PARTIAL PURIFICATION AND CHARACTERIZATION OF ACETYLCHOLINESTERASE FROM ADULT *ONCHOCERCA VOLVULUS*

BY

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A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR A MASTER OF PHILOSOPHY DEGREE IN BIOCHEMISTRY AT THE UNIVERSITY OF GHANA, LEGON.

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UNIVERSITY OF GHANA
LEGON - GHANA.

SEPTEMBER, 1992.

DECLARATION

The work presented in this report was carried out by me at the Department of Biochemistry, University of Ghana, Legon, under the supervision of Dr R.A. Acquaah.

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SEPTEMBER, 1992.

DEDICATION

To my Father,

Mr. Kwame Ankama Brobey

and

My Grandma,

Madam Afua Badu.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all who contributed in making this work a success.

I am especially grateful to my supervisor, Dr. Robert A. Acquaah for his patience, invaluable suggestions and relentless effort in seeing to the successful completion of this work. I am also greatly indebted to Dr. S.O. Asante, a member of the supervisory committee for his ~~critism~~ criticism and suggestions. My sincere thanks go to Dr F.N. Gyang, Head of Department for the keen interest he showed in the project. I also wish to thank Drs. K.K. Oduro, Department of Biochemistry. Legon and Rebecca Tarrab-Hazdai, Wiezmann Institute of Science, Israel for their criticisms and support.

A special note of thanks is due Dr. George E. Armah of Noguchi Memorial Institute for Medical Research, Legon for his help at some stages of the project. His constructive suggestions and wholehearted co-operation greatly facilitated and hastened the task of completing the project. I also owe a debt of gratitude to Dr. K. Awadzi and the entire staff of Onchocerciasis Chemotherapeutic Research Centre, Hohoe in Ghana for providing me with onchocercomata without which the project would not have been possible

Last, but not the least, I would like to thank Miss Irene Afua Obeng Agyapomaah, a good friend of mine for her moral support. Often we prayed together, and she was a source of inspiration.

Finally I wish to thank the entire technical staff at the Biochemistry Department for their assistance. The graphs were done by Mr. C. Brown from Botany Department, Legon and the typing of the manuscript was by Mrs. Christiana Nettey of Biochemistry Department, Legon. I deeply appreciate their help.

I am, however, wholly responsible for any shortcoming with regard to this work.

ABSTRACT

Acetylcholinesterase (AChE) from adult *Onchocerca volvulus* was extracted in either 50mM Tris-HCl, containing Triton X-100 (detergent buffer) or plain 50mM Tris-HCl (low-salt buffer)

Gel chromatography on Sephacryl S-300 column of both the detergent buffer and the low-salt buffer extracts gave two components each. The two components of the detergent buffer gave purification factors of 12 and 13 folds and yields of 22 and 23% respectively; those of the low-salt buffer had purification factors of 5 and 9 folds and yields of 11 and 19% respectively The two components of the detergent buffer extract had sedimentation coefficient values of 9.5S and 18S while those of the low-salt buffer extract were 11.3S, the major component in terms of activity, and >38S, the minor component.

The enzyme showed a marked preference for acetylthiocholine compared to butyrylthiocholine and it was also inhibited by high concentration of the former substrate. Moreover, the enzyme was inhibited by the general cholinesterases inhibitor, eserine.

Michaelis-menten constant (Km) of 0.22mM and 0.28mM and Vmax values of 2.7×10^{-3} and 2.5×10^{-3} µmole thiocholine min^{-1}, were found for the respective components of the detergent buffer extract. The major component of the low-salt buffer extract gave a Km value of 0.34mM and Vmax value of 1.9×10^{-3} µmole thiocholine min^{-1}

The two components of the detergent buffer extract had a molecular weight of 525 KD and 375 KD on Sephacryl S-300; >600 KD and 400 KD were the values obtained for the low-salt buffer extract. In contrast, polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions revealed molecular weight species of 560 KD, 355 KD, 290 KD and 67 KD for the enzymes in the detergent buffer extract; those of the low-salt buffer extract were 500 KD, 280 KD, 220 KD and 58 KD.

The crude enzyme was activated by Mg^{++}, Mn^{++} and Ca^{++} at concentrations ranging from $10^{-4}M$ to $10^{-2}M$. Mg^{++} at similar concentrations also activated the purified enzymes. Co^{++} moderately inhibited the crude enzyme at $10^{-2}M$. The enzyme showed maximal activity at pH 8.0 in three buffer systems. Veronal and phosphate buffers were activating.

The results indicate that adult *O. volvulus* AChE is similar to the mammalian AChE and AChE of other nematodes in its catalytic properties.

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ABBREVIATIONS

AChE	Acetylcholinesterase
ChE	Cholinesterase(s)
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
cDNA	Complimentary Deoxyribonucleic Acid
NADH	Nicotinamide Adenine Dinucleotide (Reduced)
NADP	Nicotinamide Adenine Dinucleotide Phosphate
DEC	Diethylcarbamazine
DTNB	5, 5´-dithiobis-(2-nitrobenzoic Acid)
S	Sveberg Constant (Sedimentation Coefficient)
EDTA	Ethylenediamine tetraacetic acid
PAGE	Polyacrylamide gel Electrophoresis
Km	Michaelis Constant
Vmax	Maximum Velocity
BW284C51	(1,5-bis-(-4-allyldimethyl-ammonium)-pentene-3-one diodide
ISO-OMPA	N,N,N,N´-tertraisopropylpyro-phosphoramide.
cGMP	Cyclic Guanosine Monophosphate
IL-2	Interleukin 2
GABA	Gamma Aminobutyric acid

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Onchocerciasis, also called River Blindness, is a disease caused by the parasitic nematode *Onchocerca volvulus*. The adult worms live together in human subcutaneous nodules from where the female worms release microfilariae which migrate to other parts of the body. According to the World Health Organization, onchocerciasis is the most economically devastating disease known to humans (1). The disease incapacitates both the young and the old, who should be in their most economically productive years.

Onchocerciasis can be controlled by the following methods: drug administration, eliminating the insect-vector, and immunotherapy. Although immunotherapy is an effective way of treating a disease, efforts towards this mode of treatment of onchocerciasis are in the experimental stages only Hence chemotherapy and vector control are presently the mainstay of the control programmes. For instance, aerial spraying with chemical insecticides has been used successfully to interrupt transmission of river blindness from some areas of the Volta River Basin under the auspices of the Onchocerciasis Chemo-therapy Programme (OCP) (2) For those individuals who harboured the disease before the inception of the OCP project,

chemotherapy is the only means at the moment to control the infection.

Existing drugs for combating the disease, like suramin and diethylcarbamazine, have been found to cause severe and sometimes unbearable side effects among patients (3). Although with the introduction of ivermectin the treatment of onchocerciasis has improved, several investigations have established that ivermectin is effective against the microfilariae which are the primary cause of the disease (4 7) Ivermectin also paralyses microfilariae within the uterus of the female worm for a period (7). A drug for the therapy of onchocerciasis should not only be safe and effective, it should also help interrupt the transmission of the disease. There are therefore expressions of doubt as to whether an effective microfilaricidal drug like ivermectin will completely prevent the transmission of onchocerciasis and/or lead to its eradication. Although it is safe, ivermectin has no long term or permanent effect on the reproductive capabilities of the adult worms. Once the effect of the drug has worn off, the adult worms resume the production of microfilariae.

There is still need therefore for other more suitable filaricidal agents which either have macrofilaricidal activity or will result in a permanent sterilization of the adult worms.

One approach in the search for such agents has been to study the biochemistry of the host and the parasite, with a

view to identifying parasite-specific component(s) as target for chemotherapeutic intervention.

Some of the parasite-specific component(s) such as enzyme systems responsible for metabolism of key metabolites can be exploited for vaccine production and also use to develop appropriate immunological probes for diagnostic purposes.

Unusually high activity of choline acetyltransferase [EC 2.3.1.6] has been reported in filarial parasites (8) This enzyme catalyses the synthesis of acetylcholine from choline and acetyl CoA. In some parasitic helminths, investigations have revealed that acetylcholine is a possible inhibitory neurotransmitter (9, 10). For instance, there is evidence that the conductance pathway in the trematode parasite, *Schistosoma mansoni* is dependent on transmission across synapses and acetylcholine is suggested to be an inhibitory modulator in this process (10). However in parasitic nematodes, like filariae, acetylcholine is an excitatory neurotransmitter (11). Acetylcholine might therefore play a crucial role in the motility of parasitic helminths. If acetylcholine is essential in nervous transmission of filarial parasites, then most likely an effective enzyme system exists in these parasites to inactivate acetylcholine This will prevent a possible spastic paralysis, the result of repeated depolarisation of the musculature.

Acetylcholinesterase [AChE, EC 3.1 1.7] is of particular interest because of its possible physiological role in inactivating acetylcholine at cholinergic synapses (12) It preferentially hydrolyses acetylcholine to choline and acetic acid. Bueding (13) in 1952, demonstrated the presence of AChE in *S. mansoni*. This was partially characterized histochemically by Fripp (14) and kinetically by Gear and Fripp (15). The enzyme was subsequently isolated, purified and characterized from several other sources (16-19), in both vertebrates and invertebrates. Recent studies have shown that AChE from schistosomula of *S. mansoni* (20) and secretory products of *Brugia malayi* (19) is an important antigen.

Unfortunately little is known of AChE from *O. volvulus*, due in part to the dearth of knowledge regarding this organism, which in turn is related to the scarcity of parasite material and also to lack of a good animal model for the study of this parasite in the laboratory However, where it has been possible to collect parasite material from infected patient populations, basic experiments have been initiated to increase the scope of knowledge of biochemistry and physiology of this worm. A better characterization of the onchocercal enzyme and an investigation of its role in the neuromuscular physiology of this parasite may be of value for designing specific methods for control of onchocerciasis.

In this study an effort has been made to isolate, purify and biochemically, characterize AChE of adult *O. volvulus*. It is hoped that in addition to increasing our knowledge in neurophysiology and biochemistry of this worm, the study may help in the search for more safe and effective therapeutic agents against onchocerciasis.

1.2 LITERATURE REVIEW

1.2.1 Onchocerciasis

Onchocerciasis is chiefly transmitted by the bites of female blackflies of the genus *Simulium*. Both female and male adults of the causative organism, *O. volvulus*, live in human subcutaneous nodules. The female worms shed the microfilariae, which are the developing embryoes, into the surrounding tissue from where they migrate to other parts of the body, especially those areas where bone tissue closely underlies the skin. Adults worms are large, with females being 40-60 cm in length (1)

The life-cycle of *O. volvulus* is shown in Figure 1. Onchocerciasis is essentially an immunological reaction of the host to dead microfilariae Live microfilariae are not immunogenic. The immunological reactions include itching and swellings. Sometimes the wandering microfilariae invade the delicate ocular tissue and cause the death of the optic fibres; this is followed by blindness when the infection has persisted for several years.

An estimated 20 million people in Africa and South America are known to be afflicted with onchocerciasis (2) which, according to World Health Organization (1), is the fourth leading cause of blindness in the world today

In Ghana, onchocerciasis is endemic in vast arable lands in the Volta River basin (21) By 1975, nearly 200,000 people had contracted onchocerciasis with the prevalence being high

along the basins of the Black, White and Red Volta ,the Sissili, and the Kulpawn rivers (21) Almost all the villages along these rivers had prevalence rates far above 70%. The blindness rate in the endemic areas was over 5% (21, 22). However, owing to the extensive vector control and chemotherapeutic programmes launched by the World Health Organisation, the United Nations Development Programme, the World Bank and the Food and Agriculture Organisation, significant control of onchocerciasis in these areas has been achieved (21)

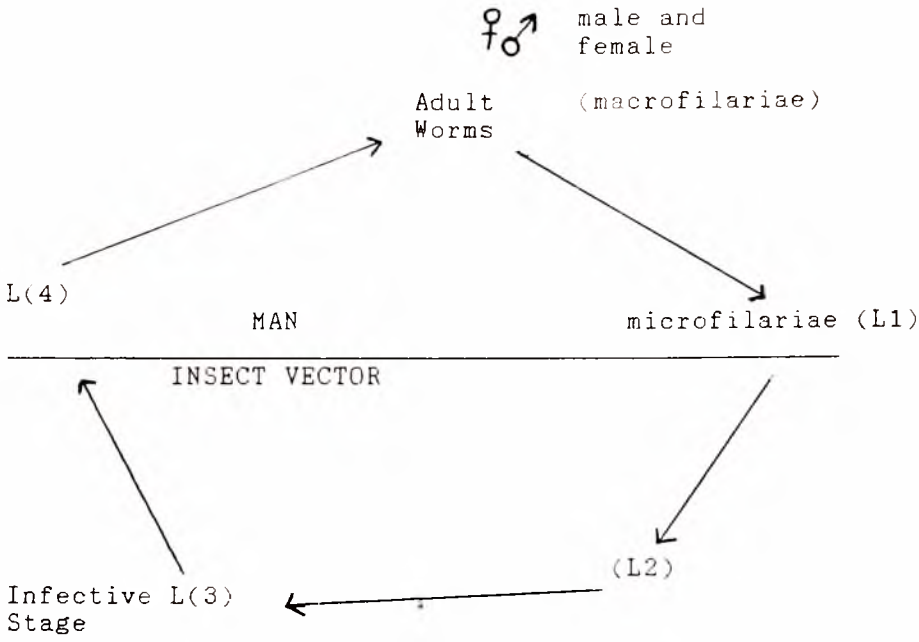


Fig. 1 Life cycle of *O. volvulus*

The insect-vector (blackfly) bites man and picks up microfilariae (L1). The microfilariae undergo two successive transformations in the vector to the infective stage (L3) Further transformations occur in man and adult worms are subsequently developed. In all, *O. volvulus* passes through five developmental stages in its life cycle (23).

