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ACETYLCHOLINESTERASE

STUDIES ON ACETYLCHOLINESTERASE OF OX BRAIN

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A Thesis
submitted to the
UNIVERSITY OF NAGPUR
for the degree of
DOCTOR OF PHILOSOPHY

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JUNE 1968

A C K N O W L E D G E M E N T

The author is grateful to Dr. V. Jagannathan, Head of the Division of Biochemistry, for his valuable guidance and encouragement throughout the course of this investigation.

He wishes to acknowledge the help and encouragement of Dr. Mira Joshi in his work. The author also would like to express his deep gratitude to all his colleagues in the Division of Biochemistry for pleasant and friendly dealings.

His thanks are also due to the Council of Scientific and Industrial Research for the award of fellowship and the Director, National Chemical Laboratory for permission to submit this work in the form of a thesis for the degree of Doctor of Philosophy.

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List of abbreviations

AchE	-	Acetylcholinesterase
DEAE-cellulose		Diethylaminoethyl cellulose
CM-cellulose		Carboxymethyl cellulose
EDTA	-	Ethylenediaminetetraacetate
Tris	-	Tris(hydroxymethyl)aminomethane
-SH	-	Sulfhydryl group
°	-	degree centigrade
hr	-	hour, hours
min	-	minute, minutes
g	-	gram, grams
mg	-	milligram, milligrams
μg	-	microgram, micrograms
xg	-	acceleration of gravity
S	-	sedimentation coefficient
O.D.	-	optical density
ΔO.D.	-	optical density change

CHAPTER 1

I N T R O D U C T I O N

INTRODUCTION

Acetylcholinesterase catalyzes the hydrolysis of acetylcholine to choline and acetate. Its existence was postulated by Dale (1914) and it was subsequently shown to be present in erythrocytes, electric organs of some fishes, nerve tissue of animals and some snake venoms. The enzyme has been recently crystallized from the electric organ of *Electrophorus electricus*. Partially purified preparations of the enzyme have also been obtained from erythrocytes, cobra venom and brain tissue. The enzyme from brain tissue has, however, not been purified to any significant extent due to its attachment to particles or to its instability. The present work deals with a new method for the solubilization of ox brain acetylcholinesterase, its purification and the study of its properties.

SECTION I

Historical and scope of the literature survey

Historical

The idea of neurohumoral transmission was put forward by Elliott[1] in 1905 and the marked pharmacological action of acetylcholine was described by Hunt and Taveau[2] in 1906. Loewi in 1921 found a compound, which was termed "Vagusstoff", in the perfusion fluid of the nerve endings[3]. This compound was later shown to be identical with acetylcholine[4]. Dale and Dudley (1929) demonstrated the presence of acetylcholine in animal tissues[5].

Acetylcholine plays an important role in the transmission of nerve impulses. The release of acetylcholine at the nerve endings gives rise to a change in the permeability of the membrane which gives rise to an ionic concentration gradient and becomes the effective source of electromotive force[6]. Earlier the role of acetylcholine was considered to be limited exclusively to the synapse, but according to a theory proposed by Nachmansohn and Meyerhof the action is not an inter- but an intra-cellular process taking place within the conducting membrane[7].

One of the objections raised against this kind of a role for acetylcholine was the absence of a mechanism for the rapid

breakdown of acetylcholine as is necessary during the transmission of a nerve impulse[8]. But Dale in 1914 had suggested the inactivation of the active ester by enzymic action. Engelhardt and Loewi in 1930 showed the presence of an esterase which hydrolyzes acetylcholine in blood serum, plasma, erythrocytes and stromata[9]. Shortly afterwards its presence in brain and electric organ of electric fish was reported. The latter is a very rich source of this enzyme and rapid progress was made in the purification and the study of the kinetics and mechanism of action of the enzyme from electric fish.

Scope of the literature survey

The systematic name of the enzyme acetylcholinesterase is acetylcholine acetyl hydrolase (EC 3.1.1.7). The abbreviation AchE or the trivial name acetylcholinesterase will be used to designate this enzyme. The earlier work on AchE is reviewed in this chapter. Only brief reference will be made to non-specific or pseudocholinesterase. This survey deals mainly with studies on the enzyme from electric organ of electric fish, erythrocytes, cobra venom and brain tissue with special reference to those aspects (purification, properties and kinetics) which form the subject of this thesis.

SECTION II

Different types of cholinesterases

Esterases are a group of enzymes with comparatively low specificity and are specific for ester linkages but hydrolyse a large number of different esters at different rates. "The simple esterases and lipases are concerned with the hydrolysis of uncharged substrates, and here the main factors influencing the specificity are the lengths of the hydrocarbon chains on either side of the ester link. These enzymes are not of high specificity, and probably each hydrolyses a range of alkyl esters of different molecular weight." (In "Enzymes" by M. Dixon and E. C. Webb). More specific esterases such as cholinesterases hydrolyse special types of esters, some of which bear charged groups.

Esterases which hydrolyse choline esters can be divided into two types, those which are relatively non-specific (pseudo-cholinesterase) and the other more specific (true cholinesterase or acetylcholinesterase). With AchE the rate of hydrolysis decreases sharply with increase of chain length of acyl group attached to choline whereas the reverse is the case with pseudocholinesterase.

Serum cholinesterase and AchE from erythrocytes of human blood were found to differ in their activities towards acetyl-β-methyl choline. This substrate is not hydrolyzed by serum cholinesterase but

is hydrolyzed by the erythrocyte enzyme. Moreover, erythrocyte AchE has a sharp substrate optimum, whereas the serum cholinesterase does not have this property[10].

The relative rates of hydrolysis of a number of substrates such as acetylcholine, acetyl-β-methyl choline, benzoyl choline and the effect of substrate concentration on enzyme activity were studied with cholinesterases from different sources[11]. It was observed that acetyl-β-methyl choline was hydrolyzed only by the brain and erythrocyte enzymes whereas benzoylcholine was hydrolyzed only by the serum enzyme. Inhibition by high substrate concentration was also observed only with erythrocyte and brain enzymes. Based on these studies the name pseudocholinesterase was introduced for the serum enzyme and true cholinesterase for the enzyme from brain and erythrocytes.

Studies with a number of substrates such as acetylcholine, butyrylcholine, propionylcholine, acetyl-β-methyl choline, benzoyl choline, tributyrin, methylbutyrate and at different substrate concentrations were made with enzymes from electric tissue, caudate nucleus and gray matter of brain from rat, mouse, cat and their sera and erythrocytes[12]. These detailed kinetic data clearly established that the rates of hydrolysis of the different substrates and the specificities of the enzymes from electric tissue, ~~and~~ from erythrocytes and brain of different species were very similar, whereas the enzymes from serum

were markedly different from the other group of enzymes. The former group of cholinesterases showed a bell-shaped activity - $\log \frac{1}{[\text{substrate}]}$ concentration curve, whereas the serum enzyme gave a S-shaped graph. These results have been confirmed and extended by other investigators[9,13,14]. Low Michaelis constants for the true enzyme and high values for the serum enzyme were also noted. Cobra venom enzyme was also shown to belong to the group of true cholinesterases[9,15]. A difference in the electrophoretic mobility of the two types of cholinesterases was also reported[16]. Serum cholinesterase was shown to be a sialoprotein[128a,b].

Differences have also been observed with respect to the inhibition of the two types of cholinesterases. Human serum cholinesterase is strongly inhibited by nupercaine, irgamide and isopropyl antipyrin but erythrocyte and brain enzymes are only weakly inhibited. Caffeine inhibits only serum cholinesterase[17].

Pseudocholinesterase from serum is inhibited by eserine with acetylcholine as the substrate and only slightly with non-choline esters (ethylchloracetate and β-chloroethylacetate), whereas inhibition of AchE by eserine is observed with both choline and non-choline esters of acetate[18]. Potent and selective inhibition of AchE <u>in vivo</u> in dog was observed by bis 2-piperidinomethyl 2-3-dihydrobenzofuran 5-yl ketone dimethiodide[19]. The erythrocyte enzyme was inhibited about 50% and the non-specific esterase only about 8% at the same concentration of the

inhibitor. β-β'-dichlorodiethyl-N-methylamine and an analogue of prostigmine, N-p-chlorophenyl-N-methyl carbamate of m-hydroxyphenyl trimethyl ammonium bromide [Nu1250] are some of the other selective inhibitors of AchE[19a]. The activity of AchE is about 1000 times more sensitive to the inhibitory action than is the pseudocholinesterase. A group of compounds containing two quaternary nitrogens linked with a complex chain of atoms forms another class of selective inhibitors of AchE[20].

Differences in the active sites of AchE and pseudocholinesterase have also been noted[21-24].

It will be seen from this that the main criteria used for distinguishing AchE from non-specific cholinesterase are the relative rates of hydrolysis of several different substrates, the effect of substrate concentration on activity, difference in the action of specific inhibitors, differences in the Michaelis constants and differences in the active sites of the two enzymes.

SECTION III

Occurrence

As indicated earlier AchE occurs in red blood cells, brain tissue, electric organ of some fishes and venoms of some snakes.

The electric organ of electric fish is the richest source of AchE[25]. The electric organ of Electrophorus electricus hydrolyzes 900 to 1400 mg of acetylcholine and the electric organ of Torpedo hydrolyzes 2000 to 3000 mg of acetylcholine per hour per g of tissue. It was shown that the voltage in the electric organs of Torpedo marmarota, Gymnotus electricus and Raja undulata closely parallels the concentration of AchE. The activity of AchE was shown to decrease progressively from the head to the caudate end of the organ[26]. High activities of AchE have also been noted in the electric organs of several other fishes[26a,26b].

Ammon and Voss studied AchE activity in the blood of different species and found snail (Helix pomatia) to be a very rich source[27]. Human red blood corpuscles can hydrolyze about 200 mg of acetylcholine per hour per g.[28] In red blood corpuscles the enzyme is not present in the cell interior but is localised exclusively in the stroma[29].

Zeller studied AchE activity in a number of dried venoms and showed it to be present in nineteen species of Colubridae but not in the venoms of Vipera[30,31]. It occurs in the secretion and not in intimate association with structural elements of the cell. Its activity is high

in venoms noted for their neurotoxic properties[15]. Some of the highly active species (activities in µl of CO_2 per mg of dried venom) of Colubridae are Naja melanoleuca (27,900), Bungarus veruleus (24,900) and Bungarus fasciatus (18,800)[30].

The occurrence of AchE activities in brain and nerve tissue of different animals and the distribution of the enzyme in different parts of brain have been the subject of several studies[33a]. A higher activity of the enzyme in brain than in blood was noted by Bernheim and Bernheim[32]. The distribution of AchE activity in different parts of human brain was studied by Nachmansohn[33b]. Caudate nucleus and putamen were shown to be the richest in AchE activity. The activities observed in occipital cortex, parietal cortex, caudate nucleus, putamen, thalamus, anterior quadrigemina and cerebellum respectively were 1.2, 1.3, 30, 46, 2.7, 5.9 and 8.2 mg acetylcholine hydrolyzed per 100 mg tissue in one hour.

The localization of AchE in brain cells was studied by Nathan and Aprison making use of rabbit brain caudate nucleus homogenate in 0.25M sucrose. More than 70% of the brain cell AchE activity was found in the cytoplasmic particulates; the enzyme was not merely adsorbed on the particles[34]. The enzyme has been stated to be bound to lipids[35].

Nachmansohn and Rothenberg compared the AchE activities of the brains of different species[36]. The activities calculated as µl of CO_2 evolved per hour per mg tissue for whole brain of rat, whole brain of

mouse, caudate nucleus of ox, cortex of ox and caudate nucleus of cat were 1, 3, 9, 2.5 and 15 respectively.

In Schisstonnsoma mansoni, AchE activities in concentrations comparable to those of mammalian brain were observed[37]. AchE has been reported to be present in cerebrum and spinal cords of insects such as Rana temporaria (840), Petromyzon flaviatilis (74) and Calliphora erythrocephala (2240) (activities in parenthesis are µmoles of acetylcholine hydrolyzed per hour per g)[38]. AchE activity was also reported in bees - Apis melliferra[41]. High AchE activity was observed in Aplysia[40].

In Niella flexis - an alga having cells capable of propagating electric currents AchE activity of 6.5 µmoles per hour per g has been reported[39].

SECTION IV

Methods of Estimation

Methods for the estimation of AchE can be broadly divided into biological and chemical methods.

Biological Methods

In the case of biological methods a known amount of acetylcholine is incubated for a definite period with the enzyme preparation, the reaction is stopped by an enzyme inhibitor and the action of the residual acetylcholine on isolated organs such as frog heart, guinea-pig intestine, frog rectus abdominus or on blood pressure is compared with that of known amounts of acetylcholine. These methods generally make use of high enzyme and low substrate concentrations whereas the reverse is the case with chemical methods. The biological methods are less accurate and more tedious than the chemical methods.

Chemical Methods

The chemical methods can be divided into four groups: gasometric, measurement of acid liberated, determination of residual acetylcholine and spectrophotometric.

I. Manometric Method - The Warburg manometric method for AchE estimation was introduced by Ammon[42] and subsequently modified by other workers[43,44]. In principle the method consists in the measurement of CO_2 evolved during enzymic hydrolysis of acetylcholine in bicarbonate-CO_2 system. One

equivalent of CO_2 is evolved for every mole of acetylcholine hydrolyzed (or acetic acid liberated) and is estimated by conventional Warburg manometry. Generally a 95% N_2 - 5% CO_2 mixture is used as the gas phase. This method is widely used for AchE estimation. It has the advantage of accuracy, sensitivity and also continuous measurement of CO_2 evolution with time whereas the reaction has to be stopped if, for instance, the residual acetylcholine is to be estimated. The method is useful for kinetic studies and is more convenient than Hestrin's method (see below) when the effect of a wide range of substrate concentrations is to be studied. This method is less convenient when the effect of pH is to be studied, since this involves a change in bicarbonate concentration if the gas phase contains 5% CO_2.

Barcroft differential technique[45], van Slyke method[46], Cartesian diver technique[47] and a simplified gasometric method[48] are the other gasometric methods used.

II. Change in pH - Methods based on measuring the acid produced can be divided into two classes: in one the change in pH ~~is~~ due to acid production is measured and in the other the acid produced is titrated with standard alkali.

The principle of Michel's method[49] is to measure the rate of change in pH as a measure of enzyme activity. pH is measured at the beginning and at the end of a certain period. The method has been used by a number

of investigators for the routine determination of blood AchE activity. A buffer with low buffering capacity is to be used and the pH of the added enzyme should not considerably affect the pH changes. Moreover, the change in pH during the reaction may affect the activity. The method has been recently modified to give a stable rate of change in pH during a five minute period[50]. Change in substrate concentration is to be held to a minimum.

Titrimetric Method - The principle of the titrimetric methods is to determine the acid liberated during the hydrolysis of acetylcholine by the enzyme. Titration is carried out at constant pH using either an indicator[51], potentiometer or by conductometric titration[52]. Use of indicators in this method has several limitations and can be used under very narrow conditions where not much exactness is required because different indicators may affect the enzyme and different buffer systems may affect the color. Electrometric titration making use of the glass electrode is the most widely used titrimetric method[53]. The use of automatic recording devices for following the enzyme reaction has made this method more convenient[54].

III. Colorimetric Method - Hestrin's colorimetric method[55] is based on the measurement of residual acetylcholine. Acetylcholine reacts with hydroxylamine in alkaline medium to form acethydroxamic acid which forms a red purple complex with ferric ions in acid solution. The intensity of

the color is proportional to the concentration of acetylcholine present over a range of 0.04 μmoles to 5 μmoles of acetylcholine per ml. The color has λ_{max} at 540 mμ.

This is probably the most widely used method because of its convenient nature and applicability over a wide range of conditions, such as pH, enzyme concentration and different buffer systems. Generally not more than about 30% of the ester is allowed to be hydrolyzed. The method is very much less sensitive at high substrate concentrations if the amount of acetylcholine hydrolyzed is small.

IV. Spectrophotometric Methods

Spectrophotometric methods for the estimation of AchE activity make use of artificial substrates such as thiocholine esters[56] and aromatic esters e.g. phenyl acetate, p-nitrophenyl acetate, indophenyl acetate, N-4'-acetoxy phenyl p-quinone amine[57].

Hydrolysis of acetylthiocholine is followed by measuring the change in optical density at 250 mμ. This method has been modified[58] by the use of 5-5'-Dithio-bis-2-nitrobenzoate (DTNB) so that the change in optical density can be followed at 412 mμ.

Phenyl acetate and p-nitrophenyl acetate are some of the widely used phenol esters[89]. Hydrolysis of phenyl acetate is followed at 270 mμ and of p-nitrophenyl acetate at 348 mμ. The use of the aromatic ester, 1-methyl-acetoxy quinolinium iodide, has also been made to follow AchE

activity[59]. In the range of 406 to 460 mµ the hydrolysis product adsorbs but not the substrate.

Because of the rapidity with which the reactions can be followed with very small quantities of enzyme the spectrophotometric methods are useful, especially for routine assays.

SECTION V

Purification of Acetylcholinesterase

Electric organ of Electrophorus electricus

This is a very rich source of AchE and most of the studies on AchE have been carried out with the enzyme from this source. Several purified preparations[60-65] are available from this source and the enzyme has been recently crystallized by Leuzinger and Baker[65].

(In the following discussion the specific activity of the enzyme refers to μmoles of acetylcholine hydrolyzed per mg protein per hour unless otherwise specified.)

The initial specific activity observed with this organ is about 2000 which is about a hundred times more than that of mammalian brain[64]. The removal of mucin from the electric organ is the essential step before purifying AchE from this source. The tissue is cut into pieces, soaked in toluene and kept in a refrigerator under a layer of toluene for six weeks. Toluene is changed from time to time and the exudate is discarded. Mucin-free tissue is then extracted with five per cent ammonium sulfate.

Rothenberg and Nachmansohn obtained a purified preparation by ammonium sulfate fractionation with a specific activity of about 400,000[60]. Lawler developed a simple procedure of ammonium sulfate fractionation at pH 4.2 but the final specific activity of this preparation was only 110,000[61]. Kremzer and Wilson developed a chromatographic procedure for purifying AchE.

They fractionated the enzyme on benezyl DEAE-cellulose, Sephadex-G-200, cellulose phosphate and DEAE-cellulose. The final specific activity was about 660,000[64].

Making use of a similar chromatographic procedure Leuzinger and Baker obtained a preparation with a final specific activity of 720,000 to 750,000 with better yields. When the enzyme was left in 35 per cent ammonium sulfate in a refrigerator for a few weeks, hexagonal crystals of the enzyme were obtained[65].

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Erythrocytes

AchE in erythrocytes resides in the stroma and therefore has to be brought into soluble form before it can be purified. Several attempts have been made to purify AchE from erythrocytes of different species[66-71]. The initial specific activity of AchE in human erythrocyte is about 2, in ox red blood cells it is about 12 and in Vineyard snail red blood cells it is about 8[68,69,70].

The purification of AchE from human red blood cells was carried out by Zittle and Monica[68]. His procedure consists of haemolysis of red blood cells in dilute acetic acid, treatment with ethanolamine, cadmium acetate and Tween-20 and fractionation with ammonium sulfate and calcium phosphate gel. A dry powder was finally obtained by extraction with acetone, ethanol and ether. The final specific activity was 440.

Mitchell and Hanahan have reported the solubilization of human red blood cells stromal AchE, making use of hypertonic sodium chloride

solution (0.2 to 1.4 M). Stromal proteins and lipids were solubilized. The solubilization was independent of pH but depends on sodium chloride concentration[72].

Cohen and Warringa[69] developed a procedure consisting of fifteen steps for the purification of ox red blood cells AchE to obtain a final specific activity of about 250.

Bockendahl and Mullar developed a purification procedure for AchE from Vineyard snail red blood cells[70]. They made use of carbowax treatment, ammonium sulfate fractionation, chromatography on cellulose-phosphate and ultracentrifugation. This preparation had a final specific activity of about 6500.

Pavzner purified AchE from red blood cells of long horned cattle making use of treatment with Tween-20, cadmium acetate, ammonium sulfate fractionation, treatment with calcium phosphate gel and chromatography on Sephadex G-50 to obtain 200-fold purification[71].

Cobra venom

AchE from Ringhals cobra venom was purified about ten-fold. The procedure consisted of chromatography on DEAE-cellulose and treatment with calcium phosphate gel[73].

Brain

AchE of brain tissue is associated with particulate material and the insolubility of the enzyme has made its purification difficult.

Many attempts have been made to solubilize and purify AchE from this tissue[74-80]. In most cases use was made of surface active agents to solubilize the enzyme. In some cases the unstable nature of the partially purified preparation made further work difficult[77]. The initial specific activity of AchE in caudate nucleus ranges between 10 to 25.

Ord and Thompson studied the effect of the homogenizing medium on the distribution of AchE activity of rat brain in the sediment and supernatant fluid[74]. They found that if the rat brain tissue is homogenized in water about 90% of the activity remains in the supernatant fluid. They used Lubrol-W, a surface active agent, as solubilizing agent and obtained a final specific activity of about 100.

A partially purified preparation was obtained by Got and Polya from sheep brain[76]. The specific activity of this preparation was about 1500. The sheep brains were frozen in liquid air and then extracted with sodium chloride and Lubrol-W. The extract was centrifuged and the supernatant was fractionated by ammonium sulfate, calcium phosphate gel and DEAE-cellulose chromatography. This preparation was accompanied by a yellow pigment.

The effects of several agents such as surface active agents, organic solvents (including toluene treatment similar to that of electric eel), enzymes and venoms and change of pH on the solubilization of AchE were studied by Lawler using acetone powder prepared from caudate

nucleus of calf brain[77]. All the solvents and surface active agents except toluene and ether inactivated the enzyme. Treatment with toluene and some enzymes was also ineffective. Better solubilization from acetone powder homogenate in 1 M glycine was observed at pH 8.8 than at 6.4. Venoms were of some use e.g. after incubation the homogenate with Agleistron p piscinnus protein 93 per cent of the AchE activity was in the supernatant fluid. The final solubilization procedure consisted of ultrasonication and incubation of the acetone powder homogenate with lipase. About 45 per cent of the activity was solubilized by this method. Fractionation with ammonium sulfate gave a final specific activity of about 93.[77]

A preparation with a specific activity of 1100 was obtained by Jackson and Aprison from calf caudate nucleus[78]. The same procedure could be applied to human and sheep caudate nucleus also. The procedure involves homogenization in water, freeze-drying, extraction with n-butanol at room temperature and with ether at 0°, extraction of the dried powder with glycine-NaOH at pH 9.0, fractionation with ammonium sulfate, treatment with toluene, gel filtration on sephadex G-200, fractionation with sodium chloride and two electrophoretic separations.

A purified preparation of AchE from human caudate nucleus was reported to have been obtained making use of Triton-X-100 as the solubilizing agent followed by fractionation on benezyl DEAE-cellulose, DEAE-cellulose,

Sephadex-G-25 and G-200. Further details of this brief abstract are awaited[79].

A preparation from cow brain with a specific activity of about 6800 was reported[80]. As in the treatment of electric organ, pieces of brain were treated with toluene in a refrigerator over a period of three months. This was followed by extraction with 5 per cent ammonium sulfate, fractionation on DEAE-cellulose and Sephadex G-200. The total purification achieved was 370-fold and the yields were 0.84%.

SECTION VI

Isoenzymes of acetylcholinesterase

Bernsohn, Barron and Hess have shown the presence of isoenzymes of AchE from human brain[81]. Homogenates of the caudate nucleus and putamen in saline were frozen and thawed and centrifuged. The supernatants were used for starch gel electrophoresis at pH 6.2. Four zones were obtained. Studies with acetylthiocholine, butyryl thiocholine and inhibitors such as eserine and mytelase suggested that one band was due to a cholinesterase and that three bands represented AchE. Further studies by the same authors on these three bands indicated no significant differences in their K_m or inhibition by DFP, eserine and mytelase[82]. On the basis of vertical zone electrophoresis studies with water soluble esterases from electric organ of <u>Electrophorus electricus</u> Ecobichon and Israel suggested the presence of at least two types of cholinesterases in this tissue[83]. These interesting observations require to be confirmed and studied in greater detail.

SECTION VII

Properties of Acetylcholinesterase

Physical properties

Electric eel enzyme - Due to the tendency of AchE molecule reversibly to aggregate or disaggregate under different conditions of ionic strength and pH values, a number of values for the sedimentation coefficients and molecular weight of AchE have been reported by different authors.

Values reported for the sedimentation coefficient are 60-80 S,[84] 4 S (0.1 M NaCl, 0.5% protein concentration)[63], 10.8 S[85], 10 S, 14 S and 65 S (at different ionic strengths, by sucrose density gradient centrifugation)[86,87]. Different S values depending on ionic strength have also been reported for AchE from the electric organ of Torpedo marmorata[88]. The values obtained are 14 S ($\Gamma/2 = 0.3$) and 10 to 80 S ($\Gamma/2 = 0.003$). Detailed studies were made recently by Grafius and Miller at different pH values (pH 4 to 10) and ionic strengths (0.1 - 0.5) using sucrose density gradient centrifugation[86,87]. The authors have concluded that there is a possibility of AchE existing in an aggregated form in the tissue and that it may be converted to smaller units by treatment by 5 to 30% ammonium sulfate in the extraction procedure. High ionic strength favors the formation of low molecular weight fractions.

The values of molecular weight reported for AchE of electric organ of Electrophorus electricus are 3×10^6,[60] 1.3×10^7 (probably an

aggregate of about 50 units)[84] and 2.5×10^5 (by Sephadex gel filtration)[85,65].

The diffusion coefficient of AchE was found to be 2.6×10^{-7} cm^2/sec. (4°) and 4.3×10^{-7} cm^2/sec. (20°)[85].

The absorption spectrum of electric eel AchE was found to be characterized by a maximum at 280 mμ and a minimum at 250 mμ. Distinct fine structures were observed at 290 mμ, 278 mu and 258 mμ. The ratio of the absorption at 280 mμ to that at 250 mμ was 1.67[85] indicating the absence of ultraviolet absorbing components other than protein in the enzyme.

Leuzinger and Baker have recently determined the amino acid analysis of purified AchE from electric organ of Electrophorus electricus[65].

Brain acetylcholinesterase - Different sedimentation coefficients observed for brain AchE are 6 to 10 S for sheep brain AchE[76] and 11.7 S and 6.6 S (for two different peaks) for calf caudate nucleus AchE[78].

Molecular weights of 284,000 to 360,000 for one peak and 161,400 to 204,000 for the second peak were reported for calf caudate nucleus AchE[78]. A molecular weight of 230,000 for human caudate nucleus AchE was found by sucrose density gradient centrifugation and gel filtration on Sephadex G-200[79].

Stability of acetylcholinesterase

The stability of AchE differs widely with the preparation but purified electric eel preparations are generally quite stable. The stability was dependent on the protein concentration and the presence of some ions. Purified AchE from electric eel is generally diluted with a solution containing Mg^{++} and 2 to 2.5 per cent gelatin.

A partially purified preparation from cattle erythrocytes lost about 14 per cent activity in 90 days at a protein concentration of 1.1 mg per ml. It was very unstable at lower protein concentrations[71].

The storage stability of cobra venom AchE was studied at different pHs and with different buffers. Maximum stability was observed in phosphate and citrate in the pH range of 7.5 to 8.9. In Tris at a similar pH range there was total loss in activity[73]. In the case of crude cobra venom preparation bicarbonate- Ringer solution showed maximum stabilizing properties as compared with water or salts (0.01 M NaCl or KCl). Ca^{++} and Mg^{++} were also shown to have some stabilizing effect[119].

A preparation of AchE obtained from the caudate nucleus of calf brain[77] was very unstable whereas a more purified preparation[78] obtained from the same source was quite stable when frozen and lost 10 to 20 per cent activity in two weeks at 4°.

Effect of organic solvents

Treatment of brain AchE with organic solvents in aqueous medium

was shown to result in complete inactivation of the enzyme.[77,114,115] Toluene and ether were the only solvents to which the enzyme was resistant. But when a lyophilized preparation from rat brain was treated with alcohol, acetone and petroleum ether there was no inactivation indicating that the lipids removed by light petroleum ether or acetone are not necessary for AchE activity[116].

Effect of surface active agents

The effect of surface active agents on brain AchE[117,118,77] depends on their concentrations and ionic nature. Generally non-ionic agents enhance the activity at concentrations in the range of 0.001 to 0.1 per cent. The activations observed with some of the non ionic agents were: Tween-80 (146%), Renex 690 (143%), Triton X-200 (133%). But the effect of Tween-80 was not observed on soluble and partially purified AchE from caudate nucleus of calf.

Other properties

Preparations of AchE from pig brain[75] and sheep brain[76] were yellow in color but further purification is required to determine whether the colored component is required for or influences activity.

Effect of sulfhydryl inhibitors

Ferricyanide, *o*-iodobenzoate, iodoacetate, *p*-chloromercuribenzoate and selenite had no effect on AchE of human erythrocytes[125].

Kinetics

Effect of substrate concentration

AchE exhibits marked substrate inhibition at higher concentrations of acetylcholine. A bell-shaped curve is obtained if activity is plotted against the log of the reciprocal of substrate concentration[12,77,78]. A sharp optimum substrate concentration is observed between 3 to 4 mM concentration of acetylcholine.

K_m determinations of AchE from electric eel, brain and erythrocytes have been made using different substrates at different temperatures and pH values. With acetylcholine as substrate K_m values of 5×10^{-4}, 1×10^{-4}, 4.5×10^{-4} and 1.4×10^{-4} M have been observed with electric eel enzyme by different authors[83, 96-98, 104]. With the same substrate K_m values of 3.3×10^{-4}, 8.7×10^{-4}, 1×10^{-4} and 8.5×10^{-4} M were observed for human, bovine and dog erythrocyte AchE respectively[99,100,101]. The K_m values for caudate nucleus AchE were 1.38×10^{-4} and 2.8×10^{-4} M for calf and human AchE respectively[78,79]. K_m values of 1.7×10^{-3}, 5.3×10^{-4} and 2×10^{-3} M have been reported for whole homogenate, supernatant and sediment of dog brain[101].

The K_m value for Electrophorus electricus enzyme with acetylthiocholine as substrate was 1.2×10^{-4} M and with acetic anhydride the K_m was 4×10^{-4} M.[98]

Effect of pH

A pH optimum between 8 to 8.5 has been observed for AchE of electric eel, cobra venom, brain and erythrocytes[78,89,102,103]. Optimum substrate concentration is a function of pH and it increases if the pH is shifted from the optimum[104].

Effect of temperature

The effect of temperature on the activity (V_{max}) and K_m have been studied using electric eel[97] and erythrocyte AchE[99,105]. Four different substrates - acetylcholine, dimethylaminoethyl acetate, methylaminoethyl acetate and aminoethyl acetate - were used to study the effect of temperature (5° to 35°) on electric eel AchE. Michaelis-Menton constants were independent of temperature. Arrhenius plots with the first two substrates showed curvature at higher temperatures but the latter two substrates gave linear plots. Very low energies of activation were observed. Between 25°-35°, Q_{10} = 1.3 was reported for human erythrocyte AchE[99].

These findings on the effect of temperature on AchE were explained on the basis of a two step mechanism of action namely acetylation and deacetylation of AchE, the deacetylation step having low energy of activation[97].

K_m was shown to be independent of temperature for erythrocyte AchE[99]. The energy of activation of human erythrocyte AchE was shown to be a function of temperature[105].

The energy of activation for calf caudate nucleus AchE was reported to be 4020 calories per mole[78].

Effect of ions and ionic strength on acetylcholinesterase

The role of monovalent ions such as Na^+ and K^+ and bivalent ions such as Ca^{++}, Mg^{++}, Mn^{++} as activators of AchE was supported by some workers[106-109]. But detailed studies by other workers using purified electric eel AchE have ruled out any effect of Mg^{++} or any other bivalent ions on AchE activity[110]. While this thesis was under preparation it was reported that crystalline AchE from E.electricus shows 40 per cent less activity in the absence of $MgCl_2$ (0.02 M)[195]. The effect of ionic strength between 0.17 to 0.56 was studied on the electric eel enzyme but no difference in activity was observed[111]. But increase in ionic strength was reported to result in the lowering of the optimum substrate concentration[106]. Activity determinations in phosphate, Tris, borate and citrate showed relative rates of 1.0, 0.83, 0.17 and 0.04 respectively. It was observed that freshly prepared AchE from electric eel is sensitized by Mg^{++} and desensitized by Mg^{++} chelating agents. This property is retained on storage as concentrated preparations but diluted preparations lose their sensitivity to Mg^{++} on storage[112]. Optimal velocity was shown to be increased with increase in ionic strength in the case of AchE of Torpedo marmorata[88]. Tris has been recently shown to inhibit electric eel AchE activity competitively[113]. AchE from calf caudate nucleus was reported to be activated by 0.5 M sodium chloride[78].

