ACETYLCHOLINESTERASE FROM THE COCKROACH PERIPLANETA AMERICANA L.: PURIFICATION, PROPERTIES AND CORRELATION WITH BEHAVIOURAL CHANGES IN THE ANIMAL

by

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Errata

triton-X-100 and lubrol-Px are more usually abbreviated as Triton X-100 and Lubrol PX respectively.

ABBREVIATIONS USED

ACh	acetylcholine
AChE	acetylcholinesterase
Ald.-2	aldrithiol-2(2,2'-dithiodipyridine)
ATCh	acetylthiocholine
ATP	adenosine 5'-triphosphate
BuCh	butyrylcholine
BuChE	butyrylcholinesterase(pseudocholinesterase)
BuTCh	butyrylthiocholine
ChAc	choline acetylase
CNS	central nervous system
cyclic AMP	adenosine 3':5'-monophosphate
DDT	dichlorodiphenyl trichloroethane
DFP	di-isopropyl fluorophosphate
DOC	deoxycholate
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
GABA	γ-amino butyric acid
IPA	indophenyl acetate
M.Wt.	molecular weight
NMR	nuclear magnetic resonance spectroscopy
R_{BPB}	migration of proteins relative to the migration of bromophenol blue
SDS	sodium dodecyl sulphate
s.e.m.	standard error of mean
TDF	p-(trimethyl ammonium) benzene diazonium fluoroborate
TEA	tetraethyl ammonium
TEMED	N,N,N',N'-tetramethylethylenediamine
TEPP	tetraethyl pyrophosphate
TMA	tetramethyl ammonium
u	micro
v:v	volume:volume
w:v	weight:volume

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ABSTRACT

FACULTY OF SCIENCE

PHYSIOLOGY AND BIOCHEMISTRY

Doctor of Philosophy

ACETYLCHOLINESTERASE FROM THE COCKROACH PERIPLANETA AMERICANA L.: PURIFICATION, PROPERTIES AND CORRELATION WITH BEHAVIOURAL CHANGES IN THE ANIMAL.

by Philip William Beesley

The enzyme acetylcholinesterase has a high specific activity and plays an important role in nervous transmission in the CNS of insects. Previous work has correlated a 50% decrease in the specific activity of the enzyme in the metathoracic ganglion of Periplaneta americana with shock avoidance learning in the insect. Therefore the properties of the enzyme from Periplaneta americana were investigated.

The enzyme rapidly loses up to 50% of its activity in crude nerve tissue homogenates. This loss is stabilised by the detergents triton-X-100, lubrol-PX and deoxycholate. Only deoxycholate will release the decayed activity into an assayable form. Two procedures for accurately determining the total acetylcholinesterase activity of such homogenates are described.

All three detergents solubilised a large fraction of the membrane bound enzyme. Gel electrophoresis and gel filtration studies showed the existence of multiple forms of the soluble enzyme. Certain properties of crude and partially purified preparations of the enzyme were investigated.

The results of experiments attempting to correlate a decrease in the specific activity of acetylcholinesterase with shock avoidance learning are equivocal. Possible reasons for this discrepancy in the results are discussed, including the effects of environmental conditions on acetylcholinesterase activity. An increase in ambient temperature resulted in a decrease in the specific activity of acetylcholinesterase in the metathoracic ganglion.

SECTION ONE

1.1. Introduction

Sir Henry Dale (1914) proposed that ACh was involved in chemical transmission at autonomic ganglia. He suggested that enzymic hydrolysis of the ester linkage would provide a method for the rapid removal of ACh from the synaptic cleft. The classical studies of Loewi (1921) and Feldberg and Gaddum (1934) confirmed Dale's original proposal. Subsequently ACh has been shown to be the chemical transmitter at many central and peripheral synapses (Eccles, 1964; Phillis, 1970). The components of the cholinergic system have been identified and studied in a wide variety of tissues.

Stedman, Stedman and Easson (1932) reported the presence, in blood, of a specific esterase responsible for the hydrolysis of ACh. Alles and Hawes (1940) demonstrated the presence of at least two types of esterase capable of hydrolysing choline esters. One was associated with erythrocytes and the other with serum. The differences between these two enzymes have formed the basis for classifying cholinesterases from all other tissues. Enzymes exhibiting the same characteristics as the erythrocyte enzyme are termed acetylcholinesterases (E.C. 3.1.1.7 acetylcholine acetyl hydrolase) whereas those enzymes exhibiting the same characteristics as the serum enzyme are termed pseudocholinesterases (E.C. 3.1.1.8 acylcholine acyl hydrolase). Augustinsson (1957) defines true acetylcholinesterases as, "eserine sensitive esterases that are inhibited by high ACh concentrations (generally 3-5 mM) and that split ACh at a much higher rate than BuCh". Not all cholinesterases fall rigidly into one category or the other.

The importance of AChE in chemical transmission at peripheral synapses has been demonstrated by Eccles and Jaeger (1958) and Takeuchi and Takeuchi

(1959) who report that the decay of the end plate current at the neuro-muscular junction is prolonged in the presence of anti-cholinesterases.

The electric eel enzyme has been purified and its properties have been extensively studied (Froede and Wilson, 1972). Changeux (1966) showed that there was a second binding site on the enzyme which had an affinity for curare-like compounds. He suggested a regulatory role for AChE at the synaptic junction. This work has been further developed (Kitz, BraswellandGinsburg, 1970), but there has been no clear demonstration that the second binding site has any physiological significance.

Much current research has been directed towards an understanding of the neuronal mechanisms of acquired behaviour and particularly towards identifying biochemical correlates of behaviour. One mechanism proposed to account for acquisition of behaviour is a change in the efficiency of synaptic transmission between neurones involved in the response. Kandel and Tauc (1965) have provided electrophysiological evidence that synaptic facilitation can take place in Aplysia. Therefore because of:

(1) the relative ease of assay of AChE,

(2) the importance of AChE in transmission at cholinergic synapses,

(3) the possibility that AChE is a regulatory enzyme,

several studies have been made, both in vertebrates and in invertebrates, attempting to correlate AChE with behavioural paradigms (Kerkut, Oliver, Rick and Walker, 1970; Kerkut, Emson, Brimblecombe, Beesley, Oliver and Walker, 1972; Aleksidze and Balavadze, 1971; Vernadakis and Rutledge, 1973).

A major problem to such studies is that of selecting a suitable preparation that will exhibit acquisition of a simple, reproducible

behavioural response and that will also be useful for neurophysiological and neurochemical studies. Horridge (1962, 1965) demonstrated that locusts and cockroaches were able to correlate leg position with shock and suggested that this represented postural learning. Kerkut, Oliver, Rick and Walker (1970) reported changes in a number of systems in the metathoracic ganglion of animals which had acquired this behaviour. They reported changes in protein synthesis, RNA synthesis and a large decrease in AChE activity (50%).

Therefore we have carried out a thorough investigation of this response and also of the properties of insect AChE in order to investigate the mechanism by which this change takes place.

I.2. Solubilisation of AChE

Most of the AChE present in both nervous and non-nervous tissue is membrane bound. Hollunger and Niklasson (1973) report that the cytosol fraction from freshly homogenised, whole rat brain contains only 7% of the total AChE activity. They state that it is probable that this small, but significant proportion of the enzyme occurs in the soluble form in the intact animal. Thus the first step in the purification of the enzyme and the study of its properties is the solubilisation of the major portion of the membrane bound enzyme. Several procedures have been used to accomplish this.

Kremzner and Wilson (1963), Leuzinger and Baker (1967) and Massoulie and Rieger (1969) have extracted the electric eel enzyme by autolysis of a tissue homogenate under toluene. This procedure ultimately releases all of the membrane bound enzyme into solution. Several groups of workers

have solubilised the enzyme by controlled digestion with proteolytic or lipolytic enzymes (Ord and Thompson, 1951; Lawler, 1964; Massoulie, Rieger and Tsuji, 1970; Kaplay and Jagannathan, 1970). Ord and Thompson report an 88% solubilisation of the rat brain enzyme after treatment with trypsin.

The most widely used method of solubilisation is treatment of the tissue with detergent (Lawler, 1964; Jackson and Aprison, 1966b; Kremzner, Kitz and Ginsburg, 1967; Ho and Ellman, 1969; Crone, 1971; Srinivasan, Karczmar and Behrnson, 1972). The thorough investigation of Jackson and Aprison (1966b) showed that anionic and cationic detergents are potent inhibitors of the mammalian brain enzyme. Nnn-ionic detergents activated the enzyme slightly at low concentrations (0.01%) but did not inhibit it at higher concentrations (up to 1%). Of the detergents used triton-X-100, Lubrol W and deoxycholate (DOC) were the most effective solubilising agents for the enzyme. Ho and Ellman (1969) report 86% solubilisation of mammalian brain enzyme with triton-X-100, whilst Fiszer and De Robertis (1967) report that the same detergent releases 75% of the enzyme from rat brain nerve endings. Tochsi (1959) reports that 0.5% DOC and Lubrol W release 77% and 73% respectively of the AChE activity from pellet material of brain microsomal fraction whereas detergents of the tween type release only 50% of the bound activity.

Wright and Plummer (1972) report that use of triton-X-100 in neutral KCl solution is more effective than use of the detergent alone in solubilising the erythrocyte enzyme, 15% more activity being released in the presence of KCl. The combination of the two agents weakens both the hydrophobic and ionic bonds that exist between AChE and other membrane components. They

state that neutral KCl solution alone is ineffective in solubilising the enzyme.

The mammalian brain enzyme is readily solubilised by incubation in an ion-free medium (Hollunger and Niklasson, 1967). Chan, Shirachi, Bhargava, Gardner and Trevor (1972) report that release of calf brain AChE into 0.32M sucrose is stimulated in the presence of EDTA. They obtain a 70% solubilisation of the enzyme by repeated homogenisation and centrifugation in 0.32M sucrose containing EDTA. Hollunger and Niklasson (1973) find that release of the calf brain enzyme into 0.32M sucrose is stimulated by both EDTA and tetracaine, but is inhibited by monovalent and divalent cations. The effects of the cations are not related to ionic strength alone and are interpreted as being due to an effect on the conformational state of the membrane. The effect of Ca^{2+} and Mg^{2+} on the solubilisation of AChE is thought to be more potent than that of Na^{+} and K^{+} because of the involvement of the divalent cations in the formation of cross-linkages in the membrane. Hollunger and Niklasson obtain an 82% solubilisation of the calf brain enzyme by two 2 hr. incubations of the homogenate in 0.32M sucrose containing EDTA and tetracaine.

The insect enzyme has been solubilised by autolysation under toluene (Krysan and Kruckeberg, 1970) and by treatment with detergents (Lord, 1961; Edwards and Gomez, 1966; Knowles and Arurkar, 1969). Dauterman, Talens and Van Asperen (1962) and Kunkee and Zweig (1963) solubilised the enzyme by preparation of a butanol dried powder. This procedure gives only a 20-30% solubilisation of the mammalian enzyme (Lawler, 1964).

1.3. The effects of solubilising agents on the properties of AChE

A major difficulty in the study of a membrane bound enzyme is that solubilisation of the enzyme may result in a considerable change in its properties. Extraction of AChE by the mild procedure of Hollunger and Niklasson (1973) releases the enzyme in a low M.Wt. (80,000) form which aggregates into higher M.Wt. (250,000 and 510,000) forms on storage. Thus even this solubilisation procedure results in a change of properties of the enzyme.

Extraction of AChE with proteolytic enzymes may result in a cleavage of the enzyme into lower M.Wt. species. Massoulie, Rieger and Tsuji (1970) report that the three native species of AChE present in electric eel are converted into a species of different M.Wt. when treated with trypsin. The rat brain enzyme has a M.Wt. greater than 200,000 when extracted with triton-X-100, but when extracted with bacterial protease the M.Wt. is only of the order of 100,000 (Ho and Ellman, 1969).

A second problem encountered with solubilisation of the enzyme is that the agents used may activate or inhibit it. The studies of Jackson and Aprison (1966b) suggest that only non-ionic detergents are of use in solubilising the mammalian brain enzyme and that these activate at low concentrations. Kremzner, Kitz and Ginsburg (1967) report similar findings for the human brain enzyme. Srinivasan, Karczmar and Behrnson (1972) report that triton-X-100 had no effect on the AChE activity in the particulate fraction of human brain homogenate despite its solubilising effect. However, the detergent increased the relative specific activity in the supernatant fraction by 360%. They conclude that this activation is due to an enhancement of the catalytic activity of the enzyme by the detergent.

They also observe marked changes in the electrophoretic pattern of the enzyme when separated by polyacrylamide gel electrophoresis in the presence of the detergent. They suggest that the change in pattern is due to progressive removal of lipid components from an AChE-lipid complex by increasing concentrations of triton-X-100. Progressive removal of lipid from the AChE-lipid complex would increase the exposure of the catalytic site thus accounting for the observed increase in the activity of the enzyme.

Fiszer and De Robertis (1967) report that triton-X-100 had no activating effect on the enzyme from rat brain synaptosomes. However, Harwood and Hawthorne (1969) found that the enzyme from guinea pig synaptosomes showed a 116% increase in activity when treated with 0.1% triton-X-100.

Preparation of a butanol dried powder of the insect enzyme may well result in its activation because both Colhoun (1961) and Kunkee and Zweig (1963) report that insect AChE is activated by a number of straight chain alcohols of which n-butanol is the most potent. This activation is specific to the insect enzyme which exhibits this property even when it has been partially purified.

The investigations described clearly show the pitfalls of studying the purified enzyme and emphasize that when considering the role of AChE in the intact nervous system its integration into the nerve membrane and its relationship with other membrane components must be considered.

I.4. The mechanism of action of detergents in solubilising AChE

The mechanism of action of detergents used to solubilise AChE has been widely studied, in particular the mode of action of triton-X-100

(Ponder, 1955; Bakerman and Wasemiller, 1967; Fiszer and De Robertis, 1967; Bonsall and Hunt, 1971). The mechanism of action of the detergent is primarily to weaken hydrophobic bonds between molecules without affecting electrostatic interactions. Putnam (1948) points out that therefore the detergent has little effect on proteins, but acts mainly on lipids. Evidence for this is provided by Ferdman, Himmelreich and Dyadusha (1970). They report that treatment of sarcolemma membranes with the anionic detergent SDS solubilises proteins and phospholipids in the same ratio as they occur in the intact membrane whereas treatment with the non-ionic triton-X-100 results in a preferential separation of phospholipids from the membrane. They interpret these results as indicating that SDS breaks protein-protein bonds, but not protein-lipid bonds, the converse being true for triton-X-100. The results of Wright and Plummer (1972) also support the view that the major part of the interaction of AChE with other membrane components is hydrophobic, the weakening of electrostatic interactions with the membrane by 1.2M KCl having only a small effect on the solubility of the enzyme.

Crone (1971) has carried out a detailed study on the dissociation of rat brain membranes by triton-X-100. He obtained a variable solubilisation of the AChE and concluded that the ratio of triton-X-100 to the actual amount of tissue present may be the important factor determining the amount of AChE solubilised. Gel filtration of the solubilised product showed that it had a M.Wt. of 5×10^5 daltons and also contained 0.95 mg phospholipid/mg protein. Removal of the detergent by dialysis resulted in aggregation and precipitation of the solubilised particles, suggesting that detergent binds to the solubilised particles, Crone proposes that these particles represent

existing lipoprotein subunits of the membrane.

These studies suggest that triton-X-100 does not truly solubilise AChE, but releases it in a form in which it is attached to other membrane components.

1.5. Purification of AChE

A prime requirement for the purification of an enzyme is a rich source of that enzyme. Nachmahnson and Lederer (1939) reported that the electroplax of the electric eel, Electrophorus electricus, contained large amounts of AChE. Therefore many of the studies on the purification of AChE have been carried out on the electric eel enzyme. Rothenberg and Nachmahnson (1947) achieved a 50% purification of AChE from this tissue (sp. act. 7mmoles ACh hydrolysed/min./mg prot.) using ammonium sulphate fractionation and high speed centrifugation. Similar results were obtained by Lawler (1959). A 90% purification of the enzyme (sp. act. 11mmoles ACh hydrolysed/min./mg protein) was obtained by Kremzner and Wilson (1963) using extensive column chromatography. The enzyme prepared by this method was crystallised and partially characterised by Leuzinger and Baker (1967).

Attempts to purify mammalian brain AChE yielded relatively impure products until recently (Jackson and Aprison, 1963, 1966a; Kremzner, Kitz and Ginsburg, 1967; Ho and Ellman, 1969). Chan, Shirachi and Trevor (1972) obtained a 400 fold purified product using ion-exchange and molecular sieve chromatography. The purification procedure has been considerably simplified by the introduction of affinity chromatography methods for the enzyme. Berman and Young (1971) describe the synthesis of inhibitors, particularly

trimethyl(m-aminophenyl)ammonium chloride, suitable for the purification of the enzyme by this method. They claim a 2,500 fold purification of a crude extract of bovine erythrocyte AChE. Chan, Shirachi, Bhargava, Gardner and Trevor (1972) have obtained a 700 fold purification of the mammalian enzyme using the method of Berman and Young. The synthesis of columns for the purification of the enzyme containing a wide variety of inhibitors coupled to agarose beads have been described (Rosenberry, Chang and Chen, 1972; Dudai, Silman, Kalderon and Blumberg, 1972; Dudai, Silman, Shinitzky and Blumberg, 1972). Ashani and Wilson (1972) describe the synthesis of an affinity column in which the AChE forms a covalent bond with 2-aminoethyl p-nitrophenyl methyl phosphonate that is subsequently broken by treatment with 2-(hydroximinomethyl)-1-methylpyridinium iodide. Jung and Belleau (1972) have used d-tubocurare to purify the enzyme by binding to a second site on its surface.

Despite these advances in technique the insect enzyme has not yet been obtained in a highly purified state. This is primarily due to the considerable amount of material required for enzyme purification. Lord (1961) obtained a 20 fold purification of the enzyme from Blatella germanica L. by acetone precipitation and ammonium sulphate fractionation of the crude extract. The fly head enzyme has been purified 157 fold by Dauterman, Talens and Van Asperen (1962) using techniques similar to those of Lord. Hellenbrand (1967) achieved a 20 fold purification of the fly head enzyme using sodium chloride fractionation and gel filtration. Kúnkee and Zweig (1963) describe the use of sucrose density gradient centrifugation to further purify the bee enzyme following ammonium sulphate fractionation. Their final product had an overall purification of 50 times.

I.6. Properties and mechanism of AChE

The only species of AChE which has been fully characterised is that from the electric eel. The turnover no. for the enzyme is reported to be 6×10^5/min. by Kremzner and Wilson (1964). The Km of the enzyme varies between 10^{-4} and 10^{-5}M depending on the source of the enzyme, the method of purification and the molecular species of the enzyme being examined.

The pH optimum of all species of AChE examined to date is near 8. Standard activity measurements are made at this pH.

The characteristic phenomenon of substrate inhibition was first observed by Nachmahnson and Rothenberg (1944, 1945). This phenomenon was observed to arise at substrate concentrations of over 2 mM in a variety of tissues. Explanations of substrate inhibition are considered on page 15.

The enzyme is activated by monovalent and divalent cations (Cohen and Oosterbaan, 1963). Nachmahnson (1940) reported activation of the electric eel enzyme by Ba, Ca, Mg and Mn ions. Van der Meer (1953) reported that the activation was a non-specific effect. Augustinsson (1948) and Myers (1950) concluded that the ions have a specific activating effect on the enzyme. The specificity of ion activation of AChE has been re-investigated by Dawson and Crone (1973) using the bovine erythrocyte enzyme. They find that the Km and Vmax parameters of the enzyme increase markedly as the concentration of cations is increased, but that there is a small but significant difference between the effects of Na, K, Ca and Mg ions on the enzyme. The anion present with the cation also has a small but significant effect on the activity of the enzyme. Crone (1973) suggests that activation of AChE by gallamine may occur by the same mechanism as cation activation as the two effects are not additive and the activation by the two types of agent are indistinguishable.

The nature of the active site of AChE, i.e., "that part of the enzyme which combines with the substrate and is responsible for the enzymic properties of the molecule", and the mechanism of hydrolysis have largely been elucidated by structure-activity studies using different substrates and inhibitors. The active site (see fig. 1) consists of an anionic site which binds the alcohol moiety of the substrate and an esteratic site which catalyses the cleavage of the ester bond (Adams and Whittaker, 1950; Wilson and Bergmann, 1950).

Both Adams and Whittaker and Wilson and Bergmann have studied the difference in binding energies of charged and uncharged isosteric analogues, e.g., isoamyl alcohol and hydroxyethyl dimethyl ammonium ions, to the enzyme. They conclude that this difference corresponds to a single negative charge at the anionic site separated from the positive charge of the quaternary ammonium group by a distance of 5Å. Other arrangements involving two or more negative charges are possible. Krupka (1966a,b) concludes from a pH study that the pKa of the anionic site is 4.5. and that it is probably a side chain carboxyl group. Wilson (1952) has studied the binding of TMA analogues in which the methyl groups are selectively replaced by hydrogen atoms. He concludes that hydrophobic bonding of the methyl groups to the anionic site makes a greater contribution to substrate binding at this site than the electrostatic interaction of the quaternary nitrogen atom with the side chain carboxyl group. Froede and Wilson (1972) state that "the anionic site aids in the binding of the substrate to the enzyme and orients it such that it is in a favourable position with respect to the esteratic site".

Cleavage of ACh at the esteratic site involves acetylation of the enzyme. This is shown in fig. 2. The bell shaped pH curve for the enzyme

FIG. 1. The active site of AChE (from Cohen and Oosterbaan, 1963)

The active site of the enzyme is proposed to consist of an anionic site which binds the quaternary ammonium group of the substrate and an esteratic site which is responsible for hydrolysis of the substrate. The esteratic site contains an acidic (Ac) and a basic (Ba) group which participate in the catalytic mechanism of the enzyme.

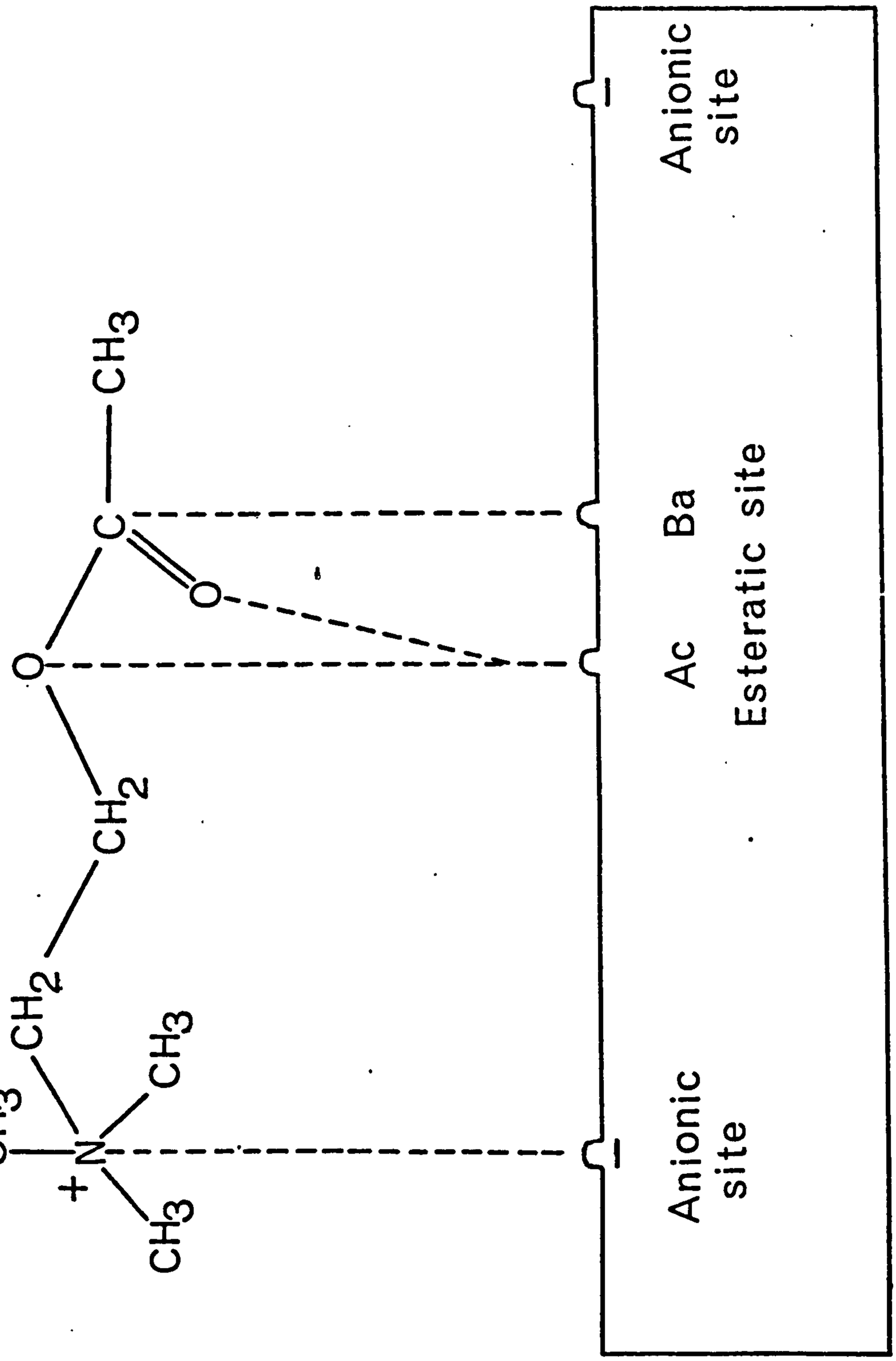

Anionic site
−
Ac
Ba
Esteratic site
Anionic site
−
O
C
CH3
O
CH2
CH2
O
C
CH3
CH3
N
+
CH3
CH3

FIG. 2. The hydrolysis of ACh by AChE (from Wilson and Harrison, 1961)

Substrate reacts at the active site of the enzyme to form a Michaelis-Menten complex. An internal electron shift takes place, acetate covalently binding to the enzyme and choline being released as the first reaction product. The acetyl-enzyme complex is rapidly hydrolysed releasing acetate and regenerating the active enzyme. The following abbreviations are used in the diagram:

E = enzyme

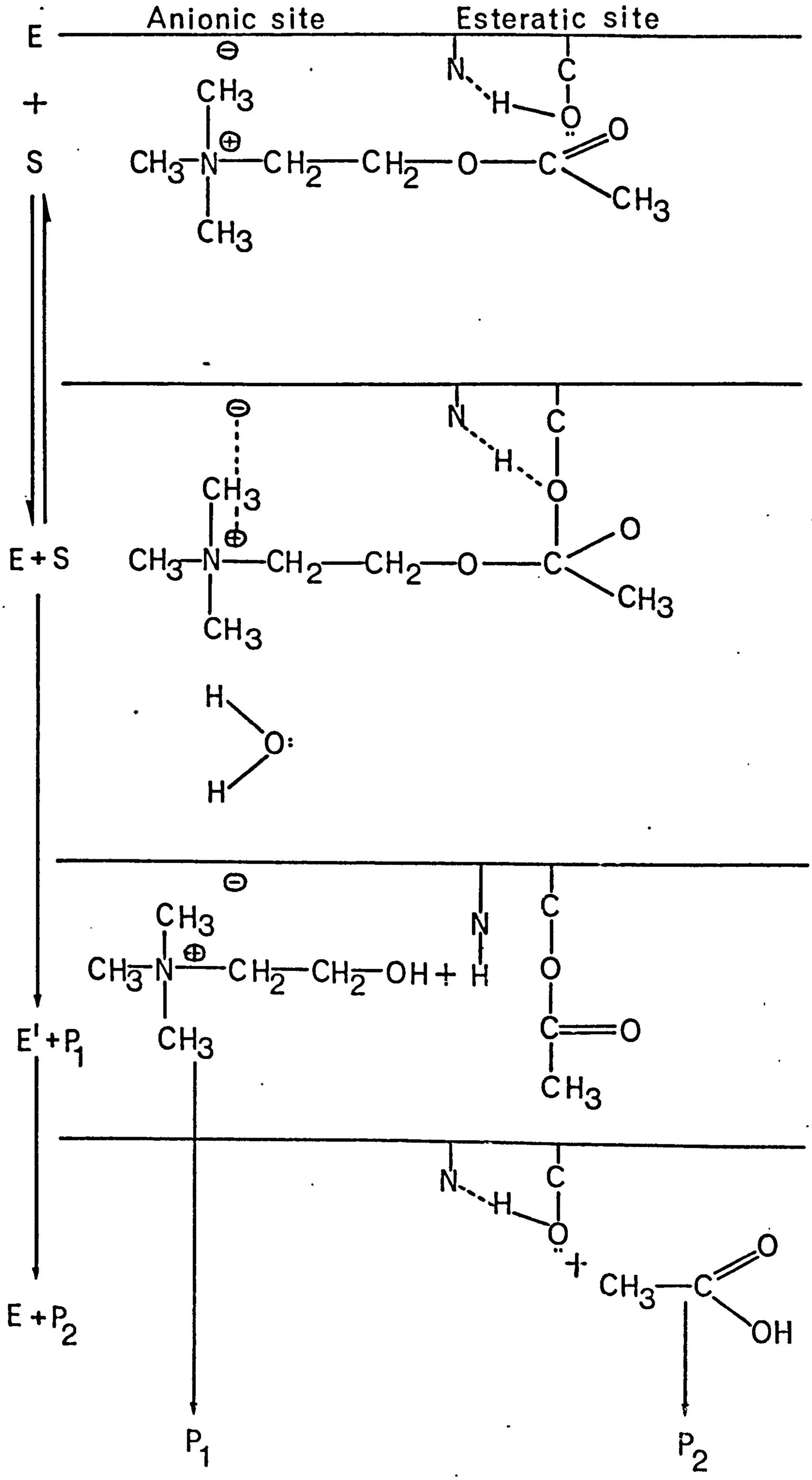
S = substrate

ES = Michaelis-Menten complex

E' = acetyl-enzyme

P_1 = choline

P_2 = acetate


Anionic site
Esteratic site
E
+
S
E+S
E'+P1
E+P2
P1
P2
H
O:
OH

suggests that ionization of two groups of pKa 5.5 and 10.5 is essential for the cleavage of ACh (Wilson and Bergmann, 1950; Krupka, 1966b). It is generally concluded that a serine residue at the esteratic site is acetylated, the imidazole group of histidine playing a secondary role in this process (Froede and Wilson, 1972). This mechanism is essentially the same as that proposed for other esterases, chymotrypsin in particular. Krupka (1966a) has produced evidence for the involvement of two basic groups of pKa 5.5 and 6.3 at the active site. These groups are a distance of 9Å and 5Å respectively from the anionic site. He has also produced evidence that the two groups are histidine residues which function sequentially in the acetylation and deacetylation of the serine residue. He proposed that the group of pKa 5.5 functions in the acetylation reaction and the group of pKa 6.3 functions in the deacetylation reaction (Krupka, 1967).

Hellenbrand and Krupka (1970, 1974) propose that the catalytic mechanism of the insect (fly head) enzyme is essentially the same as that for the mammalian enzyme. However, the pKa's of the two basic groups at the esteratic site are 6.7 and 7.0.

This information on the mechanism of the enzyme has led to proposals for the mechanism of substrate inhibition. The more traditional explanation is that an inactive E-S-S complex is formed at high substrate concentrations. Krupka and Laidler (1961) and Wilson and Alexander (1962) propose that the inhibition results from the formation of an acetyl-enzyme-substrate complex which is only slowly broken down. Kato, Tan and Yung (1972) suggest that an allosteric mechanism could account for the phenomenon.

Several groups of inhibitors for the enzyme are well known and their mechanism of action has been extensively studied. Inhibitors can exert their action by binding to the enzyme at the anionic or esteratic sites. Any substituted quaternary ammonium ion will compete with the substrate for the anionic site (Froede and Wilson, 1972). Belleau and Tani (1966) describe a second group of inhibitors, e.g., N,N-dimethyl-2-chloro-2-phenylethylamine which alkylate the enzyme at or near the anionic site.

Organophosphate and carbamate inhibitors both act at the esteratic site. The organophosphates, e.g., DFP, act as nucleophiles and phosphorylate the hydroxyl group of the serine residue (Aldridge, 1953). These active site directed inhibitors are extremely potent and not readily reversible. Several reactivators for the inhibited enzyme have been designed one of the more effective agents being pyridine-2-aldoxime methiodide (Wilson and Ginsburg, 1955). Carbamate inhibitors react with the enzyme in an analogous manner to the organophosphates resulting in the formation of a carbamyl-enzyme complex (Myers and Kemp, 1954).

1.7. Isozymes and multiple forms of AChE

Early studies of the physical properties of AChE from the electric eel gave an extremely high value for the M.Wt. Rothenberg and Nachmahnson's preparation had a sedimentation coefficient in excess of 40S indicating a M.Wt. of over 2,000,000. Lawler (1963) obtained M.Wts. in excess of 30,000,000. Sedimentation studies carried out on the pure enzyme yielded a M.Wt. of 230,000 (Kremzner and Wilson, 1964). This value agreed with the Wt. of a single active unit of the enzyme as estimated by inhibition

studies on a preparation of known specific activity (Lawler, 1961). The studies of Kremzner and Wilson revealed that their preparation gave a single symmetrical peak in sedimentation experiments with a M.Wt., measured by gel filtration, of 250,000. The friction ratio indicated the protein to be globular in nature and equivalent weight studies indicated that each 250,000 unit contained four active sites.

Leuzinger, Goldberg and Cauvin (1969) carried out a study on the molecular properties of the enzyme purified by the method of Kremzner and Wilson (1963). Sedimentation studies revealed the presence of a single protein species of M.Wt. 260,000. Treatment of this species with guanidine and mercaptoethanol yielded four inactive subunits of the enzyme. Examination of the C-terminal amino acid residues by hydrazinolysis or by enzymic hydrolysis with carboxypeptidase A revealed the presence of two types of polypeptide chain. Therefore they proposed that the enzyme consists of a dimeric hybrid structure comprising 2α and 2β chains. Froede and Wilson (1970) used sucrose density gradient centrifugation to study the enzyme and reported that treatment of the preparation with guanidine broke active units of M.Wt. 224,000 down into inactive subunits of M.Wt. 102,000. Addition of 2-mercaptoethanol to the guanidine treated AChE caused a further dissociation of the protein into subunits of M.Wt. 49,000. They also propose that the enzyme consists of four subunits with disulphide bridges linking pairs of subunits. Millar and Grafius (1970) report a M.Wt. of 260,000 for the enzyme based on sedimentation studies, but suggest that treatment with neutral guanidine does not always completely disrupt intermolecular forces. In order to completely dissociate the active enzyme into

subunits they use guanidine and dithiothreitol at pH 2.0. They obtain subunits of M.Wt. 21,500 by this method. Thus it is possible that the units of 42,200 which they obtain using the same conditions as Leuzinger, Goldberg and Cauvin are dimers. They suggest that the intact protein consists of six subunits of M.Wt. 42,200, but point out that not all sites are necessarily equivalent.

Leuzinger (1971) finds that stepwise inhibition of the enzyme with DFP, or titration with O-nitrophenyl dimethylcarbamate, yields an equivalent weight of 130,000/active site, i.e., two active sites/250,000 unit of enzyme. Froede and Wilson (1970) find, by activity inhibition studies, that each 224,000 unit contains four active sites. Further evidence that the 260,000 form of the enzyme contains four active sites is provided by Mooser, Schulman and Sigman (1972) using fluorescent probes. Millar and Grafius argue that their studies when taken together with the data of Michel and Krop (1951) indicate six active sites/260,000 unit of enzyme. Thus there is considerable discrepancy between these workers as to the number of active sites/single unit of active AChE.

Impure preparations of the electric eel enzyme have been found to contain multiple active forms of AChE. Grafius and Millar (1965, 1967) showed that partially purified extracts of electroplax tissue contained three major, but polydisperse bands of AChE when separated by sucrose density gradient centrifugation. The fast sedimenting component only appeared after dialysis of the extract against buffers of low ionic strength and then at the expense of the two slower sedimenting components. These results are similar to those of Massoulie and Rieger (1969) and Massoulie, Rieger and Tsuji (1970) who report the existence of three native species

of the enzyme, which can be converted into a fourth species on treatment with trypsin. In a later study Massoulie, Rieger and Bon (1971) find that the native species A, C and D are excluded from Sephadex G-200 and Bio-Gel P-300 molecular sieves and do not enter polyacrylamide gels. A consideration of the sedimentation coefficients of the native species of AChE indicates that the inability of these species to enter the gels mentioned is only compatible with their having an elongated structure. However, the enzyme released by tryptic digestion of the tissue, the pure enzyme and the species released on sonication of the elongated forms of the enzyme all have globular structures. Bauman, Benda and Rieger (1972) have characterised the globular species of the enzyme by polyacrylamide gel electrophoresis.

Grafius, Bond and Millar (1971) find that treatment of the higher M.Wt. forms of the enzyme with phospholipase C releases the monomeric form of the enzyme from a lipoprotein matrix. This study would suggest that the elongated forms of the enzyme are aggregates of AChE with other membrane components.

More detailed information on the structure of the various species of AChE from the electric eel has been provided by electron microscope studies of the enzyme. Changeux, Ryter, Leuzinger, Barrand and Podleski (1969) obtained electron micrographs of the pure enzyme indicating that the molecule has a globular structure consisting of four subunits. Rieger, Bon, Massoulie and Cartaud (1973) studied the structure of both the globular and elongated forms of the enzyme by electron microscopy. Their results indicate that the globular forms of the enzyme consist of two and four

subunits and that the elongated forms of the enzyme consist of four to six, six to eight and at least ten subunits associated with a semi-rigid tail structure. The subunits in the head portion of the elongated forms of the enzyme appear identical with the subunits of the globular forms of the enzyme. They propose that the species with elongated structures consist of one, two and three tetrameric units of the enzyme associated with an identical tail structure.

Both Grafius et al and Massoulie et al have proposed that reversible aggregation of some species of AChE may play an important role in regulating membrane permeability. However, AChE is not likely to be responsible for permeability changes which occur at the cholinergic synapse in response to ACh as both De Robertis and De Plazas (1970) and Changeux, Meunier and Huchet (1971) report the isolation of a receptor molecule for ACh which is distinct from the AChE molecule. They suggest that the receptor molecule controls the ionic permeability of the post-synaptic membrane, though the receptor molecule need not correspond to the ionophore.

Thus to summarise, the enzyme from the electric eel consists of an active unit of M.Wt. 260,000 which can be dissociated into at least four subunits. The 260,000 species has two, four or six active sites on its surface. Some forms of the native enzyme aggregate reversibly with both homologous and heterologous protein and lipid species. It has been suggested that some of these large complexes may represent basic integral units of excitable membranes.

The enzyme from mammalian nervous tissue exists in multiple forms similar to those which occur in the electric eel. Behrmson, Barron and Hedrick (1961) were able to separate three species of mammalian AChE by

starch gel electrophoresis. Two of these had extremely similar R_F values. These were treated as one band. No difference in Km, pH optimum and pI_{50} values with respect to eserine, mytelase and DFP could be distinguished for the two species. Bajgar and Zizkovsky (1971) separated three species of rat brain AChE by agar gel electrophoresis. They characterised two of these bands as being "true" AChE. Studies on preparations of varying degrees of purity also reveal the presence of multiple forms of the enzyme. Jackson and Aprison (1966a) report the existence of three or possibly four bands of the enzyme whilst Ho and Ellman (1969) find only two bands of activity, the lowest of which has a M.Wt. of 100,000. These reports were made on the basis of gel filtration and gel electrophoresis studies. Chan, Shirachi and Trevor (1972) report the isolation of two species of AChE by molecular sieve and ion-exchange chromatography, but in a later paper (Chan, Shirachi, Bhargava, Gardner and Trevor, 1972) they find that the enzyme purified by affinity chromatography exhibits three peaks of activity on gel filtration corresponding to species of M.Wt. 130,000, 270,000 and 390,000. They also report the isolation of three bands of activity from less purified preparations by use of polyacrylamide gel electrophoresis and isoelectric focusing. The species of M.Wt. 390,000 and 270,000 exhibited the same Km, pH optimum and sensitivity to inhibitory agents. The species of M.Wt. 130,000 differed considerably from the other two species with respect to these properties. They suggest on the basis of M.Wts. and by analogy with the electric eel enzyme that these species represent dimer, tetramer and hexamer of the same isozyme. If these species are aggregate forms of the enzyme it is of interest that the properties of the dimer should be different to those of the tetramer and hexamer. Hollunger and Niklasson (1973) report the existence of three

similar species of M.Wt. 80,000, 250,000 and 510,000 and also a high M.Wt. aggregate in excess of 1,000,000 daltons. They find that in fresh extracts the enzyme exists in a predominantly low M.Wt. form, but that on aging of the extracts the two higher M.Wt. species form in increasing amounts by aggregation of the low M.Wt. (80,000 daltons) species. The enzyme is stabilised as the monomer by DEAE-Sephadex. They propose the three bands are monomer, trimer and hexamer and question the physiological significance of the two larger aggregates. They also point out that stabilisation of the proposed monomer by treatment with the ion-exchange medium may be due to separation of the enzyme from an aggregating factor present in the homogenate. Kremzner and Fei (1971) have reported the existence of an aggregating factor for electric eel AChE which they propose to be a protein of membrane origin.

Although relatively few studies have been carried out on the insect enzyme the available evidence indicates the existence of multiple forms some of which show the property of aggregation. Menzel, Craig and Hoskins (1963) used starch gel electrophoresis to classify the electrophoretic properties of housefly esterases. They report the existence of four cholinesterases, two aliesterases and one aromatic esterase. Similar results have been obtained by Knowles and Arurkar (1969) who detected three soluble forms of housefly AChE, further possible forms existing in the particulate fraction. The cattle tick Boophilus microplus (Can.) contains five forms of AChE (Nolan, Schnitzerling and Schunter, 1972) three of which exhibit a variation in their sensitivity to anti-AChE agents between susceptible and resistant strains of the tick. Menzel, Craig and Hoskins also reported differences in the electrophoretic pattern of the enzyme from malathion sensitive and malathion resistant strains of housefly.

Eldefrawi, Tripathi and O'Brien (1970) have separated four species of AChE from the housefly, two from Periplaneta americana and one from the southern army worm, by polyacrylamide gel electrophoresis. Kerkut, Emson and Beesley (1972) have also separated two major species of AChE from P. americana. These studies would seem to indicate that true isozymes of insect AChE occur (as opposed to and in addition to forms representing aggregates of the same isozyme).

Lord (1961) and Dauterman, Talens and Van Asperen (1962) state that their partially purified preparations of insect AChE only show one diffuse band of activity on electrophoresis. Krysan and Chadwick (1966) report a M.Wt. of the order of 160,000 for the housefly enzyme as estimated by sucrose density gradient centrifugation.

Two studies have been carried out on the sedimentation properties of the insect enzyme. Kunkee and Zweig (1963) obtained a diffuse band of activity on sucrose density gradient centrifugation of the bee enzyme the pattern of sedimentation not being altered by high salt concentration, urea, mercaptoethanol, deoxycholate, butanol or sonic oscillation. Krysan and Kruckeberg (1970) report that mayfly and bee AChE sediment as a single peak (7.3S) at pH 8.0. On lowering the pH to 6.8 the mayfly enzyme sediments as forms of 7.3S, 10.2S and even larger whilst the honey bee enzyme becomes polydisperse. The 10.2S form of the mayfly enzyme is only 0.6S different from the 260,000 dalton form of the electric eel enzyme.

The molecular properties of AChE extracted from various sources are summarised in table 1.

The occurrence of true isozymes of AChE in mammals in addition to the proposed aggregated forms of the enzyme has been reported. The term

TABLE 1. Summary of some studies on the molecular properties of AChE

a) The electric eel enzyme

Study	Purification procedure	Molecular species separated	Aggregating properties and subunit structure	Additional information
Kremzner and Wilson (1964)	Repeated column chromatography (product 90% pure)	1 species of M.Wt. 230,000 - 250,000	-	M.Wts. estimated by sedimentation studies and column chromatography
Leuzinger, Goldberg and Cauvin (1969)	Pure crystalline product obtained using the method of Kremzner and Wilson	1 species of M.Wt. 260,000	Treatment with guanidine and mercaptoethanol gave 4 inactive subunits all of 60,000 M.Wt.	2 active sites/260,000 unit of enzyme. 2 different C-terminal amino acids detected. Intact molecule suggested to have $\alpha_2\beta_2$ structure
Froede and Wilson (1970)	Pure enzyme	1 species of M.Wt. 224,000	Treatment with guanidine gave 2 subunits (M.Wt. 102,000) which were further broken down by treatment with mercapto-ethanol to subunits of M.Wt. 49,000	4 active sites/single active unit of enzyme. They propose pairs of subunits are joined by disulphide bridges.
Millar and Grafius (1970)	Pure enzyme	1 species of M.Wt. 260,000	Inactive subunits of M.Wt. 42,200 obtained by treat-ment with neutral guanidine and dithiothreitol. These broken down into subunits of M.Wt. 21,500 by treat-ment with guanidine and dithiothreitol at pH 2.0	They propose 6 active sites/single active unit of enzyme
Grafius and Millar (1965, 1967) Grafius, Bond and Millar (1971)	Native species of the enzyme separated by sucrose density gradient centrifugation	Several slower (8-18S) and faster sedimenting species (53-77S) detected	At low ionic strength and low pH the slower sediment-ing species aggregate into the faster sedimenting species. Phospholipases C and D convert faster sedimenting species to the slower sedimenting species	They suggest that the faster sedimenting species are due to a complex between monomer AChE and a lipoprotein matrix
Massoulie and Rieger (1969) Massoulie, Rieger and Tsuji (1970) Massoulie, Rieger and Bon (1971) Rieger, Bon, Massoulie and Cartaud (1973)	Native species of the enzyme separated by sucrose density gradient centrifuga-tion, and characterised by electron microscopy	3 native species of enzyme A, C and D (18, 14 and 8S) obtained. A, C and D all converted to 11S form by trypsin	The 14S and 18S species aggregate at low ionic strength, the 8S and 11S species do not. 11S species is a tetramer corresponding to the pure form of the enzyme	They suggest that the 8S, 14S and 18S species represent aggregates of 1, 2 and 3 tetrameric structures attached to a common tail structure

b) The mammalian enzyme

Study	Purification procedure	Molecular species separated	Aggregating properties and subunit structure	Additional information
Jackson and Aprison (1966a)	Butanol extract partially purified by column chromatography and electrophoresis	3 (or 4) species separated. 2 have M.Wts. of 161,400-204,600 and 284,000-360,000	-	-
Ho and Ellman (1969)	Protease and detergent extracts partially purified by column chromatography	2 species separated 1 of M.Wt. 100,000 the other of M.Wt. 200,000	-	-
Chan, Shirachi, Bhargava, Gardner and Trevor (1972)	Sucrose/EDTA solubilised enzyme purified by affinity chromatography	3 species of M.Wts. 130,000, 270,000 and 390,000	They suggest that the forms represent dimer, tetramer and hexamer	Proposed dimer has a higher Km and a greater sensitivity to eserine than the other two species
Hollunger and Niklasson (1973)	Sucrose/EDTA/ tetracaine solubilised enzyme separated by column chromatography	4 species of M.Wts. 80,000, 250,000, 510,000 and > 1,000,000	80,000 M.Wt. species aggregates into the higher M.Wt. species. Aggregation prevented by treatment with DEAE-Sephadex	The 3 lower M.Wt. species suggested to be monomer, trimer and hexamer

c) The insect enzyme

Eldefrawi, Tripathi and O'Brien (1970)	Separated by polyacrylamide gel electrophoresis	4 species separated from housefly, 2 from cockroach and 1 from the southern army worm	-	-
Kunkee and Zweig (1963)	Partially purified by ammonium sulphate fractionation and sucrose density gradient centrifugation	A single diffuse band of the bee enzyme	-	No change in sedimentation pattern observed after treatment with urea, mercaptoethanol, 1-butanol, deoxycholate or sonic oscillation
Krysan and Kruckeberg (1970)	Partially purified as above	Mayfly and bee enzyme sediment as single species (7.3S) at pH 8.0	At pH 6.8 mayfly enzyme forms faster sedimenting species and the bee enzyme becomes polydisperse	-
Huang and Dauterman (1973)	Highly purified by repeated column chromatography	2 species separated by gel electrophoresis	The 2 species are interconvertible	-

isozyme is used to describe enzymes catalysing identical reactions but which are derived from different genes. Several atypical pseudoesterases exist in human blood differing in the configuration of the anionic site (Harris, 1962). These different forms of the enzyme are hereditary in nature. Wilson, Mettler and Asmundson (1970) have produced evidence that the three species of the enzyme occurring in chick muscle may be isozymes as opposed to aggregated forms of the same isozyme. They find that the activity and electrophoretic pattern of AChE vary in dystrophytic as opposed to normal chicks.

I.8. The evidence that AChE is an allosteric enzyme

There has been considerable interest in possible ways in which the activity of individual neurones and neuronal networks is controlled. The activity of any neurone will be partially determined by its synaptic inputs from other nerve cells. The efficiency of the synaptic input in producing a particular response in a neurone will be affected by the efficiency of transmission at each individual synapse, i.e., the response of a neurone to a given stimulus could be altered by synaptic facilitation/inhibition. This facilitation/inhibition response could be regulated by changes in the activity of chemicals involved in synaptic transmission, e.g., AChE at cholinergic synapses. Thus considerable attention has been given to the fact that AChE may show regulatory properties.

Monod, Wyman and Changeux (1965) proposed an elegant theory to explain how the activity of certain enzymes could be regulated by binding of an effector molecule to a second, allosteric site remote from the catalytic site of the enzyme. Koshland (see Koshland and Neet, 1968) has proposed an alternative explanation for these phenomena based on sequential changes

in enzyme conformation. Evidence that AChE is an allosteric enzyme has come from studies on the action of certain classes of inhibitory compounds on the reactions of the enzyme and also from a study of the binding of agents to the enzyme surface.

Quaternary ammonium ions, as expected, inhibit the carbamylation and methane sulphonylation reactions of AChE. Interesting exceptions to this are known. Metzger and Wilson (1963) found that quaternary ammonium ions accelerated the carbamylation reaction of the enzyme with dimethyl carbamyl fluoride. Belleau, Ditullio and Tsai (1963) have investigated the effect of a number of quaternary ammonium ions on the reaction of AChE with several carbamate and methane sulphonyl inhibitors. The acceleratory effect of the quaternary ammonium ions can be as great as 50 x the normal reaction rate. A third group of ions, including the N-methyl pyridinium ion, have no effect on the reaction of the carbamate or methane sulphonyl inhibitors with AChE, but prevent the acceleration of the reaction observed in the presence of quaternary ammonium ions. Roufogalis and Thomas (1968a,b) have explained these observations on the basis that the quaternary ammonium ions speed up the deacylation reaction. Iverson (1971) finds that the kinetics of the carbamylation reaction of AChE in the presence of TEA ions indicates simultaneous binding of both types of inhibitor to the enzyme surface. Since it is unlikely that TEA could bind to the anionic site and the carbamate inhibitor to the esteratic site the TEA ion must be binding at a second allosteric site producing a conformational change in the enzyme. Kitz and Kremzner (1968) report changes in the ORD spectrum of highly purified AChE in the presence of acetylhomocholine, 3-hydroxyphenyldimethylethyl-ammonium chloride and tetraethyl pyrophosphate thus giving further support to this theory.

Thus the evidence discussed indicates the existence of an acceleratory site on the AChE molecule which will bind certain quaternary ammonium ions. This acceleratory site is not equivalent to the anionic binding site.

Changeux (1966) made the first proposal that AChE is an allosteric enzyme. He studied the effects of pachycurares, e.g., flaxedil, and lepto-curares, e.g., decamethonium, on the kinetics of AChE. He demonstrated that at low ionic strength both of these types of compound could act as partial competitive inhibitors of the enzyme. The mechanism of partial competitive inhibition is usually indicative of a second site binding process. The pachycurares also antagonised the effects of some reversible competitive inhibitors of the enzyme, but enhanced the inhibition by the (3-hydroxyphenyl)trimethylammonium ion. On the basis of these studies he proposed an allosteric model for AChE and suggested that it might also function as the ACh receptor (Changeux, Podleski and Meunier, 1969). As already discussed on page 20 more recent evidence indicates that this model is now untenable though the data remains undisputed. However, this does not preclude the possibility that the AChE molecule and the ACh receptor may be closely linked in the membrane and exert a regulatory effect on each other.

Changeux, Leuzinger and Huchet (1968) showed, by equilibrium dialysis, that ^{14}C ACh will bind to pure AChE even in the presence of eserine. This binding shows no tendency towards saturation. The bound ACh is displaced by d-tubocurare and decamethonium. Studies with the affinity labelling reagent, TDF, indicate that this compound prevents the binding of ACh. Studies with TDF in the presence of curare-like compounds reveal that four molecules of the agent bind/260,000 unit of the enzyme (Changeux, Podleski and Meunier, 1969). These results are interpreted as indicating the presence

of two catalytic sites and two regulatory sites (capable of binding ACh) per single active unit of the enzyme.

Belleau and Ditullio (1971) have used another affinity labelling reagent, N-N-dimethyl-2-phenylaziridinium chloride, and show that two molecules of this reagent bind/65,000 unit of electric eel AChE. The labelled species are inactive to ACh, but exhibit enhanced reactivity to indophenyl acetate. They conclude that one molecule of the affinity labeller is binding to the active site and the other to the proposed regulatory site.

Kitz, Braswell and Ginsburg (1970) produce evidence for the existence of regulatory sites on AChE capable of binding gallamine. They show that the effect of gallamine is additive with the effect of choline, an inhibitor which is known to bind to the anionic site. Wombacher and Wolf (1971) report similar results for membrane bound erythrocyte AChE.

Kato and his co-workers have carried out a thorough study on the binding and kinetic effects of another anti-cholinergic agent, atropine, on AChE. Kato, Tan and Yung (1972) find that atropine relieves substrate inhibition of the enzyme, though lowering its Vmax. At suitable concentrations atropine relieves substrate inhibition without lowering the Vmax of the enzyme and turns the Michaelis-Menten plot for AChE into a sigmoid curve. At low substrate concentrations atropine inhibition is of the competitive type, but at higher substrate concentrations the Hill coefficient (1.6) is indicative of a co-operative binding effect. Kato, Yung and Ihnat (1970) have used high resolution proton NMR to study the binding of atropine and eserine to AChE. In the presence of TEPP or DFP there is a diminished binding of eserine, but not atropine. Kato (1972a) reports that gallamine

displaces bound atropine from its site, but has no effect on the binding of eserine. In a second paper, Kato (1972b) finds that hydrolysis of ACh by AChE is reduced by eserine and neostigmine, both interfering with the binding of substrate to the enzyme. Atropine exerts its effect on the hydrolysis without interfering with the binding of substrate.

