (1970). They studied the binding of ^{32}P - labelled DFP to the motor - end plates. They reported that 60% of the DFP binding sites were not acetylcholinesterase nor did they have cholinesterase activity. They are, thus, serine - dependent esterases. Barnard and Wieckowski (1970) conclude that they may be enzymes required for the maintenance of the synapse e.g. membrane renewal or that they could be an inactive precursor of acetylcholinesterase.

e) <u>The effects of inorganic ions</u>.

Many reports have been published which indicate that the activity of acetylcholinesterase is affected by inorganic ions (Cohen and Oosterbaan 1963, Changeux 1966, Kitz et al 1970, Roufogalis and Thomas 1970, Crone 1973, and Crone and Dawson 1973). The raising or lowering of ionic concentrations in the synaptic cleft, possibly via ionic pump mechanisms, would alter the properties of acetylcholinesterase on the post - synaptic membrane. This mechanism would be extremely flexible and may account for the diurnal variations in acetylcholinesterase activity that have been observed in the house - cricket (Cymborowski et al 1970).

Crone and Dawson (1973) concluded from their studies into the effects of inorganic ions on the kinetic parameters of mammalian acetylcholinesterase that ionic strength plays the more important role in the regulation of the activity of the enzyme, through its effects on protein conformation, in general, and on the state of acetylcholinesterase aggregation in particular (Changeux 1966, Grafius and Millar 1967). However, Crone and Dawson (1973) reported that the specific cation effects they observed were related to ion - dependent interactions with the nucleophilic esteratic or allosteric sites of acetylcholinesterase and were, thus, predominantly related to changes in the Km value.

f) <u>Allosterism or conformational changes in the enzyme</u>.

The observed changes in enzyme activity could be due to allosteric interactions or conformational changes in the enzyme protein. An allosteric effect has been defined as the effect of a modifier bound at a site which is topographically distinct from the active site. It is clear from the work of Crone and Dawson (1973) that, in this case, the modifiers are probably not inorganic ions since these lead to changes, predominantly, in the Km value.

If these changes are due to an allosteric or conformational change in the enzyme protein then, to account for the persistence of the activity

change, they must either:

1) be stabilised by an ion or effector

or 2) a second, metabolic, consolidation phase e.g. protein synthesis must begin to operate quite soon after the allosteric or conformational change.

g) Others.

It is possible that substrate or end - product inhibition may have a physiological role in the regulation of acetylcholinesterase activity via a build - up of their concentrations in the confined space of the synaptic cleft. This type of inhibition, however, would not be expected to persist for long periods.

It is, also, possible that the observed enzyme activity changes might be, at least in some measure, explicable in terms of the re - distribution of multiple forms of the enzyme e.g. iso - enzymes. Two iso - enzymes have been demonstrated for the rat brain by Bajgar and Zizkovsky (1971). A demonstration of at least 2 multiple forms of acetylcholinesterase from the rat brain on polyacrylamide gels is described in this study (see Appendix C).

Many studies have shown that large changes in iso - enzyme patterns and aggregation states of acetylcholinesterase occur during tissue development (Maynard 1966, Barron et al 1968, Dabich et al 1968 and Wilson et al 1969). Vijayan et al (1972) have reported that parathion treatment causes an alteration of the distribution of the iso - enzymatic patterns of the rat brain.

As to whether these changes in the activity of acetylcholinesterase have any behavioural significance, it may be suggested that the changes reflect some form of switching mechanism in the central nervous system and that this may be important for the selection of behaviourally useful pathways and for the inhibition of pathways leading to undesirable behavioural results. This view is consistent with the concept of

competing responses: a particular behavioural pattern is not elicited, by stimulation and experience, in a vacuum but in relation to all the other possible and competing responses available to the organism. This array of responses, available to the organism, is initially observed under even the most restricted environmental circumstances but many responses disappear as exposure to the situation continues. That these responses have not been destroyed but, merely, suppressed can be demonstrated by replacing the animal into a novel environment when many, if not all, of the original array re - appear.

Alterations in the acetylcholinesterase activity at the synapse may, by enhancing the suppression of competing responses, allow a dominant and behaviourally useful response to be expressed. In this situation the useful response would be more resistant to extinction. This view has been stated by Carlton (1963):

> ".....there are inferential grounds for supposing that the cholinergic system selectively antagonises the effects of activation on certain behavior and that the basis of this selectivity is the extent to which that behavior is un -rewarded".

It should, however, be again stated at this point that the acetylcholinesterase component of the central cholinergic transmission system does not function in an isolated fashion but as an integral part of a total 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, and even the neurochemical, significance of these enzyme activity changes must await further studies into the functioning of the total transmission system.

It is well established (Tables 3-7) that the concentrations of acetylcholine in the brain change as a result of behavioural treatments.

It must also be established whether and to what degree do the other components of the transmission system also alter in response to such behavioural treatments. In order to do this ultra-micro methods are necessary. Such a study may prove valuable at the nuclear level in the brain where electro-physiology and neuro-pharmacology are already providing evidence for cholinergic transmission but it may be necessary and ultimately possible to conduct such a study at the synaptic level.