Turnover number and turnover time

Different values of turnover number and turnover time have been reported by different workers for electric eel, erythrocyte and brain AchE.

Turnover numbers of 3×10^7, 7.4×10^5, 4.9×10^5, 6×10^5 and 6×10^5 per minute have been obtained by different authors for electric eel AchE[60,120-122, 85]. The difference in the values may partly be due to differences in the methods of their determination and in the assumptions regarding the molecular weight of the enzyme.

A turnover number of 3×10^5 per minute was reported for erythrocyte AchE using acetylthiocholine as substrate[121]. The turnover numbers of human, sheep and calf caudate nucleus AchE were 20×10^5 and 4.06×10^5, 4.36×10^5 and 4×10^5 per minute respectively with acetylthiocholine as substrate[78,79].

The turnover time, as calculated by different workers was 81 µsec and 100 µsec for the electric eel enzyme[120,122] and 123 µsec, 304 µsec and 220 µsec for erythrocyte AchE[121,123,124]. Turnover times of 29 µsec, 147 µsec and 137 µsec for the caudate nucleus AchE of human, sheep and calf brain respectively using acetylthiocholine as substrate[78] and 142 µsec for human caudate nucleus using acetylcholine as substrate were obtained[79].

Specificity of acetylcholinesterase

The specificity of AchE with respect to the rates of hydrolysis

of a number of substrates was studied with the electric eel enzyme[89] and calf caudate nucleus AchE[78]. The acetate esters are preferentially hydrolyzed by AchE. The relative rates observed with electric eel AchE were acetylcholine (1.4), acetylthiocholine (7.0), acetyl-β-methyl choline (0.5), p-nitrophenyl acetate (0.15), phenyl acetate (3.2) and p-methoxy phenyl acetate (1.2). The relative rates for calf brain AchE were - acetylcholine (100), acetylthiocholine (170), acetyl-β-methyl choline (19), butyryl choline (2), propionyl choline (85), benzoyl choline (0), succinyl choline (0.85).

Stereospecificity - Major and Bennett[90] and Glick[91] observed stereospecificity in the hydrolysis of acetyl-β-methyl choline by AchE. Only the d-isomer was shown to be split by AchE. These studies were later extended by Hoskin and Trick using rat brain homogenate and racemic acetyl-dl-β-methyl choline as substrate and showed that only d-isomer is hydrolyzed by AchE, l-isomer acts as an inhibitor of hydrolysis of acetylcholine and acetyl-d-β-methyl choline by AchE[92a,b]. Augustinsson used enzymes from the electric organ of Torpedo marmorata, blood plasma and cobra venom and observed that AchE does not show selective substrate specificity with the mercapto analogue (acetyl-β-methyl thiocholine). Brain AchE was shown to preferentially split the l-isomer of mandelic acid choline ester[94]. The L(+)-isomer of lactoylcholine was shown to be hydrolyzed at a faster rate than the L(-) isomer by the electric eel enzyme. Hydrolysis of L(+) isomer was inhibited by D(-)-isomer of lactoylcholine[95].

SECTION VIII

Inhibitors of acetylcholinesterase

Inhibitors of AchE may be broadly divided into reversible and irreversible inhibitors. Two classes of compounds, carbamates and organophosphates form two groups of AchE inhibitors. Carbamates are reversible and organophosphates are irreversible inhibitors. Apart from these two major classes, there are several other compounds which inhibit AchE activity. A series of AchE inhibitors have been synthesized and their kinetics studied to find the structure near the active center of the enzyme. Minimum structural requirements for a compound to be an inhibitor of AchE have been established[126] as "locus of high electron density separated by roughly a $-CH_2-CH_2-$ unit distance from a polymethylated nitrogen atom (preferably quaternary)". Mono, di and tri- quaternary ammonium compounds are inhibitors of AchE to a smaller or greater extent. Based on the concept of two step mechanism of action of AchE, Wilson has divided AchE inhibitors into prosthetic and oxydiaphoric or acid transferring inhibitors[127]. Inhibitors of the former class form reversible addition complexes with the enzyme. Quaternary ammonium ions and acetylcholine belong to this class of inhibitors. Alkyl phosphates and carbamates are the two major classes of compounds which belong to exidiaphoric type.

Reversible inhibitors

Eserine, prostigmine, isolan, Nu 1250, sevin, choline and butyryl choline are some of the common reversible inhibitors of AchE and have been studied widely. They all inhibit AchE competitively. Inhibition by eserine increases with decrease of pH but prostigmine inhibition is independent of pH. The Ki values for eserine and prostigmine are 6.1×10^{-8} and 1.6×10^{-7}M respectively[96]. The I_{50}* value for choline is 0.033 M for AchE of erythrocytes[129]. I_{50} value for butyryl choline is 2.5×10^{-3}M with the electric eel enzyme[172]. Diquaternary ammonium salts are a 100 to 1000 times more potent inhibitors of AchE as compared with monoquaternary ammonium salts[130]. I_{50} values of some of the quaternary ammonium compounds are, tetramethyl ammonium iodide (2.5×10^{-2}M), d-tubo curarin chloride (9×10^{-4}M), stilbamidine-di-isothionate (6.5×10^{-5}M) and 1:10 decane bis (trimethyl ammonium bromide) (2.5×10^{-5}M). I_{50} value for hexamethonium ions with dog brain AchE was 2.1×10^{-2}M and for dog and human erythrocytes were 3.2×10^{-2}M and 2.4×10^{-2}M respectively[131]. From the studies of inhibition by a series of methyl and n-heptyl trimethylammonium ions linearity has been established between the p_{I50}* values and number of carbon atoms in the alkyl chain[132]. Inhibition of bovine erythrocyte AchE by a homologous series of alkyl trimethyl ammonium ions for C_1 to C_{12} was studied and a gradual decrease in the Ki values was observed

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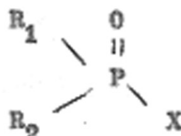
*I_{50} is concentration of the inhibitor for 50 per cent inhibition and p_{I50} is $\log \frac{1}{[I]}$.

with increase in chain length e.g. Ki values with C_1, C_5 and C_{12} were found to be 23.3×10^{-4}, 13.2×10^{-4} and 0.52×10^{-4}M respectively[133]. Anti-AchE activity of a homologous series of trimethyl (phenylalkyl) ammonium ions increases as the number of rings in the compound increases upto three and then decreases[134]. A series of mono- and diquaternary ammonium ion inhibitors were studied with erythrocyte AchE and it was observed[135] that the Ki values in both the series increased with increase in chain length e.g. monoquaternary ammonium ions series. Ki for four-CH_2 (1.7×10^{-2}M) for ten-CH_2- (Ki = 7.0×10^{-2}M) and in the case of diquaternary ammonium ions, with three- CH_2-Ki = 1.1×10^{-2}M and with twelve -CH_2- Ki = 2.1×10^{-2}M. Reversible inhibition of AchE has also been observed with tertiary amines such as banthine and meperidine[136], 1,2,3,4 tetrahydro-5-aminoacridine[137], thiophosphoric acids such as diphenyl chlorothiophosphate and di-p-tolyl chlorothiophosphate[138], nitromin, armite[139], deoxycholate (37°, 1 to 3 hr incubation)[140], muscarine and muscarone[141,142] (most potent is o-acetyl muscarine) derivatives, tubocurarine[143], cetyltrimethyl ammonium bromide[144], psychotropic compounds[145] such as chloropromazine, secergen, thiopropazate, procaine, dibucaine (anesthetics)[146], aliphatic trimethyl betaines (nitriles, amides and amines)[147], copper and nickel metal chelates (1-1-Cu and 2-1-Ni chelates) of glycine and ethylenediamine[148,149], 7-chloroheptyl phosphonate[150], dimethyl sulfoxide (0.5 M - 44% 1 M - 85% inhibition)[151] and sodium fluoride[151a].

Irreversible inhibitors

Organophosphates, compounds with a general formula



inhibit AchE irreversibly (R_1 and R_2 are alkyl groups and X is either -F, -CN, $-OC_6H_4$ or $-NO_2$). The best known compounds of this class are diisopropyl fluorophosphate (DFP), tetraethylpyrophosphate (TEPP), sarin, tubun and soman. During inhibition by organophosphates the AchE molecule gets phosphorylated[152] and dephosphorylation is an extremely slow process. Similar to the acetylated enzyme phosphorylated AchE is susceptible to nucleophilic attacks, but the compounds required to bring about dephosphorylation should be very strong nucleophilic agents e.g. reactivation of organophosphate inhibition has been so far achieved by choline, hydroxylamine[153], 2-pyridine-aldoxime methiodide (2PAM)[154] (and to a lesser extent its 3- and 4-isomers) and monoisonitrosoacetone. Reactivation by 2-PAM is depressed by the presence of sodium, potassium or magnesium chloride and acetylcholine[155]. Ageing of phosphorylated AchE is reported to decrease the reactivability of the enzyme and the rate of ageing increases on decreasing the pH from 7 to 6 and on addition of thiourea[156]. Some other reactivators of phosphorylated AchE are dichloride of bis (4-hydroxyiminomethylpyridinomethyl) ether[157], 2-hydroxyiminoformyl-N (γ-hydroxy propyl) pyridinium bromide[158], sodium fluoride[159] (for sarin inhibited AchE). Recently participation of metal ions in the reactivation of phosphorylated AchE by monoisonitrosoace-

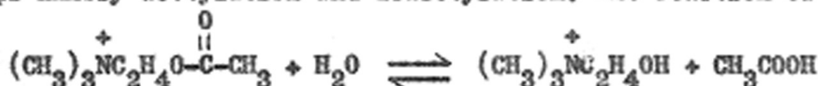
tone in cat brain has been suggested because EDTA prevented the reactivation and addition of Mg^{++}, Co^{++} or Ni^{++} stimulated it[160]. Some other compounds which inhibit AchE irreversibly are parathions and their analogues[161], tertiary amines such as chloromazine and promethazine[162], o-Et S-(2-chloroethyl) ethylphosphothionate (I_{50} = 5.8×10^{-8} M)[163], armine[164], N-N'-dimethyl-2-phenylaziridinium (DPA)[164a]. Carbomylation (by carbomylcholine)[165] and sulfonylation (by sulfonic acid esters)[166] of AchE also inhibited AchE.

SECTION IX

Mechanism of action and active sites of acetylcholinesterase

Elegant kinetic studies on AchE with a number of substrates and inhibitors under different conditions of pH and temperature have contributed to the elucidation of the inner structure of AchE and its mechanism of action.

The hydrolytic action of AchE has been shown to proceed through two steps namely acetylation and deacetylation. The reaction of AchE is:

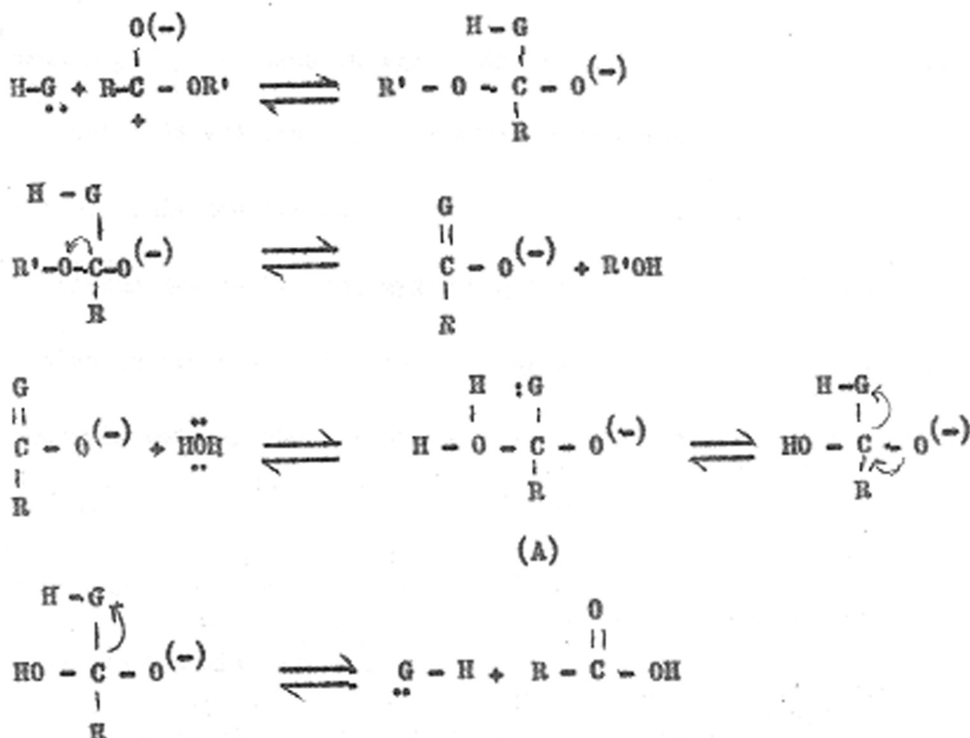


$$(CH_3)_3\overset{+}{N}C_2H_4O-\overset{O}{\overset{\|}{C}}-CH_3 + H_2O \rightleftharpoons (CH_3)_3\overset{+}{N}C_2H_4OH + CH_3COOH$$

Most of the studies were made with purified enzyme preparations either from electric eel or erythrocytes.

Wilson and Bergmann in 1950 observed the pH dependence of acetyl choline and dimethylaminoethyl acetate hydrolysis, eserine inhibition and tetraethyl pyrophosphate inhibition and suggested the presence of an esteratic site and an electrically negative site[167]. The esteratic site was also suggested to consist of basic and acidic groups, pKa values of basic group (=7.2) and acidic group (=9.3) were calculated and the presence of imidazole and ammonium groups in the esteratic site was suggested[168,172]. In contimuation of these studies inhibition of AchE by several classes of compounds such as amino acids, ketones, nitriles, amides, dipeptides and esters was studied which led to the suggestion of formation of an intermediate[169] (A - in the following Scheme-1). Based

on these studies the following probable mechanism was suggested[170].



Scheme-1

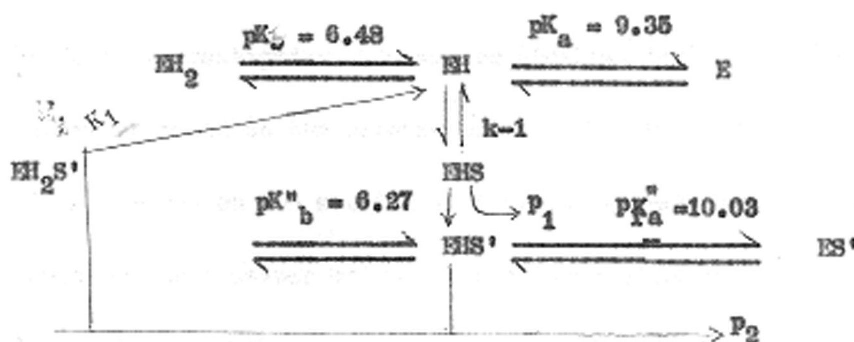
GH : Active enzyme surface containing basic group and an acid group.

G-H is part of an active enzyme surface which contains an acid and basic group and formation of an intermediate A is the rate limiting step.

Studies on mono- and diquaternary ammonium salts led to the suggestion of two anionic sites in the AchE molecule[130]. The presence of two anionic sites was taken to explain the mechanism of acetylcholine inhibition of AchE. It was suggested that one substrate molecule is attached to one anionic site and esteratic site and the second substrate

molecule attaches to the second anionic site. The enzyme substrate complex, ES, requires energy of activation for transformation to activated ES and the second substrate molecule may interfere at this stage. This concept was supported by other workers[22,171,172]. Histidine and either the hydroxy group of tyrosine or ϵ-amino group of lysine in the esteratic site and carboxyl group of either aspartic or glutamic acid in the anionic site were suggested[172]. The work of Coleman and Eley with erythrocyte AchE on inhibition by a series of mono- and diquaternary ammonium salts could not show the presence of a second anionic site[135].

Detailed studies made by Krupka and Leiderer[174,175a-d] on erythrocyte AchE using acetylcholine and N-methylaminoethyl acetate (MAEA) as substrates and cis-2-dimethylamino cyclohexanol as inhibitor have contributed to the understanding of the mechanism of action of AchE. The following mechanism was suggested[174].



Scheme 2

EH: Free enzyme; EHS: Michaelis complex; EHS': Acetylated enzyme.

pK values of ionising groups in the free enzyme are 6.48 and 9.35. These ionisations are completely suppressed in the Michaelis complex. The EHS complex is formed through EH_2S and ES which are "non-existent". In the acyl enzyme these ionisations appear again and the pK values are then 6.27 and 10.03. The group with pK = 6.5 was suggested to be imidazole and the one with pK = 9.35 is ω-amino or phenolic group. The transfer to the serine group and subsequent deacylation are presumably controlled in a very critical way by the spatial arrangement of the various groups at the active center. It is concluded that in the acetyl enzyme the anionic site is free while in the Michaelis complex it is not.

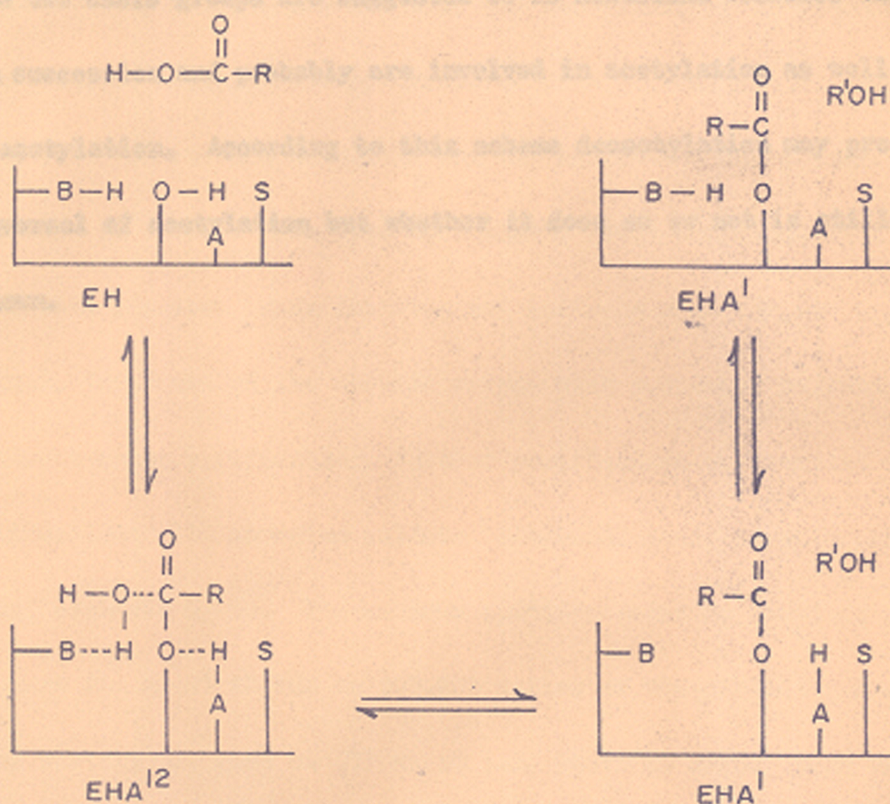
Based on these concepts it was suggested that AchE can be inhibited in two ways, one by the inhibitor getting attached to the acetyl enzyme and thus blocking deacetylation as is done by *cis*-2-dimethylamino cyclohexanol or acetylcholine, giving rise to mixed (competitive and noncompetitive) inhibition and second without blocking deacetylation as is done by prostigmine, eserine or choline. But the substrate can also become attached to the anionic site and block the deacetylation[176].

Based on the studies of inhibitors such as curare and stilbamidine the distance between two anionic sites was calculated as 14 Å. The distance of the acidic group is 2.5 Å and that of the basic group is 5 Å from the anionic site. The distance between anionic site and esteratic site was calculated to be 2.5 Å[173].

Different aspects of mechanism of AchE action have been reviewed by Krupka[177]. In summary it may be stated that the structure of AchE is complementary to acetylcholine as trimethylammonium ion vs negatively charged anionic site and an ester linkage vs an esteratic group. During acetylation, phosphorylation, carbomylation or sulfonylation of AchE, serine hydroxyl is involved. An imidazole nitrogen atom withdraws a proton from a serine hydroxyl group which then makes a nucleophilic attack on the substrate or inhibitor and as a result serine hydroxyl will become acetylated. Deacetylation may be a general imidazole catalysis of a nucleophilic attack by water upon the esterified serine resulting in its hydrolysis. The proposed mechanism of action may be presented as in Scheme-3.

Substrate inhibition is explained on the basis of only one anionic site and the substrate is suggested to bind to the acetyl enzyme[178]. It has been suggested that in acetylcholine the carbonyl oxygen atom may interact with the quaternary nitrogen atom to form a six membered ring, thus making it an inhibitor[179].

Studies on purified bovine erythrocyte AchE with phenylacetate, isoamyl acetate and number of acetylcholine analogues as substrates and tetra- and trimethyl ammonium ions as inhibitors at different pH values suggested the presence of two basic groups[180a,b]. These two groups are catalytically active when unprotonated. One of them having pK$\sim$5.5 is 9Å



SCHEME-3

B- Basic group, OH- serine hydroxyl, AH - acidic group in the esteratic site, S- anionic site, R-C(=O)-OH' - substrate, EHS[12] - Michealis complex in AchE-MAEA (N-methyl aminoethyl acetate) system. EHA', EHA[12] - acetylated enzyme.

EH - The free enzyme.

and other with pK=6.3 is within 5 Å from the anionic site. The anionic site is said to be protonated at pH 4 to 4.5 and is probably a side chain carboxyl group. A fourth group which functions catalytically when protonated is postulated to be more than 10 Å from the anionic site. The presence of an intermediate in the formation of an acetyl enzyme from enzyme-substrate complex was also suggested recently[181]. The two basic groups are suggested to be histidine residues which work in succession and probably are involved in acetylation as well as deacetylation. According to this scheme deacetylation may proceed as reversal of acetylation but whether it does so or not is still not known.

SECTION X

PRESENT WORK

The work reported in this thesis deals with the preparation of soluble AchE from the particulate enzyme of ox brain, its purification and the study of its properties and kinetics.

The solubilization procedure is based on earlier studies in this Laboratory which indicated that the hexokinase of ox brain can be obtained in soluble form by treatment with crystalline elastase.[182a,b] This method was studied in detail and the conditions for obtaining ox brain AchE in soluble form were determined. After treatment with crystalline elastase brain AchE was not sedimented at 100,000 x *g* in one hour. Treatment of the soluble enzyme with protamine sulfate facilitated subsequent purification. Further purification was carried out by fractionations with ammonium sulfate, DEAE-cellulose, calcium phosphate gel and Sephadex G-200. The maximum specific activity obtained by this procedure was about 80,000 to 100,000 μmoles of acetylcholine hydrolyzed per mg protein per hour at 37° at pH 7.5, which is higher than that reported hitherto for mammalian brain AchE.

The properties and kinetics of the enzyme which were studied include the effect of pH, temperature, concentration of acetylcholine, acetyl-β-methyl choline, the stability of the enzyme and a new observation

on its inactivation when frozen in the presence of phosphate and thioethanol. The inhibition of the enzyme by eserine, prostigmine, butyrylcholine and sodium fluoride were studied and the Ki values for these compounds were determined.

Chapter II of the thesis deals with the materials and experimental methods used in these studies.

Chapter III deals with the preparation of soluble AchE from the particulate enzyme of ox caudate nucleus and the purification of soluble AchE.

Chapter IV deals with the properties and kinetics of purified ox brain AchE.

Chapter V deals with the discussion of the results of these studies.

Chapter VI contains a summary of the results and conclusions of this work.

A bibliography is presented at the end.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

Materials

The following chemicals were of analytical grade: potassium dihydrogen phosphate, sodium pyrophosphate, ammonium sulfate, magnesium chloride, sodium chloride, Tris, sodium acetate, sodium barbiturate, acetic acid, potassium chloride, EDTA, 2-mercapto ethanol, sodium fluoride, hydrochloric acid, hydroxylamine hydrochloride. Acetylcholine bromide was of "Biochemical" grade supplied either by British Drug Houses or Koch-Light & Co. Acetyl-β-methyl choline iodide and butyrylcholine iodide were from Sigma Chemical Co. Phenylacetate was synthesized in this laboratory. Prostigmine bromide was obtained from Hoffman-La Roche and eserine sulfate from E.Merck. 2-Pyridine-aldoxime methiodide was from Sigma Chemical Co. Protamine sulfate was from Sigma Chemical Co., and salmine sulfate from British Drug Houses. Protamine sulfate and salmine sulfate were interchangeably used.

Calcium phosphate gel was prepared according to the procedure of Swingle and Tiselius[183]. The gel was finally suspended in water (60 mg per ml dry weight). DEAE-cellulose was either prepared according to Peterson and Sober[184] (100 to 200 mesh 0.5 meq per g) or was obtained from Bio-Rad Laboratories ("Cellex-D", 0.69 meq per g). Amberlite IRC-50 (XE-64) (mesh 200 to 400) was obtained from Rohm and Haas. Carboxymethylcellulose and cellulose phosphate were obtained from Bio-Rad Laboratories. DEAE-Sephadex A-50 and Sephadex G-200 were from Sigma Chemical Co.

Twice crystallized elastase from pancreas was obtained from Sigma Chemical Co.

The author wishes to thank Dr. B. V. Ramachandran, Dr. D. R. Sanadi (Boston), Dr. A. D. Joshi, Dr. S. S. Subramanian, Dr. J. H. Balwani; Hoffman La-Roche and Sigma Chemical Co. for generous gifts of chemicals.

The author is also grateful to the authorities of Bombay Slaughter House for their cooperation and Dr. Mysorekar for demonstrating the isolation of caudate nucleus.

Methods

Spectrophotometric determinations were carried out in a model DU Beckman spectrophotometer with silica cuvettes of 10 mm light path. pH estimations were carried out with the glass electrode. The pH of ammonium sulfate solutions was determined after diluting four times with water.

Ammonium sulfate saturations refer to 0° and were calculated according to Jagannathan *et al.*[185] The following equations were used for convenience to calculate ammonium sulfate saturations:

1. For solid ammonium sulfate-

$$X = \frac{50(S_2-S_1)}{1 - 0.28S_2}$$

2. For saturated ammonium sulfate solution -

$$Y = \frac{100(S_2-S_1)}{1 - S_2}$$

where X is the g of solid ammonium sulfate to be added for every 100 ml, Y is the ml of saturated ammonium sulfate solution to be added for every 100 ml. S_1 is the initial saturation and S_2 is the required saturation of ammonium sulfate at 0°. Solid ammonium sulfate was slowly added over a period of about half an hr with gentle stirring but without allowing the liquid to froth. It was then kept for about 30 min and centrifuged at 14,000 x g for 45 min. The precipitates were dissolved in a known volume of buffer and the final volume was then noted. The increase in volume was assumed to be due to ammonium sulfate at the saturation at which the

precipitate was obtained and a correction was made for the ammonium sulfate concentration in the enzyme.

All the chromatographic columns were prepared under gravity and packed only by hydrostatic pressure, no external pressure being applied during operation of the column.

Centrifugations were carried out at 0° in an International Centrifuge (Model PR-1 and PR-2), Sorvall (Model SS-1) and Spinco (Model-L). Chromatographic fractions were collected on a Technicon Automatic fraction collector. Warburg apparatus of Gilson Medical Electronics was used.

All glassware was routinely washed with sodium carbonate and then with nitric acid, rinsed successively with tap water, distilled water and glass-distilled water and dried. The use of chromic acid or detergents was avoided. No grease or silicone was used for ground glass joints and stopcocks in chromatographic columns. All purification operations were carried out at 0° unless otherwise stated. Glass-distilled water was used for the preparation of all solutions.

Protein was routinely determined by the following method. A modification of the method of Warburg & Christian was used[186]. A correction for nucleic acid and other ultraviolet absorbing impurities was made by the following empirical equation[185]. It was assumed that a 0.1 per cent protein solution has an optical density of 1 at 280 mμ.

$$\frac{4}{7} \times \left[2.3\,(\text{O.D.}_{280\ m\mu} - \text{O.D.}_{340\ m\mu}) - (\text{O.D.}_{260\ m\mu} - \text{O.D.}_{340\ m\mu})\right]$$

$$= \text{mg protein per ml}$$

Enzyme solutions were diluted (if necessary) in 0.1 M phosphate, pH 7.5 and the optical densities at 340, 280 and 260 mμ were determined. A buffer of the same composition was used as blank.

As a check for the above method protein was also estimated by the method of Lowry *et al.*[187] Crystalline bovine serum albumin was used as the standard. The concentration of serum albumin was calculated from its extinction coefficient at 280 mμ according to Long[188]. Enzyme free from ammonium sulfate and containing only low concentrations of phosphate was used for this method to avoid interference from these substances. The protein values obtained by the two methods were in good agreement with each other.

Definition of unit of activity and specific activity

The unit of AchE activity is defined as the amount of enzyme required to hydrolyze one μmole of acetylcholine in one hour at 37° and pH 7.5 under the experimental conditions given in the text. The specific activity of the enzyme is defined as the activity per mg of protein.

The results of some activity determinations, especially on enzyme kinetics, are presented as amount of CO_2 evolved in 30 min instead of μmoles of acetylcholine hydrolyzed.

Dilution of enzyme for estimation

Crude AchE was diluted at 0° with 0.05 M phosphate buffer, pH 7.5, and the purified enzyme in 0.1 M phosphate buffer, pH 7.5, containing 0.1 per cent serum albumin unless otherwise stated.

Estimation of acetylcholinesterase from brain

The following three methods were used for the estimation of AchE activity. A few of the precautions, controls and other salient features of each method are also noted here in order to avoid repeated reference to these details for each experiment in the section on enzyme kinetics.

1. Hestrin's colorimetric method[55] (Assay-I)

This method has been used routinely for assaying AchE during purification of the enzyme and for the study of some of its properties. The details of the method are as follows except when stated otherwise.

The assay system consisted of potassium phosphate buffer, pH 7.5 (75 μmoles), NaCl (195 μmoles), $MgCl_2$ (45 μmoles), acetylcholine bromide (8 μmoles) and enzyme in a final volume of 1.5 ml. The final pH of the reaction mixture was 7.5 and the temperature 37°. The reaction was initiated by adding enzyme and the reaction mixture was incubated for 30 min. The amount of acetylcholine hydrolyzed was not greater than 25 to 30% of the initial quantity. The reaction was stopped by adding 3 ml of alkaline hydroxylamine (prepared by mixing equal volumes of 3.5 M NaOH and 2M hydroxylamine hydrochloride). This was followed after a minimum of one min by 1.5 ml of hydrochloric acid (concentrated HCl diluted 1.2 to 3.0 ml) and 1.5 ml of 0.37 M ferric chloride (prepared in 0.1 N HCl). The color was read at 540 mμ. If the optical density readings exceeded 0.4, the solutions were diluted 2-fold with 0.074 M ferric chloride in 0.1 N HCl before measuring the color. An optical density change of 0.105 was taken as equivalent to one μmole of acetylcholine hydrolyzed. A blank with substrate alone without enzyme was

always run (blanks with enzyme alone were negligible). Controls for non-enzymic hydrolysis were also run and were negligible except at pH above 9. For each variation in experimental conditions (e.g.determining the effect of some compounds on activity) controls were run to ensure that the compound did not interfere with the color. In the case of reaction mixtures containing more than or equal to 100 µg of protein, the samples were filtered before taking the readings. In experiments with different pHs acetate buffer was used at pH 6, phosphate at pH 7 to 7.5 and barbiturate buffer at pH 8.5 to 9.5. The concentrations of buffer in all the cases were the same as stated for the standard assay system. This method will be referred to as Assay-I.

2. Manometric method (Assay-II)

This method was used for all the kinetic studies of brain AchE with change in substrate concentration. The reaction mixture consisted of $NaHCO_3$ (93 µmoles), NaCl (390 µmoles), $MgCl_2$ (90 µmoles), acetylcholine bromide (9 µmoles) and enzyme in a final volume of 3 ml. Acetylcholine was in the side arm. The final pH was 7.5 and the temperature was 30°. The flasks were gassed with 95% O_2 + 5% CO_2 for 5 to 10 min and equilibrated for 5 min before the substrate was tipped in. Readings were taken every 10 min. Controls for non enzymic hydrolysis of substrate were always run and the results corrected for the blanks. This method will be referred to as Assay-II.

3. Spectrophotometric method (Assay-III)

The hydrolysis of phenylacetate results in an increase in absorption at 270 mµ. This is made use of for a rapid and convenient method to test the activity routinely during fractionation of the enzyme.

The reaction mixture consisted of potassium phosphate buffer (20 μmoles), NaCl (100 μmoles), $MgCl_2$ (30 μmoles), phenyl acetate (1 μmole) and enzyme in a final volume of 1.0 ml. The pH was 7.5 and the temperature 30°. The stock solution of 5 mM phenylacetate in water was freshly prepared everyday. The change in optical density per minute was recorded and was kept between 0.015 to 0.025. Readings were taken only upto the Δ O.D. of 0.5 as they were not linear beyond this.