Thus evidence exists for both an acceleratory site and a regulatory site on the AChE molecule in addition to the catalytic site. Neither of these "second sites" has been shown to have any physiological significance and the relationship between them is not known at present.

The situation has been further complicated by two recent reports. The first, by Chiu and O'Brien (1971), suggests that the neutral substrate indophenyl acetate, IPA, binds to a special site on the insect enzyme rather than to the esteratic site. Their evidence for this special binding site is that TEA and decamethonium are competitive inhibitors for ATCh, but are non-competitive for IPA. Further, TDF reduces the activity of the enzyme for all substrates except IPA. The second report, by Roufogalis and Quist (1972), proposes that there are three binding sites on bovine erythrocyte AChE, the active site and two regulatory sites. It is proposed that one regulatory site binds decamethonium and that the other binds gallamine. They do not comment on whether either of these regulatory sites is coincident with the acceleratory site which binds simple quaternary ammonium ions.

1.9. Histochemical localisation of AChE

The principal histochemical technique for the localisation of AChE depends on the production of copper thiocholine from ATCh and its subsequent conversion to copper sulphide (Koelle, 1950, 1951). This

technique has been used to study the distribution and localisation of AChE in the mammalian peripheral and central nervous systems (Hubbard, 1973; Lewis and Shute, 1966; Shute and Lewis, 1967). At the neuromuscular junction the enzyme is localised mainly on the post-junctional membrane, though recent evidence indicates that it is situated on the basement membrane (Betz and Sakmann, 1971). BuChE and a number of other eserine insensitive esterases are also present at the neuromuscular junction (Barnard, Rymaszweska and Wieckowski, 1971). In the CNS the enzyme is localised on both the pre- and post-synaptic membranes. There is a close correlation between the regional distribution of AChE and ChAc in the mammalian CNS which suggests that AChE is mainly associated with cholinergic tracts (Hebb and Silver, 1956).

Histochemical studies on the distribution and localisation of AChE in insect tissues have been carried out using both light and electron microscopy. Iyatomi and Kanehisa (1958) have used the copper thiocholine method to localise ChE in P. americana. They find ChE activity in the peripheral and central nervous system, and in the digestive and reproductive systems, but not in the fat bodies, trachea and haemolymph. The activity in the nerve tissue was AChE, that in other systems being due to both AChE and other esterases. They report that the AChE in the nervous system is localised on nerve sheaths and neurone surfaces. Frontali, Piazza and Scopelliti (1971) have found that AChE is present in high concentrations in the neuropil regions of the brain of P. americana, but is less concentrated or absent in other brain regions.

Treherne and Smith (1965b) have made electron microscope studies of the distribution of AChE in P. americana. Eserine sensitive esterase activity was localised in the synaptic regions of the neuropil and in the glial sheaths which encapsulate the cell bodies of the neurones. The glial enzyme occurs principally in the more peripheral regions of the ganglia and is situated close to the extensive extracellular spaces of the glial lacunar system (Wigglesworth, 1960; Smith and Treherne, 1963). Wigglesworth (1958) reports that the distribution of AChE in Rhodnius prolixus is the same as that in P. americana. He was not able to find any evidence for the presence of AChE at the neuromuscular junction.

Thus AChE has a much wider distribution in the insect nervous system than in the mammalian nervous system.

1.10. The role of AChE in nervous transmission

The importance of AChE in hydrolysing ACh released at mammalian peripheral cholinergic synapses and thus maintaining transmission at these sites has been clearly demonstrated. However, the mammalian central cholinergic system is less well understood and ACh has been unequivocally identified as the transmitter at only a few central synapses. Some evidence has been obtained that AChE is important in removing ACh from the synaptic cleft at these sites (Eccles, 1964; Phillis, 1970). The lack of a firm understanding of the central cholinergic system has led Krnjevic (1969) and Roberts and Thesleff (1969) to suggest alternative roles for ACh and AChE in the mammalian CNS.

Early studies attempting to show that ACh is a neurotransmitter in insects were unsuccessful. Roeder (1948) could find no effect of ACh on neurones in the insect central nervous system. Subsequently it has been shown that this is due to difficulty of applied ACh penetrating a barrier localised on the sheath of connective tissue surrounding the ganglia (Twarog and Roeder, 1956). Harlow (1958) and Treherne (1962) find that changes in electrical activity are observed when large doses of ACh are injected under the sheath surrounding the sixth abdominal ganglion. Intracellular recordings show that neurones in the sixth abdominal ganglion of P. americana are excited by ACh applied locally by iontophoresis or micropipette (Callec and Boistel, 1967; Kerkut, Pitman and Walker, 1969). Pitman and Kerkut (1970) have compared the action of iontophoretically applied ACh with electrically driven EPSP's. Kerkut et al. have also shown that the effects of iontophoretically applied ACh are potentiated by eserine.

The nature of the barrier to the penetration of ACh has been extensively studied. Treherne and Smith (1965a) report that ^{14}C ACh rapidly penetrates ganglia, but is rapidly destroyed by AChE. In the presence of eserine the distribution of ACh reaches the values predicted by the Gibbs-Donnan equilibrium. They argue that penetration of ganglia by ACh is not limited by the presence of the ion barrier demonstrated by Twarog and Roeder (1956). Treherne and Smith (1965b) suggest that the high AChE activity in the insect nervous system, particularly that portion of the activity localised on peripheral glia, forms an effective barrier to the penetration of ACh. This suggestion would infer that only a small portion of AChE present in the insect CNS is associated with synaptic events.

Treherne, Lane, Moreton and Pichon (1970) have shown that mechanical treatment of the preparation has a marked effect on the accessibility of the CNS to small ions. They report that there is a barrier to small ions associated with tight junctions at the inner margin of the intercellular perineural cleft (Pichon, Moreton and Treherne, 1971). This barrier has only a small, but finite permeability for choline ions. These more recent results cast doubt on the earlier work of Treherne and Smith.

In contrast to the vertebrate neuromuscular junction ACh is not the transmitter at the insect neuromuscular junction (Usherwood, 1963; Kerkut and Walker, 1966). Kerkut and Walker (1967) have produced evidence that glutamate is the excitatory transmitter at this site.

I.11. The cholinergic system in insects

The occurrence of ACh in insects was first reported by Gautrelet (1938) and Cortegianni and Serfaty (1939). Means (1942) showed the presence of AChE in a species of Orthoptera. Tobias, Kollros and Savit (1946) showed that ACh accumulated in insect nervous tissue following treatment with DDT. Thus evidence existed for the occurrence of the three primary components of the cholinergic system in insects. Hopf (1952) claimed that Tobias *et al.* had only demonstrated the presence of a substance with ACh-like action and not unequivocally identified it as ACh. Lewis (1953) and Cherfurka and Smallman (1955, 1956) identified ACh in houseflies and blowflies. Chang and Kearns (1955) demonstrated the presence of ACh in *Periplaneta*. However, other choline esters are also present in insect tissues (Prosser, 1952; Augustinsson and Grahn, 1954). One of these esters has been identified as B,B-dimethylacrylylcholine (Bisset, Frazer, Rothschild and Schachter, 1960) and is almost as potent as ACh when tested on guinea pig ileum.

Insects have a dietary need for choline and its absence from their diet prevents normal growth and maturation. Symptoms of choline deficiency may be relieved by supplementing the diet with certain related compounds, e.g., carnitine. It has been suggested that these analogues could be acetylated in nervous tissue and be capable of functioning as transmitters (Bridges, 1972).

The only insect species thoroughly investigated biochemically for the distribution of ChAc, ACh and ChE is P. americana. Colhoun (1959) reports that the activity of all three components is restricted to the nervous system and that correlation between ChAc activity and ACh content in various regions of the nervous system is good (see table 2). Other workers find that components of the cholinergic system do occur in other tissues. Dry venom sacs of Vespa crabro contain 50 mg/g of ACh, royal jelly contains 1 mg/g of ACh and the reproductive organs of Lepidoptera contain extremely high amounts of ACh (Colhoun, 1963). These findings suggest that ACh may function as a local hormone in these tissues.

The pathway for the synthesis of ACh in insects involves two steps, the rate-limiting step being the synthesis of acetyl CoA (Smallman, 1956, 1961). The reactions are:

ATP + CoA + acetate = acetylCoA + AMP + PPi

acetylCoA + choline = acetylcholine + CoA

Toppozoda, Eldefrawi and O'Brien (1970) have isolated a muscarone binding protein from housefly brain. This binding is blocked by a number of cholinergic agents. They conclude that this is the ACh receptor protein.

In summary the available evidence indicates that ACh is an excitatory transmitter in the insect CNS and may also function as a local hormone in

other tissues. Insect AChE is localised both on neurones and glia. It may protect neurones from excitation by extraneous ACh as well as functioning in synaptic transmission.

TABLE 2. The distribution of ACh, ChAc and ChE in the conductive tissue of Periplaneta americana (after Colhoun, 1959)

The ACh content is expressed as ug/g wet weight of tissue.
QChAc is expressed as mg ACh synthesised/g wet weight of tissue/hr.
QChE is expressed as mg ACh hydrolysed/g wet weight of tissue/hr.

TISSUE	ACh	QChAc	QChE
Brain	143.6 ± 4.0	50.6	137.7 ± 15.4
Brain and suboesophageal ganglion	135.2 ± 2.0	53.0	153.4 ± 21.2
Ventral cord	63.2 ± 4.5	10.6	270.4 ± 16.3
Thoracic cord	79.0 ± 6.1	11.4	221.3 ± 15.9
Thoracic ganglia	95.4 ± 4.3	20.8	331.8 ± 12.4
Thoracic connectives	31.3 ± 2.4	2.6	238.7 ± 18.5
Abdominal cord	65.2 ± 1.6	6.2	187.5 ± 10.1
Sixth abdominal ganglion	63.0 ± 1.5	18.0	314.9 ± 20.6
Fifth leg nerve	1.21*	2.0	176.5 ± 26.8
Cercal nerves	1.43*	3.6	150.8 ± 30.3
Coxal muscle	0	0	0
Flight muscle	0	0.08	?
Heart	0	0	0
Blood serum	0	0	?
Blood cells	0	0	?

* expressed as ug/60 nerves

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QChAc is expressed as mg ACh synthesised/g wet weight of tissue/hr.
QChE is expressed as mg ACh hydrolysed/g wet weight of tissue/hr.

TISSUE	ACh	QChAc	QChE
Brain	143.6 ± 4.0	50.6	137.7 ± 15.4
Brain and suboesophageal ganglion	135.2 ± 2.0	53.0	153.4 ± 21.2
Ventral cord	63.2 ± 4.5	10.6	270.4 ± 16.3
Thoracic cord	79.0 ± 6.1	11.4	221.3 ± 15.9
Thoracic ganglia	95.4 ± 4.3	20.8	331.8 ± 12.4
Thoracic connectives	31.3 ± 2.4	2.6	238.7 ± 18.5
Abdominal cord	65.2 ± 1.6	6.2	187.5 ± 10.1
Sixth abdominal ganglion	63.0 ± 1.5	18.0	314.9 ± 20.6
Fifth leg nerve	1.21*	2.0	176.5 ± 26.8
Cercal nerves	1.43*	3.6	150.8 ± 30.3
Coxal muscle	0	0	0
Flight muscle	0	0.08	?
Heart	0	0	0
Blood serum	0	0	?
Blood cells	0	0	?

* expressed as ug/60 nerves

SECTION TWO

THE PROPERTIES OF AChE FROM *Periplaneta americana* IN NERVE TISSUE HOMOGENATES

2.1. Plan of experiments

The activity of many enzymes decays rapidly in tissue homogenates or when treated with a variety of agents routinely used to solubilise or activate enzymes, for example, detergents. Therefore, the stability of cockroach AChE in total nerve cord homogenates was tested over a period of hours. The nature of the observed decay of AChE activity was investigated.

Several detergents were tested for their effectiveness in stabilising and activating/re-activating AChE in such preparations. These experiments lead to the proposal of two methods for measuring the total AChE activity in cockroach nerve tissue homogenates.

The effectiveness of a variety of detergents in solubilising the membrane bound AChE was also investigated.

2.2. Methods

2.2.1. Dissection of nerve cords

Nerve cords were routinely dissected by removing the legs from the animal as near as possible to where the coxa joins the body of the animal. Prior to this operation the animal was sometimes rapidly frozen on dry ice depending on the purpose of the experiment (Willner and Mellanby (1974) found that the specific activity of AChE in homogenates of the metathoracic ganglion was the same whether the animals were frozen in this way or not). The animal was then pinned out on a wax block, ventral surface uppermost.

The abdominal cord was exposed by cutting the sternites on either side of the cord, carefully peeling the middle section of the sternites back to the tip of the abdomen and cutting it free from the rest of the animal. The sixth abdominal ganglion was exposed by displacing the overlying mushroom shaped gland to one side (male animals only were used in all experiments). The thoracic cord was exposed by cutting the T-shaped middle section of the thoracic sternites laterally and dissecting each one from the animal individually. The sixth abdominal ganglion was dissected free from nerves, cleaned free of surrounding fatty tissue and the abdominal cord lifted back as far as the metathoracic ganglion. It was cleaned free of surrounding fatty tissue. Each of the three thoracic ganglia was cut free from nerves other than the connectives and cleaned free of tracheoles and fatty tissue. The connectives were cut anterior to the prothoracic ganglion and the whole cord lifted out and immediately placed in buffer on ice.

In some experiments only parts of the cord were used, particularly the metathoracic ganglion. In this case the operations were modified such that the appropriate part of the cord was exposed and cleaned free of other tissue as previously described. Both anterior and posterior connectives were cut close to the ganglion to be removed and the ganglion lifted out.

Male animals only were used in these experiments as they have less fatty tissue surrounding the nerve cord, particularly around the thoracic ganglia.

2.2.2. Homogenising fluid and homogenisation procedure

The dissected nerve cord was placed in 1 - 2 ml of homogenising fluid. This was most commonly 0.1M Na_2HPO_4/NaH_2PO_4 buffer, pH 8.0, but sometimes other buffers, e.g., Tris/HCl buffer, were used and sometimes the medium was Pringle's cockroach Ringer. All samples were stored on ice at a temperature of 2-4°C. Depending on the design of the individual experiment small amounts of detergent were added to the buffer.

Homogenisation was achieved using a Voss overhead homogeniser fitted with a Teflon head and operated at a speed of 2,000 R.P.M. The best way found to achieve a constant homogenisation of tissue was to subject each extract to a constant number of vertical strokes delivered with approximately the same speed so that the whole process occurred over a reasonably constant time (about 1 min.). Routinely fifteen upward/downward strokes were given to each extract. Care was taken that no excessive local heating took place during the procedure. Each extract was homogenised immediately prior to the initial assay of AChE in order to reduce the effect of any decay process to a minimum.

2.2.3. AChE assay

AChE activity was routinely assayed on either a Pye Unicam SP 600 spectrophotometer connected to a Vitatron UR401 pen recorder or a Pye Unicam SP 1800 spectrophotometer connected to a Pye Unicam AR 25 recorder. The latter system was used for the more accurate and sensitive work or for assays in which aldrithiol-2 was used.

The assay used was that of Ellman, Courtney, Andres and Featherstone (1961) in which the false substrate ATCh is hydrolysed to acetate and thiocholine. The thiocholine then undergoes a coupled reaction with DTNB.

The yellow coloured anion of 5,5'-dithiobis-(2-nitrobenzoic acid) produced in the reaction is measured by its absorption at a wavelength of 412 nm. This anion has an extremely large molar extinction coefficient (1.36 x 10^4 L. $mole^{-1}$ cm^{-1}) thus accounting for the sensitivity of the assay.

Assays were carried out in 0.4, 1 and 3 ml cuvettes depending on the AChE activity of the sample. The concentrations of reagents used are as described by Ellman et al. The proportions of constituents added are listed for a typical assay using a 3 ml cuvette.

10mM DTNB	0.1 ml
10mM ATCh	0.3 ml
Nerve cord homogenate	0.1 ml
0.1M phosphate buffer, pH 8.0	2.5 ml

The DTNB was made up in 0.1M phosphate buffer, pH 7.0. It was stored on ice and made up fresh every 3-4 days. The ATCh was made up in 0.1M phosphate buffer, pH 8.0, was stored on ice and made up fresh daily. Cockroach AChE exhibits substrate inhibition at ATCh concentrations of 2-3mM. Therefore a final substrate concentration of 1mM was used in experiments in which the total AChE activity of a homogenate was being measured. In experiments to determine the kinetic parameters of the enzyme a range of substrate concentrations from 1 to 0.01mM was used.

Brownson and Watts (1973) suggest that the kinetic parameters of AChE are altered by DTNB and propose the use of aldrithiol-2(2,2'-dithiodipyridine) as an assay reagent which does not affect the kinetics of the enzyme. The principle of the reaction is exactly the same as that for DTNB. However, the reagent is less sensitive (molar extinction coefficient 7.06 x 10^3 L. $mole^{-1}$ cm^{-1}). The reaction is monitored at a wavelength of 343 nm. Therefore in certain experiments the enzyme was assayed with this reagent.

Ald-2 only dissolves at acid pH's and was dissolved in distilled water to which the minimum amount of concentrated HCl to bring about effective solubilisation of the reagent (1-2 drops) was added.

A slow,but appreciable hydrolysis of ATCh takes place at alkaline pH's. Therefore, in all experiments this rate was measured in a blank assay in which no enzyme was added to the cuvette. If the value obtained was significant as compared with the rate of enzymic hydrolysis the blank value was subtracted from the rate measured in the presence of enzyme. If the rate was measured using an SP 1800 spectrophotometer the blank rate was automatically adjusted for by adding substrate to the reference cell with the machine set for double beam readings.

Chemicals were stored in a vacuum dessicator at -20^{o}C prior to being dissolved for use.

2.2.4. Assay of protein content of samples

The protein concentration of samples was determined by taking an aliquot of the sample, diluting it with buffer if necessary, and assaying it for protein content by the method of Lowry, Rosebrough, Farr and Randall (1951). The total volume of each assay was 0.375 ml, a volume suitable for estimating total protein content in the range 2-20 ug. All protein measurements were carried out in duplicate. All glassware used for protein assays was thoroughly washed in chromic acid and rinsed in distilled water. This procedure removed grease and dirt from the surface of the glass and prevented it from contributing to the measured O.D. A standard calibration curve in the region 2-20 ug/sample was constructed using known concentrations of bovine serum albumin, Cohn fraction 5.

2.2.5. Calculation of the specific activity of AChE in samples

The specific activity of AChE (umoles ATCh hydrolysed/min./ug protein) was calculated from the following formula:

$$\text{umoles substrate hydrolysed/min./uL} = \frac{\text{O.D. change/min.}}{1.36 \times 10^4} = V_o$$

Therefore, the specific activity of AChE is given by:

$$\text{umoles substrate hydrolysed/min./ug prot.} = V_o \times \frac{\text{Vol. of assay}}{\text{protein content}}$$

The protein content refers to the total amount of protein added to the AChE assay.

2.2.6. Addition of detergents to nerve cord homogenates

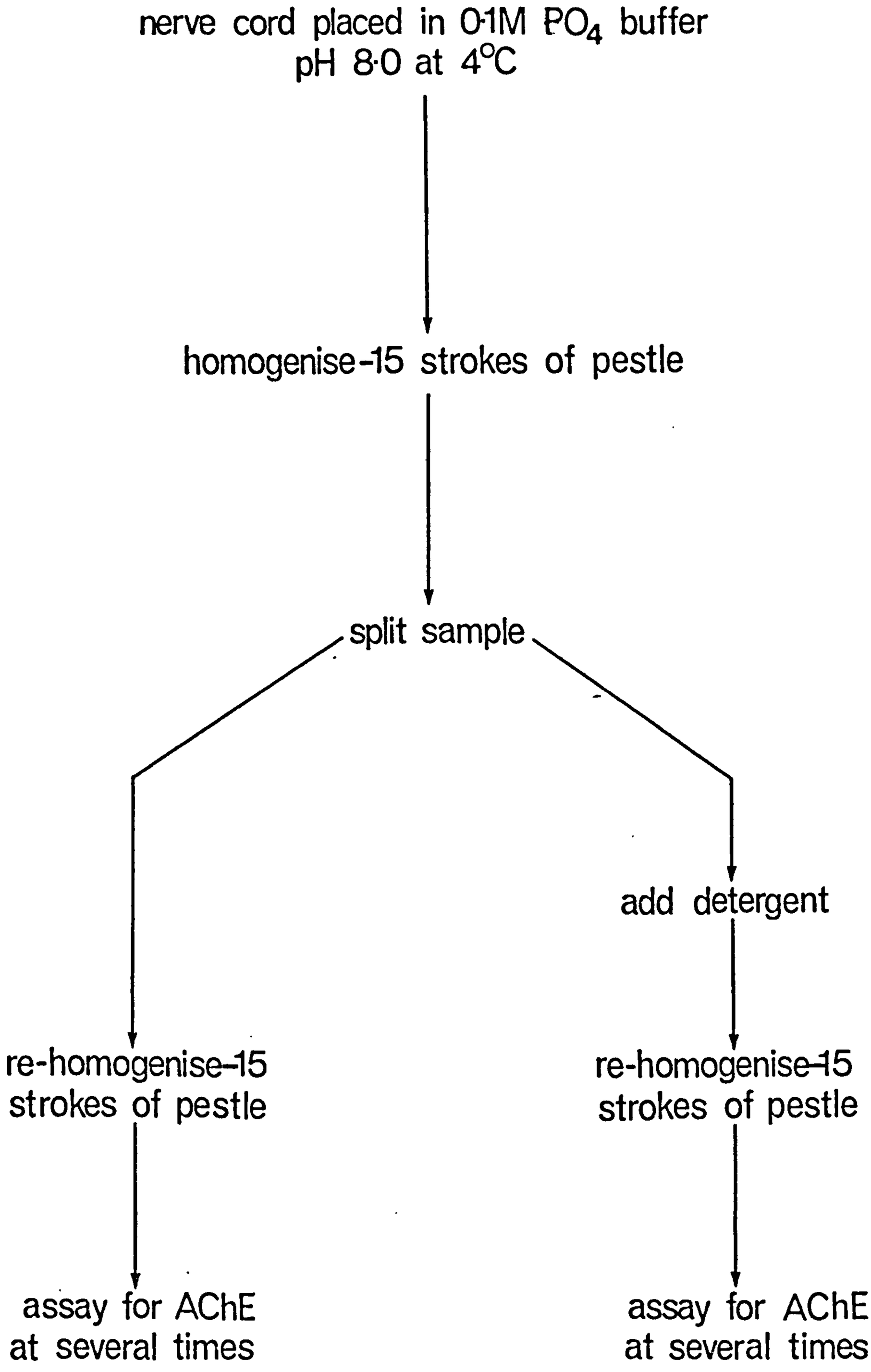
Detergents were normally added to each sample prior to homogenisation. When detergent was added to a sample after homogenisation, e.g., in re-activation experiments, the sample was re-homogenised. Detergents used in this study were principally lubrol-PX, triton-X-100 and DOC, the first two being added at a concentration of 1% (v:v).DOC was found to inhibit the enzyme at this concentration and was therefore added at a concentration of 0.1% or 0.25% (w:v).

In experiments in which the activity of a detergent activated sample was being compared with the activity of the untreated sample the homogenates were prepared according to the scheme shown in fig. 3.

In order to determine the effectiveness of the detergents in solubilising AChE, treated samples were separated into supernatant and particulate fractions by centrifugation. Each sample was centrifuged at 100,000 g for 1 hr. on an MSE 65 ultracentrifuge. The supernatant fraction was removed with a Pasteur pipette, the tip of which was placed below the layer of

FIG. 3. Procedure used to compare AChE activity in detergent treated and untreated nerve cord homogenates

In order to obtain an accurate comparison of AChE activity in detergent treated and untreated samples, the samples were initially homogenised and then divided into two equal fractions. Detergent was added to one fraction only. Both fractions were re-homogenised and assayed for AChE activity. In this way it was possible to eliminate differences due to the variation of AChE activity between animals and to obtain an accurate measure of any activation of the enzyme produced by the detergent.


nerve cord placed in 0·1M PO_4 buffer pH 8·0 at 4°C
homogenise-15 strokes of pestle
split sample
re-homogenise-15 strokes of pestle
assay for AChE at several times
add detergent
re-homogenise-15 strokes of pestle
assay for AChE at several times

lipoprotein which floated on the top of the supernatant. In this way the supernatant fraction was separated from the lipoprotein and pellet material. The pellet material was washed with a small volume of 0.1M phosphate buffer, pH 8.0, which was subsequently discarded. The pellet material was then resuspended in an equal volume of buffer to the supernatant and re-homogenised by the standard procedure. It was then possible to assay the AChE activity in the two fractions and determine the % of the enzyme that had been solubilised. In some experiments the lipoprotein fraction was also separated by removing it from the top of the supernatant with a Pasteur pipette. This could then be assayed for AChE activity.

2.3. Materials

Periplaneta americana used in these experiments were purchased from Gerrard and Haig, Gerrard House, Worthing Road, East Preston, Sussex.

The chemicals used in these experiments were obtained from BDH Biochemicals, Poole, Dorset with the following exceptions:

N-ATCh iodide, DTNB and lubrol-PX were obtained from the Sigma Chemical Company, St. Louis, Missouri, U.S.A.

Aldrithiol-2 was obtained from Ralph Emanuel Ltd., Wembley, Middlesex.

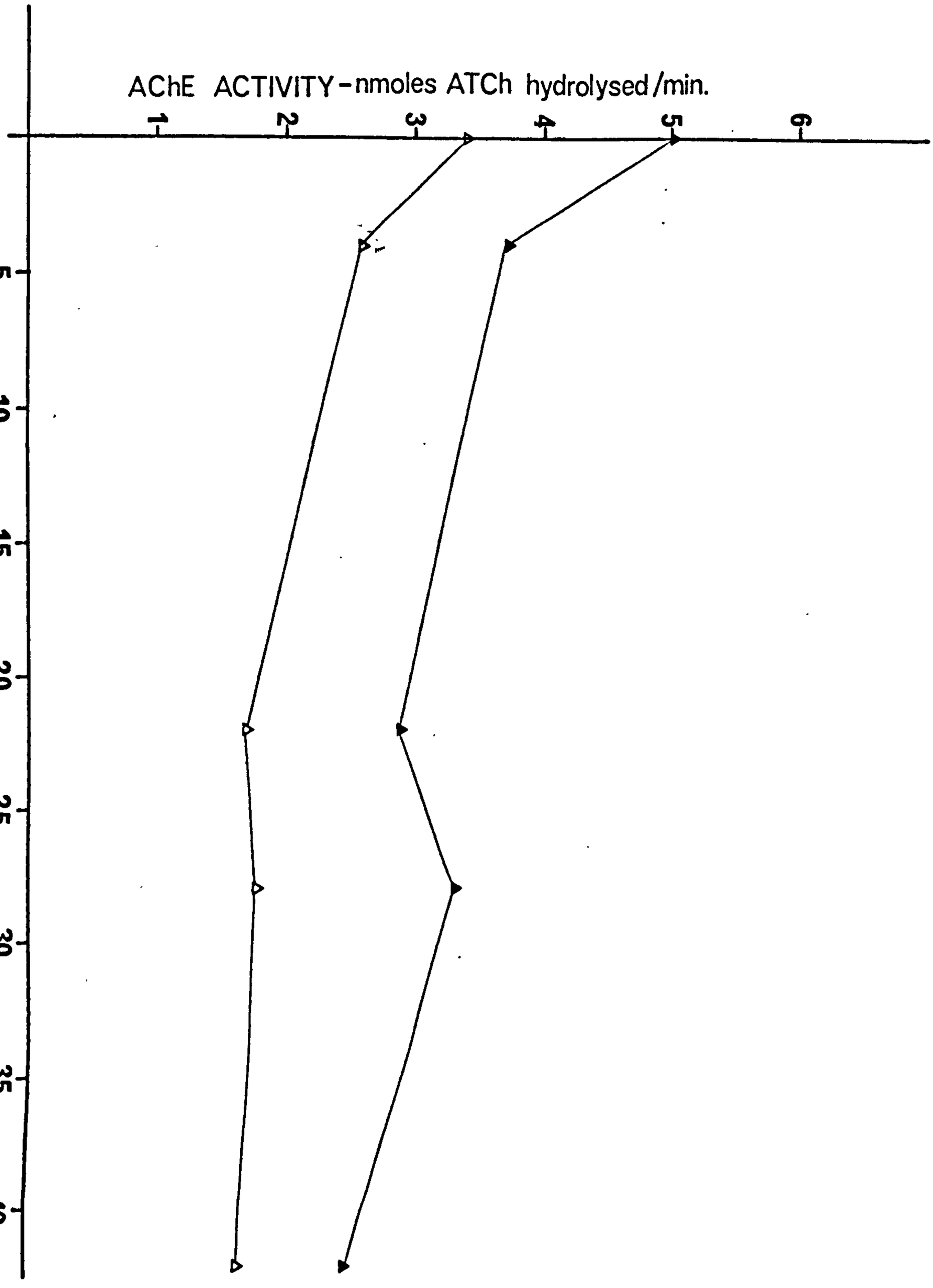
2.4. Results

2.4.1. Decrease of AChE activity in total nerve cord homogenates

The results of the first experiment (fig. 4) show the AChE activity in two homogenates of nerve cord tissue assayed at 0, 4, 22, 28 and 42 hrs. after homogenisation. During this time the samples were stored

FIG. 4. A graph showing the decrease in AChE activity in total nerve cord homogenates at various times after homogenisation

AChE activity is plotted against time after homogenisation of the sample. The enzyme activity decreases rapidly, both samples losing 40% of the initially measured AChE activity in the first 4 hrs. after homogenisation. After this the activity in the samples is relatively stable, only a further 10% of enzyme activity being lost in the next 36 hrs. The samples were stored on ice at 2-4^{o}C in sealed containers.


AChE ACTIVITY - nmoles ATCh hydrolysed /min.
1
2
3
4
5
6
TIME AFTER HOMOGENISATION (hrs.)
5
10
15
20
25
30
35
40

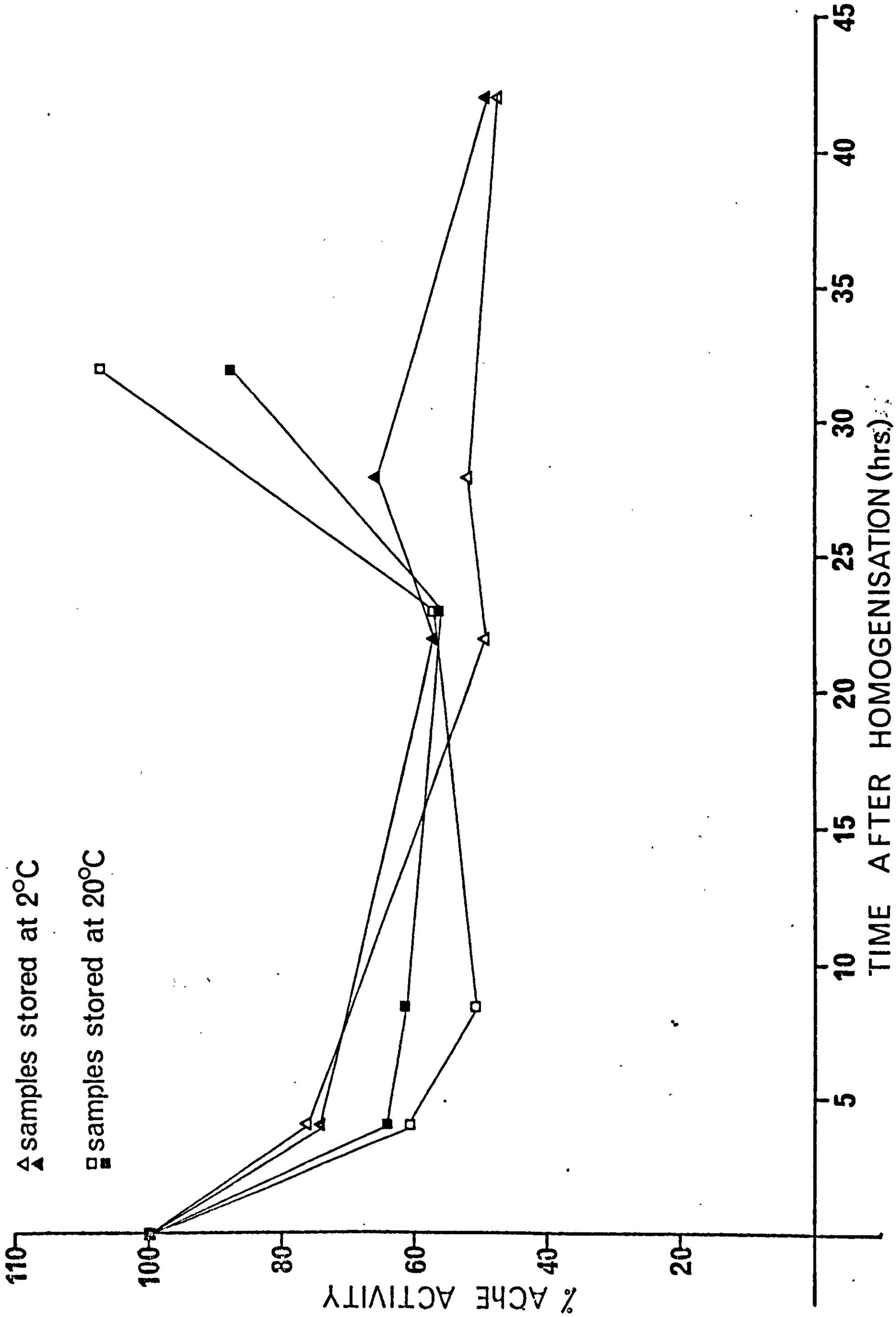
on ice at a temperature of 2-4°C in sealed containers. The graphs show that even though the samples were stored at ice temperature the measurable AChE activity was unstable. Both samples lost 40% of their initial AChE activity in the first 4 hrs. after homogenisation. During the next 36 hrs. only a further 10% of the initial AChE activity was lost.

This rapid loss of measurable AChE activity was observed in all samples tested. The phenomenon was found to be temperature dependant. Fig. 5 shows the decay of enzyme activity in two samples stored on ice and two samples stored at room temperature (20°C). The samples stored at room temperature lost 40% of their initial activity in the first 4 hrs. following homogenisation, whereas the samples stored at 2-4°C lost only 25% of their initial activity over the same time period. It should be noted that AChE activity is not significantly increased if the aged sample is re-homogenised.

Two features of the effect of temperature on the process are of special interest. The first is that although the rate of decay of activity was faster at room temperature than at 2-4°C at both temperatures a constant fraction (40-50%) of the initially determined AChE activity was lost. The second is that the AChE activity in the samples stored at 20°C began to increase 22-30 hrs. after homogenisation and increased to between 85-100% of the original activity. This would indicate that recovery of the decayed activity is possible, though the possibility that the increase in AChE activity may be due to another process must be borne in mind, e.g., an activation of the fraction of the enzyme remaining after loss of initial activity.

FIG. 5. A graph showing the % of the initial AChE activity in total nerve cord homogenates measured at various times after homogenisation and the effect of temperature on this

The AChE activity (expressed as % of the initially determined value) in the samples is plotted against time after homogenisation. Samples were stored at 2-4°C or at 20°C. Samples stored at both temperatures lost 40-50% of their initial AChE activity. This loss of activity occurred more rapidly in the samples stored at 20°C than in the samples stored at 2-4°C. The AChE activity in the samples stored at 20°C increased to 85-100% of their initial activity 24-32 hrs. after homogenisation. This increase was not observed in the samples stored at 2-4°C.


% ACHE ACTIVITY
110
100
80
60
40
20
TIME AFTER HOMOGENISATION (hrs.)
5
10
15
20
25
30
35
40
45
samples stored at 20°C
samples stored at 2°C

This rapid loss of AChE activity was further investigated by determining the AChE activity in homogenates over a much shorter time period, i.e., the phase of rapid decay in the first 2-3 hrs. after homogenisation. The time of the initial homogenisation was noted and all other times were related to this. Several types of graphical analysis of the results were tested. A plot of log. AChE activity against log. time after homogenisation of sample is linear during the period of rapid loss of AChE activity (fig. 6). Thus during this period

$$\text{log. AChE activity} \propto \frac{1}{\text{log. time after homogenisation}}$$

The validity of this relationship has several important consequences.

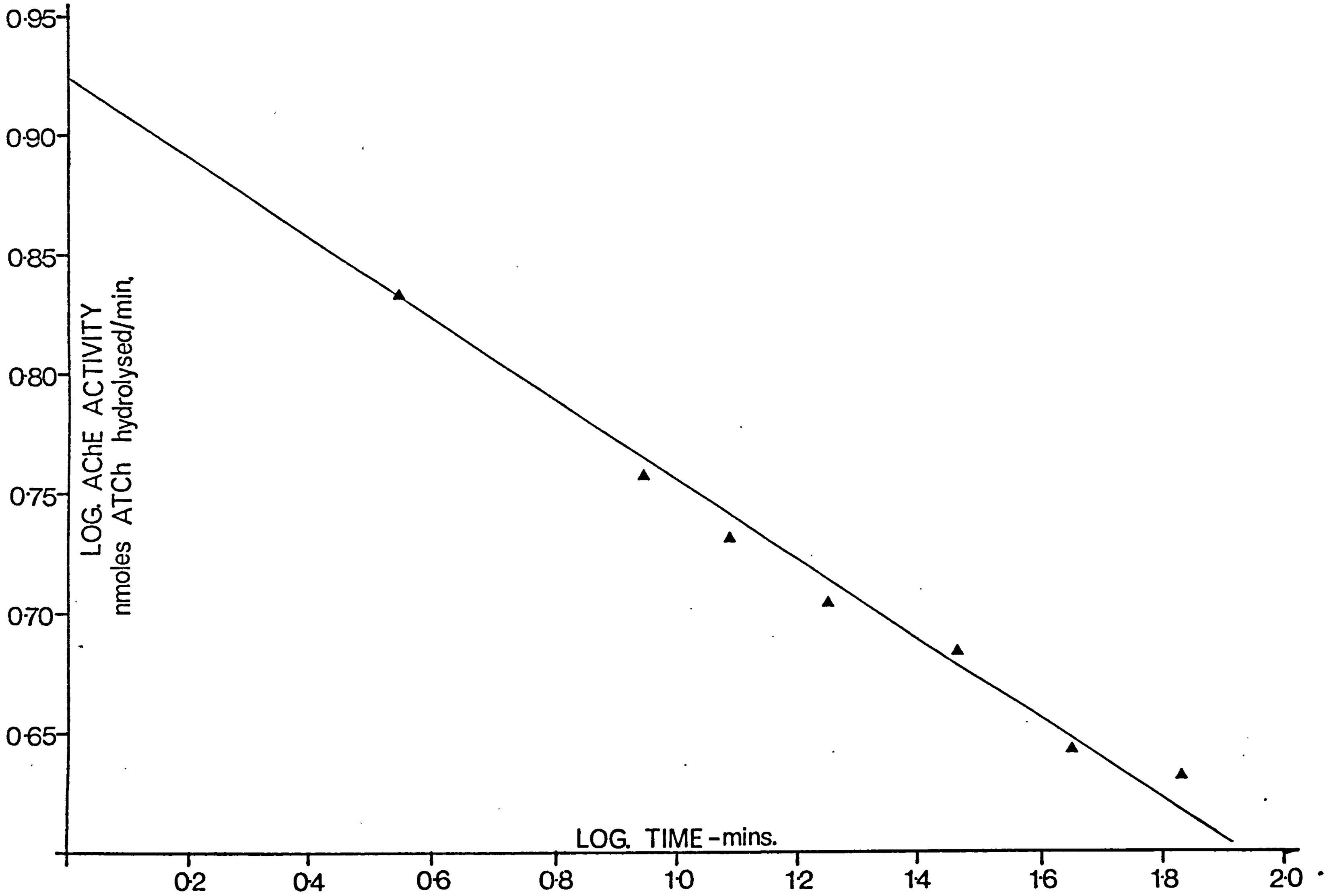
(1) By extrapolation of the graph to the time of homogenisation of the sample, i.e., t = 0, the total AChE activity of the sample at this time can be measured from the intercept on the Y axis.

(2) The loss of AChE activity in the first few minutes after homogenisation is extremely rapid. For example, for the sample shown in fig. 6 the AChE activity decayed from 8.3 nmoles ATCh hydrolysed/min. to 6.8 nmoles ATCh hydrolysed/min. during the first three mins. after homogenisation, i.e., a decrease of 19% in three mins.

(3) The decay of AChE activity is not a typical denaturation process in which a constant fraction of the pool of active enzyme is denatured over constant time periods. This type of denaturation process is described by an exponential, and not a log.-log., relationship. Thus the loss of AChE activity in cockroach nerve cord homogenates is not due to denaturation of a single species of the enzyme.

FIG. 6. A plot of log. AChE activity against log. time after homogenisation of the sample

The log.-log. plot shows that during the period of rapid loss of AChE activity in total nerve cord homogenates log. AChE activity is inversely proportional to log. time after homogenisation of the sample. A regression line for this analysis was plotted on a Diehl calculator and a correlation coefficient of -0.9615 between the two variables was obtained. The sample was stored at 2-4°C.


0·95
0·90
0·85
0·80
0·75
0·70
0·65
LOG. AChE ACTIVITY
nmoles ATCh hydrolysed/min.
LOG. TIME - mins.
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0

2.4.2. Stabilisation and activation of AChE in total nerve cord homogenates

In order to obtain an accurate determination of an enzyme activity in a sample it is necessary to ensure that total enzyme activity is being assayed. Further, the activity in the sample should be stable, at least for the period during which the experiment is being conducted. Clearly AChE activity in cockroach nerve cord homogenates does not fulfil these criteria. It is desirable that the enzyme activity should be stable for a period of several days so that the sample may be subjected to further biochemical operations, e.g., purification procedures. Therefore the stabilising and activating effects of several agents on AChE activity in total nerve cord homogenates were investigated.

Many workers have observed that detergents stabilise and activate enzyme preparations, e.g., Fonnum (1969) reports use of triton-X-100 to activate ChAC and AChE from mammalian nervous tissue. The effects of the non-ionic detergent triton-X-100 and the anionic detergent DOC on the stability of cockroach AChE are illustrated in figs. 7 and 8 respectively. At concentrations of 0.025%, 0.1% and 1% (v:v) triton-X-100 was extremely effective in stabilising the AChE activity for a period of at least six days following homogenisation of the samples. At the two higher concentrations the detergent also produced a slight increase in measured AChE activity. This increase was of the order of 10-15% during the first 20 hrs. after homogenisation. The increase of measurable AChE activity was probably due to the release of occluded enzyme from membranous structures rather than a specific activation of the enzyme.

DOC was less effective in stabilising AChE activity and at a concentration of 1% produced rapid and complete denaturation of the enzyme. At a concentration of 0.25% it produced a 15% activation of the enzyme followed by a slow

FIG. 7. The stability of AChE activity in nerve cord homogenates treated with triton-X-100

AChE activity decreased rapidly in the sample to which no detergent had been added (45% of the initial activity was lost in the first 24 hrs. following homogenisation). In those samples treated with triton-X-100 the enzyme activity was quite stable over a period of days. Note the slight activation (10-15%) in samples containing 0.1% and 1% triton-X-100, but not in the sample containing 0.025% of the detergent.

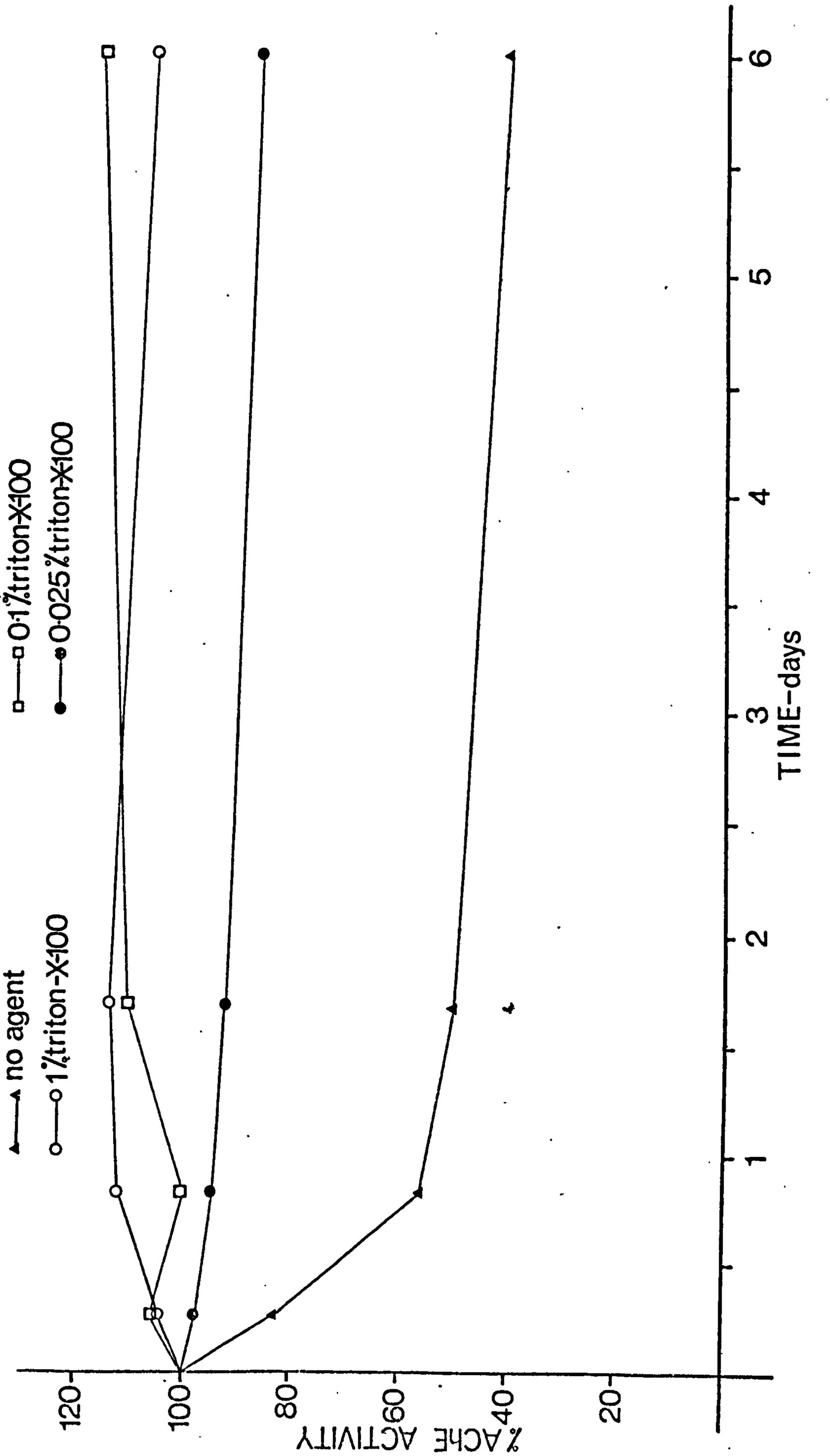
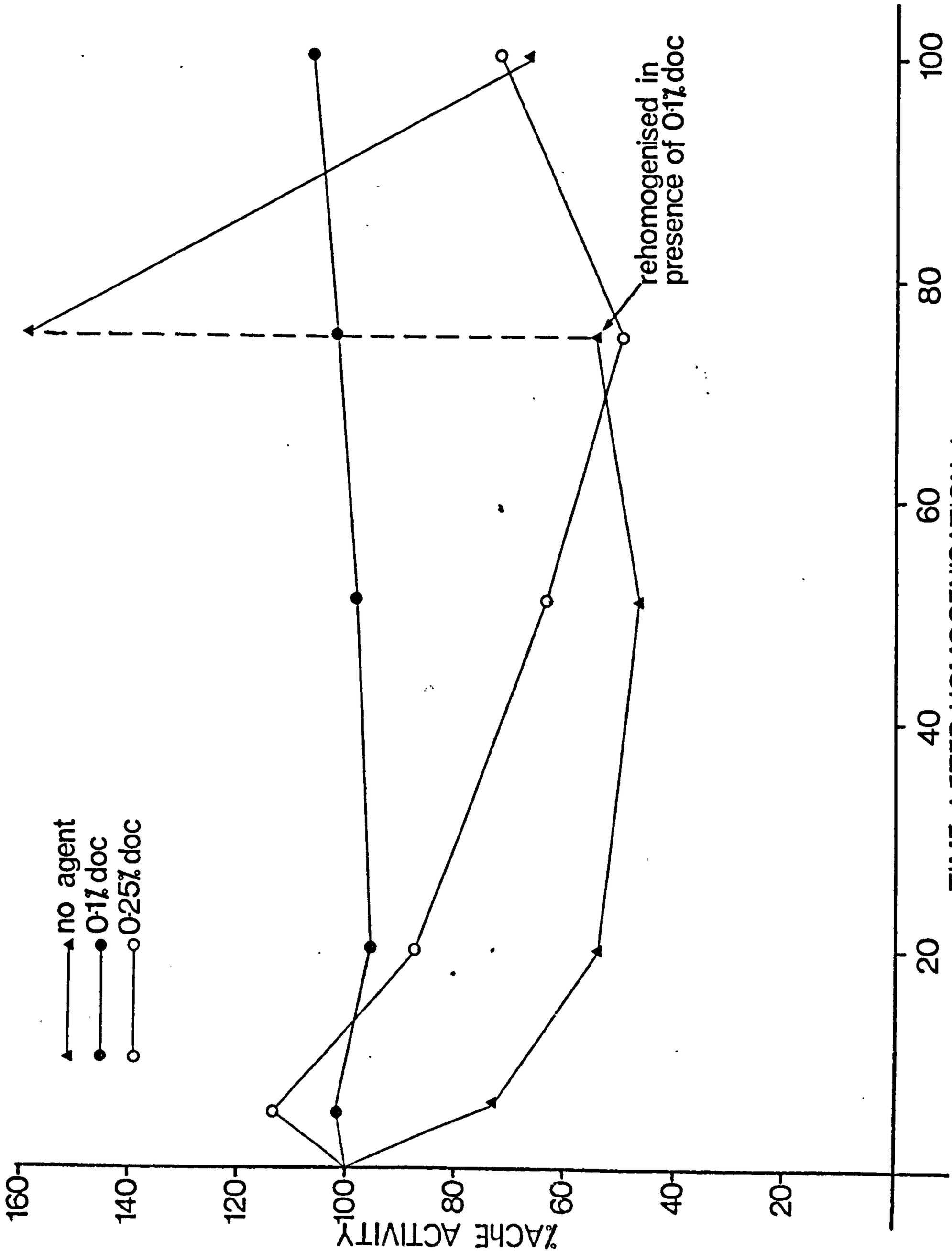

0·1%triton-X100
0·025%triton-X100
no agent
1%triton-X100
%ACHE ACTIVITY
120
100
80
60
40
20
TIME-days
1
2
3
4
5
6

FIG. 8. The stability of AChE activity in nerve cord homogenates treated with DOC

0.25% DOC produced an activation of the enzyme followed by a slow denaturation. Higher concentrations of the detergent resulted in rapid and complete denaturation of the enzyme. 0.1% DOC effectively stabilised the enzyme and also produced a slight (10%) increase in its activity. Treatment of the control sample, which had lost 45% of its original activity, with 0.1% DOC produced a large increase in the AChE activity to 160% of the initially measured value.


%ACHE ACTIVITY
160
140
120
100
80
60
40
20
TIME AFTER HOMOGENISATION-hrs.
20
40
60
80
100
no agent
0·1% doc
0·25% doc
rehomogenised in presence of 0·1% doc

denaturation. The highest concentration of DOC which would effectively stabilise the AChE activity was 0.1%. At this concentration DOC stabilised AChE activity for several days.

These results are similar to those reported by Jackson and Aprison (1966b) for the effects of non-ionic and anionic detergents on AChE from the mammalian CNS.

Having established that the detergents tested were effective in stabilising AChE activity in total nerve cord homogenates, the activating properties of these agents were investigated more thoroughly. Both the activation of the enzyme in freshly homogenised samples and the re-activation of the enzyme in sampleswhich had lost 40-50% of their initial activity were investigated. The scheme used to carry out these studies is that illustrated in fig. 3. The results of these studies are summarised in tables 3 and 4.

The results presented in table 3 show that addition of 0.1% DOC to one half of a freshly homogenised nerve tissue sample produces a 25% activation of AChE compared with the enzyme activity in the untreated half of the same sample. A similar experiment in which 1% lubrol-PX was used showed that this agent neither activated nor inhibited the enzyme in the treated as compared to the untreated half of the sample. In both of these experiments the time between the initial homogenisation of the sample and the assay of the untreated fraction was 2½-3 mins. The activating effect of triton-X-100 on a freshly homogenised sample was less thoroughly investigated, but the available results indicate that it had no immediate activating or inhibiting effects on the enzyme.

TABLE 3. The effects of 0.1% DOC and 1% lubrol-PX on AChE activity in freshly homogenised nerve cord samples

(AChE activity is expressed as nmoles ATCh hydrolysed/min./ug prot.).

3a. These results show that treatment of a fraction of each sample with 0.1% DOC produced a 24.6% activation of AChE as compared with the untreated fractions of the same samples.

Sample No.	AChE activity in untreated fraction	AChE activity in fraction treated with 0.1% DOC
1	1.153	1.691
2	1.180	1.378
3	1.343	1.482
4	1.475	1.965
5	1.645	1.956
mean	1.359	1.694
s.e.m.	± 0.0824	± 0.1069

3b. These results show that treatment of a fraction of each sample with 1% lubrol-PX did not produce activation or inhibition of AChE as compared with the untreated fractions of the same samples.

Sample No.	AChE activity in untreated fraction	AChE activity in fraction treated with 1% lubrol-PX
1	1.30	1.13
2	1.09	1.20
3	1.20	1.11
4	0.97	1.02
5	1.10	1.15
mean	1.13	1.12
s.e.m.	± 0.1581	± .02645

TABLE 4. Re-activation of AChE in total nerve cord homogenates by treatment with triton-X-100, lubrol-PX or DOC

Samples which had lost 40% of their initially measured AChE activity were treated with detergent. The effect of the detergent on the AChE activity of the sample was determined. Only DOC was totally effective in restoring AChE activity. The action of DOC was blocked by pre-treatment of the sample with triton-X-100. AChE activity is expressed as a % of the activity measured immediately after the initial homogenisation of the sample.