1.2.2 Control and Treatment Measures

In the absence of a reliable immunotherapeutic agent, drug treatment and insect-vector control programmes have been the widely used measures for the treatment, control and prevention of transmission of onchocerciasis.

1.2.2.1 Vector Control

This can be divided into spraying with chemical insecticides, and the application of biological agents to control the insect vector The target of vector control has always been the larvae of the blackfly, *S. damnosum*.

The chemical insecticides that have been used include organochlorides and organophosphates (24). In their actions, these chemical agents are known to interfere with the nervous system of the insect-vector by inhibiting certain specific enzymes and also disrupting ion transport (24).

Generally, chemical agents used as insecticides are liable to pollute the environment resulting in serious environmental degradation (25). Aside from this, the period for application must at least be as long as the life span of the adult filariae, to ensure complete irradication of the microfilariae, the primary cause of most filarial infections (7) During this time the possibility of emergence of resistant forms of the insect-vector to the chemical being applied cannot be over-emphasized. All these limitations make the development of alternative methods of control essential.

Biological agents like bacteria, have been used in experimental trials with immense success as an alternative method to control the vector population. Success has primarily been achieved with the bacteria of the genus *Bacillus* (26 - 30), although other useful organisms such as *Coelomomyces* species and nematode *Romanomerrius* have been employed (26).

Notwithstanding these achievements, biological agents may cause damage to the environment which may indirectly affect man by: (i) displacement of more desirable organisms through competition, (ii) infestation of and/or predation on beneficial organisms, (iii) interference with some other elements in the control programme, and (iv) undesirable effects emanating from association with impurities or from eventual modification of the agent (26). Thus thorough studies of the organism and its careful application is very essential when using biological organisms to control the insect-vector.

In spite of the general drawbacks in insect-vector control, considerable success has been achieved in eliminating the blackfly population. Aerial spraying with chemical agents alone to combat the blackfly vector has scored a remarkable success, at least, in areas covered by the Onchocerciasis Chemotherapy Programme (2). However, there is still the problem of those who contracted the disease before the inception of the vector control programme; these people will still harbour the parasites. Chemotherapeutical method will

need to be used to ensure the reduction in the incidence of the disease and the reservoir of parasites.

1.2.2.2 Chemotherapy

Of the drugs commonly used against filarial infections namely suramin, diethylcarbamzine, mebendazole, flubendazole and ivermectin, only the last drug is well tolerated by patients with little or no Mazzotti reactions (6) However, ivermectin is only microfilaricidal (4-6); it has no long term or permanent toxic effect on the adults of *O. volvulus*.

Interestingly, all these drugs were introduced into the therapy of onchocerciasis after being proven efficacious against other infectious diseases. Thus, none of the commonly used drugs has been shown to exploit any known targets in *O. volvulus*. The chemical structures of the drugs used against filarial infections are shown in Figure 2.

(i) Suramin : Of the filaricidal drugs currently available, only suramin exhibits activity against adults of *O. volvulus*. However, it is extremely toxic and the need for intravenous administration under medical supervision for up to six weeks (31) has limited its use at present. Adverse effects associated with the use of suramin are due both to reactions to the drug itself (31) and to allergic reactions resulting from antigens released from the dead and /or dying parasites (31).

Suramin has widespread antiparasitic action. It shows toxic effect on many enzymes, including RNA polymerases and glycerolphosphate oxidase in trypanosomes (32, 33). In filarial parasites, it is known to affect the epithelium of the intestinal lumen and greatly alters the absorption and permeability characteristics of the gut (34) Suramin also inhibits malate and lactate dehydrogenases of *Dirofilaria immitis* and *O. volvulus* (35). These enzymes are involved in the reoxidation of NADH produced in glycolysis. The drug might therefore interfere with energy metabolism of these worms. In addition, suramin inhibits dihydrofolate reductases and NADP-dependent 10-formyltetrahydrofolate ($f^{10}FH_4$) dehydrogenase enzyme in folate metabolism (36). The inhibition of dihydrofolate reductases deprives filarial worms of the ability to reduce folic acid to tetrahydrofolate - initial step in folate metabolism, while the inhibition of NADP-dependent $f^{10}FH_4$ dehydrogenase stops the worms from deformylating $f^{10}FH_4$ to FH_4 cofactors.

The extreme toxicity of suramin has necessitated the search for alternative filaricidal drugs.

(ii) Diethylcarbamazine (DEC) :

DEC has been used as an antifilarial drug for both lymphatic filariasis and onchocerciasis. DEC has little or no antiparasitic action against filarial worms *in vitro*, but it is active *in vivo* against micro-filariae

of *O. volvulus* and adults of a variety of filarial species (37)

The mode of action of DEC is not properly understood. In part, it resembles suramin by showing toxicity towards NADP-independent activity of the filarial $f^{10}FH_4$ dehydrogenase enzyme (38), thus interferring with folate metabolism of these worms. DEC is known to inhibit glucose uptake in the cotton rat filaria, *Litomosoides carini* and reduces the activities of phosphoenolpyruvate carboxykinase, fumarate reductase and succinate dehydrogenase in this worm (7) It appears that DEC might interfere with energy metabolism of this parasite. DEC causes opsonization of *O volvulus* by unmasking its antigenic determinants. It therefore causes the wandering microfilariae to be trapped in the liver sinosoids where they are destroyed by phagocytosis. This action of DEC has also been shown for the cotton rat filaria, *L. carinii* (39).

The drug is known to cause spastic paralysis in filarial worms (37). It might therefore be expected to interfere with acetylcholine hydrolysis by AChE, resulting in an increased acetylcholine concentration within the neuromuscular junctions of the parasite.

DEC, like suramin, has unbearable adverse effects on patients. The most severe side effect produced appears to be the Mazzotti reaction which occurs immediately after treatment and is most frequently seen in patients

with onchocerciasis (40) The Mazzotti reaction is a pronounced allergic response caused by dying and/or dead microfilariae and is manifested by oedema, conjuctivitis, intense itching, dermatitis and fever. This reaction invariably limits the use of DEC as a potent filaricide and has prompted the search for alternative antifilarial drugs.

(iii) Mebendazole and Flubendazole :

These compounds are benzimidazoles. They are potent anthelmintics against several nematodes (41, 42)

The exact mode of action of these drugs is not fully understood. They are thought to disrupt embryogenesis in *O. volvulus* (43), and exhibit some promise as filaricidal agents that do not produce a severe Mazzotti reaction. It has been proposed that they bind avidly to the parasite tubulin, and prevent polymerization into microtubules (31) This postulate is based on the findings that benzimidazole-resistant nematodes show both alterations in tubulin structure and in the rate of tubulin synthesis (31)

The benzimidazoles are known to cause several other effects in parasites, for example reduction in glucose transport across membranes and depletion of glycogen levels (31)

However, Mebendazole is poorly absorbed when taken orally and it also requires prolonged administration.

These factors may make its routine use somewhat impracticable.

(iv) Ivermectin :

The introduction of this compound, a macrocyclic lactone, as an antifilarial drug, provides an agent that is not only relatively safe to use but also shows immense filaricidal action against the microfilariae of *O. volvulus*, in dosage regimens that make it possible for patients to be treated only once or twice yearly (5,6,44,).

The exact mechanism of action of ivermectin remains unknown; it is thought to selectively activate gamma aminobutyric acid (GABA) pathways in the parasite via an effect on the GABA receptor-chloride ion channel complex (3, 31) without interference with the mammalian GABA system. Specifically ivermectin potentiates the release of GABA from presynaptic inhibitory terminals of the parasite, and also causes a dose-dependent increase in chloride ion permeability. The effects of the drug on GABA-mediated events in the parasite have been shown not to affect the viability of adults of *O. volvulus* (3, 45)

The microfilaricidal-dependent drug(s) are indispensable since microfilariae are responsible for both the pathology and spread of onchocerciasis (7). However these filaricidal agents are unable to destroy the adult worms which can live for several years (1) and continue to produce the

microfilariae Therefore there is an urgent need for macrofilaricides or alternative filaricides to kill or permanently sterilise the adult worms.

1.2.3. Antifilarial Drug Development

The search for safer and more effective filaricides has not been easy. This is due partly to the paucity of knowledge of the biochemistry and physiology of the causative organisms. Paucity of knowledge is attributed to the difficulty in getting parasite material for research.

Two approaches are being pursued for antifilarial drug development:

(i) Rational approach which involves comparative study of the parasite and the vertebrate host to discover parasite specific component(s) as target for therapeutic intervention.

(ii) Empirical approach which involves screening of chemical compounds for antifilarial activity

The latter approach has resulted in the discovery of a number of promising compounds. One of these, CGP6140 (amocarzine) possesses both micro- and macrofilaricidal activity against *O. volvulus* (31, 46). Another agent CGP20376 (a benzothiazole derivative) is active against both micro- and macrofilariae of a number of filarial species (31, 47, 48). Although its mechanism of action remains unknown, it is known to cause degeneration of parasite musculature in *L. carinii* (47) and muscle cells of microfilariae of *O. volvulus* (49)

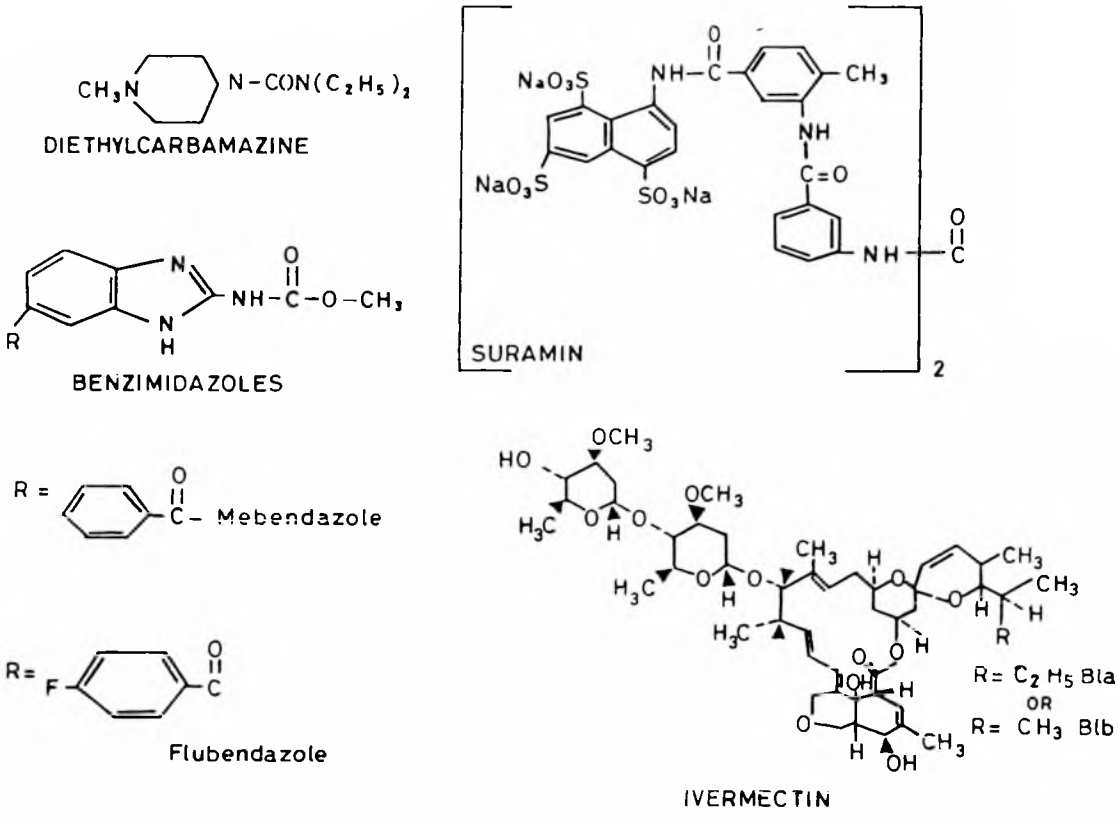


Fig. 2 Structures of some commonly used antifilarial drugs (31)