Electro-physiological studies of the changes that take place at the synapse have already been carried out. Repetitive stimulation across spinal cord synapses leads to a facilitation of the synapses (Eccles 1964, Spencer and Wigdor 1965 and Beswick and Conroy 1965). Opposite effects have been demonstrated by Doty and Feutress (1965). Neurochemical studies are necessary because, although changes occur at the synapse, there is no evidence that they are ultimately utilised for information storage in the central nervous system. Sharpless (1964) has pointed out that learning is not due to the simple use of a neuronal pathway and he questions whether the phenomena studied by Eccles (1964) have anything to do with learning in the intact organism. Neurochemical studies, at this level, combined with electrophysiological approaches would be invaluable in resolving these problems. The interpretation of the behavioural significance of these changes in the activity of acetylcholinesterase would be a little clearer.

Weiss and Heller (1969) summarised the problems facing future neurochemical investigations into the relations between the cholinergic nervous system and behaviour when they stated:

> "We cannot avoid the fact that the problems of securing control of the fundamental processes of behavior and eliminating spurious variables are much more formidable than we recognise. We are confronted with the total nervous system, not an isolated component. And it is a system, moreover, with a history thatplays a major

role in determining the consequences of altering the normal physio - chemical processes that sustain it. The anatomic complexity of brain, its relative inaccessibility and the presence of a blood - brain barrier add to the complexity of the problem. But the obstacles are not insuperable. Scaling them demands mainly that we confront the problems directly, rather than fallaciously eliminating them by pretending that they do not exist".

* * * *

5 .2. General summary.

The experiments, described in this study have demonstrated that the activity of acetylcholinesterase from the brains of young adult female rats is responsive to environmental stimulation. Automated semi - micro methods for the assay of acetylcholinesterase and total protein were developed and used to assay the enzyme activity in twelve regions of the brain following a variety of behavioural treatments, designed to incorporate a wide spectrum of physiological stimulation. The results indicate that:

a) The activity of acetylcholinesterase appears to vary directly with the state of activation of the animal in the cerebellum, ventral cortex, thalamus and the caudate nucleus.

b) The increases in enzyme activity, exhibited in the various brain regions from both the trained and the yoked - control groups, appear to be further increased when the assays are conducted both after 24 hours and 4 days following the treatments.

c) In the 3 dorsal cortical regions no significant changes in enzyme activity were observed, as a result of any behavioural treatment, except following 2 - way shock - avoidance conditioning when all 3 regions exhibited increased enzyme activities.

d) The medulla appears to be an exception to this pattern. It exhibits increased enzyme activity following anaesthesia and decreased

activity following stimulation. Thus, in general terms, the response of the enzyme from the medulla appears to be a "mirror - image" of the trend in a). This may reflect differences in the operation of the cholinergic system in the medulla as opposed to those in other regions of the central nervous system and merits further investigation.

e) In the superior colliculus, pons and hypothalamus the enzyme activity changes, where any occur, do not appear to conform to any pattern with respect to other regions of the brain and, hence, may reflect seasonal variations in baseline activity levels. This factor also warrants further investigation.

f) No enzyme activity changes were observed following any of the behavioural treatments in either the olfactory tubercle or hypothalamus.

g) A unifying feature of both sets of kinetic investigations into the observed enzyme activity changes was that, using the methods of Ellman et al (1961) and Brownson and Watts (1973), in all samples the activity changes were explicable in terms of an altered V max. value of the enzyme and that no change in Km was observed.

h) The activation effect of DTNB on acetylcholinesterase (Brownson and Watts 1973) was investigated and the effect confirmed. Subsequent kinetic investigations using aldrithiol - 2, while altering the Km value of the rat brain enzyme as compared to DTNB, gave highly similar results to those found with DTNB.

i) An experiment, conducted to test the hypothesis that the activity of acetylcholinesterase could be affected directly by pentobarbitone - sodium, showed that no effect on enzyme activity was observed <u>in vitro</u> as a result of enzyme incubations carried out in the presence of varying concentrations of the anaesthetic.

j) Experiments, carried out to determine the effects of two - way shock - avoidance conditioning on the acetylcholine esterase activity of

animals injected with cycloheximide give a preliminary indication that the changes in enzyme activity are not so great as for untreated animals. This may be taken to indicate a significant contribution of de novo enzyme protein synthesis to the observed enzyme activity changes.

* * * *

5 . 3. Future experiments.

Further experiments could be directed at particular problems which have arisen:

1) To determine whether the enzyme activity changes are reversible and the time-course of the reversal.

2) To determine whether the enzyme activity changes occur in the absence of de novo protein synthesis.

3) Since regional effects have been demonstrated it could prove valuable to conduct a study using micro - methods on specific brain nuclei.

4) Attempts to stabilise, extract and purify the altered enzyme would prove very valuable for further comparative kinetic studies.

5) Studies to determine the nature and degree of any adaptive changes in pseudo-cholinesterase activity in regions of the rat brain, as a result of similar behavioural manipulations, would provide valuable data to compare the effects on the two enzymes.

6) In order to interpret the significance of the enzyme activity changes it is necessary to determine whether, and to what degree, do the other components of the cholinergic transmission system also alter in response to such behavioural manipulations. Studies on acetylcholine concentrations, choline acetylase activity and receptor kinetics would, therefore, be valuable.

7) A general investigation, perhaps using labelled precursors, into alterations of protein synthesis and its time-course may provide evidence for the hypothesis that changes in protein synthesis are responsible for the consolidation of functional pathways. This could possibly occur via growth of the synaptic terminals as electron - microscopic evidence has already suggested.

In the wider aspects of the work a definitive interpretation must await the results of continuing electro - physiological, neuro - anatomical and neuro - pharmacological investigations into the behavioural functions of the cholinergic pathways in the C.N.S.