Detergent added	% initial AChE activity in untreated fraction of sample	% initial AChE activity in detergent treated fraction of sample
0.1% DOC	56	118
	55	122
	53	133
1% triton-X-100	56	63
	55	68
	50	70
1% lubrol-PX	56	80
	54	74
	52	77
1% triton-X-100 followed by 0.1% DOC	57	82
	55	77
1% lubrol-PX followed by 0.1% DOC	54	121
	52	137

Thus of the three detergents tested only DOC produced any immediate activation of AChE. The activation produced by DOC in this experiment (24.6%) is of the same order of magnitude as the estimated loss of activity by samples during the first 3 mins. after homogenisation (a value of 19% - see fig. 6). The similarity of these two values would suggest that treatment with 0.1% DOC releases the total AChE activity present in samples into a measurable form.

The results presented in table 4 show the effects of all three agents on re-activating samples which have lost 40-50% of their initial AChE activity. The results indicate that only DOC was effective in re-activating such preparations. In this experiment the AChE activity in the DOC treated samples was 24.3% higher than the initially measured AChE activity in the untreated samples, i.e., the same % increase in enzyme activity (compared with the initially determined value) is produced by treatment with DOC whether the sample is freshly homogenised or has been allowed to age and lose 40-50% of its initial AChE activity.

The re-activating effect of DOC is also shown in fig. 8.

The two non-ionic detergents had some re-activating effect on the aged preparations, lubrol-PX being slightly more effective than triton-X-100. Even after treatment of the aged samples with lubrol-PX the AChE activity was still 23% lower than the initially determined activity in the same samples.

It is of interest that re-activation of AChE in aged samples by 0.1% DOC is almost entirely blocked if the samples are first treated with 1% triton-X-100. However, pre-treatment of samples with 1% lubrol-PX does not block the re-activating effect of DOC. This indicates a difference in the mode of action of these three detergents.

2.4.3. The non-linearity of the rate of hydrolysis of ATCh by AChE and the effect of detergents on this

Routine assays of total nerve cord homogenates for AChE activity showed that the progress curves for the assay are non-linear. The rate of enzymic hydrolysis of ATCh increased considerably as each assay proceeded. The sample plots illustrated in fig. 9 indicate that in a freshly homogenised sample the measured rate of hydrolysis of ATCh increased by 33% during the first 4 mins. of the progress curve. When the same sample was aged for 2 hrs. after homogenisation and its AChE activity re-determined the rate of hydrolysis of ATCh increased by 45% during the same time period. Thus as the sample aged and lost activity the non-linearity of the progress curve for the reaction increased. This point is also illustrated in fig. 10.

Fig. 10 shows the rate of hydrolysis of ATCh measured at 1 minute intervals during each assay plotted against the time course of each assay. If a straight line with a slope of 0 is obtained using this plot then the rate of the reaction is constant. The results show that the rate of hydrolysis of ATCh increased by 25% during the first 3 mins. of the progress curve when the sample was freshly homogenised. However, when the sample was aged for 100 mins. after homogenisation the rate increased by 48% during the same time period. Fig. 10 also shows that the hydrolysis of ATCh approached a constant rate after each assay had proceeded for 3 mins.

A possible explanation for this increase in the rate of hydrolysis of ATCh could be that DTNB slowly activates the enzyme. The results of Brownson and Watts (1973) suggested that this reagent might affect the activity of AChE. To test this possibility aged samples of nerve cord homogenates were pre-incubated with DTNB for varying periods of time prior to determination of AChE activity. The rate of hydrolysis of ATCh increased by 59% during the

FIG. 9. Typical progress curves for the hydrolysis of ATCh by AChE in total nerve cord homogenates

The graph shows nmoles ATCh hydrolysed plotted against the time course of the assay. The progress curves show that the rate of hydrolysis of ATCh is non-linear. The rate of hydrolysis of ATCh increased by 33% during the first 4 mins. of the progress curve when the sample was freshly homogenised. When the sample was aged for 2 hrs. after homogenisation the rate of hydrolysis increased by 45% during the same time period. Treatment of the aged sample with 0.1% DOC re-activated the AChE in the sample and also resulted in a linear progress curve.

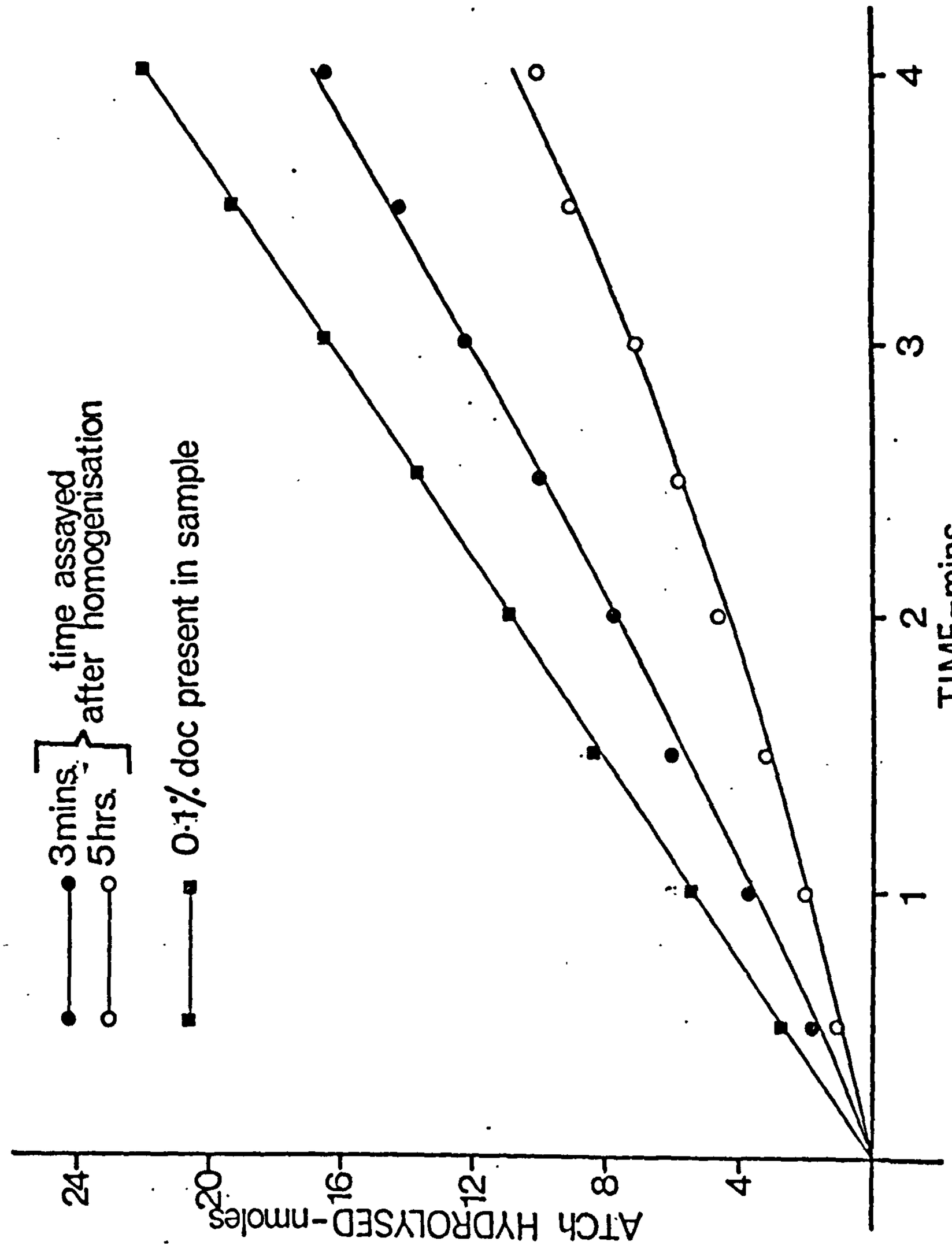
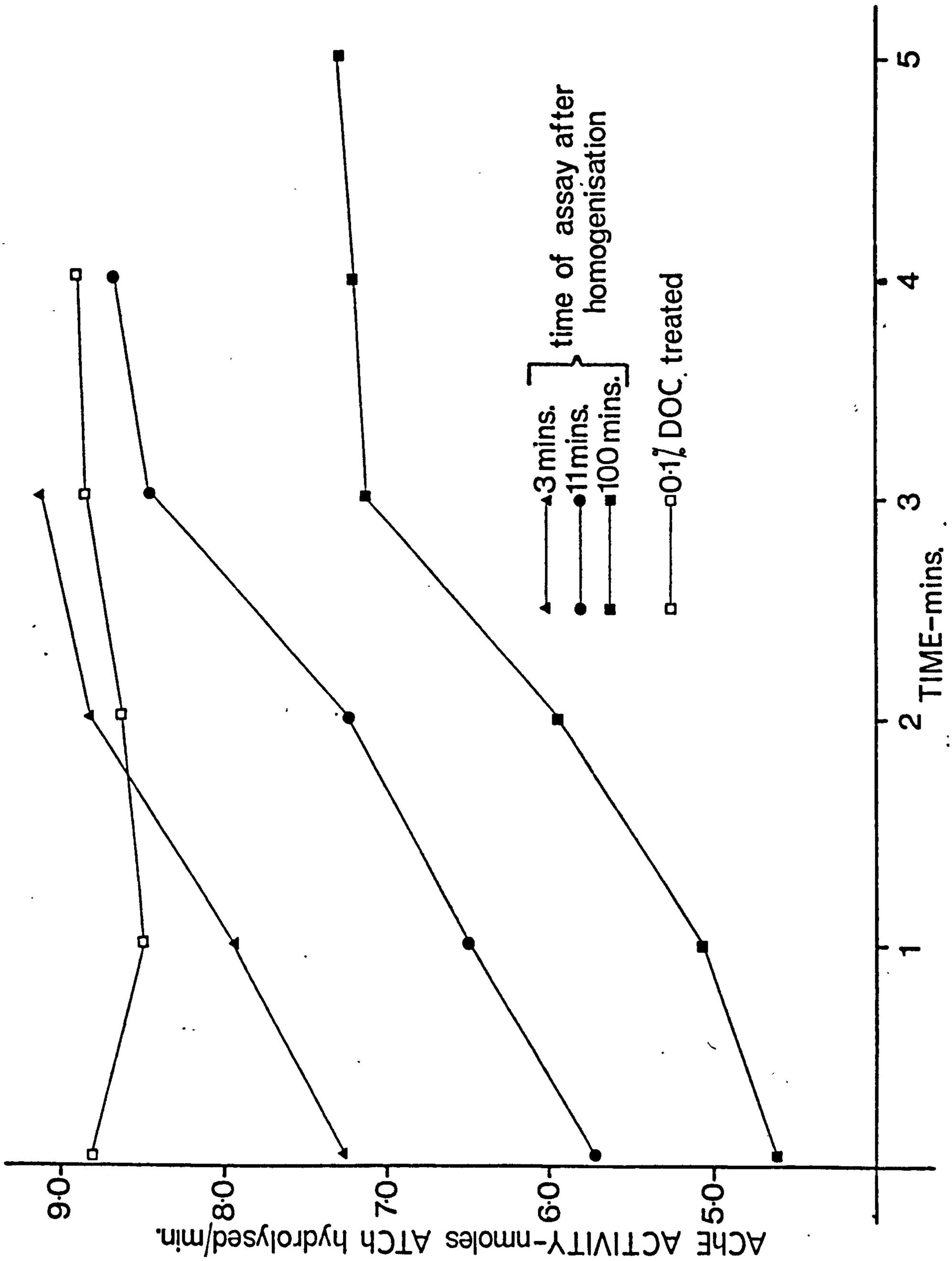

ATCh HYDROLYSED-nmoles
24
20
16
12
8
4
TIME-mins
1
2
3
4
3mins.
5hrs.
time assayed after homogenisation
0·1% doc present in sample

FIG. 10. A graph showing the rate of hydrolysis of ATCh (nmoles ATCh hydrolysed/min.) as a function of time

This plot will give a straight line with a slope of 0 if the rate of reaction is constant. Clearly for the untreated sample this is not so. The increase in the observed rate of reaction during the first 3 mins. of the progress curve is 25% for the freshly homogenised sample. This figure increased to 48% when the sample was aged for 100 mins. after homogenisation. The hydrolysis of ATCh approached a constant rate after each assay had proceeded for 3 mins.

Treatment of the sample with 0.1% DOC resulted in the progress curve for the assay becoming linear.


ACHE ACTIVITY-nmoles ATCh hydrolysed/min.
9·0
8·0
7·0
6·0
5·0
TIME-mins.
1
2
3
4
5
0·1% DOC. treated
3 mins.
11 mins.
100 mins.
time of assay after homogenisation

first 3 mins. of the progress curve when the sample was not pre-incubated with DTNB. Pre-incubation of the sample with DTNB for 15 mins. reduced this figure to 17%. Pre-incubation of the sample with DTNB for longer times did not reduce this figure any further.

The possible activation of AChE by DTNB was further investigated by assaying aliquots of the same sample with DTNB and Ald.-2, and comparing the results (fig. 11). The results show that the progress curve for the reaction is linear when Ald.-2 is used as the assay reagent. A comparison of the rates of hydrolysis obtained with the two reagents shows that the value obtained using Ald.-2 (2.18 nmoles ATCh hydrolysed/min.) is considerably lower than even the initial rate as measured with DTNB (3.30 nmoles ATCh hydrolysed/min.). The rate after the assay had proceeded for 3 mins. (as measured with DTNB) increased to 4.85 nmoles ATCh hydrolysed/min. These results do not necessarily mean that DTNB was exerting a direct activating effect on the AChE.

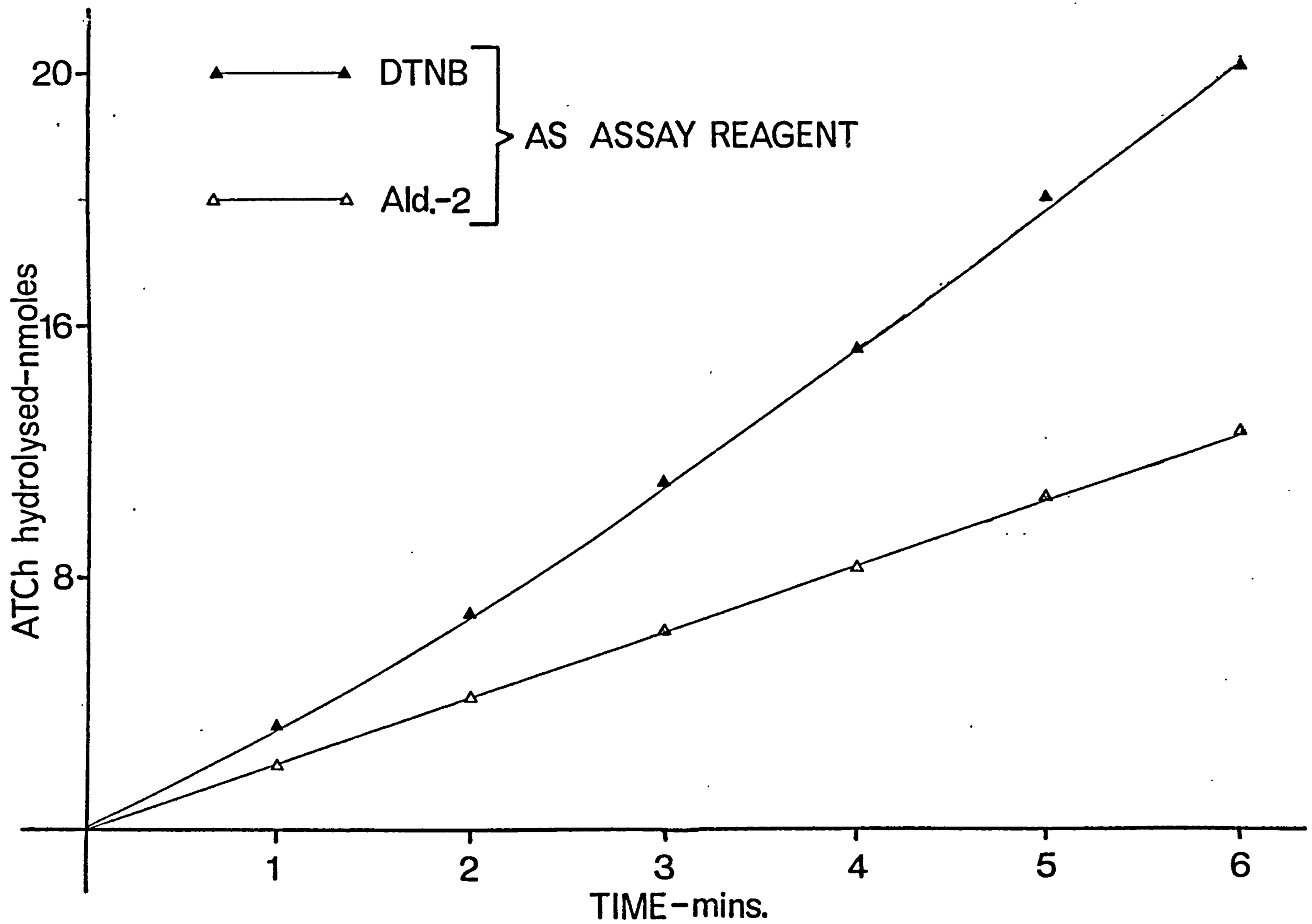
Finally the effects of triton-X-100, lubrol-PX and DOC on the non-linearity of the progress curve for the reaction were investigated. The results are summarised in table 5. Treatment of samples with 1% triton-X-100 had little effect on the non-linearity of the progress curve. In samples treated with 1% lubrol-PX the rate of hydrolysis of ATCh increased by only 8.5% during the first 3 mins. of the progress curve as compared with an increase of 46.5% in untreated fractions of the same samples. Treatment of samples with 0.1% DOC resulted in the progress curve for the assay becoming linear. This point is also illustrated in figs. 9 and 10. It is of interest that when samples were first treated with 1% triton-X-100 and subsequently with 0.1% DOC the rate of hydrolysis of ATCh did not become constant. However, treatment of samples with 1% lubrol-PX prior to treatment

FIG. 11. Progress curves for the hydrolysis of ATCh by AChE in total nerve cord homogenates obtained using DTNB and Ald.-2 as assay reagents

The graph shows nmoles ATCh hydrolysed plotted against the time course of the assay. The sample was aged for 2 hrs. after homogenisation prior to assay. Note that:

1) The progress curve for the reaction, using Ald.-2 as assay reagent, is linear.

2) The rate of hydrolysis of ATCh obtained using Ald.-2 (2.18 nmoles ATCh hydrolysed/min.) is considerably lower than even the initial rate of hydrolysis of ATCh obtained using DTNB (3.30 nmoles ATCh hydrolysed/min.).


DTNB
Ald.-2
AS ASSAY REAGENT
ATCh hydrolysed-nmoles
20
16
8
1
2
3
4
5
6
TIME-mins.

(61)

TABLE 5. The effect of detergents on the non-linearity of the progress curve for the hydrolysis of ATCh by AChE in total nerve cord homogenates.

The table summarises the effects of 1% triton-X-100, 1% lubrol-PX and 0.1% DOC on the non-linearity of the progress curve for the enzymic hydrolysis of ATCh by nerve cord homogenates. The samples were aged for 2 hrs. before being treated with detergent. Only treatment with DOC results in a constant rate of hydrolysis of ATCh. The % increase in the rate of hydrolysis is calculated as follows:

1) The rate of hydrolysis is measured at the start of each assay and this value is taken to be 100%.

2) The increase in the rate of hydrolysis during the first 3 mins. of each progress curve is measured.

3) The observed increase is related to the initial rate of hydrolysis thus allowing the results to be expressed as the % increase in the rate of hydrolysis.

n = 3 for each group

Detergent added	% increase in rate of hydrolysis of ATCh
none (samples freshly homogenised)	22
none (samples aged for 2 hrs.)	46.5
0.1% DOC	0
1% triton-X-100	41
1% lubrol-PX	8.5
1% triton-X-100 then 0.1% DOC	40
1% lubrol-PX then 0.1% DOC	0

with 0.1% DOC did not block the effect of DOC. As triton-X-100 also prevents DOC activating the enzyme this would imply that the non-linearity of the progress curve is associated with the decay of AChE activity, a finding which is also supported by the fact that the degree of curvature of the rate of hydrolysis increases with the age of the sample.

Possible explanations for the non-linearity of the progress curve are discussed on pages 74-75.

2.4.4. Procedures for accurately measuring AChE activity in total nerve cord homogenates

It has already been suggested that the total AChE activity in total nerve cord homogenates can be estimated by the following procedure:

1) Measuring the AChE activity in samples at accurately noted times after homogenisation,

2) Plotting the results on a graph of log. AChE activity against log. time after homogenisation of sample,

3) Extrapolating the graph to the time of homogenisation of the sample (t = 0) and measuring the AChE activity at this time. Two points for each graph would be sufficient for this determination.

However, as the progress curve for the assay is not linear under these conditions this will clearly affect the determination of total AChE activity by such a method. The effect due to the non-linearity of the progress curve was determined by assaying samples at various times after homogenisation and measuring the rate of hydrolysis at 0, 1 and 3 mins. after the start of each assay. The three sets of results were plotted on a graph of log. AChE activity against log. time after homogenisation of sample. The results

from one such experiment are shown in fig. 12. All three log.-log. plots are linear, but extrapolation to the time of homogenisation gives widely differing values for the total AChE activity present in the sample. Thus the AChE activity as determined from the initial rates of reaction is 8.61 nmoles ATCh hydrolysed/min., the value determined from the reaction rates after the assays had proceeded for 1 min. is 9.66 nmoles ATCh hydrolysed/min., whilst the value obtained after the assays had been allowed to proceed for 3 mins. is 10.59 nmoles ATCh hydrolysed/min. There is a difference of 23% between the three values.

The hydrolysis of ATCh by AChE approaches a constant rate after each assay has proceeded for 3 mins. Therefore it is suggested that to measure the total AChE activity of samples by the method outlined above the rates of hydrolysis of ATCh should be measured after each assay has proceeded for at least a 3 minute period.

It has been suggested that treatment of samples with 0.1% DOC releases all of the AChE present in the sample into a form in which it can be aasayed. Thus treatment with DOC offers an alternative method of assaying the total AChE activity in nerve cord homogenates. If both these methods do measure the total AChE activity then there should be good agreement between the values obtained by each method. In order to test this homogenates were split into two fractions one of which was assayed by the time dependant method, the other being assayed after treatment with 0.1% DOC. The values for AChE activity obtained by the two methods are compared in table 6a. The mean result for five such determinations shows that there is only a 4.5% difference in the values obtained by the two methods. Further evidence that both methods give an accurate determination of the total AChE activity in homogenates of cockroach nerve cord tissue was obtained by measuring the specific activity

FIG. 12. The effect of the non-linearity of the rate of hydrolysis of ATCh on the determination of the total AChE activity of nerve cord homogenates

The graph shows log. AChE activity plotted against log. time after homogenisation of sample. Three sets of values were obtained for each assay. These represent the initial rates of hydrolysis of ATCh and the rates of hydrolysis measured 1 and 3 mins. after the start of each assay. The correlation coefficients for log.-log. plots of the three sets of values, in the order listed above, are -0.9710, -0.9599 and -0.9369. The values for AChE activity measured from the three plots are:

1) 8.61 nmoles ATCh hydrolysed/min. as measured from the initial rates of hydrolysis,

2) 9.66 nmoles ATCh hydrolysed/min. as measured from the rates of hydrolysis 1 min. after the start of each assay,

3) 10.59 nmoles ATCh hydrolysed/min. as measured from the rates of hydrolysis 3 mins. after the start of each assay.

There was an overall difference of 23% in the values for AChE activity.

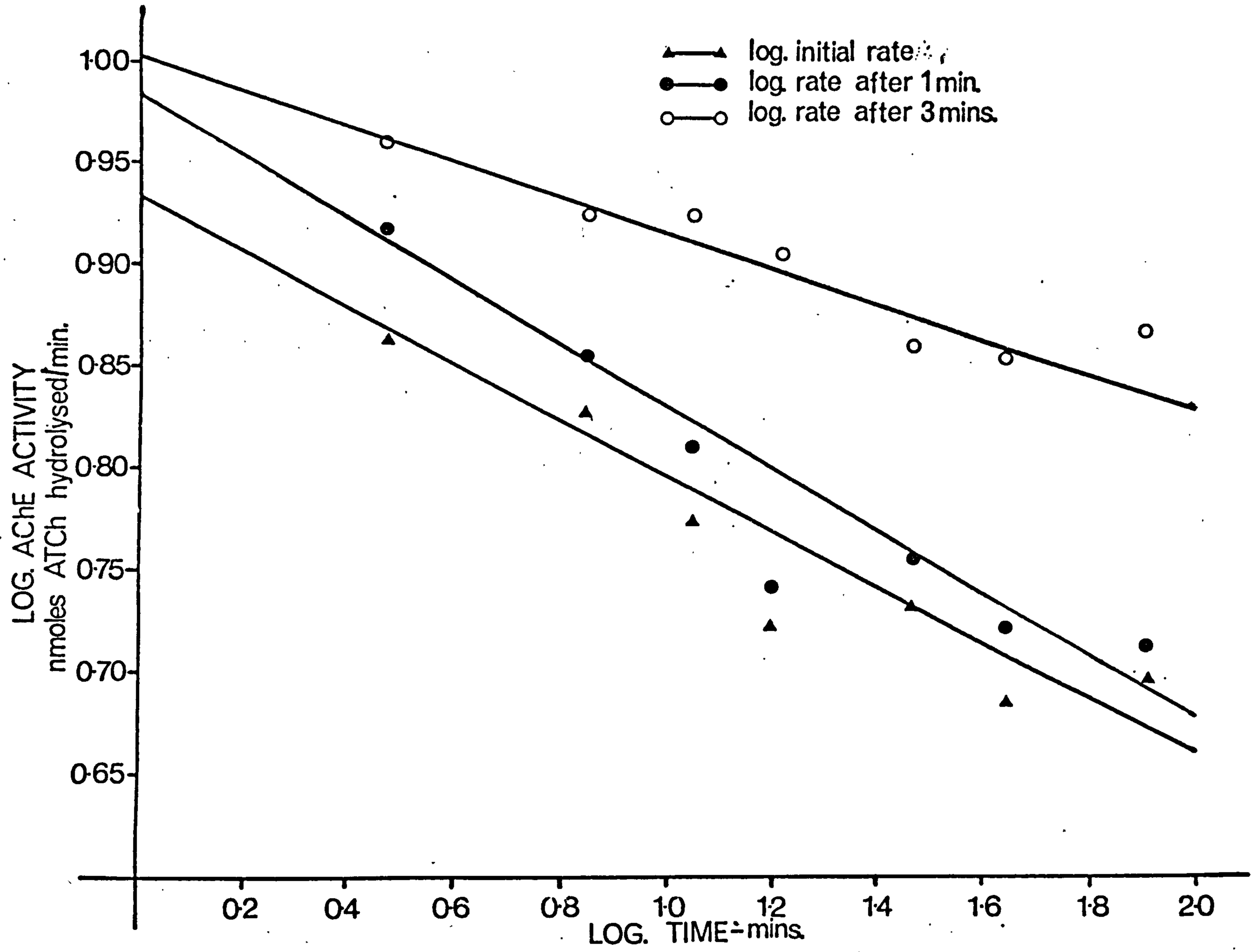

log. initial rate
log. rate after 1 min.
log. rate after 3 mins.
LOG. AChE ACTIVITY
nmoles ATCh hydrolysed/min.
1·00
0·95
0·90
0·85
0·80
0·75
0·70
0·65
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0
LOG. TIME - mins.

TABLE 6. Values for the total AChE activity present in nerve cord tissue from Periplaneta americana

The total AChE activity of samples was determined either following treatment with 0.1% DOC (method 1) or from a graph of log. AChE activity plotted against log. time after homogenisation of the sample (method 2). When AChE activity was determined by method 2 each assay was allowed to proceed for 3 mins. before the reaction rate was measured.

6a. Comparison of AChE activity from a group of five nerve cord samples as determined by the two methods. The difference between the two values for mean AChE activity is 4.5%.

	AChE activity - nmoles ATCh hydrolysed/min.	
	Determined by method 1	Determined by method 2
Total AChE activity	24.60	25.70
Mean AChE activity	4.92	5.15
s.e.m.	± 0.692	± 0.602

6b. Comparison of the specific activity of AChE in the metathoracic ganglion of P. americana as determined by the two methods. Two separate groups of five animals were used in this experiment. The AChE activity of the metathoracic ganglia from each group of animals was determined by only one of the two methods. The difference between the values for the specific activity of AChE as measured by each of the methods is 1.4%

	Specific activity of AChE - nmoles ATCh hydrolysed/min./ug prot.	
	Determined by method 1	Determined by method 2
Total AChE activity	7.37	7.45
Mean AChE activity	1.47	1.49
s.e.m.	0.108	0.082

of AChE in the metathoracic ganglia of two groups of animals (n = 5). The activity of each group of ganglia was determined by only one of these methods. The two sets of results (table 6b) are in excellent agreement.

From these results a value for the specific activity of AChE in the metathoracic ganglion of P. americana of 1.48±.095 nmoles ATCh hydrolysed/ min./ug prot. was obtained. This is considerably higher than previously reported values (see discussion).

2.4.5. Solubilisation of nerve cord AChE

The effectiveness of several non-ionic and anionic detergents in solubilising nerve cord AChE was investigated (table 7a). The criterion of solubility was the % of the enzyme activity remaining in the supernatant fraction after centrifugation of the sample at 100,000 g for 1 hr. A considerable fraction (17.8±5.6%) of the enzyme was soluble in samples that had not been treated with any agent. This is a considerably larger fraction than is soluble in untreated mammalian brain homogenates (e.g., see Hollunger and Niklasson, 1973).

Of the detergents tested the anionic types SDS and sodium cetrimide caused a rapid inhibition of AChE activity when present in concentrations of 1% (w:v). 1% triton-X-100, 1% lubrol-PX and 0.1% DOC all gave approximately a 70% solubilisation of the enzyme. The other non-ionic detergent, tween 80, solubilised only 42% of the AChE activity. The results of a more detailed investigation of the solubilisation of AChE by the three most effective agents are shown in table 7b. DOC, triton-X-100 and lubrol-PX solubilised 58-66% of the AChE activity. The results indicate that these agents are less effective in solubilising the cockroach enzyme than they are in solubilising the mammalian enzyme. Ho and Ellman (1969) report that triton-X-100

TABLE 7a. The effect of detergents on the solubility of AChE

The % AChE solubilised by several detergents is shown. All detergents were present at a concentration of 1%, except for DOC which was present at a concentration of 0.1%.

Detergent added	% solubilisation of AChE
no agent	30
lubrol-PX	65
triton-X-100	70
tween 80	42
DOC	72
SDS	inhibition of AChE
sodium cetrimide	inhibition of AChE

TABLE 7b. An investigation of the effect of lubrol-PX, triton-X-100 and DOC on the solubility of AChE

This table shows the results of a more detailed study on the % solubilisation of AChE produced by 1% lubrol-PX, 1% triton-X-100 and 0.1% DOC (n = 4 for each detergent tested).

Detergent added	% solubilisation of AChE
no agent	17.8 ± 5.6
lubrol-PX	65.4 ± 4.7
triton-X-100	66.5 ± 2.1
DOC	58.5 ± 6.1

solubilised 85% of the mammalian enzyme, i.e., 20% greater solubilisation than for the cockroach enzyme. Further, there is a large variation in the solubilisation produced by a given detergent. For example, in the case of DOC the s.e.m. was $\pm$6% (n = 4). Crone (1971) suggests that this variation may be due to differences in the ratio of tissue to detergent between samples.

There is some evidence to suggest that triton-X-100 releases the mammalian enzyme in a form in which it is still attached to other membrane components (Crone, 1971). This may also be so for the cockroach enzyme. Evidence suggesting this is as follows:

1) Preliminary studies indicate that AChE activity is 40-60% higher in the lipoprotein material (which separates at the top of the supernatant fraction) than in the rest of the supernatant fraction.

2) A portion of the AChE activity present in the lipoprotein fraction is occluded from the surrounding medium.

3) Treatment of nerve cord samples with 1% triton-X-100 solubilised 65% of the AChE activity, but did not significantly re-activate the enzyme in aged nerve cord homogenates (i.e., it did not disrupt the occlusion barrier).

The available evidence suggests that DOC did produce a true solubilisation of the enzyme. For example, in DOC treated samples AChE activity was evenly distributed in the lipoprotein and supernatant fractions. Further results which support these conclusions are presented in section three.

2.4.6. Summary of results

The results presented in this section show that:

1) The AChE activity in nerve cord samples decays by 40% during the first 4 hrs. after homogenisation.

2) This decay in activity follows the relationship

$$\text{log. AChE activity} \propto \frac{1}{\text{log. time after homogenisation of sample}}$$

during the period when there is a rapid loss of AChE activity.

3) 1% triton-X-100, 1% lubrol-PX and 0.1% DOC all prevent this rapid loss of AChE activity. Only treatment of samples with 0.1% DOC will release the decayed enzyme activity into a measurable form.

4) The progress curve for the hydrolysis of ATCh by AChE (measured using DTNB as assay reagent) is non-linear, the rate of the reaction increasing during the first 3 mins. of the progress curve. The deviation of the progress curve from linearity increases as the AChE activity of each sample decreases.

5) The progress curve for the reaction is linear when Ald.-2 is used as the assay reagent, although the rate of hydrolysis of ATCh is considerably lower than that measured with DTNB.

6) Treatment of homogenates with 0.1% DOC results in the rate of hydrolysis of ATCh becoming constant.

7) The total AChE activity present in such homogenates may be determined by two methods. The first involves assaying each sample at two or three known times after homogenisation, the rates being measured 3 mins. after the start of each assay. The results are plotted on a graph of log. AChE activity against log. time after homogenisation. The total AChE activity

is measured by extrapolation of the graph to the time of homogenisation. The second method is to treat the sample with 0.1% DOC before assay.

8) The agreement between values for AChE activity as measured by the two methods is good. The specific activity of AChE in the metathoracic ganglion of the cockroach was determined by the first method to be 1.49 ± 0.082 nmoles ATCh hydrolysed/min./ug prot. and by the second method to be 1.47 ± 0.108 nmoles ATCh hydrolysed/min./ug prot.

9) 1% triton-X-100, 1% lubrol-PX and 0.1% DOC produce a 60-65% solubilisation of cockroach AChE.

10) Triton-X-100 may not give a true solubilisation of the enzyme, but may release it in a form in which it is attached to other membrane components.

2.5.1. Discussion

The results presented suggest that the rapid loss of AChE activity in homogenates of cockroach nerve cord tissue is due to the gradual occlusion of a fraction of the enzyme from the surrounding medium. The occluded fraction of the enzyme cannot be assayed as the occlusion barrier is not freely permeable to the components of the assay system. The main evidence to support this suggestion is:

1) The decay of AChE activity follows a log.-log. relationship and not an exponential relationship. This suggests that the loss of activity is not caused by denaturation of the enzyme.

2) The fraction of AChE activity which is rapidly lost after homogenisation of samples can be recovered in a measurable form. This point is illustrated in the experiment in which aged samples were stored at 25°C (fig. 5) and in experiments in which samples were treated with 0.1% DOC (fig. 8 and tables

3, 4 and 6). There is good agreement between values for the total AChE activity of samples as estimated by the two suggested methods. This supports the proposal that DOC is acting by releasing the decayed fraction of the enzyme rather than exerting a direct activating effect on AChE.

It is well knownthat detergents disrupt lipid and membranous structures by interacting with the hydrophobic bonds between the components of such structures (see section 1.4). Therefore it is probable that the occlusion barrier is of a lipid or membranous nature. The effects of detergents on the stabilisation and activation of AChE suggest that the two non-ionic detergents differ from the anionic detergent with respect to their mechanism of action. Triton-X-100 and lubrol-PX will prevent the formation of the occlusion barrier, but only DOC will disrupt it once it has formed. Ferdman, Himmelreich and Dyadusha (1970) propose that the anionic detergent SDS and the non-ionic detergent triton-X-100 have different actions on sarcolemma membranes. They suggest that the former agent breaks only protein-protein bonds whilst the latter agent breaks only protein-lipid bonds. The results presented on pages 67-69 suggest that triton-X-100 releases AChE still attached to other membrane components whereas DOC releases the enzyme in a truly soluble form. This is evidence that triton-X-100 and DOC break different bonds between the components of cockroach nerve tissue membranes.

Two possible models which could account for the formation of an occlusion barrier around membrane bound AChE are illustrated in fig. 13. In the first type membrane fragments containing AChE molecules fold back on each other thus forming a small vesicle. A fraction of the AChE which is bound to the outer face of the membrane in intact tissue is now enclosed within the vesicle. In the second type of model free lipid or lipoprotein molecules in the surrounding medium form hydrophobic bonds with membrane components

FIG. 13. Schematic representations of two models for the occlusion of AChE activity in homogenates of cockroach nerve tissue

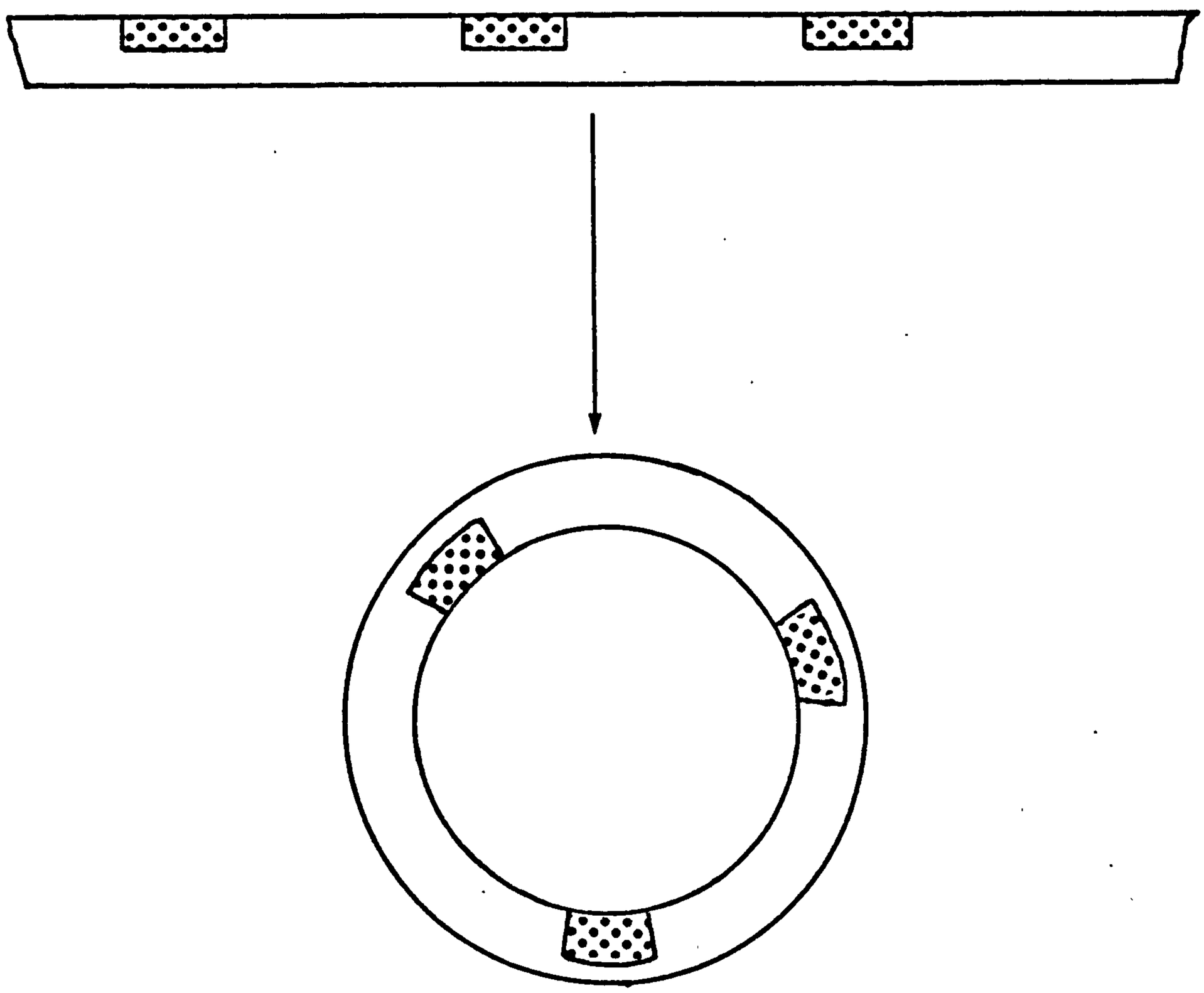
FIG. 13a. Sites of AChE activity are located on the outer face of membrane fragments. Some of the fragments gradually close together to form inverted sacs or vesicles with sites of AChE activity now located inside the vesicles. This activity is occluded from assay thus lowering the measurable AChE activity present in the sample.

FIG. 13b. AChE is attached to the outer face of membrane fragments which form hydrophobic bonds with molecules from the surrounding medium. These attach to membrane components adjacent to AChE molecules and overlap a fraction of the active sites of AChE thus occluding them from assay.

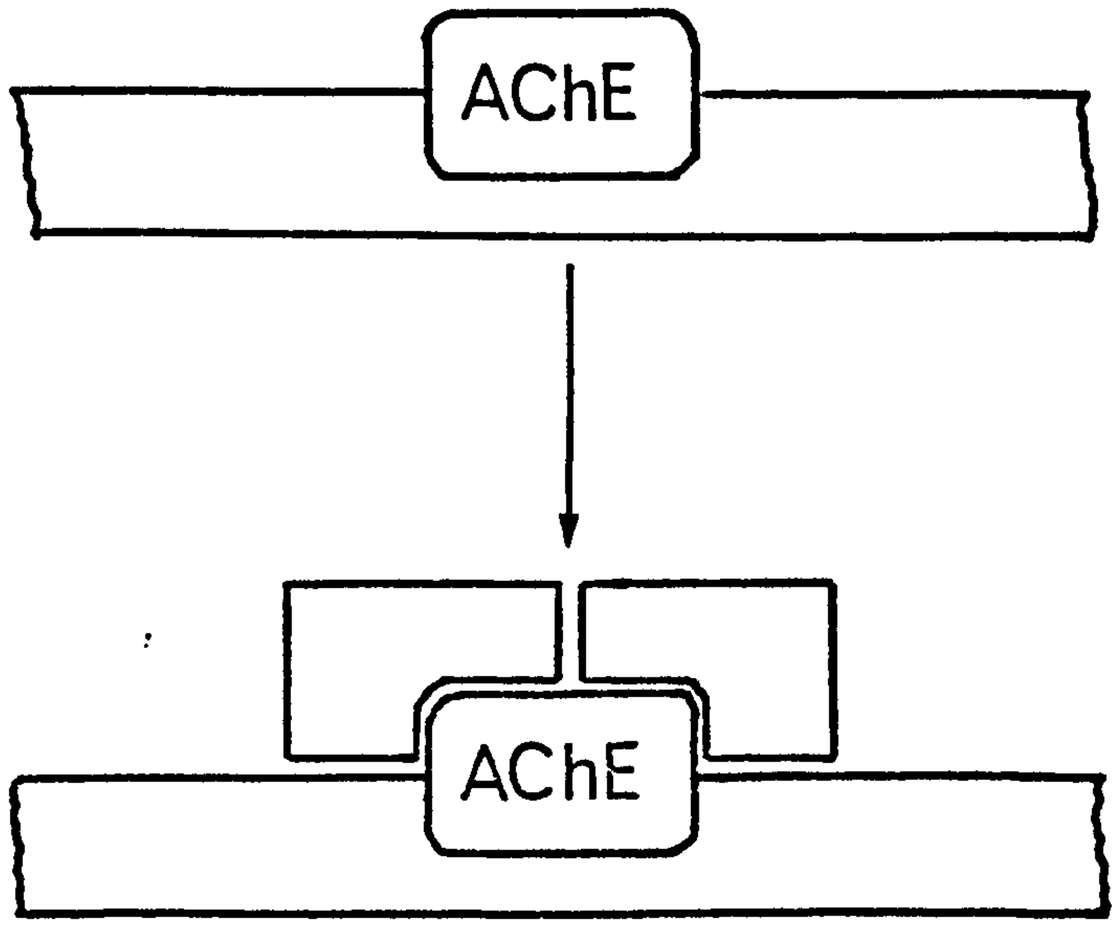
These two representations of the occlusion of AChE activity are only schematic and other models are also possible.

 = sites of AChE activity

A



B



adjacent to the AChE molecule thus forming a micellar structure. A fraction of the AChE activity is enclosed within the micelle. The barrier formed in both models would be permeable only to lipid soluble molecules. The occluded fraction of the enzyme may not be accessible to some components of the assay medium and therefore would not be assayable.

There is no direct evidence to show which, if either, of these models is correct. Clearly other types of model could also explain the formation of the occlusion barrier. Preliminary electron microscope studies of pellet material from nerve cord homogenates centrifuged at 100,000 g for 1 hr. show the presence of small vesicles in aged samples, but not in freshly homogenised samples. These studies suggest that the first type of model is correct. The occlusion barrier is not disrupted by treatment with triton-X-100. This suggests that the components of the barrier are strongly bound, i.e., a model of the first type is likely to be correct.

Any model attempting to explain the decay of AChE activity must also account for the non-linearity of the progress curve for the hydrolysis of ATCh. The experiments described in section 2.4.3. establish that:

1) A slow, apparent activation of AChE occurs during the course of each assay.

2) The activation is an indirect effect associated with the occlusion barrier since, if the latter is disrupted, a constant rate of hydrolysis of ATCh is observed.

3) DTNB, or one of its reaction products, is responsible for the activation since a constant rate of hydrolysis of ATCh is observed if Ald.2 is used as the assay reagent.

These findings suggest that the permeability of the occlusion barrier to the assay reagents increases as the reaction proceeds, i.e., the occluded fraction of the enzyme becomes more accessible to the assay reagents thus accounting for the non-linearity of the progress curve.

Experiments in which samples were pre-incubated with DTNB never resulted in a completely linear progress curve. Therefore it is unlikely that DTNB itself produces the increase in the permeability of the occlusion barrier. It is more probable that 5-thio-2-nitrobenzoic acid released during the assay causes this increase. Since DTNB will react with sulphydryl groups on proteins a small amount of 5-thio-2-nitrobenzoic acid could have been released in the pre-incubation experiments. The amount released would have been sufficient to reduce the degree of non-linearity of the progress curve, but not sufficient to result in a constant rate of hydrolysis of ATCh. This explanation could be tested by observing the effect of 5-thio-2-nitrobenzoic acid on the rate of hydrolysis of ATCh in nerve cord homogenates.

It should be emphasised that the above explanation is only a tentative attempt to account for events occurring in what is obviously a complex system.

A further point to consider is that only 40-50% of the total AChE activity present in homogenates becomes occluded from assay. The enzyme would appear to exist in two types of environment, one of which becomes occluded from assay. The electron microscope studies of Treherne and Smith (1965b) indicate that AChE is bound to both neuronal and glial membranes in the ganglionic tissue of P. americana. It is possible that the two types of membrane are composed of different lipids and proteins and that only one type of membrane will form an occlusion barrier in nerve cord homogenates.

Further evidence for the existence of two pools of AChE is provided by Willner and Mellanby (1974). They find that the AChE activity in fresh nerve tissue homogenates from cockroaches which had been frozen in liquid nitrogen is lower than the activity in similar samples from animals not subjected to this procedure. On aging the samples the AChE activity in the homogenates from both groups of animals decayed to the same level. These results suggest that only the fraction of AChE activity which becomes occluded from assay is denatured by freezing the animals in liquid nitrogen.

One other type of explanation that could account for the observed decrease in AChE activity is that a lipid/lipoprotein moiety which is strongly bound to the AChE molecule slowly alters its conformation following homogenisation of samples. This alteration results in steric hindrance of the interaction between substrate and the active site of the enzyme. DOC treatment would separate the AChE from the lipid/lipoprotein moiety thus relieving the steric hindrance. Similarly 5-thio-2-nitrobenzoic acid could also act by relieving the steric hindrance. This type of explanation has been proposed by Colhoun (1961) to account for the activation of insect AChE by treatment with cholate.

The solubilisation studies indicate that of the detergents tested only DOC is effective in releasing the enzyme in a form in which it is not attached to other membrane components. Both the electric eel enzyme and the mammalian brain enzyme are reported to be released still attached to other membrane components (Grafius, Bond and Millar, 1971; Rieger, Bon, Massoulie and Cartaud, 1973; Ho and Ellman, 1969; Crone, 1971).

If the results obtained in this study of P. americana apply to other species of insect then many of the reported values for insect AChE activity may be incorrect. Also a critical re-evaluation of differences in AChE activity observed in insects in response to parameters such as circadian

rhythm and shock avoidance learning is necessary. A slight difference in the age of homogenates could cause a considerable, spurious difference in AChE activity between test and control groups of animals.

2.5.2. Summary of discussion

It is suggested that the decrease of AChE activity in cockroach nerve cord homogenates is due to the formation of a membranous or lipoprotein barrier around a fraction of the enzyme. Two models for the barrier are described. The barrier is not permeable to the assay reagents. Therefore the occluded enzyme cannot be assayed. Triton-X-100 and lubrol-PX prevent the formation of the barrier, but only DOC will disrupt it once it has formed. The non-linearity of the progress curve for the reaction can be explained by an increase in the permeability of the occlusion barrier to the assay reagents as the reaction proceeds. This increase in permeability may be caused by 5-thio-2-nitrobenzoic acid released during the course of the assay. There may be at least two pools of AChE surrounded by different lipoprotein environments. Only one of these becomes occluded from assay. An alternative explanation for the decrease in AChE activity is that a lipid/lipoprotein moiety alters its conformation resulting in steric hindrance of the interaction between substrate and the active site of the enzyme.

It is concluded that DOC is the only agent of those tested which solubilises AChE in a form in which it is not attached to other membrane components.

If the results obtained for AChE from P. americana can be extrapolated to other species of insect then many of the reported values for the activity of insect AChE are subject to the errors discussed.

SECTION THREE

PARTIAL PURIFICATION OF AChE FROM THE NERVE CORD OF *Periplaneta americana* AND AN INVESTIGATION OF SOME OF ITS PROPERTIES

3.1. Plan of experiments

The experiments described in this section were designed to achieve a partial purification of cockroach AChE. The amounts of enzyme available were too small to obtain a highly purified product by conventional methods. Therefore small amounts of the enzyme were partially purified by sucrose density gradient centrifugation, analytical polyacrylamide gel electrophoresis or gel filtration. Some of the basic properties of the partially purified product were investigated, particularly the different molecular species of the enzyme which were isolated and their relationship to one another. Some of the properties of the enzyme in crude nerve cord homogenates were also investigated.

3.2. Methods

3.2.1. Preparation of density gradients

The method used was essentially that described by Kerkut, Oliver, Rick and Walker (1970). A continuous density gradient (5-20% sucrose) was prepared by placing 7.5 ml each of 5% and 20% sucrose solutions separately in the two compartments of a gradient former. The solution of 5% sucrose was gently stirred and the tap connecting it to the compartment containing 20% sucrose was opened. The mixed product was fed into three 5 ml polycarbonate centrifuge tubes simultaneously by a peristaltic pump. The gradient was fed into the tubes through three identical sets of polythene tubing connected

to finely drawn out glass tubing. The system was designed so that the flow rate in the three sets of tubing was the same, i.e., three identical gradients could be set up at the same time.

Samples were routinely prepared by centrifuging total nerve cord homogenates at 100,000 g. for 1 hr. A 0.1 ml aliquot of the supernatant was layered on the gradient by gently running the sample down the side of the centrifuge tube, care being taken not to disturb the boundary between the sample and the top of the gradient. The gradients were centrifuged for 18 hrs. at 80,000 g on an MSE 65 centrifuge. 25x 0.2 ml fractions were collected by piercing the bottom of the polycarbonate tubes and collecting the appropriate volume. The AChE activity in each fraction was assayed. The sucrose concentration in every fifth fraction was measured using a Zeiss-Jena refractometer.

3.2.2. Preparation of polyacrylamide gels

The method used was essentially that of Davis (1964). The preparation of stock acrylamide solutions and gel and reservoir buffers is described in appendix 1. Acrylamide was recrystallised prior to use. The quantity of TEMED used was lowered from the 0.46% used by Davis to 0.40-0.42%. This modification increased the polymerisation time of the gels to 30-60 mins. at room temperature, thus giving more time to handle the solutions. All solutions were de-aerated on a water pump immediately prior to use. Samples were initially run on both 7.5% and 5% gels, but it was decided that the 5% gels gave the best separation of different species of AChE. No stacking gel was used as this was found to be unnecessary in achieving a good resolution of protein species.

Gels were set up in Plexiglass tubes 7 cm long and of 0.6 cm internal diameter. The bottoms of the tubes were sealed with parafilm. The tubes were filled with unpolymerised acrylamide solution to a mark 1 cm from the top. Distilled water was carefully layered on the top of the un-polymerised gel mixture, care being taken not to disturb the gel/water interface. This procedure ensured that the surface of each gel was flat. After polymerisation had occurred the distilled water was discarded and the surface of each gel was rinsed twice with the Tris/glycine running buffer. The tubes were placed in the disc gel electrophoresis apparatus and the upper and lower reservoirs were filled with Tris/glycine running buffer, pH 8.3, i.e,, a discontinuous buffer system was used.

The electrophoresis tanks and the power pack were supplied by Shandon Ltd. and the Plexiglass tubes were supplied by Joyce Loebl Ltd.

3.2.3. Preparation and layering of samples on gels

Samples were prepared as described in section 2.2.2. except that the homogenising medium was Tris/glycine buffer, pH 8.3 (either with or without detergent). The homogenising medium contained 20% sucrose so that the sample layered as a thin disc on the top of the gel. A small amount of bromophenol blue was added to the medium to act as a marker dye. A 50 or 100 ul aliquot of the sample was routinely layered on the surface of each gel using a 0.1 ml serological pipette. The tip of the pipette was placed below the surface of the reservoir buffer near the top of each gel. By allowing the solution to flow gently from the tip of each pipette the sample stacked as a thin disc on the surface of the gel. This operation was carried out as rapidly as possible to allow the minimum time for the diffusion of the layered samples.

Samples were run into the gels at a current of 0.5 mA/tube for 15 mins. The electrophoresis was continued at a constant current of 3 mA/tube until the bromophenol blue had migrated three quarters of the length of the gels. Lambertsen (1972) reports that the running pH of the lower quarter of the gels shifted from 8.9 to 8.4 during the electrophoresis. Thus if the samples had been allowed to migrate further another parameter affecting the migration of the protein species would have been introduced into the system. The normal running time was 2-2½ hrs. The electrophoresis was carried out in the refrigerator to prevent heating effects causing denaturation of the protein species.