The rational approach, which exploits the differences between the parasite and the host, has not yielded any promising compound(s) at the moment, despite several efforts being put in this direction. However, the situation seems to be improving as investigators continue to employ advanced technology to study filarial biochemistry and physiology

Thus, studies by Barrett and colleagues for example have shown that the energy-generating pathways in both *Onchocerca* and *Brugia* species contain the complete complement of enzymes necessary for aerobic as well as anaerobic energy production (50) But it is not yet known whether these enzymes show significant differences in activity from their mammalian counterparts so as to be potentially useful targets for chemotherapy In another experiment, Mackenzie and co-workers used nuclear magnetic resonance (NMR) spectroscopy to study filarial energy metabolism. They discovered that *O. volvulus* and *B. pahangi* were able to assimilate labelled glutamine ([^{13}C]-glutamine) as an energy source (51) While both organisms could metabolise [^{13}C]-enriched glutamine to succinate, the turnover of glutamine was two times as great for *O. volvulus* as for *B. pahangi* (51) In addition, it was also discovered that the pathway of glutamine metabolism in these parasites as shown in Figure 3 differs slightly from that of proliferating tumour cells (31)

The recent introduction of recombinant DNA strategies to filarial biochemistry and physiology opens the way for successful isolation and characterization of drug receptors or

other valuable proteins in these and other parasites, which cannot be obtained in large quantities. At present genomic and complementary DNA (cDNA) libraries exist for both *O. volvulus* and *Brugia* species (31). It is highly probable that the genes coding for biologically important molecules, such as enzymes, neuromodulators and drug receptors, may be present within these libraries.

As more become known about the biochemistry and physiology of the worms, the prospects of understanding and controlling the diseases caused by these worms will become brighter. It would even be possible for antifilarial drug development to be more target-oriented than it has been hitherto.

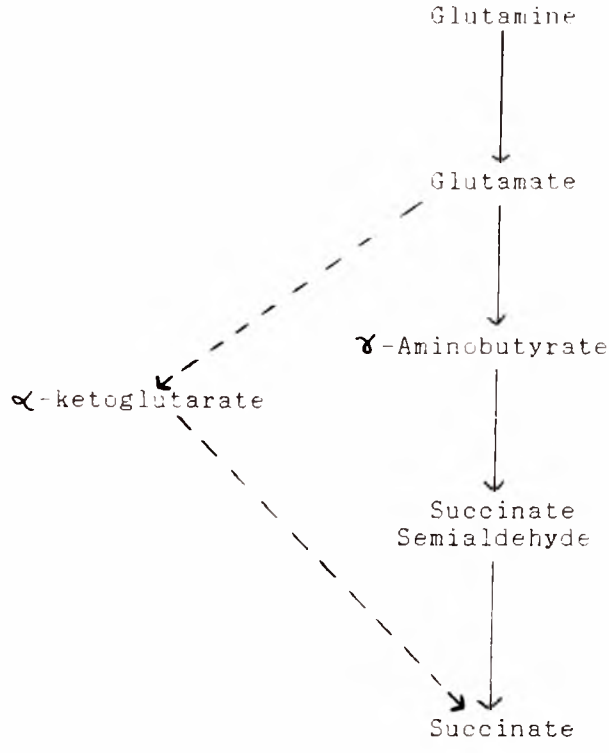


Fig. 3 Metabolic pathways of glutamine metabolism in *O. volvulus* and *B. pahangi* as determined by NMR spectroscopy using [^{13}C]-enriched glutamine (51) [The broken arrows show alternative pathway observed in mammalian proliferating tumour cells (31)]

1.2.4 Cholinesterases

Cholinesterases catalyse the hydrolysis of choline esters to choline and the corresponding acid. Unlike other esterases, cholinesterases have a marked affinity for quaternary ammonium ions (52). They are also markedly inhibited by anticholinesterases (52). Two distinct types of cholinesterases are now recognized, namely: acetylcholinesterase [EC 3.1.1.7] and pseudocholinesterase, also called cholinesterase or butyrylcholinesterase [EC 3.1.1.8] (17).

Acetylcholinesterase (AChE) is the predominant acetylcholine-decomposing enzyme of the nervous tissue and the erythrocytes of most species, whereas pseudocholinesterase chiefly resides in blood plasma of most species (52)

These enzymes can further be differentiated by their substrate specificities. However, substrate specificity studies are sometimes hampered by the fact that most of the esters in question are relatively insoluble in water This makes them difficult to study under comparable conditions. Generally, both types of enzyme have higher substrate affinity for cationic esters relative to neutral esters. The activity of AChE for carboxylic acid ester substrates is sharply decreased for acyl groups larger than propionyl moiety (53)

In a study, Goldlust *et al* (54) observed a sevenfold difference between the rate of hydrolysis of acetylthiocholine and butyrylthiocholine by crude acetylcholinesterase of *S. mansoni*. Acetylthiocholine and butyrylthiocholine are artificial substrates of cholinesterases. They are suitable

substrates for colorimetric determinations of the activity of cholinesterases. The thiocholine produced enzymatic hydrolysis of acetylthiocholine or butyrylthiocholine reacts with 5, 5´-dithio-bis-2-nitrobenzoic acid (DTNB) in a disulphide exchange reaction to give a yellow anion 5 -thio -2 -nitrobenzoate (52). The rate of formation of this coloured compound can be followed colorimetrically. The specificity of AChE for acetylthiocholine and acetylcholine is almost the same; the Michaelis constant (Km) of the enzyme for both substrates are similar (53). Similar ratio of the rate of hydrolysis of acetylthiocholine to butyrylthiocholine was found for crude enzyme (AChE) fractions of the parasitic nematode *Stephanurus dentatus* (55). However in other parasites, the rate of hydrolysis of acetylthiocholine is quite different from that of butyrylthiocholine. In the nematode *Nippostongylus brasilliensis* (56) and in the trematode *Fasciola hepatica* (57) acetylthiocholine was hydrolysed at a rate only 50% greater than butyrylthiocholine.

A Km value of 1.8mM for the substrate acetylthiocholine was reported for AChE from cercaciae of *S. mansoni* (54); those of the adult worm were 2.4 - 5.2 mM (15). In addition, Km values of 0.012 - 0.08 mM have been found for AChE of the nematode *Caenorhabditis elegans* (58).

The AChE from electric eel also gave a Km value of 0.46 mM for acetylthiocholine (59). The Km with respect to butyrylthiocholine for the eel AChE has not been well studied.

With choline esters of aliphatic acids, acetylcholinesterase, but not pseudocholinesterase, shows substrate inhibition (60). Goldlust and coworkers (54) have reported an optimal substrate concentration of 2 x 10^{-2} M for AChE of cercaria of *S. mansoni*. This is similar to the value previously reported for the adult worm (13, 15). Very comparable optimal substrate concentrations have also been reported for certain parasitic nematodes such as *S. dentatus* (55), and *N. brasiliensis* (56) where it was found that respective concentrations of acetylcholine greater than 1 x 10^{-2} M and 2 x 10^{-2} M inhibited AChE activity. However, since an optimum substrate concentration is dependent on the amount of enzyme used for the reaction, the results from different experiments should be interpreted with caution. It has been proposed that at high substrate concentration, there is an ineffective enzyme-substrate complex formation at the active site due supposedly to competition by the excess substrate molecules (61). During catalysis, it is thought that the alcohol moiety of the substrate is released, with the formation of an acylated enzyme which is later deacylated (61). At high substrate concentration, a second substrate molecule may combine with the alcohol-binding site of the acylated enzyme, leading to retardation of the deacylation process (61)

Certain classes of inhibitors have specific action on cholinesterases. For instance, AChE and pseudocholinesterase can be specifically inhibited by 1, 5 - bis - (-4- allyldime-

thylammonium) - pentene - 3 - one diodide (BW284C51) and N, N, N´, N -tetraisopropylpyrophosphoramide (ISO-OMPA). Whereas BW284C51 causes appreciable inhibition of AChE, ISO-OMPA has little or no inhibitory effect on the enzyme under similar conditions (17, 54) The reverse is true for pseudocholinesterase (52), that is, ISO-OMPA inhibits pseudocholinesterase much more than AChE. These inhibitors are therefore normally used to distinguish between the two enzymes.

1.2.5 Acetylcholinesterase

This enzyme has been well studied because of its possible physiological role in neuromuscular transmission (12, 17) AChE is a glycoprotein (17, 62-64). *Torpedo* AChE has four asparagine-linked carbohydrate chains (63). The carbohydrate moieties differ for AChE from different tissues. This was suggested by Meflah *et al* (65) who reported differences in lectin binding for AChE from bovine lymphocytes, erythrocytes and brain membrane. Interaction with lectins also distinguishes foetal calf serum AChE from bovine erythrocyte AChE (66) Thus glycosylation in AChE can be very specific.

In addition, there is strong cumulative evidence from vertebrates and some invertebrates that AChE is a membrane-bound enzyme (67, 68) but the exact relationship of the enzyme to the membrane is not clearly understood yet.

The enzymes from many vertebrate sources are stable at very low temperatures. Freezing has been shown not to affect

the enzyme's activity (59, 69) However, AChE of vertebrates is reported to be susceptible to inhibition at relatively high temperatures (52). This is very true for many mammalian enzymes. A maximum catalytic activity at 37°C has been observed for the AChE of mammalian erythrocyte (70) Vertebrate AChE has a broad pH optimum ranging from 7.5 to 9.0 (52) This might reflect the type of amino acid residues present at the active site of this enzyme. The diagrammatic model of AChE catalytic site is shown in Figure 4. The catalytic site is composed of two ligand-binding areas, the esteratic and the anionic subsites. The anionic site is thought to be an ionized carboxyl group which forms an ionic bond with the positively charged cationic head of substrates and effectors of the enzyme. The esteratic site combines with the ester group of the substrate. This is where presumably acylation reaction occurs. It contains the amino acid sequence glutamic acid - serine - alanine and probably a histidine group (71). In addition, there is a second anionic site (peripheral sites) which is believed to act as a regulatory site (53). It allows simultaneous interaction by multiquaternary ligands with the catalytic site. Such interaction is known to bring about conformational change in the enzyme (53). The function of the acidic group (HZ) in the esteratic subsite is not understood yet; it may be required for enzyme acylation (53) The hydrophobic areas in the catalytic site contribute primarily to the binding of ligands with substantial hydrophobic moieties, thus allowing for

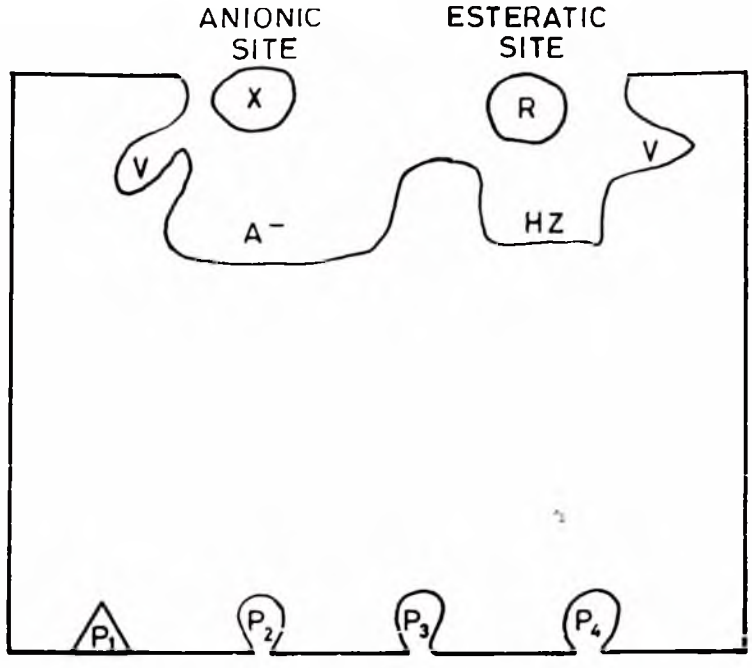


Fig. 4 Diagrammatic model of the AChE Catalytic site (53) A^- anionic group ; HZ, an acidic group; V, hydrophobic areas ; P_1 , P_2 , P_3 , P_4 , peripheral sites .

effective enzyme-substrate interaction (53)

Catalysis apparently proceeds by transfer of the acyl group from substrate to the enzyme to form an acyl-enzyme intermediate which is then deacylated (71) It is believed that the oxygen of the serine hydroxyl group donates electrons to the electrophillic carbon of the carboxyl group of the substrate. The hydrogen atom of the serine hydroxyl group is probably accepted temporarily by a histidine imidazole group in the enzyme. Choline is then released leaving an acylated enzyme. The transient acylated enzyme reacts with water to yield the acid and regenerates the active enzyme (71). The mechanism of enzyme action is illustrated in Figure 5, using acetylcholine as substrate.