From the optical density values of different concentrations of phenol and phenylacetate at 270 mμ and also from the change in optical density on complete hydrolysis of one μmole of phenylacetate enzymically, it was calculated that an optical density change of 1.65 corresponds to the hydrolysis of one μmole of phenylacetate under the assay conditions.

This method will be referred to as Assay-III.

EXPERIMENTAL AND RESULTS

CHAPTER 3

SOLUBILIZATION AND PURIFICATION

SECTION I

Solubilization

Crystalline elastase was earlier shown to be effective in bringing hexokinase from ox heart and brain in solution from the particulate preparations[185]. Its use for bringing AchE from brain tissue in solution was, therefore, studied. From this point of view several experiments were carried out to explore the conditions which will bring AchE from brain tissue in solution by treatment with crystalline elastase.

All operations were carried out at 0°. Phosphate buffers were of potassium salts unless stated otherwise.

The effect of composition of extracting media on sedimentation of acetylcholinesterase activity

70 g of gray matter of ox brain were homogenized in a Waring blendor for 3 min with 210 ml of media of different compositions and in one case with 700 ml of glass distilled water. The homogenates were centrifuged at 800 x *g* for 20 min. The sediments and the supernatants were tested for activity. The results are presented in Table 1.

The ratio of the activity of AchE in the sediment to that in the supernatant was about 2:1 in the case of (0.1 M phosphate, pH 7.6), (0.1 M phosphate + Tris pH 8.0) and (0.1 M phosphate pH 6.8 + 0.005 M EDTA). The ratio was about 2.5:1 in the case of (0.1 M phosphate pH 6.8), (0.1 M phosphate + 0.01 M magnesium chloride pH 7.6) and (0.2 M sodium bicarbonate). The ratio in the case of 0.3 M sucrose was 3.3:1. There was more activity in the supernatant liquid than in the sediment only in the case of the water extract.

TABLE 1

EFFECT OF COMPOSITION OF EXTRACTING MEDIUM ON SEDIMENTATION OF BRAIN ACETYLCHOLINESTERASE ACTIVITIES

70 g of gray matter from ox brain were used in each case

No.	Medium of extraction	Total activity Sediment	Supernatant	Ratio of activities sed/supt.
		units	units	
1.	Water	1680	3220	1:2
2.	0.1 M phosphate pH 7.6	3220	1680	2:1
3.	0.1 M phosphate, Tris to make pH 8.0	3300	1600	2:1
4.	0.1 M phosphate pH 6.8 + 0.005 M EDTA	3220	1600	2:1
5.	0.1 M phosphate pH 6.8	3560	1340	2.5:1
6.	0.1 M phosphate + 0.01 M $MgCl_2$ pH 7.6	3500	1400	2.5:1
7.	0.2 M sodium bicarbonate	3680	1300	2.5:1
8.	0.3 M sucrose	3680	1100	3.3:1

Preparation of particulate enzyme

The following procedure was routinely used for the preparation of the particulate enzyme. The tissue (ox or sheep gray matter) was homogenized in a Waring blendor with 3 ml of 0.1 M phosphate buffer, pH 6.8 per g of tissue for 3 min and the homogenate was centrifuged at 800 x g for 20 min. The sediment was washed with the same volume of buffer and centrifuged as before. The sediment was then suspended in 0.1 M phosphate, pH 7.5, and was homogenized in a glass homogenizer to break up the lumps. The final volume of the suspension was made up to 3 ml per g of initial tissue. It was then mixed with one-fifth of its volume of 0.09 M sodium pyrophosphate-HCl, pH 8.5. This served as a standard particulate preparation for further studies.

Solubilization

Treatment with elastase - 3.2 mg of crystalline elastase were added to the particulate preparation obtained from 50 g of the tissue (sheep or ox brain gray matter). This was kept at 0° for 24 hr and then frozen and thawed 5 or 6 times over a period of about a week. Freezing was done at -20° and thawing was carried out slowly at room temperature taking care that the temperature did not exceed 5°. It was centrifuged at 14,000 x g for 1 hr and the supernatant was tested for activity (Table 2). About 12 per cent of the activity from ox brain and 8 per cent from sheep brain was present in the supernatant liquid. The two supernatant liquids from ox and sheep brain were recentrifuged at 100,000 x g for one hr and the supernatant liquids were retested for activity. All the activity was present in the supernatant liquid. A part of AchE of brain from the two species was, therefore, obtained in soluble form by elastase treatment. Preliminary tests also showed that the soluble enzyme was stable on

TABLE 2

SOLUBILIZATION OF ACETYLCHOLINESTERASE FROM GRAY MATTER OF OX AND SHEEP BRAIN

Source	Fraction	Volume	Activity	Total activity	Protein	Specific activity
		ml	units/ml	units	mg/ml	
Ox	Insoluble	10	25	250		
	Soluble	6	5	30	4.4	1.1
Sheep	Insoluble	10	72	720		
	Soluble	6	10	60	4.6	2.5

storage at -20° for several days.

In the following description "insoluble enzyme" refers to the standard particulate preparation and "soluble enzyme" to the 14,000x *g* supernatant liquid.

Activity of acetylcholinesterase in ox and sheep brain

The activities of AchE in gray matter and caudate nucleus of ox and sheep brain were tested. For this purpose caudate nucleus was removed first from the whole brain and then gray matter was removed by scraping out from the brain. Connective tissue was removed from the gray matter as much as possible.

The tissues were homogenized separately with three volumes of 0.1 M phosphate buffer, pH 6.8, for 3 min in a Waring blender. The homogenates were used for determining the activity. The results are shown in Table 3. In view of the high activity of caudate nucleus of ox brain this material was chosen for most of the subsequent work.

Solubilization of acetylcholinesterase from caudate nucleus of ox

The particulate preparation from caudate nucleus was prepared by the same procedure as described earlier in the case of gray matter. The particulate suspension was divided into two parts. To one part elastase was added (in the proportion of 3.2 mg of elastase for particulate fraction from 50 g of tissue) and to the other no elastase was added. Both were kept at 0° for 24 hr and then frozen and thawed 5 to 6 times as described earlier. The enzyme treated with elastase during the course of freezing and thawing changed its appearance and became more viscous and jelly-like than the one without elastase.

10 ml of each of the preparations were then centrifuged at 14,000 x *g* for 1 hr and the supernatant fluids were tested for activity.

TABLE 3

ACTIVITY OF ACETYLCHOLINESTERASE IN BRAIN

Source		Activity
		units/g
Ox	Gray matter	70
	Caudate nucleus	1900
Sheep	Gray matter	420
	Caudate nucleus	840

It can be seen from Table 4a that 14,000 x g supernatant contains about 2 per cent of the activity from the preparation to which no elastase was added whereas about 8 per cent activity was present in the 14,000 x g supernatant from the preparation to which elastase was added. Both the 14,000 x g supernatant fluids were then centrifuged at 100,000 x g for 1 hr and the supernatant fluids were tested for activity. The results are shown in Table 4b. It is evident from this experiment that the activity obtained in the 14,000 x g supernatant without addition of elastase was not a truly soluble enzyme and was sedimented completely when centrifuged at 100,000 x g for 1 hr. The activity obtained in the 14,000 x g supernatant after treatment with elastase on the other hand did not sediment after centrifugation at 100,000 x g for 1 hr and may be regarded as a soluble enzyme.

The AchE activity which was brought into solution was low (about 7% to 10% of the particulate fraction). Several different experiments were, therefore, tried to increase the yields of soluble enzyme.

Successive treatments with elastase

In one batch the particulate enzyme obtained after the first treatment was given two more similar treatments with elastase. The 14,000 x g sediment was suspended in 0.1 M phosphate pH 7.5 and homogenized in a glass homogenizer. Final volume was made upto three volumes of the original weight of the tissue and the suspension

TABLE 4a

SOLUBILIZATION OF ACETYLCHOLINESTERASE FROM CAUDATE NUCLEUS OF OX

Fraction	Volume	Activity	Total activity	Protein	Specific activity
	ml	units/ml	units	mg/ml	
Insoluble	10	448	4480		
Elastase treated	6	53	318	2.2	24
Without elastase treatment	6	14	84	1	14

TABLE 4b

SOLUBILIZATION OF ACETYLCHOLINESTERASE FROM CAUDATE NUCLEUS OF OX

Fraction	Activity of AchE in the supernatant fluid					
	14,000 x *g*			100,000 x *g*		
	Volume	Activity	Total activity	Volume	Activity	Total activity
	ml	units/ml	units	ml	units/ml	units
Elastase treated	10	70	700	9.5	70	665
Without elastase	10	17	170	9.5	0	0

was mixed with one-fifth volume of 0.09 M sodium pyrophosphate pH 8.5. 3.2 mg of elastase per 50 g of tissue were added and it was kept at 0° for 24 hr. It was then frozen and thawed as stated earlier. A total of about 28% of the activity was brought into solution in three such treatments. It may be noted that successively lesser amounts of AchE activity were solubilized in successive treatments with elastase. The results are presented in Table 5.

Only two treatments with elastase were routinely given. The soluble enzyme from both was pooled together and used for further work. Further treatment was not used routinely due to the danger of bacterial contamination.

Though the enzyme was obtained in soluble form by elastase treatment, the yield was rather low and was only 28% after three elastase treatments. Several modifications were tried either in the conditions for digestion with elastase or for preparation of the particulate enzyme. These results are summarized in the following sections.

(1) Concentration of enzyme and elastase

In one experiment the particulate preparation was diluted five times with 0.1 M phosphate-pyrophosphate and divided into two parts. To one was added the usual quantity of elastase and to the other ten times more elastase was added. They were kept at 0° for 24 hr, then frozen and thawed as usual and the activity of the soluble enzyme was determined as before.

The dilution of the particulate preparation was carried out since the thick particulate suspension may not mix thoroughly

TABLE 5

EFFECT OF SUCCESSIVE TREATMENTS WITH ELASTASE ON SOLUBILIZATION OF ACETYLCHOLINESTERASE

Fraction	Volume	Activity	Total activity	Protein	Specific activity	Yield of soluble activity
	ml	units/ml	units	mg/ml		%
Insoluble	2040	344	701,760			
Elastase treated						
I	1500	71	106,500	4.4	16	15
II	1250	53	66,250	3	18	9
III	1000	30	30,000			4

with the insoluble crystalline elastase. The suspension was also shaken occasionally. The results are presented in Table 6.

A greater amount of enzyme was solubilized with a large excess of elastase. Due to the high cost of elastase the usual amount of elastase (3.2 mg for 50 g of tissue) was, however, used in later work. The amount of enzyme activity solubilized with the diluted enzyme was higher than that with undiluted enzyme, but due to the inconvenience of handling large volumes, further work was carried out only with undiluted particulate enzyme.

(2) Insoluble enzyme obtained by water extraction

It will be seen from Table 1 that if the tissue is extracted with water instead of a salt solution about 70 per cent of the AchE activity of brain remains in the 800 x *g* supernatant. An attempt was made to sediment the enzyme by the addition of salt to this supernatant liquid to obtain an insoluble fraction purer than the 800 x *g* sediment.

In one experiment 25 g of ox caudate nucleus were homogenized for 3 min with 75 ml water in a Waring blendor. The homogenate was centrifuged at 800 x *g* for 20 min. The sediment was washed once with 75 ml of water and centrifuged similarly. To the pooled supernatants was added 14 ml of 1 M phosphate buffer pH 6.8 (to make the final concentration of phosphate 0.1 M). This was centrifuged at 4000 x *g* for 30 min. The 4000 x *g* sediment was suspended in 0.1 M phosphate pH 7.5 and mixed with one-fifth its volume of 0.09 M sodium pyrophosphate pH 8.5 and treated with elastase as before. The results are presented in Table 7.

TABLE 6

EFFECT OF DILUTION AND INCREASED QUANTITY OF ELASTASE ON SOLUBILIZATION OF ACETYLCHOLINESTERASE

Fraction	Volume	Activity	Total activity	Soluble activity
	ml	units/ml	units	%
Insoluble	27	360	9720	
Soluble				
Control	17	48	816	8
x 5 diluted	125	11	1375	14
x 5 diluted + (x10 elastase)				
I elastase	125	20	2500	26 } 33
II elastase	140	5	700	7 }

TABLE 7

EFFECT OF EXTRACTION WITH WATER ON SOLUBILIZATION OF ACETYLCHOLINESTERASE

Control: Particulate preparation by usual method (800xg sediment)

Fraction	Volume	Activity	Total activity	Protein	Specific activity	Soluble activity
	ml	units/ml	units	mg/ml		%
Control						
Insoluble	90	400	36,000			
Soluble	54	55	2,970	4.4	12	8
Experimental						
Insoluble (4000xg sediment)	45	260	11,700			
Soluble	40	56	2,240	4.6	12	20

It is evident from Table 7 that AchE obtained by this procedure (extraction with water and sedimenting at 4000 x *g*) can also be solubilized by elastase treatment. By using this type of preparation about 2.5 times more enzyme could be solubilized by elastase treatment as compared with that from 800 x *g* sediment. But the amount of AchE obtained by water extraction and 4000 x *g* sedimentation was much lower than by 800 x *g* sedimentation. The yield of the total soluble enzyme (obtained by processing the same quantity of liquid) was lower than that obtained by the usual procedure and there was no difference in the specific activities of the two soluble enzymes. It was also inconvenient to handle much large volumes in the aqueous extraction method. This procedure was, therefore, not further used.

The following additional experiments on solubilization were carried out to obtain better yields of soluble enzyme. Since these results were negative, they are only briefly described and the results shown in Table 8.

1. Reblendorizing before elastase treatment - To ensure further breaking up of the particles in the particulate preparation, it was homogenized in a Waring blendor for 3 min before adding elastase, but there was no effect on solubilization.

2. Increase in the phosphate concentration - Increase in the phosphate concentration to 0.5 M instead of 0.1 M gave slightly more soluble activity than the control. This was however only due to better packing of the precipitate during centrifugation at 14,000 x *g* thus giving more supernatant fluid and not due to more units of activity solubilized per ml.

3. Washing of the particulate enzyme at pH 5.7 - In order to remove any interfering components present in the particulate enzyme,

TABLE 8

EFFECT OF DIFFERENT CONDITIONS ON SOLUBILIZATION OF BRAIN ACETYLCHOLINESTERASE

Control: Elastase 3.2 mg/50 g tissue.
Insoluble activity: Suspension of 800 x g sediment in 0.1 M phosphate + pyrophosphate.

Soluble activity: Activity in the 14,000 x g supernatant

Treatment	Fraction	Volume	Activity	Total activity	Activity soluble
		ml	units/ml	units	%
1. Reblendorising before adding elastase	Insoluble	144	264	38,016	
	Soluble				
	Control	80	54	4,320	11
	Experi-mental	80	52	4,160	11
2. Increasing phosphate concentration to 0.5 M	Insoluble	5	240	1,200	
	Soluble				
	Control	1.7	49	80	6
	Exptl.	4	31	124	10
3. Washing of the 800xg sediment with 0.1M phosphate pH 5.7	Insoluble	10	310	3,100	
	Soluble				
	Control	6	56	316	10
	Exptl.	12	26	312	10
4. Liquid air freezing & thawing	Insoluble	20	343	6,860	
	Soluble				
	Control	10	60	600	9
	Exptl.	11	50	550	8
5. Toluene treatment A.	Insoluble	10	350	3,500	
	Soluble				
	Control	6	58	348	10
	Exptl.	6	41	246	7

continued......

TABLE 8 (continued)

Treatment	Fraction	Volume	Activity	Total activity	Activity soluble
		ml	units/ml	units	%
5. Toluene treatment. B.	Insoluble	10	350	3,500	
	Soluble				
	Control	6	58	348	10
	Experimental	6	44	264	7
6. Treatment of acetone powder with elastase	Control				
	Insoluble	10	270	2,700	
	Soluble	5	52	260	10
	Acetone powder				
	Insoluble	20	46	920	
	Soluble	17	13	221	23
7. 10% Ammonium sulfate	Insoluble	10	520	5,200	
	Soluble				
	Control	6	69	414	8
	Experimental	7	64	448	8
8. Treatment with DNA	A. Insoluble	15	300	4,500	
	Soluble				
	Control	10	53	530	11
	Experimental	12	46	552	12
	B. Insoluble	12	300	3,600	
	Soluble				
	Control	8	30	240	7
	Experimental	8	30	240	7

continued....

TABLE 8 (continued)

Treatment	Fraction	Volume	Activity	Total activity	Soluble activity
		ml	units/ml	units	%
9. Dialysis for 24 hr during elastase treatment	Insoluble	10	520	5,200	
	Soluble				
	Control	6	69	414	8
	Experimental	6	61	366	7
10. Increasing the pH to 8.5 with K_2HPO_4	Insoluble	10	400	4,000	
	Soluble				
	Control	6	60	360	9
	Experimental	6	60	360	9
11. 0.1 M cysteine	Insoluble	5	370	1,850	
	Soluble				
	Control	3	64	192	10
	Experimental	4	64	256	14

NB: A - Treatment was given after elastase treatment.
B - Treatment was given during elastase treatment.

it was washed in one experiment with 0.1 M phosphate pH 5.7 and then suspended as usual in 0.1 M phosphate pH 7.5 - pyrophosphate and treated with elastase. The soluble activity obtained was identical with that of the control.

4. Freezing in liquid air - Very rapid freezing at the temperature of liquid air and then a slow thawing may break up the particles better than freezing at -20°. Therefore in one experiment the particulate preparation after 24 hr treatment with elastase was frozen and thawed three times in liquid air, then centrifuged at 14,000 x g and tested for the soluble activity. The solubilization however was almost the same as that of the control.

5. Treatment with toluene - Toluene treatment of the electric organ is a well known procedure for the purification of AchE from this source. The effect of toluene on our particulate preparation was studied in two ways. In one case toluene was added to the preparation after elastase treatment and in the second case it was added during the elastase treatment. In both the cases the suspension was kept mixed with the thawed preparation six to seven hours every day. The layer of toluene was removed after freezing. The toluene was changed four to five times. The toluene treated preparation gave three distinct layers on centrifugation at 14,000 x g. The middle clear layer was taken as the soluble enzyme and tested for activity. Both types of experiments gave somewhat lower soluble activities compared to the control.

6. Elastase treatment of the acetone powder - An acetone powder of the homogenate of caudate nucleus was prepared. This resulted in about 80 per cent inactivation of the AchE activity. But when this acetone powder was suspended in 0.1 M phosphate pH 6.8, centrifuged

at 800 x g and the sediment treated with elastase by the standard procedure about 23 per cent of the activity was brought into solution. It may be that if interference of lipids in the action of elastase is removed by preparing acetone powder better solubilization can be achieved. But due to excessive loss in activity while preparing the acetone powder this procedure was not adopted.

7. Extraction with 10% ammonium sulfate - The use of 5% ammonium sulfate for extraction of the electric eel AchE is routinely made and recent reports on reversible aggregation of AchE and show the importance of the ionic strength of the medium[86,87]. But our studies on the effect of adding 10 per cent ammonium sulfate to the particulate preparation during elastase treatment did not give any difference in the soluble activity as compared with the control. But the particulate preparation was packed better than the controls when centrifuged at 14,000 x g.

8. Effect of DNA - Alivisatos and Woolley in 1956 described the effect of DNA in solubilizing beef spleen diphosphopyridine nucleotidase[194] (DPNase). The effect of DNA on solubilization of AchE by elastase was, therefore, tested. The effect was examined under two different conditions. In the first experiment 15 ml of particulate enzyme were treated with 10 mg of DNA (in 1 M NaCl and 0.1 M phosphate pH 7.5) after treatment with elastase and freezing and thawing. In another experiment identical treatment with DNA was given to the 10 ml of particulate preparation while it was digested with elastase for 24 hr at 0°. The preparation was then frozen and thawed as usual.

Better packing and clearer supernatants as compared with the control were obtained with the DNA treated enzyme, but there was no increase or decrease in soluble activity.

9. <u>Dialysis</u> - It was considered possible that some low molecular weight products of elastase action on the particulate preparation may be inhibitory to further action of elastase, thus limiting the solubilization. Therefore in one experiment 10 ml of the particulate preparation after adding elastase were dialyzed against 0.1 M phosphate - 0.09 M pyrophosphate (1/5th volume) for 24 hr. This gave only the same soluble activity as the control.

10. <u>Effect of increasing pH</u> - Elastase is known to have better action at pH 8.5 than at pH 7.5 and hence in one experiment the pH of the particulate preparation was adjusted to 8.5 with K_2HPO_4 before elastase was added. The soluble activity obtained was not different from that of the control.

11. <u>Effect of cysteine</u> - The effect of a sulfhydryl compound on solubilization of AchE with elastase was tested by adding 0.1 M cysteine to the particulate preparation during elastase treatment. When it was centrifuged at 14,000 x g the supernatant was very clear and packing of the sediment was much better than that of the control. But there was no difference in the units per ml of the soluble activity. More soluble activity was however obtained because of the greater volume of the supernatant fluid.

Final procedure for solubilization

Based on the above studies following final procedure for the solubilization of ox caudate nucleus AchE was adopted.

Preparation of the particulate enzyme - 50 g of caudate nucleus were isolated from 5 to 6 ox brains. They were homogenized in a Waring blendor for 3 min with 150 ml of 0.1 M phosphate pH 6.8 and the homogenate centrifuged at 800 x g for 20 min. The sediment was washed once with the same buffer and centrifuged similarly. The sediment was then suspended in 0.1 M phosphate pH 7.5 and lumps were broken up by homogenizing in a glass homogenizer. The final volume of the suspension was made upto 150 ml and it was then mixed with 30 ml of 0.09 M Na-pyrophosphate-HCl, pH 8.5.

Elastase treatment - 3.2 mg of crystalline elastase were added for every 50 g of tissue and the suspension was kept at 0° for 24 hr. Elastase is insoluble at this pH and has a tendency to settle. The suspension was, therefore, stirred intermittently. At the end of 24 hr it was frozen at about -20° to -30° and then thawed. Freezing and thawing was repeated 5 to 6 times over a period of a week. The temperature while thawing was not allowed to exceed 5° to 6°. The enzyme at this stage became highly viscous and jelly-like. It was centrifuged at 14,000 x g for 1 hr. Approximately 7 to 10 per cent of the activity was obtained in the supernatant.

The residue obtained after centrifugation was once more treated with elastase and then frozen and thawed in a similar manner and finally centrifuged at 14,000 x g for 1 hr. The soluble enzyme solutions from the two treatments were pooled and used for further work. A total of about 15 to 20 per cent of activity was brought into solution by two

TABLE 9

SOLUBILIZATION OF OX CAUDATE NUCLEUS ACETYLCHOLINESTERASE BY ELASTASE TREATMENT

Data from batches of 50 g of caudate nucleus

		Particulate preparation				Elastase treated enzyme				
		Volume	Activity	Total activity	Activity	Volume	Activity	Total activity		Activity
		ml	units/ml	units	units/g	ml	units/ml	units		%Soluble
Maximum value	I	180	335	60,300	1,200	108	62	6696	11756	20
	II					110	46	5060		
Minimum value	I	180	458	82,440	1,650	102	53	5406	9711	12
	II					105	41	4305		
Average value	I	180	397	71,500	1,430	108	56	6048	10888	15
	II					110	44	4840		

elastase treatments. The soluble enzyme was not sedimented on centrifugation at 100,000 x g for 1 hr. In the sixty-two batches of ox caudate nucleus which were used for the preparation of the enzyme the specific activity of the enzyme ranged between 15 to 24 which was about twenty times more than that obtained with gray matter. The results of all the preparations of the soluble enzyme are summarized in Table 9. Fluctuations in the per cent soluble activity were due to variations in the initial activity of the particulate preparations.

SECTION II

Purification of Acetylcholinesterase

Preliminary experiments on fractionation of the soluble enzyme with calcium phosphate gel or ammonium sulfate gave little or no increase in purity. It was found that treatment with protamine sulfate converts the viscous jelly-like enzyme into a non-viscous fluid material. There was increase in specific activity by fractionation procedures after protamine sulfate treatment, apparently due to the removal of some interfering material. The following procedure for protamine sulfate treatment was adopted on the basis of the preliminary studies.

Step I - Protamine sulfate treatment

The soluble enzyme was mixed with 0.1 volume of 1 M phosphate buffer, pH 7.5 followed by 0.2 volume of a 2.5 per cent solution of protamine sulfate or salmine sulfate (in 0.05 M phosphate pH 7.5) with rapid stirring. It was allowed to stand for about 10 min and centrifuged at 14,000 x g for 30 min and the sediment was discarded. The supernatant fluid thus obtained was water clear. The ratio of optical density at 280 mμ to that at 260 slightly increased from about 0.8 to 1.0. Table 10 shows the results of a typical experiment. Yields of the enzyme in this step were almost quantitative in many experiments but there was no significant increase in the purity. Protamine sulfate and salmine sulfate gave similar results. The viscous enzyme became quite fluid and lost its high viscosity after this treatment.

Step - II - Ammonium sulfate fractionation

The elastase and protamine sulfate treated enzyme was fractionated with ammonium sulfate between 0.3 and 0.6 saturation. To every 100 ml of

TABLE 10

PROTAMINE SULFATE TREATMENT OF BRAIN ACETYLCHOLINESTERASE

	Volume	Activity	Total activity	Protein	Total protein	Specific activity	$\frac{O.D. 280\ m\mu}{O.D. 260\ m\mu}$	Recovery
	ml	units/ml	units	mg/ml	mg			%
Elastase treated enzyme	210	64	13,440	3.8	798	17	0.87	
Protamine sulfate treated enzyme	260	46	11,960	2.5	650	18	1.06	90

enzyme solution 16.4 g of powdered ammonium sulfate were added with stirring and after 30 min at 0° the solution was centrifuged at 14,000 x g for 40 min. The sediment was discarded and to every 100 ml of the supernatant fluid 18 g of ammonium sulfate were added to increase the saturation from 0.3 to 0.6. After about 40 min standing the precipitate was collected by centrifugation at 4000 x g for 45 min. The precipitate was dissolved in 0.05 M phosphate pH 7.5. Ammonium sulfate fractionations were carried out by adding small amounts of solid at a time without too rapid stirring. 0.05 ml of 2N ammonia solution was added intermittently for every 2 g of ammonium sulfate to maintain the pH of the solution at 7.5.

The results of a typical experiment are presented in Table 11. It is evident from the table that about 2.5-fold purification is achieved in this fractionation giving a final specific activity of 55. The final specific activity with different batches ranged between 40 to 70. The yields of the activity were between 80 to 85 per cent.

The results of several different fractionation procedures, which were tested, are described below. The enzyme after solubilization, protamine sulfate treatment and ammonium sulfate fractionation (0.3 to 0.6) was used for these experiments unless otherwise stated.

Fractionation with solvents - Attempts to fractionate AchE either with alcohol or acetone at pH 6.5 or 7.5 and at 0° resulted in complete inactivation of the enzyme.

Refractionation with ammonium sulfate at pH 5.5 - The enzyme was dialyzed against 0.02 M sodium acetate-acetic acid buffer pH 5.5. The insoluble material formed during dialysis was removed by centrifugation and 5.5 ml of the clear supernatant were fractionated with solid ammonium sulfate between 0 - 0.40, 0.40 - 0.45, 0.45 - 0.50 and 0.50 - 0.55 saturation. The results are presented in Table 12. About 2.5-fold

TABLE 11

AMMONIUM SULFATE FRACTIONATION OF BRAIN ACETYLCHOLINESTERASE

	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Recovery
	ml	units/ml	units	mg/ml	mg		%
Protamine sulfate treated enzyme	950	60	57,000	3	2850	20	
0.3 - 0.6 ammonium sulfate fraction	46.4	1056	49,020	19.2	890	55	86

TABLE 12

REFRACTIONATION OF BRAIN ACETYLCHOLINESTERASE WITH AMMONIUM SULFATE AT pH 5.5

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Yield
	ml	units/ml	units	mg/ml	mg		%
1. Dialyzed 0.3–0.6 fraction at pH 5.5	5.5	134	740	2.7	14.85	49	
2. 0 – 0.4 fraction	1	30	30	1.6	1.6	20	4
3. 0.4 – 0.45 fraction	1	34	35	0.8	0.8	40	5
4. 0.45 – 0.5 fraction	2	150	300	1.3	2.6	107	40
5. 0.5 – 0.55 fraction	2	194	388	1.1	2.2	170	52

purification was achieved in the 0.45 - 0.50 fraction and about 4-fold in the 0.50 - 0.55 fraction. This method was not further used owing to the better results obtained by fractionation on DEAE-cellulose which is described later.

<u>IRC - 50</u> - The cation exchange resin IRC-50 (XE-64) was washed and equilibrated as described by Hirs[189]. Detailed studies were made at different pH values (viz., 5.5, 6.5 and 7.5) and 0.005 M and 0.01 M phosphate concentration using the sodium form of the resin and at pH 6.8, 0.005 M phosphate using the ammonium form of the resin. About 1 g of resin was used for 200 to 250 units of the enzyme. No purification was obtained under any of these conditions. An attempt to fractionate the enzyme on IRC-50 column (1.5 x 36 cm) equilibrated with 0.005 M phosphate pH 6.8 also did not result in any increase in specific activity.

<u>Carboxymethyl cellulose</u> - The enzyme was fractionated on calcium phosphate gel to give a specific activity of 200 and used for studies with carboxymethyl cellulose. The adsorbent was washed according to the procedure of Peterson and Sober[190] and was equilibrated with 0.005 M phosphate pH 6. 1.4 ml of the dialyzed enzyme solution containing 200 units was mixed with about 1 g of CM-cellulose. The adsorbent was successively washed with 0.02 M phosphate pH 6, 0.05 M phosphate, pH 7.5. Only about 50 per cent of the activity could be recovered from all the fractions and there was no increase in the specific activity of any of the fractions. The results of this experiment are given in Table 13. A similar experiment with CM-cellulose was also carried out at pH 7.5 and similar results were obtained. The adsorbent was not used for

TABLE 13

ADSORPTION OF BRAIN ACETYLCHOLINESTERASE ON CM-CELLULOSE

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Yield
	ml	units/ml	units	mg/ml	mg		%
1. Dialyzed enzyme	1.4	143	200	0.70	1	200	
2. Supernatant after adsorption	2	23	46	0.11	0.22	200	23
3. 0.02 M phosphate pH 6 eluate	5	5	25	0.09	0.45	55	12
4. 0.05 M phosphate pH 6 eluate	5	4	20	0.03	0.15	130	10
5. 0.05 M phosphate pH 7.5 eluate	5	0	0	0.02	0.10		

TABLE 14

PRECIPITATION OF BRAIN ACETYLCHOLINESTERASE AT pH 5

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Yield
	ml	units/ml	units	mg/ml	mg		%
1. Dialyzed 0.3–0.6 ammonium sulfate fraction	4.5	186	830	4.5	20.2	41	
2. pH 5 supernatant	4.6	80	372	2.8	13	28	44
3. pH 5 precipitate	0.5	300	150	14.4	7.2	20	18

further work.

Precipitation at low pH - In one experiment the enzyme was dialyzed against 0.02 M phosphate pH 7.5 and was adjusted to pH 5 by adding 1 M acetic acid. It was kept for 15 to 20 min and then centrifuged clear. The pH of the supernatant fluid was adjusted back to 7.5 by KOH solution and it was assayed for activity and protein. The sediment was dissolved in 0.05 M phosphate pH 7.5 and tested for activity and protein. The results are presented in Table 14. It is evident from the Table 14 that there was no purification achieved by this method and that there was about 40 per cent inactivation of the enzyme.

Step III - DEAE-Cellulose

In a preliminary batchwise experiment about 200 units of dialyzed 0.3 - 0.6 ammonium sulfate fraction were found to be adsorbed completely by 1 g of DEAE-cellulose in 0.01 M phosphate pH 7.5. The activity could be eluted by 0.07 M phosphate pH 7.5 with about 5-fold purification. Column chromatography of the enzyme on DEAE-cellulose was then carried out.

About 5 g of DEAE-cellulose were washed according to the procedure of Peterson and Sober[193]. It was then equilibrated with 0.01 M phosphate pH 7.5. A column (1.5 x 10 cm) was prepared and washed with 0.01 M phosphate containing 0.002 M EDTA. 1.7 ml of the enzyme obtained after protamine sulfate treatment and ammonium sulfate fractionation was dialyzed against 0.01 M phosphate, pH 7.5, centrifuged to remove insoluble material and then loaded on the column. The column was washed with

about 80 ml of 0.04 M phosphate pH 7.5 and the activity was then eluted with 0.07 M phosphate pH 7.5. 3 ml fractions were collected every 10 min. Activity and protein were determined in each fraction. The results are presented in Table 15 and the activity and protein patterns are shown in Fig.1. It can be seen from Table 15 that 18 to 20-fold increase in specific activity was obtained in two of the fractions. Maximum activity and purity were obtained in the earlier fractions. The protein content of the individual fractions was difficult to determine accurately because of the low protein and blanks due to UV absorbing material from the DEAE-cellulose. The first three fractions were pooled together and precipitated with ammonium sulfate (0.90 saturation) and the activity and protein of the precipitated enzyme were determined. About one-third of the initial activity was recovered with a specific activity of about 800. Only 50 per cent of the total enzyme could be accounted for in the different fractions. Since the purification obtained by this method was much higher compared to that from other methods which were described earlier, DEAE-cellulose fractionation was routinely used after protamine sulfate treatment and ammonium sulfate fractionation as the Step-III in the fractionation procedure.