3.2.4. Removal and staining of gels

At the end of each run the gels were removed from the Plexiglass tubes by inserting the nozzle of a syringe, fitted with a rubber bung of the appropriate size, into the top of the tubes. The gels were eased out of the tubes with distilled water from the syringe. The gels were placed either into a solution of coomassie brilliant blue if they were to be stained for protein or into a mixture of acetone/dry ice if they were to be assayed for AChE activity.

The protein bands on the gels were stained with 0.1% (w:v) coomassie brilliant blue in methanol/acetic acid/water in the ratio 5:5:1 (v:v:v). Staining was usually allowed to take place overnight. The concentration of coomassie brilliant blue is lower than that used by other workers. It was found that at this concentration background stain was easier to remove, whilst all bands of protein still stained adequately.

Destaining of the gels was accomplished by placing them in several changes of 7% acetic acid. Gels were destained until all background stain was removed from the region beyond the buffer front. This process was normally completed in 24 hrs. A scan of the separated protein bands was obtained using a Joyce Loebl Polyfrac gel scanner.

The migration of bands of protein was calculated relative to the movement of bromophenol blue (R_{BPB}).

3.2.5. Assay of AChE activity from gels

Two methods were routinely used to assay the AChE activity present on gels. In the first method gels frozen rapidly in acetone/dry ice were sliced transversely into 1.6 mm strips using a gel slicer. The gel slicer consisted of razor blades mounted in a Perspex holder. The razor blades were spaced 1.6 mm apart by placing washers between successive razor blades. The washers and razor blades were mounted on long bolts. Corresponding slices from several gels were placed in test tubes containing 1 ml of 0.1M phosphate buffer, pH 8.0. Protein was eluted from the gel slices by standing the test tubes in the refrigerator overnight. Aliquots of each sample were assayed spectrophotometrically for AChE activity. This method had the disadvantage that minor variations in the migration of proteins from gel to gel caused a slight broadening of the bands of AChE activity. It had the advantage that the properties of the eluted enzyme could be investigated further.

In the second method bands of AChE activity were stained on the intact gels. This method was used to obtain a rapid localisation of bands of the enzyme. Thawed gels were placed in a 1mM solution of ATCh for 2 mins. and

then transferred to a 10mM solution of DTNB for 1 min. Regions of AChE activity stained yellow. The gels were scanned on the Polyfrac at a wavelength of 420 nm both before and after the reaction. The whole procedure had to be carried out rapidly because the yellow 5-thio-2-nitrobenzoate anion diffuses rapidly through the gel. From the two scans the bands of AChE activity could be identified and a comparison of the relative amount of activity in each band could be made. The bands of activity could be accurately sliced out from the gels and the enzyme eluted into phosphate buffer. The properties of the eluted enzyme could then be investigated in more detail. The accuracy of the method could be improved by fixing the coloured product in acetic acid, but this would denature the enzyme, i.e., it could not be used for further studies. Chiu, Tripathi and O'Brien (1972) report the estimation of the Km of AChE on intact polyacrylamide gels using the Gomori method.

3.2.6. Preparation and use of gel filtration columns

The different molecular species of AChE present in nerve cord samples were separated by gel filtration using Bio-Gel A-0.5m(200-400 mesh). Sephadex G-200 and Sepharose 6B were found unsuitable for the purpose as they did not give a good resolution of the different species present. The Bio-Gel has a M.Wt. exclusion limit of 500,000. The column used was designed to give a good separation of proteins present in small samples of relatively impure homogenates. It was 1 metre long. The bed volume of the column, determined by direct measurement and by separation of a low M.Wt. molecule (DOC), was 49 ml. The void volume of the column, determined by separation of a sample of blue dextran, was 14 ml. The dead volume below the sintered glass filter was small so that mixing of the eluate was minimal. Care was

taken to pack the column properly before the first sample was separated on it. The column was of a downward flow type, the pressure being applied from a 1 litre reservoir placed 60 cm above the top of the column. The height of the buffer in the reservoir was maintained constant so that a constant flow rate of 3 ml/hr. was obtained. The column was kept in the cold room (temperature $4^{o}C$) at all times and the phosphate running buffer was de-aerated prior to use to prevent air bubbles forming in the Bio-Gel. When not in use the column was stored by washing with buffer containing 0.02% (w:v) sodium azide as an anti-bacterial agent. The sodium azide was washed off the column with fresh buffer prior to re-use.

Samples were prepared as previously described (in 0.1M phosphate buffer, pH 8.0, containing 20% sucrose to facilitate the layering of samples on the surface of the Bio-Gel). A 0.1 - 0.5 ml aliquot of each sample was layered on the surface of the gel. The technique used was the same as that described for layering samples on polyacrylamide gels. 1 ml fractions of the eluate were collected using a Gilson fraction collector. Protein concentrations were determined by measuring the absorption of the fractions at 280 nm on a Pye Unicam SP1800 spectrophotometer. AChE activity was assayed in the usual manner. No attempt was made to calibrate the column in order to determine the M.Wts. of the different species of AChE separated. The impurity of the samples would have resulted in any such values obtained being inaccurate due to interaction of the enzyme with other components of the sample. Approximate values for the M.Wts. of the species of AChE were estimated from a knowledge of the separating characteristics of the Bio-Gel.

Fractions corresponding to a single band of AChE activity were pooled and concentrated in a Chemlab C50 concentration cell. This procedure enabled the activity present in 10 ml to be concentrated into 1 ml. The

concentrated sample was separated on the column for a second time. The re-separation ensured that bands of activity were due to different molecular species of AChE and not caused by artefacts such as the layering of the sample. The re-separation was also used to give information on the inter-convertibility of the various bands of activity.

3.2.7. Calculation of Km and Vmax for AChE

Values for the Km and Vmax of the enzyme were computed using a Hewlett-Packard calculator. The programme used computes the Michaelis-Menten curve which best fits each set of data. Vmax is calculated from the assymptote to the curve. The Km value is also calculated directly from the Michaelis-Menten plot. This method avoids the errors associated with the estimation of these parameters from a Lineweaver-Burk plot. The results can also be displayed as a Lineweaver-Burk plot using a graph plotter connected to the calculator. Though the points representing the experimental data are displayed on the plot the line is drawn through the reciprocals of the calculated Km and Vmax values.

3.3. Materials

Chemicals were obtained from BDH Biochemicals, Poole, Dorset, with the following exceptions:

Bio-Gel A-0.5m was obtained from Bio-Rad Laboratories Ltd., 27, Homesdale Road, Bromley, Kent.

Sephadex G-200, Sepharose 6B and blue dextran were obtained from Pharmacia (Great Britain) Ltd., Paramount House, 75, Uxbridge Road, London W5 5SS.

Coomassie brilliant blue was obtained from G.T. Gurr, Searle Scientific Services, Coronation Road, Cressex Industrial Estate, High Wycombe, Bucks. Horse serum BuChE was obtained from the Sigma Chemical Company, St. Louis, Missouri, U.S.A.

3.4. Results

3.4.1. Separation of cockroach AChE by sucrose density gradient centrifugation

Fig. 14 shows the separation, by sucrose density gradient centrifugation, of AChE activity from the supernatant fraction of a homogenate of metathoracic ganglia. The homogenate was not treated with detergent. The supernatant fraction was separated by centrifugation at 100,000g for 2 hrs.

A single sharp peak of enzyme activity separated at a sucrose concentration of 9.5-11%. The activity profile suggests that the peak may contain a shoulder representing a species of slightly higher M.Wt. This single peak of naturally soluble cockroach AChE may correspond to the single peak of activity obtained by Krysan and Kruckeberg (1970) on sucrose density gradient centrifugation (at pH 8.0) of mayfly and honey bee AChE. The cockroach enzyme had a slightly lower sedimentation coefficient than a sample of pure horse serum BuChE separated on an identical gradient.

In contrast to this result a crude post-mitochondrial supernatant fraction (obtained by centrifuging a homogenate of metathoracic ganglia at 10,000 g for 30 mins) resolved into four peaks of AChE activity when separated on a density gradient (fig. 15). A large portion of the activity separated as pellet material at the bottom of the gradient. Also a considerable fraction of the activity did not enter the gradient, but remained in the lipoprotein fraction at the surface. Of the four peaks of activity separated on the gradient the slowest sedimenting species corresponded to the peak of

FIG. 14. The separation of soluble AChE activity from cockroach metathoracic ganglia by sucrose density gradient centrifugation

The naturally soluble cockroach enzyme was prepared from a homogenate of metathoracic ganglia (not treated with detergent) by centrifugation at 100,000 g for 2 hrs. 0.1 ml of the supernatant fraction was separated on a 5-20% sucrose gradient. The gradient was spun at 80,000 g for 18 hrs. on an MSE65 centrifuge. 0.2 ml fractions of the gradient were collected and assayed for AChE activity. A single, sharp peak of AChE activity, sedimenting between 9.5-11% sucrose, was obtained. There may be a slight shoulder due to the presence of a slightly faster sedimenting species of the enzyme.

Note: The experiments involving separation of AChE by sucrose density gradient centrifugation, gel filtration and polyacrylamide gel electrophoresis were each separately repeated several times. For example the results shown in fig. 14 are from one separation which is typical of three separate experiments carried out.

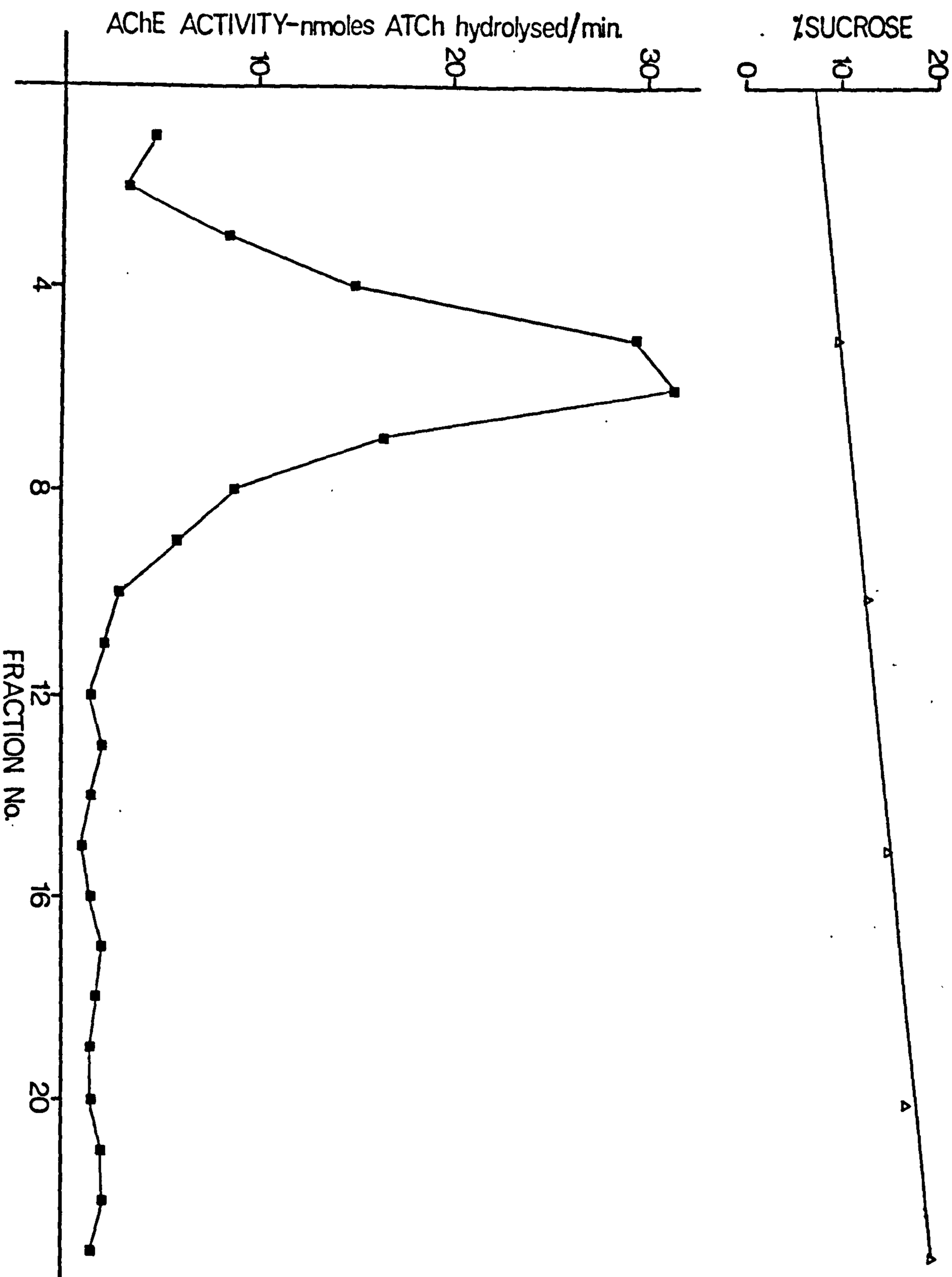
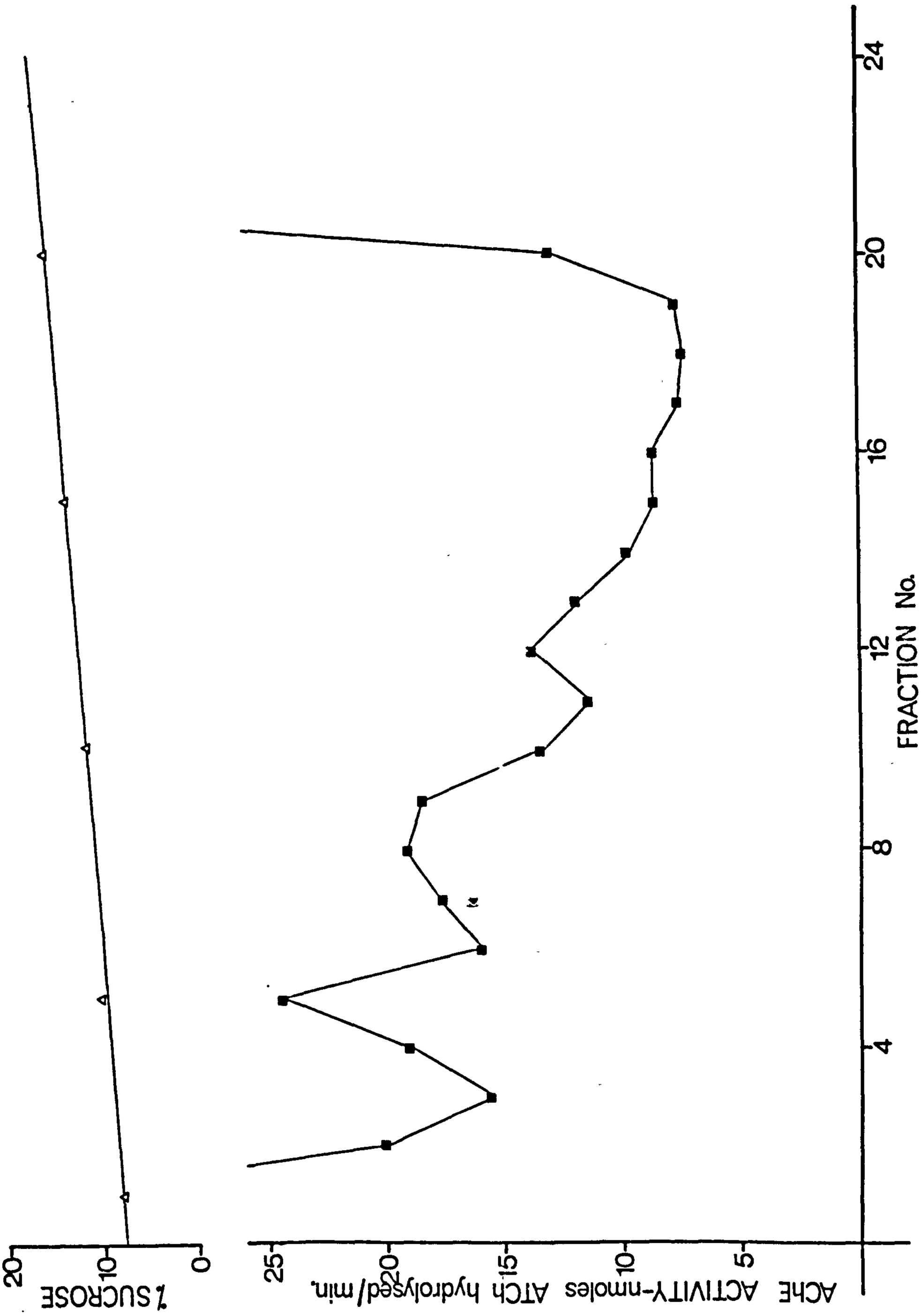

AChE ACTIVITY-nmoles ATCh hydrolysed/min.
10
20
30
4
8
12
16
20
24
FRACTION No.
%SUCROSE
0
10
20

FIG. 15. Separation of AChE activity from a post-mitochondrial supernatant fraction of cockroach metathoracic ganglia by sucrose density gradient centrifugation

The fraction was prepared from a homogenate of cockroach metathoracic ganglia (not treated with detergent) by centrifugation at 10,000 g for 30 mins. on an MSE minor centrifuge. 0.1 ml of the supernatant was separated on a 5-20% sucrose gradient as described in fig. 14. Much of the AChE activity remained in the lipoprotein fraction at the top of the gradient or separated as pellet material at the bottom of the gradient. Four peaks of AChE activity separated on the gradient. The slowest moving peak corresponded to the soluble form of the enzyme. The other three peaks were all polydisperse.


ACHE ACTIVITY-nmoles ATCh hydrolysed/min.
25
20
15
10
5
0
% SUCROSE
20
10
0
FRACTION No.
4
8
12
16
20
24

activity obtained in fig. 14. The three faster sedimenting species were all polydisperse in nature and contained less activity than the band of soluble enzyme. Whether they were aggregate forms of the enzyme or species of the enzyme which were still attached to other membrane components is not known.

It would be of interest to make a further study of these species of AChE using the other techniques described in this section.

3.4.2. Separation of cockroach AChE by analytical polyacrylamide disc gel electrophoresis

Routine electrophoresis of the supernatant fraction from homogenates of cockroach metathoracic ganglia gave a clear separation of 16 major species of protein (fig. 16a). A scan of a typical separation of ganglionic proteins is shown in fig. 17. The scan was obtained using a Vitatron modular spectrophotometer fitted with a densitometer unit. Direct staining of the gels with ATCh and DTNB showed the presence of two bands of AChE activity (fig. 16b). The first band (R_{BPB} = 0.29) was well defined and gave a sharp peak of activity suggesting that it corresponded to a single molecular species of the enzyme. The second band (R_{BPB} = 0.40-0.56) was diffuse indicating a poor resolution of enzyme activity. Several reasons could account for this. Some of these possibilities are:

1) The molecular species of AChE which accounts for band 2 activity does not separate well by this technique because of its physical properties.

2) Two molecular species of AChE contribute to band 2 activity, their properties being sufficiently similar to cause them to migrate to overlapping positions on the gel.

FIG. 16. Electrophoretic separation of ganglionic proteins and AChE activity.

Proteins were solubilised with 1% triton-X-100 and the supernatant fraction separated by centrifugation at 100,000 g for 1 hr. Aliquots of the supernatant were separated by gel electrophoresis.

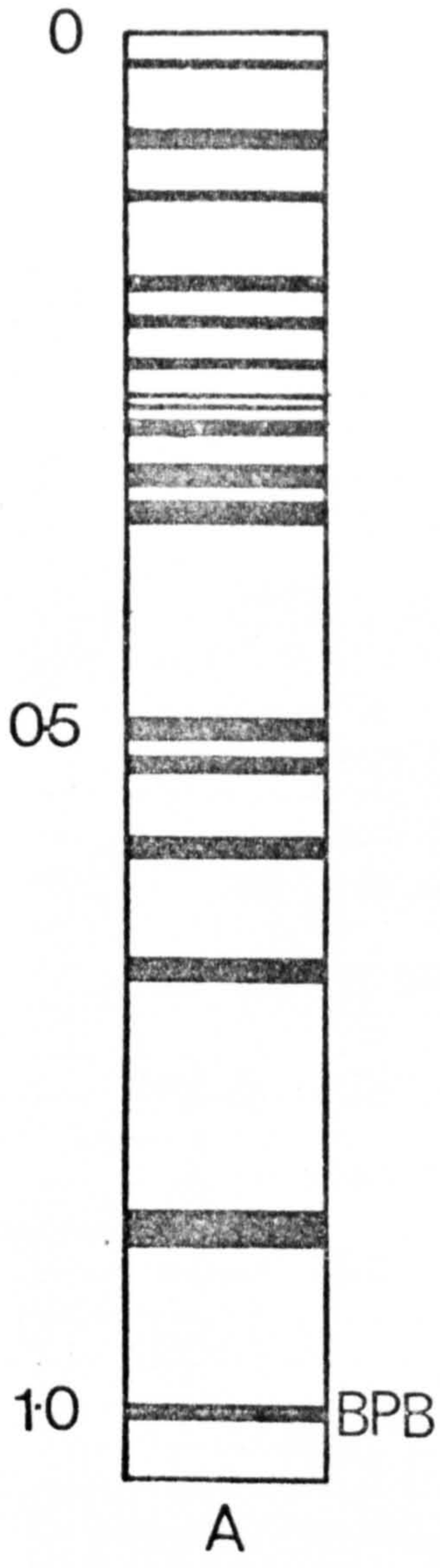
FIG. 16a. Typical separation of proteins from the metathoracic ganglion. The bands of protein were stained with coomassie brilliant blue. 16 bands of protein were distinguishable.

FIG. 16b. Typical separation of AChE activity from the metathoracic ganglion. Gels were stained with ATCh and DTNB. Two bands of soluble AChE activity were detected by this method. Band 1 was sharply defined, whereas band 2 was diffuse.

R_{BPB} BAND 1 = 0.25

R_{BPB} BAND 2 = 0.30-0.43

A considerable fraction of the AChE activity remained at the top of the gel, i.e., the species responsible for this activity were too large to enter the gel.


0
05
1·0
BPB
A

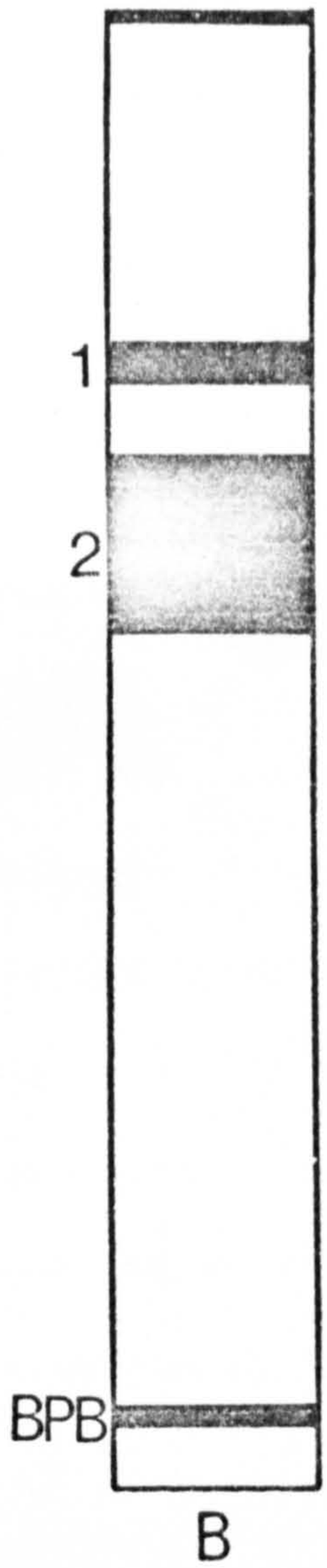
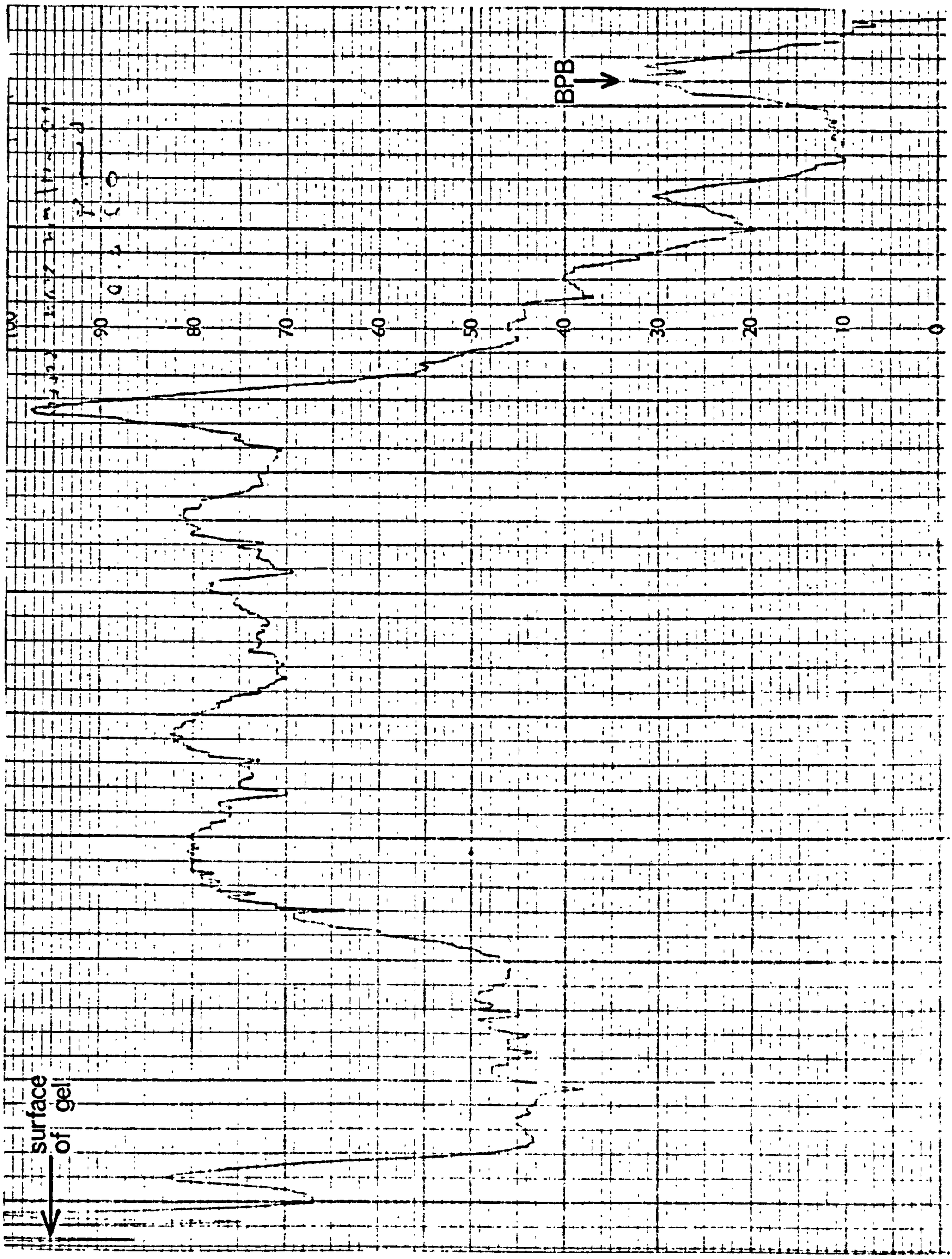

1
2
BPB
B

FIG. 17. Scan of a typical separation of proteins from cockroach metathoracic ganglia

Samples were prepared as described in fig. 16. The electrophoresis was carried out as described in the text. The gels were stained with coomassie brilliant blue. A scan of a typical separation is shown. The scan was made on a Vitatron gel scanner at a speed of 10mm/sec. A full scale deflection represents an O.D. change of 0-2.


BPB
surface of gel

3) The enzyme species which accounts for band 2 activity may be unstable and the enzyme may slowly denature as a result of the electrophoresis.

4) The band 2 enzyme may be breaking down into smaller subunits as the electrophoresis proceeds.

5) The band 2 enzyme may interact with other proteins present in the sample to form a complex. This complex may break down during the course of the electrophoresis thus causing the band of activity to broaden.

Evidence that AChE band 2 activity consists of two molecular species of the enzyme is provided by a comparison of figs. 16a and 16b. Whereas AChE band 1 corresponds to only one band of protein AChE band 2 corresponds to at least two bands of protein. These bands of protein may not be the molecular species responsible for the AChE activity though it would seem probable that they are.

In addition to these two bands of AChE a third intensely staining band of enzyme activity occurred at the top of the gels. This third band probably consisted of high M.Wt., aggregate forms of the enzyme which were too large to enter the gel matrix.

A quantitative estimation of the AChE activity separated on polyacrylamide gels is shown in figs. 18 and 19. Results obtained by eluting AChE activity from 1.6 mm slices of the gels show that 46% of the soluble AChE activity was present in band 1 and 54% was present in band 2 (fig. 18). Results obtained by direct staining of the gels showed that of the soluble AChE activity 37% was associated with band 1 and 63% was associated with band 2. The bands of activity measured by direct staining of the gels were more diffuse than those measured by the gel slicing method. This is due to the rapid diffusion of the 5-thio-2-nitrobenzoate anion. For this

FIG. 18. Quantitative estimation of AChE activity from cockroach metathoracic ganglia separated by gel electrophoresis

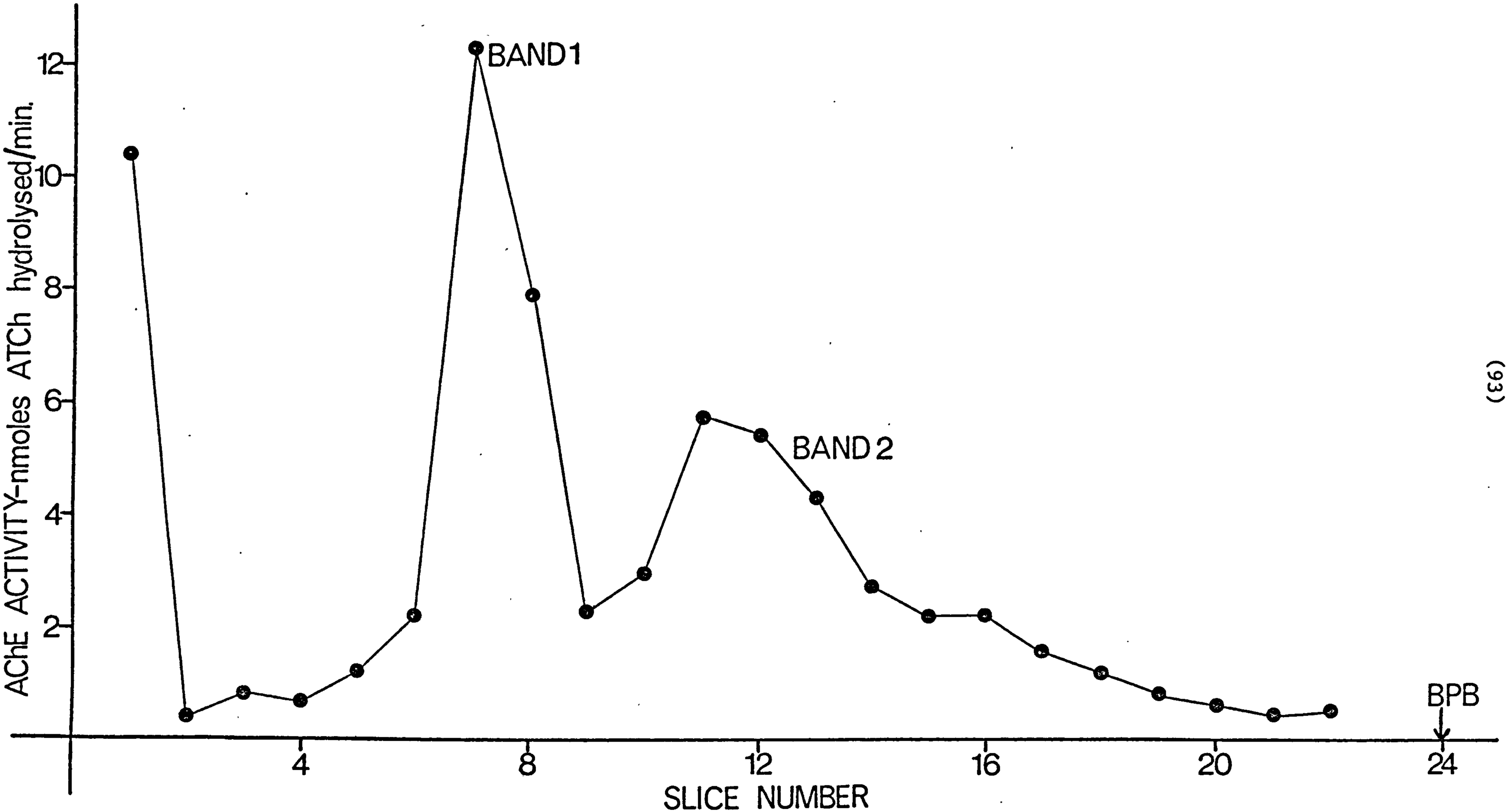
Gels were frozen in acetone/dry ice and sliced transversely into 1.6 mm discs. Corresponding slices from six gels were pooled and protein eluted overnight into 1 ml of 0.1M phosphate buffer, pH 8.0. Aliquots were assayed for AChE activity by the method of Ellman et al. (1961). Two soluble bands of activity were detected. Band 1 activity was sharply defined. Band 2 activity was diffuse. 46% of the soluble enzyme activity was localised in band 1 and 54% in band 2.

The R_{BPB} values of the two bands were:

R_{BPB} BAND 1 = 0.29

R_{BPB} BAND 2 = 0.40 - 0.56

Note that a large fraction of the AChE activity did not enter the gels.


AChE ACTIVITY-nmoles ATCh hydrolysed/min.
12
10
8
6
4
2
BAND1
BAND2
BPB
4
8
12
16
20
24
SLICE NUMBER

(93)

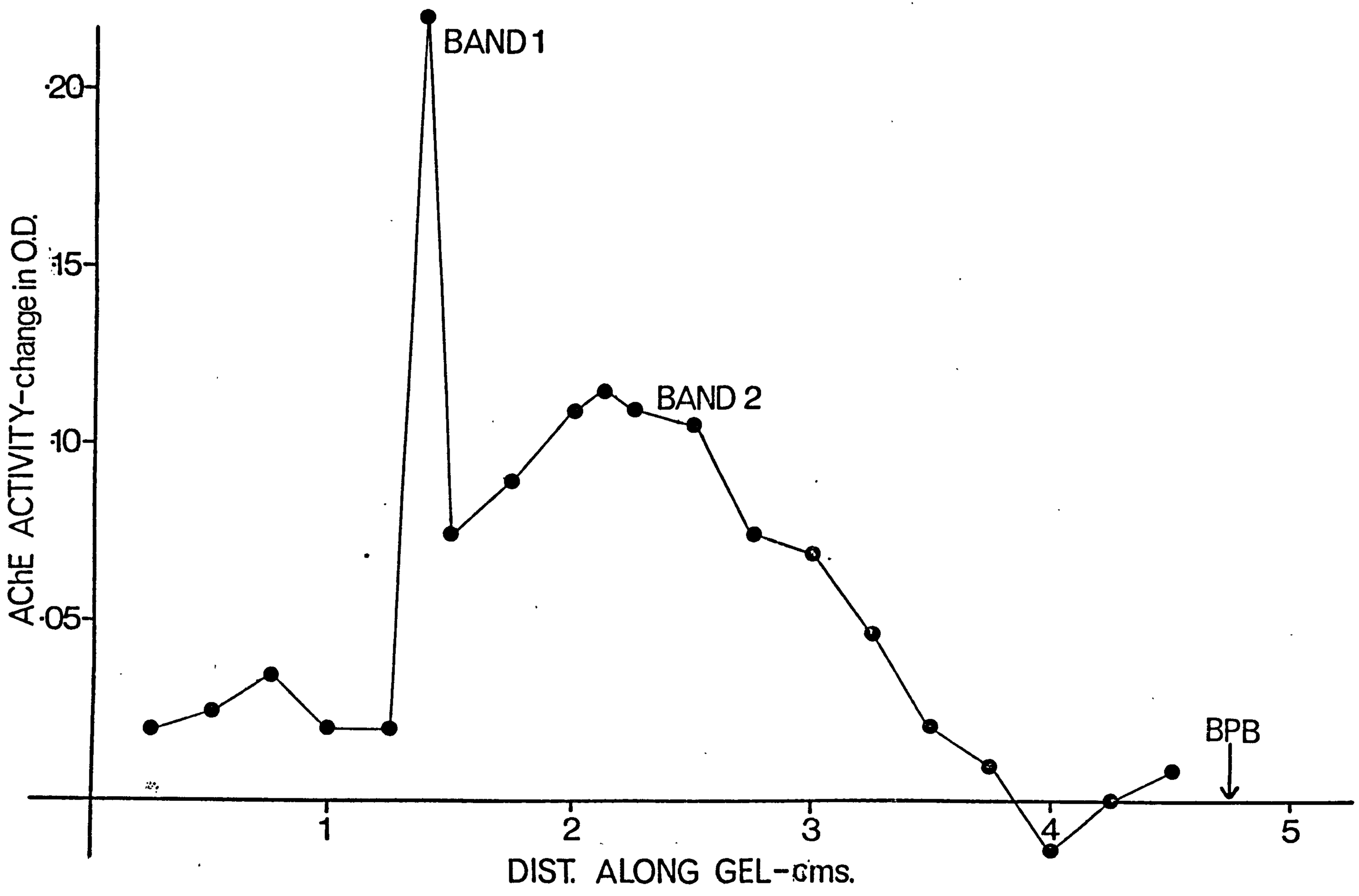
FIG. 19. Scan of a single gel of separated ganglionic proteins stained for AChE activity

Proteins from cockroach metathoracic ganglia were separated by gel electrophoresis. The gels were stained for AChE activity by incubation in media containing ATCh and DTNB. The gels were scanned on a Joyce-Loebl Polyfrac gel scanner at a wavelength of 420 nm. The typical activity profile shown represents the difference in the absorbance of the scans before and after staining of the gel. The points plotted were measured at successive 0.25 cm intervals along the gel. The R_{BPB} values of the two bands were:

$$R_{BPB}\ \text{BAND 1} = 0.29$$

$$R_{BPB}\ \text{BAND 2} = 0.42\text{-}0.65$$

37% of the soluble AChE activity was associated with band 1 and 63% with band 2.


BAND 1
BAND 2
BPB
AChE ACTIVITY-change in O.D.
·20
·15
·10
·05
1
2
3
4
5
DIST. ALONG GEL-cms.

reason the values for % AChE activity in the two bands is more accurately determined by the gel slicing method than by the direct staining method.

The properties of the two bands of AChE activity were further investigated by a second separation of the electrophoretically pure products (fig. 20). The two bands of activity, localised by direct staining of the gels, were sliced out and the activity eluted. The eluted activity was concentrated by negative pressure dialysis and the purified species separately re-run on polyacrylamide gels.

A second electrophoretic separation of the AChE band 1 activity (fig. 20a) gave only one band of protein (R_{BPB} = 0.21). This R_{BPB} value is considerably lower than the R_{BPB} of 0.29 for AChE band 1 obtained from the first electrophoretic separation. Two possible reasons for this discrepancy are:

1) The molecular species responsible for AChE band 1 activity aggregated prior to the second electrophoresis.

2) The migration of the molecular species responsible for AChE band 1 activity was affected by interaction with other components of the homogenate during the first electrophoresis. These components would not be present in the purified sample used for the second electrophoresis.

A second electrophoretic separation of AChE band 2 activity gave six bands of protein, all staining with approximately equal intensity (fig. 20b). The R_{BPB} values of these bands are given below:

PROTEIN BAND No.	R_{BPB}
1	0.19
2	0.33
3	0.42
4	0.50
5	0.55
6	0.64

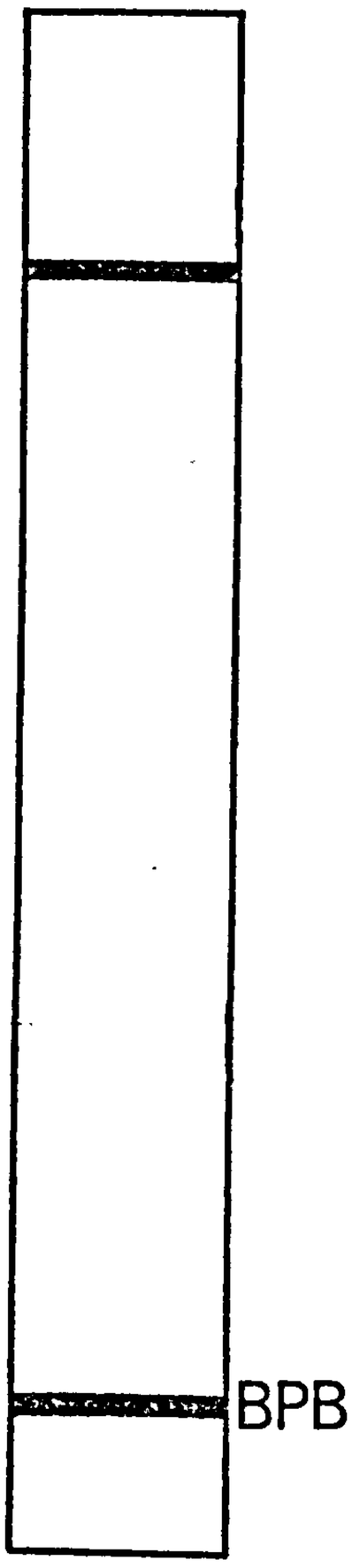
FIG. 20. Protein bands detected following a second electrophoretic separation of purified AChE band 1 and band 2 activity

AChE band 1 and band 2 activity, separated by electrophoresis, was detected by direct staining of the gels. The two bands of activity were eluted, concentrated and subjected to a second electrophoretic separation. The second set of gels was stained for protein with coomassie brilliant blue.

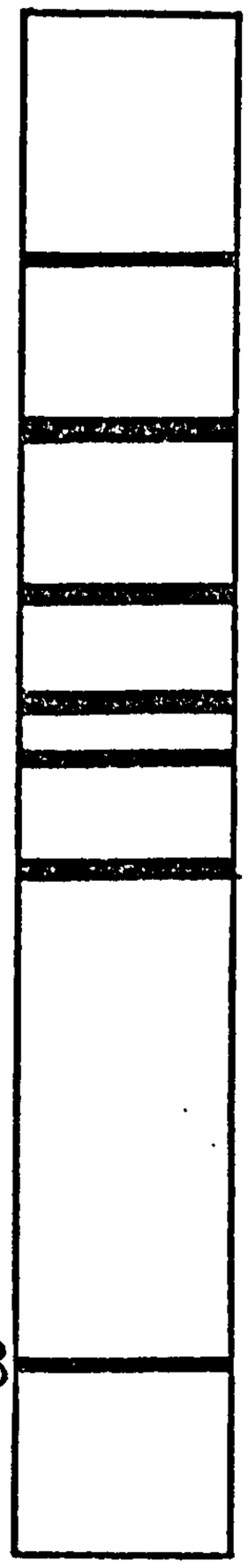
FIG. 20a. A second electrophoretic separation of AChE band 1 activity revealed the presence of only one band of protein (R_{BPB} = 0.21).

FIG. 20b. A second electrophoretic separation of AChE band 2 activity revealed the presence of six bands of protein. The significance of these bands is discussed fully in the text. The results suggest that band 2 activity may aggregate into higher M.Wt. forms and may also break down into subunits.

Note that the AChE activity detected on the second set of gels was minimal.



A



B

Staining of these gels with ATCh and DTNB showed the presence of only minimal AChE activity. The small amount of enzyme activity detected was associated with protein bands 1, 2 and 3, but not with protein bands 4, 5 and 6. Because of the low AChE activity on the gels it is not possible to draw any definite conclusions as to the relationship of the protein bands to the original AChE band 2 activity. For example, some of the protein bands may be due to the presence of contaminating species. Nevertheless the results obtained from the second electrophoretic separation of AChE band 2 indicate that:

1) The R_{BPB} values of protein bands 3 and 4 are well within the range of R_{BPB} values obtained for AChE band 2 activity. Thus these two proteins may correspond to the two molecular species of AChE which have been proposed to account for the diffuse nature of AChE band 2 activity.

2) The R_{BPB} value of protein band 2 is very similar to the R_{BPB} value for AChE band 1 activity obtained from the first electrophoretic separation. Thus the band 2 enzyme may aggregate to form the band 1 enzyme.

3) The R_{BPB} value of protein band 1 is almost identical to the R_{BPB} value for the protein band obtained from a second electrophoresis of AChE band 1 activity, i.e., both the band 1 and band 2 enzymes may aggregate to form the same slower migrating species.

Protein bands 5 and 6 may be breakdown products of AChE band 2 activity.

The information obtained from the second electrophoretic separation suggests that reasons 2, 3 and 4 (pages 89 and 92), either separately or in combination, account for the diffuse nature of AChE band 2 activity.

3.4.3. Separation of cockroach AChE by gel filtration

Separation of ganglionic proteins (solubilised with 0.1% DOC) on Bio-Gel A-0.5m showed the presence of two major bands of AChE activity (fig. 21). The first major band of activity occurred between fractions 18-26 and the second between fractions 27-33. The second band eluted as a sharp peak whereas the first was more diffuse and always contained a shoulder at its slower eluting end. The latter point is better illustrated in the expanded scale of fig. 22. Thus the results indicate the existence of three different molecular species of AChE which can be resolved by this method. These species have been designated peak 1, peak 2 and peak 3 AChE activity. Peak 1 is the fastest eluting species and peak 3 the slowest. Peak 3, the lowest M.Wt. species, gave the sharpest elution profile. The recovery of AChE activity from the column was good (better than 90%).

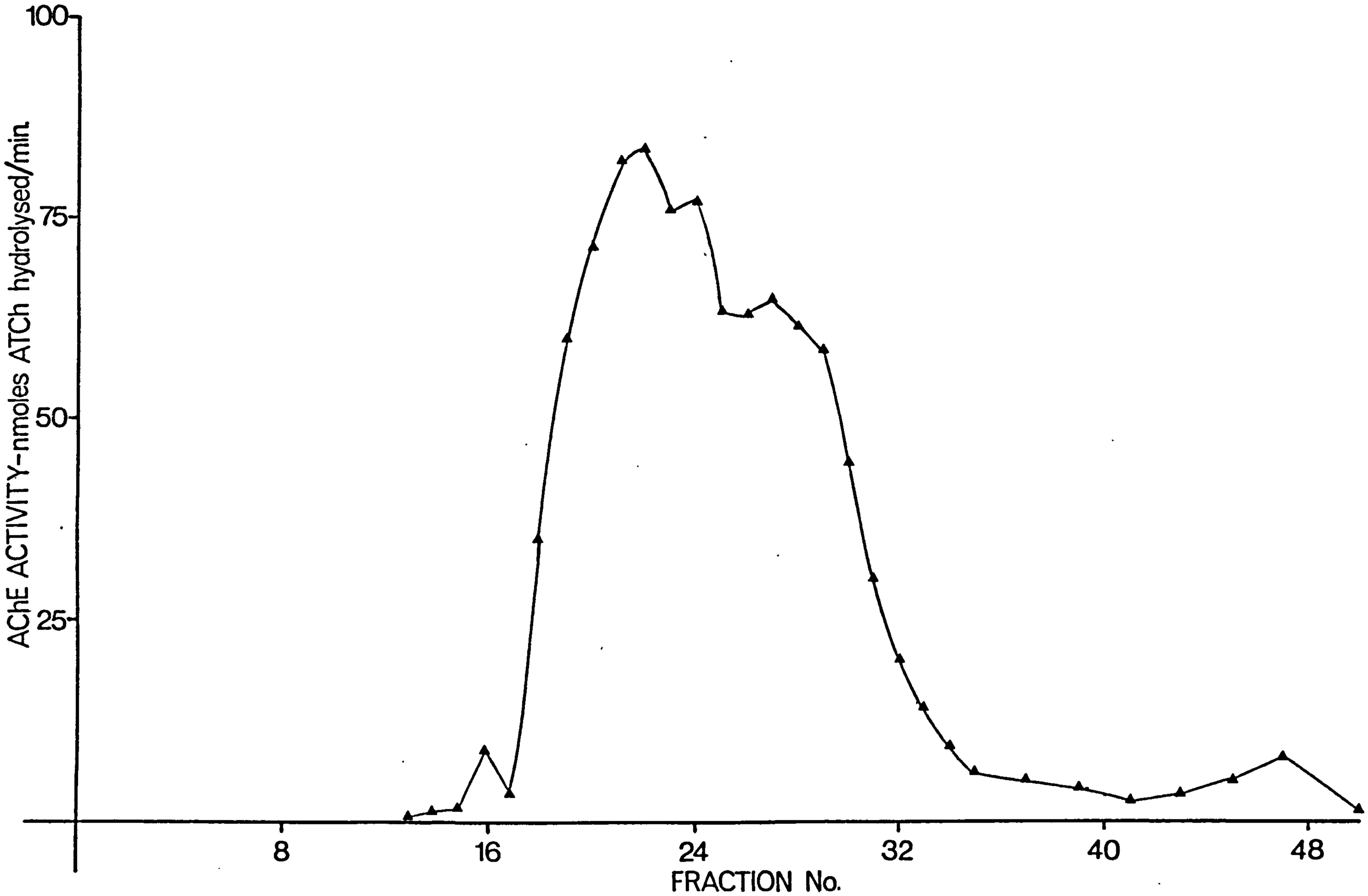
Further information about the three species was obtained by re-separating them on the column. Fractions 17-24 (peaks 1 and 2) and fractions 27-33 (peak 3) were pooled separately, concentrated and re-run on the column. Fig. 23 shows the elution profiles for the two re-separated samples. The concentrate from peaks 1 and 2 gave an elution profile that was polydisperse, but consisted of at least two species of AChE. The position of the two peaks of activity corresponded well with those obtained from the first separation. Re-separation of peak 3 gave a single sharp peak of activity containing a distinct shoulder corresponding to peak 2 activity. Peak 3 activity eluted more slowly from the column on re-separation than during the initial gel filtration. This slight difference was probably due to the removal of contaminating species of protein during

FIG. 21. Elution profile for AChE activity from metathoracic ganglia separated by gel filtration on Bio-Gel A-0.5m

The AChE activity was solubilised with 0.1% DOC. The characteristics of the column used are described in the text. Two major bands of AChE activity were eluted from the column. The faster eluting band of activity contained a distinct shoulder at its slower eluting end. Thus three peaks of AChE activity were separated by this method. The three peaks of activity eluted in the following fractions:

PEAK I	FRACTIONS 18-22	)	
		)	BAND 1
PEAK 2	FRACTIONS 23-26	)	
PEAK 3	FRACTIONS 27-33	)	BAND 2

Note that nearly all the AChE activity entered the gel, only a minimal amount of activity eluting in the void volume.


100
75
50
25
AChE ACTIVITY-nmoles ATCh hydrolysed/min.
8
16
24
32
40
48
FRACTION No.

(66)

FIG. 22. Elution profile for AChE activity from metathoracic ganglia separated by gel filtration (expanded scale)

AChE activity from a second sample was separated by gel filtration. Details are exactly as described for fig. 21. The scale is expanded to illustrate that the faster eluting band of activity contains a shoulder, i.e., this band can be resolved into two peaks of activity.