1.2.5.1 Multiple Molecular Forms

The presence of distinguishable forms of AChE using fractionation techniques such as sucrose gradient centrifugation, gel-exclusion chromatography. polyacrylamide gel electrophoresis, and also by histochemical methods has been reported in extracts of a variety of tissues (17, 53).

Studies on purified AChE from the electric organs of the eel, *Electrophorus electricus*, and the ray, *Torpedo californica* (72), show that the various molecular forms of this enzyme are asymmetric and contain large numbers of subunits with a common catalytic subunit. Several forms of AChE have also been shown to contain a collagen-like tail (17, 53, 73)

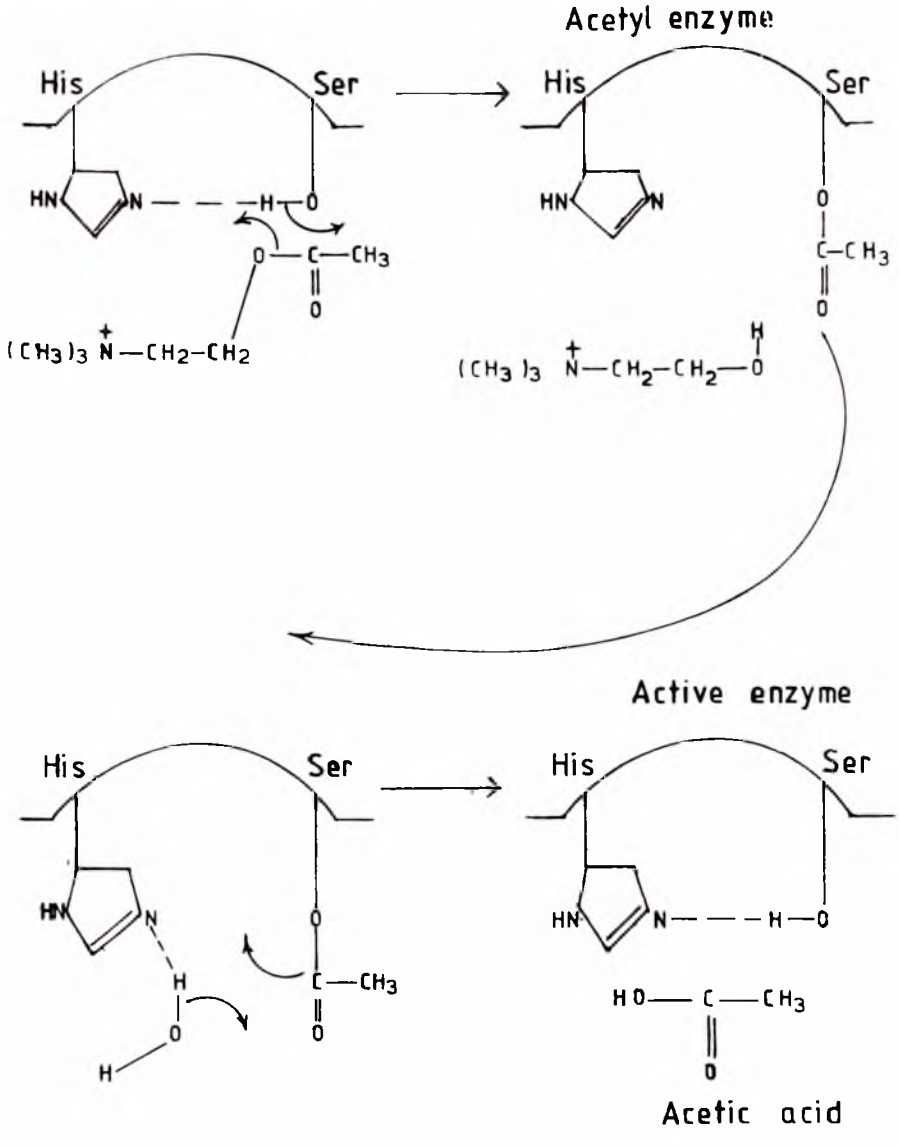


Fig. 5 Hydrolysis of acetylcholine at the esteratic site of AChE (71)
For further description see test.

It is observed that the various molecular species of electric eel AChE appear to have the same Km for acetylthiocholine. Also no differences in inhibitor constant (Ki) values among these species have been reported (74). Similar observations have been made of the mammalian erythrocytes, nerve and muscle enzymes where it was found that the various molecular forms isolated have highly similar catalytic site and the same general catalytic mechanisms (75).

The extraction medium employed has been shown to have a strong effect on the release and on the molecular forms of this enzyme. Thus a heterogenous AChE population in high ionic-strength extracts of fresh electric organ from *E. electricus* has been described by Massouli and Rieger (74). Three molecular forms of AChE with sedimentation coefficients of 8S, 14S, and 18S were observed in sucrose gradient centrifugation studies. Higher aggregates were obtained by extraction in low-salt buffer solutions (69, 72).

Multiple molecular forms of AChE have also been reported in parasite systems, such as *S. mansoni* (54, 69,). Enzyme forms corresponding to molecular species with sedimentation coefficients of 8S and 18S were observed in sucrose gradient centrifugation when the adult parasites were extracted in high ionic-strength buffer or detergent buffer (69). However, the situation is quite different in the larval form of the parasite. The enzyme extracted with detergent behaved as a monodispersed species with a sedimentation coefficient of 8S; in the absence of detergent, the low-salt soluble extract is

polydispersed, containing both 10S and an aggregated 32S component (69). Recently, the presence of various molecular forms of AChE has been also reported for the nematodes *N. brasilensis* (76) and *B. malayi* (19). It appears therefore that the existence of multiple molecular forms of AChE is a universal phenomenon.

Although several studies have confirmed the existence of multiple molecular forms of AChE, the exact number of the polymorphic forms is not certain yet. Moreover, the apparent molecular weight of the native AChE has been a focus of controversy An attempt to determine the apparent molecular weight of 8S, 14S and 18S species of electric eel AChE on Sephadex G-200 or Biogel P-300 ended up with all the enzymes eluting with the void volume (77) This could suggest extreme asymmetry of these enzymes (53, 78). The apparent molecular weight of AChE can also be determined by the use of polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions. But care must be taken when interpreting the results. This is because proteolytic degradation of AChE under non-denaturing conditions has been reported (53). However, Bon and co-workers (79) have been able to determine the molecular weight of the eel AChE by calculation based on their sedimentation coefficients. Values of 430,000, 780,000 and 1,100,000 daltons were obtained for the 8S, 14S and 18S species respectively The mass difference between the molecular weights of the 14S and 8S and 18S and 14S enzymes is very close to the molecular weight of the 11S species, 335,000

(78) This molecular species was isolated from the eel after extraction in low-salt buffer (78). In a recent study, Tarrab-Hazdai and others (69) have estimated the apparent molecular weight of the 8S AChE species from the trematode parasite *S mansoni* on Sephacryl S-300. A value of 450,000 daltons was found. Using polyacrylamide gel electrophoresis under non-denaturing conditions, a value of 500,000 daltons was observed (54). In contrast, relatively lower molecular weight forms of the enzyme have been reported from mammalian sources (80, 81). Various brain extracts appear to give apparent molecular weights of AChE species in the region of 80,000 - 180,000 daltons (82). However, in situations where the extraction procedure led to solubilization of the enzyme, molecular weights greater than 270,000 have been observed (82).

Purification of AChE has long been fraught with problems. Some of the factors that have hindered the successful purification of the enzyme include:
(i) the existence of multiple molecular forms of the enzyme, (ii) the ability of the enzyme to aggregate at low ionic strength (72, 83) and (iii) relatively low activity of the enzyme in tissues (84). These have presented a unique problem to the choice of purification method. Purification of AChE enzyme from most vertebrate sources has primarily been carried out with conventional chromatographic techniques, such as gel filtration and ion-exchange chromatography (16, 85). Although a high degree of purity has been reported in some cases

(16, 85), the yield obtained is rather low. Kremzner and Wilson (16) first purified AChE from the electric organ of the eel, using conventional chromatographic procedure and obtained a yield which was less than 10% of the initial activity With the introduction of affinity chromatographic technique into the purification of AChE, however, an extremely high degree of purity with higher yields have been obtained (54, 81, 84). But affinity chromatographic procedure also requires a thorough knowledge of the catalytic structure of the enzyme, and the nature of extraction medium (84)

1.2.5.2 Metal Ion Activation

Enzymes generally do not exhibit an absolute requirement for any metal ion. In all cases studied, more than one metal ion has a pronounced effect on the catalytic activity of the protein. Nevertheless, there are usually distinct differences in the efficiency with which different metal ions act as activators for a given enzyme (86).

Reliable data do not appear to exist on the specificity of metal ion activation of enzymes. This is because different studies have been made with enzymes of highly varying degrees of purity, and employing methods of varying sensitivity In addition, there were many reported studies in which metal ion activation has been carried out at a single concentration. The difficulty here is that no meaningful conclusion can be drawn from the results, as the concentration range in which activation occurred is markedly dependent on the metal ion

used, and since high metal ion concentration usually leads to enzyme inactivation (86) In spite of these drawbacks, the activation of AChE by divalent cations has been reported by several investigators (87 - 89)

In his studies on AChE from the electric organ of *Torpedo*, Nachmansohn in 1940 (89) reported the activation effect of Mn^{++}, Mg^{++}, Ca^{++} and Ba^{++} ions on the crude enzyme extract, with Mn^{++} being the most activating. Activity of AChE from the red blood cell or the caudate nucleus of rat is also reported to be greatly enhanced by Ca^{++} ion (90) Purified AChE from the electric organ of the *E. electricus* was shown to be highly activated by Mg^{++} ion (88). This resulted in an erroneous conclusion that Mg^{++} is essential for catalytic activity of the enzyme (88). It was later shown that Mg^{++} is not very essential for the catalytic activity of the electric eel AChE (88), and that it is very improbable that any metallic ion is required for the catalytic function of AChE.

However, it is now known that inorganic cations exert a biphasic effect on electric eel AChE activity, accelerating activity at low ionic-strength, and inhibiting at relatively high ionic-strength by competing with substrates for the catalytic site (53)

(I) Factors Involved in Metal Ion Activation

Several factors have been proposed to explain the observed specificities in metal ion activation Three of

these namely complex stability, size and charge of the metal ion and electronic properties have been well studied (86, 91, 92). Whereas these factors have been extensively investigated in some enzyme systems, for example, peptidases (91) and enolase (93), there is scanty information on the cholinesterases.

(II) Mechanism of Action of Metal Ion Activators:

Metal ions may produce activating effects on enzymes by a variety of mechanisms. Three of these mechanisms have been postulated to explain metal ion activation. These are discussed below.

(a) Induction of Conformational Changes in Enzymes:

It is anticipated that the binding of metal ion to the neighbouring groups may cause changes in the conformation of the enzyme Such association is expected to expose a concealed group for binding to the substrate (94). Even though experimental evidence has been advanced to account for this effect, a direct influence on the configuration at the active site of the enzyme is considered more probable (95)

(b) Induction of Electronic Transformation in Substrate:

Polarization effect due to the metal ion on the substrate has been proposed in this mechanism. Smith and co-workers used polarization effect to explain metal ion

catalysis in peptidases (96). With regard to cholinesterases, such information is lacking.

(c) Ternary Complex

The formation of a ternary complex involving metal ion, substrate and peripheral anionic site (see Figure 4) of acetylcholinesterase has been reported (53) It has been proposed that the metal ion acts as a bridge in the ternary complex, bridging the substrate, and the protein.