* * * *

APPENDIX A.

The morphology of the brain region samples and the histochemical localisation of acetylcholinesterase.

Electron - microscopic investigations of the morphology of the samples and the histochemical localisation of acetylcholinesterase were conducted in the following manner:

a) morphology. A rat was killed by stunning and exsanguination. The brain was exposed and treated in situ with 2 or 3 mls. of 3% glutaraldehyde in cacodylate (plus 4.5% w/v. dextrose) pH 7.4 buffer. The brain was then removed and the samples dissected. The samples were then immersed in the same fixative for 4 hours at 0°C. The samples were then cut into small blocks and washed twice in buffer. The tissue blocks were then drained and post - fixed in 2% Os04 in cacodylate buffer (plus 0.25 M. sucrose) for 1 hour at room temperature. The blocks were then dehydrated in ethyl alcohol and embedded in araldite. Thin silver sections were then cut on a Sorvall Porter - Blum ultra - microtome using glass knives. The sections were then briefly counterstained in lead citrate and examined in a Phillips E.M. 300 electron microscope. A photograph showing the representative morphology of the brain tissue samples is shown in Fig.30.

b) histochemistry. A rat was anaesthetised with 3% methoxyfluorane followed by 4 mls. chloral hydrate (3.1% soln.). The rat was then perfused intra - aortically with 2% glutaraldehyde - 1% formaldehyde in cacodylate (plus 4.5% w/v. dextrose) pH7.4 buffer. The brain was then removed and the caudate nucleus sample dissected. The sample was then chopped into small blocks and immersed in the same fixative for 30 mins. at 0°C. The blocks were then washed several times in buffer over a period of 3 hours at 0°C and finally, once, in sodium hydrogen maleate buffer pH 6.5. The samples were then divided into two groups: one was incubated in the presence of acetylthiocholine substrate and the other was incubated in the absence of substrate as a control.

Incubation medium:

Sodium hydrogen maleate buffer pH 6.5 .....6.5 mls.

N. acetylthiocholine iodide .....6.5 mg.

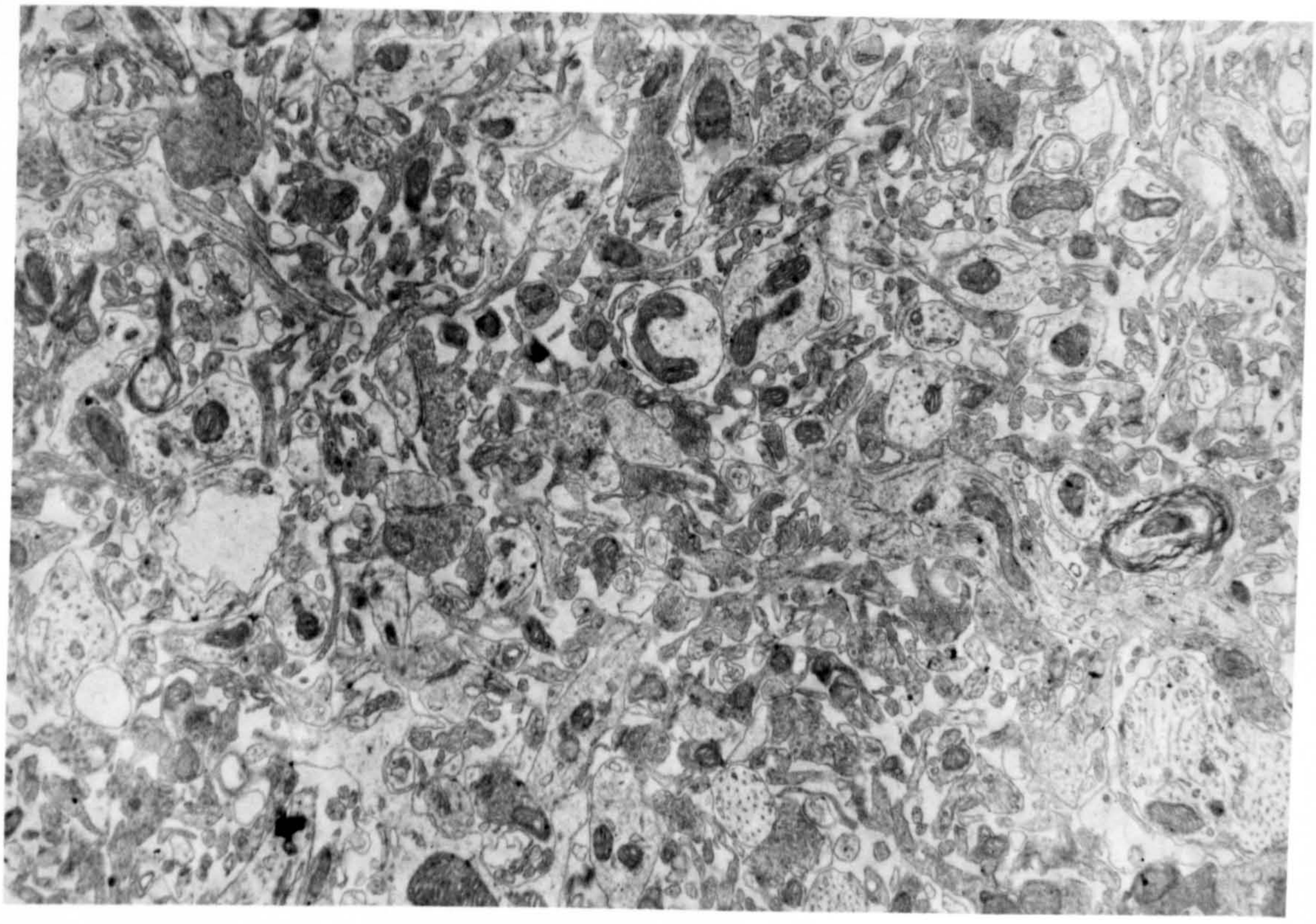
Sodium tartrate (0.6 M.) .....0.5 ml.