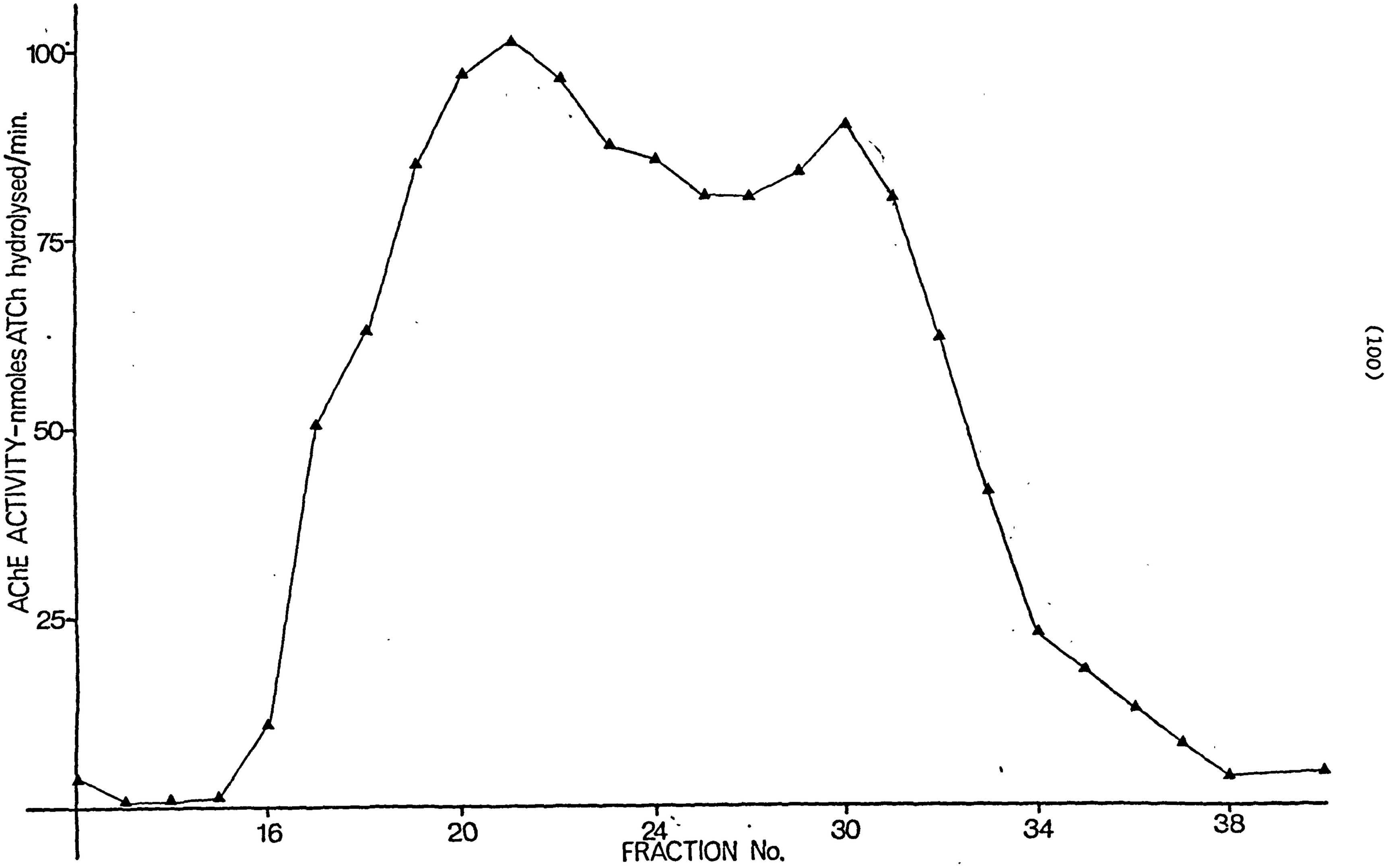
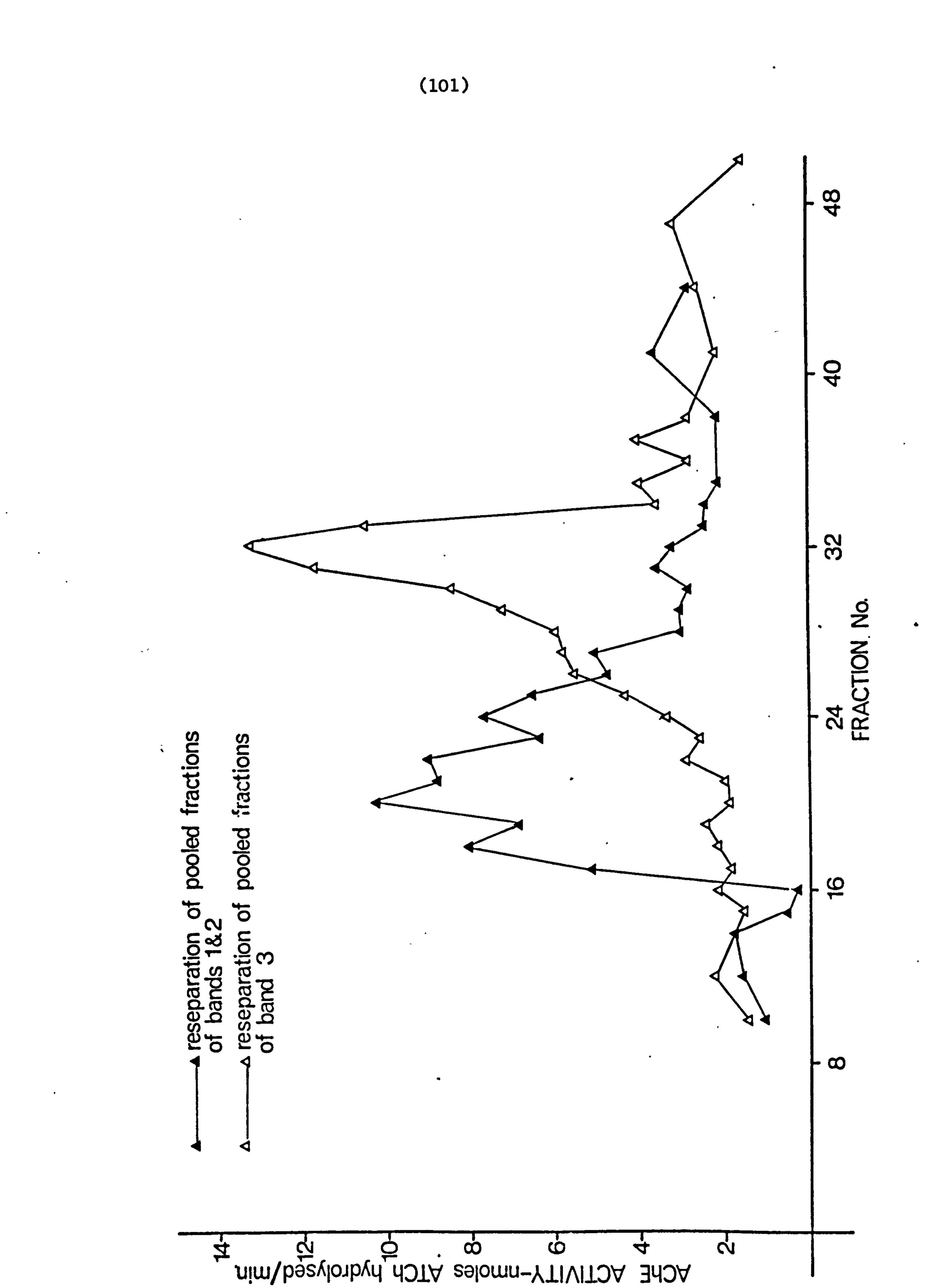

100
75
50
25
AChE ACTIVITY-nmoles ATCh hydrolysed/min.
16
20
24
30
34
38
FRACTION No.
(100)

FIG. 23. Re-separation by gel filtration of AChE activity from peaks 1 and 2 (combined) and peak 3

AChE activity from peaks 1 and 2 combined (fractions 17-24) and peak 3 (fractions 27-33) were pooled separately and concentrated. The concentrated extracts were re-separated on Bio-Gel A-0.5m. Re-separation of the concentrate from peaks 1 and 2 gave a polydisperse elution profile that consisted of at least two species of AChE. Peak 3 re-separated as a sharp band of activity containing a shoulder of peak 2 activity. The re-separation confirms the existence of three species of AChE of differing M.Wts. Little information was obtained concerning the inter-convertibility of the three species.


▲ reseparation of pooled fractions of bands 1&2
△ reseparation of pooled fractions of band 3
ACHE ACTIVITY-nmoles ATCh hydrolysed/min
FRACTION No.
2
4
6
8
10
12
14
8
16
24
32
40
48

the first separation. The shoulder corresponding to peak 2 activity could either have been due to contamination or due to conversion of peak 3 activity into peak 2 activity.

These results confirm the existence of three species of AChE of differing M.Wt. They also suggest that it is unlikely that any of the peaks is a result of protein-protein interactions between AChE and other protein species present in the original sample. The elution profiles for the three species indicate M.Wts. in the region of 400,000, 300,000 and 200,000 daltons. Thus it is possible that the three species are aggregate forms of the enzyme. Little information was obtained which indicates whether the three species are inter-convertible or not. The separation of three species of AChE by gel filtration supports the suggestion that AChE band 2 activity, separated by gel electrophoresis, consists of two different species of the enzyme.

3.4.4. Some properties of cockroach AChE when present in crude nerve cord homogenates

The effects of monovalent and divalent cations on the activity of cockroach AChE present in crude nerve cord homogenates were investigated. The effects of Ca^{2+} and Mg^{2+} on the apparent Km and Vmax of the enzyme are shown in figs. 24 and 25. Values for these parameters measured at different concentrations of Ca^{2+}, Mg^{2+} and also Na^{+} are summarised in table 8.

The effects of the two divalent cations on the enzyme were complex. An increase in the concentration of Ca^{2+} or Mg^{2+} from 1 to 10 mM produced an activation of AChE at high substrate concentrations, but inhibited the enzyme at low substrate concentrations. Both the Km and Vmax parameters of the enzyme were affected. An increase in Ca^{2+} concentration from 1 to

FIG. 24. Lineweaver-Burk plots showing the effects of Ca^{2+} ions on the kinetic parameters of AChE in total nerve cord homogenates

The plots show that an increase in Ca^{2+} concentration from 1-10 mM produced an activation of AChE at high substrate concentrations, but inhibited the enzyme at low substrate concentrations. Both the apparent Km and apparent Vmax of the enzyme were affected. The inhibitory effect of Ca^{2+} could be an indirect effect caused by interaction of Ca^{2+} with membrane fragments containing AChE. However, the linearity of the plots suggests that the ions were acting directly on the enzyme. The anion present with Ca^{2+} was Cl^{-}.

Note: All the kinetics experiments involving determination of the K_m and V_{max} of AChE were each separately repeated several times. For example the results shown in fig. 26 are from one set of results which is typical of three separate experiments carried out, whilst the results shown in fig. 30 are typical of the results obtained in six completely separate experiments.

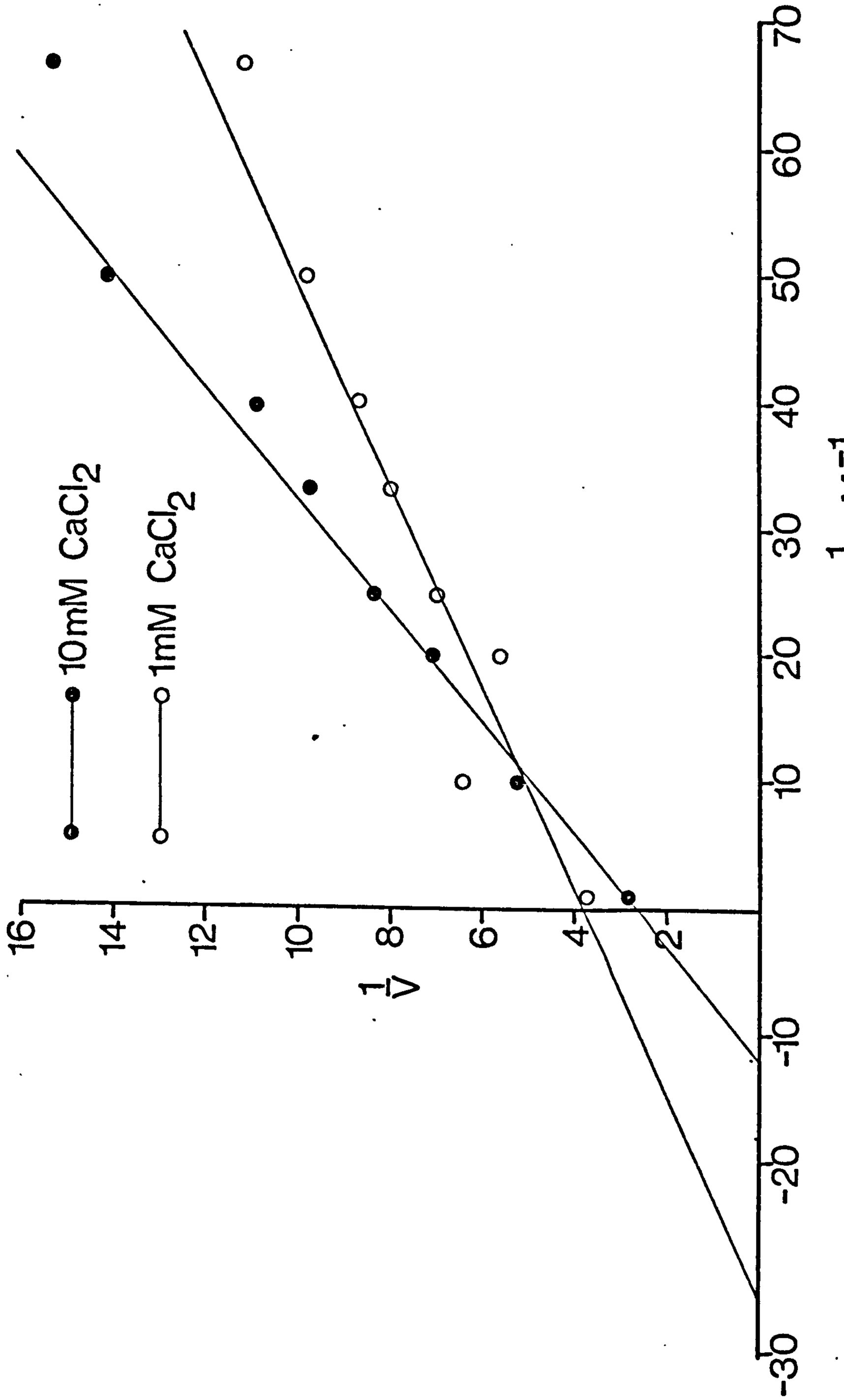

● 10mM $CaCl_2$
○ 1mM $CaCl_2$
$\frac{1}{V}$
$\frac{1}{S}mM^{-1}$
16
14
12
10
8
6
4
2
-10
-20
-30
10
20
30
40
50
60
70

FIG. 25. Lineweaver-Burk plots showing the effects of Mg^{2+} ions on the kinetic parameters of AChE in total nerve cord homogenates

The plots show that an increase in Mg^{2+} concentration from 1-10 mM increased the apparent Km of the enzyme by 35%, but had no significant effect on apparent Vmax. The pattern of activation/inhibition is similar to that produced by an increase in Ca^{2+} concentration from 1-10 mM. 100 mM Mg^{2+} activated the enzyme at all substrate concentrations tested. The apparent Vmax was increased by 120% as compared with the value measured at a Mg^{2+} concentration of 1 mM. The anion present with Mg^{2+} was Cl^{-}.

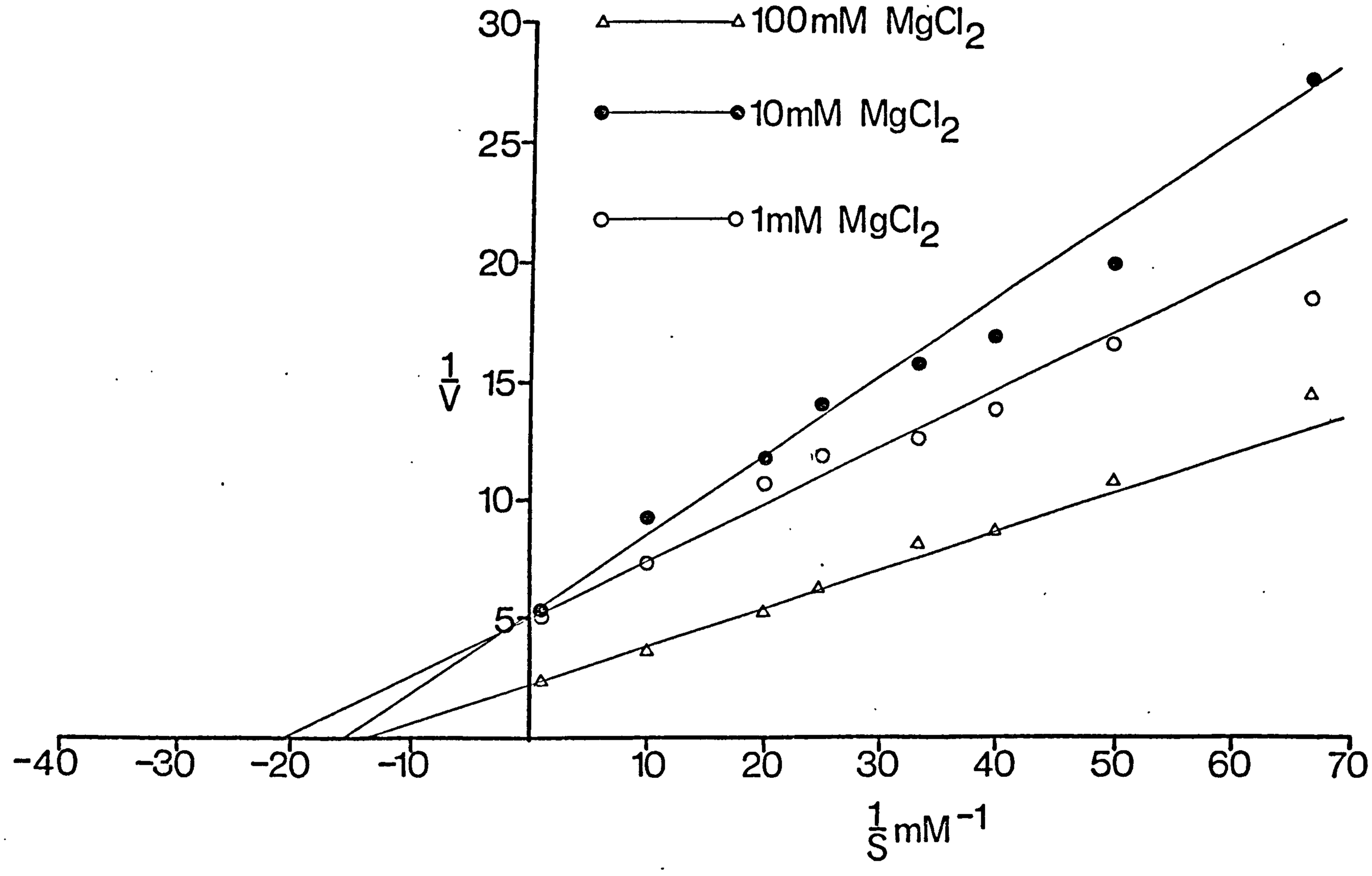

100mM MgCl2
10mM MgCl2
1mM MgCl2
30
25
20
15
10
5
1/V
-40
-30
-20
-10
10
20
30
40
50
60
70
1/S mM-1

TABLE 8. Summary of the effects of monovalent and divalent cations on the kinetic parameters of cockroach AChE in total nerve cord homogenates

All values for the apparent Km and apparent Vmax of the enzyme were calculated as described in section 3.2.7. The anion added with each cation was Cl^-.

Cation added	Concentration of cation (mM)	Apparent Km ($\times 10^{-5}$M)	S.E. of apparent Km	Apparent Vmax (nmoles ATCh hydrolysed/ min./ug prot.)	S.E. of apparent Vmax
Na^+	1	4.050	$\pm$ 0.726	0.201	$\pm$ 0.013
	10	4.254	$\pm$ 0.437	0.329	$\pm$ 0.041
	100	9.235	$\pm$ 0.517	0.477	$\pm$ 0.017
Mg^{2+}	1	4.773	$\pm$ 0.356	0.198	$\pm$ 0.006
	10	6.301	$\pm$ 0.458	0.189	$\pm$ 0.006
	100	6.977	$\pm$ 0.335	0.432	$\pm$ 0.009
Ca^{2+}	1	3.017	$\pm$ 0.689	0.256	$\pm$ 0.021
	10	8.397	$\pm$ 0.466	0.371	$\pm$ 0.009

(S.E. = Standard error)

10 mM resulted in a 180% increase in apparent Km and a 45% increase in apparent Vmax. A similar increase in Mg^{2+} concentration resulted in only a 35% increase in apparent Km and produced no significant change in the value for apparent Vmax. Thus at these two concentrations Ca^{2+} exerted a greater effect on AChE activity than Mg^{2+}. The increase in apparent Km is probably due to competition of the ions with substrate for binding at the anionic site. 100 mM Mg^{2+} activated cockroach AChE at all substrate concentrations tested. The apparent Vmax was increased by 120% as compared with the value measured at a Mg^{2+} concentration of 1 mM.

Increases in Na^{+} concentration also increased the apparent Km and Vmax of the enzyme (table 8). However, increasing concentrations of Na^{+} did not inhibit AChE at any of the substrate concentrations tested. Thus the action of the monovalent ion on the enzyme differed from that of both divalent cations in this respect. Dawson and Crone (1973) have produced evidence that cation activation of mammalian AChE is not exclusively related to ionic strength. Ion activation of both electric eel and mammalian AChE has been reviewed on page 11. Inhibition of the enzyme from these sources by divalent cations has not been reported. It is possible that the inhibitory effects of Ca^{2+} and Mg^{2+} on cockroach AChE are indirect being caused by interaction of the ions with membrane fragments containing AChE. Monovalent and divalent cations are known to have different effects on membrane conformation. Any ion induced change in membrane conformation could alter the activity of membrane bound AChE.

Other studies carried out on the enzyme in total nerve cord homogenates were directed towards clarifying the changes which take place during the

decay of AChE activity as discussed in section 2. Lineweaver-Burk plots for freshly homogenised samples and the same samples aged for 2 hrs. after homogenisation (fig. 26) show that the apparent Km of the enzyme increased by 12% whilst the apparent Vmax decreased by 20% during the decay phenomenon. Thus the major effect of the formation of the occlusion barrier is on the apparent Vmax of the enzyme.

The apparent Km of the soluble and membrane bound enzyme was investigated by separating the supernatant and pellet material of samples centrifuged at 100,000 g for 1 hr. Only 29% of the AChE activity was soluble as the samples had not been treated with detergent. The apparent Km of the soluble enzyme was 3.7×10^{-5}M whereas that of the membrane bound enzyme was considerably higher, being 6.0×10^{-5}M (fig. 27). This difference is attributed to the fact that the activity of the membrane bound AChE was decreasing during the centrifugation. The apparent Vmax of the membrane bound enzyme had also probably decreased during this process, but this could not be ascertained. These results raise the question as to whether the kinetic parameters of the enzyme can be altered by the conformational state of the membrane <u>in vivo</u> as well as <u>in vitro</u>.

Colhoun (1961) reports that cockroach AChE is activated by aliphatic alcohols, n-butanol being the most effective of the alcohols tested. The effect of n-butanol on the activity of the enzyme in total nerve cord homogenates was investigated by adding 10 µl of the alcohol to the assay medium (assays carried out in 3 ml cuvettes). The results summarised in table 9a show that n-butanol produced a 60% activation of the enzyme. This effect was specific to the insect enzyme as n-butanol produced no activation of AChE present in rat brain homogenates. The alcohol activated

FIG. 26. Lineweaver-Burk plots for AChE in fresh and aged nerve cord homogenates

The plots show that on aging of the sample for 2 hrs. after homogenisation the apparent Km of the enzyme increased by 12% whilst the apparent Vmax of the enzyme decreased by 20%. Thus the major effect of the proposed occlusion barrier was on the apparent Vmax of the enzyme. The values for the kinetic parameters were calculated as described in section 3.2.7.

	Apparent Km ($\times 10^{-5}$M)	S.E. of apparent Km	Apparent Vmax (nmoles ATCh hydrolysed/ min./ml homogenate	S.E. of apparent Vmax
Freshly homogenised sample	5.13	$\pm$ 0.21	33.5	$\pm$ 0.55
Sample aged for 2 hrs.	5.78	$\pm$ 0.34	26.9	$\pm$ 0.67

(S.E. = Standard error)

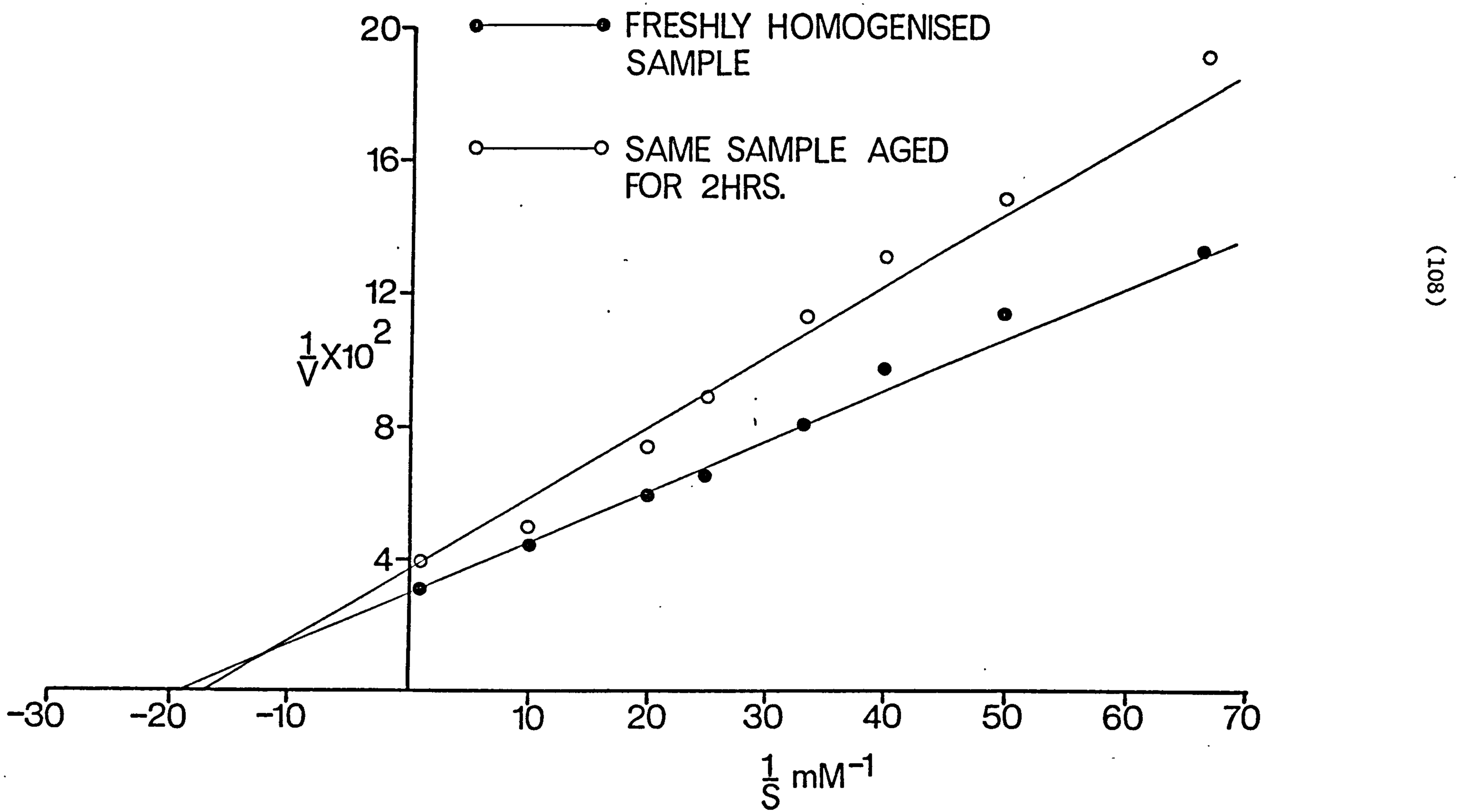
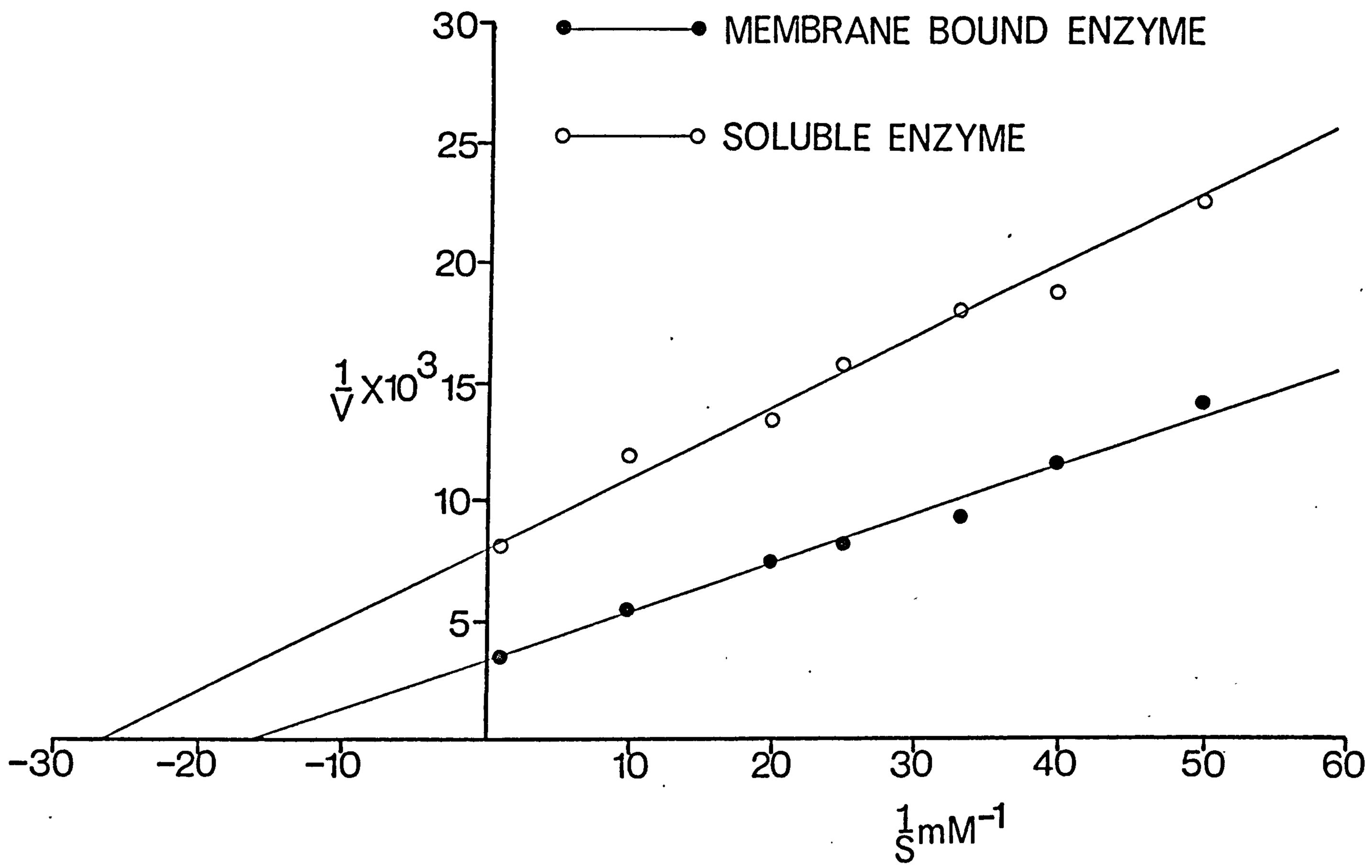

FRESHLY HOMOGENISED SAMPLE
SAME SAMPLE AGED FOR 2HRS.
20
16
12
8
4
$\frac{1}{V}X10^2$
-30
-20
-10
10
20
30
40
50
60
70
$\frac{1}{S}$ mM^{-1}

FIG. 27. Lineweaver-Burk plots for soluble and membrane bound AChE

The membrane bound and soluble fractions were separated by centrifugation of homogenates at 100,000 g for 1 hr. Only 29% of the enzyme activity was soluble as the sample was not treated with detergent. The apparent Km of the soluble fraction was considerably lower than that of the membrane bound enzyme. This difference is attributed to the occlusion of a portion of the membrane bound enzyme from assay. Values for the apparent Km were calculated as described in section 3.2.7.

$$\text{Km SOLUBLE ENZYME} = 3.68 \pm 0.33 \times 10^{-5} M$$

$$\text{Km MEMBRANE BOUND ENZYME} = 6.00 \pm 0.26 \times 10^{-5} M$$


MEMBRANE BOUND ENZYME
SOLUBLE ENZYME
30
25
20
15
10
5
$\frac{1}{V} X10^3$
-30
-20
-10
10
20
30
40
50
60
$\frac{1}{S} mM^{-1}$

the enzyme in 0.1% DOC treated samples as well as in untreated samples. The effects of DOC and n-butanol on the enzyme activity were additive. Thus n-butanol was not activating samples by disrupting the occlusion barrier. This finding is supported by the observation that the rate of hydrolysis of ATCh was still non-linear in n-butanol treated samples. If samples were treated with triton-X-100 or lubrol-PX before treatment with n-butanol a constant rate of hydrolysis of ATCh was obtained (table 9b). Thus it is possible that a combination of triton-X-100 or lubrol-PX and n-butanol disrupts the occlusion barrier.

The activation mechanism of the alcohol is unknown, but presumably it alters the conformation at the active site of the enzyme. Colhoun speculates that it may affect the binding to the AChE molecule of a lipid moiety which hinders interaction of the substrate with the active site.

3.4.5. Some properties of partially purified preparations of cockroach AChE

Brownson and Watts (1973) report that DTNB activates mammalian AChE by competing with the substrate for a second binding site on the enzyme surface. The presence of DTNB causes a lowering of the Km value of the enzyme, but does not affect its Vmax. Brownson and Watts also report that DTNB lowers the Km and increases the Vmax of pseudocholin-esterase. Augustinsson and Eriksson (1974) dispute these conclusions and present evidence that Ald.-2 has an inhibitory effect on cholinesterases.

TABLE 9. Summary of the effect of n-butanol on AChE activity in homogenates of metathoracic ganglia

TABLE 9a. The effect of n-butanol on AChE activity in nerve cord homogenates in the presence and absence of 0.1% DOC (n=5 for each result). Note that the effects of n-butanol and 0.1% DOC on enzyme activity were additive.

Agent added	Specific activity AChE-nmoles ATCh hydrolysed/min./ug prot.		% increase in AChE activity
	- n-butanol	+ n-butanol	
None	$1.51^{\pm}0.074$	$2.44^{\pm}0.097$	61
0.1% DOC	$2.09^{\pm}0.119$	$3.24^{\pm}0.143$	55

TABLE 9b. The effect of n-butanol on the non-linearity of the progress curve for the enzymic hydrolysis of ATCh. Note that a constant rate of hydrolysis of ATCh was obtained after treatment of samples with triton-X-100 or lubrol-PX and n-butanol.

	Agent added	% increase in rate of hydrolysis of ATCh during the first 2 mins. of assay
No n-butanol present	none	21.4
	1% triton-X-100	17.6
	1% lubrol-PX	10.6
	0.1% DOC	0
n-butanol present	none	24
	1% triton-X-100	0
	1% lubrol-PX	0
	0.1% DOC	0

The effect of DTNB on a preparation of soluble cockroach AChE was investigated by assaying fractions with both DTNB and Ald.-2. The enzyme was solubilised with 1% lubrol-PX. The Lineweaver-Burk plots obtained (fig. 28) show that the Km of the enzyme was 3.36×10^{-5}M when measured using DTNB and 6.54×10^{-5}M when measured using Ald.-2. The Vmax parameter of the enzyme was also affected. A value of 1.08 nmoles ATCh hydrolysed/min./ug prot. was obtained with DTNB and a value of 0.87 nmoles ATCh hydrolysed/min./ug prot. was obtained with Ald.-2. The initial interpretation of the results is that the cockroach enzyme behaves more like mammalian pseudocholinesterase than mammalian AChE with respect to the two assay reagents. However, a second explanation of the results is possible, namely that a lipid or lipoprotein component is still attached to a fraction of the enzyme. This component blocks assay of that fraction of the enzyme by Ald.-2, but not by DTNB. The lipoprotein component could be responsible for the formation of the occlusion barrier during the decay of AChE activity in nerve cord homogenates.

To test this hypothesis the lipoprotein material separated from the supernatant fraction after centrifugation was added back to it. The kinetic parameters of the preparation were re-determined using both DTNB and Ald.-2. Fig. 29 shows that the Km for AChE in this preparation was 3.10×10^{-5}M when measured with DTNB and 5.41×10^{-5}M when measured with Ald.-2. The two values are not significantly different from those obtained when the lipoprotein material was not present. The Vmax value for the enzyme was 1.49 nmoles ATCh hydrolysed/min./ug prot. when measured with DTNB and 1.07 nmoles ATCh hydrolysed/min./ug prot. when measured with Ald.-2.

FIG. 28. Lineweaver-Burk plots for cockroach AChE measured using DTNB and Ald.-2 as assay reagents

The sample was prepared by treatment with lubrol-PX and centrifugation at 100,000 g for 1 hr. The supernatant fraction was assayed at substrate concentrations from 1 -0.01 mM using both DTNB and Ald.-2 as assay reagents. The plots show differences in both the Km and Vmax of the enzyme as measured with the two assay reagents. The Km and Vmax values were calculated as described in section 3.2.7.

Assay reagent	Km ($x10^{-5}$M)	S.E. of Km	Vmax (nmoles ATCh hydrolysed/ min./ug prot.)	S.E. of Vmax
DTNB	3.36	$\pm$0.16	1.08	$\pm$0.02
Ald.-2	6.54	$\pm$0.25	0.87	$\pm$0.01

Note the curvature of the plot obtained using DTNB at low substrate concentrations.

(S.E. = Standard error)

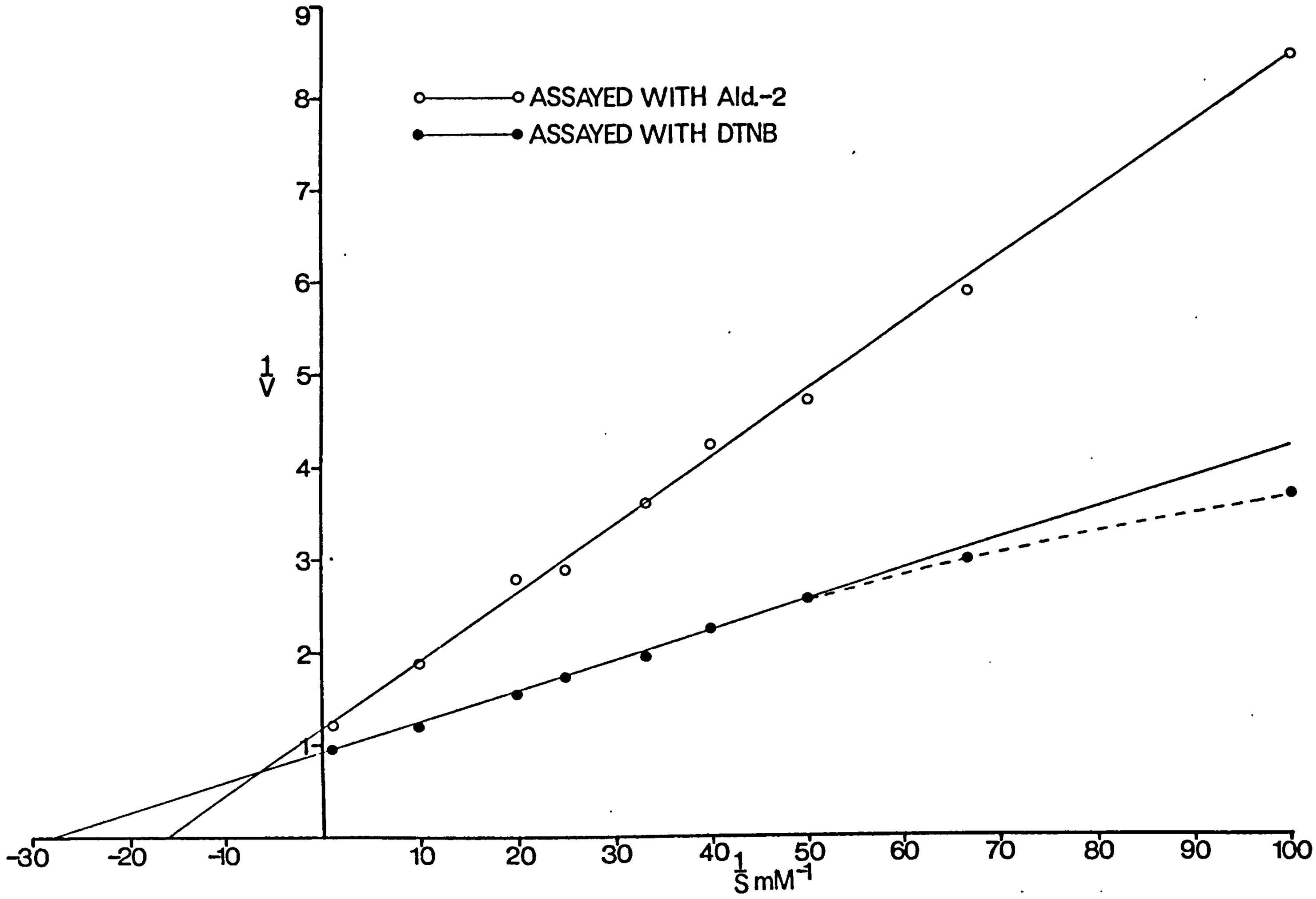

ASSAYED WITH Ald.-2
ASSAYED WITH DTNB
1/V
1/S mM⁻¹
9
8
7
6
5
4
3
2
1
-30
-20
-10
10
20
30
40
50
60
70
80
90
100

FIG. 29. Lineweaver-Burk plots for cockroach AChE measured using DTNB and Ald.-2 as assay reagents (lipoprotein material present in sample)

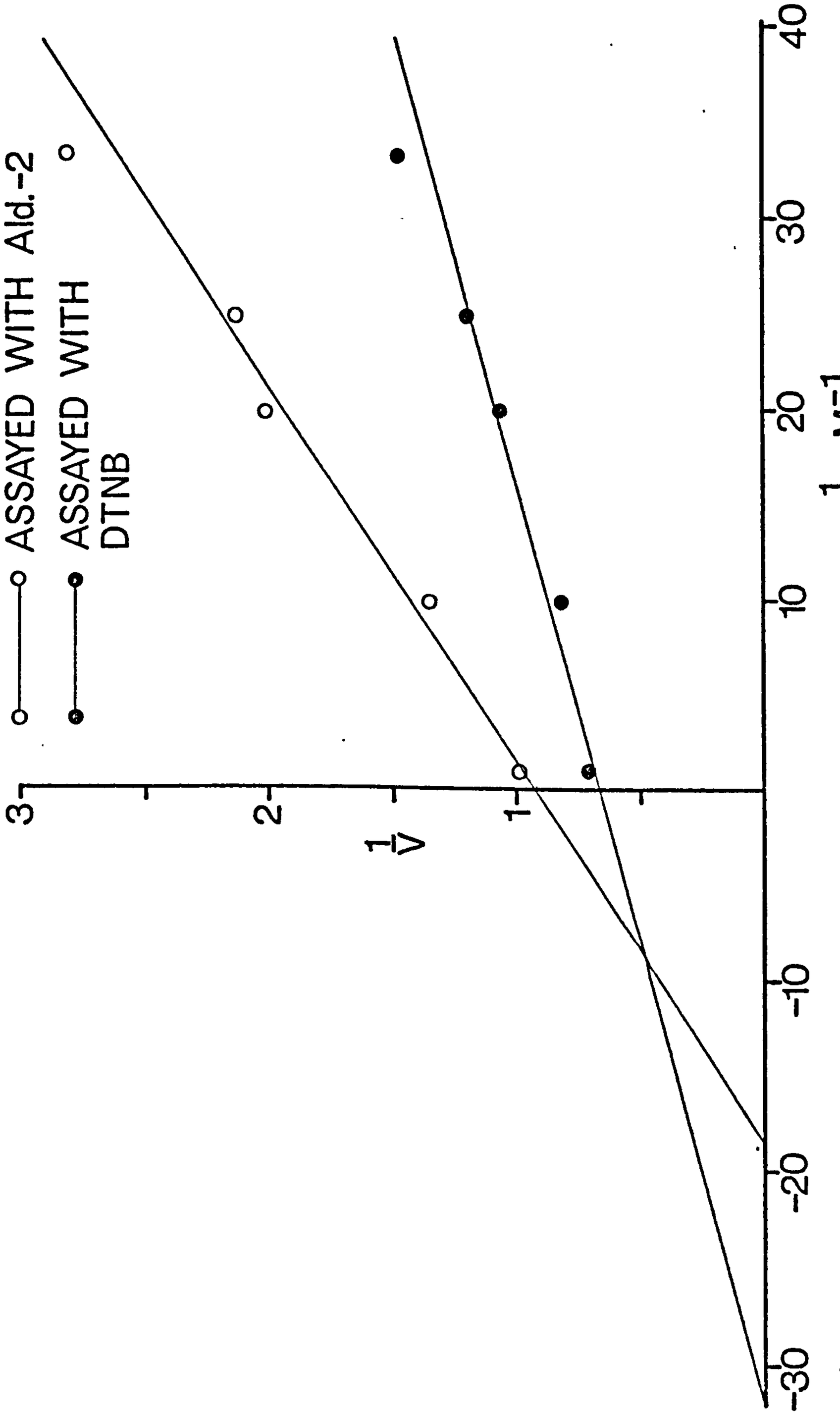
The same sample was used as in fig. 28, but the lipoprotein material was added back to the rest of the supernatant. Again differences in the Km and Vmax of the enzyme were observed depending on the assay reagent used. Note:

1) The % difference between the two Km values measured using DTNB and Ald.-2 is not significantly different to that measured in fig. 28.

2) The % difference between the two Vmax values measured using DTNB and Ald.-2 is considerably higher than that measured in fig. 28.

Assay reagent	Km ($\times 10^{-5}$M)	S.E. of Km	Vmax (nmoles ATCh hydrolysed/ min./ug prot.)	S.E. of Vmax
DTNB	3.10	$\pm$0.43	1.49	$\pm$0.003
Ald.-2	5.41	$\pm$0.52	1.07	$\pm$0.006

(S.E. = Standard error)


ASSAYED WITH Ald.-2
ASSAYED WITH DTNB
3
2
1
1-V
40
30
20
10
-10
-20
-30
1/S mM-1

In the absence of lipoprotein material the difference between the Vmax of the enzyme as assayed with the two reagents was 19% whereas in the presence of lipoprotein the difference between the two values was 28%. These results are interpreted as indicating that the difference between the Vmax values of the enzyme as measured with DTNB and Ald.-2 is accounted for by the second explanation.

A final feature of interest illustrated in fig. 28 is that the Lineweaver-Burk plot obtained using DTNB is curved at low substrate concentrations. The curvature indicates a faster reaction rate than would be expected at these substrate concentrations. This effect was routinely observed when DTNB was used as the assay reagent, even when AChE samples were electrophoretically pure. The curvature was not observed when Ald.-2 was used as the assay reagent. The magnitude of the effect is such that the rate of hydrolysis of 0.01 mM ATCh is 15% faster than would be expected if Michaelis-Menten kinetics were operating. This is further evidence that DTNB activates the enzyme and that substrate competes with DTNB for a binding site on the enzyme surface.

It is tentatively concluded that DTNB activates cockroach AChE and that the reagent would affect only the Km parameter of a pure preparation of the enzyme.

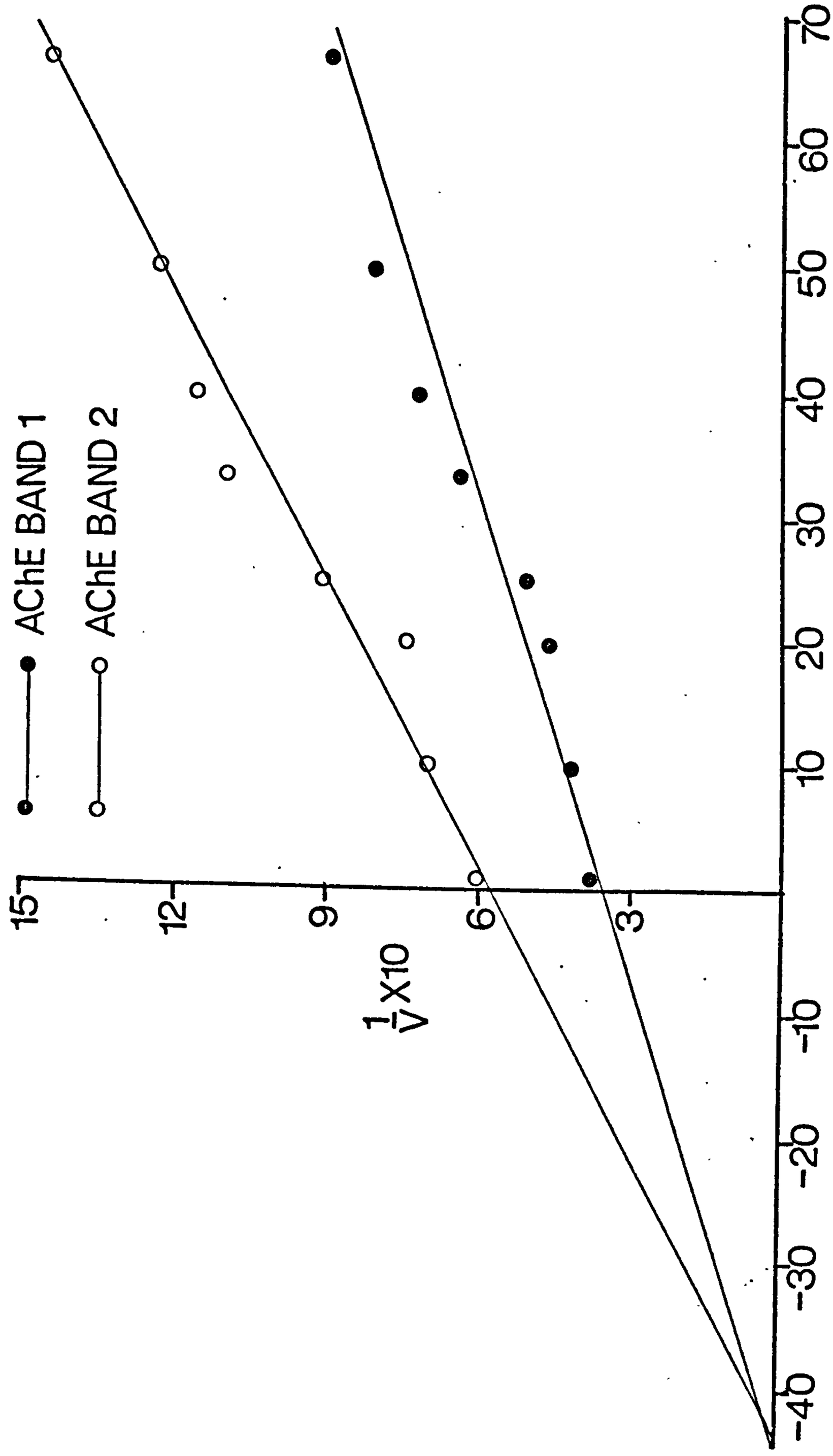
The kinetic properties of the two bands of cockroach AChE eluted from polyacrylamide gels were investigated (fig. 30). DTNB was used as the assay reagent. The Km value for AChE band 1 in the sample plot shown is 2.25×10^{-5}M. The Km value for AChE band 2 in the sample plot shown is 2.17×10^{-5}M. There is no significant difference between the two values.

FIG. 30. Lineweaver-Burk plots for electrophoretically pure AChE (DTNB used as assay reagent)

AChE band 1 and band 2 activity was eluted from gel slices as described in the text. The Km value of each band was calculated as described in section 3.2.7. No significant difference between the Km values of the two bands was obtained.

Band of AChE activity	Km ($\times 10^{-5}$M)	S.E. of Km
Band 1	2.25	$\pm$ 0.27
Band 2	2.17	$\pm$ 0.30

(S.E. = Standard error)


● AChE BAND 1
○ AChE BAND 2
15
12
9
6
3
$\frac{1}{V} X10$
70
60
50
40
30
20
10
-10
-20
-30
-40
$\frac{1}{S}$ mM^{-1}

The Km values obtained for six separate samples of electrophoretically pure AChE band 1 and AChE band 2 activity were analysed using a Diehl calculator. The results obtained were:

	Mean Km ($\times 10^{-5}$M)	s.e.m.
AChE BAND 1	3.02	$\pm$ 0.30
AChE BAND 2	2.60	$\pm$ 0.22

It is concluded that the Km values of the two bands are not significantly different.

The onset of substrate inhibition of AChE band 1 and AChE band 2 activity was observed at an ATCh concentration of 1-2 mM. Thus the two bands of AChE activity have the same Km and exhibit substrate inhibition at the same concentration of ATCh.

The properties of the enzyme which were investigated are summarised in table 10.

3.4.6. Summary of results

1) Three methods for partially purifying cockroach AChE were investigated. Polyacrylamide gel electrophoresis and gel filtration gave a better resolution of the molecular species of AChE present than did sucrose density gradient centrifugation.

2) Two distinct bands of soluble AChE activity were resolved by gel electrophoresis. The second, faster moving band was diffuse and may consist of two different species of the enzyme. A fraction of the triton-X-100 solubilised AChE activity did not enter the gel.

TABLE 10. Summary of the properties of cockroach AChE investigated in section 3

Property investigated	Comments on experimental procedure	Results obtained
Occurrence of multiple forms of the enzyme	Sucrose density gradient centrifugation (samples not treated with detergent)	Only one species of the naturally soluble enzyme resolved. Technique useful for separating membrane fragments containing AChE.
	Analytical polyacrylamide gel electrophoresis (5% gels used. AChE solubilised with 1% triton-X-100)	Two bands of AChE activity detected. AChE band 1 (R_{BPB} = 0.29) sharply defined, AChE band 2 (R_{BPB} = 0.40 - 0.56) diffuse. Electrophoretic re-separation of bands 1 and 2 shows: 1) Band 2 activity may aggregate into band 1 activity, 2) Band 2 activity may consist of two molecular species of AChE, 3) The band 2 enzyme may be unstable.
	Gel filtration (Bio-Gel A-0.5m used. AChE solubilised with 0.1% DOC).	Three species of AChE resolved. M.Wt. of largest component ~ 400,000 daltons.
Effect of decay of AChE activity on the kinetic parameters of the enzyme	Total nerve cord homogenate used.	Major effect on apparent Vmax. On aging apparent Km increased from 5.13×10^{-5}M to 5.78×10^{-5}M and apparent Vmax decreased from 33.5 to 26.2 nmoles ATCh hydrolysed/min./ml homogenate.

Table 10 continued

Property investigated	Comments on experimental procedure	Results obtained
Michaelis constant of the enzyme	Membrane bound AChE	$6.00 \pm 0.26 \times 10^{-5}$M
	Naturally soluble AChE	$3.68 \pm 0.33 \times 10^{-5}$M
	Electrophoretically pure AChE (mean of six experiments)	AChE BAND 1: $3.02 \pm 0.30 \times 10^{-5}$M AChE BAND 2: $2.60 \pm 0.22 \times 10^{-5}$M
Effect of divalent cations Ca^{2+} and Mg^{2+} on AChE activity	Total nerve cord homogenate used	Increasing concentrations of Ca^{2+}/Mg^{2+} increase apparent Km and Vmax of enzyme. 10 mM Ca^{2+}/Mg^{2+} has inhibitory effect on AChE at low substrate concentrations
Effect of Na^{+} on AChE activity	Total nerve cord homogenate used	Increasing concentrations of Na^{+} increase apparent Km and Vmax of enzyme. No inhibitory effects of Na^{+} observed
Substrate inhibition	Electrophoretically pure AChE used	Onset of substrate inhibition of both AChE band 1 and AChE band 2 observed between 1-2 mM ATCh
Effect of DTNB on AChE activity	Soluble preparation (enzyme solubilised with 1% lubrol-PX)	Km measured with DTNB lower than that measured with Ald.-2. Vmax measured with DTNB higher than that measured with Ald.-2. Effect on Vmax may be due to interaction of AChE with lipoprotein
Activation by n-butanol	Total nerve cord homogenate used	AChE activated 60% by n-butanol. The alcohol probably acts directly at the active site.

Note that the electrophoretically pure AChE was true AChE (as judged by the relative rates of hydrolysis of ATCh and BuTCh).

3) Two methods for estimating AChE activity separated by gel electrophoresis were developed. In the first method gels frozen in acetone/dry ice were sliced into 1.6 mm discs and the activity eluted into phosphate buffer. The eluted activity was assayed spectrophotometrically. In the second method intact gels were stained for AChE activity with ATCh and DTNB. The stained gels were scanned for a change in absorbance at a wavelength of 420 nm. This method enabled the enzyme activity to be rapidly localised, but was only semi-quantitative.

4) A second electrophoretic separation of AChE band 1 showed the presence of only one protein band. A second electrophoretic separation of AChE band 2 showed the presence of six protein bands. The R_{BPB} values of the six proteins suggest that AChE band 2 activity may aggregate into higher M.Wt. forms and also dissociate into lower M.Wt. forms.

5) Three distinct peaks of cockroach AChE were resolved by gel filtration. Re-separation of concentrated fractions of the eluate confirmed these results.

6) The elution profiles obtained from gel filtration studies suggest that the forms of AChE may be aggregates of one another, but no conclusive evidence was obtained for their inter-convertibility.