Other studies involving model systems have clearly demonstrated that metal ions can mediate the binding of ligands to proteins (86, 97, 98). For instance, Roufogalis and Wickson (99) have reported the mediatory role of metal ions in the formation of ternary complex with the cation ligand gallamine triethiodide on peripheral anionic sites of acetylcholinesterase. Such interaction may stabilize the enzyme considerably and probably protect the protein against heat denaturation (53)

1.2.5.3 Effect of Inhibitors

Inhibitors may act reversibly or irreversibly on enzymes.

(I) Irreversible Inhibition

This is characterized by a progressive increase in inhibition with time, ultimately reaching complete inhibition

even at low inhibitor concentrations, provided that the inhibitor is in excess of the amount of enzyme present. The potency of the inhibitor is expressed by a velocity constant, which determines the fraction of the enzyme inhibited in a given period of time by a certain concentration of inhibitor (100)

Organic fluorophosphates, for example diisopropylphosphofluoridate (DPF), are thought to inhibit cholinesterases irreversibly by forming a very stable covalent phosphoryl-enzyme complex with the active site serine residue (101)

The organophosphate therefore effectively poisons the enzyme by phosphorylation and thus blocks efficient hydrolysis of acetylcholine. The reaction with organophosphate is not unique to cholinesterases. Serine proteases give similar reactions with organophosphates (101).

(II) Reversible Inhibition

This type of inhibition is characterized by an equilibrium between enzyme and inhibitor. The potency of the inhibitor is here, normally, expressed by an equilibrium constant (Ki) which is a measure of affinity of the inhibitor for the enzyme. Sometimes, the molar concentration of the inhibitor necessary to effect 50% inhibition, denoted by IC_{50}, is used to express the potency of the inhibitor (69). The degree of inhibition depends on the inhibitor concentration.

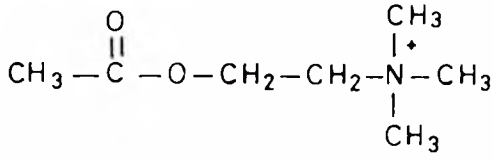
Reversible inhibition can be competitive in which the inhibitor, normally structural analogue of the substrate,

binds to the same site of the enzyme as the substrate; non-competitive, in which the inhibitor binds to a site far removed from the substrate-binding site of the enzyme or un-competitive in which the inhibitor can only combine with the enzyme-substrate complex. Uncompetitive inhibition, however, seems to be exceedingly rare (101).

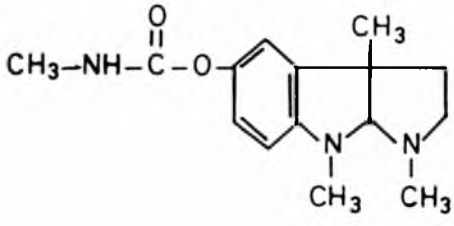
A great many substances, which include physostigmine (Eserine) and prostigmine appear to inhibit cholinesterases in a competitive manner by behaving as substrate analogues of acetylcholine (101, 102) However, a careful study shows that eserine and prostigmine, carbamoyl-ester compounds, behave similarly to the organophosphorous compounds by forming a very stable covalent carbamoyl-enzyme complex which is very slowly hydrolysed (101). Especially eserine is a powerful and specific inhibitor of cholinesterases such that inhibition by it is used to generally distinguish between cholinesterases and other esterases (103) Compounds such as decamethionium and succinylcholine (suxamethionium) inhibit acetyl-cholinesterase at all concentrations by competing with substrate for binding to the catalytic site (102)

Multi-quaternary aromatic cations, for example gallamine triethiodide on the other hand, have been observed to play a dual role in acetylcholinesterase catalytic activity (104) At low ionic-strength, gallamine triethiodide at a concentration less than or equal to 10^{-7} M (53, 105) binds tightly to the peripheral anionic site of the enzyme and forms a stable enzyme-ligand complex. In this form, the enzyme remains

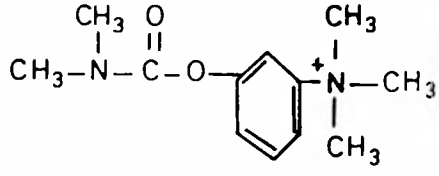
insensitive to the inhibitor or is slightly activated by it (83). However, such stabilization effect is abolished at gallamine concentrations greater than 10^{-7}M (104). At these concentrations, gallamine preferentially binds to the catalytic site and inhibits enzyme activity Gallamine triethiodide also reverses enzyme inhibition due to decamethionium (104). Such antagonistic behaviour of gallamine might suggest that, under certain conditions, catalytic activity of acetylcholinesterase can be modified by ligand binding at sites, other than the catalytic site. The behaviour of AChE toward gallamine seems to suggest that the enzyme has allosteric properties (53). This deduction holds for the electric eel AChE (104, 105), mammalian brain (83), and erythrocyte (83), enzymes. The inhibition of acetylcholinesterases of the nematodes, *N. americanus* (106) and *N. brasiliensis* (106) by Concanavalin A has been reported. This points to the fact that, like other cholinesterases, these enzymes are glycoproteins (64). The specific action on cholinesterases by the inhibitors BW284C51 and ISO-OMPA has already been described in section 1.2.4. BW284C51 inhibitor contains a substantial hydrophobic moiety The preferential inhibitory action of BW284C51 on AChE may therefore point to the fact that the catalytic site of AChE might contain enormous hydrophobic inhibitor binding area which facilitate effective interaction of the enzyme with the inhibitor (53). Similar hydrophobic binding site is not likely to be present in large amount in pseudocholinesterase. BW284C51 is known to



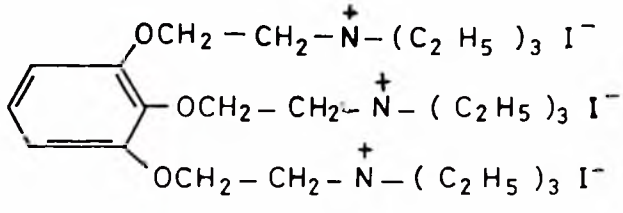
Acetylcholine



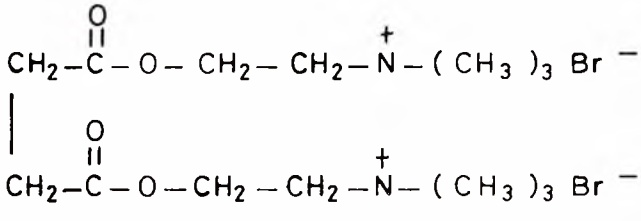
Physostigmine (Eserine)



Prostigmine



Gallamine triethiodide (Flaxedil)



Suxamethonium Bromide

Fig. 6 Structures of some specific inhibitors of Cholinesterases and the Substrate, acetylcholine (101).

have little or no inhibitory effect on pseudocholinesterase (52, 54).

A similar explanation has been proposed for the difference in magnitude of inhibition observed between *N. americanus* and *N. brasileinsis* AChE by BW284C51 (106). Here, it was discovered that the inhibition of the *N. brasiliensis* enzyme by BW284C51 was greater than that of the *N. americanus* enzyme by a factor of ten. The above observation suggests that BW284C51 may be useful for differentiating among the acetylcholinesterases.

1.2.5.4 Biological Significance of Acetylcholinesterase

Life depends upon the existence of an ordered array of chemical reactions. Order can be created because nearly all the reactions in the living system are catalysed by enzymes, which are highly specific in their actions. Properties of enzymes can therefore be adjusted to control rates of chemical reactions. This explains why enzymes can be of diagnostic importance as well as therapeutic agents. For instance amino-transferases are used in liver function tests and for monitoring myocardial infarction (107). Phosphatases are found at high levels in obstructive and hepatic jaundice and in cancer of the prostate gland (107). Aminopeptidases are potent therapeutic agents in hypertension (108). In addition, physiologically essential processes in the body, such as nerve conduction are under the direct influence of specific enzymes.

A nerve transmits a stimulus from one point in the body

to another through a travelling wave of changes in concentration of ions. In many nerves the stimulus is carried beyond the terminus of the neuron in the form of acetylcholine. The arrival of the ionic wavefront at the terminus causes the release of acetylcholine which diffuses into the intimately associated receptor cell, such as a muscle fiber or another neuron. This causes another travelling wave of excitation to begin in the receptor Once the acetylcholine has provoked the desired response, it must be removed to permit recovery of the receptor for the next stimulus or to prevent repeated and uncontrolled responses after a single stimulus. The removal is accomplished by hydrolysis of the compound and AChE is known to catalyse this reaction (12, 102, 109) Attention to the physiological role of AChE in synaptic transmission therefore developed in parallel with the concept of the importance of acetylcholine at the neuromuscular junction (102).

Ionic currents in postsynaptic membrane were reported to be highly sensitive to inhibitors which blocked the access of acetylcholine to either the active site of AChE or of acetylcholine receptor (53). Such information confirmed the idea that AChE can control the ionic currents produced in postsynaptic membrane at cholinergic synapses by hydrolyzing acetycholine, thus destroying its potent agonist action on the receptor Relatively recent work suggests that the enzyme at the synapse may also help regulate the level of presynaptic acetylcholine, perhaps by providing part of the choline used

for the resynthesis of the acetylcholine (110)

Studies have shown that excretory-secretory AChE from certain nematodes plays important roles in the parasitic adaptation. However, there is no convincing evidence at the moment to support any of them. One plausible proposition is that the secretory AChE acts as a kind of local anaesthetic on the gut of the host to prevent parasite expulsion (106) This is probably the result of the degradation of acetylcholine. Degradation of acetylcholine would lead to the inhibition of both intestinal peristalsis (111) and the secretion of mucus by the goblet cells (112). These events might serve to promote worm survival in the gut. It has also been reported that acetylcholine stimulates glycogen synthesis (106). If this effect is important in the gut, another possible role of AChE might be that of neutralizing the action of acetylcholine. This would indirectly make glucose available to the parasite In this way the enzyme could help the nutrition of the parasite.

Neurochemical transmitters are known to influence immune cell activation (113) One pathway to accomplish this is by alteration of the intracellular concentration of cyclic nucleotides (114). Experimentally, acetylcholine and related agonists enhance the release of histamine and leukotrienes from mast cells (115) and promote the ability of cytotoxic lymphocytes to kill target cells (116) They have also been implicated in numerous effects of polymorphonuclear cell function. These include lysosomal enzyme secretion,

phagocytosis and antibody-dependent cellular cytotoxicity (114). Moreover, acetylcholine, by raising intracellular cyclic guanosine monophosphate (cGMP) levels, can release T cells from their dependence upon interleukin 2 (IL-2) for interferon gamma production (117) If these effects are important in the gut, another aspect of secretory and/or excretory AChE might be to reduce the immune response of the host to the parasite as a result of its hydrolytic action on the neurochemical transmitter.

In effect if AChE secretion does constitute one level of immune modulation by parasites, this enzyme may be an important target of the host immune response. Truely, the AChE secreted by the gastrointestinal nematodes (118, 119), some tissue dwelling worms (19), and some worms inhabiting the lymphatic system (55), are important antigens.

Furthermore AChE is the target for several antiparasitic drugs that have specific toxic activities, for example hycanthone (120) and metrifonate (121).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Parasites

Onchocercomata were obtained fresh from onchocerciasis patients at the Onchocerciasis Chemotherapeutic Research Centre (OCRC) at Hohoe in Ghana. The freshly extirpated human nodules were quickly placed in special bottles containing RPMI medium 1640 on ice and immediately transported in the cold to Accra. On arrival, the nodules were either immediately digested (see section 2.2.4) and the freed worms stored at -71°C or kept frozen at the same temperature until needed.

2.1.2 Chemicals and Reagents

The following chemicals were obtained from Sigma Chemical Company. St. Louis, Mo., U.S.A.: acetylthiocholine iodide and S-butyrylthiocholine iodide, 5,-5′ dithiobis - (2 - nitro-benzoic acid) (DTNB), Sephacryl S-300, collagenase enzyme (from *Clostridium histolyticum*; 230 units/mg solid), commercial acetylcholinesterase from electric eel (1270 units/mg protein) and Eserine hemisulfate. The protein markers used for the gel filtration namely beef liver catalase, *Escherichia coli* β-galactosidase and bovine serum albumin were also from Sigma. Gentamycin used was a product of M.C.A Medical and Chemical AG. Milan, Italy N,N-methylene

bisacrylamide, 2 times crystallized, acrylamide, 4 times crystallized, and the alcohol dehydrogenase were obtained from Serva Feinbiochemica, Heidelberg, Germany Molecular weight markers used for the polyacrylamide gel electrophoresis, namely thyroglobulin, ferritin, catalase and bovine albumin (monomer) were products of Pharmacia Fine Chemicals, Uppsala, Sweden.