Supric sulphate (0.03 M.) .....1.0 ml.

Potassium ferricyanide (0.005 M.) .....1.0 ml.

Distilled water .....1.0 ml.

The samples were incubated at 0^{o}C. for 15 mins. - 1 hour. The medium was then de - canted and the blocks washed twice in cacodylate buffer. The blocks were then fixed in 2% OsO_4 in cacodylate (plus 0.25 M. sucrose) pH 7.4 buffer for 1 hour at room temperature. The blocks were then dehydrated in ethyl alcohol and processed for electron - microscopy as before. Photographs showing the localisation of acetylcholinesterase in the caudate nucleus sample are shown in Fig.31.



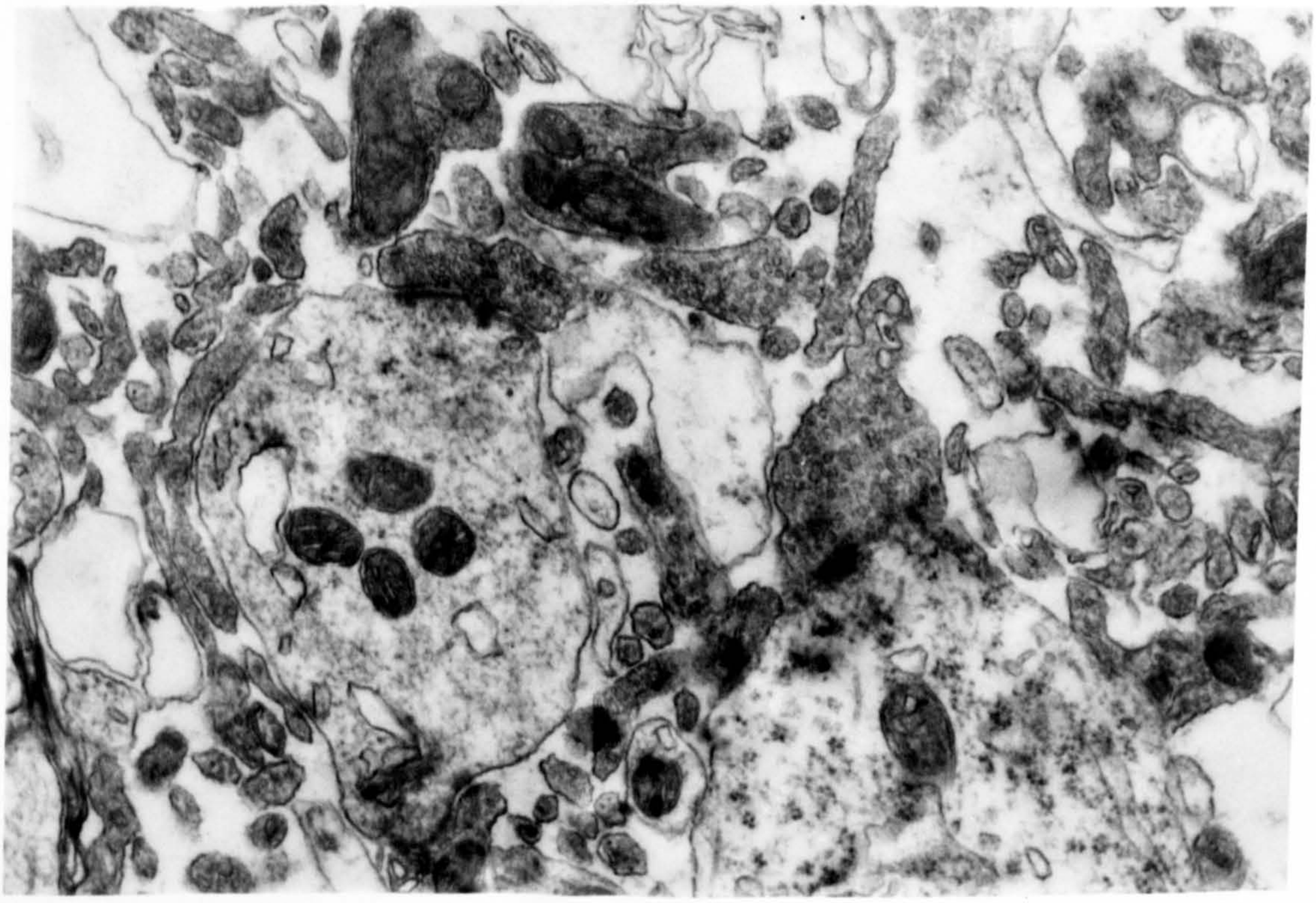
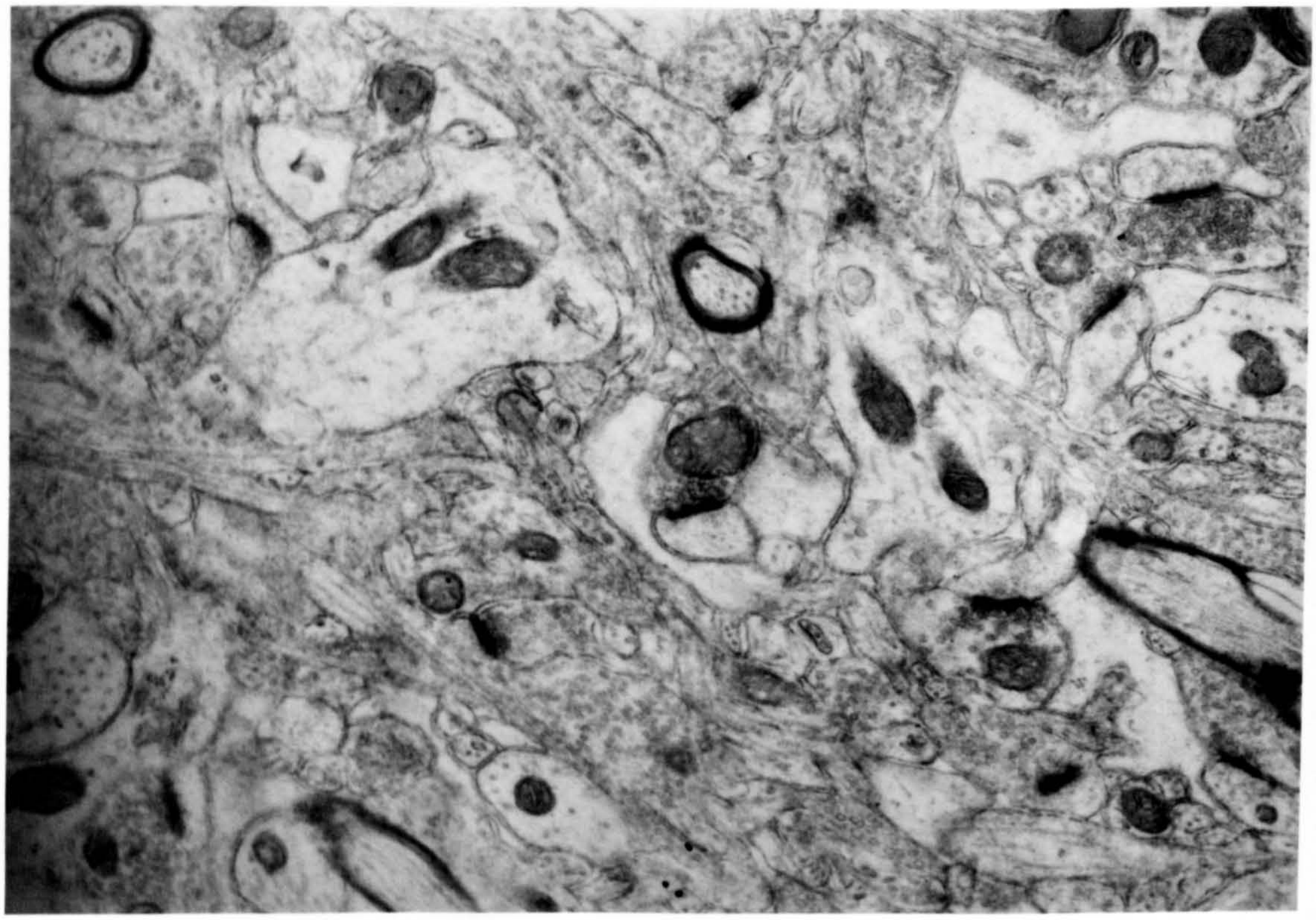