7) Increasing concentrations of Ca^{2+}, Mg^{2+} and Na^{+} increased the apparent Km and apparent Vmax of AChE present in crude homogenates. Lower concentrations of the divalent cations exerted an inhibitory effect on the enzyme at low substrate concentrations whereas increasing concentrations of Na^{+} activated the enzyme at all substrate concentrations tested.

8) Aging of nerve cord homogenates resulted in a decrease in the apparent Vmax of AChE. Only a small increase in apparent Km was observed.

9) Naturally soluble AChE has a lower Km than the membrane bound enzyme.

10) The enzyme is specifically activated by n-butanol.

11) Kinetic analysis of lubrol-PX solubilised AChE gave a lower Km when DTNB was used as assay reagent than when Ald.-2 was used as assay reagent. Also the Vmax of the enzyme was higher when measured using DTNB than when measured using Ald.-2.

12) The Km values for the two bands of AChE activity eluted from polyacrylamide gels were not significantly different. The onset of substrate inhibition of both bands of activity was observed at the same substrate concentration.

3.5.1. Discussion

The methods of partial purification described gave valuable information on the molecular properties of AChE from the nerve cord of Periplaneta americana. A combination of these methods could also be used to obtain a considerable purification of the enzyme.

Kunkee and Zweig (1963) obtained a 50 fold purification of honey bee AChE by a combination of sucrose density gradient centrifugation and ammonium sulphate fractionation. It was not possible to measure the degree of purification of the cockroach enzyme obtained in this study because of the small amount of protein layered on each gradient. The single species of naturally soluble cockroach AChE separated on gradients may correspond to the 7.3S species of the bee or mayfly enzyme reported by Krysan and Kruckeberg (1970). Thus the sedimentation coefficient of insect AChE (measured at pH 8.0) is considerably lower than the value of 10.8S reported for the 260,000 M.Wt. form of electric eel AChE by Massoulie, Rieger and Tsuji (1970). Krysan and Kruckeberg also find that the honey bee and mayfly enzymes aggregate into

faster sedimenting forms when the pH of the medium is lowered or its ionic strength increased. Thus, though sucrose density gradient centrifugation did not give a good resolution of the different molecular species of cockroach AChE it could be used to study the aggregation of the enzyme into higher M.Wt. forms, e.g., following solubilisation of the enzyme with different detergents. The method could also be used to purify different membrane fractions to which the enzyme is bound.

The resolution of two bands of soluble AChE activity by gel electrophoresis agrees with the results of Eldefrawi, Tripathi and O'Brien (1970) and also with earlier results reported by this group (Kerkut, Emson and Beesley, 1972). However, the kinetic properties of the two bands reported in section 3.4.5. do not agree with those of Kerkut et al. who report Km values of 5.88×10^{-5}M for the slower migrating band of AChE and 1.90×10^{-4}M for the faster migrating band of AChE. They also found that whilst the slower migrating band of activity exhibited substrate inhibition at a concentration of 2 mM ATCh the faster migrating band did not exhibit this phenomenon at concentrations of ATCh below 3-5 mM. It should be noted that different concentrations of polyacrylamide were used in the two studies (5% in the present study and 7.5% in the earlier study). It is likely that one of two possibilities could account for the differences between the two sets of results:

1) Evidence has been presented that AChE band 2 consists of two molecular species of the enzyme. One of these species could be a monomer of AChE band 1 and have the same kinetic properties as the band 1 enzyme. The other species could be a distinct isozyme with different kinetic properties from the two aggregate species. If, in the present study, this second isozyme

accounted for only a small fraction of the AChE activity localised in band 2 then both AChE band 1 and band 2 would have extremely similar Km values, as was observed. In the study of Kerkut, Emson and Beesley it is possible that the faster migrating band of AChE activity consisted exclusively of the second isozyme, the remainder of the soluble activity having aggregated into the band 1 enzyme. Thus, to summarise, the discrepancies between the two sets of results could be due to a difference in the degree of aggregation of one of the two suggested isozymes of cockroach AChE.

2) The number of molecular forms of AChE shows a marked variation between different species of insect. Edwards and Gomez (1966) report the existence of eight species of enzyme with ChE activity in the housefly. Three of these are AChE. Eldefrawi et al. (1970) report the existence of four species of AChE in the housefly, two in the cockroach and one in the southern army worm. Evidence exists that the number and properties of the molecular forms of AChE vary between strains of the same species of insect with differing susceptibilities to insecticides (Menzel, Craig and Hoskins, 1963; Nolan, Schnitzerling and Schunter, 1972). Thus it is possible that the discrepancy in the results is due to a strain difference in the animals used in the two studies.

Of these two explanations the available evidence favours the first suggestion. Clearly further work to clarify this discrepancy is desirable.

Approximate values for the M.Wts. of the three species of cockroach AChE resolved by gel filtration suggest that they may be aggregate forms of the enzyme. The kinetic properties of the two bands of AChE resolved by gel electrophoresis suggest that these two bands may be aggregates of one another.

This evidence indicates that the properties of cockroach AChE are similar to those of mammalian brain AChE in this respect (Chan, Shirachi, Bhargava, Gardner and Trevor, 1972; Hollunger and Niklasson, 1973). It is not known whether the aggregate forms of cockroach AChE are due to interaction of the enzyme with homologous or heterologous protein species. The gel filtration results indicate the existence of at least one active species of the cockroach enzyme with a M.Wt. considerably greater than the value of 260,000 reported for a single active unit of the electric eel enzyme (Leuzinger, Goldberg and Cauvin, 1969). The sedimentation studies discussed on page 121 suggest that insect AChE also occurs in an active form with a M.Wt. lower than 260,000. Recently Huang and Dauterman (1973) have purified the fly head enzyme 1,300 fold. They separated only one form of the enzyme (M.Wt. 208,000) by gel filtration. Gel electrophoresis indicated that their preparation contained two inter-convertible species of AChE. As yet no information is available concerning the number and type of polypeptide chains which make up the different species of insect AChE.

The results presented in section 3 are subject to the criticism that the methods used to study the enzyme may result in a change in its properties For example, Shafai and Cortner (1971) find that the properties of the erythrocyte enzyme are altered by ion-exchange chromatography. This criticism is particularly true of membrane bound enzymes which are solubilised with detergents. The existence of high M.Wt. forms of AChE does not give any information as to the state of the membrane bound enzyme, e.g., as to how many monomeric units of the enzyme may be linked together on the membrane surface. In a recent study Levinson and Ellory (1974) have estimated the M.Wt. of AChE bound to electroplax membranes by irradiation inactivation of the enzyme. They report a value of approximately 75,000 daltons. This

value differs considerably from the M.Wt. of the purified enzyme. Furthermore, studies of the purified enzyme do not give any information on the effects of other membrane components on the activity of the enzyme.

The kinetic studies of fresh and aged nerve cord homogenates, and of the soluble and membrane bound enzyme suggest that other membrane components have a considerable effect on the activity of cockroach AChE in vitro. Similar effects may also modify the activity of the enzyme in vivo. The differences in the kinetic parameters of the enzyme as measured with DTNB and Ald.-2 suggest that there may be a tightly bound lipid or lipoprotein component which affects the measurable activity of the enzyme. These results complement those presented in section 2 and suggest that the formation of the occlusion barrier is due to this lipid/lipoprotein component. A key experiment which would test this hypothesis would be to assay the DOC solubilised enzyme with DTNB and Ald.-2. DOC disrupts the occlusion barrier. Therefore, if the lipid/lipoprotein component is responsible for the occlusion barrier no difference in the Vmax values of the enzyme as measured with DTNB and Ald.-2 should be observed.

Non-ionic detergents interact exclusively with hydrophobic bonds whereas an anionic detergent, such as DOC, may also interact with ionic bonds (see section 1.4). Thus it is possible to propose a model in which cockroach AChE is bound to some membrane components by hydrophobic interactions alone, but is bound to others by either a mixture of ionic and hydrophobic bonds or by ionic bonds alone (fig. 31). Non-ionic detergents, such as triton-X-100, would release the AChE from components which were bound to it mainly by hydrophobic interactions. DOC, on the other hand, would release the enzyme from all components to which it was bound by either hydrophobic or ionic bonding. This would account for the disruption of the

FIG. 31. A model for the interaction of cockroach AChE with other membrane components

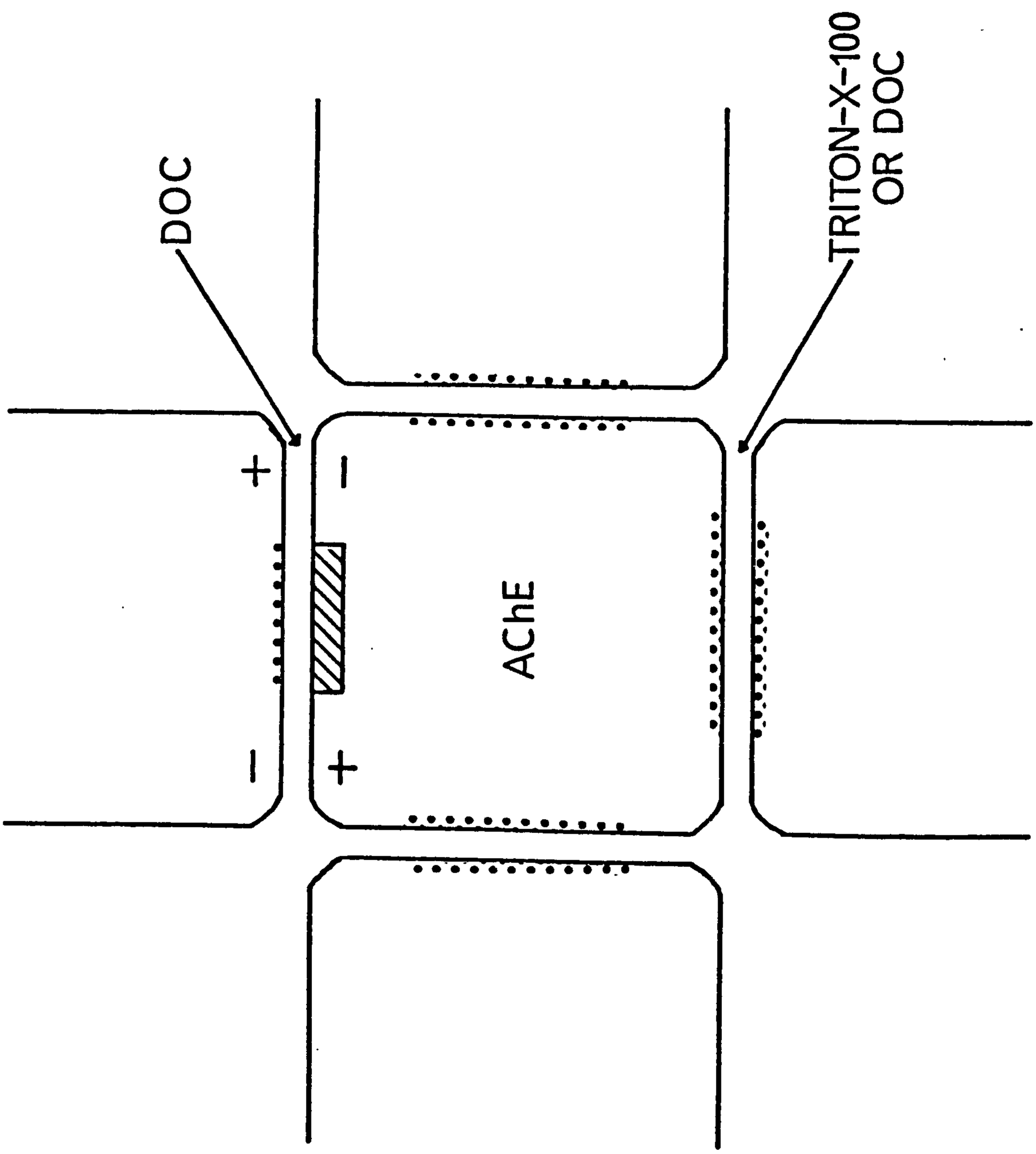
In this schematic representation membrane bound AChE interacts with other membrane components by both hydrophobic and ionic bonding. Interaction of AChE with the majority of these components is mainly due to hydrophobic bonding. Treatment with either triton-X-100 or DOC will disrupt the bonds between AChE and such components. Ionic bonding plays an important role in the binding of at least one membrane component to AChE. The component is probably a lipid or lipoprotein and is close to the active site of the enzyme. A change in configuration of this lipid/lipoprotein could hinder the interaction of substrate with the active site of the enzyme. This would result in a decrease in AChE activity.

KEY


= active site of AChE

+, - = regions of ionic bonding


= regions of hydrophobic bonding


DOC
TRITON-X-10
OR DOC
+
1
AChE
−
+

occlusion barrier by DOC, but not by triton-X-100. The model is supported by the evidence that DOC, but not triton-X-100, releases the enzyme in a form in which it is not attached to other membrane components. The work of Wright and Plummer (1972) suggests that ionic interactions play a role in the binding of AChE to the erythrocyte membrane.

The second aspect of the results obtained using DTNB and Ald.-2 is the effect on the Km of the enzyme. The results indicate that a second binding site for substrate may exist on the surface of cockroach AChE. Other evidence for the existence of a second binding site on the enzyme isolated from other sources has been reviewed in section 1.8. It would be of interest to know whether the site which binds DTNB is equivalent to the second binding site proposed by Changeux, Leuzinger and Huchet (1968). and Changeux, Podleski and Meunier (1969) for the electric eel enzyme. The data of Brownson and Watts shows that low concentrations of ATCh displace DTNB from its binding site. Therefore it is unlikely that the site has any physiological significance.

The properties of the cockroach enzyme are similar in many respects to those of AChE from other species, but there are also some distinct differences. The cockroach enzyme has a lower Km value than the mammalian brain enzyme (2.95×10^{-5}M as compared with 1.0×10^{-4}M). The pH optimum of the cockroach enzyme is of the same order as that for the mammalian brain enzyme. A specific property of cockroach AChE is its activation by n-butanol The mechanism of this activation has not been studied, but clearly it differs from the activation of AChE present in homogenates by DOC. The interaction between the effects of n-butanol and triton-X-100 on samples in which the AChE activity has decayed is puzzling. This is because the effect of n-butanol

is interpreted as being a direct effect on the enzyme. The activation of the cockroach enzyme by monovalent and divalent cations is similar to that observed in other species. The unusual aspects of the effects of the divalent cations on the enzyme have already been discussed.

3.5.2. Summary of discussion

It is concluded that at least three molecular species of AChE are obtained from the nerve cord of P. americana and that these may be aggregate forms of the enzyme. Insect AChE exists in forms of M.Wt. both lower and higher than the 260,000 form of the electric eel enzyme. There is some evidence from gel electrophoresis studies for the existence of a separate isozyme distinct from the two aggregate forms of the enzyme. The kinetic properties of the enzyme are modified in vitro by other membrane components. It is suggested that DTNB may activate the enzyme by binding to a second site on its surface. The difference between the Vmax values of the enzyme measured with DTNB and Ald.-2 is explained in terms of a lipid/lipoprotein component attached to the AChE molecule. This component may be the site of the occlusion barrier æsponsible for the observed decay of AChE activity. A model describing the interactions of AChE with other membrane components is discussed. The properties of the cockroach enzyme which were investigated are compared with those of the mammalian enzyme. There are some distinct differences between the properties of the enzyme isolated from the two sources, notably the activation of cockroach AChE by n-butanol. This activation is interpreted as being a direct effect of the alcohol on the enzyme.

SECTION FOUR

AN INVESTIGATION INTO THE POSSIBLE CORRELATION OF AChE ACTIVITY WITH BEHAVIOURAL CHANGES IN *Periplaneta americana*

4.1.1. General introduction

It has been realised for a considerable time that changes in the behavioural pattern of an organism must result in concommitant biochemical changes within the organism, i.e., there should be biochemical correlates of behaviour. Of prime interest are the biochemical changes that take place during the learning of a new task. Learning is defined as a change in the behaviour of an organism due to experience. Memory is the retention of this change.

A variety of approaches have been used to investigate the nature of the biochemical changes that take place in particular, defined learning situations. Many such studies have made use of invertebrate preparations because of their relative simplicity compared with the CNS of vertebrates. It has been disputed, however, that invertebrates exhibit true learning.

4.1.2. The use of invertebrates as preparations for behavioural studies

Invertebrates exhibit a wide range of behavioural responses including complex associative learning. The subject has been reviewed by McConnell (1966) and Eisenstein (1967). Simple behavioural responses are exhibited by non-cellular animals such as *Spirostomúm ambiguum*. Osborn, Blair, Thomas and Eisenstein (1973) report that this organism exhibits habituation of bodily contraction in response to mechanical vibration

(habituation is defined as a decrease in the response of an organism to a monotonously repeated stimulus). Gelber (1965) claims that Paramecia exhibit associative learning in response to a stimulus involving the presentation of bacteria on a wire loop as food.

The platyhelminthes are the first phylogenetic group which have been used for neurochemical studies of learning. Their neurones, however, are too fine to permit electrophysiological studies. Thompson and McConnell (1955) report that planarians exhibit classical conditioning behaviour in response to light as the conditioned stimulus and electric shock as the unconditioned stimulus. Best (1965) suggests that planarians also exhibit complex associative learning. Though neurochemical studies have been carried out on Planaria subjected to conditioning behaviour the repeatability of the results has been disputed (see review by Eisenstein).

The arthropods and molluscs provide more viable systems for electrophysiological studies. For example, Kandel and Spencer (1968) describe the use of Aplysia to study electrophysiological models of learning. Both homosynaptic and heterosynaptic facilitation have been observed to occur in this organism, the heterosynaptic facilitation lasting up to 90 mins. after stimulation.

Two other molluscan systems are of particular interest. The first is the snail, Physa. Wells (1973) reports that under no conditions yet tried does this organism show any associative or conditioned learning responses. It relies only on habituation or sensitisation which it exhibits to a number of responses such as light or electric shock. This is in contrast to the results of Emson (1971) who reports that Helix aspersa exhibits classical type two conditioning in response to electric shock received through the

tentacle. The second is the octopus which readily exhibits complex associative learning in response to a punishment/reward situation (Young, 1961). These differences in the complexity of behaviour of the two molluscs are interesting in view of their differences in life style. Young has also postulated the basic neural circuit involved in the acquisition of complex associative learning by the octopus which he terms the mnemon.

Two interesting behavioural systems in the arthropods have been described by Horridge. In the first system a locust or cockroach is arranged so that when it lowers its leg below a specified level it receives an electric shock. The animal learns to hold the test leg up for long periods thus avoiding the shock (Horridge, 1962, 1965). Horridge terms this type of learning postural learning. The test animal is connected to a yoke animal which acts as a control for the stressful effects of the electric shock, but does not learn to associate leg position with the shock. However, the results of Kerkut, Oliver, Rick and Walker (1970) suggest that the yoke animal exhibits a learning response, namely not to associate leg position with shock. Other criticisms of procedures which involve the use of yoke control animals are presented in the discussion.

The second arthropod preparation involves learning of visual discrimination by the crab Carcinus maenas (Horridge, 1966). The crab is placed in a visual field consisting of vertical black and white stripes. The animal orientates itself with respect to these stripes. If the light is switched off and the drum on which the stripes are supported is rotated when the light is restored the animal will learn to move its eyes in relation to the distance the drum is rotated. This response is termed the opto-kinetic response.

There has been considerable dispute as to whether invertebrate preparations exhibit learning in the same sense of the word as is implied when considering learning in vertebrate preparations. Wells (1965) states that "the octopus exhibits learning in whatever sense one considers the word". Even if the other invertebrate preparations discussed are not considered to exhibit true learning it is clear that several preparations exhibit responses that are useful as analogues of learning in more complex systems. For example, the leg-lifting response of insects may represent the simplest form of learning involving modification of a simple reflex arc.

4.1.3. The value of invertebrate preparations in the study of learning and behaviour

A number of properties of invertebrate nervous systems make them particularly valuable in the study of the mechanisms by which a particular behavioural response is acquired and retained. These include the following:

1) The invertebrate CNS contains relatively few neurones compared with the mammalian cerebral cortex, for example. This results in a considerable simplification of the system being studied. Even the simplest behavioural response acquired by an organism, however, involves a system of considerable complexity.

2) The simplicity of the invertebrate nervous system makes it relatively easy to map the neurones and their interconnections and to define their functions. Thus it may be possible to identify the individual neurones which are involved in the acquisition and retention of a particular behavioural response.

3) The organisation of the invertebrate nervous system into ganglia allows easy dissection of a small portion of nerve tissue, a large part of which may be involved in the acquisition of the response being studied. This is of great value in neurochemical studies.

4) A number of invertebrates, particularly molluscs, have large neurone cell bodies which readily lend themselves to electrophysiological studies. Many of these cells are identifiable and are thus easily studied from animal to animal.

4.1.4. Neurochemical approaches to learning and behaviour

In order to elucidate the underlying mechanism of a given behavioural response it is necessary to integrate the results from a number of different approaches and synthesise them into a testable model. Thus neurochemical changes which take place during acquisition and retention of a behavioural response must be integrated with the neurophysiological changes which also take place.

Katz and Halstead (1950) made the first proposal of a system of biochemical changes to account for learning and memory. Their hypothesis implicated RNA and protein synthesis in the laying down of the memory trace. The subject has been reviewed by Glassman (1969). Many studies have involved the use of drugs which block RNA and protein synthesis (see review by Agranoff, 1967). The results, though complex, indicate that drugs which block protein synthesis do not affect short term memory formation, but prevent the formation of long term memory. Drugs which block RNA synthesis markedly impair retention of a behavioural response when administered immediately after the training procedure. The effect of these agents on

leg-lifting behaviour in P. americana have been investigated by Brown and Noble (1968) and Kerkut, Oliver, Rick and Walker (1970). Both groups of workers report that these agents inhibit learning of the shock avoidance behaviour. Glassman, Henderson, Cordle, Moon and Wilson (1969) suggest that this is due to the effect of the drugs on the performance of the animals.

Another approach has been to investigate the incorporation of labelled precursors into protein and RNA during various behavioural and learning situations (see review by Glassman). A major drawback to many such studies is that the apparent increase in precursor incorporation into macromolecules is an artefact caused by an increase in the size of the precursor pool in the test animal. These problems have been overcome by Hyden and Lange (1970) and by Richardson and Rose (1973). Hyden and Lange report a correlation between the level of the brain specific S-100 protein in the hippocampus and transfer of handedness in rats. Richardson and Rose report an increase in the incorporation of amino acids into specific cortical proteins in rats in response to first exposure to light. The functions of these proteins arenot yet known.

The other major neurochemical approach to the changes that take place during learning has been to investigate possible changes in neurotransmitter systems. These studies are related to the suggestion that learning may involve synaptic facilitation/inhibition. A limited amount of evidence for synaptic facilitation exists. The work of Kandel on Aplysia has already been mentioned. Bliss and Lomo (1973) have demonstrated long lasting potentiation in the dentate area of the rabbit brain following stimulation of the perforant pathway. The potentiation lasted for 30 mins. to 10 hrs.

Physical enlargement of cat spinal cord synapses in response to repetitive stimulation of a single posterior root has been reported by Illis (1969).

This approach has been used to implicate changes in AChE activity with behavioural changes both in vertebrates and invertebrates.

4.1.5. Evidence that AChE activity changes in response to behavioural and environmental stimuli

The first direct evidence that AChE activity in the CNS could change in response to experience was provided by Krech, Rosenzweig and Bennett (1960). They demonstrated small, but significant increases in measurable AChE and BuChE activity in the brains of rats exposed to an experience rich environment as compared with the brains of rats placed in an impoverished environment. Subsequently Mollgaard, Diamond, Bennett, Rosenzweig and Lindner (1971) showed significant changes in the size and number of synapses between the experience enriched and impoverished groups of rats. They calculated that the total area of synaptic contact was 40% greater in the experience enriched rats. The results of Krech, Rosenzweig and Bennett have been confirmed by La Torre (1968) using two different strains of mice.

The majority of subsequent studies have involved the administration of anticholinesterase agents to animals and the observation of their effect on the behaviour of the animal. For example, the studies of Russell (1954), Glow and Rose (1965) and Deutsch and Liebowitz (1966) all infer that ChE activity is important in the acquisition and retention of behaviour. However, the results of such studies must be treated with caution as

anticholinesterases would be expected to have widespread, non-specific effects which may result in changes in patterns of behaviour. Deutsch (1971) has extended his studies to other components of the cholinergic system. He concludes that the cholinergic synapse is modified as a result of learning, the exact locus of the change being the sensitivity of the post-synaptic membrane to ACh which increases during memory formation and gradually declines during forgetting.

Several other studies directly implicate changes in AChE activity with behavioural changes in vertebrates. Aleksidze and Balavadze (1971) report both specific and non-specific changes in AChE activity in regions of the cerebral cortex of rats trained to reach food with the unpreferred paw. Vernadakis and Rutledge (1973) find changes in the activity of rat brain AChE and BuChE in response to pentobarbital and ether anaesthesia. Durkin and Kerkut (1974) also find changes in AChE activity in several regions of the rat brain in response to a variety of treatments including pentobarbital anaesthesia, non-specific electrical stimulation and two-way shock avoidance learning. Rose (1974) reports changes in the AChE activity of the forebrain roof of chicks trained to imprint on a flashing light.

There is also evidence which suggests that AChE activity can alter in invertebrates in response to various behavioural paradigms. Kerkut, Oliver, Rick and Walker (1970) report a decrease of the order of 50% in AChE activity in the metathoracic ganglion of the cockroach in response to shock avoidance learning (the metathoracic leg being used as the test leg). They have correlated the return of the enzyme activity to normal with the "forgetting" of the response. Woodson, Schlapfer and Barondes (1972) and Willner and Mellanby (1974) have been unable to repeat these results.

Emson (1971) has reported an increase in the AChE activity of the brain of Helix aspersa in response to a similar learning situation. The shock is received by the animal in response to extension of the tentacle.

Several workers have demonstrated rapid changes in AChE activity in response to a variety of natural conditions in both vertebrates and invertebrates. Perhaps the most striking demonstration of this is provided by the results of Oba, Ota and Yokoyama (1971) who report a rapid decrease and restoration of AChE activity in the rat hypothalamus in response to suckling. The decrease in AChE activity was observed after only 2 mins. of suckling and returned to normal within 15 mins.

An interesting adaptation of AChE in the trout brain in response to thermal acclimatization has been reported by Baldwin and Hochachka (1969). They find that a different isozyme of AChE is present in the brain of trout kept at $2^{o}C$ and $17^{o}C$. At temperatures intermediate between these values both isozymes are present. The Km of each isozyme is lowest at the temperature at which that isozyme is induced. They conclude that in many acclimatization responses new enzyme variants are synthesised which are better adapted to their catalytic function under the new conditions.

In the invertebrates Cymborowski, Skangiel-Kramska and Dutkowski (1970) report a correlation of AChE activity with circadian rhythm in the house cricket, Acheta domesticus Their results indicate a sharp 40% decrease in AChE activity exactly coincident with the animals peak locomotor activity (approximately the first three hours of exposure to dark).

Van der Kloot (1955) found that the ChE level in the brain of the silkworm fell rapidly during the larval-pupal moult. The loss of ChE activity was accompanied by a loss of spontaneous nervous activity in the brain which

became electrically inexcitable. Following diapause the ChE activity slowly increased followed by a return of electrical activity. These effects were specific for the brain and absent in the nerve cord.

Murali-Mohan and Murali K-Dass (1969) report that AChE activity decreased in the Indian apple snail, Pila globosa, during aestivation. This change was accompanied by a decrease in the electrical activity in the nervous system of the aestivating snails.

Thus there is considerable evidence that AChE activity can alter in response to behavioural and environmental stimuli. The functional significance of these changes in the activity of the enzyme and the mechanism(s) by which they occur are not understood at present.*

4.2. Plan of experiments

The experiments in this section were designed to investigate the nature of the decrease in AChE activity in response to shock avoidance learning reported by Kerkut et al. (1970). The results obtained, together with those of other workers, suggested that animals can acquire and retain the shock avoidance response without exhibiting concommitant changes in AChE activity. Some of the possible reasons for this discrepancy were investigated. These included the effects of environmental factors on AChE activity present in the metathoracic ganglion of Periplaneta americana. The conditions studied were ambient temperature and light/dark.

* A note on the rationale for studying the activity of AChE in relation to shock avoidance learning is presented on page 139a.

4.3. Methods

4.3.1. Training of the animals

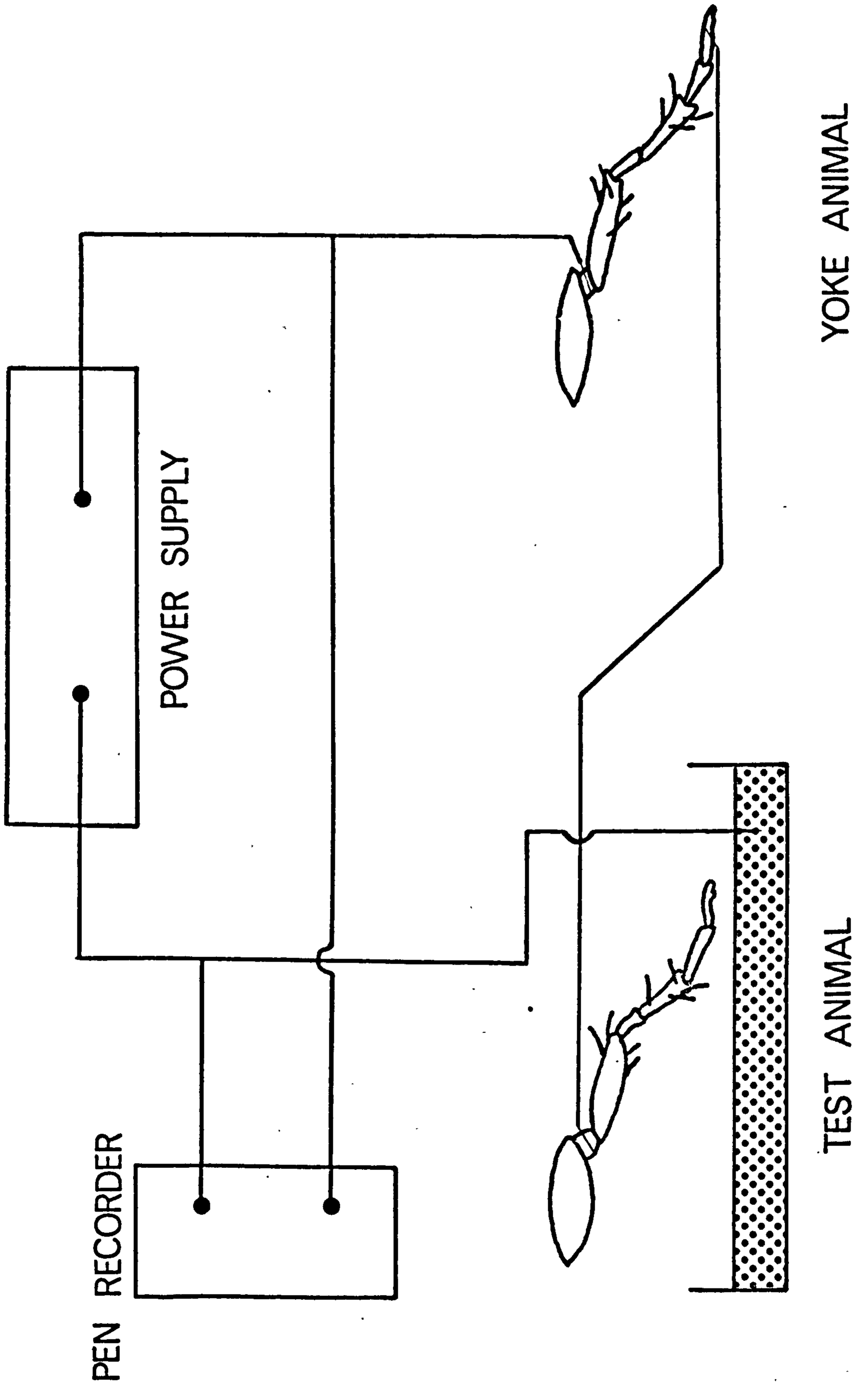
The method used was essentially the same as that described by Kerkut et al. (1970). Headless, adult, male cockroaches were attached to a horizontal sheet of Perspex by means of an elastic band placed between the pro- and mesothoracic segments of the animals. All the legs except the test metathoracic leg had previously been removed from each animal at the coxal joint. The abdomen of each animal was supported by a cotton loop so that it remained in contact with the Perspex and did not dip into the bath of saline. Both the yoke and test animals were attached to the same sheet of Perspex. The test animal was wired in series to the yoke animal and to a Grass SD9 stimulator. The stimulator was set to deliver 100 volt pulses of 2 msec. duration at a frequency of 1 stimulus/sec. The experimental animal was placed over a bath of saline so that its leg, when held freely, dipped into the saline. The height of the animal was adjusted so that only its tarsus was covered by the saline. The circuit was wired so that the test animal received the 100 volt pulses supplied by the stimulator during the times it dipped its leg into the saline. The number of shocks received by the test animal was recorded on a George Washington 400 MD2 pen recorder. The details of the circuit are illustrated in fig. 32. The yoke animal received a shock each time the test animal did regardless of the position of its own leg. In this way it was possible to distinguish the stressful effects of the electric shock from the effects due to acquisition of the shock avoidance behaviour.

The following precautions were taken prior to commencing the training procedure:

The rationale for studying the activity of AChE in relation to the shock avoidance learning paradigm is that a change in the activity of the enzyme at a cholinergic synapse could alter the efficiency of synaptic transmission. For example, if we consider a single cholinergic synapse in the cockroach metathoracic ganglion at which a constant amount of ACh is released into the synaptic cleft on arrival of one action potential at the pre-synaptic nerve terminal then a change in the activity of the synaptic AChE could have two effects on synaptic transmission. If the level of ACh in the synaptic cleft is low and the activity of AChE is decreased then the efficiency of synaptic transmission will be potentiated as the decreased rate of hydrolysis of ACh will enable the level of the transmitter to remain sufficiently high to trigger a response in the post-synaptic neurone. If, however, the level of ACh present in the synaptic cleft is high and AChE activity is decreased the level of ACh will remain sufficiently high to produce a prolonged post-synaptic event, i.e., a depolarising block will be observed. Thus a change in the activity of AChE due to shock avoidance learning could result in a change in the efficiency of synaptic transmission in the metathoracic ganglion and could thus be an integral part of the biochemical changes underlying the learning behaviour. Evidence that such mechanisms may be involved in learning in the mammalian CNS is provided by Deutsch (1971) and is also discussed by Biederman (1974).

FIG. 32. Diagram of the test circuit for shock avoidance learning

The animals were connected into the circuit shown with fine silver wire which was tied in loops aroumd the legs as shown in the diagram. The test or P animal received 1 shock/sec. during the times it dipped its leg into the saline and was thus able to associate leg position with shock. The yoke or R animal received a shock each time the test animal did regardless of the position of its own leg. Thus it received the same number of shocks as the test animal without being able to associate them with leg position.


YOKE AND ANIMAL
POWER SUPPLY
ANIMAL
TEST
PEN RECORDER

1) The legs of both yoke and test animals were connected into the circuit by fine silver wire. The junctions between the wires and the leg of each animal were moistened with a mixture of glycerol/saline to ensure good electrical contact throughout the training procedure.

2) The apparatus was carefully adjusted so that the test animal could not avoid dipping its leg into the saline, for example, by resting it on the elastic band used to attach the animal to the Perspex.

3) Initially on contact with the saline the test animal withdrew its leg even though it received no electric shock. To eliminate this effect the test animal was placed over the bath of saline with the circuit switched off for a period of 15 mins. During this period the animal overcame its aversion to the saline and allowed its leg to dip freely into the saline.

Subsequent to these procedures the circuit was switched on and the animals were trained for a period of 35 mins. It was not possible to train the animals to a criterion as their performance varied considerably (particularly between batches of animals). Horridge (1962) was also unable to train animals to a criterion, though Oliver (1970) claims that he could train animals to a criterion of receiving less than four shocks in a 3 min. period. This point is discussed further in the results though it is clear that during the period of training used in this investigation the animal had reached the peak of its performance.

Following the training procedure the metathoracic ganglia from both yoke and test animals were removed and stored separately, unhomogenised in ice cold buffer. The ganglia were only homogenised immediately prior to assay of AChE activity. The AChE activity and protein content of each ganglion was estimated as previously described.

Occasionally animals were subject to re-test 1 hr. after the initial training period to check for retention of the behaviour. These animals were also tested for AChE activity.

The animals used were stored at normal laboratory temperatures (18-20^{o}C) under conditions of constant light. They were kept well supplied with water and also with dried milk and ground rat food as nutrients. The animals survived well under these conditions.

4.3.2. Control of environmental conditions

The two environmental conditions of the animals which were controlled were light/dark and ambient temperature. For the first condition the animals were exposed to continuous bright light or complete darkness for a period of at least 24 hrs. prior to sacrifice. Both groups of animals were kept in a constant temperature room (30^{o}C). This controlled for the possible effect any variation in ambient temperature might have had on the measured AChE activity in the two groups of animals.

For the second condition the ambient temperature was varied by keeping the animals in constant temperature rooms or by suspending the container in which the animals were stored in a water bath set at the required temperature. The animals were stored at temperatures of 16, 23, 30 and 37^{o}C. All the groups of animals were kept under conditions of bright light and were kept at the appropriate ambient temperature for at least 24 hrs. prior to sacrifice.

In certain of the experiments described in this section the values of AChE were not corrected for decay of activity, but in these cases the AChE activity was determined immediately following homogenisation.

4.4. Results

4.4.1. Acquisition and retention of the shock avoidance response by Periplaneta americana

The typical learning behaviour of groups of animals is illustrated in figs. 33-35. Fig. 33 shows a composite learning curve for a group of nine animals. There is a sharp decrease in the average number of shocks received/animal/3 min. period during the first 15 mins. of the training procedure. This is followed by a rise and then another fall in the number of shocks received/animal/3 min. period. The temporary deterioration in the performance of the animals has also been reported by Oliver (1970). The final number of shocks received/animal/3 min. period was 20. It was not possible to reduce this number to the criterion proposed by Oliver (1970). The probable reason for this is that the performance of the animals varies considerably. This point is illustrated in fig. 35. Thus only in some cases did the animal reach a criterion of less than 4 shocks received/3 min. period. Some animals showed no acquisition of the response at all. Such animals were discarded as were animals which acquired the response in less than 10 mins. (these animals took less than 100 shocks to acquire the response and received very few shocks thereafter). Because of this variation in performance the animals were all trained for a period of 35 mins. Any animals which received more than 20 shocks/3 min. period at the end of this time were discarded.

Figs. 34 and 35 show typical learning curves from four animals and illustrate two points. The first is that the animals not only acquire the response, but also retain it. In figs. 34a and b the animals were left suspended over the saline for 1 hr. after training with the stimulator

FIG. 33. Composite learning curve for the headless cockroach

The graph shows a composite learning curve for a group of nine animals. The average number of shocks received/ animal/3 min. period is plotted against the time for which the animals were subjected to the training procedure. The average number of shocks received by the animals dropped from over 100/3 min. period to less than 20/3 min. period after the training had proceeded for 27 mins. Note the deterioration in the performance of the animals between 15-21 mins. after the training procedure was commenced.

The animals were suspended over the bath of saline for 15 mins. before the circuit was switched on.

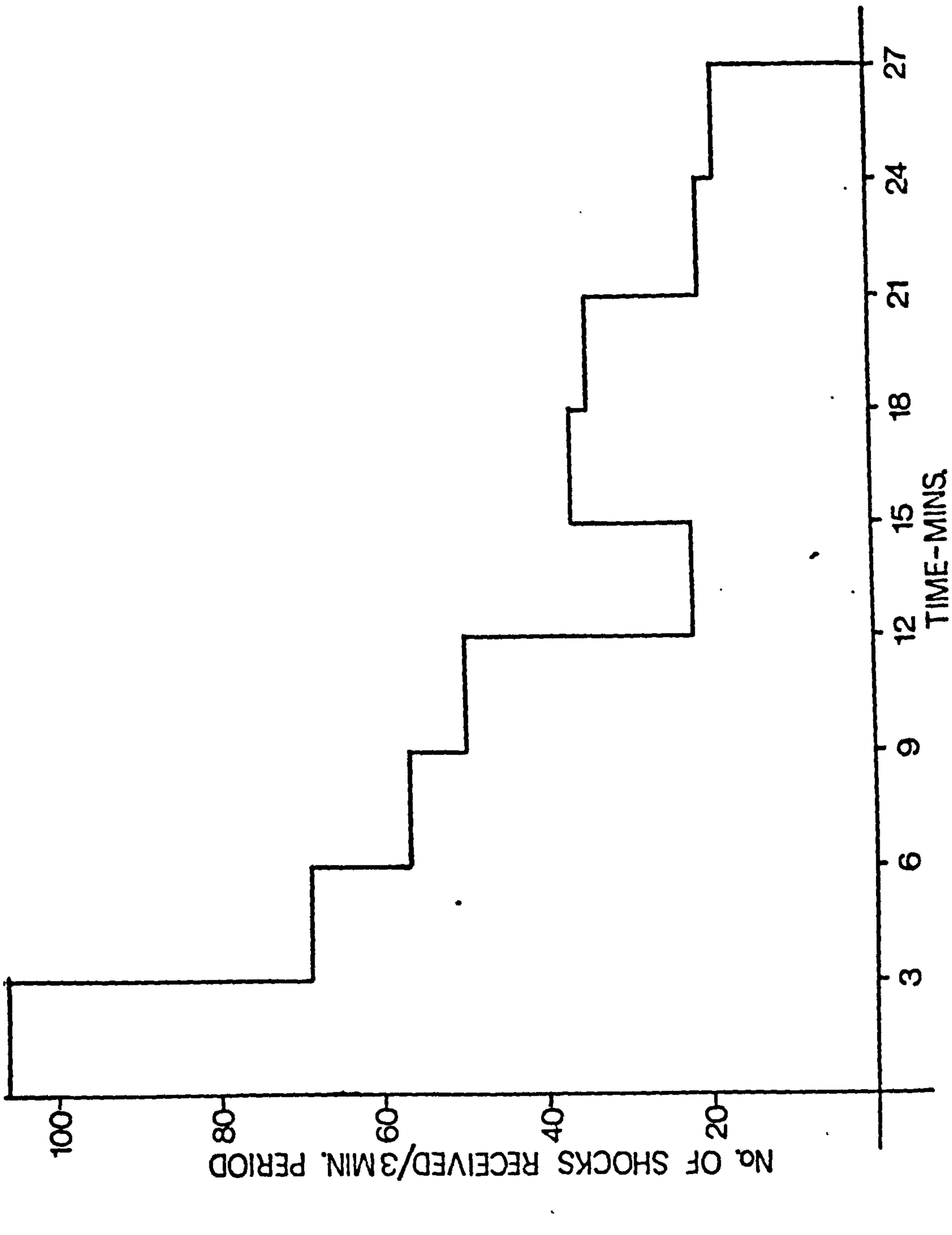
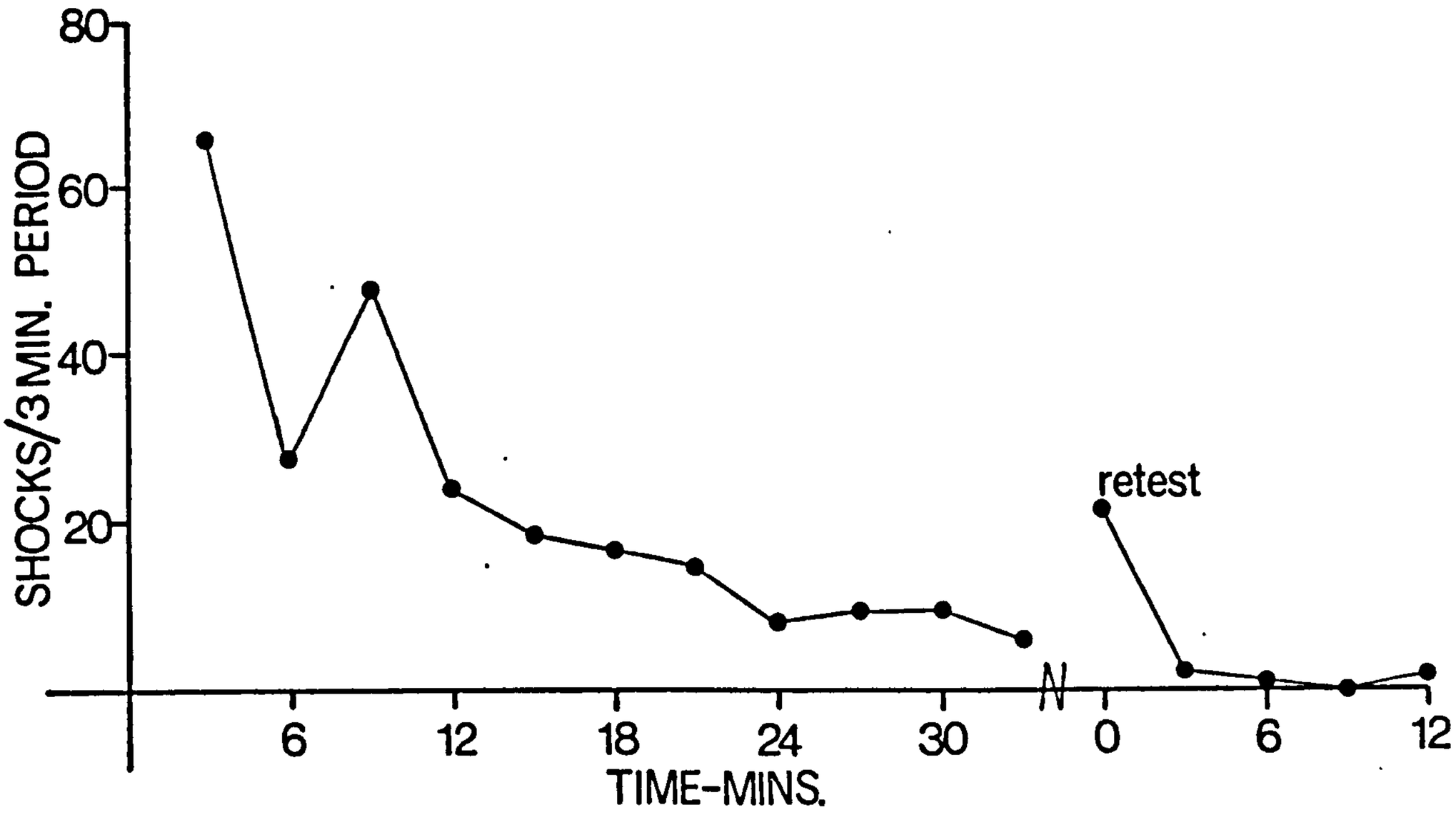

No. OF SHOCKS RECEIVED/3 MIN. PERIOD
100
80
60
40
20
TIME-MINS.
3
6
9
12
15
18
21
24
27

FIG. 34. Two typical learning curves to illustrate retention of the shock avoidance response

Figs. 34a and b show two typical learning curves for headless cockroaches. In both cases the stimulator was switched off after the animal had been trained for a period of 36 mins. At the end of this period the animals were receiving only 10 shocks/3 min. period. The test animals were left suspended over the bath of saline for a further hour. During this time the response was extinguished. The animals were then re-tested and showed rapid recall of the shock avoidance behaviour quickly receiving less than 5 shocks/3 min. period. Thus the animals used in these experiments showed good retention of the response.


80
60
40
20
SHOCKS/3MIN. PERIOD
6
12
18
24
30
N
0
6
12
retest
TIME-MINS.

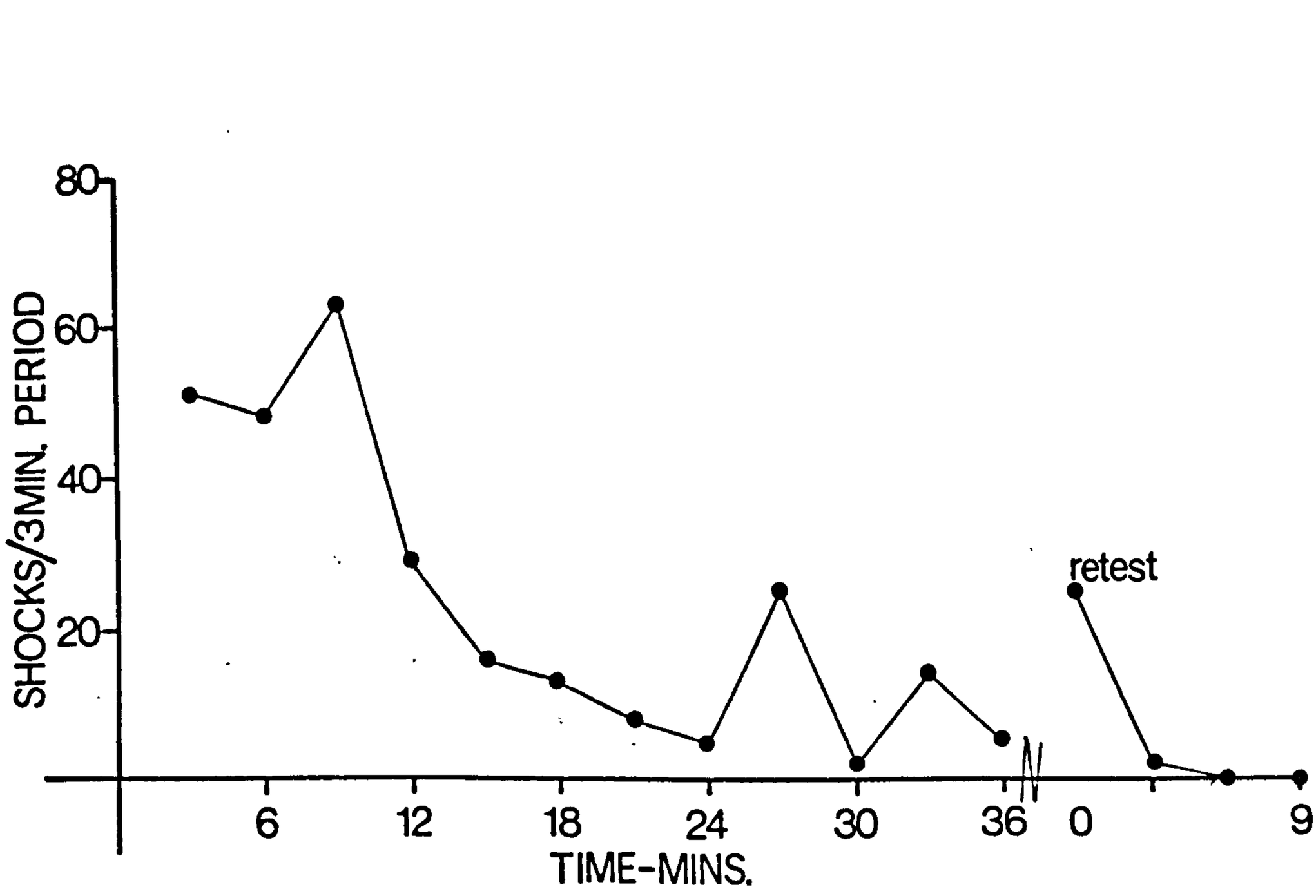
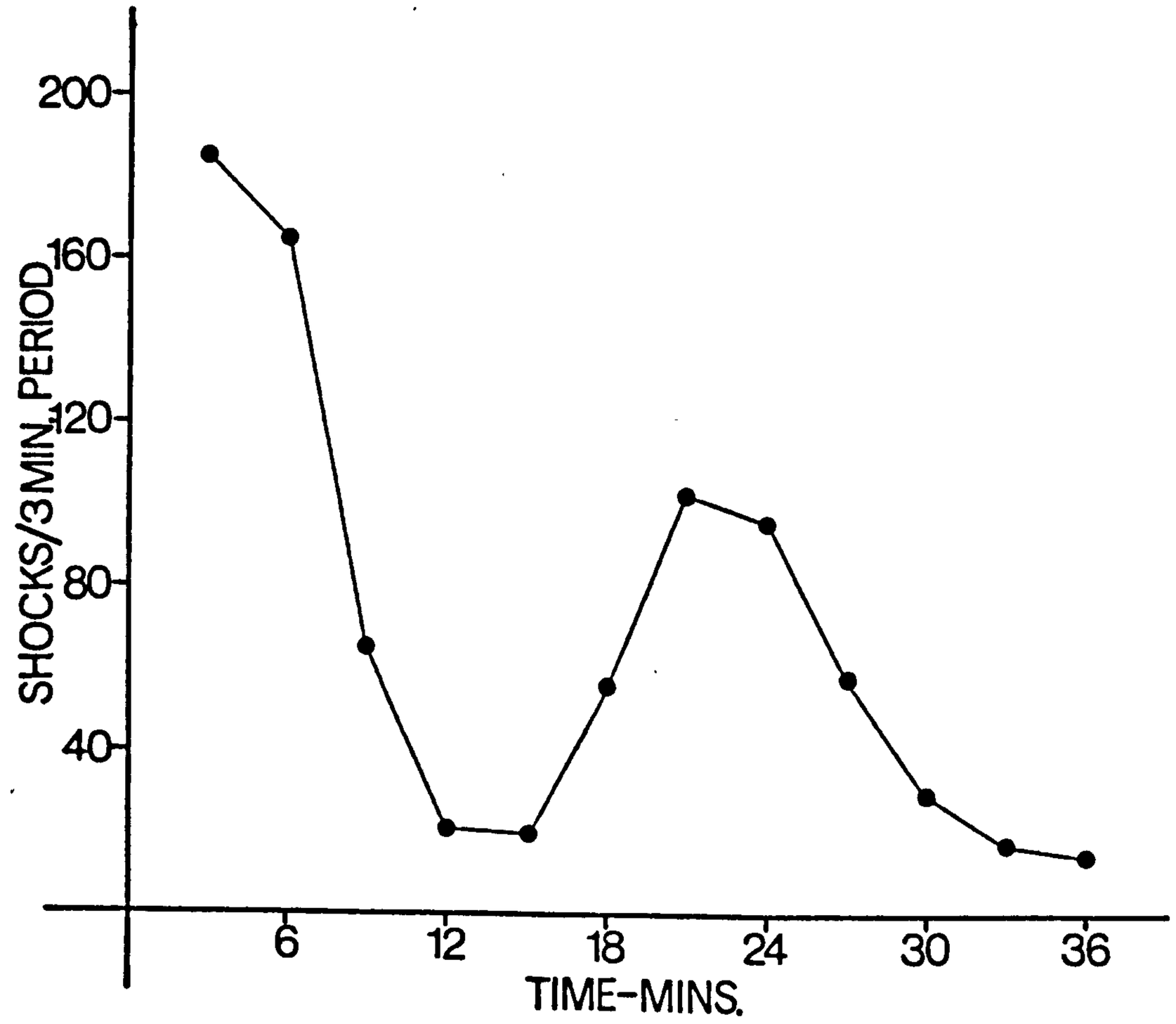
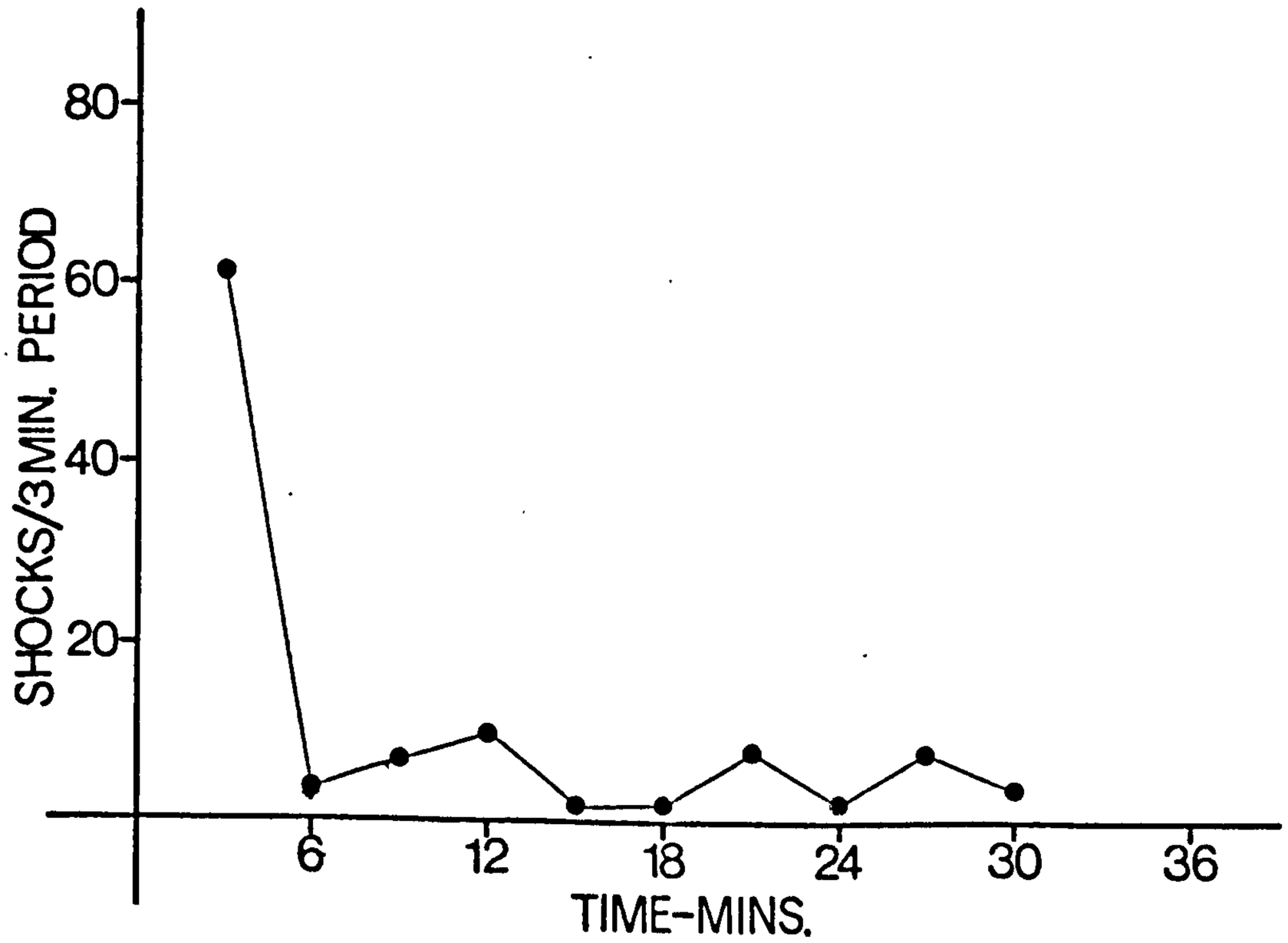

80
60
40
20
SHOCKS/3MIN. PERIOD
6
12
18
24
30
36
0
9
retest
TIME-MINS.