Columns (4.5 x 10 cm) were routinely made using 25 g of DEAE-cellulose. For large-scale preparations of the enzyme two such columns were run together. About 100 to 110 mg of protein equivalent to about 5,000 to 6000 units were loaded on each. This step has been repeated about 50 to 60 times with reproducible results. The specific activity ranged from 600 to 1100.

Better results with respect to recoveries were obtained with DEAE-cellulose prepared in this laboratory. In some of the experiments

TABLE 15

COLUMN CHROMATOGRAPHY OF BRAIN ACETYLCHOLINESTERASE ON DEAE-CELLULOSE

(column: 1.5 x 10 cm)

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Yield
	ml	units/ml	units	mg/ml	mg		%
Dialyzed enzyme	1.7	686	1166	15.6	26.5	45	
Eluted fractions							
1 - 7	21						
8	3	18	54	0.02	0.06	900	
9	3	84	252	0.10	0.30	840	
10	3	46	138	0.11	0.33	410	
11	3	19	57	0.07	0.21	260	
12	3	10	30	0.05	0.15	200	
13	3	7	21				
14	3	6	18				
15	3	3.5	10				
Fractions 8 to 15	24		530				50
Fractions 8 to 10 pooled	9		444				38
Ammonium sulfate precipitate of pooled fraction	1	330	330	0.4	0.4	825	30

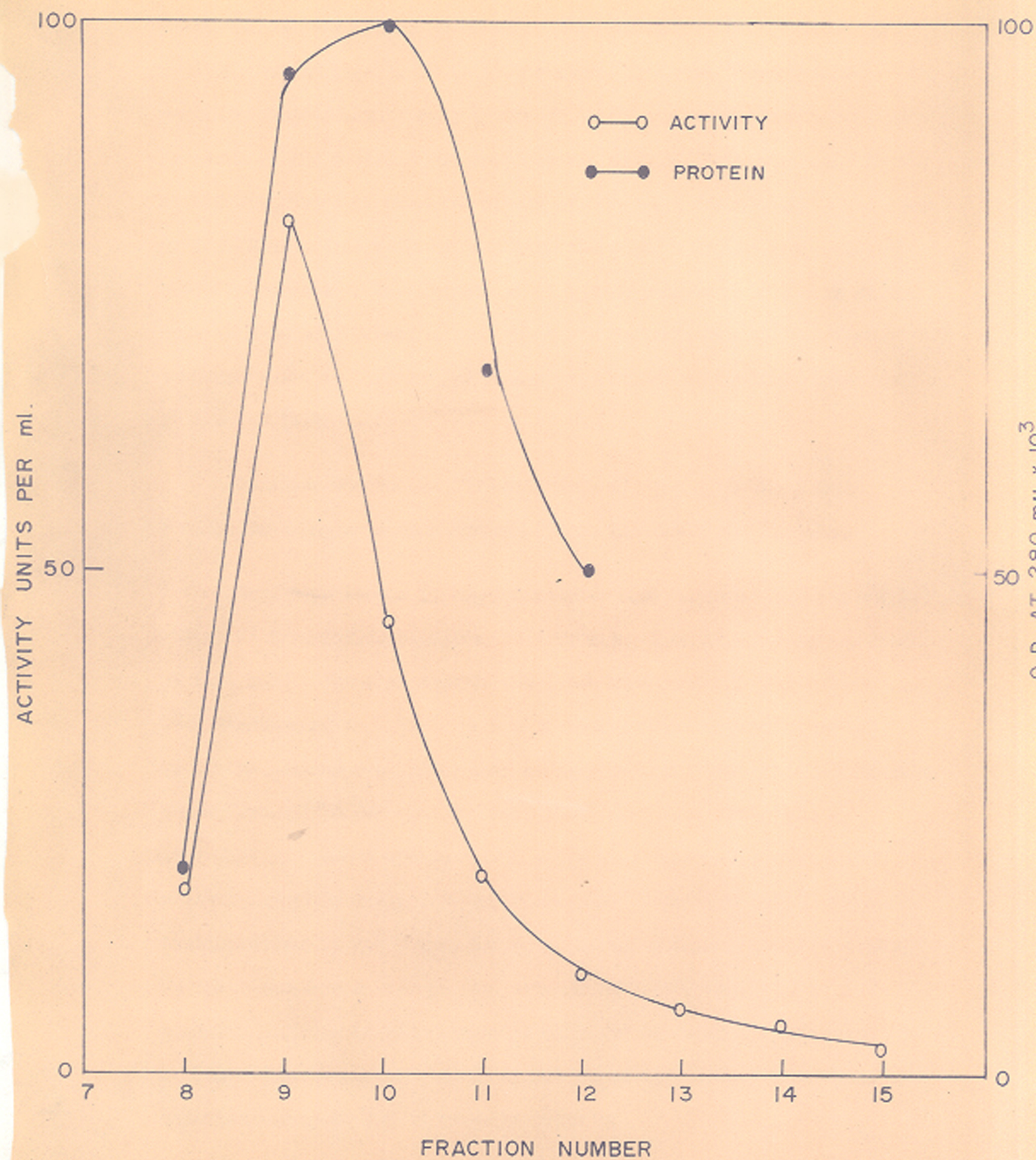


FIG.1. ELUTION PATTERN OF BRAIN ACETYLCHOLINESTERASE ACTIVITY AND PROTEIN ON FIRST DEAE-CELLULOSE COLUMN (1.5 x 10 cm). Fraction = 3 ml.

using the DEAE-cellulose prepared in this laboratory the recovery of activity was as high as 70 to 75 per cent. But with Bio-Rad DEAE-cellulose yields always ranged from 45 to 55 per cent. About 10 to 15 per cent of the activity was found in the 0.04 M buffer washing. Most of the work has been done making use of Bio-Rad DEAE-cellulose. Moreover with DEAE-cellulose made in this laboratory the activity was eluted by 0.05 M phosphate pH 7.5 instead of at 0.07 M phosphate pH 7.5 as in the case of Bio-Rad DEAE-cellulose. This was probably due to the lower exchange capacity (0.5 meq/g) of our preparation as compared with that of Bio-Rad (0.69 meq/g).

Gradient elution on DEAE-cellulose did not give any better results than the procedure described here and hence was not used.

Very high losses in enzyme activity were observed while precipitating it from dilute solutions at 0.90 saturation of ammonium sulfate at this stage. Several experiments (such as increasing the buffer concentration of the eluate to 0.2 M, adding EDTA, adjusting the pH to 5.5 by acetate and adding 0.05 M acetylcholine) were tried to decrease these losses. It was observed that the presence of about 0.05 M acetylcholine in the eluate before addition of the ammonium sulfate results in better recoveries of the enzyme. Therefore, all further precipitations of the dilute solutions by 0.9 saturation with ammonium sulfate were carried out in the presence of 0.05 M acetylcholine. Ammonium sulfate was added immediately after the addition of acetylcholine. The pH was maintained at 7.5 by adding 0.05 ml of 2N ammonia solution for every 2 g of ammonium sulfate.

This method of precipitation of the dilute enzyme solutions with ammonium sulfate in the presence of acetylcholine was used in all subsequent work both for DEAE-cellulose eluates as well as those obtained from other adsorbents in subsequent work.

DEAE-Sephadex - At this stage one column chromatography was carried out with DEAE-sephadex A-50. A column (1.5 x 48 cm) was prepared and equilibrated with 0.05 M phosphate pH 7.5. About 1 ml of concentrated and dialyzed DEAE-cellulose fractionated enzyme (2640 units/ml) was loaded on this column. It was washed with about 100 ml of 0.05 M phosphate pH 7.5 and the activity was eluted by 0.1 M phosphate pH 7.5. 3.5 ml fractions were collected every 10 min. Only about 50 per cent of the activity could be recovered and there was no increase in the specific activity. No further work was carried out by this method.

Step IV - Calcium phosphate gel

The conditions for the adsorption and elution of the enzyme on calcium phosphate gel were determined in preliminary experiments. It was found that 0.05 mg of gel (dry wt) was required for the adsorption of 1 unit of enzyme from 0.01 M phosphate pH 6.0. Adsorption was found to be the best in 0.01 M phosphate pH 6.0 as the enzyme was eluted at very low concentration of the phosphate (0.003 M) at a higher pH. Therefore, these conditions of adsorption were used in all subsequent experiments. The enzyme could be eluted at 0.003 M phosphate pH 7.5 but the units per ml were less than those with 0.005 M phosphate and there was also no difference in the specific activity of the two eluates. Therefore in all subsequent experiments elutions were always made with 0.005 M phosphate. More units per ml could be eluted at higher concentrations of phosphate, but the specific

activity of such eluates was very low. Elution with more concentrated buffer was used for concentrating the dilute 0.005 M eluate by readsorbing the activity on gel after adjusting the pH to 6.0 and adding the calculated amount of gel and then eluting with 0.2 M phosphate in small volume. Based on these results the following procedure was adopted for adsorption and elution from calcium phosphate gel.

Enzyme obtained in Step-III was dialyzed against 0.01 M phosphate pH 6.0. 5 ml of the dialyzed enzyme (1800 units per ml) were mixed with 450 mg of gel (60 mg/ml) and 150 ml of 0.01 M phosphate pH 6.0. After keeping for 30 min it was centrifuged, the supernatant was discarded and the gel was washed with 150 ml of 0.01 M phosphate pH 6.0. The activity was then eluted twice with 0.005 M phosphate pH 7.5. 500 ml of the buffer were used for the first elution and 300 ml for the second. The gel was kept suspended in the buffer for about 30 min for each elution. It can be seen from Table-16 that the mixed 0.005 M eluate was dilute and had only 5.6 units per ml. 50 per cent of the activity was eluted. To the 800 ml of the mixed eluates were added 5.6 ml of 1 M acetate buffer pH 4.5 with constant stirring and the calculated amount of gel was then added. It was kept for about 30 min and centrifuged. The enzyme was eluted from the gel with 6.3 ml of 0.2 M phosphate pH 7.5. This enzyme was then precipitated by adding ammonium sulfate to 0.7 saturation. The precipitate was dissolved in 0.1 M phosphate buffer. It can be seen that the final recovery was 40 per cent with 3.7-fold purification giving a final specific activity of 4800.

TABLE 16

CALCIUM PHOSPHATE GEL TREATMENT OF BRAIN ACETYLCHOLINESTERASE

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Yield
	ml	units/ml	units	mg/ml	mg		%
1. Dialyzed DEAE-cellulose enzyme	5	1800	9000	1.4	7.2	1300	
2. Supernatant after adsorption on gel	160	0					
3. 0.01 M phosphate pH 6.0 washing	150	0					
4. 0.005 M phosphate pH 7.5 Eluate I + II	800	5.6	4480				50
5. 0.2 M phosphate pH 7.5 eluate after readsorption	6.3	672	3930	0.14	0.88	4800	44
6. 0.7 saturated ammonium sulfate precipitated enzyme	0.3	12,000	3600	2.5	0.75	4800	40

Fractionation on calcium phosphate gel was regularly used for purification and was repeated about 12 times with reproducible results. The specific activity after this fraction ranged from 4000 to 6000.

Step V - Rechromatography on DEAE-cellulose

The enzyme obtained after calcium phosphate gel fractionation was used for refractionation on DEAE-cellulose. The DEAE-cellulose used for this step was exclusively the one prepared in this laboratory. The adsorption or elution conditions were the same as in the first DEAE-cellulose fractionation. Similar amounts of protein were loaded on the column but the total units loaded were obviously more because of the higher purity at this stage.

A column (1.5 x 15 cm) was prepared with about 5 to 6 g of DEAE-cellulose and was equilibrated with 0.02 M phosphate pH 7.5. 2 ml of enzyme obtained after Step-IV were dialyzed against 0.02 M buffer and loaded on this column. It was washed with about 80 ml of 0.02 M phosphate pH 7.5 and the activity was eluted by 0.05 M phosphate pH 7.5. 4 ml fractions were collected every 7 min.

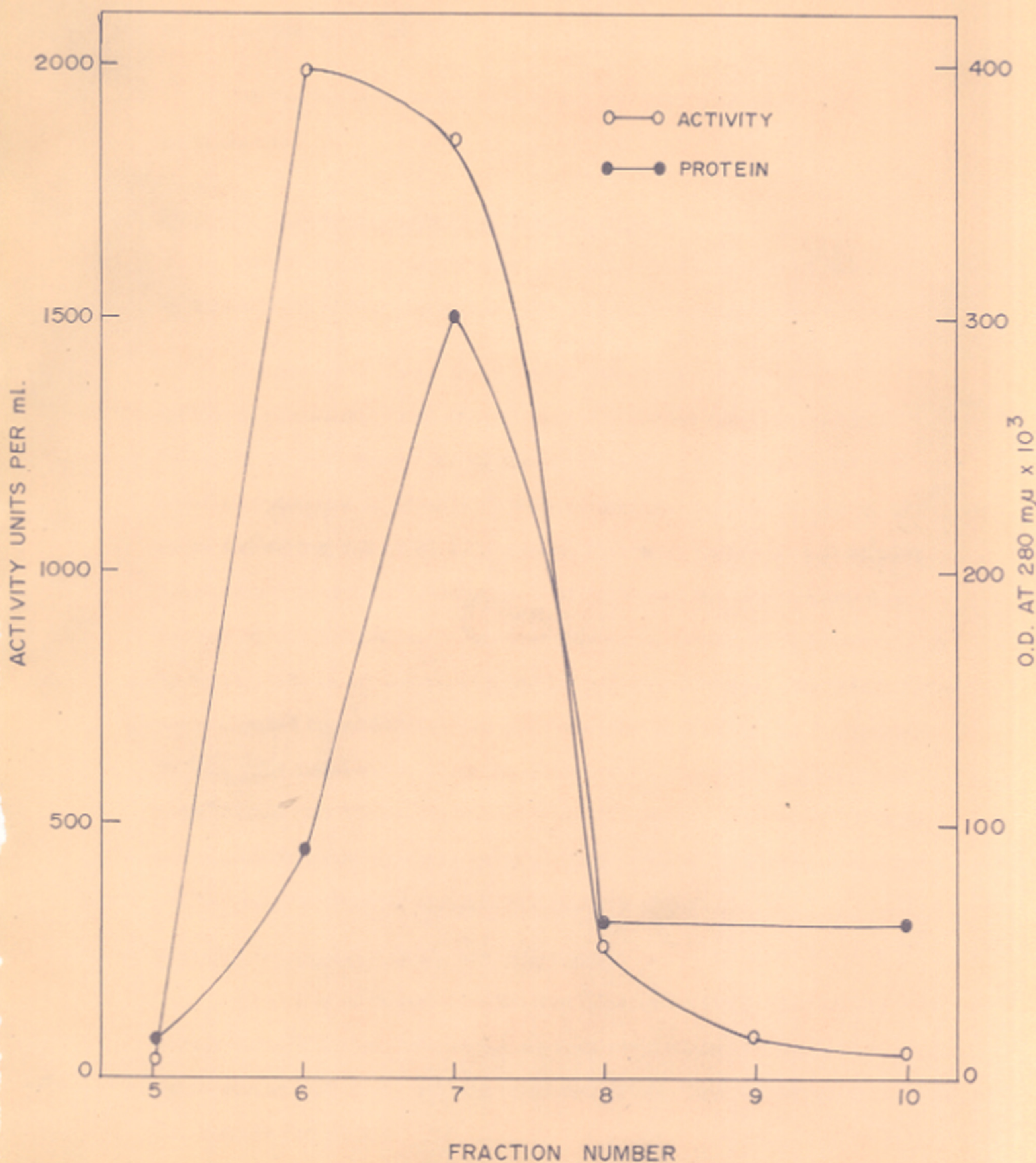
The results of a typical chromatographic fractionation are given in Table 17. The activity and protein patterns are shown in Fig. 2. As in the first fractionation on DEAE-cellulose, there was high specific activity in the initial fractions and a sharp decrease in the specific activity in the subsequent fractions. Those fractions with a specific activity of above 6000 were pooled. This fractionation has been repeated twice with reproducible results and the specific activity was about 10,000. The recovery of the activity was about 70 per cent. The pooled fractions

TABLE 17

REFRACTIONATION OF BRAIN ACETYLCHOLINESTERASE ON DEAE-CELLULOSE

Column: 1.5 x 15 cm

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Yield
	ml	units/ml	units	mg/ml	mg		%
1. Concentrated and dialyzed calcium phosphate gel eluate	2	12,000	24,000	2.7	5.4	4400	
2. Eluate fractions							
5	4	12	48				
6	4	1990	7960	0.09	0.36	22,000	
7	4	1860	7440	0.30	1.20	6,200	
8	4	240	960	0.06	0.24	4,000	
9	4	90	360	0.09	0.36	1,100	
10	4	66	264	0.06	0.24	1,100	
Total of fractions 5 to 10	24		17,030				70
Pooled fractions 6 and 7	8		15,400				62
Fractions 6 and 7 precipitated with ammonium sulfate	0.6	25,000	15,000	2.5	1.5	10,000	62



[FI]G. 2. ELUTION PATTERN OF BRAIN ACETYLCHOLINESTERASE ACTIVITY AND PROTEIN ON SECOND DEAE-CELLULOSE COLUMN (1.5 x 15 cm). Fraction = 4 ml.

were precipitated by 0.7 saturation of ammonium sulfate after addition of acetylcholine to 0.05 M.

Refractionation on DEAE-cellulose was used as Step-V of the purification.

<u>Step VI</u> - <u>Refractionation with ammonium sulfate</u>

On the basis of preliminary studies on purification of the enzyme by ammonium sulfate fractionation the following procedure was adopted.

The enzyme obtained after second chromatography on DEAE-cellulose was fractionated with saturated ammonium sulfate solution. To 0.6 ml of the enzyme (25,000 units/ml) 0.25 ml of ammonium sulfate solution (prepared by dissolving 72 g of ammonium sulfate in 100 ml of water and adding 1.8 ml of 2 M ammonium hydroxide to get pH 7.5 at room temperature) to get 0.3 saturation. After 30 min the precipitate was removed by centrifugation. The supernatant was raised to 0.55 saturation by adding 0.35 ml of saturated ammonium sulfate solution. The precipitate was collected by centrifugation and dissolved in 1 ml of 0.1 M phosphate pH 7.5. The results are shown in Table 18. The specific activity was about 23,000 and 73% of the activity was recovered. This fractionation gave about 2.5-fold increase in purity with good yield of the activity and was used as Step-VI of the fractionation procedure.

<u>Step-VII</u> - <u>Sephadex G-200 chromatography</u>

About 5 g of Sephadex G-200 were allowed to swell in a sufficient volume of 0.05 M phosphate pH 7.5 at room temperature for about 3 to 4 days and a column (1.3 x 100 cm) was prepared. The column was equilibrated with the same buffer at 0°.

TABLE 18

REFRACTIONATION OF BRAIN ACETYLCHOLINESTERASE WITH AMMONIUM SULFATE

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Yield
	ml	units/ml	units	mg/ml	mg		%
1. Second DEAE-cellulose fractionation	0.6	25,000	15,000	2.5	1.5	10,000	
2. 0.3 - 0.55 ammonium sulfate fraction	1.1	10,300	11,330	0.45	0.5	22,660	73

1.1 ml of the ammonium sulfate fractionated enzyme was loaded on this column and 0.05 M phosphate pH 7.5 was passed through it. 2.8 ml fractions were collected every 20 min and the fractions were tested for activity. It was not possible to determine the protein in each fraction due to high dilution. Hence the fractions with high activities were pooled together and those with lesser activities were pooled separately. The two pooled eluates were separately precipitated by 0.9 saturation of ammonium sulfate in the presence of 0.05 M acetylcholine as before. The precipitates were dissolved in 0.1 M phosphate pH 7.5 and tested for activity and protein. The results are shown in Table 19. The lesser activity fraction had a specific activity of about 11,000 whereas the high activity fraction had a specific activity of about 100,000. The recovery of the activity in the low specific activity fractions was only about 10% and the recovery in the high specific activity (100,000) fraction was about 45 per cent of that loaded on the column.

This fractionation gave about 4-fold purification and was used as the last step (Step VII) in the fractionation procedure for AchE.

Further purification of the enzyme was not attempted since the amount of enzyme obtained after Sephadex G-200 fractionation was only about 0.1 mg of protein. This amount of enzyme was required for studies on the properties and kinetics of the enzyme. Since the number of ox brains available daily in Poona was only 4, it was necessary to obtain larger quantities from Bombay about 120 miles away. From 60 ox brains it was possible to obtain only about 0.1 mg of purified enzyme. Due to the difficulty of obtaining and handling larger quantities of tissue,it was not possible to study further purification of the enzyme and all further work on the properties and kinetics of the enzyme was carried out with preparations of specific activity of about 100,000.

TABLE 10

FRACTIONATION OF BRAIN ACETYLCHOLINESTERASE ON SEPHADEX G-200

Column: 1.3 x 100 cm in 0.05 M phosphate pH 7.5

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Yield
	ml	units/ml	units	mg/ml	mg		%
1. Ammonium sulfate fractionated enzyme	1.1	10,300	11,330	0.45	0.5	23,000	
Fractions							
1 - 16	45						
17	2.8	100	280				
18	2.8	500	1,400				
19	2.8	1,080	3,020				
20	2.8	1,320	3,830				
21	2.8	626	1,430 } 11,300				100
22	2.8	293	820				
23	2.8	150	390				
24	2.8	70	182				
25	2.8	21	54				
Pooled 17+22+23+24+25	14		1,720				15
Ammonium sulfate precipitated (17+22 to 25)	1.1	990	1,100	0.09	0.10	11,000	10
Pooled 18 to 21	11		9,670				85
Ammonium sulfate precipitated (18 to 21)	1.2	4,170	5,000	0.04	0.05	100,000 (approx.)	46

On the basis of the above findings the following final procedure was adopted for the purification of acetylcholinesterase from caudate nucleus of ox brain.

The soluble enzyme was obtained by the procedure described in the first section of this chapter and it was fractionated through the following steps. The term buffer in the following refers to potassium phosphate buffer, pH 7.5. The temperature was 0°-5° unless stated otherwise.

Step I - Protamine sulfate treatment

The soluble enzyme was slowly mixed with 0.1 volume of 1M phosphate buffer and then with 0.2 volume of 2.5 per cent protamine sulfate solution. It was kept for 10 to 15 min and centrifuged at 14,000 x *g* for 40 min. The clear supernatant was collected.

Step II - Ammonium sulfate fractionation

The protamine sulfate treated enzyme was fractionated with ammonium sulfate. 16.4 g of ammonium sulfate were added for every 100 ml of the enzyme solution to get 0.3 saturation. After 30 min it was centrifuged at 14,000 x *g* for 40 min. The supernatant-fluid was raised to 0.6 saturation by adding 18 g of ammonium sulfate for every 100 ml. The precipitate was collected after 40 min by centrifugation at 4,000 x *g* and dissolved in 0.05 M buffer. It was dialyzed against 0.01 M buffer and centrifuged clear to remove the turbidity which appeared during the dialysis.

Step III - DEAE-cellulose column chromatography

DEAE-cellulose (Bio-Rad) was washed and equilibrated with 0.01 M buffer and was routinely washed with buffer containing 0.002 M EDTA. A 4.5 x 10 cm column (25 g adsorbent) was prepared with a layer of acid

washed glass-wool at the bottom. The column was equilibrated with 500 ml of 0.01 M buffer. The enzyme from Step-II was dialyzed against 0.01 M phosphate buffer pH 7.5 and centrifuged clear. About 5 to 7 ml of this enzyme containing 5000 to 7000 units and a total of 100 to 110 mg protein were loaded on this column. Two such columns were generally run simultaneously. The column was washed with 500 ml of 0.04 M buffer and the activity was eluted with 0.07 M buffer. 10 to 12 ml fractions were collected every 10 min. The active fractions with specific activity higher than 500 were pooled together and acetylcholine was added to a final concentration of 0.05 M. 60 g of ammonium sulfate were added immediately afterwards for every 100 ml of the eluates. 0.05 ml of 2N ammonia solutions were added for every 2 g of the ammonium sulfate to maintain the pH at 7.5. The precipitate was collected after 1 hr by centrifugation at 14,000 x g for 1 hr and dissolved in 0.1 M buffer and preserved at -20° .

Step IV - Calcium phosphate gel treatment

Enzyme from Step-III was dialyzed against 0.01 M phosphate buffer pH 6, centrifuged clear and was mixed with 0.05 mg of calcium phosphate gel per unit of activity. About 8000 to 10,000 units were processed at a time and enough 0.01 M phosphate pH 6 was added to make the volume about 150 ml during adsorption. The enzyme was kept for about 30 min and centrifuged. The supernatant was discarded. The gel was washed once with 0.01 M phosphate pH 6 and the enzyme eluted successively with 500 ml and 300 ml lots of 0.005 M buffer, pH 7.5.

The dilute eluate was adjusted to pH 6 by adding 3.5 ml of 1 M acetate buffer pH 4.5 for every 500 ml with mixing and the calculated amount of gel was added to readsorb the eluted activity. The enzyme was then eluted from the gel with 5 to 6 ml of 0.2 M phosphate buffer.

The enzyme was precipitated by adding 4.3 g of ammonium sulfate for every 10 ml of the enzyme (0.7 saturation). The precipitate was collected by centrifugation as before and dissolved in 1.5 ml of 0.1 M buffer.

Step V - Rechromatography on DEAE-cellulose column

A column (1.5 x 15 cm) was prepared using about 5 g of washed DEAE-cellulose (prepared in this laboratory). The column was equilibrated with 0.02 M buffer. Enzyme from Step-IV was dialyzed against 0.02 M phosphate buffer pH 7.5 and centrifuged clear after dialysis. 2 ml of dialyzed enzyme containing about 25,000 units were loaded on this column. The column was washed with 80 to 100 ml of 0.02 M buffer and the activity was eluted with 0.05 M buffer. 4 ml fractions were collected every 7 min. The fractions with specific activity more than 6000 were pooled together, mixed with acetylcholine (0.05 M final concentration) and precipitated by adding 4.3 g of ammonium sulfate for every 10 ml of the eluate (0.7 saturation). The precipitate was collected in 0.1 M buffer after 1 hr by centrifugation at 14,000 x *g* for 45 min.

Step VI - Refractionation with ammonium sulfate

About 25,000 units per ml enzyme obtained from the previous step was further fractionated by adding 0.43 ml of ammonium sulfate solution (prepared by dissolving 72 g of ammonium sulfate in 100 ml water and adding 1.8 ml of 2N ammonium hydroxide solution to adjust the pH to 7.5 at room temperature) at pH 7.5 per ml of the enzyme to get 0.3 saturation. The precipitate was centrifuged at 14,000 x *g* for 30 min and discarded. The ammonium sulfate saturation of the supernatant liquid was raised to 0.55 by adding 0.55 ml of saturated ammonium sulfate solution per ml of

the supernatant. The precipitate was collected after 30 min by centrifugation at 14,000 x *g* for 40 min. It was dissolved in 1 ml of 0.1 M buffer.

<u>Step VII</u> - <u>Sephadex G-200 column chromatography</u>

A column (1.3 x 100 cm) of Sephadex G-200 was prepared and equilibrated with 0.05 M buffer. About 11,000 units in 1.1 ml were loaded on this column. 2.8 ml fractions were collected every 20 min. The activities of the fractions were determined. The fractions with maximum activities per ml were pooled, those with lower activities were separately pooled and each pooled fraction was precipitated separately by adding 6 g of ammonium sulfate for every 10 ml of the solution in the presence of 0.05 M acetylcholine. After keeping for 1 hr the precipitates were collected by centrifugation at 14,000 x *g* for 1 hr. They were dissolved in 0.1 M buffer and assayed for protein and activity. The fraction with higher amount of total activity had a specific activity of approximately 100,000. It was stored at -20°. The final specific activity obtained was approximately 100,000 by this procedure.

This procedure upto the third step has been repeated 60 to 70 times, upto the fourth step about 12 times and up to the last step twice with reproducible results.

Approximately 100,000 was the highest specific activity obtained. No further studies on the purification of the enzyme were carried out. The results of a typical fractionation procedure are given in Table 20.

TABLE 20

PURIFICATION PROCEDURE OF BRAIN ACETYLCHOLINESTERASE

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity	Yield
	ml	units/ml	units	mg/ml	mg		%
Whole homogenate	3100	477	1,482,500				
Particulate enzyme	2380	400	954,000				
Elastase treated - I	1620	72	116,640	4.2	6825	17	
-II	1200	60	72,000	3.5	4200	17	
Total soluble enzyme	2820		188,640		11025	17	100
Step							
I Protamine sulfate treatment	3420	51	174,420	3	10260	17	92
II Ammonium sulfate fractionation-I	125	1210	151,250	25	3125	48	80
III DEAE-cellulose chromatography-I	13	3680	57,000	4.3	56	850	30
IV Calcium phosphate gel treatment	2	14500	29,000	2.6	5.2	5500	16
V DEAE-cellulose chromatography-II	0.5	30000	15,000	2.6	1.3	11600	9
VI Ammonium sulfate fractionation-II	1.5	9600	14,400	0.44	0.66	21800	9
VII Sephadex G-200 chromatography	1.5	4800	7,200	0.05	0.075	100,000 (approx.)	4

Note: Activity of soluble enzyme is taken as 100 per cent.

CHAPTER 4

PROPERTIES AND KINETICS

SECTION I

Properties

Solutions of purified enzyme were water clear and colorless. But enzyme of maximum purity (about 100,000 units/mg) was available only in dilute solution and it is not possible to state definitely whether any colored material is associated with it.

Molecular weight

The molecular weight of AchE was determined by gel filtration on Sephadex G-200. A column (1.2 x 100 cm) was equilibrated with 0.05 M phosphate pH 7.5. Catalase and crystalline alcohol dehydrogenase (yeast) were used as reference proteins. AchE with specific activity of about 1000 was used. 2.2 ml fractions were collected every 20 min. The protein appeared in the following order of fractions: catalase: (21 to 24), AchE (23 to 28) and alcohol dehydrogenase (32 to 36). By Andrew's method for calculations of molecular weight[191], about 200,000 was tentatively estimated to be the molecular weight of AchE.

Stability of the enzyme on storage

The enzyme was remarkably stable at different stages of purification. Enzyme of specific activity of 20 to 20,000 could be stored at -20° for several days without loss in activity. The soluble enzyme was generally treated with protamine sulfate within a day or two after preparation. It could then be stored at -20° for about 2 to 3 weeks without any loss in activity. Enzyme of specific activity about 1000 (Step III) could be preserved for about one month at 0° without any loss in activity. This enzyme could also be kept at 30° for 24 hr without any loss in activity. Enzyme having a specific activity of about 5000, did not lose any

activity in 2 to 3 weeks at -20° and at specific activity of 9000 the loss in activity even after 4 months was only about 50 per cent.

Enzyme of specific activity of about 100,000 was more unstable and lost about 50 per cent activity in 5 days at -20°. This was probably due to the high dilution (about 0.05 to 0.1 mg/ml) in which it was stored. But when 2-3 mg per ml of serum albumin was added to it, AchE of maximum specific activity could be stored in 0.1 M phosphate pH 7.5 for about a month without any loss in activity. For kinetic studies enzyme of the highest purity was always diluted in 0.1 M phosphate pH 7.5 containing 1 mg per ml serum albumin and under these conditions 100 per cent activity was retained at 0° for at least two days.

Thermal stability

Studies were made on the thermal stability of AchE. Enzyme of low specific activity (about 60 to 70) was used. About 50 units of the enzyme were taken in 1 ml of 0.05 M phosphate pH 7.5 and heated in a water bath at 60° for a period of 5 min. There was 100 per cent loss in activity. But when heating was done in the presence of 0.005 M and 0.05 M acetylcholine the losses were 75 per cent and 4 per cent respectively showing the protecting action of acetylcholine against heat inactivation. (In the case of experiments with acetylcholine (0.05 M), 0.1 ml of the heated enzyme was added to the assay system (Assay-I) containing all other components except the substrate. Zero min reading of acetylcholine was taken by adding alkaline hydroxylamine at zero min and to another tube after 30 min incubation at 37°). At 65° for 5 min there was complete loss in activity even in the presence of 0.2M acetylcholine.