FIGURE 30. Electron-micrographs to show:

a) (top left) The morphology of the caudate nucleus sample used in the study. The final magnification is 17, 500^{x}.

b) (bottom left) Control incubation for the localisation of acetylcholinesterase activity in the caudate nucleus sample. The final magnification is 33, 500^{x}.



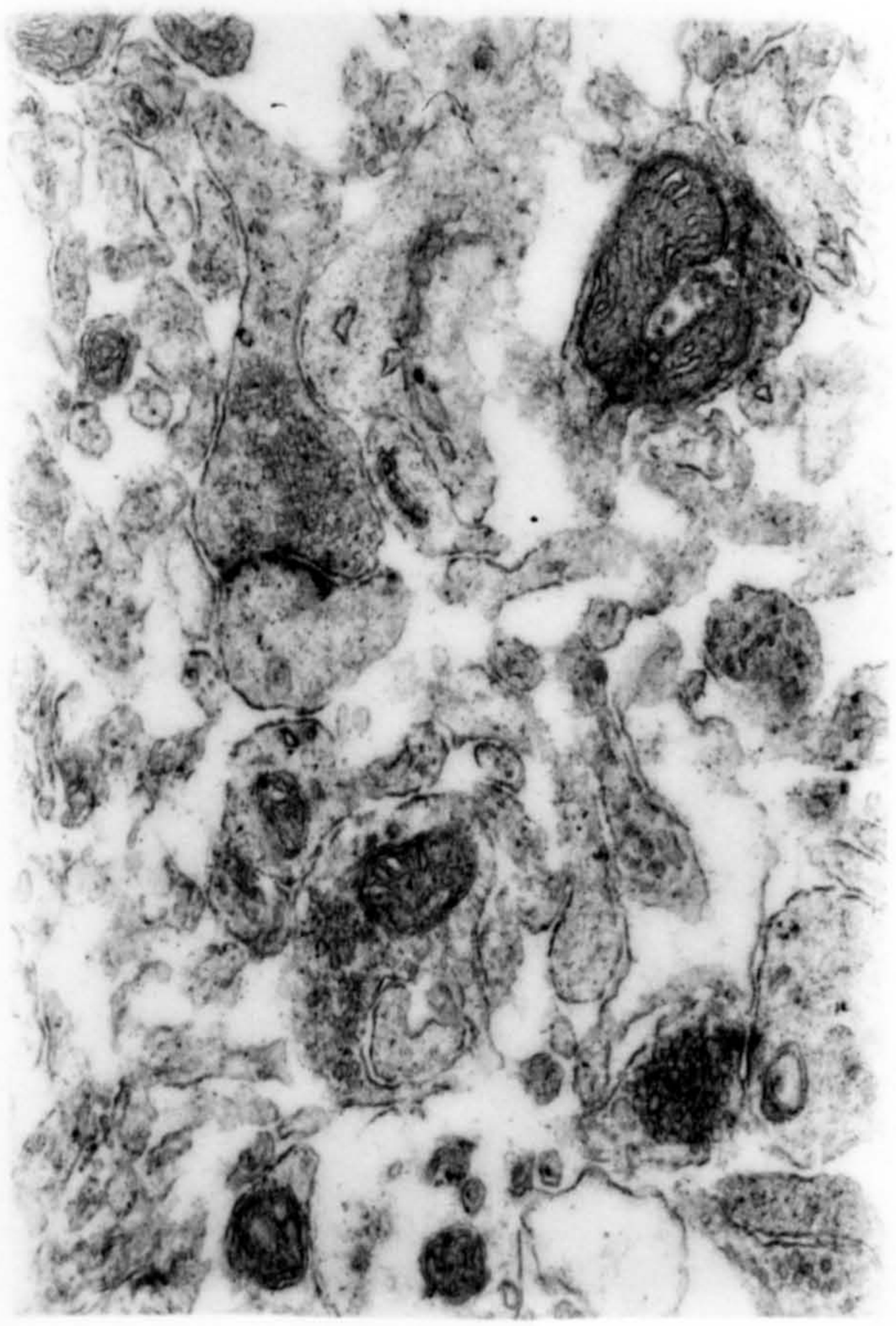


FIGURE 31. Electron-micrographs to show:

a) (top left) The histochemical localisation of acetylcholinesterase in the caudate nucleus sample. Several synapses can be seen with a significant darkening of the post-synaptic membrane. This indicates the localisation of the acetylcholinesterase. The control incubation is shown in Figure 30. The final magnification is 33, 500x.

b) (bottom left) This electron-micrograph is a detail from the plate in a) above. The nerve ending can be seen to contain vesicles, the synapse is clearly visible and the reaction product indicating the localisation of acetylcholinesterase is clearly visible on the post-synaptic membrane. The final magnification is 33, 500x.

APPENDIX B.

Details of the preparation of reagents and buffers. All solutions were made up in glass distilled water.

1) Sodium barbital buffer (pH 8.2)

Barbitone sodium ..... 1.237 g

Potassium dihydrogen phosphate ..... 0.136 g

Sodium chloride .....17.535 g

Water (distilled) .....to 1 litre

2) D.T.N.B. (for the conventional assay)

5, 5' dithiobis 2 - nitrobenzoic acid (39.0 mg.) and Sodium bicarbonate (15.0 mg.) were dissolved in 10.0 mls. of 0.1M Sodium phosphate pH 7.0 buffer.

3) D.T.N.B. (for the automated assay)

5, 5' dithiobis 2 - nitrobenzoic acid (198.0 mg.) and Sodium bicarbonate (75.0 mg.) were dissolved in 50.0 mls. of 0.1 M. Sodium phosphate pH 7.0 buffer.

4) Sodium phosphate buffer (pH 7.0)

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

5) Lowry reagent for protein estimation (conventional assay)

Sodium carbonate (anhydr.) 20g and Sodium hydroxide 4.0g were dissolved in distilled water and made up to 1 litre. To 100 mls of this reagent were added 1.0 ml cupric sulphate (1.0% w/v.) and 1.0 ml. sodium potassium tartrate (2% w/v.) This was reagent D of which 5.0 ml. was added to the protein sample. 0.5 ml. of Folin - Ciocalteau phenol reagent, diluted x $\frac{1}{2}$ was then added with immediate and thorough mixing.

6) Lowry reagent for protein estimation (automated assay)

Sodium carbonate (anhydr.) 20g and Sodium hydroxide 4.0g were dissolved in distilled water and made up to 1 litre. To 500 mls of this reagent were

added 8.0 ml cupric sulphate (1.0% w/v.) and 8.0 ml sodium D - tartrate (2.0% w/v.) and 1.0 ml. Levor 4 detergent. This mixture was pumped, with the protein sample into the auto - analyser. To this mixture was added Folin and Ciocalteaus phenol reagent diluted x 2.33 with mixing.

7) Protein solubilising fluid

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

8) Aldrithiol - 2

2, 2' dithiodi - pyridine (22.8 mg.) was dissolved in hydrochloric acid (0.2N) neutralised with sodium hydroxide (0.2N) and made up to 10.0 ml. with 0.1M sodium phosphate pH 7.0 buffer.