RMPI medium 1640 was a product of Gibco Europe Limited, Scotland, U.K.; Folin-ciocalteu phenol reagent was from Hopkin and Williams, England; and Tris (hydroxymethyl aminomethane) base was from Eastman Kodak Company Rochester, NY, USA.

Suxamethionium bromide and gallamine triethiodide were products of May and Baker Limited, Dagenham, England.

All other chemicals and reagents used were from the British Drug House (BDH) Chemicals Limited, Poole, England.

2.2 METHODS

2.2.1 Enzyme Assays

2.2.1.1 Acetylcholinesterase

Acetylcholinesterase activity was routinely determined colorimetrically according to the method of Ellman *et al* (122). The reaction mixture (final volume 1.57 ml) was made up of 100 mM Tris-HCl, pH 8.0 or 100 mM phosphate buffer, pH 8.0, containing 0.3 mM DTNB and either acetylthiocholine iodide or S-butyrylthiocholine iodide as substrate(s), at a final concentration of 0.47 mM. The reaction was performed at 25°C.

The thiocholine released reacted with DTNB in a disulphide exchange reaction to give a yellowish anion, 5-thio-2-nitrobenzoate (122), which was monitored continuously for 10 minutes at 410 nm, using a Shimadzu UV-190 double beam Spectrophotometer. Enzyme activity was calculated from a standard curve. Activity is defined as µmole or nmole thiocholine liberated per min. under the standard assay conditions specified above.

2.2.1.2 Catalase

Catalase activity was assayed by following the decrease in absorbance at 240 nm of a 3-ml reaction mixture containing 50 mM of potassium phosphate buffrer, pH 7.0, 10 mM of H_2O_2 and 50 µl of enzyme fraction (123). Activities were calculated in terms of change in absorbance per sec. per 50 µl of enzyme fraction.

2.2.1.3 β-Galactosidase

β-Galactosidase activity was determined according to the method described by Dahlquist (124). The reaction mixture comprised 50 mM maleate buffer, pH 6.0, 28 mM D-lactose, and 10 µl enzyme fraction. The enzymatic reaction was stopped at 10 minutes by adding 300 µl of 0.5 M Tris-HCl, buffer, pH 7.0. The glucose released was determined by the use of Glucose Diagnostic Kit No. 510-A (125). Absorbance was read at 450 nm, against the appropriate blank.

2.2.2 Preparation of Standard Curve for Determination of acetylcholinesterase Activity

A standard curve of thiocholine (0 - 0.10 µmole) was prepared as follows: 1.0 ml of 100 mM Tris-HCl buffer, pH 8.0, was added to different volumes of 0.10 µmole/ml of thiocholine. Each mixture was brought to 1.57 ml with distilled water Further treatment was as described under the enzyme assay (section 2.2.1.1). Appropriate blank was used.

Thiocholine used in this study was obtained by enzymatic hydrolysis of acetylthiocholine to thiocholine and acetic acid. The standard AChE enzyme (1270 units/mg protein) was reconstituted in 10 ml of Tris-HCl buffer, pH 8.0. Enzyme solution (0.1 ml) was added to the substrate mixture. This amount of the enzyme had enough activity to completely hydrolyse the amount of substrate used within the reaction period. The substrate-enzyme mixture was incubated at 37°C for 20 mins. The amount of thiocholine formed was used to prepare the standard curve as described above.

The hydrolysis of acetylthiocholine to form thiocholine is essentially a 1:1 mole ratio. Therefore moles of acetylthiocholine reacted is equal to moles of thiocholine formed.

2.2.3 Protein Determination

Protein concentration of the enzyme fraction(s) was determined according to the method of Lowry (126) Crystalline bovine serum albumin (0.2 mg/ml) was used as

standard. Absorbance measurements were made at 750 nm in a Shimadazu UV-190 Double Beam Spectrophotometer in a 10 mm light path cuvette.

2.2.4 Isolation of Worms

Collagenase digestion technique (127) was used to isolate adult worms from human nodules. Fresh onchocercomata were carefully freed of foreign tissues. The nodules were then placed individually or in groups of two, depending on their sizes, in appropriate bottles. Nodules were covered with collagenase solution, consisting of 0.5%(w/v) collagenase enzyme in RPMI medium 1640, supplemented with 0.2 mg/ml gentamycin to prevent bacterial growth. The reaction mixtures were incubated in a waterbath maintained at 34°C for approximately 24 hours. At the end, and during the incubation period where the worms were visible, the digests were poured into petri dishes and repeatedly washed with 0.9%(w/v) NaCl solution until no human tissue was seen on the worms. The worms were blotted dry and wrapped in aluminium foil. These were kept at -71°C until needed or were quickly used to prepare worm extract.

2.2.5 Preparation of Worm Extract

Unless otherwise stated, three batches of adult worms were used throughout the studies. These weighed 1.6, 3.2 and 2.12 grams (wet weight) respectively The worms were crushed manually, using mortar and pestle Approximately 500 mg of

treated sand (abrasive) was added to the homogenization medium to facilitate this procedure. Homogenization was done at 4°C in 2.5 ml of 50 mM Tris-HCl buffer, pH 7.5, containing either 1M NaCl or 1%(v/v) Triton X-100. The resulting homogenate was centrifuged at 1,000 x g for three minutes to sediment the sand. A TOMY CX-250 high speed refrigerated centrifuge was utilized. Thereafter, the extract was recentrifuged at 10,000 x g for five minutes followed by centrifugation of the resultant supernatant at 60,000 x g for three hours. The pellets were resuspended in 2-ml of buffer and the various fractions were used as enzyme source for subsequent studies.

AChE activity and protein concentrations were determined in each fraction, as described above.

2.2.8 Effect of Extraction Medium on AChE Activity of Adult *O. volvulus*.

The different media used for the parasite extraction were tested to see if they would affect the level of *O. volvulus* AChE activity. The procedure employed is as described by Tarrab-Hazdai *et al* (69).

Three different worm extract solutions (A, B and C) were prepared as follows:

Approximately 60.0 mg of adult parasite material were homogenised in;

(a) 1.0 ml of 50 mM Tris-HCl, pH 7.5 (solution A)

(b) 1.0 ml of 50 mM Tris HCl, pH 7.5 containing 1M NaCl, (solution B) or

(c) 1.0 ml of 50 mM Tris-HCl, pH 7.5 containing 1% (v/v) Triton X-100 (solution C)

Each portion was incubated with stirring for 30 min at room temperature. The homogenates (1.5 ml each) were centrifuged at 4°C for 5 min at 10,000g. The resultant supernatant fractions were recentrifuged at 60,000 x g for 3 hours, while the pellet resulting from each centrifugation step was resuspended in 1-ml volumes of 50 mM Tris-HCl buffer, pH 7.5. AChE activity in each fraction was assayed.

2.2.7 Purification of the Enzyme by Gel Filtration

(i) Preparation of Gel Filtration Column

Sephacryl S-300 suspension (130 ml) was repeatedly washed in deionised water for several minutes until the preservative (alcohol) could not be detected by odour It was then left to stand for approximately 1 hour. The gel was afterwards equilibrated with 50 mM Tris-HCl buffer, pH 7.5, or buffer containing 0.1% Triton X-100. It was then packed into a glass tube of dimension 100 cm long x 1.5 cm diameter (from Pharmacia Fine Chemicals, Uppsala) to a height of 60 cm.

Proteins of known molecular weight were used to calibrate the column.

(ii) Purification Procedure

The 60,000 x g supernatant fraction of the detergent buffer extract or the low-salt buffer extract was concentrated to a small volume (1.5 ml) by passing it through dry Sephadex G-25.

The 1.5 ml enzyme fraction was applied to the column equilibrated in either 50 mM Tris-HCl buffer, pH 7.5 or 50 mM Tris-HCl buffer, pH 7.5, containing 0.1%(v/v) Triton X-100. The protein was eluted with the buffer used to equilibrate the column. Fractions of 2.5 ml were collected at a flow rate of 17 ml/hr , using an LKB 7000 A Ultro Rac Fraction Collector.

A quick qualitative assay of all the fractions was performed by adding 0.1 ml of each fraction to 0.3 ml of buffered DTNB solution, containing 0.47 mM acetylthiocholine on tiles. Tubes which exhibited enzyme activity were investigated quantitatively by the colorimetric method (see section 2.2.1.1) Protein content of each fraction was evaluated at 280 nm in Shimadzu UV-190 double beam Spectrophotometer Fractions with enzyme activity were pooled and kept at -71°C.

2.2.8 PAGE under Non-denaturing Conditions

Polyacrylamide gel electrophoresis (PAGE) under non-denaturing condition was carried out in slab gels with a linear gradient of 3 - 20% polyacrylamide (54). The gels were prepared in 0.3M Tris-HCl buffer, pH 8.6 containing 80 mM boric acid, 2.5 mM EDTA and 0.1%(v/v) Triton X-100. For the low-salt buffer enzyme extract, Triton was omitted.

The electrophoresis was performed in the same buffer at a constant voltage of 150V for 16 hours at 4°C.

The proteins run under non-denatured conditions were stained for AChE activity by precipitating the thiocholine released from acetylthiocholine as copper salt (128). After the electrophoresis, the gels were gently shaken for 24 hours at room temperature in a medium containing 0.01 M acetylthiocholine iodide, 0.0003 M glycine, 0.025 M copper acetate and 2M sodium acetate. After staining, and destaining in 7%(v/v) acetic acid, the AChE bands became white. The gels were either traced on paper or processed for photography For photography, the gels were reshaken in Coomasie blue and destained. The AchE bands picked up the Coomasie blue and became faint blue while the other protein(s) freely picked up the dye. This provided a convinient background during photography of the electrophoragram.

2.2.9 Estimation of Sedimentation Coefficents

The apparent sedimentation coefficients (S) of the various molecular forms of *O. volvulus* AChE were estimated according to the method of Martin and Ames (123), by sedimentation in 5 - 20%(w/v) sucrose gradient. The 60,000 x g supernatant enzyme fraction in two different extraction media (50 mM Tris-HCl buffer, pH 7 5 and 50 mM Tris-HCl buffer, pH 7.5 containing 1%(v/v) Triton X-100) were used in the experiment.

Standard proteins, namely, bovine liver catalase (11.4 S) and *E. coli* ß-galactosidase (16 S) were run alongside for comparison Also it was assumed that there is a linear

relationship between migration and sedimentation. Sedimentation was conducted at 4°C by spinning in a swing-out rotor (SW 56.1) at 40,000 rpm for 4 hours in Hitachi 80P-7 automatic ultracentrifuge. The volume of the sucrose gradient was 4.2 ml. A 0.2 ml volume of protein extract (2.2 mg/ml) was layered on top of the gradient. Fractions (200 µl) were collected

2.2.10 Time-Course Experiment

The reaction catalysed by *O. volvulus* AChE was investigated to find out whether it varied linearly with time. The system used for this assay is shown in Table 1.

Table 1. Incubation System for Time-Course Experiment

Reagents	Volume of Reagents in (ml) Test	Blank
100 mM Phosphate Buffer, pH 8.0	1.40	1.40
0.47 mM Acetylthiocholine Iodide	0.01	0.01
0.3 mM DTNB	0.05	0.05
Water	0.10	0.11
Enzyme Extract	0.01	-
Final Volume	1.57	1.57

The reagents (minus enzyme) were pipetted successively into the cuvette and incubated at 25°C for 10 min. Thereafter the enzyme extract (1.2 mg/ml protein) was added and the formation of thiocholine monitored at 2 minutes intervals for 20 minutes at 410 nm.

2.2.11 Effect of Enzyme Concentration

The system employed in this experiment is shown in Table 2. Appropriate blanks were set up The addition of the enzyme extract to initiate the reaction was preceded by the incubation of the reagents for 10min. Further treatment is as described under section 2.2.1.1.

Table 2: Incubation System for Enzyme Concentration Experiment

Reagents	Volume of Reagents in (ml) 1	2	3	4	5
100 mM Phosphate Buffer, pH 8.0	1.3	1.3	1.3	1.3	1.3
0.47 mM Acetylthiocholine Iodide	0.01	0.01	0.01	0.01	0.01
0.3 mM DTNB	0.05	0.05	0.05	0.05	0.05
Water	0.185	0.160	0.135	0.110	0.085
Volume of Enzyme Extract	0.025	0.050	0.075	0.100	0.125
Final Reaction Volume	1.57	1.57	1.57	1.57	1.57

2.2.12 Effect of Ionic Strength

Phosphate buffers of pH 8.0 covering a range of ionic concentrations from 0 - 0.5 M were prepared. The activity of the enzyme in each concentration of buffer used was determined, under the normal assay conditions (see section 2.2.1.1) The experiment was done in duplicate.