The effect of phosphate (0.2 M, pH 7.5), NaCl (0.15 M, 0.5 M), KCl (0.5 M), $MgCl_2$ (0.03 M) and NaF (0.1, 0.2 and 0.5 M) on heat inactivation

TABLE 21

THERMAL STABILITY OF BRAIN ACETYLCHOLINESTERASE

50 units of enzyme of specific activity 60 were used. Enzyme was in 0.05 M phosphate pH 7.5.

Compound	Temperature	Time	Loss
	°	min	%
1. a) 0.05 M phosphate	60	5	100
b) 0.05 M phosphate	55	10	100
2. 0.005 M Acetylcholine	60	5	75
3. 0.05 M Acetylcholine	60	5	4
4. 0.2 M Acetylcholine	65	5	100
5. 0.2 M Phosphate	60	5	100
6. a) 0.5 M NaCl	55	10	55
b) 0.5 M NaCl	60	5	100
7. a) 0.5 M KCl	55	10	60
b) 0.5 M KCl	60	5	100
8. 0.1 M NaF	60	5	50
9. 0.2 M NaF	60	5	25
10. 0.5 M NaF	60	5	25

of AchE were studied (Table 21). Phosphate and $MgCl_2$ at the concentrations stated above showed no stabilizing effect. NaCl was ineffective at lower concentration, but showed a protective action at 0.5 M.KCl had the same effect as NaCl. NaF was the best protective agent of the three. In the case of experiments with NaF the activity determinations were made after dialyzing the enzyme free of NaF.

SECTION II

Kinetics

Enzyme of the highest purity (specific activity about 100,000) was used for all kinetic studies unless stated otherwise. $\triangle$ O.D. refers to the decrease in optical density at 540 mμ due to the hydrolysis of acetylcholine in Assay-I.

Enzyme concentration and activity

It will be seen from the Figs. 3 and 4 that enzyme activity is proportional to enzyme concentration by both Assays I and II. In the case of Assay-I, the proportionality was observed till 25 to 30 per cent hydrolysis of acetylcholine and with Assay-II till about 60 Cmm of CO_2 were evolved.

Time and enzyme activity

Enzyme activity was proportional to time by both the methods (Figs.5 and 6). The limits of the proportionality with time were the same as in the case of enzyme concentration and there was a fall in activity beyond that range.

Effect of temperature

The effect of temperature on AchE activity was determined by Assay-I (Table 22, Fig.7) over the range 0° to 37°. There was much less increase in activity between 12° and 30° and negligible increase between 30° and 37°. It was not possible to calculate the energy of activation by the use of the Arrhenius equation. For many enzymes there is a two-fold increase in activity for a temperature rise of 10°. As can be seen from Fig.5, there was no inactivation of the enzyme under the experimental conditions (temperature 37°, pH 7.5)

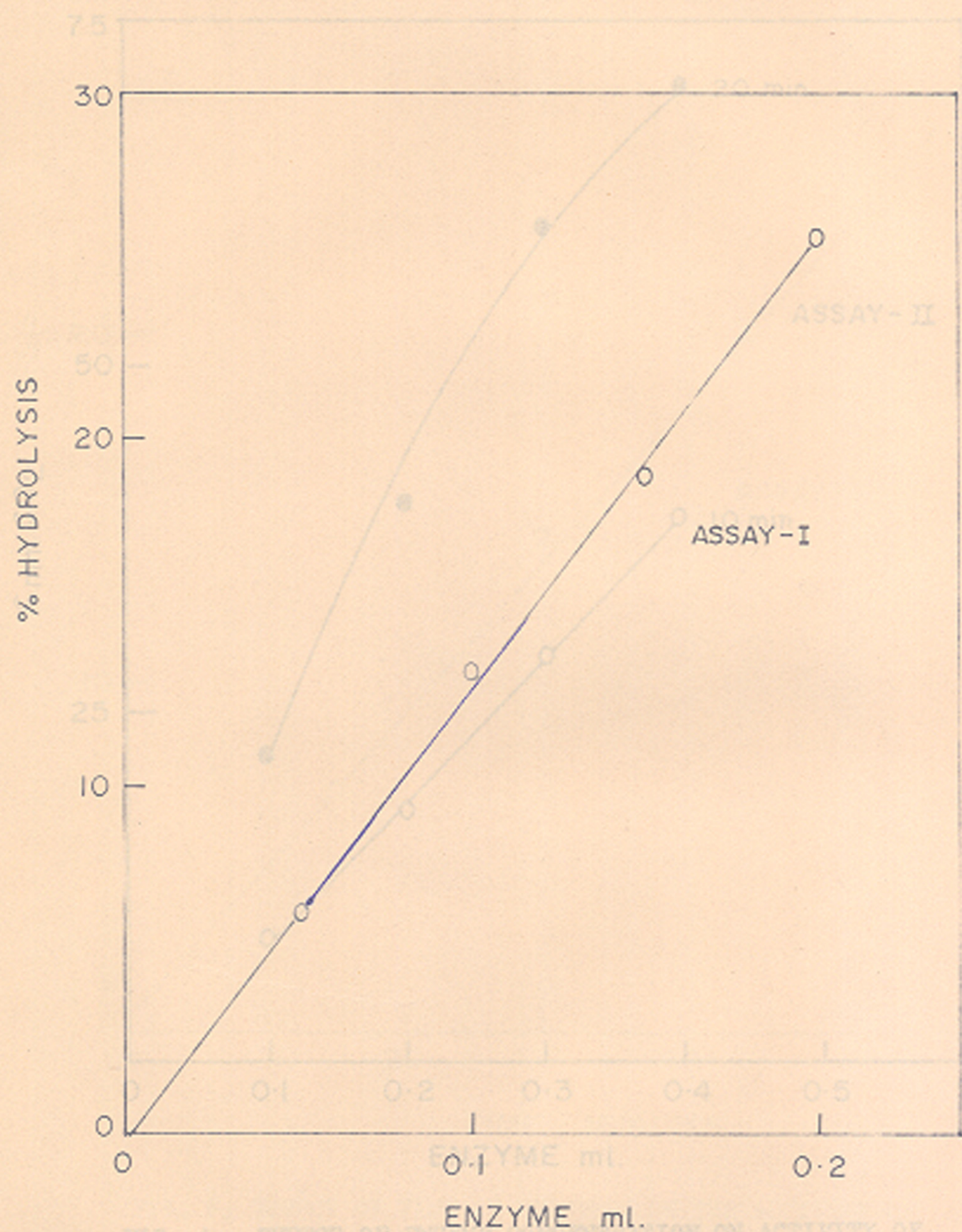


FIG. 3. EFFECT OF ENZYME CONCENTRATION ON ACTIVITY OF BRAIN ACETYLCHOLINESTERASE.
ASSAY-I.

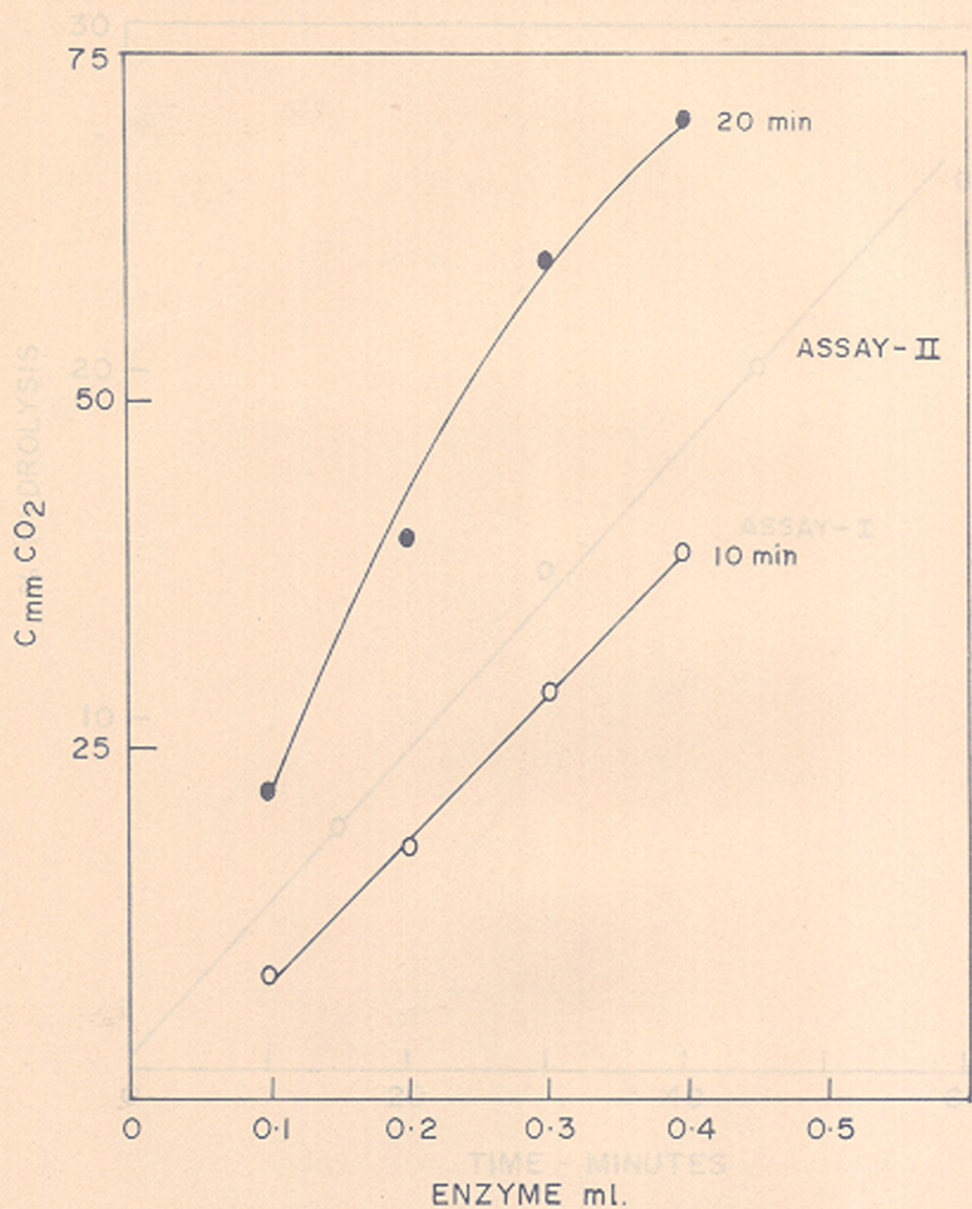


FIG. 4. EFFECT OF ENZYME CONCENTRATION ON ACTIVITY OF BRAIN ACETYLCHOLINESTERASE.

ASSAY-II.

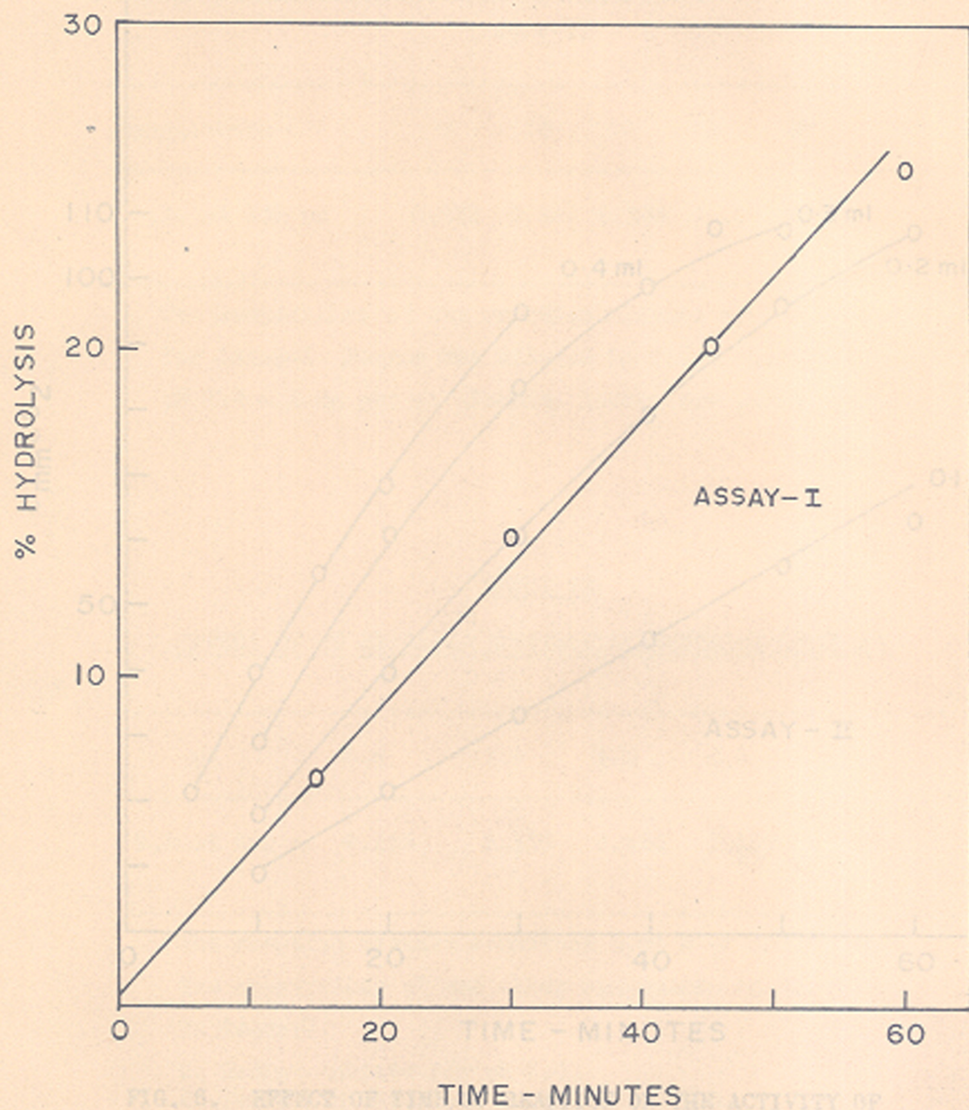


FIG. 5. EFFECT OF TIME OF REACTION ON THE ACTIVITY OF BRAIN ACETYLCHOLINESTERASE.

ASSAY-I.

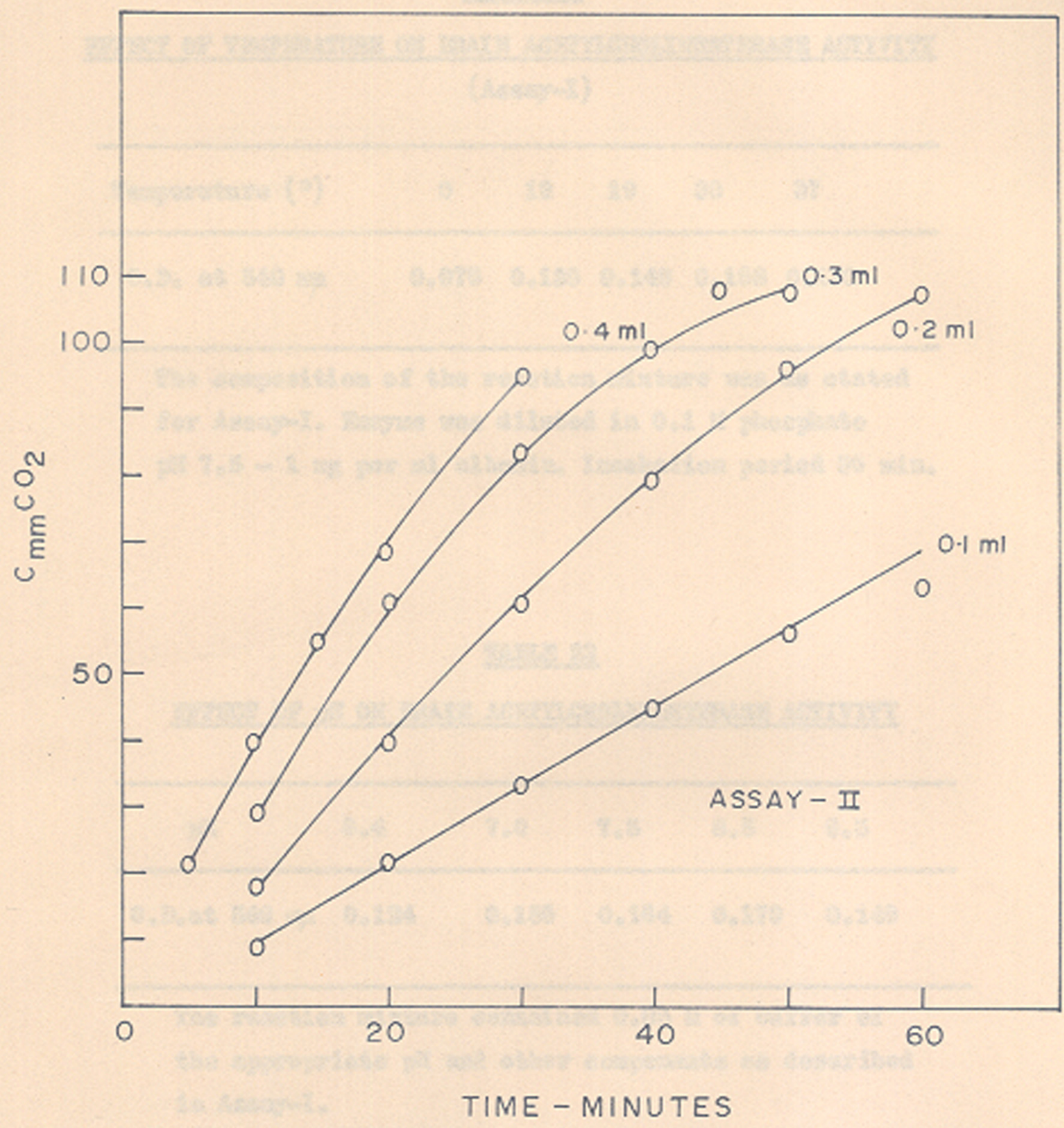


FIG. 6. EFFECT OF TIME OF REACTION ON THE ACTIVITY OF BRAIN ACETYLCHOLINESTERASE. ASSAY-II.

TABLE 22

EFFECT OF TEMPERATURE ON BRAIN ACETYLCHOLINESTERASE ACTIVITY (Assay-I)

Temperature (°)	0	12	19	30	37
O.D. at 540 mμ	0.076	0.120	0.140	0.168	0.176

The composition of the reaction mixture was as stated for Assay-I. Enzyme was diluted in 0.1 M phosphate pH 7.5 - 1 mg per ml albumin. Incubation period 30 min.

TABLE 23

EFFECT OF pH ON BRAIN ACETYLCHOLINESTERASE ACTIVITY

pH	6.4	7.0	7.5	8.5	9.5
O.D.at 540 mμ	0.124	0.156	0.164	0.170	0.140

The reaction mixture contained 0.05 M of buffer of the appropriate pH and other components as described in Assay-I.

1. Sodium acetate for pH 6.4.
2. Potassium phosphate for pH 7 and 7.5.
3. Sodium barbiturate for pH 8.5 and 9.5.

Incubation period was 30 min.

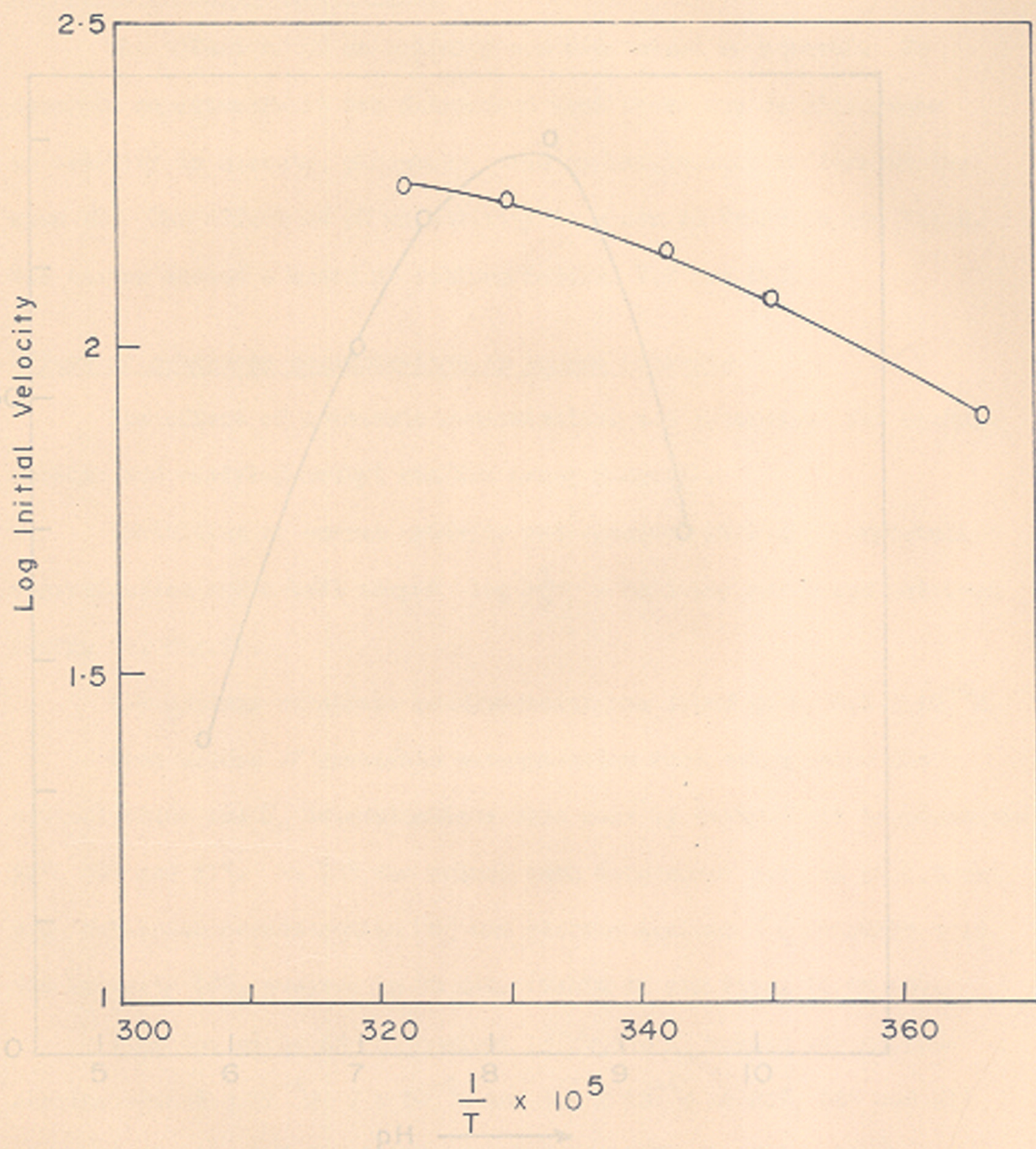


FIG. 7. ARRHENIUS PLOT FOR THE ACTIVITY OF BRAIN ACETYLCHOLINESTERASE.
ASSAY-I.

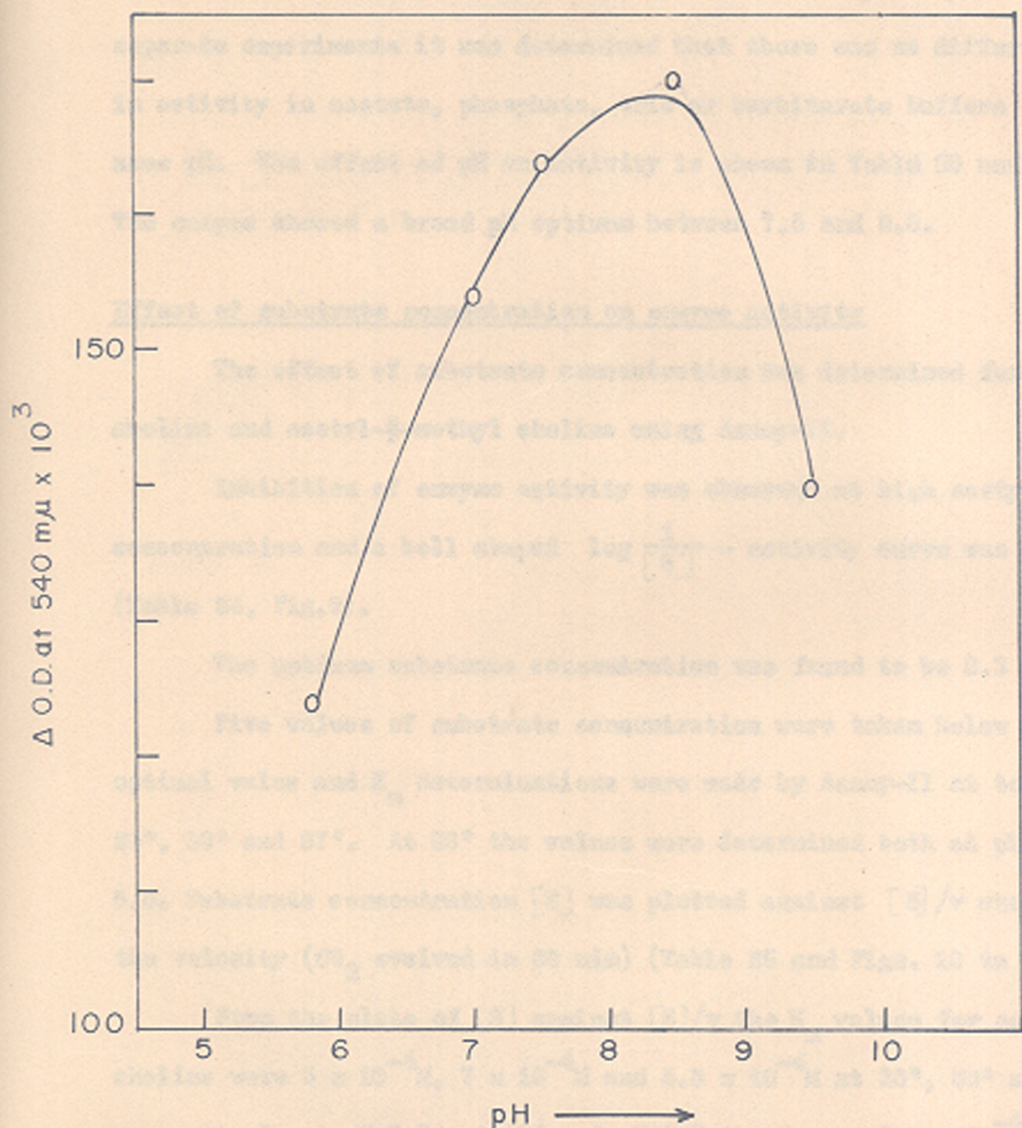


FIG. 8. EFFECT OF pH ON THE ACTIVITY OF BRAIN ACETYLCHOLINESTERASE.

ASSAY-I.

and the activity was linear up to 60 min (Assay-I). Therefore, the observed effect of temperature is not due to inactivation during the 30 min period of reaction.

Effect of pH on enzyme activity

The effect of pH on activity was determined by Assay-I. In separate experiments it was determined that there was no difference in activity in acetate, phosphate, tris or barbiturate buffers at the same pH. The effect of pH on activity is shown in Tablé 23 and Fig.8. The enzyme showed a broad pH optimum between 7.5 and 8.5.

Effect of substrate concentration on enzyme activity

The effect of substrate concentration was determined for acetylcholine and acetyl-β-methyl choline using Assay-II.

Inhibition of enzyme activity was observed at high acetylcholine concentration and a bell shaped $\log \frac{1}{[S]}$ - activity curve was obtained (Table 24, Fig.9).

The optimum substrate concentration was found to be 3.3×10^{-3}M.

Five values of substrate concentration were taken below the optimal value and K_m determinations were made by Assay-II at temperatures 20°, 30° and 37°. At 30° the values were determined both at pH 7.5 and 6.5. Substrate concentration [S] was plotted against [S]/v where v is the velocity (CO_2 evolved in 30 min) (Table 25 and Figs. 10 to 13).

From the plots of [S] against [S]/v the K_m values for acetylcholine were 5×10^{-4}M, 7×10^{-4}M and 5.5×10^{-4}M at 20°, 30° and 37° respectively at pH 7.5; at 30° and pH 6.5 the K_m was 3.5×10^{-4}M.

Acetyl-β-methyl choline - The effect of substrate concentration was determined at pH 7.5 and 30° using different concentrations of acetyl-β-methyl choline by Assay-II. The results are shown in Table 26 and

TABLE 24

EFFECT OF HIGH ACETYLCHOLINE CONCENTRATION ON BRAIN ACETYLCHOLINESTERASE ACTIVITY

Substrate concentration mM	0.33	1.1	3.3	11	33	110
Cmm CO_2 in 30 min	15	25	30	19	15	12

The composition of the reaction mixture, except acetylcholine, was as stated for Assay-II. NaCl was only 0.03 M in case of the highest substrate concentration. pH was 7.5 and temperature 30°.

TABLE 25

EFFECT OF ACETYLCHOLINE CONCENTRATION ON BRAIN ACETYLCHOLINESTERASE

Temp.	pH		Acetylcholine concentration mM 3	2	1	0.75	0.47	K_m
		$[S] \times 10^4$	30	20	10	7.5	4.7	
°			Cmm CO_2 in 30 min					M
20	7.5		16	18	16	12	10	5×10^{-4}
30	7.5		30	26	16	19	15	7×10^{-4}
30	6.5		16	15	13	12	9	3.5×10^{-4}
37	7.5		22	29	14	14	12	5.5×10^{-4}

The composition of the reaction mixture was the same as described for Assay-II except for the acetylcholine concentration and in the case of pH 6.5 0.003 M $NaHCO_3$ was used and the enzyme was one and half times the amount used at pH 7.5. Enzyme was diluted in 0.1 M phosphate pH 7.5 - 1 mg/ml albumin.

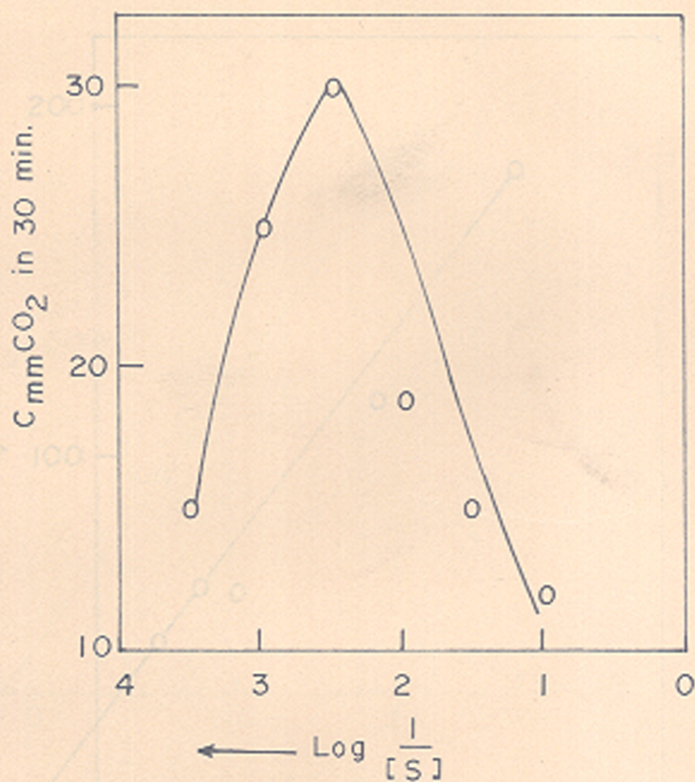


FIG. 9. LOG $\frac{1}{[S]}$-ACTIVITY PLOT OF BRAIN ACETYLCHOLINESTERASE. ASSAY-II.