* * * *

Pump tubing sizes for the auto - analyser. (Please refer to Figs. 7 and 9).

a) acetylcholinesterase assay.

N acetylthiocholine iodide (76.368 mg. in 100 mls. sodium barbital pH 8.2 buffer) ..... 0.23 ml/min

D.T.N.B. (see no.3) ..... 0.10 ml/min

Sodium barbital buffer pH 8.2 (plus 2% v/v Brij - 35) ..... 0.23 ml/min

Saline (0.9% w/v. plus 2% v/v Brij - 35) ..... 1.00 ml/min

Enzyme sample ..... 0.42 ml/min

Distilled water (plus 2% Brij - 35) ..... 2.0 ml/min

b) Total protein assay.

Lowry D (see no. 6) page ..... 1.0 ml/min

Protein sample ..... 0.16 ml/min

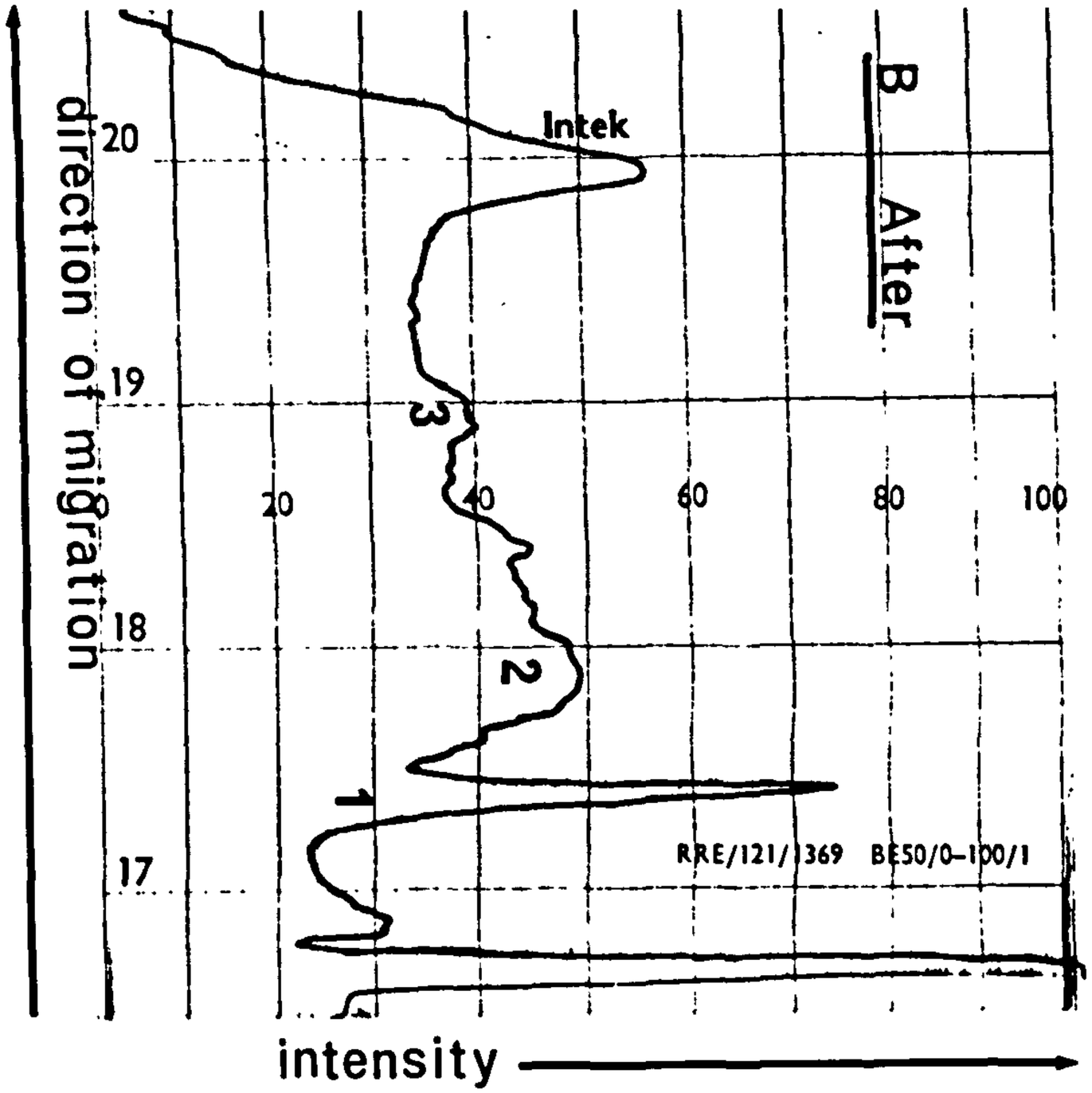
Folin and Ciocalteaus phenol reagent (see no. 6)..... 0.10 ml/min

Distilled water (plus 2% v/v levor 4) ..... 2.0 ml/min

APPENDIX C

The demonstration of multiple forms of acetylcholinesterase from the rat brain.