2.2.13 Effect of Temperature on the Activity of AChE of *O. volvulus*

Temperature effect on the enzyme activity was investigated as described below:

(A) The enzymic reaction was performed at the following temperatures namely, 15, 25, 30, 37, 40, 50, 60 and 70°C The reagents (minus enzyme) were preincubated for 10 min at each of the temperature stated above. The reaction was initiated by addition of the enzyme extract to the respective reaction mixtures. The amount of thiocholine formed was estimated as described under section 2.2.1.1.

(B) The enzyme extract in 1.41 ml 0.1M phosphate buffer, pH 8.0, was exposed for 10 min to each of the temperatures stated in (A) Afterwards the tubes were quickly cooled in ice. The residual enzyme activity was subsequently determined under the normal assay conditions (section 2.2.1.1)

In both experiments, the 60,000 x g supernatant of the detergent buffer extract (1.7 mg/ml protein) was utilized.

2.2.14 Effect of Substrate Concentration

Substrate concentrations ranging from 0 - 0.1 M, each in a 1.57 ml reaction mixture, were used in this experiment. The Sephacryl S-300 enzyme fractions, containing approximately (0.06 - 0.1) mg/ml protein were utilized. Experiments were done in duplicate.

2.2.15 Effect of Buffers

Buffer employed in an assay system has been observed to exert profound effect on enzyme activity (129) The following buffers with their effective buffering regions within which AChE is thought to exhibit optimum activity (52) were employed in this assay

(1) 100 mM Tris-HCl Buffer, pH 7.1 - 8.9

(2) 100 mM Phosphate Buffer (Sorensen s), pH 5.8 - 8.0

(3) 100 mM 5:5-diethylbarbiturate-HCl (Veronal) buffer, pH 6.8 - 9.6.

0.1 ml of the 60,000 x g supernatant fraction of the solubilized enzyme was preincubated with 1.41 ml of the buffers for 15 minutes before the substrate was added. Further treatment is as detailed in section 2.2.1.1.

2.2.16 Effect of Inhibitors and Metal Ions on AChE Activity

(a) Inhibitors:

Three anticholinesterase drugs namely, Eserine hemisulfate, suxamethonium bromide and gallamine triethiodide were tested for their inhibitory effect on

O volvulus AChE. The procedure employed is described below.

0.1ml of the dialysed crude extract or the partially purified enzyme was preincubated with the inhibitor(s) in the concentration range 10^{-8} M to 10^{-3} M in 100 mM Tris-HCl buffer, pH 8.0 for 20 minutes. This was followed by the addition of the substrate, acetylthiocholine or S-butyrylthiocholine. Enzyme activity was subsequently determined as described previously under section 2.2.1.1.

(b) Metal Ions

0.1 ml of the dialysed crude extract or the partially purified enzyme extract was preincubated with the metal ions ranging in concentration from 10^{-5} M - 10^{-1} M in 0.1 M Tris-HCl buffer, pH 8.0, for 20 minutes, before the substrate, acetylthiocholine, was added. Afterwards the normal reaction procedure was followed as described in section 2.2.1.1.

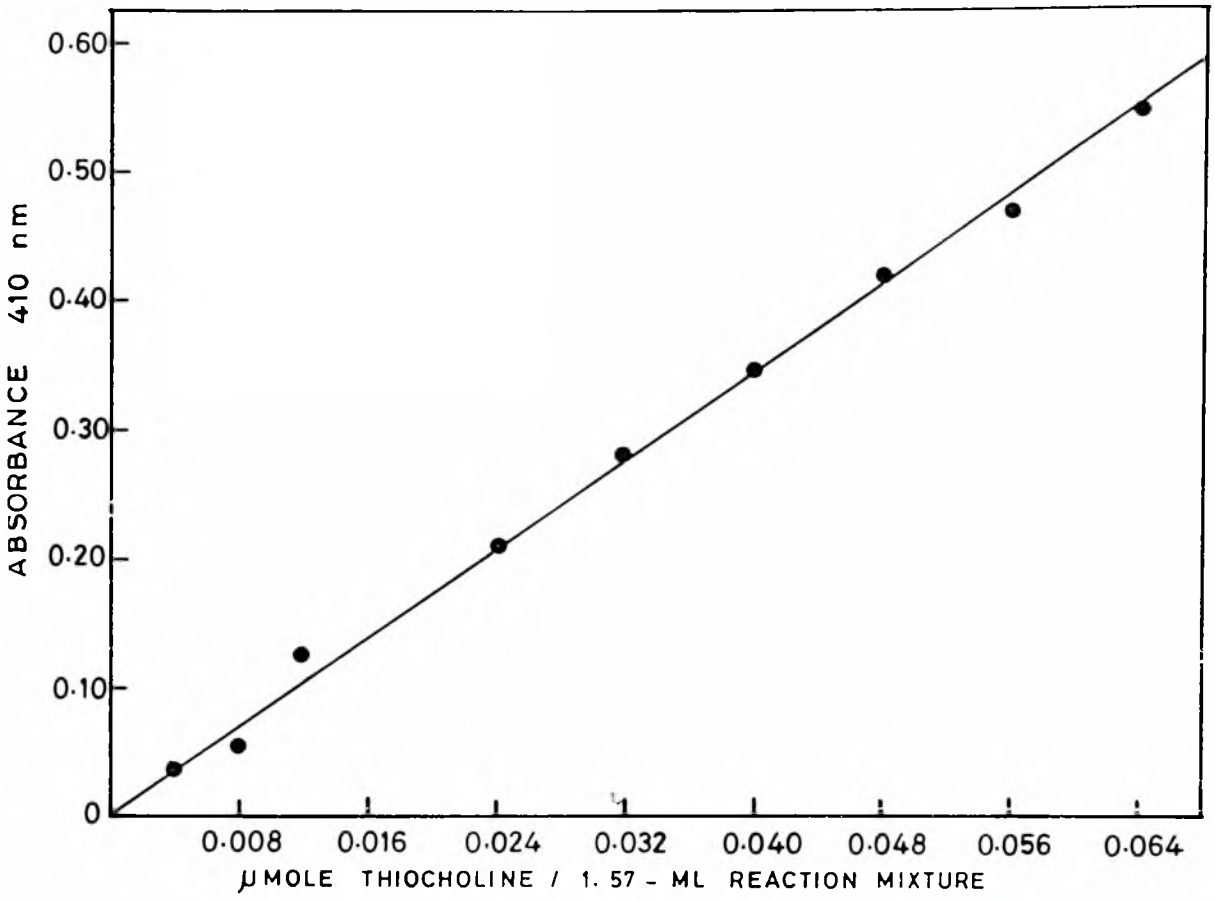


Fig. 7 STANDARD CURVE FOR ACETYLCHOLINESTERASE ACTIVITY DETERMINATION.

Different volumes of 0.10 micromole/ml of stock solution of thiocholine in a 1.57 ml reaction mixture were set up. Further treatment was as detailed in the standard assay.

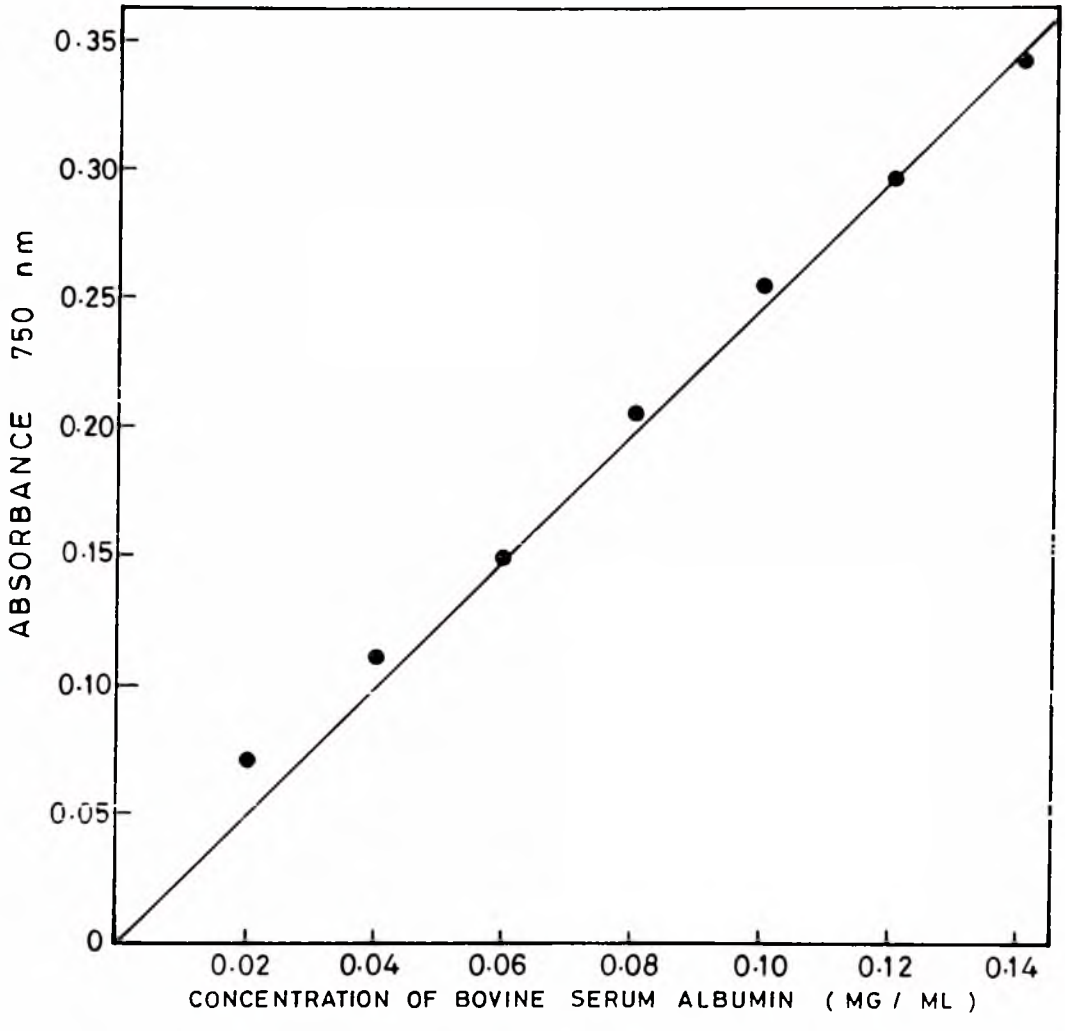


Fig. 8 STANDARD CURVE FOR PROTEIN DETERMINATION.

Determination of protein was based on the method described by Lowry (126)

CHAPTER THREE

RESULTS

3.1 Effects of the Extraction Medium on Activity of Acetylcholinesterase of *O. volvulus*

The use of different media for extraction of the parasite enzyme was found to have a marked effect on the observed level of activity of acetylcholinesterase activity isolated from *O. volvulus* (Table 3). It can be seen from the results that the highest enzyme activity was obtained when the homogenate was prepared in the presence of Triton X-100, a non-ionic detergent. The level of AchE activity in this fraction was about two times higher than that obtained from homogenates prepared in 50 mM Tris-HCl buffer, pH 7.5 or in 50 mM Tris-HCl buffer, pH 7.5 containing 1M NaCl. In addition, almost all the enzymatic activity remained in the supernatant fraction even after centrifugation at 60,000 x g for 3 hours. This fraction accounted for about 88% of the total activity. But appreciable AChE activity was also retained in the supernatant fractions of the 50 mM Tris-HCl and the 1M NaCl + 50 mM Tris-HCl extraction media.

Incorporation of 1M NaCl in the extraction buffer also had a positive solubilizing effect, but the enzyme activity was less than that of the detergent-containing buffer

Table 3: Acetylcholinesterase Activity in Homogenates of Adult *O. volvulus*

Medium of Extraction	Total Extract	Acetylcholinesterase Activity (nmoles of thiocholine formed per min) in different fractions			
		Supernatant 10,000 x g	Pellet 10,000 x g	Supernatant 60,000 x g	Pellet 60,000 x g
50 mM Tris-HCl buffer, pH 7.5 (A)	1.60	1.30	0.80	1.00	0.75
50 mM Tris-HCl + 1 M NaCl pH 7.5 (B)	2.00	1.40	0.70	1.15	0.80
50 mM Tris-HCl + 1% Triton X-100 pH 7.5 (C)	3.40	3.10	0.60	3.00	0.50

Acetylcholinesterase was extracted according to the procedure described in section 2.2.6. The enzyme activity was assayed as described in the standard enzyme assay (see section 2.2.1.1). The values are average of two experiments. Approximately 120 mg of adult parasite were used for the study.