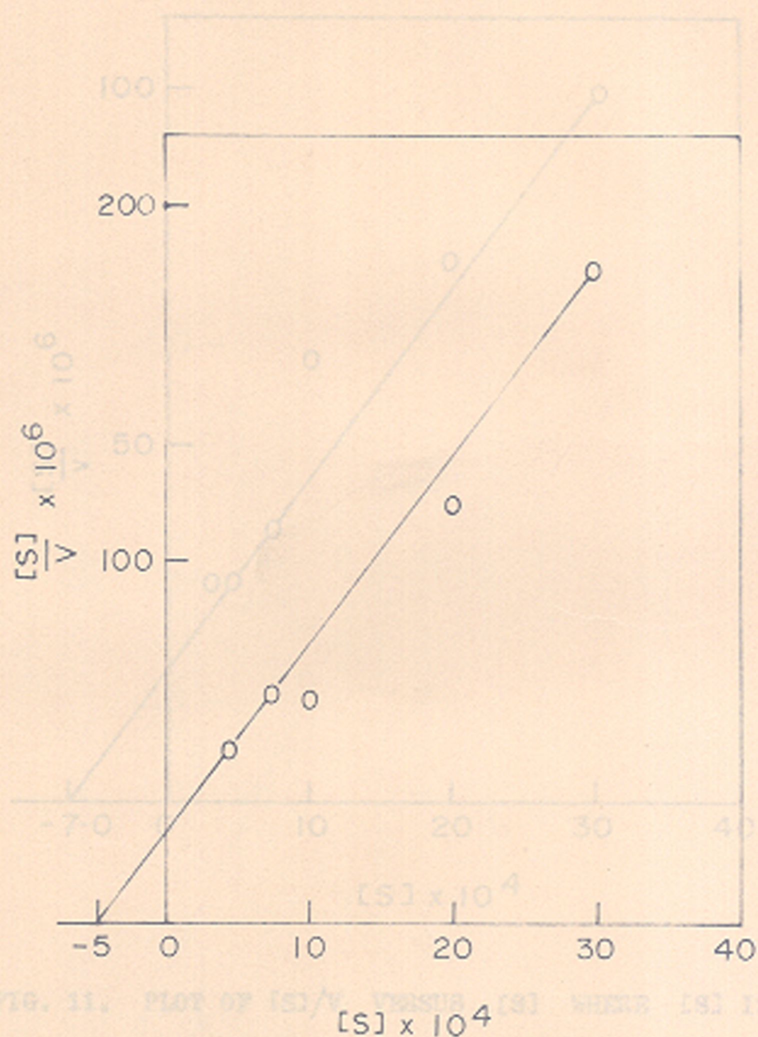


FIG. 10. PLOT OF [S]/V VERSUS [S] WHERE [S] IS THE MOLAR CONCENTRATION OF ACETYLCHOLINE AND V IS THE INITIAL VELOCITY. TEMPERATURE 20°, pH 7.5. ASSAY-II.

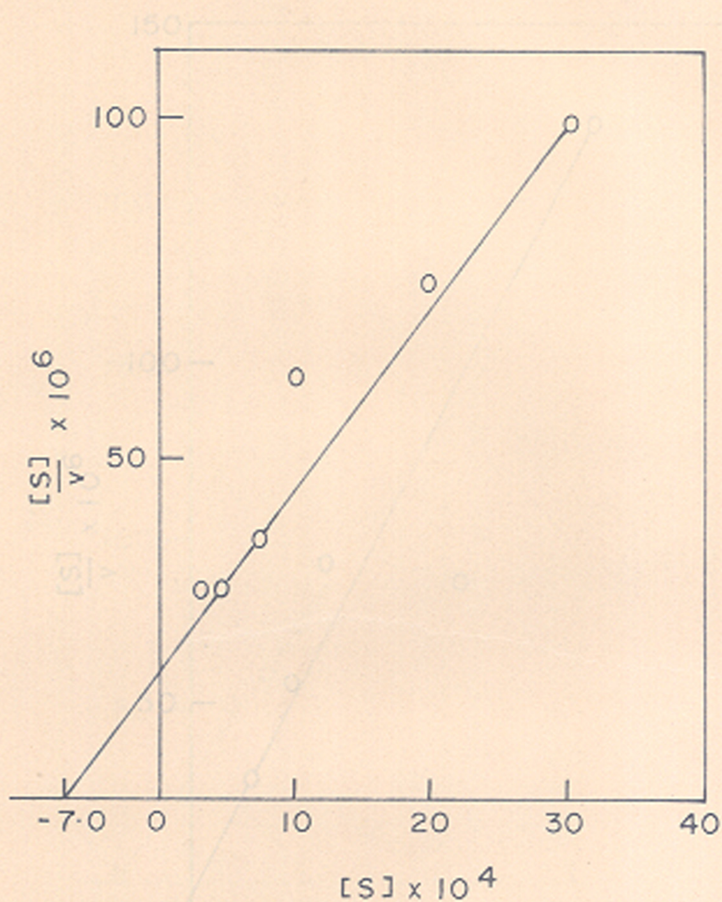


FIG. 11. PLOT OF [S]/V VERSUS [S] WHERE [S] IS THE MOLAR CONCENTRATION OF ACETYLCHOLINE AND V IS THE INITIAL VELOCITY. TEMPERATURE 30°, pH 7.5 ASSAY-II.

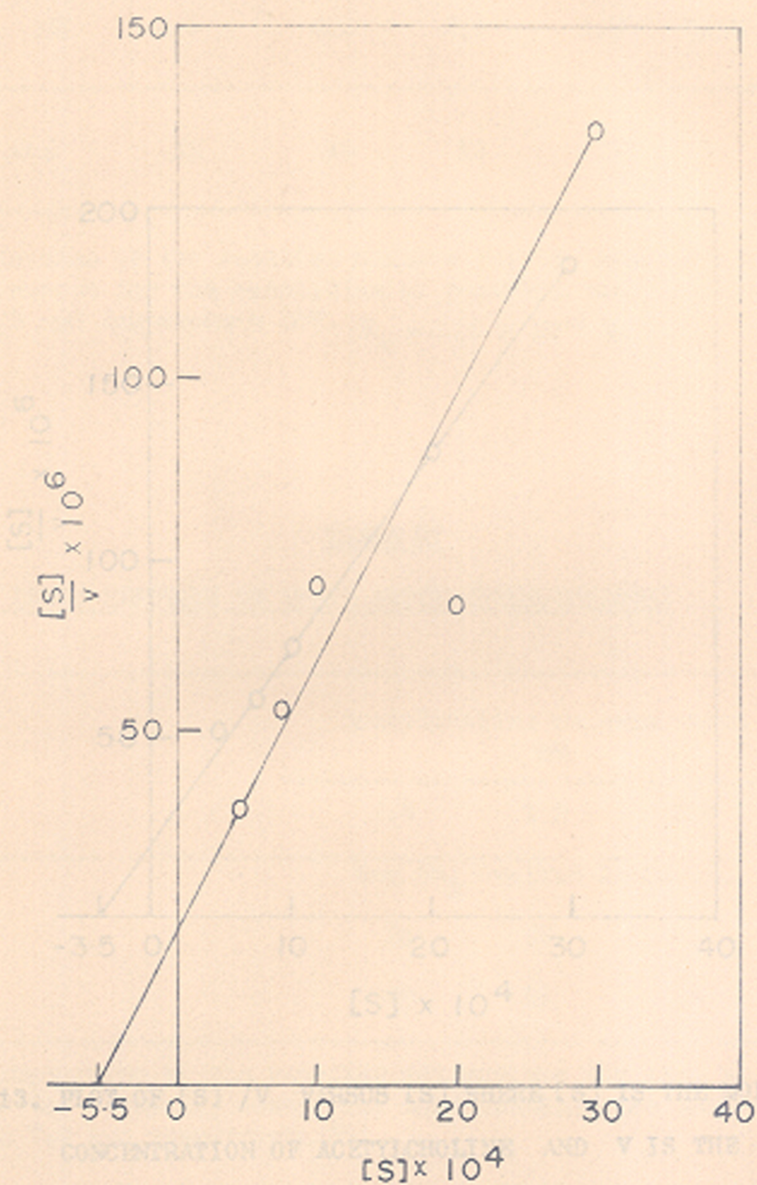


FIG. 12. PLOT OF [S] /V VERSUS [S] WHERE [S] IS THE MOLAR CONCENTRATION OF ACETYLCHOLINE AND V IS THE INITIAL VELOCITY. TEMPERATURE 37°, pH 7.5. ASSAY-II.

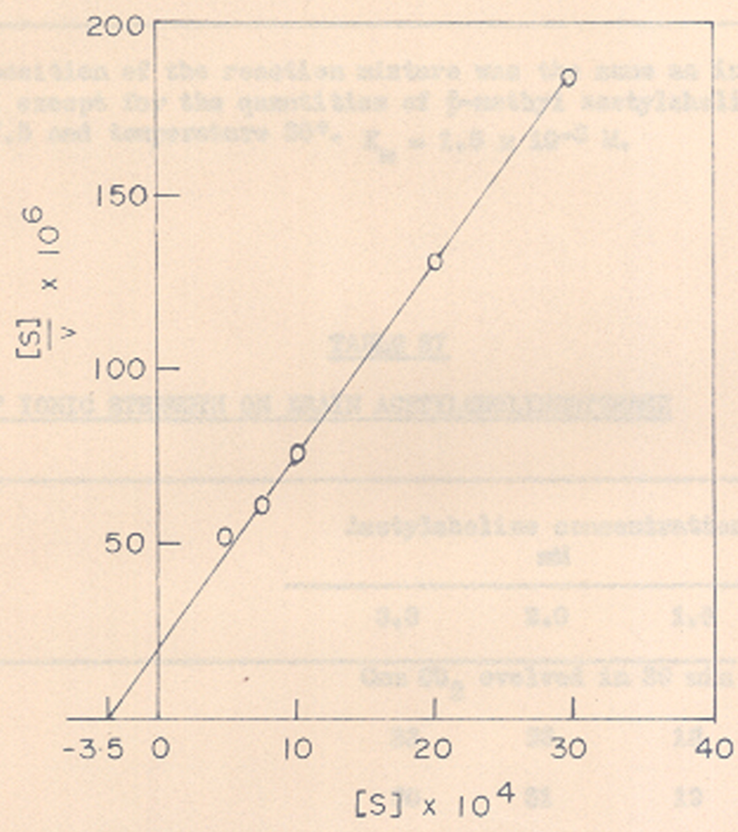


FIG. 13. PLOT OF [S] /V VERSUS [S] WHERE [S] IS THE MOLAR CONCENTRATION OF ACETYLCHOLINE AND V IS THE INITIAL VELOCITY. TEMPERATURE 30°, pH 6.5 ASSAY-II.

TABLE 26

EFFECT OF ACETYL-β-METHYL CHOLINE CONCENTRATION ON BRAIN ACETYLCHOLINESTERASE

Concentration of acetyl-β-methyl choline mM	5.0	3.0	2.0	1.0	0.75	0.5
Cmm CO_2 in 30 min	18	15	13	10	8	6

The composition of the reaction mixture was the same as in the Assay-II except for the quantities of β-methyl acetylcholine. pH was 7.5 and temperature 30°. $K_m = 1.5 \times 10^{-3}$ M.

TABLE 27

EFFECT OF IONIC STRENGTH ON BRAIN ACETYLCHOLINESTERASE

NaCl	Acetylcholine concentration mM		
	3.0	2.0	1.0
M	Cmm CO_2 evolved in 30 min		
0.1	33	28	18
0.5	30	31	19

The composition of the reaction mixture was the same as stated for Assay-II, except for acetylcholine and NaCl, pH was 7.5 and temperature 30°.

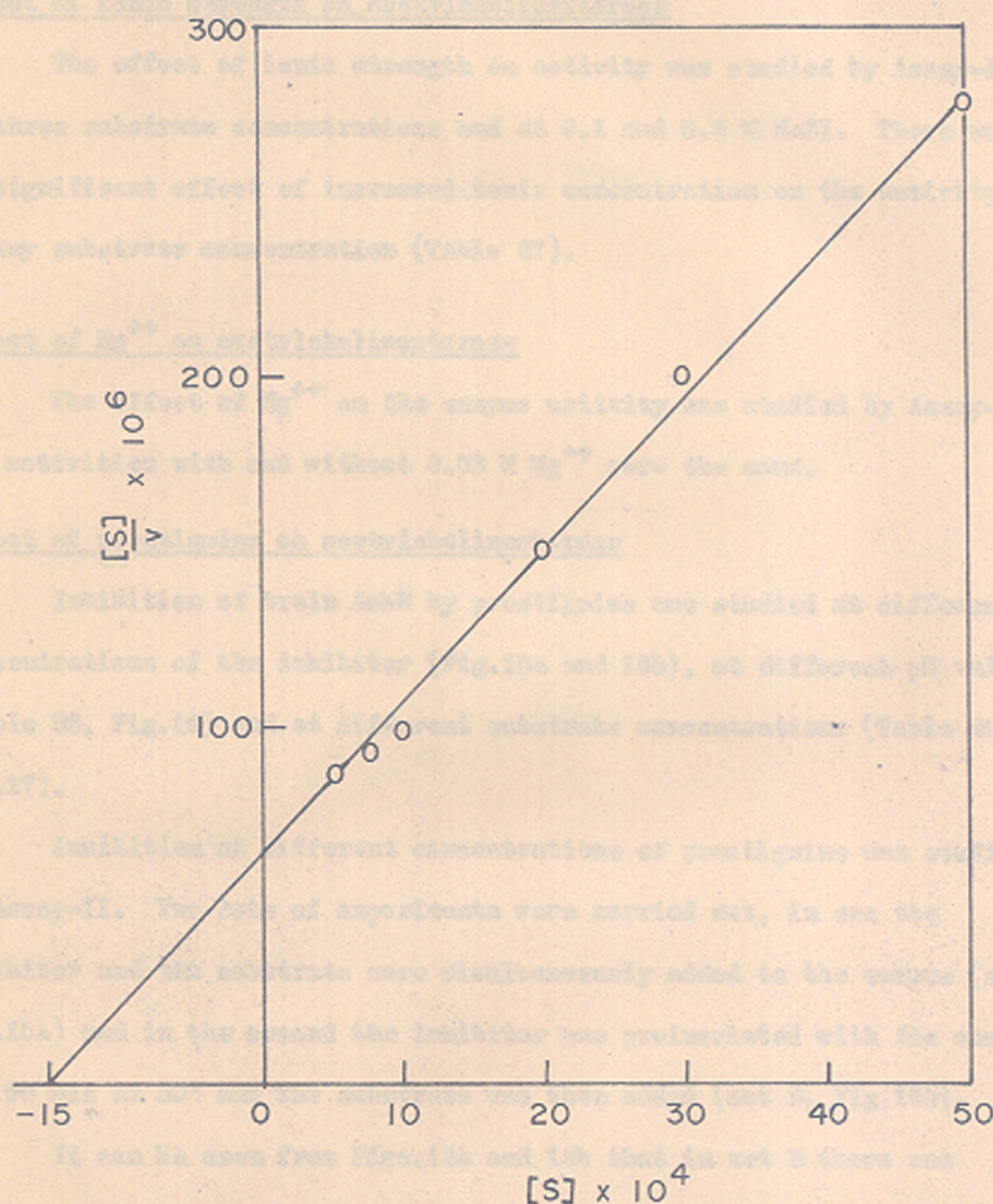


FIG. 14. PLOT OF [S] /V VERSUS [S] WHERE [S] IS THE MOLAR CONCENTRATION OF β -METHYL ACETYLCHOLINE AND V IS THE INITIAL VELOCITY. TEMPERATURE 30°, pH 7.5. ASSAY-II.

Fig.14. From the plot of $[S]$ vs $[S]/v$ the K_m value for acetyl-β-methyl choline was 1.5×10^{-3}M.

Effect of ionic strength on acetylcholinesterase

The effect of ionic strength on activity was studied by Assay-II at three substrate concentrations and at 0.1 and 0.5 M NaCl. There was no significant effect of increased ionic concentration on the activity at any substrate concentration (Table 27).

Effect of Mg^{++} on acetylcholinesterase

The effect of Mg^{++} on the enzyme activity was studied by Assay-II. The activities with and without 0.03 M Mg^{++} were the same.

Effect of prostigmine on acetylcholinesterase

Inhibition of brain AchE by prostigmine was studied at different concentrations of the inhibitor (Fig.15a and 15b), at different pH values (Table 28, Fig.16) and at different substrate concentrations (Table 29, Fig.17).

Inhibition at different concentrations of prostigmine was studied by Assay-II. Two sets of experiments were carried out, in one the inhibitor and the substrate were simultaneously added to the enzyme (set A, Fig.15a) and in the second the inhibitor was preincubated with the enzyme for 60 min at 30° and the substrate was then added (set B, Fig.15b).

It can be seen from Figs.15a and 15b that in set B there was progressive decrease in degree of inhibition with progress of time and it approached the degree of inhibition of set A. In set A there was no significant change in degree of inhibition with progress of time after 20 min. This may probably be because equilibrium between enzyme and inhibitor was attained before this period in this set. The inhibition was reversible.

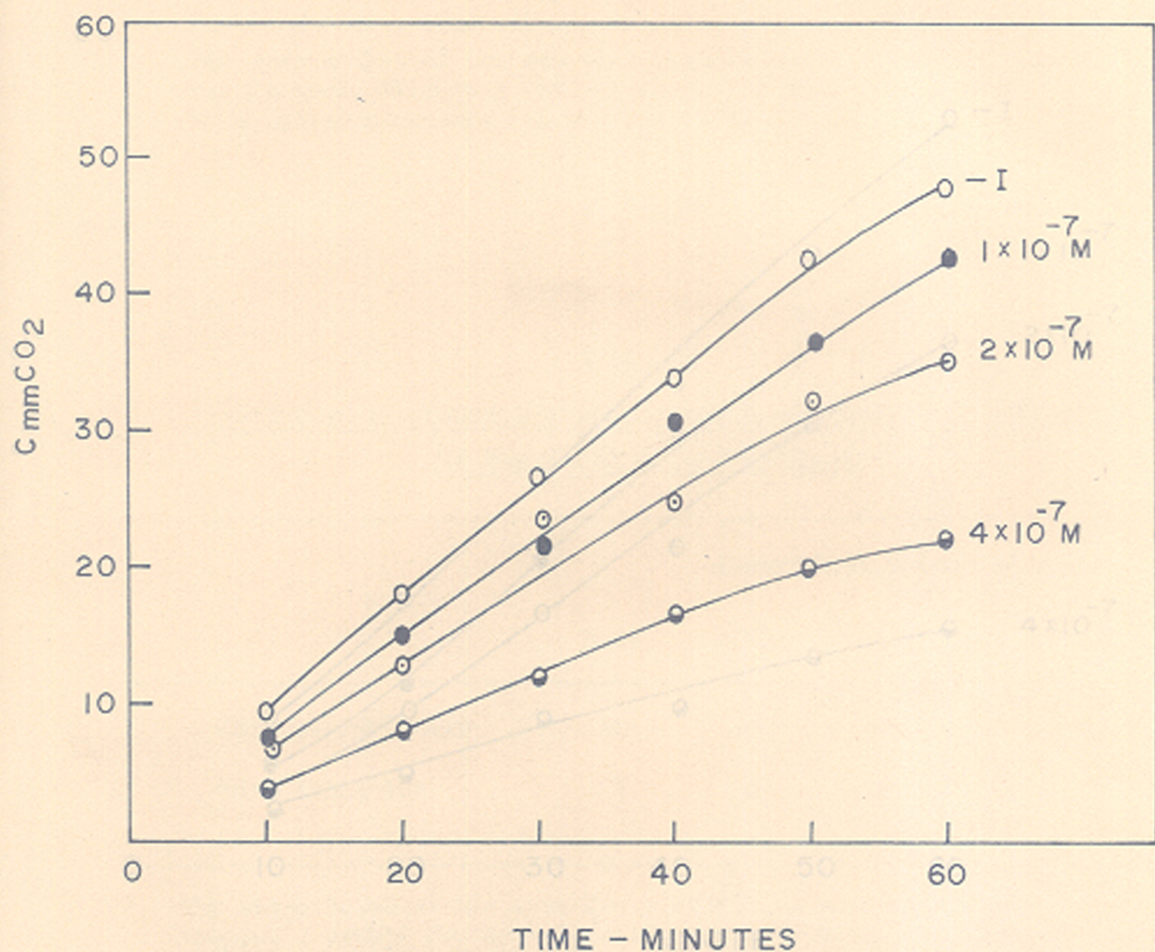


FIG. 15a. INHIBITION OF BRAIN ACETYLCHOLINESTERASE AT DIFFERENT CONCENTRATIONS OF PROSTIGMINE (1×10^{-7}, 2×10^{-7} AND 4×10^{-7}M). INHIBITOR AND SUBSTRATE SIMULTANEOUSLY ADDED TO THE ENZYME. TEMPERATURE 30°, pH 7.5. (SET A). ASSAY_II.

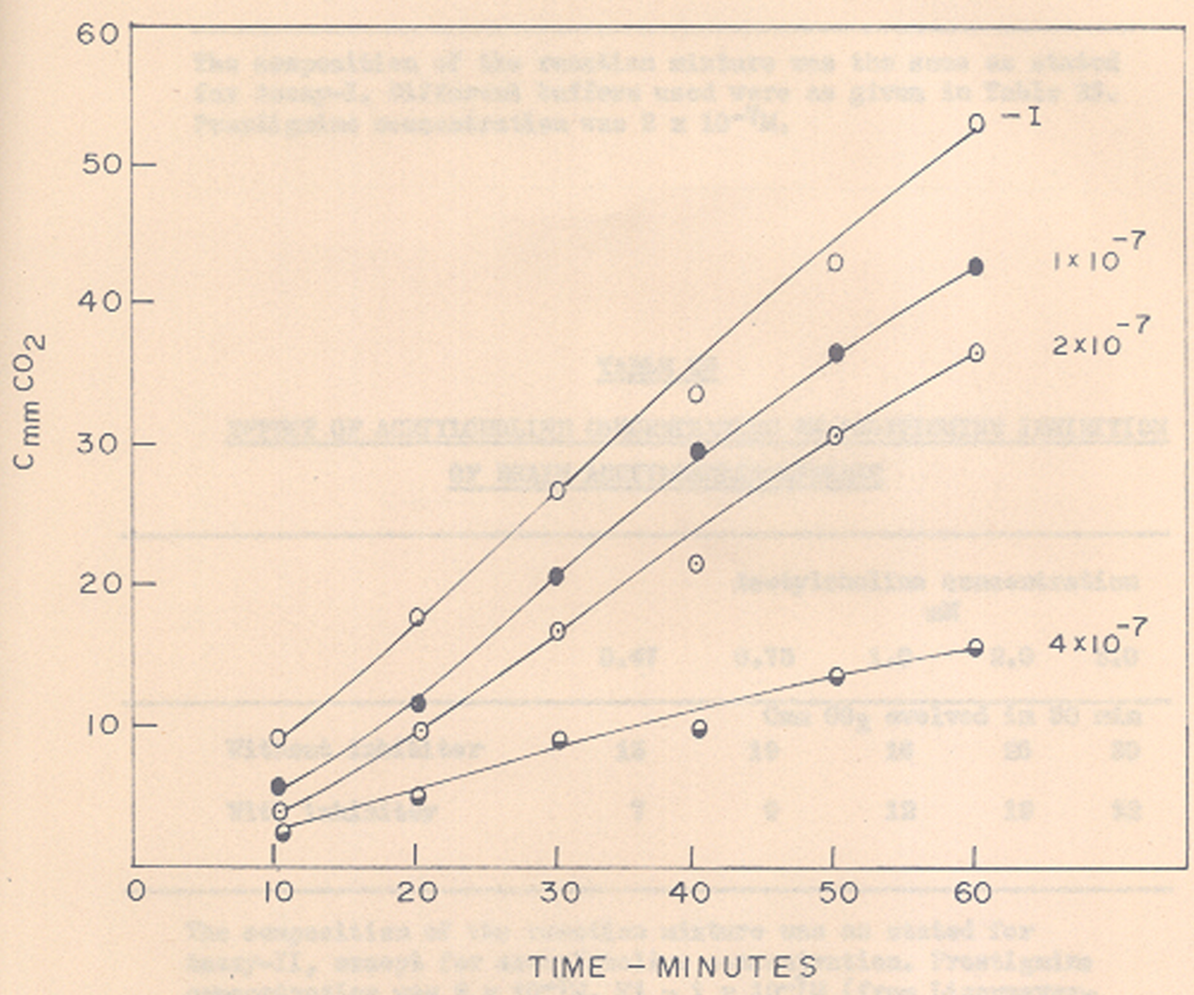


FIG. 15b. INHIBITION OF BRAIN ACETYLCHOLINESTERASE AT DIFFERENT OF PROSTIGMINE (1×10^{-7}, 2×10^{-7} AND 4×10^{-7} M).

INHIBITOR AND ENZYME PREINCUBATED AT 30° FOR 60 MIN.

TEMPERATURE 30°, pH 7.5 (SET B).

ASSAY-II.

TABLE 28

EFFECT OF pH ON PROSTIGMINE INHIBITION OF BRAIN ACETYLCHOLINESTERASE

pH	6	7.5	8.5	9.5
Per cent inhibition	55	55	55	60

The composition of the reaction mixture was the same as stated for Assay-I. Different buffers used were as given in Table 23. Prostigmine concentration was 2 x 10^{-7}M.

TABLE 29

EFFECT OF ACETYLCHOLINE CONCENTRATION ON PROSTIGMINE INHIBITION OF BRAIN ACETYLCHOLINESTERASE

	Acetylcholine concentration mM				
	0.47	0.75	1.0	2.0	3.0
	Cmm CO_2 evolved in 30 min				
Without inhibitor	15	19	16	26	30
With inhibitor	7	9	12	19	22

The composition of the reaction mixture was as stated for Assay-II, except for acetylcholine concentration. Prostigmine concentration was 2 x 10^{-7}M. Ki = 1 x 10^{-7}M (from Lineweaver-Burk plot) pH was 7.5 and temperature 30°.

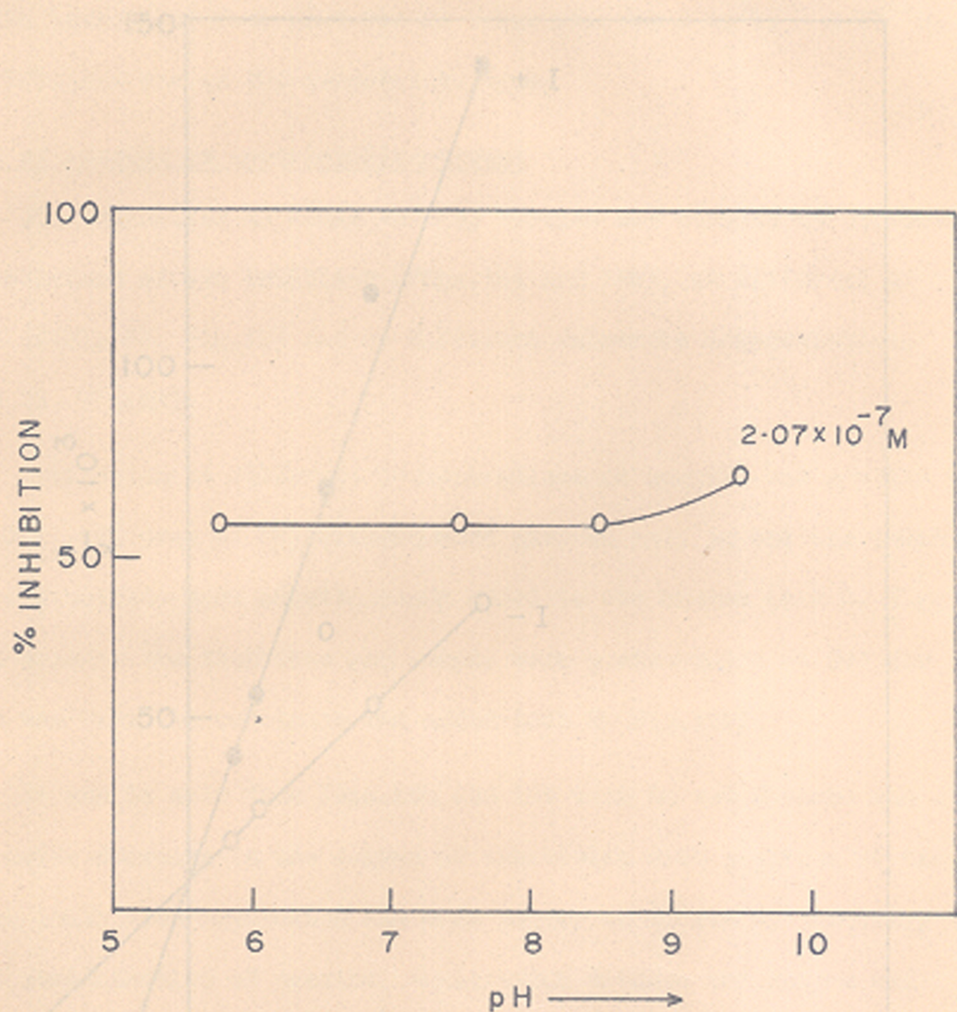


FIG. 16. EFFECT OF pH ON INHIBITION OF BRAIN ACETYLCHOLINESTERASE BY PROSTIGMINE.

ASSAY-I.

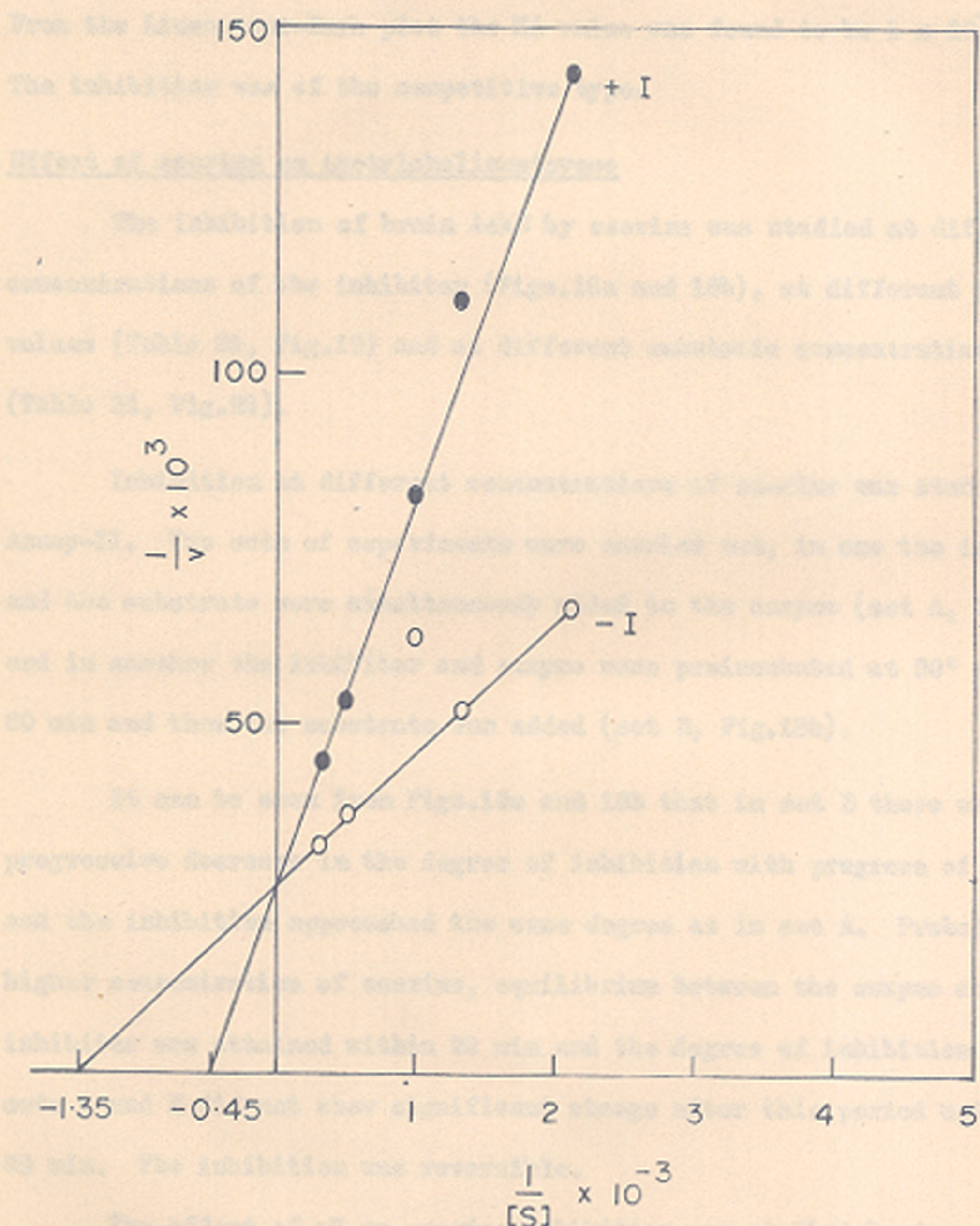


FIG. 17. LINEWEAVER-BURK PLOT OF BRAIN ACETYLCHOLINESTERASE WITHOUT (-I) AND WITH (+I) PROSTIGMINE. INHIBITOR CONCENTRATION 2 x 10^{-7}M. TEMPERATURE 30°, pH 7.5. ASSAY-II. Ki = 1 x 10^{-7}M.

Effect of pH on prostigmine inhibition was studied by Assay-I. The inhibition was observed to be independent of pH between pH 6 and 9.5 (Table 28, Fig.16).

The effect of substrate concentration on prostigmine inhibition was studied by Assay-II. The results are shown in Table 29 and Fig.17. From the Lineweaver-Burk plot the Ki value was found to be 1×10^{-7}M. The inhibition was of the competitive type.

Effect of eserine on acetylcholinesterase

The inhibition of brain AchE by eserine was studied at different concentrations of the inhibitor (Figs.18a and 18b), at different pH values (Table 30, Fig.19) and at different substrate concentrations (Table 31, Fig.20).

Inhibition at different concentrations of eserine was studied by Assay-II. Two sets of experiments were carried out, in one the inhibitor and the substrate were simultaneously added to the enzyme (set A, Fig.18a) and in another the inhibitor and enzyme were preincubated at 30° for 60 min and then the substrate was added (set B, Fig.18b).