FIG. 35. Learning curves for two headless cockroaches to show the variability of the preparation

Figs. 35a and b show the number of shocks received by the animal/3 min. period plotted against the time for which the animal was trained. The animal used in fig. 35a received over 900 shocks before it showed good acquisition of the response, whereas the animal used in fig. 35b received less than 100 shocks before it showed good acquisition of the shock avoidance behaviour. These animals could be termed low and high activity animals and any population of cockroaches would be expected to contain a distribution of animals of varying activities as judged by their performance in acquiring the shock avoidance behaviour.


200
160
120
80
40
SHOCKS/3 MIN. PERIOD
6
12
18
24
30
36
TIME-MINS.


80
60
40
20
SHOCKS/3 MIN. PERIOD
6
12
18
24
30
36
TIME-MINS.

switched off. During this period the response was extinguished. The animals were then re-tested. They required only 20-30 shocks to reach criterion compared with the 200-300 shocks required initially. The second point is the variability in the response of the test animals as illustrated in figs. 35a and b. In fig. 35a the animal received over 900 shocks before it acquired the response whereas in fig. 35b the animal received only of the order of 100 shocks before it acquired the response. Both these animals were trained on the same day. In both figs. 34 and 35, however, all of the animals received a very low number of shocks at the end of the training period (5-10/3 min. period). These results suggest that in a given group of animals there is a distribution with respect to activity. High activity animals would acquire the response quickly whereas low activity animals would acquire the response slowly or not at all. In addition to the distribution of high/low activity animals in any population of P. americana a number of other factors may affect the performance of the animals (see discussion pages 168-176).

4.4.2. A critical investigation of the role of AChE in shock avoidance learning

The first experiment carried out to confirm the suggestion of Kerkut et al. (1970) that AChE is a biochemical correlate of shock avoidance learning was to investigate whether AChE activity dropped on both contra- and ipsilateral sides of the metathoracic ganglion with respect to the test leg. It has been reported that when the response is acquired by the metathoracic leg it is partially transferred to the corresponding contralateral leg and

also to the legs attached to the other thoracic segments (see Eisenstein, 1972). Kerkut et al. (1970) report a decrease in AChE activity in the pro- and mesothoracic ganglia of test animals. Therefore, if AChE is a true correlate of the behaviour its activity should decrease in both ipsilateral and contralateral sides of the test metathoracic ganglia. Animals were trained for periods of 35 mins. and subsequently their metathoracic ganglia were divided in half longitudinally. The specific activity of AChE in both halves of the ganglia was estimated (fig. 36). The results show that the specific activity of AChE decreased by 40% on both sides of the ganglia from test animals compared with the corresponding sides of the ganglia from yoke animals.

Kerkut et al. (1970) also suggest a correlation between the return of AChE activity to the normal resting level with the "forgetting" of the response. Therefore we attempted to correlate the degree of acquisition of the shock avoidance response with the % decrease in AChE activity in the test animals relative to the yoke animals. Groups of 5 animals were sacrificed after training for periods of 5, 10, 15, 20, 25 and 35 mins. The average number of shocks received by each group of animals was compared with the average % decrease in the AChE activity of each group of test animals with respect to its own group of yoke control animals. Fig. 37 shows that as the animals are trained for progressively longer periods of time and exhibit a greater acquisition of the response so the % drop in AChE activity progressively increases. There is a good correlation between the two curves. This is illustrated in fig. 38 which shows the % AChE activity of test animals relative to yoke control animals plotted against the average number of shocks received/animal. This plot shows that as the

FIG. 36. A histogram showing the specific activity of AChE in the ipsilateral and contralateral halves of the metathoracic ganglia of test and yoke animals

The metathoracic ganglia of test and yoke animals were divided in half longitudinally and the specific activity of AChE in each half of the ganglion was determined. A decrease in the specific activity of AChE of 40% was observed in both halves of the ganglia from test animals as compared with the corresponding halves of the ganglia from yoke animals ($P < 0.05$). All other differences were not significant.

(n = 5)

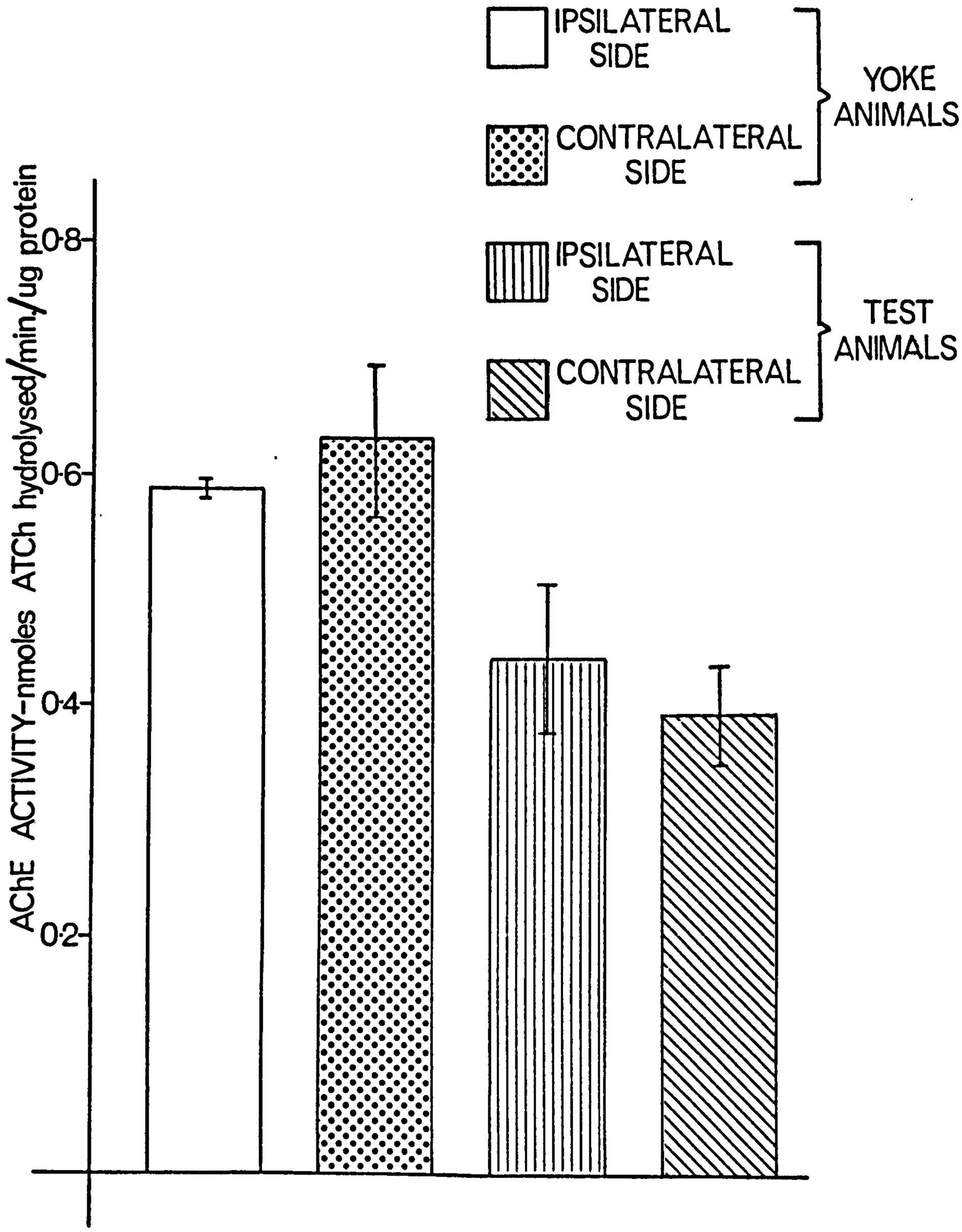

IPSILATERAL SIDE
CONTRALATERAL SIDE
YOKE ANIMALS
IPSILATERAL SIDE
CONTRALATERAL SIDE
TEST ANIMALS
AChE ACTIVITY-nmoles ATCh hydrolysed/min/ug protein
0·2
0·4
0·6
0·8

FIG. 37. Correlation of the acquisition of shock avoidance behaviour with the decrease in AChE activity in the metathoracic ganglia of headless cockroaches

Groups of 5 test and 5 yoke animals were subjected to the training procedure for periods of 5, 10, 15, 20, 25 and 35 mins. The degree of acquisition of the response was measured by the average number of shocks received by each group during this period. The animals were subsequently sacrificed and the specific activity of AChE in the metathoracic ganglia of yoke and test animals was determined. The two curves represent:

1) The average number of shocks received/group of animals plotted against the time for which the group was subjected to the training procedure.

2) The average % AChE activity in each group of test animals relative to its own group of yoke control animals plotted against the time for which the animals were subjected to the training procedure.

There is a good correlation between the two curves.

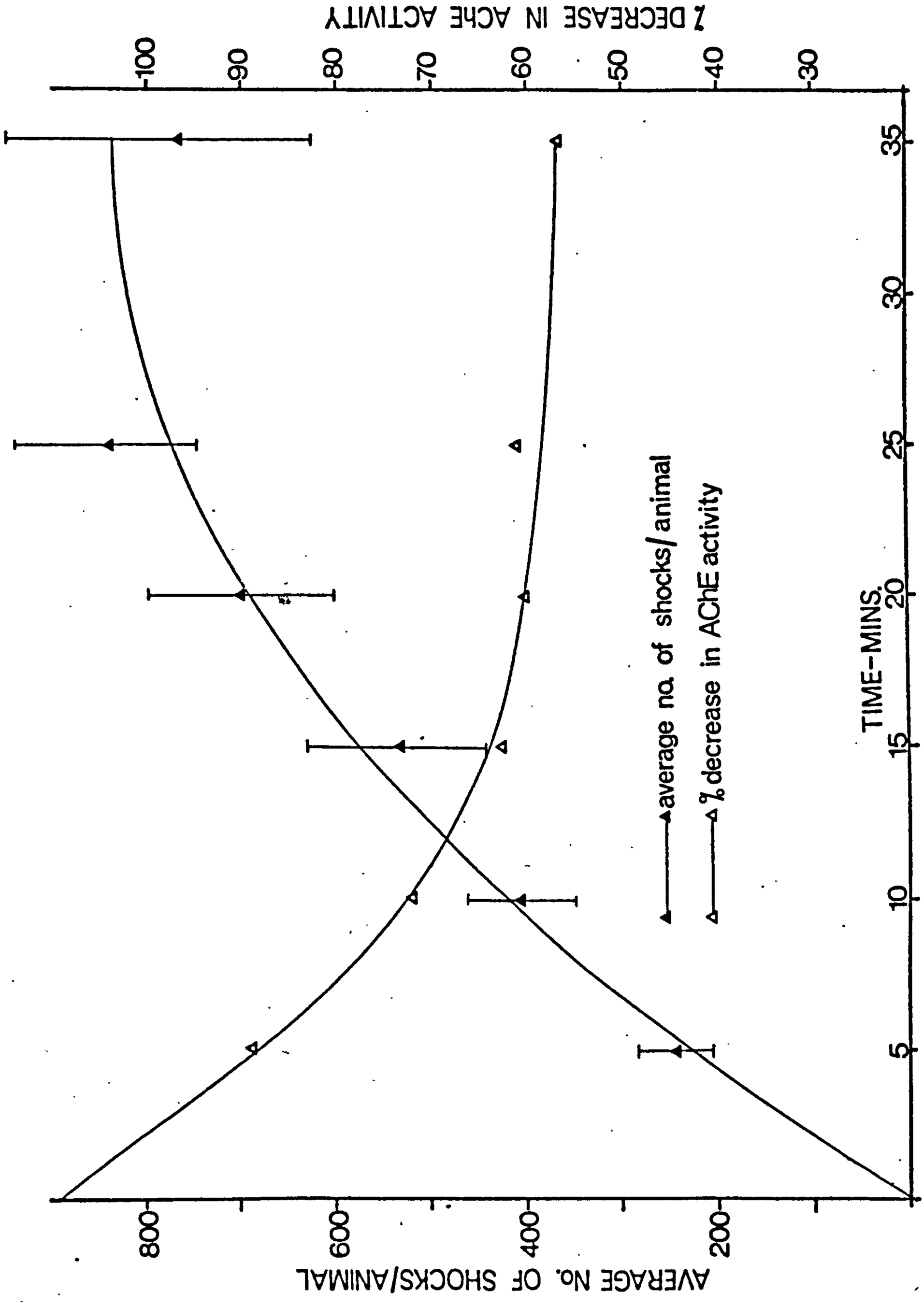
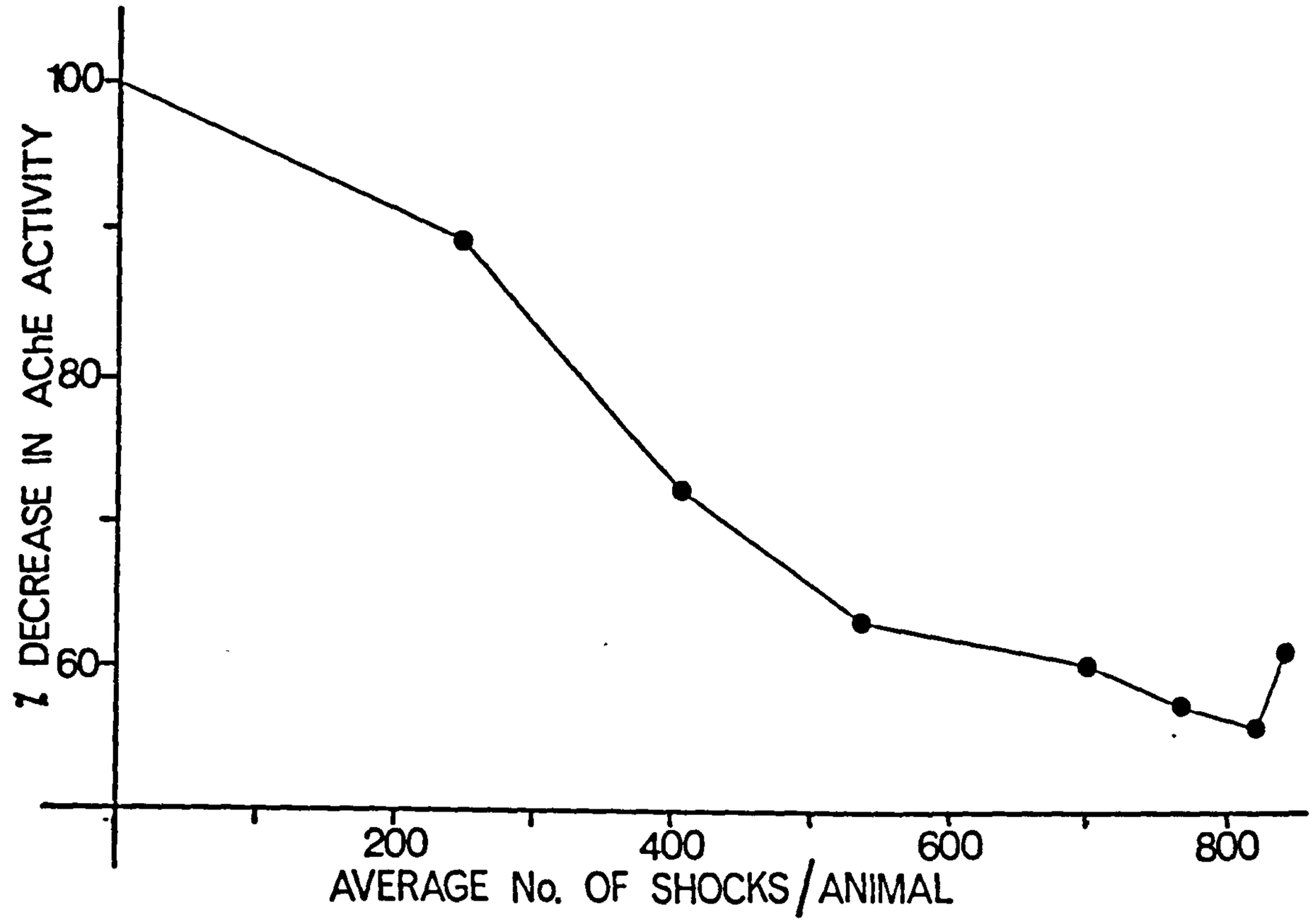

% DECREASE IN ACHE ACTIVITY
100
90
80
70
60
50
40
30
average no. of shocks/animal
% decrease in AChE activity
TIME-MINS.
5
10
15
20
25
30
35
AVERAGE No. OF SHOCKS/ANIMAL
800
600
400
200

FIG. 38. Correlation of the decrease in AChE activity in the metathoracic ganglia of test animals with the number of shocks received by the animals during the training procedure

The results from the previous experiment were re-plotted as % decrease in AChE activity in the test relative to the yoke animals against the average number of shocks received by the test animals. As the number of shocks received increases (indicating a longer period of exposure to the training procedure) so there is a larger % decrease in the AChE activity of the test animals relative to the yoke control animals.


% DECREASE IN AChE ACTIVITY
100
80
60
200
400
600
800
AVERAGE No. OF SHOCKS / ANIMAL

number of shocks received by the test animals increases (a measure of the acquisition of the shock avoidance behaviour) so the AChE activity in the metathoracic ganglia of the test relative to the yoke animals decreases. The relationship between the two parameters, however, may not be linear. Absolute values for the specific activity of AChE obtained from this type of experiment are shown in table 11.

It is concluded that these studies indicate a good correlation between the decrease in the AChE activity of the metathoracic ganglia of the test animals and the acquisition of the behavioural response.

Several approaches were adopted to investigate the mechanism of the decrease in AChE activity. A kinetic analysis of the enzyme in homogenates of the metathoracic ganglia from test and yoke animals was attempted. If these studies showed that a decrease in the Vmax of the enzyme was associated with the drop in AChE activity this would suggest that the number of active sites available was being reduced. If, however, the Km of the enzyme increased in the test animals this would indicate that the affinity of the enzyme for its substrate was being altered. The results from this type of experiment (fig. 39) show that the Km of the enzyme from the test animals was 8.14×10^{-5}M compared with a value of 6.30×10^{-5}M for the enzyme from yoke animals. The Vmax parameter of the enzyme from the test animals was 0.51 nmoles ATCh hydrolysed/min./ug prot. whereas that from the yoke animals was 0.68 nmoles ATCh hydrolysed/min./ug prot. Thus the Km of AChE from test animals had increased by 29% with respect to the Km of AChE from yoke animals whilst the Vmax of the enzyme had decreased by 25% in the test animals compared with the yoke animals.

TABLE 11. The specific activity of AChE in the metathoracic ganglia of test and yoke animals exposed to the training procedure for varying lengths of time

Groups of 5 test and 5 yoke animals were trained for periods of 5, 10, 15, 20 and 25 mins. The AChE activity in the metathoracic ganglia of the test and yoke animals was determined immediately following the training procedure. The mean specific activity of AChE (nmoles ATCh hydrolysed/min./ug prot.) in all groups of test and yoke animals is summarised in the table together with the average number of shocks received by each group of animals during the training procedure. The results are also expressed as the % difference in AChE activity between corresponding groups of test and yoke animals.

Time for which animals were trained (mins.)	Average number of shocks received/ animal	Mean specific activity of AChE (nmoles ATCh hydrolysed/min./ug prot.)		% difference test/yoke animals
		Test animals	Yoke animals	
5	175	0.216	0.208	+ 3.8
10	365	0.158	0.180	- 10
15	770	0.176	0.192	- 8.3
20	720	0.152	0.198	-23.2
25	750	0.136	0.238	-42.8

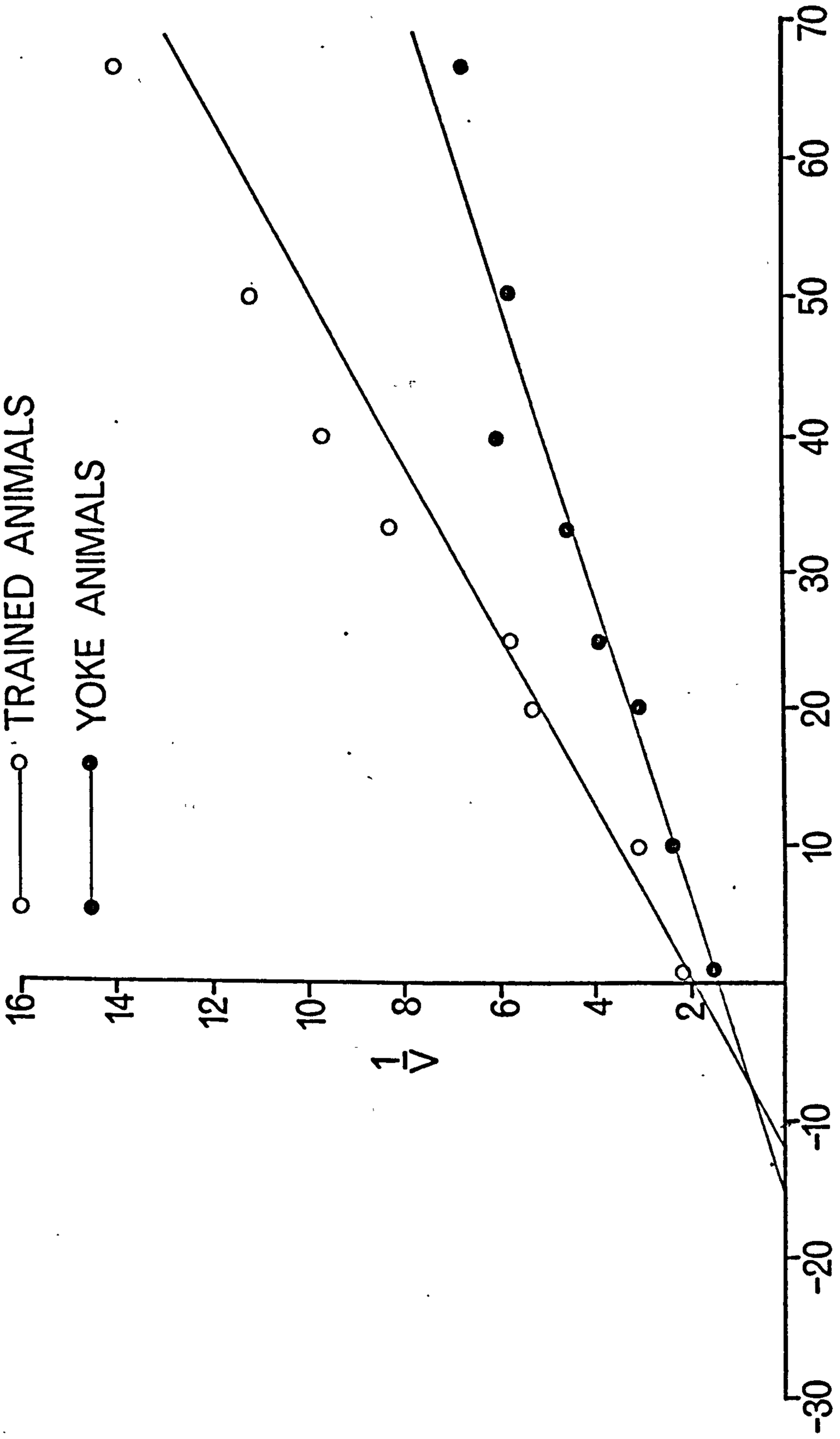
FIG. 39. Lineweaver-Burk plots for AChE from the metathoracic ganglia of test and yoke animals

The graphs show Lineweaver-Burk plots for AChE from the metathoracic ganglia of test and yoke animals. The results were obtained from total tissue homogenates not treated with detergent. The kinetic parameters of the enzyme present in each sample were calculated using a Hewlett-Packard graph plotter. The values obtained were:

	Km ($\times 10^{-5}$M)	S.E. of Km	Vmax nmoles ATCh hydrolysed/ min./ug prot.)	S.E. of Vmax
Test animals	8.14	$\pm$ 0.95	0.508	$\pm$ 0.020
Yoke animals	6.30	$\pm$ 0.46	0.684	$\pm$ 0.025

(S.E. = Standard error)

Thus the Km of the enzyme from test animals increased by 29% with respect to that of the yoke animals whilst the Vmax decreased by 25% in the test animals with respect to yoke animals.


TRAINED ANIMALS
YOKE ANIMALS
1/V
1/S mM⁻¹
16
14
12
10
8
6
4
2
-10
-20
-30
10
20
30
40
50
60
70

An initial evaluation of these results using a less accurate method of analysis indicated that the Km of the enzyme from test animals had increased by 123% with respect to the Km of the enzyme from yoke animals whereas the Vmax of the enzyme from test animals had decreased by only 10% with respect to the Vmax of the enzyme from yoke animals. This led Beesley, Emson and Kerkut (1971) to suggest that the change in the Km of the enzyme was the important factor in the acquisition of the shock avoidance behaviour. The analysis of the results presented here indicates that the original interpretation of the results is incorrect.

Two other pieces of evidence suggest that the initial interpretation of the data is incorrect. Firstly, the change in the Km of the enzyme originally reported is not large enough to account for the change in AChE' activity observed as a result of shock avoidance learning. Substitution of the appropriate Km values into the Michaelis-Menten equation reveals that at a substrate concentration of 1 mM the increase in the Km of the enzyme from test animals would result in only a 10-12% decrease in their AChE activity. This value is considerably lower than the 50% decrease in AChE activity reported by Kerkut et al. (1970). Secondly, the differences in the kinetic parameters of the enzyme from test and yoke animals parallel the changes in the kinetic properties of the enzyme observed on aging of total tissue homogenates. Even though a difference between the Km of AChE from test and yoke animals was obtained in five separate experiments it is not possible to rule out the effect that even a slight aging of the homogenates may have had on the results. Three important factors which could have contributed to the observed difference in Km are:

1) The order in which the samples from test and yoke animals were assayed (though the selection of this order was random).

2) The actual rate of decrease of AChE activity caused by aging of the sample varies markedly between samples. It is possible that the AChE activity in the samples from the test animals decayed more rapidly than that in the samples from the yoke animals.

3) At the time these experiments were carried out it was not realised that the rate of hydrolysis of ATCh was not linear under the assay conditions used. This could lead to considerable discrepancies in the measured rates of hydrolysis of ATCh.

For these reasons the initial conclusions of Beesley, Emson and Kerkut (1971) are not regarded as valid.

Other approaches directed towards elucidating the mechanism of the decrease in AChE activity associated with shock avoidance learning have been presented elsewhere (Emson, 1971). These results suggest:

1) That only AChE band 1 activity (as separated by gel electrophoresis) decreased in the metathoracic ganglia of test animals. Lambertsen (1972) was unable to repeat this type of experiment. His results never conclusively showed a decrease in AChE band 1 or band 2 activity from test animals as compared with yoke animals. It should also be noted that Lambertsen was unable to reproduce the electrophoretic pattern for AChE obtained by Emson.

2) That AChE activity increased when homogenates of metathoracic ganglia from test animals were aged for 24 hrs. This increase was not observed in similar homogenates from yoke animals. In view of the results discussed in section 2, together with the fact that aging of homogenates results in the release of AChE activity into an assayable form due to autodigestion of the tissue, this increase cannot be directly attributed to the shock avoidance training.

Thus, for the reasons explained, these investigations into the mechanism of the proposed decrease in AChE activity are not considered valid and a more critical design for experiments of this kind is desirable.

In some of the earlier experiments it was observed that test animals which had acquired the shock avoidance behaviour showed no concommitant decrease in AChE activity. Woodson, Schlapfer and Barondes (1972), Moffitt and Rick (1972) and Willner and Mellanby (1974) all report that they could find no evidence for the correlation of AChE activity with shock avoidance behaviour.

Over an eight month period from June 1972 to January 1973 a systematic attempt was made to repeat the original results which correlated a decrease in AChE activity with shock avoidance behaviour. In none of these experiments (fig. 40) was there a significant drop in the specific activity of AChE in the metathoracic ganglia from test animals as compared with yoke or resting animals. The learning curves obtained in these later experiments appeared to be the same as those obtained in the earlier experiments indicating that AChE was a biochemical correlate of shock avoidance learning. It is concluded that shock avoidance learning can occur without concommitant changes in AChE activity in the ganglion controlling movement of the test leg. *

4.4.3. The effect of environmental factors on AChE activity in the metathoracic ganglion

It is possible that many factors could cause an alteration in AChE activity in the nervous system of P. americana some of these factors may be responsible for the discrepancy in the results attempting to correlate

* A table showing that the protein content of the metathoracic ganglion did not vary between test and yoke animals is presented on page 157a.

In the experiments carried out in this section to investigate the possibility that AChE is a biochemical correlate of shock avoidance learning it is possible that either the difference or lack of difference in the specific activity of AChE between test and yoke animals could be related to rapid changes in the protein content of the metathoracic ganglia of either group of animals. The table below summarises the mean protein content of the metathoracic ganglia of animals used to obtain the data for figs. 36 and 40. The data indicates that for each experiment there was no significant difference in protein content of the meta-thoracic ganglia between groups of test and yoke animals.

a) Data relating to fig. 36.

	Mean protein content of whole metathoracic ganglion ug	s.e.m.
Yoke animals	82.5	± 6.02
Test animals	105.25	±12.04

(n = 4)

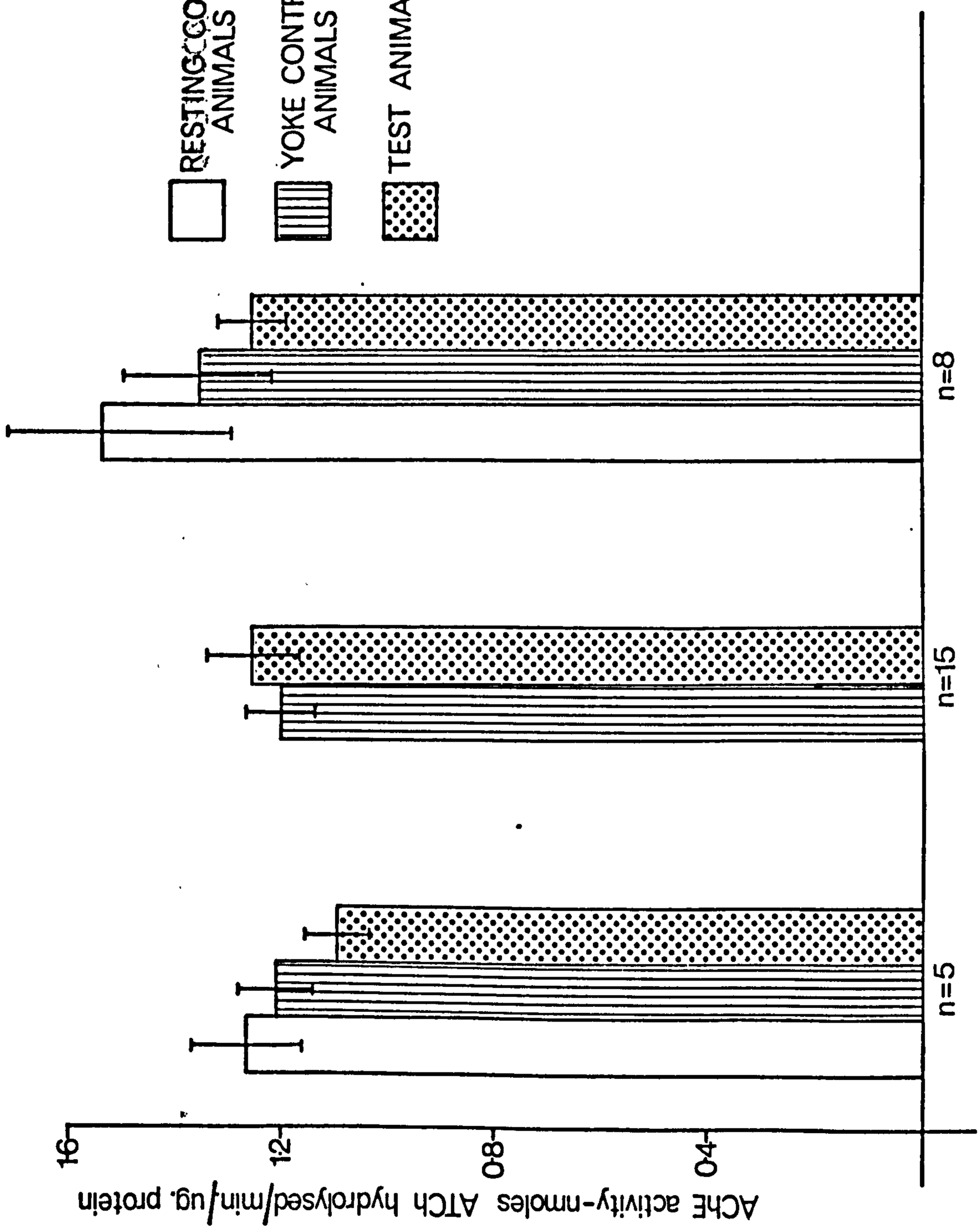
b) Data relating to fig. 40

Date of experiment	Group of animals	Mean protein content of metathoracic ganglia ug	s.e.m.
21/6/72	Test (n= 5)	98.6	±13.5
	Yoke (n= 5)	100.8	± 5.4
18/9/72	Test (n=11)	102.0	± 6.1
	Yoke (n=11)	96.7	± 6.4
8/1/73	Test (n= 8)	77.6	± 4.0
	Yoke (n= 8)	75.6	± 4.2

The mean protein content of the metathoracic ganglia of a typical group of nine quiet control animals was 85.0 ± 5.9 ug.

FIG. 40. Histogram of the specific activity of AChE in the metathoracic ganglia of test, yoke and resting animals

The histogram shows the specific activity of AChE in 3 groups of animals. All groups contain test and yoke animals and in two cases resting animals. In none of these groups was there a significant difference between the specific activity of AChE in the ganglia from test and yoke or test and resting animals. Note that the specific activity of the enzyme measured in these groups of animals is considerably higher than the values shown in fig. 36. The experiments were carried out on the 21-23/6/72, 16-18/9/72 and 8-10/1/73.


RESTING CONTROL ANIMALS
YOKE CONTROL ANIMALS
TEST ANIMALS
n=8
n=15
n=5
ACHE activity-nmoles ATCh hydrolysed/min/ug. protein
16
12
08
04

AChE activity with shock avoidance learning. Harker (1964) reports that cockroaches exhibit a circadian rhythm. Thus AChE activity in P. americana may exhibit a similar variation to the circadian rhythm of the enzyme activity observed in the house cricket by Cymborowski et al. (1970). This possibility was investigated experimentally by varying the conditions under which animals were kept.

The first condition varied was the amount of light received by animals. The specific activity of AChE in the metathoracic ganglia of groups of animals kept under conditions of constant light or constant darkness was determined. The results (table 12) show that there is no significant difference between the specific activities of AChE for the two groups of animals. Therefore it is concluded that light/dark has no effect on the specific activity of AChE in the metathoracic ganglion. This does not preclude the possibility that AChE activity might exhibit a cyclic diurnal rhythm which is suppressed under conditions of constant light and constant temperature.

The second condition varied was the ambient temperature at which the animals were kept. Results from two experiments investigating the effect of ambient temperature on the specific activity of AChE are shown in table 13 and fig. 41. The samples used in the first experiment were not treated with DOC prior to assay of AChE activity, but were assayed for enzyme activity immediately following homogenisation. The samples used in the second experiment were treated with DOC prior to assay of AChE activity. Both experiments indicate that as the ambient temperature at which the animals were kept was increased so the specific activity of AChE in the metathoracic ganglion decreased. In the second experiment (table 13b) an increase in ambient temperature from 16-37^{o}C was associated with a 43% decrease in the specific activity of AChE. An increase in ambient temperature also resulted in a marked increase in the activity of the animals. From this

TABLE 12. The effect of exposure to continuous light/dark on the specific activity of AChE in the metathoracic ganglion

Animals were exposed to continuous light or dark for at least 24 hrs. prior to sacrifice. The animals were kept at a constant temperature of 30°C. The samples were not activated with DOC, but were assayed for AChE activity immediately after homogenisation. No significant difference in the specific activity of AChE between the two groups of animals was detected.

Condition to which animals were exposed	Specific activity of AChE in the metathoracic ganglion (nmoles ATCh hydrolysed/min./ug prot.)
Light	2.294 ± 0.207
Dark	2.374 ± 0.195

(n = 8)

TABLE 13. The effect of ambient temperature on the specific activity of AChE in the metathoracic ganglion

Animals were kept at different ambient temperatures for at least 24 hrs. prior to sacrifice. All groups were kept under conditions of constant light. The samples used to obtain the data shown in table 13a were not treated with DOC, but AChE activity was assayed immediately following homogenisation. The samples used to obtain the data shown in table 13b were treated with 0.1% DOC prior to assay of AChE activity.

TABLE 13a.

Ambient temperature at which animals kept (^{o}C)	Specific activity of AChE in the metathoracic ganglion (nmoles ATCh hydrolysed/min./ug prot.)
30	1.738 ± 0.089
19	2.080 ± 0.077
10	2.174 ± 0.106

(n = 8) $P < 0.02$ between the 19^{o}C and the 30^{o}C groups of animals

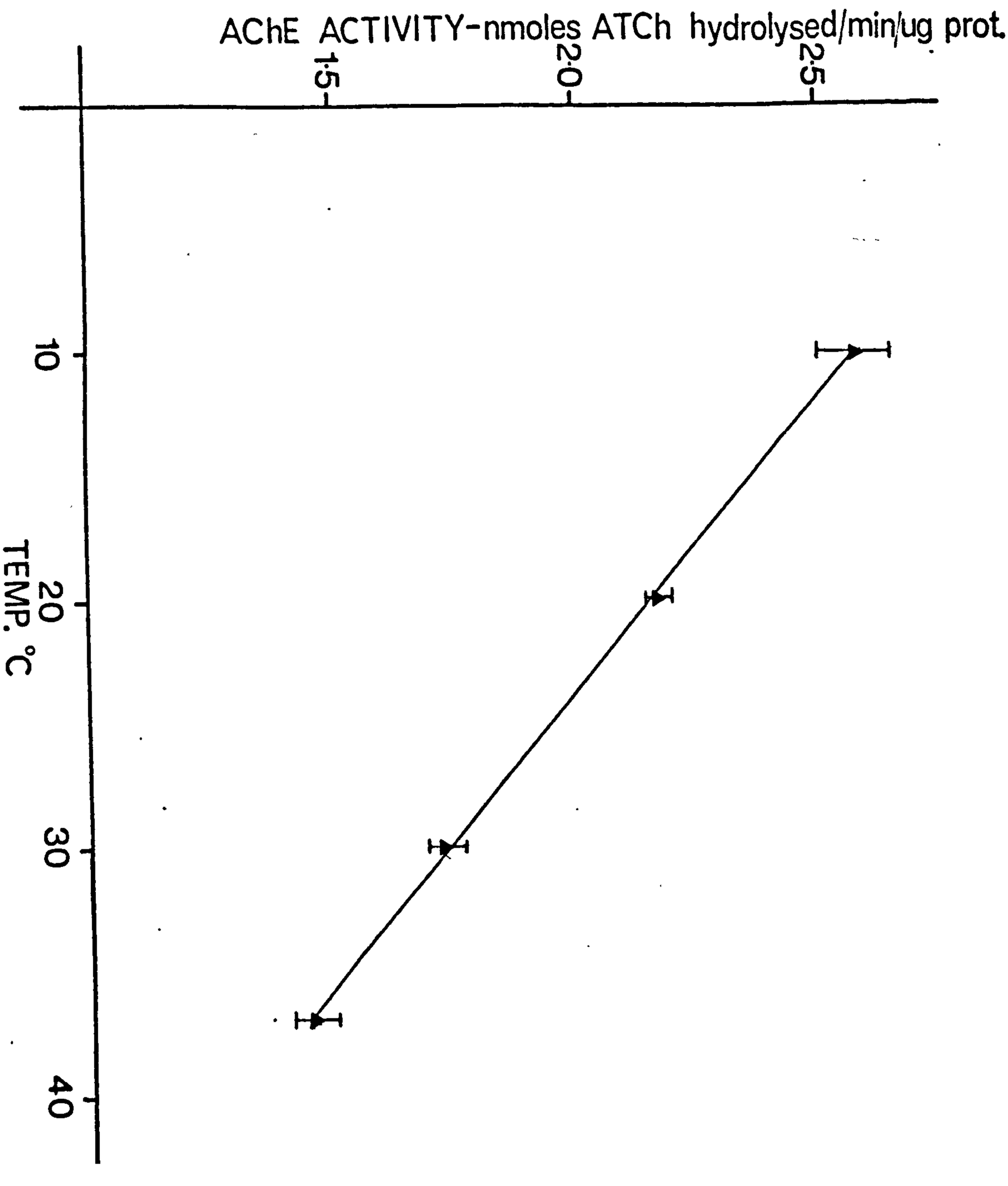
TABLE 13b.

Ambient temperature at which animals kept (^{o}C)	Specific activity of AChE in the metathoracic ganglion (nmoles ATCh hydrolysed/min./ug prot.)
37	1.460 ± 0.048
30	1.733 ± 0.021
23	2.170 ± 0.011
16	2.585 ± 0.079

(n = 6) $P < 0.001$ between the 16^{o}C and the 37^{o}C groups of animals

FIG. 41. The effect of ambient temperature on the specific activity of AChE in the metathoracic ganglion

The figure shows the specific activity of AChE in the metathoracic ganglion plotted against the ambient temperature at which the animals were kept. Animals were kept at the ambient temperatures stated under conditions of continuous light for at least 24 hrs. prior to sacrifice. Samples were treated with 0.1% DOC prior to assay of AChE activity. The results suggest a correlation between AChE activity and ambient temperature. When the ambient temperature was increased from 16°C to 37°C there was a concommitant 43% decrease in AChE activity.


AChE ACTIVITY-nmoles ATCh hydrolysed/min/ug prot.
1·5
2·0
2·5
10
20
30
40
TEMP. °C

data it is concluded that AChE activity in the metathoracic ganglion does vary with ambient temperature. These findings are only considered to be preliminary and further confirmation of the results is desirable.

4.4.4. Summary of results

1) The test animals used in these experiments learned to associate leg position with electric shock.

2) The test animals showed good retention of the response.

3) The performance of the animals varied considerably. The variation was particularly marked between different batches of animals. The animals could not be trained to the criterion suggested by Oliver (1970).

4) The initial results obtained indicated that the specific activity of AChE decreased by 40% in both ipsilateral and contralateral halves of the metathoracic ganglion with respect to the test leg.

5) The initial results obtained also indicated that as the test animals were exposed to the training procedure for increasing lengths of time and showed greater acquisition of the shock avoidance response so the decrease in AChE activity in the metathoracic ganglion of the test animals with respect to the yoke animals became larger.

6) Later results definitely showed that test animals could acquire and retain the shock avoidance behaviour without concommitant changes in the specific activity of AChE.

7) Several approaches directed towards elucidating the mechanism of the decrease in AChE activity observed as a result of shock avoidance learning are not considered valid because of the peculiar properties of cockroach AChE when present in homogenates of nerve tissue.

8) Exposure of animals to conditions of continuous light or continuous darkness had no effect on the specific activity of AChE in the metathoracic ganglia of the animals tested.

9) A rise in the ambient temperature at which animals were kept was associated with a decrease in the specific activity of AChE in the metathoracic ganglia of these animals. This finding requires further investigation.

4.5.1. Discussion

A summary of the available data from experiments attempting to correlate AChE activity with shock avoidance learning in P. americana is presented in table 14. Two features of particular interest emerge from this data:

1) There is considerable variation in the specific activity of AChE in the metathoracic ganglion of P. americana as measured by the different groups of workers. A large fraction of this variation may be accounted for by the effects discussed in section 2, i.e., the rapid decrease in measurable AChE activity in nerve tissue homogenates. This effect, which could account for a 50% variation in results depending on the time after homogenisation at which the samples were assayed, is not sufficient to account for all of the 70% variation observed. Though different methods of assaying AChE were used by several workers Woodson et al. (1972) and Willner and Mellanby (1974) used the same assay method as was used to obtain the results presented in this thesis. Other factors which could contribute to the observed variation in AChE activity are differences in the conditions under which the animals were kept and differences in the strains of animals used. Willner and Mellanby suggest that the latter

TABLE 14. Summary of data on the effect of shock avoidance learning on AChE activity in the metathoracic ganglia of test, yoke and resting animals

Source of data	AChE activity			Comments
	Resting animals	Yoke animals	Test animals	
Kerkut, Oliver, Rick and Walker (1970)	2.86 ± 0.39	2.08 ± 0.06	1.12 ± 0.06	n = 31 50% drop test/ resting animals. Results expressed as umoles HAc produced/hr./100 ug prot.
Woodson, Schlapfer and Barondes (1972)	2.058 ± 0.108	-	2.166 ± 0.06	n = 12
	7.5 ± 0.48	-	7.68 ± 0.6	radiochemical assay used
Willner and Mellanby (1974)	6.2 ± 0.25	6.1 ± 0.3	6.5 ± 0.25	n = 12 expressed as umoles ATCh hydrolysed/hr.
	3.2 ± 0.15	2.8 ± 0.25	3.2 ± 0.2	assayed as before, but samples aged 24 hrs.
Beesley, Emson and Kerkut (1971)	-	3.6 ± 0.20	2.46 ± 0.15	n = 5 See fig 3c
Beesley and Kerkut, unpublished observations	9.22 ± 1.44	7.53 ± 0.88	8.09 ± 0.36	n = 8 see fig 4c
Moffitt and Rick (1972)	They find no significant differences in AChE activities between test and yoke animals (personal communication).			

All values stated as μmoles ATCh hydrolysed/hr./100 μg prot. unless otherwise stated.

$$\frac{9.6 \times 60 \times 100}{10^3} = 3.6$$

reason may account for the difference between their results and those of Woodson et al.

2) Of all the reported results only those of Kerkut, Oliver, Rick and Walker (1970) and Kerkut, Beesley, Emson, Oliver and Walker (1971) correlate a decrease in the specific activity of AChE in the metathoracic ganglion of test animals with shock avoidance learning in these animals. It is of interest that in these two reports the specific activity of AChE in the resting animals is considerably lower than that reported in later experiments showing no such correlation. Willner and Mellanby assayed AChE activity in test, yoke and resting animals immediately after the tissue had been homogenised and also 24 hrs. later. Though the activity measured after 24 hrs. had decreased considerably neither set of measurements showed any correlation between AChE activity and shock avoidance learning.

A consideration of the data in table 14, together with the results presented in this thesis, leads to the conclusion that shock avoidance learning can take place in P. americana without concommitant changes in measurable AChE activity. As this is the case the problem is to explain the observations which suggest that AChE is a biochemical correlate of shock avoidance learning. A consideration of this data reveals the following facts:

1) The specific activity of AChE has been observed to drop in the metathoracic ganglion of test animals as compared with yoke control animals by up to 50% (Kerkut, Oliver, Rick and Walker, 1970).

2) As the test animal progressively acquires the response so the AChE activity in the metathoracic ganglion decreases with respect to the

AChE activity of the yoke control animal (Kerkut, Beesley, Emson, Oliver and Walker, 1971).

3) As the test animal "forgets" the response over a period of three days so the AChE activity in its metathoracic ganglion returns to that of the resting animal (Kerkut, Oliver, Rick and Walker, 1970).

It is difficult to dismiss these facts particularly as in the experiments of both Kerkut, Oliver, Rick and Walker and Kerkut, Beesley, Emson, Oliver and Walker the samples were assayed in a random mix. Other results obtained, however, which indicate that AChE is a correlate of shock avoidance learning are considered doubtful. Thus results obtained from a kinetic analysis of the enzyme from test and yoke animals (Beesley, Emson and Kerkut, 1971) are considered invalid due to the decrease in AChE activity which takes place in samples following homogenisation. The results obtained by Kerkut, Emson and Beesley (1972) using electrophoretically pure AChE from test and yoke animals are also considered invalid as it has not been possible subsequently to separate the same molecular species of AChE as were obtained in the original experiments. This suggests the possibility that different molecular species of AChE were present in animals used in studies which showed a correlation of AChE activity with shock avoidance learning from those present in animals used in studies which showed no such correlation. Finally the report by Emson (1971) that AChE activity in homogenates from the metathoracic ganglia of test animals increases with respect to the AChE activity in similar homogenates from yoke animals when stored for 24 hrs. may well be explained by the release of occluded enzyme into an assayable form. Therefore the increase may not be a direct result of the shock avoidance learning.

The validity of the first three points discussed indicates possible involvement of AChE in the learning response under some conditions and also the necessity for resolving the discrepancy between these results and those which suggest that AChE is not involved in the shock avoidance response. Factors which may account for this discrepancy can be considered under three headings, i.e., differences between the animals used in the experiments, differences in the conditions under which the animals used were kept and differences in the training procedure used in the different experiments.

Inherent differences between animals used

It is possible that different strains of cockroach may exhibit differences in the mechanism by which they learn the shock avoidance response. Thus, as has already been mentioned, the properties and isozymic forms of AChE may vary between strains of P. americana. Hence changes in the activity of the enzyme in response to shock avoidance learning may be observed only in strains which have a particular pattern of AChE activity. This statement would also imply that other factors are important in the acquisition and retention of the behaviour. Kerkut, Oliver, Rick and Walker (1970) report that changes in RNA and protein synthesis occur in test animals with respect to yoke animals. Oliver, Taberner, Rick and Kerkut (1971) report that changes in GABA levels and GAD activity are correlated with the learning behaviour. Thus the changes in AChE activity originally proposed only play a part in the learning behaviour in relation to changes which take place in other systems.

A second important factor is that learning may take place by different mechanisms in different strains of P. americana regardless of the characteristics of AChE, i.e., only in certain strains of cockroach will a change in AChE activity be associated with shock avoidance learning.

Miller (1970) stresses the importance of considering strain differences in cockroaches in this type of experiment. He points out that it is important to preserve genetically homogenous strains of rats to distinguish, for example, between maze-bright and maze-dull strains. Bullock (1966) has reported difficulty in repeating the work of Luco and Aranda (1964) on Blatta orientalis because of strain differences in the animals used. Individual strains of cockroaches can develop individual traits so that considerable variations in learning ability may occur between strains.

A third important factor which may account for the discrepancy in results has been pointed out by Church (1964). He states that inherent differences between the activity of the yoke animal and the activity of the test animal may result in comparisons between the two groups of animals not being valid (see fig. 42). Thus unless the yoke and test animals have similar activities a comparison between the two animals may negate any difference between them, either of a behavioural or neuro-chemical nature. These considerations lead to the desirability of using an internal control. Bateson, Rose and Horn (1973) have overcome this difficulty by using a split-brain preparation to study neurochemical changes associated with imprinting in chicks. A similar procedure could be used in the cockroach by severing the connectives between the meta- and mesothoracic ganglia, the metathoracic leg being used as the test leg

FIG. 42. Comparisons between yoke and test animals which are valid and those which may not be valid

In any population of Periplaneta americana there is a distribution of higher and lower activity animals. The higher activity animals will tend to learn faster than the lower activity animals. Comparison of neurochemical parameters between a high activity test animal and a low activity yoke animal and vice versa may not be valid as these parameters may also vary between high and low susceptibility animals.