A rat was killed by stunning and exsanguination. The brain was removed and frozen. A sample of caudate nucleus tissue was dissected (approx. 5 mg.) and homogenised in 0.1M pH 8.0 sodium phosphate buffer at 0°C using a Voss vertical homogeniser 2,000 r.p.m. for 1 min. 0.2 ml. aliquots were then taken and layered onto 5% polyacrylamide gels in a Shandon disc - gel electrophoresis apparatus. 16 gel tubes were used in this experiment. A voltage of 100v was applied at a current per tube of 5 mAmps. The gels were run for approximately 3 hours at 4°C. At the end of this period they were removed and divided into 2 groups. One group was taken and scanned, in a Joyce - Loebl gel scanner at a wavelength of 420 nm. The gels were then removed and incubated with 1.0 mM acetylthio - choline / DTNB for 2.0 mins. The gels were then re - scanned at 420 nm. Any increase in extinction at this wavelength on the recording trace from gels following incubation with acetylthiocholine / DTNB, thus, reflects cholinesterase activity. The traces, shown in Fig.32 indicate that there are at least 2 multiple forms of acetylcholinesterase from this preparation.


B After
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2
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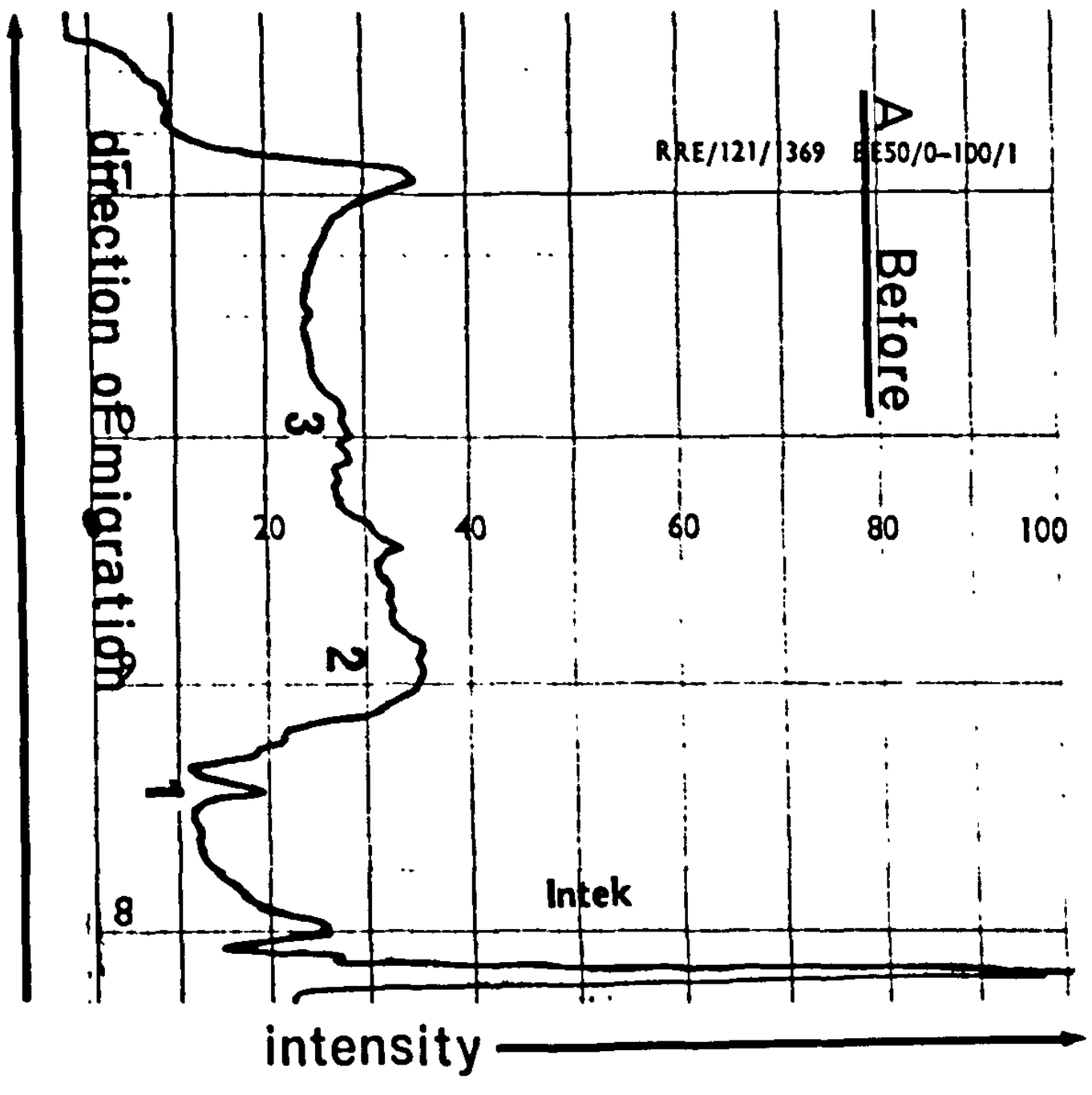
RRE/121/1369 BE50/0-100/1
20
19
18
17
20
40
60
80
100
direction of migration
intensity


A Before
RRE/121/1369 BE50/0-100/1
3
2
1
Intek
8
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40
60
80
100
direction of migration
intensity

FIGURE 32. <u>Traces to show that there are at least two multiple forms of acetylcholinesterase from the rat brain</u>.

The top trace is a gel-scan at 420 nm. obtained from a Joyce - Loebl scanner taken prior to the incubation of the gels in a medium containing acetylthiocholine/ DTNB.

The bottom trace shows a re-scan of the gel taken following a two minute incubation of the gel in the acetylthiocholine/ DTNB medium.

Three peaks have significantly increased their absorbance and this may be taken to indicate three peaks of cholinesterase activity. Of these three peaks at least two must be acetylcholinesterase forms.

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