It can be seen from Figs.18a and 18b that in set B there was a progressive decrease in the degree of inhibition with progress of time and the inhibition approached the same degree as in set A. Probably at higher concentration of eserine, equilibrium between the enzyme and inhibitor was attained within 20 min and the degree of inhibition in sets A and B did not show significant change after this period upto 60 min. The inhibition was reversible.

The effect of pH on eserine inhibition was studied by Assay-I. The inhibition increased with lowering of the pH in the range studied (Table 30, Fig.19) and was negligible at pH 9.5.

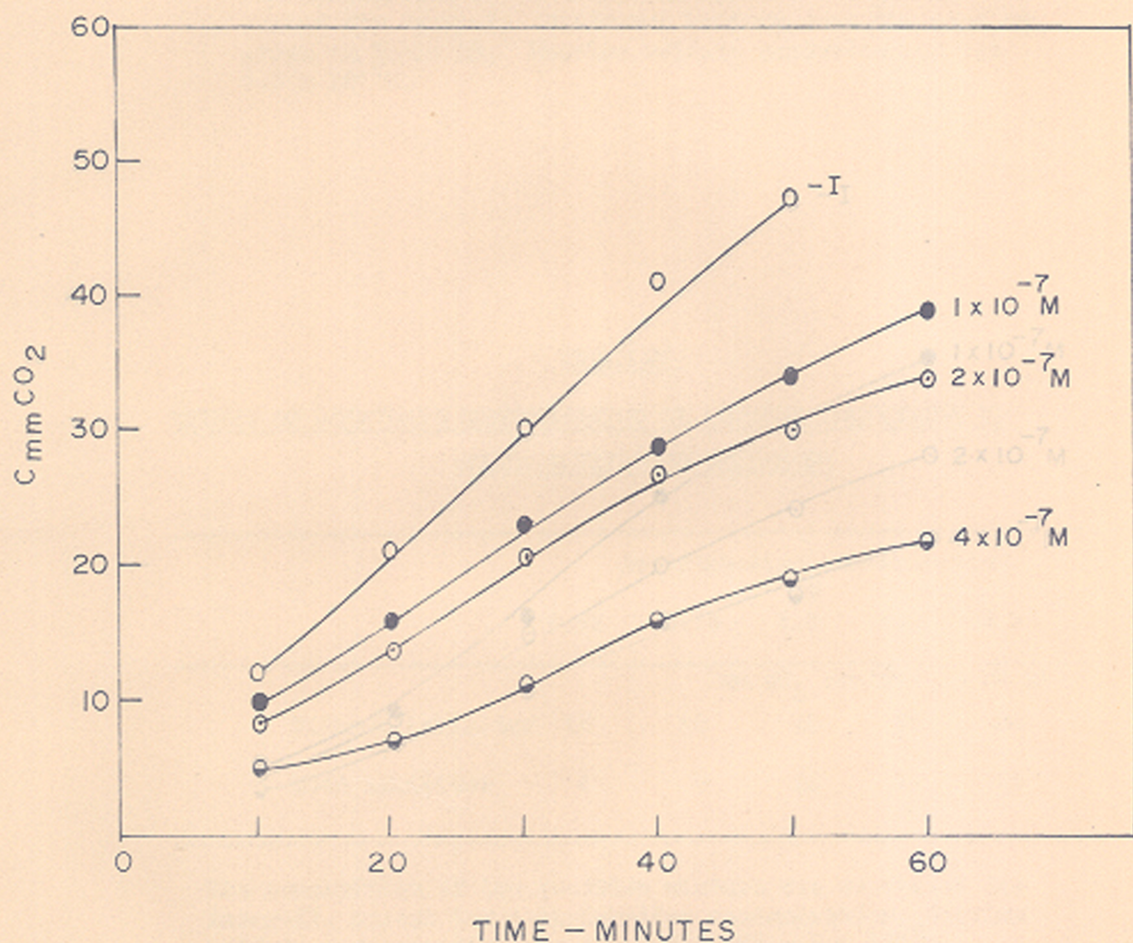


FIG. 18a. INHIBITION OF BRAIN ACETYLCHOLINESTERASE BY DIFFERENT CONCENTRATIONS OF ESERINE (1×10^{-7}, 2×10^{-7} AND 4×10^{-7}M). INHIBITOR AND SUBSTRATE SIMULTANEOUSLY ADDED TO THE ENZYME. TEMPERATURE 30°, pH 7.5. (SET-A).

ASSAY-II.

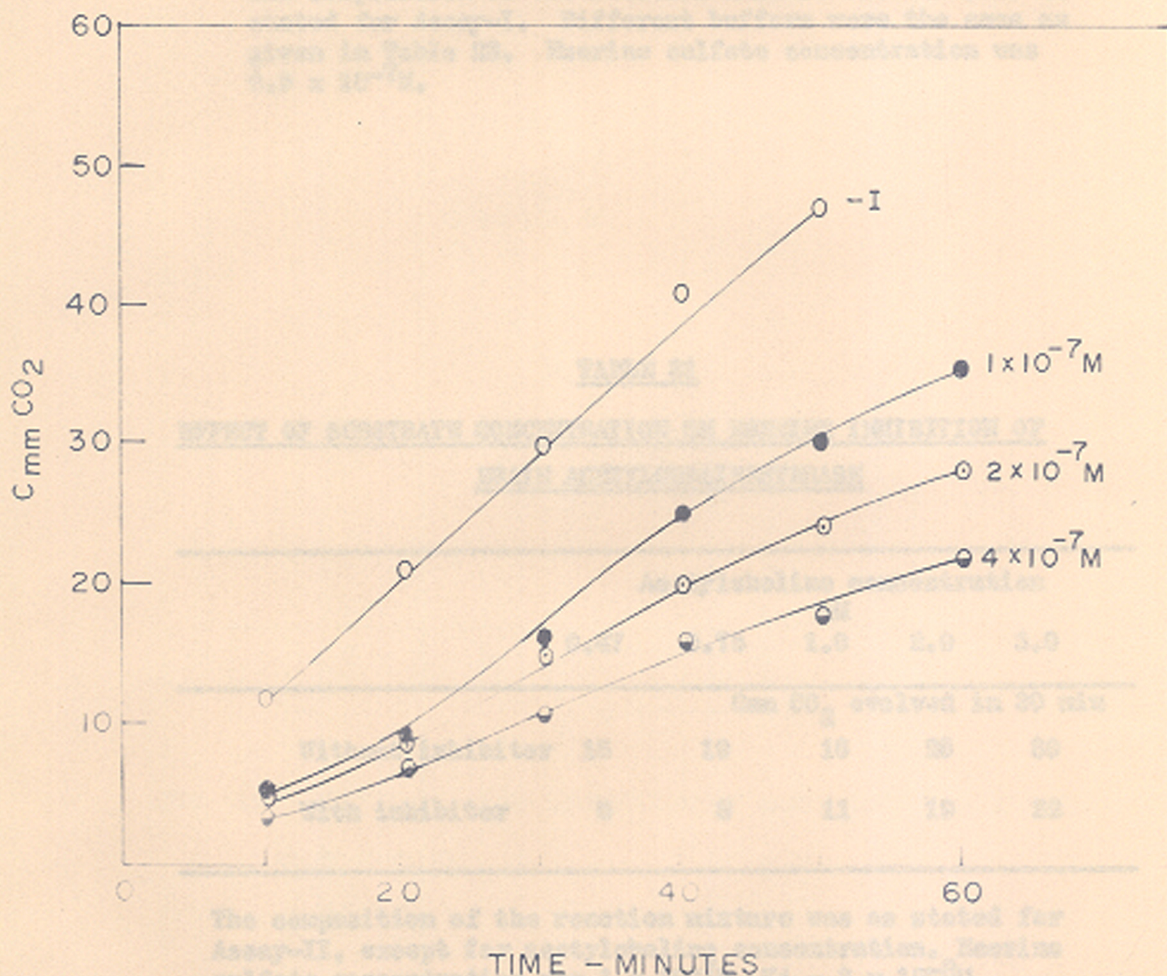


FIG. 18b. INHIBITION OF BRAIN ACETYLCHOLINESTERASE AT DIFFERENT CONCENTRATIONS OF ESERINE (1×10^{-7}, 2×10^{-7} AND 4×10^{-7}M). INHIBITOR AND ENZYME PREINCUBATED AT 30° FOR 60 MIN. TEMPERATURE 30°, pH 7.5. (SHT-B)

ASSAY-II.

TABLE 30

EFFECT OF pH ON ESERINE INHIBITION OF ACETYLCHOLINESTERASE

pH	6	6.5	7.0	7.5	8.5	9.5
Per cent inhibition	53	38	28	21	23	0

The composition of the reaction mixture was the same as stated for Assay-I. Different buffers were the same as given in Table 23. Eserine sulfate concentration was 0.9×10^{-7}M.

TABLE 31

EFFECT OF SUBSTRATE CONCENTRATION ON ESERINE INHIBITION OF BRAIN ACETYLCHOLINESTERASE

	Acetylcholine concentration mM				
	0.47	0.75	1.0	2.0	3.0
	Cmm CO_2 evolved in 30 min				
Without inhibitor	15	19	16	26	30
With inhibitor	5	8	11	19	22

The composition of the reaction mixture was as stated for Assay-II, except for acetylcholine concentration. Eserine sulfate concentration was 1×10^{-7}M. Ki = 3×10^{-8}M (from Lineweaver-Burk plot), pH was 7.5 and temperature 30°.

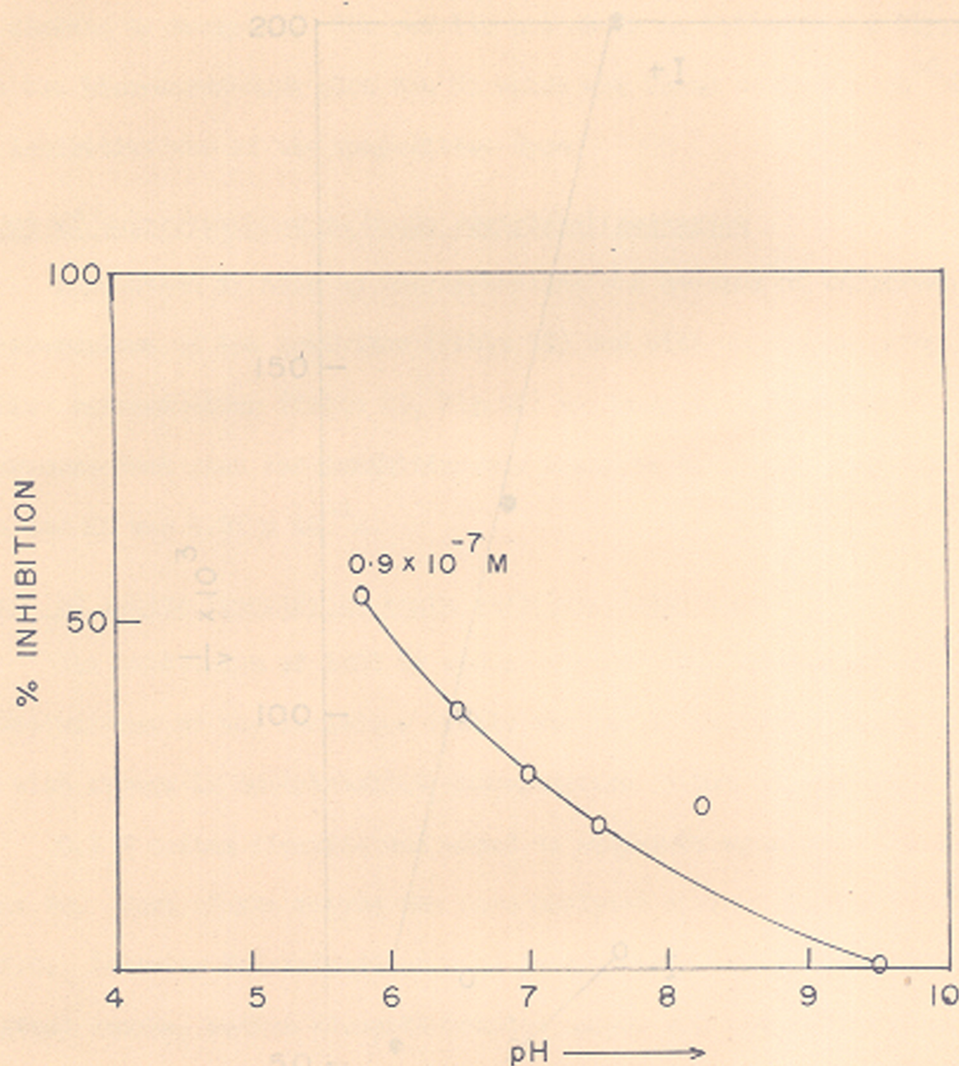


FIG. 19. EFFECT OF pH ON INHIBITION OF BRAIN ACETYLCHOLINESTERASE BY ESERINE.

ASSAY-I.

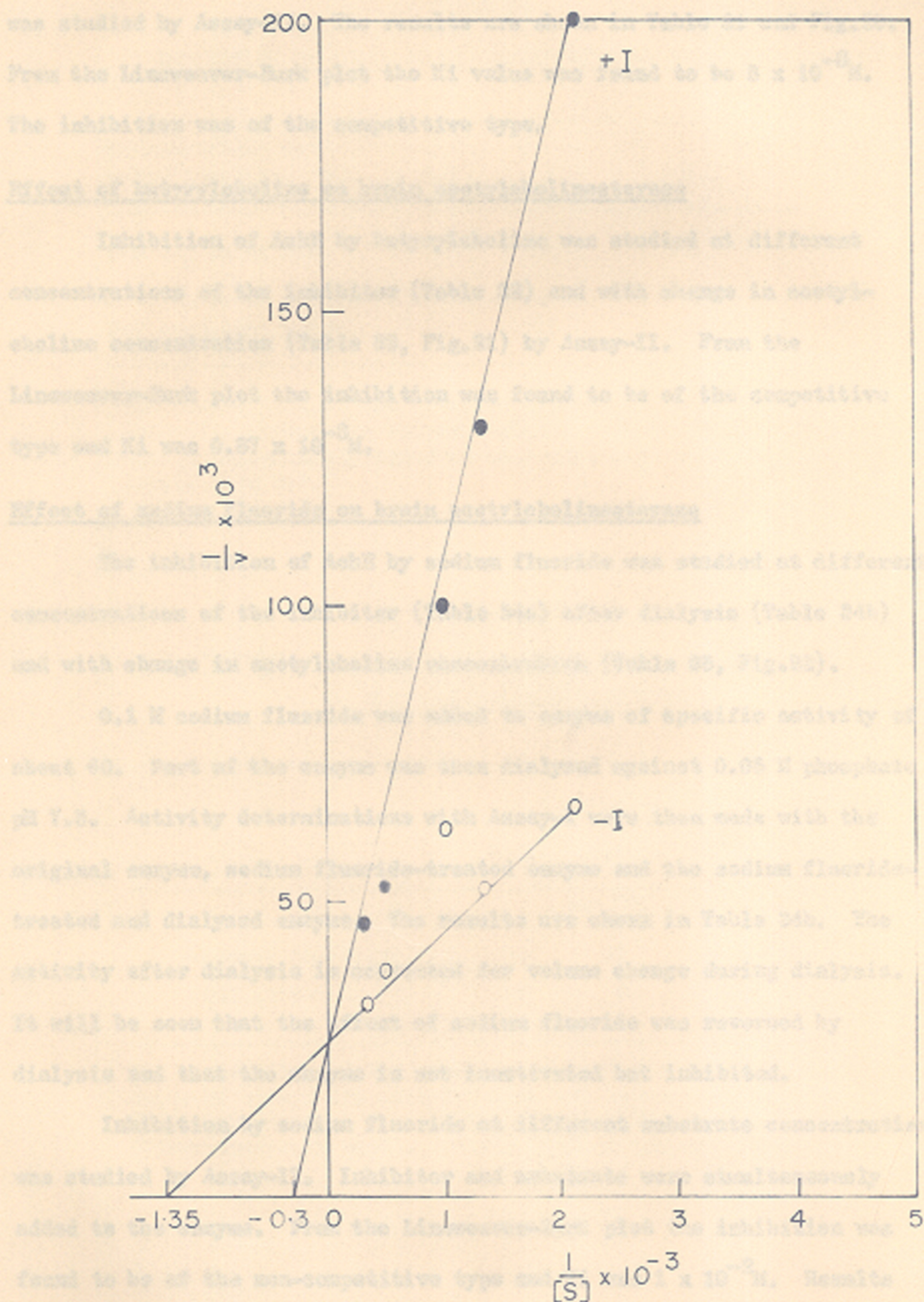


FIG. 20. LINEWEAVER-BURK PLOT OF BRAIN ACETYLCHOLINESTERASE WITHOUT (-I) AND WITH (+I) ESERINE. INHIBITOR CONCENTRATION 1×10^{-7}M. TEMPERATURE 30°, pH 7.5. ASSAY-II.

Ki = 3×10^{-8}M.

Inhibition by eserine at different concentrations of acetylcholine was studied by Assay-II. The results are shown in Table 31 and Fig.20. From the Lineweaver-Burk plot the Ki value was found to be 3×10^{-8}M. The inhibition was of the competitive type.

<u>Effect of butyrylcholine on brain acetylcholinesterase</u>

Inhibition of AchE by butyrylcholine was studied at different concentrations of the inhibitor (Table 32) and with change in acetylcholine concentration (Table 33, Fig.21) by Assay-II. From the Lineweaver-Burk plot the inhibition was found to be of the competitive type and Ki was 0.87×10^{-3}M.

<u>Effect of sodium fluoride on brain acetylcholinesterase</u>

The inhibition of AchE by sodium fluoride was studied at different concentrations of the inhibitor (Table 34a) after dialysis (Table 34b) and with change in acetylcholine concentration (Table 35, Fig.22).

0.1 M sodium fluoride was added to enzyme of specific activity of about 60. Part of the enzyme was then dialyzed against 0.05 M phosphate pH 7.5. Activity determinations with Assay-I were then made with the original enzyme, sodium fluoride-treated enzyme and the sodium fluoride-treated and dialyzed enzyme. The results are shown in Table 34b. The activity after dialysis is corrected for volume change during dialysis. It will be seen that the effect of sodium fluoride was reversed by dialysis and that the enzyme is not inactivated but inhibited.

Inhibition by sodium fluoride at different substrate concentrations was studied by Assay-II. Inhibitor and substrate were simultaneously added to the enzyme. From the Lineweaver-Burk plot the inhibition was found to be of the non-competitive type and Ki was 1×10^{-2}M. Results are shown in Table 35 and Fig.22.

TABLE 32

INHIBITION OF BRAIN ACETYLCHOLINESTERASE BY BUTYRYLCHOLINE

Concentration of butyrylcholine mM	0.0	1	2	4
Cmm CO_2 in 30 min	27	24	16	12
Per cent inhibition		11	41	53

The composition of the reaction mixture was as stated for Assay-II, except for butyrylcholine. Substrate and inhibitor were simultaneously added to the enzyme. pH was 7.5 and temperature 30°.

TABLE 33

EFFECT OF SUBSTRATE CONCENTRATION ON BUTYRYLCHOLINE INHIBITION OF BRAIN ACETYLCHOLINESTERASE

	Acetylcholine concentration mM				
	0.47	0.75	1.0	2.0	3.0
	Cmm CO_2 in 30 min				
Without inhibitor	15	19	16	26	30
With inhibitor	6	8	11	16	20

The composition of the reaction mixture was as stated for Assay-II except for acetylcholine and butyrylcholine. Butyrylcholine was 2 x 10^{-3}M. Ki was 0.87 x 10^{-3}M. (Lineweaver-Burk plot). pH was 7.5 and temperature 30°.

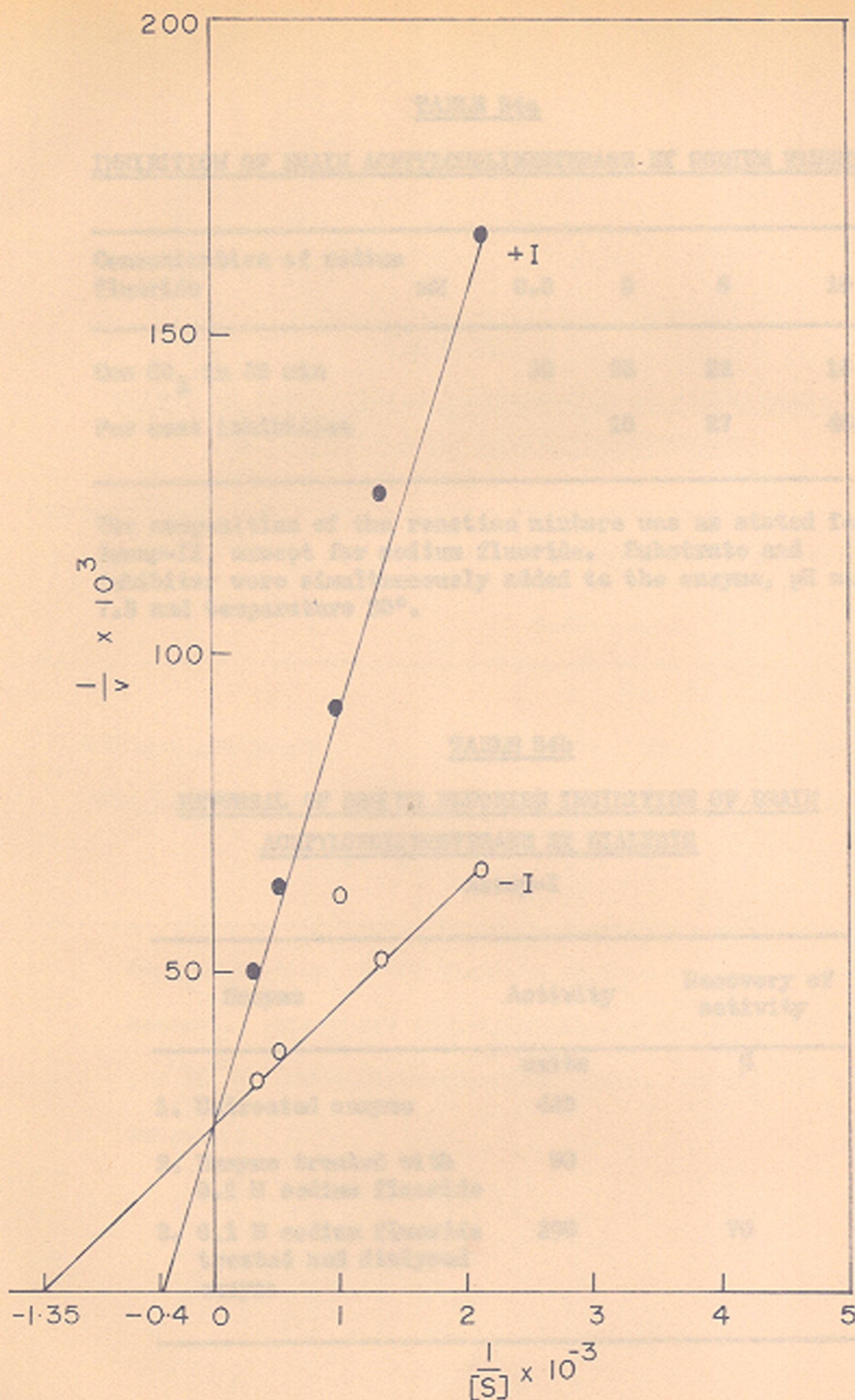


FIG. 21. LINEWEAVER-BURK PLOT OF BRAIN ACETYLCHOLINESTERASE WITHOUT (-I) AND WITH (+I) BUTYRYLCHOLINE. INHIBITOR CONCENTRATION 2×10^{-3}M. TEMPERATURE 30°, pH 7.5. ASSAY-II.

Ki = 0.87×10^{-3}M.

TABLE 34a

INHIBITION OF BRAIN ACETYLCHOLINESTERASE BY SODIUM FLUORIDE

Concentration of sodium fluoride	mM	0.0	3	6	10
Cmm CO_2 in 30 min		30	25	22	16
Per cent inhibition			16	27	46

The composition of the reaction mixture was as stated for Assay-II, except for sodium fluoride. Substrate and inhibitor were simultaneously added to the enzyme, pH was 7.5 and temperature 30°.

TABLE 34b

REVERSAL OF SODIUM FLUORIDE INHIBITION OF BRAIN ACETYLCHOLINESTERASE BY DIALYSIS

Assay-I

Enzyme	Activity	Recovery of activity
	units	%
1. Untreated enzyme	440	
2. Enzyme treated with 0.1 M sodium fluoride	90	
3. 0.1 M sodium fluoride treated and dialyzed enzyme	390	70

TABLE 35

EFFECT OF ACETYLCHOLINE CONCENTRATION ON SODIUM FLUORIDE INHIBITION OF BRAIN ACETYLCHOLINESTERASE

	Acetylcholine concentration mM				
	0.47	0.75	1.0	2.0	3.0
	Cmm CO_2 in 30 min				
Without inhibitor	15	19	16	26	30
With inhibitor 6×10^{-3}M	10	12	16	19	22
1×10^{-2}M	7	11	10	13	16

The composition of the reaction mixture was as stated for Assay-II, except for acetylcholine and fluoride. Ki was 1×10^{-2}M (from Lineweaver-Burk plot). pH was 7.5 and temperature 30°.

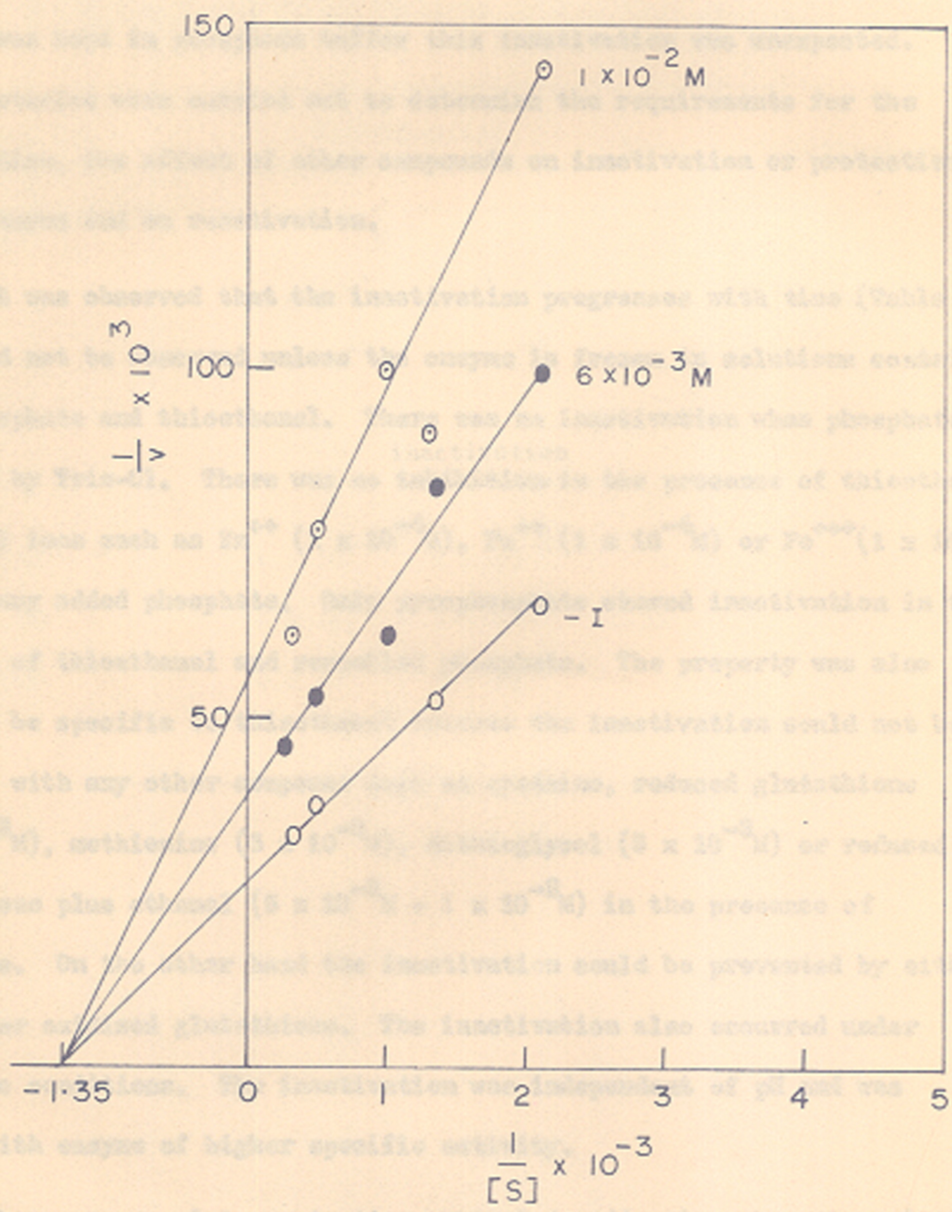


FIG. 22. LINEWEAVER-BURK PLOT OF BRAIN ACETYLCHOLINESTERASE WITHOUT (-I) AND WITH (+I) SODIUM FLUORIDE. INHIBITOR CONCENTRATION 6×10^{-3} AND 1×10^{-2} M. TEMPERATURE 30°, pH 7.5. ASSAY-II.

$Ki = 1 \times 10^{-2}$ M.

Effect of freezing in the presence of phosphate and thioethanol

Sulfhydryl agents like thioethanol, glutathione and cysteine were without any effect on the activity of AchE at 0°. But when the enzyme was frozen in 0.05 M phosphate buffer containing 5×10^{-3}M thioethanol it was found to be completely inactivated. As the enzyme was extremely stable when it was kept in phosphate buffer this inactivation was unexpected. Further studies were carried out to determine the requirements for the inactivation, the effect of other compounds on inactivation or protection of the enzyme and on reactivation.

It was observed that the inactivation progresses with time (Table 36) and could not be observed unless the enzyme is frozen in solutions containing both phosphate and thioethanol. There was no inactivation when phosphate was replaced by Tris-Cl. There was no ~~inhibition~~ inactivation in the presence of thioethanol and metal ions such as Zn^{++} (1×10^{-4}M), Fe^{++} (1×10^{-4}M) or Fe^{+++}(1×10^{-4}M) without any added phosphate. Only pyrophosphate showed inactivation in the presence of thioethanol and resembled phosphate. The property was also found to be specific to thioethanol because the inactivation could not be observed with any other compound such as cysteine, reduced glutathione (5×10^{-3}M), methionine (5×10^{-3}M), dithioglycol (6×10^{-3}M) or reduced glutathione plus ethanol (5×10^{-3}M + 1×10^{-2}M) in the presence of phosphate. On the other hand the inactivation could be prevented by either reduced or oxidized glutathione. The inactivation also occurred under anaerobic conditions. The inactivation was independent of pH and was faster with enzyme of higher specific activity.

There was complete protection against inactivation when the solution containing phosphate and thioethanol was supplemented with acetylcholine (5×10^{-2} M) or EDTA (1×10^{-2} M). But potassium cyanide (1×10^{-2} M),

TABLE 36

EFFECT OF TIME AND CONCENTRATION ON INACTIVATION OF BRAIN ACETYLCHOLINESTERASE ON FREEZING IN PHOSPHATE AND THIOETHANOL

Specific activity	Concentration Phosphate	Thioethanol	Time	Inactivation
	M		hr	%
60	1×10^{-2}	1×10^{-3}	12	20
700	1×10^{-2}	1×10^{-3}	1	0
			2.5	25
			3.5	50
			12	100
3000	1×10^{-2}	1×10^{-3}	2	70
60	5×10^{-2}	5×10^{-3}	12	100
700	5×10^{-2}	5×10^{-3}	2.5	100

Assay-III was used

sodium fluoride (5×10^{-2}M) and arsenate (5×10^{-3}M) were ineffective.

The following experiments were carried out to reactivate the inactivated enzyme. Dialysis against either Tris-HCl (0.05 M, pH 7.5), Tris-HCl-reduced glutathione (0.05 M and 0.01 M, pH 7.5) or Tris-HCl-EDTA (0.05 M, 0.01M, pH 7.5), keeping the inactivated enzyme with arsenate (5×10^{-2} M), lecithin (1.6 mg/ml), acetic anhydride (1×10^{-2}M), reduced glutathione (1×10^{-2}M), oxidized glutathione (1×10^{-2}M), reduced glutathione + EDTA (1×10^{-2}M, 1×10^{-2}M), 2-pyridine-aldoxime-methiodide (1×10^{-3}M), sodium fluoride (1×10^{-2}M) and acetylcholine (5×10^{-2}M). But none of these could reactivate the enzyme.

Therefore, in summary it may be stated that the enzyme was completely inactivated irreversibly when frozen in phosphate (or pyrophosphate) and thioethanol. The reaction was not instantaneous but progressed with time. The inactivation could be prevented by glutathione, EDTA or acetylcholine.