		TEST ANIMALS	
		HIGH ACTIVITY	LOW ACTIVITY
YOKE CONTROL ANIMALS	HIGH ACTIVITY	VALID	NOT VALID
	LOW ACTIVITY	NOT VALID	VALID

and the mesothoracic leg being used as the yoke leg. Alternatively a method of selecting yoke and test animals of similar activities could be used so that a comparison between them would be valid. Differences in the activity of animals could, in part, account for the large differences in AChE activity observed in populations of P. americana.

Menn and Mcbain (1968) have suggested the occurrence of natural cholinesterase inhibitors in the German cockroach, Blatella germanica. These inhibitors could be important in accounting for differences in AChE activity between batches of cockroaches. They could also possibly be involved in any decrease in AChE activity associated with shock avoidance learning.

Differences caused by conditions under which the animals are kept

Evidence that AChE activity in invertebrates can change in response to environmental conditions has already been mentioned (Cymborowski et al., 1970; Murali-Mohan et al., 1969; Van der Kloot, 1955). Harker (1964) reviews the subject of circadian rhythms and reports the existence of a circadian rhythm in P. americana with respect to locomotor activity. This rhythm, unlike that of many other species, is abolished if the animals are kept under conditions of continuous light. Under such conditions the animals will exhibit a rhythm in response to temperature. A phase shift in the rhythm of animals kept under a regime of 12 hrs. light/12 hrs. dark can be induced by an alteration in the timing of the light/dark cycle. Such phase shifts are interpreted as being due to the interaction of two clocks, one being hormonal in nature the other being neuronal in nature. Harker has carried out a thorough investigation of the hormonal clock in P. americana and has shown that it is probably localised in four neuro-

secretory cells of the sub-oesophageal ganglion of the brain.

Several biochemical parameters have been shown to exhibit circadian rhythms in P. americana. For example, Brady (1974) reports that cockroaches exhibit a circadian rhythm with respect to oxygen consumption, chitin deposition, susceptibility to organophosphate and carbamate insecticides, and the concentrations of uric acid and sugars present in the haemolymph. In contrast to Acheta domesticus no circadian rhythm of AChE activity has yet been reported for P. americana.

In addition to a circadian rhythm it is possible that neurochemical parameters in arthropods also exhibit seasonal variation. Smith (1939) reports that in Cambarus limosus the acetylcholine content of ganglia taken from animals in the Autumn averages about three times that obtained in the Spring. Van der Kloot (1955) reports that cholinergic activity in the brain of the Cecropia silkworm increases during diapause although the level in the nerve cord ganglia remains at a uniform level. These findings have been disputed by other workers (see Smallman and Mansingh, 1969).

The results correlating a decrease in the specific activity of AChE with an increase in the ambient temperature at which animals are kept indicate that environmental factors can modulate the activity of the enzyme. The activity of the animals also increased with increasing temperature. The results of Cymborowski et al. (1970) suggest that AChE activity in the house cricket is inversely related to locomotor activity. The results of Oliver (1970) show that the time taken by test animals to learn the shock avoidance response decreases as the ambient temperature at which the animals are kept is increased. Thus AChE activity may be

related to the overall activity of the animal and will therefore change in response to environmental stimuli which cause a change in the activity of the animal. Such changes in AChE activity may well affect any decrease in the activity of the enzyme in response to shock avoidance learning. Indeed the original decrease in AChE activity associated with shock avoidance learning may have been due to an activation of the test animal with respect to the yoke animal rather than being associated with the engram of the response.

Differences between training procedures used in shock avoidance learning experiments

Several differences may exist between the training procedures used by the various groups of workers investigating shock avoidance learning.

The height at which the insect is suspended above the saline may be critical in determining the time taken by the test animal to learn the response, the type of leg movements it makes in learning to avoid receiving a shock and the degree of retention of the response it exhibits. Willner (1973) reports that if the insect is suspended at a height of 1.2 cm above the saline the average learning time is 30-40 mins. and also the animal shows good retention of the response. If the animal is suspended at a height of 1.6 cm above the saline it only takes 10 mins. to learn to avoid receiving frequent shocks, but shows little retention of the response. The retention is measured by reversing the test situation (i.e., when the animal lifts its leg it receives a shock) and measuring the delay in the reversal of the learning.

A second variable factor is the nature of the shock received by the test animal. In the system we have used the test animal received pulses of 2 msec. duration delivered at a frequency of 1/sec. A study of the shock received by the animal showed that each shock is of 60-80 volts p.d. and of 1 mA current. Pritchatt (1970) applied pulses of 300 msec. duration delivered to the test leg at a frequency of 2/sec. Two intensities of shock were used, 20 uA and 60 uA. Learning took place at both intensities, but was impaired by the higher intensity stimulus. Woodson et al. (1972) obtained their results using pulses similar to those used by us, i.e., 2 msec. duration, 1.5 pulses/sec., 1 mA intensity. Willner and Mellanby (1974) varied the applied shock from 0.8 mA lasting 2 msecs. to 0.4 mA lasting 0.2 msecs. Both levels of shock were delivered at a frequency of 1/sec. A difference in the leg movements produced by the two levels of shock was observed the higher magnitude shock producing a twitch in the tarsal region of the leg. At both shock levels no difference in AChE activity between yoke and test animals was observed. The animals they used received approximately the same number of shocks as those used by Kerkut, Beesley, Emson, Oliver and Walker (1971).

A consideration of the leg movements used by test animals to avoid receiving the shocks is of interest. Oliver (1970) reports that three types of leg movement were commonly observed:

1) The leg was raised and bent beneath the body.
2) The leg was raised and held sideways at 90^{o} to the long axis of the body.
3) The leg was held in a straight condition below the abdomen.

Sometimes combinations of these three types of movement were used.

At present there has been no reported investigation of the actual step in the learning process which involves the changes in AChE activity. Hence it is possible that any change in AChE activity in response to shock avoidance learning may be related to the performance of the animals, i.e., the pattern of leg movements used to avoid receiving the shock. Clearly each type of movement used to avoid the shock will involve different neuronal circuits. Thus a correlation of AChE activity with shock avoidance learning would not be observed unless the test animal learnt by a particular pattern of leg movement. In turn this would be affected by the magnitude, frequency and duration of the applied shock and the height of the test animal above the saline. The type of leg movement used by individual animals to avoid the response could also be related to their inherent activity.

A final point in this context is that made by Disterhoft (1972) who reports that test animals avoid the shock by passive avoidance learning rather than escape learning. It may be possible that test animals learn by both of these mechanisms and the factors already discussed influence the exact nature of the learning response. Furthermore AChE activity may only be correlated with one of these types of learning response.

In conclusion, the available evidence indicates that shock avoidance learning is possible in <u>P. americana</u> without concommitant changes in AChE activity. The learning response, however, may not be as simple as was originally suggested by Horridge. A critical investigation of the effects of the factors discussed on shock avoidance learning in <u>P. americana</u> is

necessary before involvement of changes in AChE activity with the behaviour can be discounted. Future experiments must be designed to take these factors into account.

4.5.2. Summary of discussion

The results obtained in studies attempting to correlate changes in AChE activity with shock avoidance learning are equivocal. The initial results obtained support the proposals of Kerkut, Oliver, Rick and Walker (1970). Later results obtained clearly showed that test animals could acquire and retain the shock avoidance behaviour without concommitant changes in ganglionic AChE activity. The results of kinetic and electrophoretic studies attempting to correlate AChE activity with shock avoidance learning are not conclusive due to other factors such as the decay in enzyme activity. It is difficult, however, to dismiss the original experiments correlating AChE activity with shock avoidance learning. The effects of a large number of factors on the response of the test animals have been discussed including, strain differences between the animals used, differences between yoke and test animals used, differences in environmental conditions under which the animals used in these experiments were kept and differences in the characteristics of the training system used. Data has been presented which indicates that the specific activity of AChE in the metathoracic ganglion of P. americana varies with the ambient temperature at which the animals are kept. It is concluded that a critical investigation of the effects of the factors discussed on shock avoidance learning is necessary before correlation of AChE activity with shock avoidance learning can be dismissed. Finally, in support of the original results correlating a change in AChE activity with shock avoidance learning, the activity of

the enzyme has been shown to change in a number of other species in response to various stimuli (for review see pages 135-138).

SUGGESTIONS FOR FUTURE EXPERIMENTS

The suggestions for future experiments can be divided into two groups, namely studies on the purified enzyme and studies on the role of AChE in shock avoidance learning together with the effects of environmental conditions on AChE activity in the intact animal.

1. Studies on the purified enzyme

The following experiments are designed to solve the problems raised by the work presented in this thesis:

1) Synthesis of an affinity chromatography column to achieve rapid and complete purification of the enzyme.

2) A study of the different molecular forms of the purified enzyme to show whether they are aggegate forms of one another or are true isozymes. The study would include an investigation of the forms of the enzyme released by different methods of solubilisation including solubilisation in an ion-free medium. This study should show whether solubilisation of the enzyme with triton-X-100 does release the enzyme in a form in which it is still attached to other membrane components.

3) An investigation into the nature of the membrane components to which the enzyme is attached and whether they do modify the activity of the enzyme.

4) An investigation into the effect of DTNB on the enzyme to show that it only affects the Km parameter of the truly solubilised enzyme. Also it would be desirable to investigate the nature of the site which binds DTNB.

5) An investigation of the effects of atropine and curare-like substances on the enzyme to see whether they do modulate its activity and if they do to show whether they bind to the enzyme at a site distinct from the active site.

6) An investigation of the subunit structure of the purified enzyme to determine firstly the number of polypeptide chains comprising a single active unit of the enzyme and secondly the number of active sites/single active unit of the enzyme.

2. Studies on the role of AChE in the intact animal

In the following experiments it would be preferable to use an inbred strain of animals:

1) Confirmation of the effect of temperature on the specific activity of AChE in the metathoracic ganglion of the animal. If positive results are obtained this study could be extended to see whether ambient temperature affects AChE activity in other regions of the nervous system, e.g., the brain. Experiments would be carried out to investigate the mechanism of any observed effect of temperature on AChE activity.

2) An investigation into the possibility of a circadian rhythm in AChE activity both in the thoracic ganglia and in the brain of the animal.

3) A critical re-investigation of the learning behaviour of the animal to establish the effect on the shock avoidance learning of:

a) height of the insect above the saline,

b) the magnitude, duration and frequency of the applied shock,

c) the environmental conditions under which the animals are kept.

4) Measurement of AChE activity in animals subjected to shock avoidance learning varying the parameters mentioned in 3. In such experiments it would be preferable to use an internal control by cutting the connectives between the meso- and metathoracic ganglia and using a metathoracic leg as the test leg and a pro- or mesothoracic leg as the yoke leg.

5) An investigation of other systems which could be correlated with the learning behaviour, e.g., cyclic AMP, GABA/glutamate.

APPENDIX 1. Methods of polyacrylamide gel electrophoresis used to separate cockroach nerve cord proteins.

Three polyacrylamide gel electrophoresis systems have been used to investigate protein components of the cockroach CNS. The first method uses the buffer system of Davis (1964). Routinely 5% gels were used. The scheme for making up these gels is given below.

The other two systems used were those of Schapiro, Vinuela and Maizels (1967) and Neville (1971). Both methods require the use of SDS so that the M.Wts. of the proteins may be determined from the gels (Weber and Osborne, 1969; Neville, 1971). SDS, however, denatures AChE and therefore these methods are of little use in characterising AChE isozymes unless the starting material is pure. The method of Schapiro *et al*. uses a continuous phosphate buffer system whereas that of Neville uses a discontinuous borate/sulphate/chloride buffer system in which the borate/sulphate discontinuity is the important factor in the stacking and unstacking of proteins in the gel. Separationsof ganglionic proteins by both of these methods is shown in fig. 43. Only 11 bands were resolved by the method of Schapiro *et al*. whereas the method of Neville gave an excellent resolution of 52 bands of protein, 5 of which were in the stacking gel. Photographs of separations of ganglionic proteins by the method of Neville are shown in fig. 44 and a scan of the proteins separated by this method is shown in fig. 45. Thus the method of Neville gave a far greater resolution of ganglionic proteins than did the method of Schapiro *et al*. The details of the gel system used for this separation are given overleaf.

Details of the Davis gel electrophoresis system

1) Gel buffer

Tris-HCl, 3.0M, pH 8.9

Tris(hydroxymethyl)methylamine	36.3 g
0.1N hydrochloric acid	48.0 ml.
N,N,N',N'-tetramethylethylenediamine (TEMED)	0.40 ml

H_2O to 100 ml

2) Acrylamide solution (suitable for 4.0-7.5% gels)

Acrylamide	30.0 g
Bis (N,N'-methylenebisacrylamide)	0.8 g

H_2O to 100 ml

3) Reservoir buffer

Tris-glycine, 0.387M, pH 8.3

Tris(hydroxymethyl)methylamine	6.0 g
Glycine	28.8 g

H_2O to 1 litre

Prior to use dilute 1 part stock buffer to 9 parts distilled water.

4) Catalyst

Ammonium persulphate - 0.14% (w:v) in distilled water.

In order to make up 5% gels 1.0 parts of gel buffer, 1.33 parts of stock acrylamide solution, 2.69 parts stock catalyst solution and 2.98 parts distilled water were mixed and de-aerated. The concentration of TEMED was such that this mixture took 20-30 mins. to polymerise after de-aeration.

Details of the Neville SDS gel electrophoresis system

1) Stacking gel buffer

0.0267M H_2SO_4-0.0541M Tris, actual pH 8.64, running pH 6.1

1.638 g Tris dissolved in 90 ml H_2O, titrate with concentrated H_2SO_4 to pH 8.64. Make up to 100 ml with H_2O. Check pH.

2) Stacking gel

Acrylamide	7.5%)	
		) w:v
Bis(N,N'-methylenebisacrylamide)	0.5%)	

Made up in distilled water.

3) Lower gel buffer

0.0954M Tris-0.0954M HCl, actual pH 5.77, running pH 8.64

2.848 g Tris dissolved in 90 ml H_2O, titrate with concentrated HCl to pH 5.77. Make up to 100 ml with H_2O. Check pH.

4) Lower gel

Acrylamide	27.5%)	
		) w:v
Bis(N,N'-methylenebisacrylamide)	0.25%)	

The final solutions for both the stacking gel and the lower gel were made up of 2 parts buffer, 2 parts acrylamide solution and 1 part 0.25% (w:v) ammonium persulphate. 7 ul of TEMED was added/5 ml final solution after de-aeration of the latter.

5) Upper reservoir buffer

0.04M boric acid-0.041M Tris, pH 8.64

2.47 g/litre boric acid

4.965 g/litre Tris

pH checked and adjusted if necessary.

0.1% (w:v) SDS added to final solution.

6) Lower reservoir buffer

0.0308N HCl-0.424M Tris, actual pH 9.5, running pH 9.18

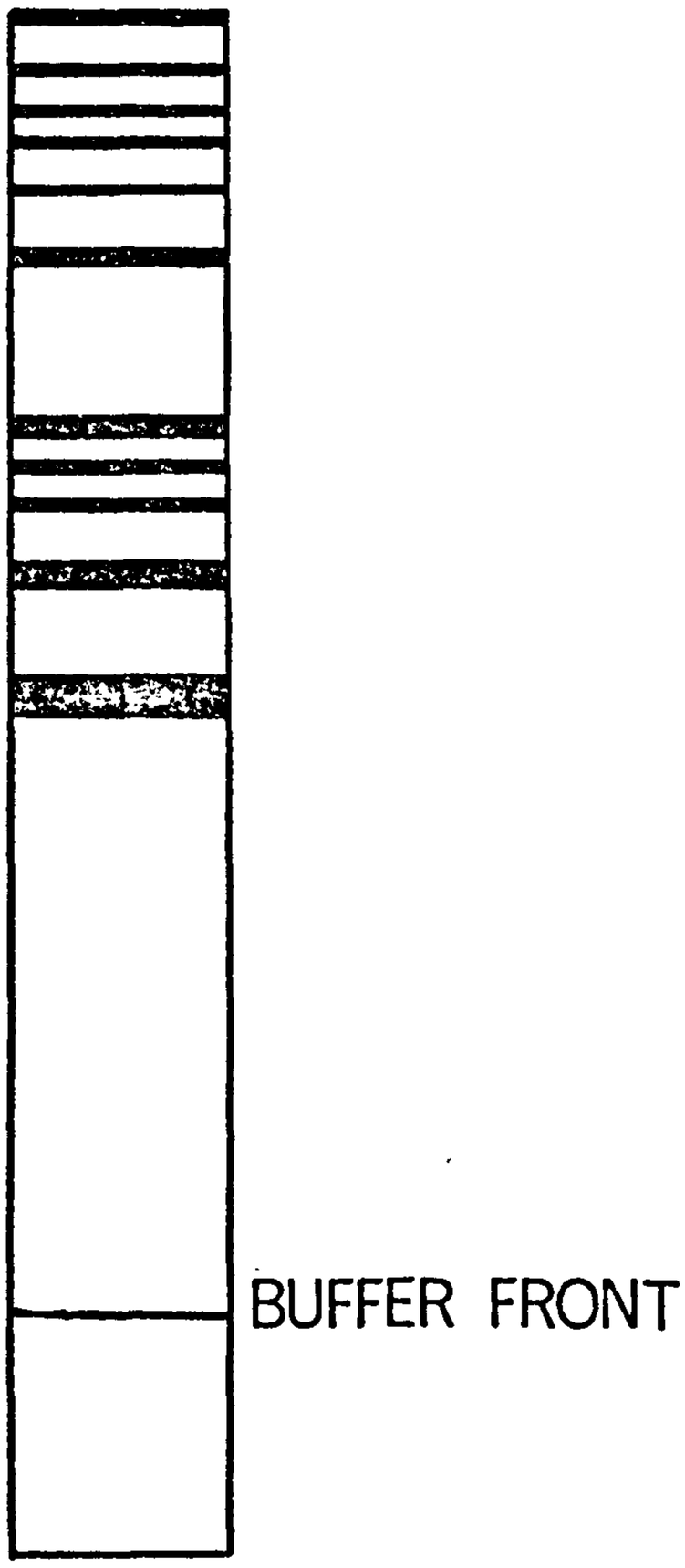
51.39 g Tris dissolved in H_2O, titrate to pH 9.5 with concentrated HCl. Dilute to 1 litre with H_2O and check pH.

7) Samples

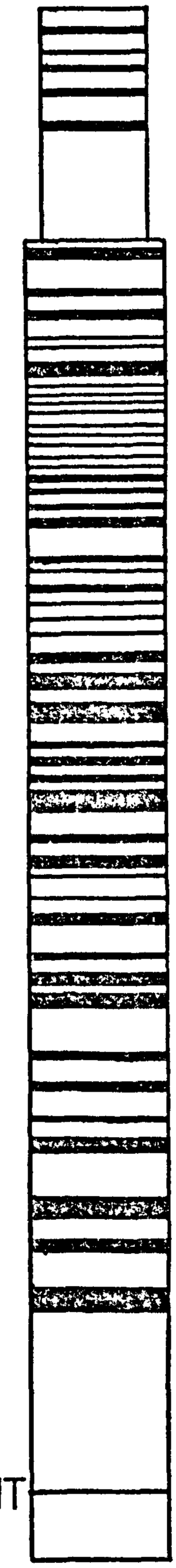
Samples were made up in 50 mM sodium carbonate containing 10% 2-mercaptoethanol and 8 mg SDS/mg protein. Samples were incubated at 37°C for 1 hr. prior to layering on the gels.

FIG. 43. A comparison of the separations of ganglionic proteins from Periplaneta americana obtained using the methods of Schapiro et al. (1967) and Neville (1971)

The figure shows drawings of typical separations of proteins from the metathoracic ganglion achieved using the two methods of SDS gel electrophoresis described. Only 11 bands were resolved by the method of Schapiro et al. whereas 52 bands were resolved by the method of Neville. Note that the drawings are not to scale, the gels used in the method of Neville being only slightly longer than those used in the method of Schapiro et al.



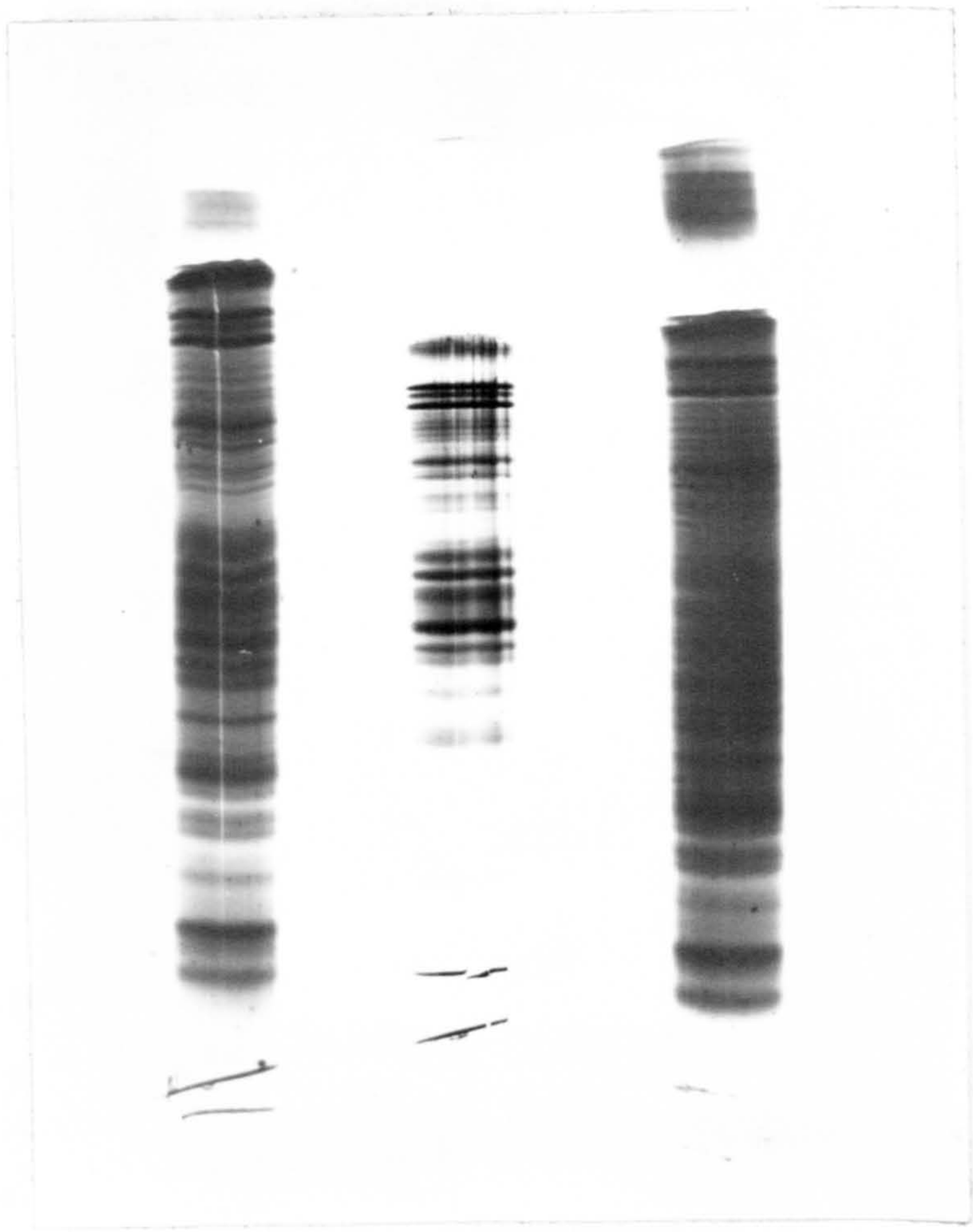
A



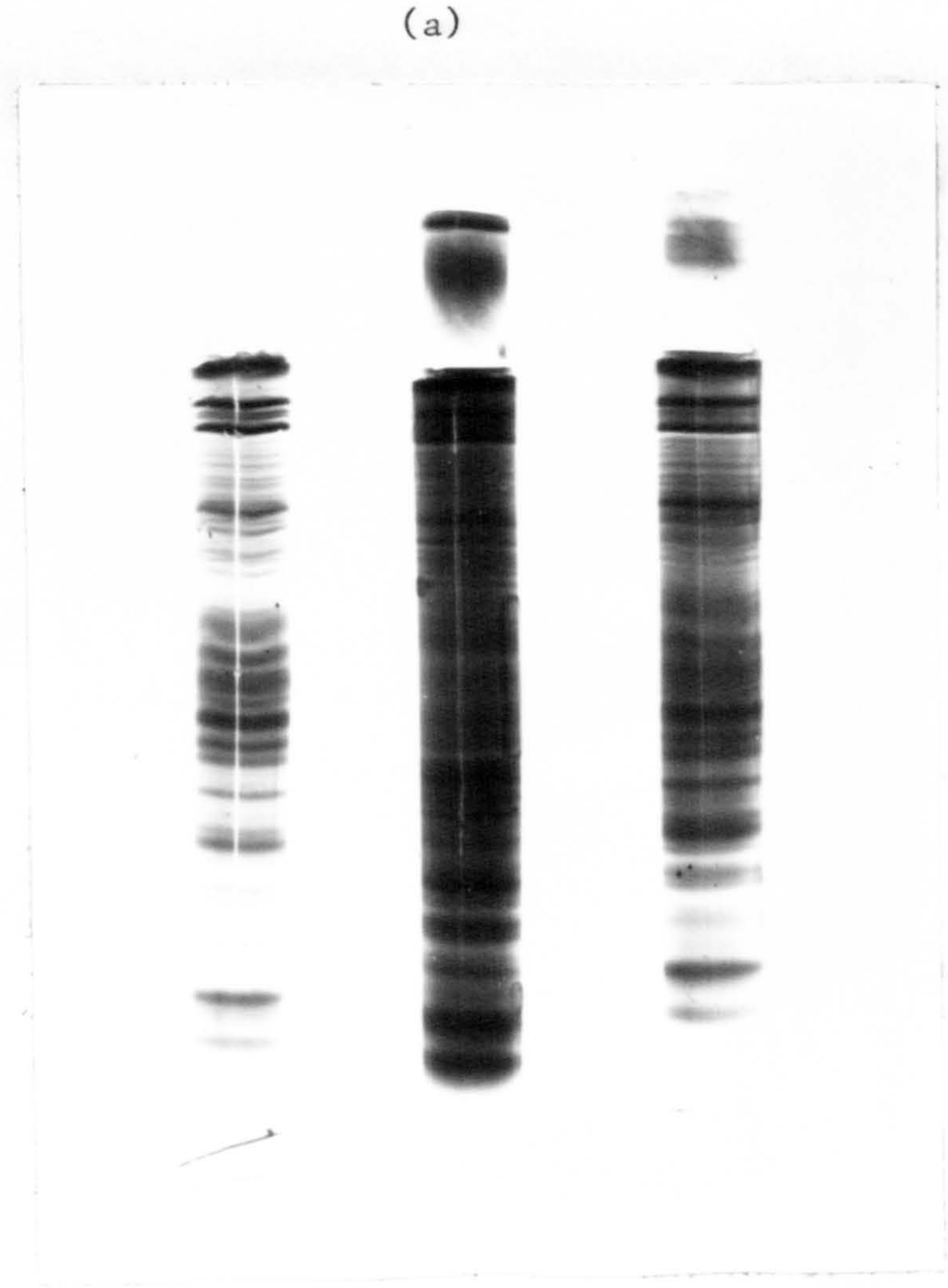
B

FIG. 44. Photographs of typical separations of ganglionic proteins obtained using the method of Neville (1971)

The two photographs show the separation of proteins from the metathoracic ganglion of P. americana obtained using the method of Neville. 52 separate bands of protein can be distinguished, 5 of which occur in the stacking gel. The maximum amount of protein loaded on to any gel was 150 ug.



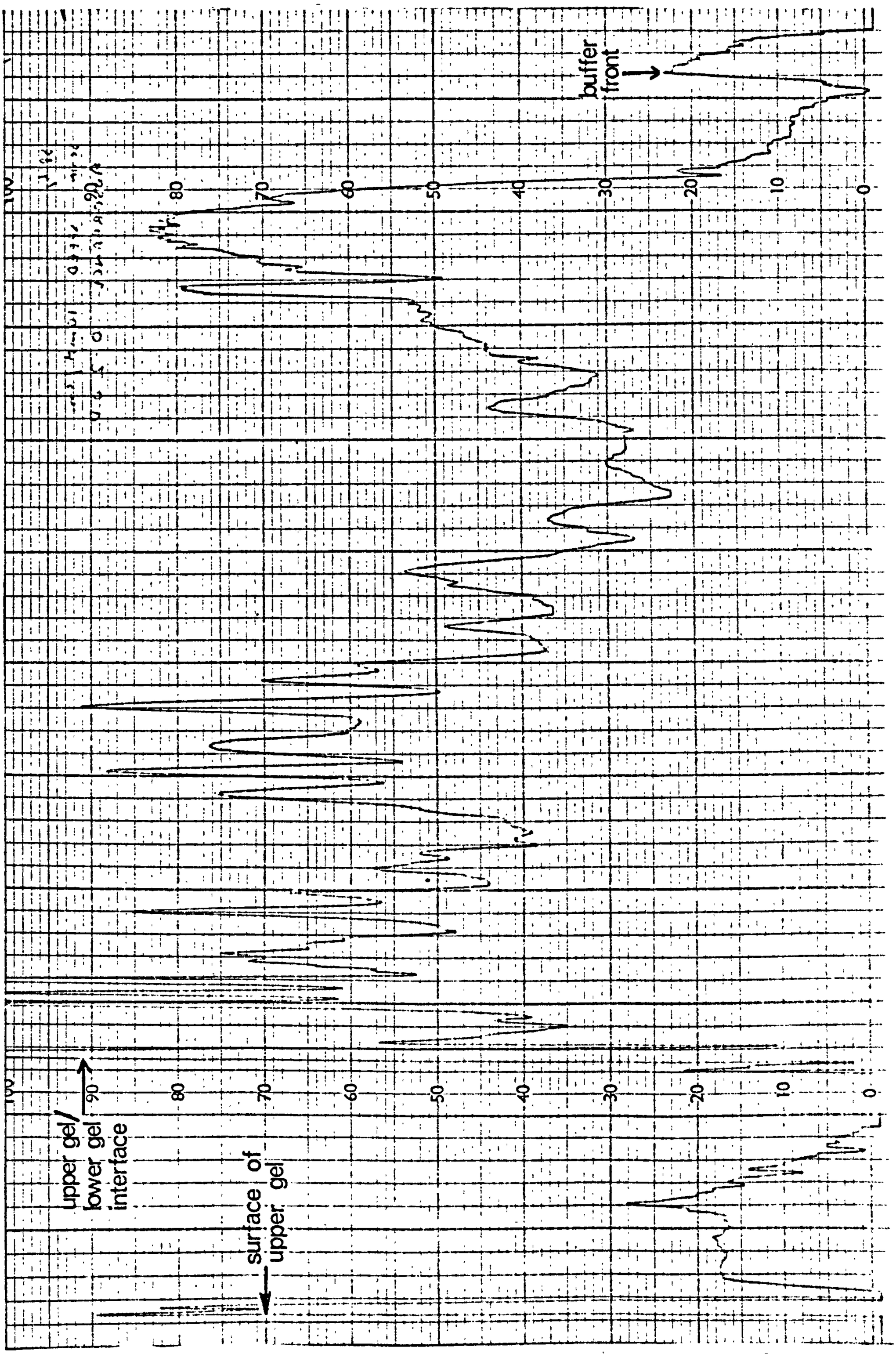
(a)



(b)

FIG. 45. A scan of a typical separation of ganglionic proteins obtained using the method of Neville (1971)

The figure shows a scan of a typical separation of ganglionic proteins obtained using the method of Neville. Note the resolution of the method. The scan was made using a Vitatron modular spectrophotometer fitted with a gel scanning attachment. A full scale deflection corresponds to an O.D. change of 2 units. The scan was made at a speed of 10 mm/sec.


buffer front
upper gel/lower gel interface
surface of upper gel
100
90
80
70
60
50
40
30
20
10
0

APPENDIX 2. Electron microscope studies of the morphology of the metathoracic ganglion and the electron microscopic localisation of AChE in the ganglion

Ganglia were removed as previously described and placed in 3% glutaraldehyde in cacodylate buffer, pH 7.4, for 4 hrs. at $0^{o}C$ in order to fix the tissue. The ganglia were cut into small blocks and washed twice in buffer. Subsequently the blocks of ganglionic tissue were post-fixed in 2% OsO_4 in cacodylate buffer (plus 0.25M sucrose) for 1 hr. at room temperature. The blocks were then dehydrated in ethyl alcohol and embedded in araldite. Thin silver sections were cut on a Sorvall-Blum ultra-microtome using glass knives. The sections were counterstained in lead citrate and examined in a Phillips E.M. 300 electron microscope. Representative electron micrographs showing the morphology of the meta-thoracic ganglion are shown in fig. 46.

The above method was modified as described below in order to study the localisation of AChE in the metathoracic ganglion. The ganglia were only fixed in 3% glutaraldehyde (plus 1% formaldehyde) in cacodylate buffer for 30 mins. as a longer time denatured nearly all of the AChE. The blocks of ganglionic tissue were washed several times in buffer over a period of 3 hrs. at $0^{o}C$ and finally once in sodium hydrogen maleate buffer, pH 6.5. The samples were split into a control group which was incubated in the absence of substrate and a test group which was incubated in the presence of ATCh as substrate. The incubation medium was as follows:

Sodium hydrogen maleate buffer, pH 6.5	6.5 ml
N-acetylthiocholine iodide	6.5 mg
0.6M sodium tartrate	0.5 ml
0.03M cupric sulphate	1.0 ml
0.005M potassium ferricyanide	1.0 ml
Distilled water	1.0 ml

The samples were incubated at $0^{o}C$ for 1 hr. The blocks of ganglionic tissue were subsequently washed twice in cacodylate buffer and then fixed in 2% OsO_4 in cacodylate buffer, pH 7.4 (plus 0.25M sucrose) for 1 hr. at room temperature. The blocks were then dehydrated in ethyl alcohol and processed for electron microscopy as previously described. Electron micrographs showing the localisation of AChE in the metathoracic ganglion are presented in fig. 47.

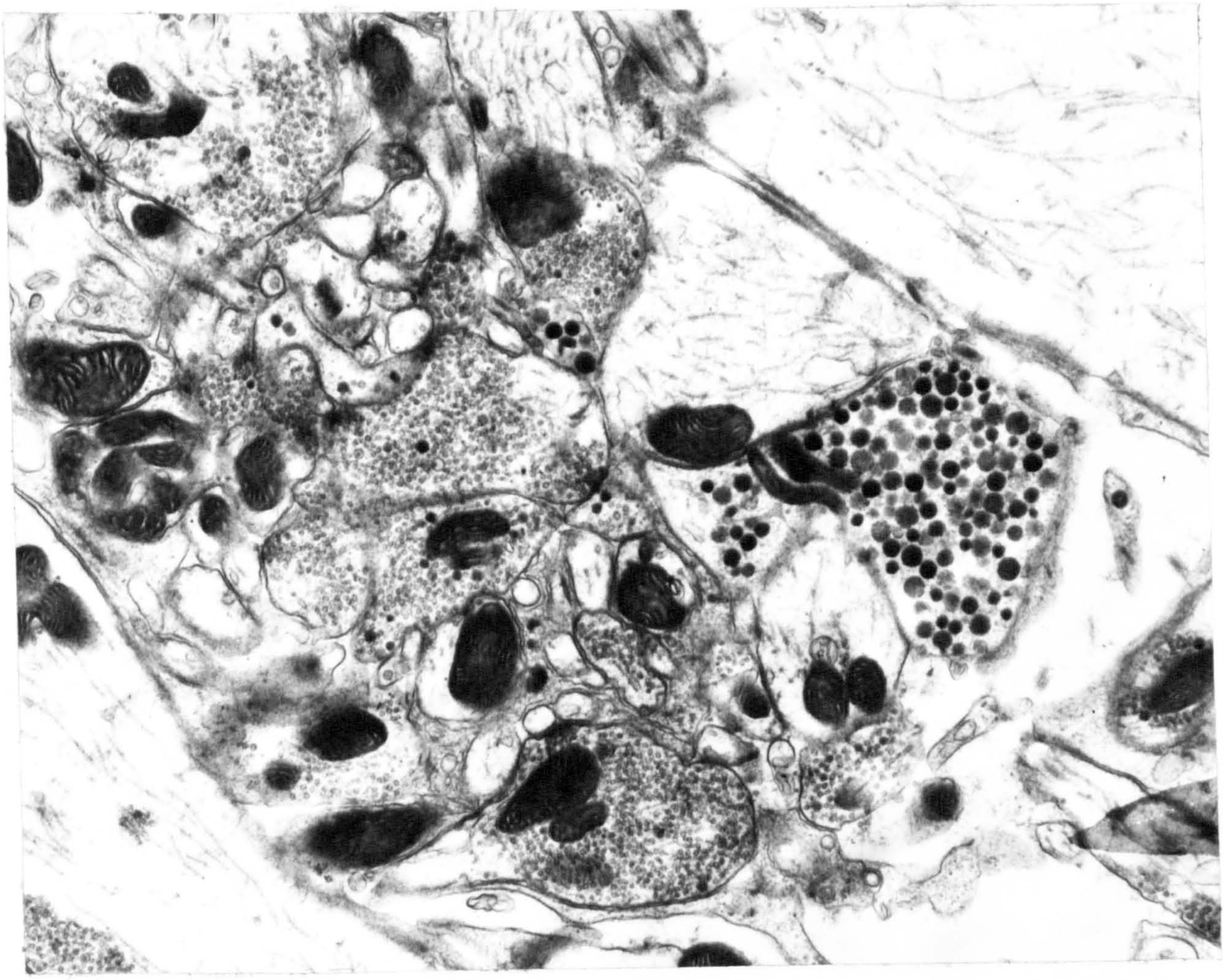
FIG. 46. Electron micrographs showing the morphology of the metathoracic ganglion

FIG. 46a. The electron micrograph shows several features of the morphology of the ganglion. Note the nerve ending in the bottom right hand corner of the micrograph. The cell bodies contain numerous vesicular structures. Microtubular elements can be seen in some of the axons.

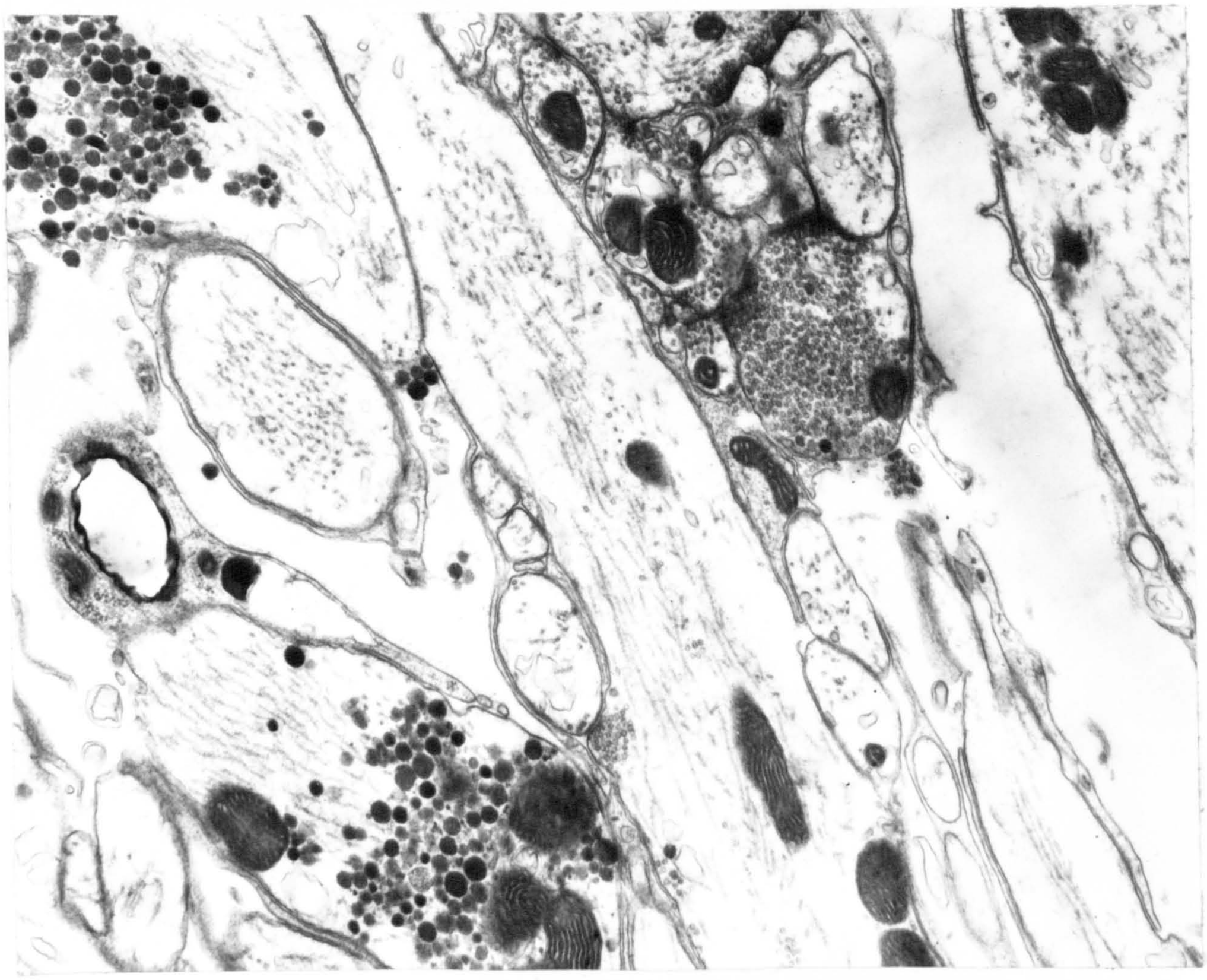
MAG. x 31,000

FIG. 46b. Many of the same features shown in fig. 46a can also be seen in this electron micrograph. Note the presence of two types of vesicle of different size and electron density (particularly in the bottom left hand corner of the micrograph). The larger vesicles may be associated with neurosecretory cells.

MAG. x 31,000



(a)



(b)

FIG. 47. Electron micrographs showing the distribution of AChE in the metathoracic ganglion

FIG. 47a. The electron micrograph shows part of the connective tissue sheath surrounding the ganglion. Note the densely staining region of AChE activity at the boundary between the sheath and the outer edge of the region of cell bodies. This region is associated with the barrier to penetration of the ganglion by ACh proposed by Treherne and Smith (1965b). Difficulty in staining AChE in other regions of the ganglion may be due to hydrolysis of substrate by the enzyme localised at the edge of the ganglion.

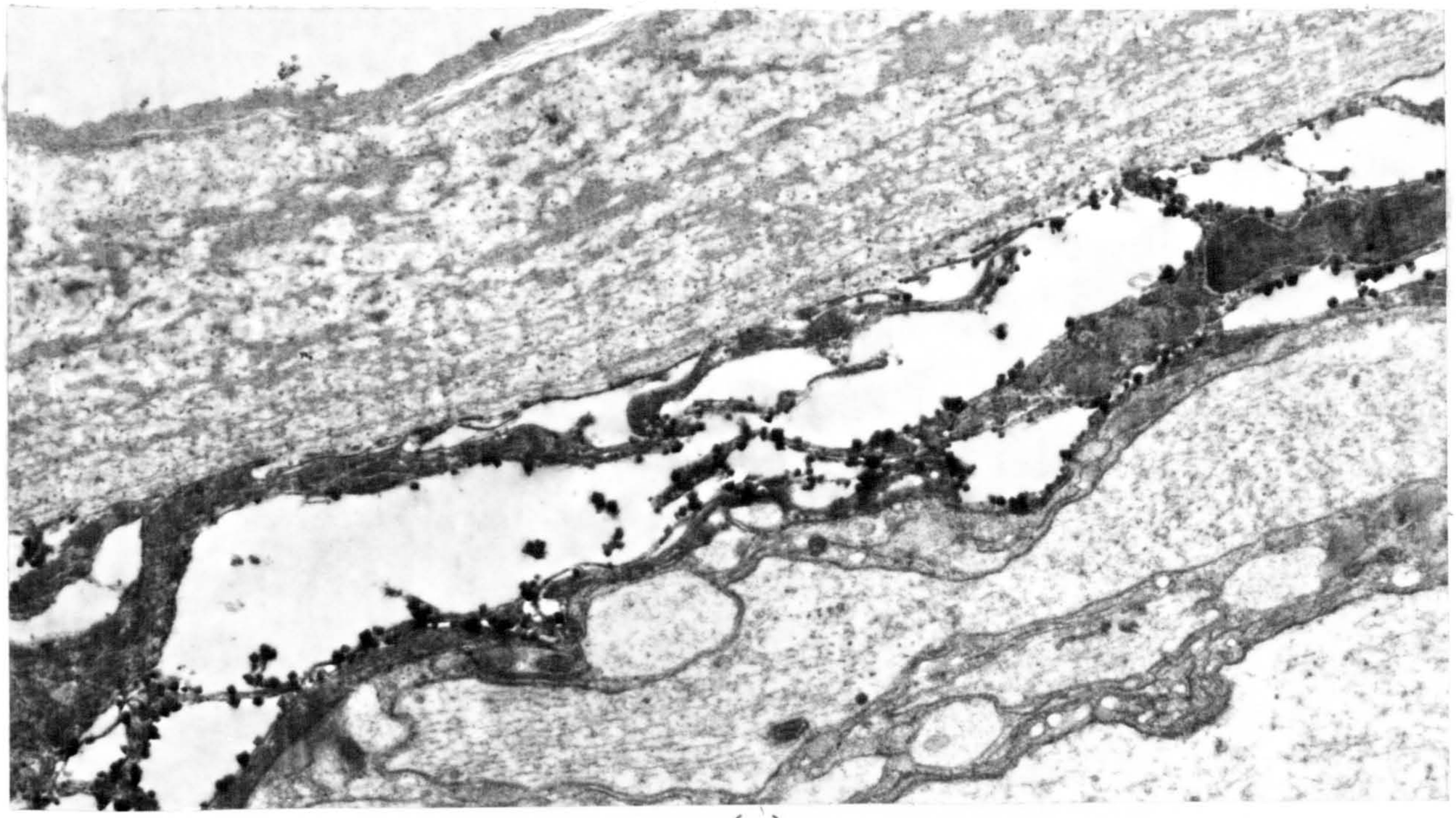
MAG. x 11,400

FIG. 47b. A synapse which has stained for AChE activity is present in the bottom right hand corner of the electron micrograph.

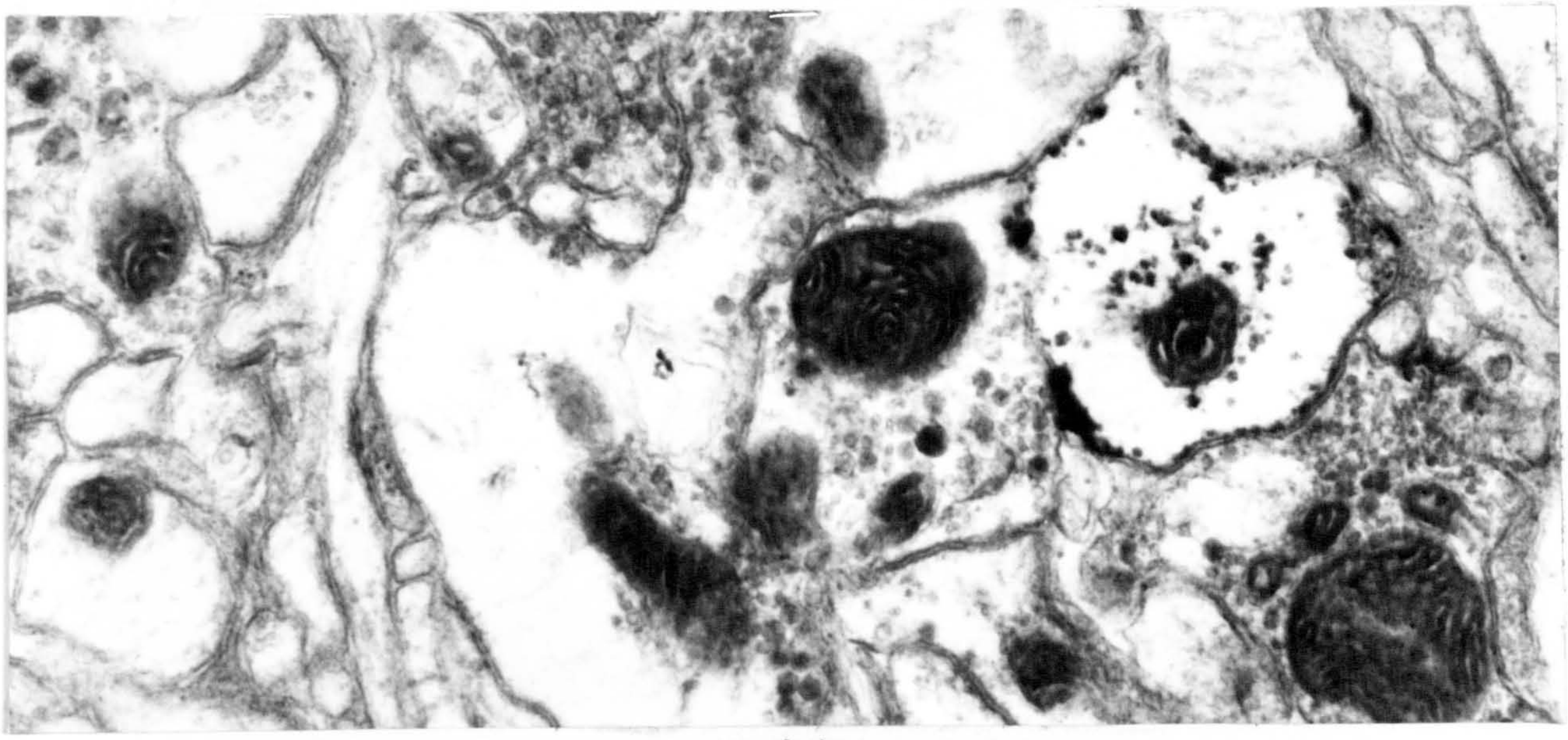
MAG. x 17,000

Fig. 47c. AChE appears to be localised on both the pre- and post-synaptic membranes of the synapse present in the centre of the micrograph. The enzyme is also distributed throughout the rest of the tissue, but in lower concentrations than at the synapse. These results agree with those of Treherne and Smith (1965b).

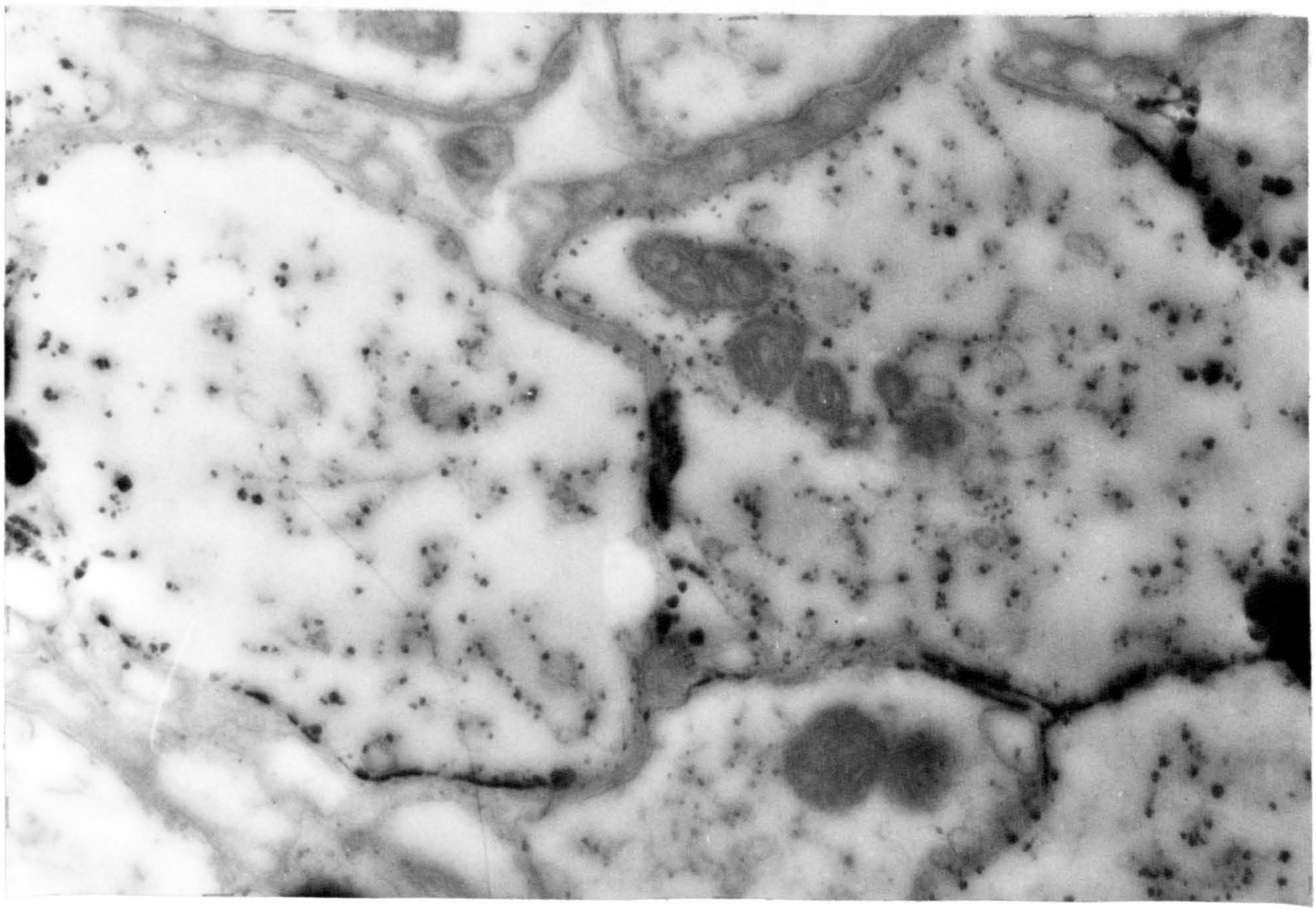
MAG. x 31,000



(a)



(b)



(c)

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