Effect of different compounds on acetylcholinesterase activity

There was no inhibition or activation of AchE activity by the compounds listed in Table 37 at the concentrations shown. As there was no inhibition by p-chloromercuribenzoate or iodoacetate there does not seem to be any SH-group requirement in AchE action. All the SH-agents tested were without any effect on AchE activity. Neither EDTA nor KCN had any effect indicating the absence of a heavy metal requirement for AchE action. The effect of γ-aminobutyric acid, serotonin, nucleotides and acetyl CoA were tested to determine if they have a regulatory role but all of them were without any effect. Semicarbazide, hydrazine sulfate and hydroxylamine had no effect on AchE activity. Acetic anhydride was also without any effect on activity. None of the cations (Zn^{++}, Cd^{++}, Fe^{++}, Fe^{+++}, Ca^{++}) tested had any effect on activity except Cu^{++} which inhibited the enzyme.

TABLE 37

FOLLOWING COMPOUNDS HAD NO EFFECT ON BRAIN ACETYLCHOLINESTERASE ACTIVITY AT THE GIVEN CONCENTRATIONS

In each case the compound was preincubated with the enzyme for 20 min at 0°. Assay-I was used.

Compound	Concentration
	M
1. Sulfhydryl inhibitors	
(i) Parachloromercuribenzoate	1×10^{-3}
(ii) Iodoacetate	1×10^{-3}
2. Sulfhydryl agents	
(i) Reduced glutathione	1×10^{-2}
(ii) Cysteine	1×10^{-2}
(iii) Thioethanol	1×10^{-2}
(iv) Oxidized glutathione	1×10^{-2}
3. Metal chelates	
(i) EDTA $\pm$ Mg	2×10^{-3}
(ii) KCN	2×10^{-3}
4. Other compounds	
(i) γ-Aminobutyric acid	1×10^{-3}
(ii) Serotonin	10 γ/ml
(iii) Acetic anhydride	2×10^{-3}
(iv) Hydrazine sulfate	5×10^{-3}, 0.1
(v) Semicarbazide	5×10^{-3}
(vi) Hydroxylamine hydrochloride	5×10^{-3}
(vii) L-Histidine	1×10^{-2}
(viii) Dithioglycol (1,2 Ethane dithiol)	6×10^{-3}
(ix) DL-Carnitine	1×10^{-3}
(x) Acetyl CoA	
(xi) ATP, ADP	1×10^{-2}
(xii) AMP and cyclic AMP	1×10^{-2}

continued

TABLE 37 contd.

Compound	Concentration
	M
Cations	
Ca^{++}	1×10^{-2}
Zn^{++}	1×10^{-3}
Fe^{++}	1×10^{-4}
Fe^{+++}	1×10^{-4}
Cd^{++}	1×10^{-4}
Cu^{++}	1×10^{-3} (66% inhibition)
Anions	
HCO_3^-	5×10^{-2}
$HASO_4^{--}$	5×10^{-3}
Enzymes	
Sialidase	
Lysozyme	

Bicarbonate and arsenate were also without any effect. Incubation of the enzyme with sialidase or lysozyme had no effect on its activity.

Specificity of acetylcholinesterase

Relative rates of hydrolysis of different substrates - The relative rates of hydrolysis of acetylcholine, acetyl-β-methyl choline, butyrylcholine and phenyl acetate were studied. Assay-I was used for the first three substrates whereas Assay-III was used for phenylacetate (Table 38). The same pattern of relative rates was observed with enzyme of low or high purity.

TABLE 38

RELATIVE RATES OF HYDROLYSIS OF DIFFERENT SUBSTRATES BY BRAIN ACETYLCHOLINESTERASE

Substrate	Relative rates of hydrolysis
1. Acetylcholine	1.0
2. Butyrylcholine	0.0
3. Acetyl-β-methyl choline	0.3
4. Phenylacetate	0.7 to 0.75

The composition of the reaction mixture was the same as stated for Assay-I for substrates 1,2 and 3. The same substrate concentrations were used in all the three cases. Assay-III was used for phenylacetate at 1 mM concentration of the substrate.

CHAPTER 5

D I S C U S S I O N

DISCUSSION

Purification

AchE from ox caudate nucleus was purified about 5000-fold and the final specific activity (about 100,000 units/mg) obtained by this procedure is higher than that of any preparation obtained hitherto from brain or from any source other than the electric organ of *Electrophorus electricus*. The hitherto available preparations from mammalian brain have specific activities of 110[74], 1500[75], 93[77], 1100[78] and 6800[80]. A new method for the preparation of human caudate nucleus AchE with high specific activity was recently described in a brief note, the details of which are awaited[79]. Another noteworthy feature of our preparation of brain AchE is the high stability of the enzyme after solubilization and at different levels of purification.

Solubilization

Attachment of enzymes to cell particles offers difficulty in their purification. About 70 per cent of brain AchE was shown to be attached to cytoplasmic particles by Nathan and Aprison[34]. Association and tight binding of *E.electricus* AchE with fragments of the cell membrane and with fragments of nerve fibre and glial cells was shown by Karlin[192]. The earlier work on brain AchE and the prolonged toluene treatment which was required to extract electric eel AchE also suggest attachment of this enzyme to cell particles. The methods of solubilization used today are mainly empirical and it is not possible to predict which methods are likely to be useful in any particular instance. Methods generally

used for the solubilization of enzymes include mechanical breaking of cells by such means as the Hughe's press, shaking with glass beads or ultrasonication, use of butanol[192] or surface active agents and digestion with proteolytic or lipolytic enzymes. In most of the earlier work surface active agents (lubrol-W or Triton X-100) were used for the solubilization of brain AchE[74-76,79]. Extraction of a lyophilized preparation was made with butanol and ether by Jackson and Aprison for AchE from caudate nucleus of calf and sheep[78]. Lawler prepared acetone powder of calf caudate nucleus and treated it with lipase after ultrasonication[77]. Prolonged treatment with toluene was given by Kartsheva _et al_ to cow brain similar to that of electric eel. But all these different procedures gave enzymes either with low specific activities, with low yields[80] or unstable preparations after partial purification[77].

The use of surface active agents for solubilization of enzymes has certain disadvantages. It is difficult later to remove the detergent from the enzyme. If it is removed from the enzyme the latter in some cases again becomes insoluble or unstable. Moreover the presence of such an agent in the enzyme may affect its properties.

Crystalline pancreatic elastase was previously shown to solubilize ox brain and heart hexokinases which are attached to mitochondrial particles[182a,b]. However it was not possible to solubilize rat liver glucose-6-phosphatase (unpublished findings of this laboratory) which is attached to microsomal particles. These findings however are not sufficient to indicate any particular

site of attachment of an insoluble enzyme which can be solubilized by elastase and further work is required to clarify its mode of action.

The use of elastase has certain advantages as compared with the methods used hitherto to solubilize brain AchE. Elastase is an insoluble enzyme at neutral pH and can be very easily removed by centrifugation and thus does not interfere with further work. Another advantage is that even prolonged incubation of brain AchE (or hexokinase) with elastase does not inactivate the enzyme whereas trypsin and other proteolytic enzymes not only solubilize but also destroy the enzyme on prolonged incubation.

Though a maximum of only about 25 to 30 per cent AchE could be solubilized from caudate nucleus of ox by elastase treatment, the soluble enzyme was established by all its properties to be a specific or true AchE, e.g. its properties such as molecular weight, relative rates of hydrolysis of acetylcholine, acetyl-β-methyl choline and butyrylcholine, inhibition at high substrate concentration and K_m value were comparable to those of either electric eel or erythrocyte AchE. Treatment with elastase does not seem to have altered the properties of the enzyme.

After elastase treatment and freezing and thawing the particulate preparation undergoes a physical change and the suspension becomes highly viscous and jelly-like. A similar observation has been made with mitochondrial particulate preparation of hexokinase from ox heart or brain. The nature of this change and its cause are not known.

Several experiments described in Chapter II to increase the yields of solubilization and explore the reasons for low solubilization showed negative results and thus no direct explanation could be obtained in this respect. Several possibilities were considered. The use of high salt concentrations or the use of a purer particulate preparation by extracting the caudate nucleus with water was attempted. The experiment in which acetone powder of caudate nucleus was used for solubilization shows that the removal of lipids has an influence on the solubilizing action of elastase and brings more enzyme in solution. But as initially there was about 80% inactivation of AchE this method could not be used. There also does not seem to be formation of any low molecular weight inhibitors of elastase as a product of its action on the particulate preparation, because dialysis during elastase treatment did not give any better results.

These studies do not indicate any definite reason for the low solubilization of brain AchE by elastase treatment. The suggestion about different types of cholinesterases in _Electrophorus electricus_[83] requires further confirmation and the possibility exists that there are more than one AchE in caudate nucleus which differ in the ease with which they can be solubilized. A second possibility is that AchE may be layered in brain between several layers of lipids and only a limited part of the enzyme is accessible to elastase. Removal of the lipids enhances the solubilization. These hypotheses are entirely speculative at present and further work is needed on the localization and attachment of AchE to the insoluble fragments of brain.

In addition to treatment with elastase and freezing and thawing, protamine sulfate treatment also appears to be essential for further purification of the enzyme. Without protamine sulfate treatment very little purification of the enzyme could be obtained by conventional methods whereas after protamine sulfate treatment the enzyme could be purified by a variety of methods. Protamine sulfate also converts the viscous soluble enzyme solution to a water clear solution which is as fluid as water. The high viscosity may be due to DNA but this was not further studied.

After the protamine sulfate treatment AchE was purified by conventional methods. They require no special comment except that no purification of the enzyme could be obtained on the cation exchange adsorbents (CM-cellulose or IRC-50) studied.

This is the first time that AchE of such high purity could be obtained from brain, but due to unavailability of enough quantity of enzyme of the highest specific activity it was not possible to establish its purity by ultracentrifugal and other studies. Our studies indicate that further purification may be possible and requires to be attempted but unfortunately this could not be carried out.

Though the enzymes from electric eel and brain may not necessarily have the same final specific activity, it will be of interest to compare the two. The initial specific activity of electric eel AchE is about 2000 and the crystalline preparation has a specific activity of about 750,000. In the case of ox caudate nucleus the initial specific activity was about 20 and the final specific activity about 100,000. It would be interesting to know

whether a further purification of about 7-fold is required to obtain the homogeneous enzyme from ox brain.

An unexplained finding during purification was the loss in activity during some of the fractionation procedures (such as on DEAE-cellulose or CM-cellulose). The losses were difficult to explain especially in view of the high stability of the enzyme. The enzyme loaded on DEAE-cellulose or CM-cellulose was never eluted completely. It may be noted that good recoveries of activities were obtained in several other steps. During fractionation on Sephadex, for instance, the recovery of enzyme of maximum specific activity was only about 50 per cent but the remaining enzyme was recovered in other fractions of lower purity. However with DEAE-cellulose and CM-cellulose only a part of the enzyme loaded on the adsorbent could be recovered in all the eluates. It is not known whether a part of the enzyme is irreversibly adsorbed on the celluloses. One of the reasons for these losses may be the presence of some impurities in the commercial adsorbents. This is supported by the fact that better recoveries of activity were obtained on some samples of DEAE-cellulose prepared in this laboratory. Further work is needed to determine the reason for the loss of enzyme in these steps. This loss of enzyme is one of the main reasons for the relatively low yield of enzyme of maximum specific activity.

<u>Properties</u>

<u>Stability</u>

Its remarkable stability even at higher levels of purity is one of the important feature of this preparation especially when compared with the low stabilities of some of the earlier preparations[77,78]

of the enzyme from the same source even at lower degrees of purity. The decrease in stability of enzyme of specific activity 100,000 units/mg is probably due to storage of the enzyme in extremely dilute solution (only about 0.05 mg protein per ml). This is also evident from the observation that the activity could be stabilized for more than one month by adding 2-3 mg/ml of serum albumin to it. The decrease in stability of dilute enzyme solutions is well known and if more enzyme of maximum purity is available it could possibly be stored without loss of activity in a more concentrated solution or as an ammonium sulfate precipitate.

Acetylcholine and sodium fluoride had a distinct protective action against heat inactivation of brain AchE. Some effect was also observed with 0.5 M NaCl and KCl but this was less than that with NaF.

<u>Molecular weight</u> - The molecular weight of brain AchE as tentatively determined by gel filtration on Sephadex G-200 was about 200,000. This value is of the same order as that of AchE from <u>Electrophorus</u> <u>electricus</u> and human caudate nucleus in which cases values of 250,000 and 230,000 respectively were obtained using the same method as ours for the determination of molecular weights[65,79]. As was indicated in the Introduction the molecular weight of AchE under different conditions of ionic strength and pH varies considerably because of its tendency to aggregate reversibly. It must, however, be noted that it was not possible to carry out molecular weight determinations with enzyme of maximum specific activity and the above value must be regarded as only a tentative one, which requires confirmation by more than one method at different pHs, temperatures and ionic strengths with highly purified enzyme.

Kinetics

Temperature

The effect of temperature on K_m and V_{max} of AchE showed that K_m was unaffected by temperature within the limits of experimental error. Between 12° and 30° there was markedly less increase in activity than between 0° and 12° and there was negligible increase in activity between 30° and 37°. It was established that there was no inactivation of the enzyme during the period of reaction at 37°. The Arrhenius plot was not a straight line with a definite slope but showed curvature which implies low energy of activation. Similar observations have been reported with AchE from Electrophorus electricus, and erythrocytes on the effect of temperature on K_m and V_{max}[97,99]. In the case of studies with AchE from E. electricus on the effect of temperature four different substrates were used by Wilson and Cabib[97]. Two of them - methylaminoethyl acetate and aminoethyl acetate - were hydrolyzed at slower rate as compared with acetylcholine. With these two substrates the Arrhenius plots were not curved. The phenomenon was explained on the basis of a two step mechanism of action of AchE - namely acetylation and deacetylation. The deacetylation step was stated to have low energy of activation.

As our studies on this aspect do not include the effects on different substrates it is difficult to say whether the same explanation is applicable to our observations also. In a preliminary experiment (which is not described in this thesis) when gel filtration on Sephadex G-200 was carried out at 37° there was a decrease in molecular weight as compared with 0°. Though the data are not conclusive the effect of temperature on activity may also partly be

due to dissociation of the AchE molecule at higher temperature or due to a two-step mechanism or by a combination of both mechanisms.

Effect of pH

A broad pH optimum between 7.5 and 8.5 was observed for brain AchE. This is similar to the pH optimum of 8 to 8.5 reported by several workers for AchE from *E.electricus*, erythrocytes and brain.

Substrate concentration

A sharp optimum substrate concentration at 3.3×10^{-3}M for acetylcholine was observed with brain AchE. This may be compared to values of optimum acetylcholine concentration obtained for AchE from *E.electricus* and erythrocytes which range from 3 to 4×10^{-3}M. Inhibition of AchE activity was observed at high acetylcholine concentration and a bell-shaped log $\frac{1}{[S]}$ vs activity curve was obtained. This is also in complete correspondence with the observations made with AchE from other sources.

K_m values with acetylcholine as substrate obtained by us for brain AchE and for AchE from different sources under different conditions of pH, temperature and methods of estimation are listed in the Table 39.

Comparison of K_m values (Table 39) from the same and different sources shows that the K_m values reported in this work are higher than those reported for AchE from caudate nucleus of calf and man and from human erythrocytes and are closer to those for the electric eel enzyme, if the values obtained by manometric estimation are compared. It can also be seen from the Table 39 that the K_m values for electric eel ~~and erythrocyte~~ AchE obtained by the method of automatic titration are lower than those obtained

TABLE 39

COMPARATIVE K_m VALUES FOR ACETYLCHOLINESTERASE FROM DIFFERENT SOURCES

Acetylcholine was the substrate in all cases.

Source	Temperature	pH	Method of estimation	K_m	Reference
	°			M	
Caudate nucleus of ox	20	7.5	Manometric	5×10^{-4}	Present work
	30	7.5		7×10^{-4}	
	37	7.5		5.5×10^{-4}	
	30	6.5		3.5×10^{-4}	
Caudate nucleus of calf	37	7.8	Automatic titration	1.38×10^{-4}	78
Caudate nucleus of human (using Triton X-100 for solubilization)	not stated			2.8×10^{-4}	79
Dog brain whole homogenate		7.5	Manometric	1.7×10^{-3}	101
supernatant		7.5	Manometric	5.3×10^{-4}	
sediment		7.5	Manometric	2.0×10^{-3}	
Electrophorus electricus	23-24	7.2	Manometric	4.5×10^{-4}	96
	"	"	"	5×10^{-4}	97
	-	7.0	Automatic titration	1×10^{-4}	104
Human erythrocytes	20	7.5	Manometric	3.2×10^{-4}	99
	30	7.5	Manometric	2.9×10^{-4}	
	37	7.5	Manometric	3.7×10^{-4}	

by the manometric method.

It may be concluded that the differences in the K_m values in the case of caudate nucleus AchE may either be due to different methods of estimation in one case (calf caudate nucleus) or due to the use of Triton X-100 and difference in source in case of human caudate nucleus. The difference from erythrocyte AchE may be attributed to the difference in source of the two enzymes.

K_m value obtained for acetyl-β-methyl choline at 30° and pH 7.5 was 1.5×10^{-3} M for brain AchE. The value is markedly higher than that for acetylcholine.

Ionic strength and ions

No significant effect of change in ionic strength (0.1 M and 0.5 M NaCl) was observed on the activity of brain AchE at the three concentrations of acetylcholine studied. A change in ionic strength from 0.17 to 0.56 in the case of electric eel AchE also did not show any effect on activity[111]. In the case of the same enzyme increase in ionic strength was reported to result in lowering of the optimum substrate concentration[106]. AchE from calf caudate nucleus was reported to be activated by 0.5 M NaCl[78] but we did not observe any such effect. The differences from the electric eel enzyme may either be due to difference in the sources of the two enzymes but it would be desirable to study K_m and V_{max} at different ionic strengths.

The same activities of brain AchE were observed in phosphate, Tris, barbiturate and acetate at the same pH. Decreasing activities were observed when activity determinations of electric eel enzyme

were made in phosphate, Tris, borate and citrate[111]. The reason for this difference is not known.

Brain AchE did not require either Mg^{++}, Ca^{++} or any other bivalent ion for its activity. Earlier a requirement of Mg^{++}, Ca^{++} or some other bivalent ion for the activity of electric eel AchE was suggested[107,108] but later work has established that there is no bivalent metal requirement for AchE activity[110]*. In the case of cobra venom AchE it was observed that the enzyme dialyzed against barbiturate buffer could be inactivated by a metal chelating agent and was reactivated by Ca^{++}, Mg^{++} or Mn^{++}.[73]

Substrate specificity

The relative rates of hydrolysis of acetylcholine, acetyl-β-methyl choline and butyrylcholine by the purified ox brain enzyme were 100:30:0 respectively. Similar relative rates of hydrolysis for these substrates were reported for the electric eel enzyme, whereas for partially purified calf caudate nucleus AchE the relative rates were 100:20:2 respectively.

The rate of hydrolysis of phenyl acetate was about 75% of that of acetylcholine by our enzyme whereas it was hydrolyzed about 2.3 times more rapidly than acetylcholine by electric eel AchE[89]. No reason for these differences can be given at present.

Inhibitors

Prostigmine - Inhibition of brain AchE by prostigmine was studied in two sets. In one (set A) inhibitor and substrate were simultaneously added to the enzyme and in the other enzyme and the inhibitor were preincubated for 60 min at 30° and substrate was then added (set B). In case of set A there was no significant change in degree

* Crystalline AchE from E.electricus showed 40% less activity in the absence of $MgCl_2$ (0.02 M).[195]

of inhibition observed with progress of time after 20 min whereas in similar studies with *E.electricus* AchE it was observed that under these conditions the degree of inhibition increased with progress of time and approached the same level as in set B. In set B, the degree of inhibition decreased progressively with time and gradually approached the level of that in set A in case of our enzyme. The inhibition is thus reversible. With respect to this set our results are similar to those for *E.electricus* AchE. The difference in set A is probably because the equilibrium between the enzyme and the inhibitor might have been attained within 20 min and hence there was no significant change in the degree of inhibition after this period. Moreover the lesser amount of enzyme used in our studies as compared to that in the case of *E.electricus* AchE might have contributed to the early attainment of the equilibrium and unfortunately we did not have sufficient readings between 0 and 20 min. Moreover the temperature at which the studies were carried out with electric eel enzyme (23°-24°) was lower than the temperature used by us (30°). Difference in the sources of the two enzymes may also be a contributory factor.

Inhibition of brain AchE by prostigmine was independent of pH in the range of 6 to 9.5. A similar result was reported on pH independence of prostigmine inhibition in the case of electric eel AchE.[167]

The Ki value for prostigmine for brain AchE was 1×10^{-7}M and is of the same order as for electric eel AchE (1.6×10^{-7}M)[96]. The inhibition was of the competitive type from the Lineweaver-Burk plot.

<u>Eserine</u> - As has been described in case of prostigmine, eserine inhibition of brain AchE was also studied in two sets A and B. Here at the 1×10^{-7}M concentration of eserine in set B, progressive decrease in degree of inhibition was observed with progress of time. This is similar to that in case of <u>E.electricus</u> AchE[96]. But at 2×10^{-7}M and 4×10^{-7}M concentrations of eserine both in sets A and B there was no significant change in degree of inhibition observed between 20 min and 60 min and the degree of inhibition in set B was higher than that in set A till 60 min. This was probably because at these inhibitor concentrations equilibrium between the enzyme and inhibitor was attained before 20 min with no change in the degree of inhibition after this period. In the case of the electric eel enzyme for set A the degree of inhibition increased progressively with time and finally inhibition in both the sets reached the same level. The reasons for these differences are probably the same as those discussed for prostigmine inhibition.

Inhibition of brain AchE decreased with increase in pH and was negligible at pH 9.5. This observation is similar to that for electric eel AchE[167].

The Ki value at 30° and pH 7.5 was found to be 3×10^{-8}M for eserine with brain AchE. The inhibition was of the competitive type. Ki value for electric eel AchE at pH 7.5 and 23-24° was 6.1×10^{-8}M which is twice that for brain enzyme[96].

The studies on the effect of pH on AchE activity, prostigmine inhibition and eserine inhibition contributed to the suggestion of an anionic and an esteratic site (with acidic and basic group) in AchE.

Observations with brain AchE are similar in this respect to the earlier observations with electric eel enzyme. This may indicate the similar nature of active sites in the two enzymes but further work is needed on this aspect of the ox brain enzyme.

Butyrylcholine - A competitive type of inhibition of brain AchE was observed with butyrylcholine. The Ki value was found to be 0.87×10^{-3}M.

Sodium fluoride - A non-competitive type of inhibition of brain AchE was observed with sodium fluoride. The Ki value was found to be 1×10^{-2}M.

In studies on the inhibition of erythrocyte AchE by sodium fluoride the inhibition was found to be of the "uncompetitive" type and it was stated that the inhibition of AchE by sodium fluoride occurs with the probable exclusion of the active sites though the evidence was not conclusive.[151a]

Inactivation of brain acetylcholinesterase on freezing with phosphate and thioethanol

The inactivation of some enzymes at low temperatures or on freezing is well-known. The noteworthy features of the inactivation of brain AchE are as follows. There was no inactivation at 0° or on freezing the enzyme with thioethanol alone or with phosphate buffer alone. There was also little inactivation on keeping the enzyme with phosphate and thioethanol at 0° even at much higher concentrations of the two substances and for longer periods than those required for complete inactivation in the frozen condition. Inactivation at -20° took place progressively with time and was not instantaneous. The effect was specific for thioethanol since other

sulfhydryl compounds (cysteine, reduced glutathione, dithioglycol) had no inactivating effect and reduced glutathione actually protected the enzyme against inactivation in the presence of thioethanol. The effect was also specific for phosphate and pyrophosphate whereas Tris and some anions had no effect. The inactivation was probably not due to impurities in thioethanol since similar results were obtained with redistilled thioethanol. Sodium or potassium phosphate and different commercial samples gave similar results. Anaerobic and aerobic conditions gave similar results but these require rigorous repetition under better conditions of exclusion of oxygen.

None of the methods tried for reactivation was successful. The enzyme could not be reactivated by 2-pyridine aldoxime methiodide which is a potent reactivator of phosphorylated AchE. But certain phosphorylated AchEs are not reactivable by this compound and it cannot be said definitely whether the enzyme is phosphorylated on freezing with phosphate and thioethanol or not. The probable involvement of an aldehyde group in the enzyme to bring about this type of inactivation was considered and the effect of semicarbazide, hydrazine sulfate and hydroxylamine was tested on the enzyme. But there was no effect of these compounds.

These results are difficult to explain at present. A conformational change in the enzyme is possible. Oxidation of thioethanol or any similar chemical reaction between enzyme, thioethanol and phosphate is less likely since it should be faster at 0° than at -20°. A change in conformation due to freezing is, therefore, tentatively suggested. The participation of a trace metal is also indicated by the protective action of EDTA. Protection by acetylcholine suggests that in the presence of substrate the unfolding of the enzyme is

prevented. The reason for the specific requirement for thioethanol is not known, unless both -SH and -OH groups are required (the addition of both reduced glutathione and ethanol along with phosphate had no inactivating effect on the enzyme on freezing). A puzzling feature is that this enzyme is not at all affected by sulfhydryl inhibitors such as iodoacetate and *p*-chloromercuribenzoate, so that the effect of thioethanol and the protective action of reduced glutathione cannot be explained on the basis of any effect on -SH groups in the enzyme. The possibility that on freezing -SH groups buried in the enzyme may be uncovered however requires investigation. Further work with purified enzyme is needed to explain these observations. It would also be of interest to determine whether AchE from other sources shows similar inactivation.

Effect of other compounds - The effect of several compounds on brain AchE was tested but none of them except Cu^{++} showed any effect.

para-Chloromercuribenzoate and iodoacetate had no effect on brain AchE showing that the enzyme does not require -SH groups for its activity. Reduced glutathione, cysteine and thioethanol also had no effect on brain AchE. Earlier work on AchE from other sources has also shown that AchE activity was unaffected by sulfhydryl inhibitors[125] or sulfhydryl agents.

As indicated above semicarbazide, hydrazine sulfate and hydroxylamine showed no effect on the enzyme.

γ-Aminobutyric acid and serotonin play an important role in brain metabolism. The effects of these compounds and of ATP, ADP, AMP and acetyl-CoA on brain AchE were tested to determine if any regulation of the enzyme by these compounds is possible but no effect could be observed. But only one substrate concentration was used for these tests.

EDTA and KCN were without any effect on brain AchE thus making improbable the involvement of heavy metal ions in brain AchE action.

Zn^{++}, Fe^{++}, Fe^{+++}, Ca^{++} & Cd^{++} did not affect brain AchE activity except Cu^{++} which was inhibitory.

The activity of brain AchE was also unaffected by HCO_3^{-} and $HAsO_4^{--}$.

Incubation of brain AchE with sialidase and lysozyme had no effect on its activity. Incubation of serum cholinesterase with sialidase also did not have any effect on its activity but it changed its electrophoretic mobility.[128b] This enzyme after incubation with sialidase was also shown to have similar electrophoretic mobility on starch gel as that of AchE of *E.electricus*.[83]

CHAPTER 6

SUMMARY AND CONCLUSIONS

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The particulate AchE from ox caudate nucleus was obtained in soluble form. The method of solubilization consisted of digestion of the particulate enzyme by crystalline pancreatic elastase followed by repeated freezing and thawing. The yield of soluble enzyme was about 20 per cent by two treatments with elastase. The soluble enzyme was not sedimented by centrifugation at 100,000 x g for one hr.

The soluble enzyme was purified by treatment with protamine sulfate followed by fractionations with ammonium sulfate, DEAE-cellulose, calcium phosphate gel and Sephadex G-200. The purification was about 5000-fold relative to the soluble enzyme.

The purified enzyme had a specific activity of about 100,000 μmoles of acetylcholine hydrolyzed per hr per mg protein at 37° and pH 7.5. This enzyme is the purest AchE obtained hitherto from brain tissue.

The purified enzyme was shown to be a true AchE by the similarity of its properties with those of electric eel or erythrocyte enzyme with respect to molecular weight, relative rates of hydrolysis of acetylcholine, acetyl-β-methyl choline, and butyrylcholine, sharp substrate optimum, effect of substrate concentration and inhibitors.

The enzyme was remarkably stable. Enzyme of specific activity of 20 to 20,000 could be stored at -20° for several days without loss of activity. However enzyme of specific activity 20,000 or higher was less stable probably due to its being present in very dilute solution. However enzyme of highest specific activity (about 100,000 units/mg) could be stored without any loss in activity for about one month when 2-3 mg/ml serum albumin was added to it.

Acetylcholine (0.05 M) and sodium fluoride (0.1 to 0.5 M) protected the enzyme against thermal inactivation. NaCl and KCl (both 0.5 M) had also a stabilizing effect which was less than that of NaF.

The molecular weight of the enzyme as tentatively determined by gel filtration on Sephadex G-200 was about 200,000.

The enzyme showed an increase of activity between 0° and 12° but there was relatively less increase in activity between 12° and 37°. It was established that there was no inactivation of the enzyme even at 37° during the period of reaction.

The purified enzyme showed a broad pH optimum between 7.5ta 8.5.

No requirement for Mg^{++} or any other bivalent ion was observed for the activity of brain AchE. Activity determinations in phosphate, Tris, acetate and barbiturate gave the same values. Change in ionic strength (0.1 M and 0.5 M NaCl) was without any effect on activity.

The optimum substrate concentration was found to be 3.3×10^{-3}M of acetylcholine. A bell shaped log $\frac{1}{[S]}$ vs activity curve was obtained.

From the Lineweaver-Burk plots, the K_m values with acetylcholine at pH 7.5 were found to be 5.0×10^{-4}M, 7×10^{-4}M and 5.5×10^{-4}M at 20°, 30° and 37° respectively. K_m at pH 6.5 and 30° was 3.5×10^{-4}M.

The K_m value of acetyl-β-methyl choline was found to be 1.5×10^{-3}M.

The relative rates of hydrolysis of acetylcholine, acetyl-β-methyl choline/butyrylcholine and phenyl acetate were 1:0.3:0.0:0.7 to 0.75. Butyrylcholine was hydrolyzed at a negligible rate.

Inhibition by prostigmine was competitive and reversible. The Ki value was 1×10^{-7}M at 30° and pH 7.5. The inhibition of AchE by prostigmine was independent of pH in the range 6 to 9.5.

Inhibition by eserine was competitive and reversible. The Ki value was 3×10^{-8}M at 30° and pH 7.5. Inhibition by eserine decreased with increase in pH and was negligible at pH 9.5.

Butyrylcholine inhibited AchE competitively and Ki was found to be 0.87×10^{-3}M.

Sodium fluoride inhibition of AchE was non-competitive and reversible. The Ki value was 1×10^{-2}M.

The enzyme was completely inactivated irreversibly when frozen in phosphate (or pyrophosphate) and thioethanol. The reaction was not instantaneous but increased with time of incubation at -20°. It was faster with the purer enzyme. The effect was specific to thioethanol and could not be obtained if thioethanol was replaced by reduced glutathione, cysteine, methionine or metal ions such as Fe^{++} or Fe^{+++} in the presence of phosphate. The inactivation could be obtained only when phosphate (or pyrophosphate) was present and could not be observed when the enzyme was frozen in thioethanol with Tris-HCl or metal ions such as Zn^{++}, Fe^{++} or Fe^{+++}. It also occurred in phosphate and thioethanol under anaerobic conditions.

The enzyme was protected against this inactivation by acetylcholine, glutathione and EDTA. But it could not be protected by sodium fluoride or KCN.

The inactivated enzyme could not be reactivated either by dialysis or by treatment with oxidized or reduced glutathione with or without EDTA, arsenate, lecithin, acetic anhydride, acetyl choline and 2-pyridine aldoxime methiodide. The mechanism of this inactivation is at present unknown.

p-Chloromercuribenzoate and iodoacetate did not show any effect on AchE activity. The enzyme activity was also unaffected by reduced and oxidized glutathione, cysteine and thioethanol showing the non-involvement of -SH groups in AchE action.

There was no effect on activity of either EDTA or KCN.

Semicarbazide, hydrazine sulfate and hydroxylamine, bicarbonate, arsenate, γ-aminobutyric acid, serotonin, ATP, ADP, AMP and acetyl Co-A did not affect AchE action in any way.

None of the metal ions tested (Zn^{++}, Ca^{++}, Fe^{++}, Fe^{+++} and Cd^{++}) had any effect except Cu^{++} which inhibited the activity (66% at 1×10^{-3} M).

It may be concluded that the soluble and highly purified enzyme from ox caudate nucleus is a true AchE and its properties, which were studied, closely resemble those of the insoluble brain enzyme and the enzyme from electric eel and erythrocytes.

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