3.2 Purification of *O. volvulus* Acetylcholinesterase

Figures 9 and 10 show the respective elution profiles of Sephacryl S-300 gel chromatography of the 60,000 x g supernatant fraction of detergent buffer and low-salt buffer extracts of *O. volvulus*. Two enzyme peaks were obtained with the Triton X-100 treated extract (Fig. 9) One of the enzyme peaks, marked Peak 1, occurred just after the void volume (arrowed). Tubes with these activities were pooled separately

With the enzyme extract prepared in the low-salt buffer, two enzyme peaks were also observed (Fig. 10). The minor enzyme peak, marked Peak 3, was eluted with the void volume. Tubes corresponding to these enzyme peaks were also pooled separately

Summaries of the purification procedures for extracts prepared in the detergent buffer and low-salt buffer are given in Table 4, A and B. Samples from peaks 1 and 2 respectively shared twelve- and thirteen fold AChE activity, with overall yields of 22% and 23% as compared with the original homogenates. In the case of the low-salt buffer enzyme extract, ~~a~~ purification factors of 5 and 9 were achieved for Peaks 3 and 4 respectively, their overall yields being 11% and 19%.

Table 4 A: Summaries of the Purification Procedures of Acetylcholinesterase from Adult *O. volvulus*

Purification Step	Volume (ml)	Enzyme Activity (units/ml)	Total Activity	Protein Conc. (mg/ml)	Specific Activity (units/mg protein)	Yields (%)	Degree of Purification
Total Extract	6.0	7.04	42	5.15	1.36	100	1
60,000 x g Supernatant	4.8	6.88	33	1.70	4.05	78	3.0
Concentration	2.4	9.60	23	2.20	4.36	55	3.2
Sephacryl S-300:							
Enzyme Peak 1	5.2	1.75	9.1	0.11	15.9	22	11.7
Enzyme Peak 2	5.5	1.76	9.7	0.10	17.6	23	13.0

A: Detergent buffer enzyme extract

Table 4 B: Summaries of the Purification Procedures of Acetylcholinesterase from Adult *O. volvulus*

Purification Step	Volume (ml)	Enzyme Activity (units/ml)	Total Activity	Protein Conc. (mg/ml)	Specific Activity (units/mg protein)	Yields (%)	Degree of purification
Total Extract	6.0	3.44	20.6	2.2	1.56	100	1
60,000 x g Supernatant	5.0	2.40	12.0	0.8	3.0	58	1.9
Sephacryl S-300:							
Enzyme Peak 3	3.2	0.72	2.3	0.09	8.0	11	5.1
Enzyme Peak 4	4.5	0.83	3.96	0.06	13.8	19	8.8

B: Low-salt buffer enzyme extract

With the exception of the total extracts, 0.05 M $MgCl_2$ was added to each assay mixture. In both studies (that is A and B), acetylthiocholine was used as substrate. Unit of activity is defined as nmole of thiocholine produced per minute.

Figure 9: Elution Profile of Detergent Buffer Extract of AChE of *O. volvulus* from Sephacryl S-300

The 60,000 x g supernatant fraction of the detergent buffer extract was applied on the column, equilibrated with 50mM Tris-HCl buffer, pH 7.5 containing 0.1% (v/v) Triton X -100. The column was eluted at 4°C with the same buffer at a flow rate of 17 ml/hr Two and a half millilitre fractions were collected.

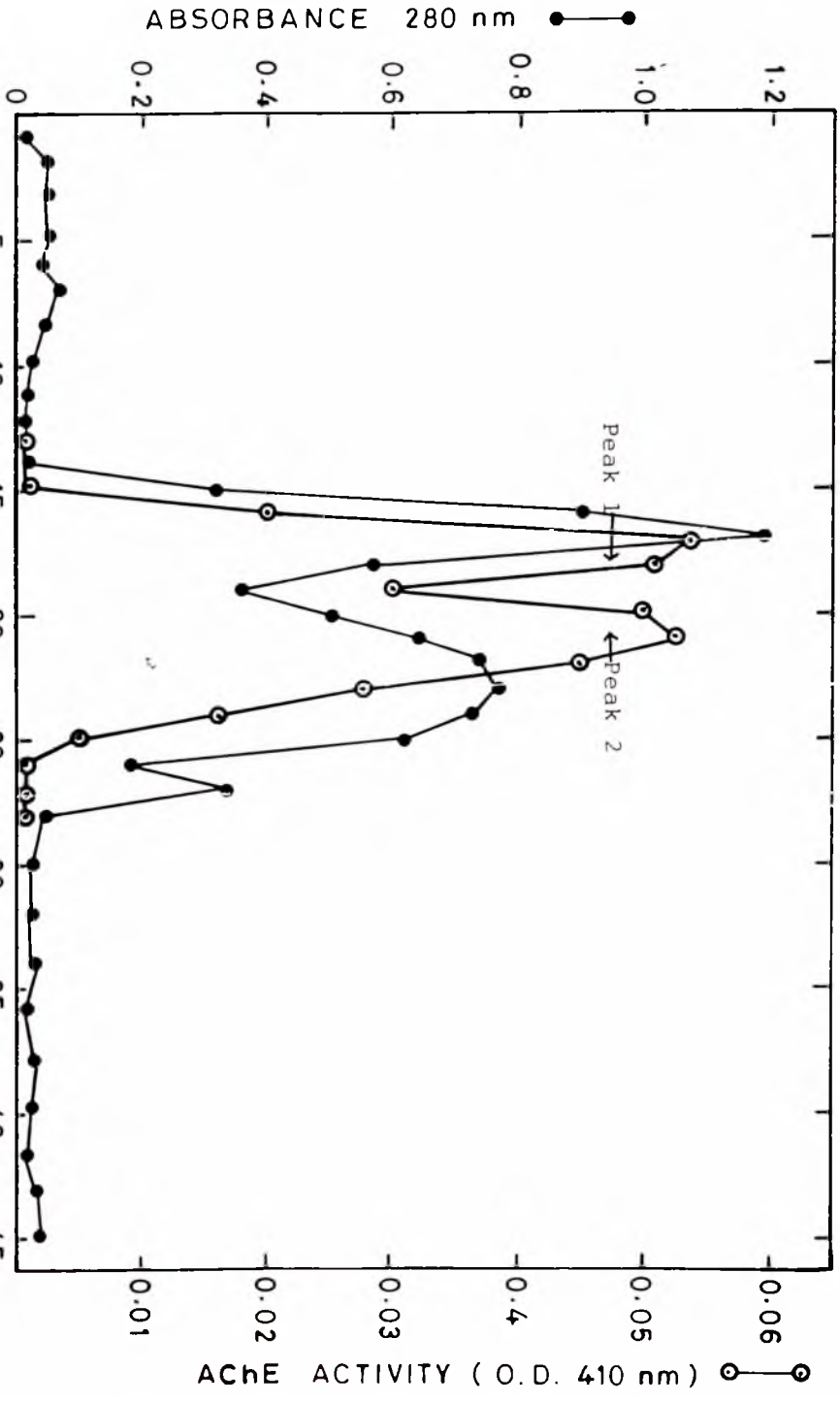


Fig. 9 ELUTION PROFILE OF DETERGENT BUFFER EXTRACT OF AChE OF O. VOLVULUS FROM SEPHACRYL S - 300.

Figure 10: Elution Profile of Low-salt Buffer Extract of AChE of *O volvulus* from Sephacryl S-300.

The 60,000 x g supernatant fraction of the low-salt buffer extract was applied on the column, equilibrated with 50mM Tris-HCl buffer, pH 7.5. The column was eluted at 4°C with the same buffer at a flow rate of 17 ml/hr. Two and a half millilitre fractions were collected.

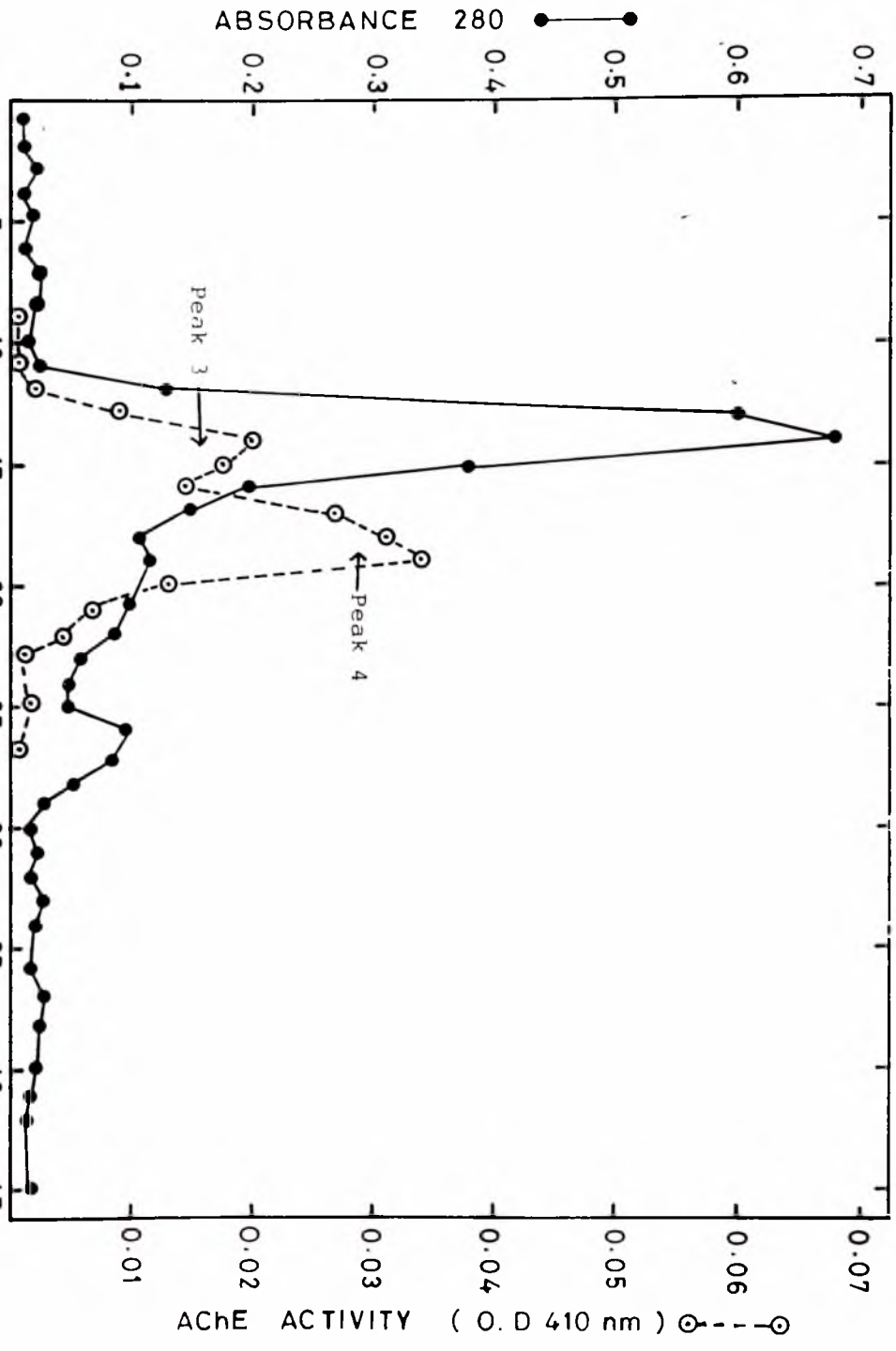


Fig. 10 ELUTION PROFILE OF LOW - SALT BUFFER EXTRACT OF AChE OF O. VOLVULUS FROM SEPHACRYL S - 300

3.3 Multiple Molecular Forms of *O. volvulus* Acetylcholinesterase on Sucrose Density Gradient

The presence of different molecular forms of acetylcholinesterase in the two extraction media is shown in Fig. 11. As is evident from the results, the enzymes extracted in plain 50 mM Tris-HCl buffer, pH 7.5 existed predominantly as an 11.3 S species with 75% of the enzyme activity Approximately 25% of the enzyme activity was present in the aggregated form with a sedimentation coefficient of about 38 S.

On the other hand, the enzyme extracted with the buffer containing detergent (Triton X-100) existed essentially as two predominant AChE species with sedimentation coefficients of 9.5 S and 18 S, the former having a slightly higher total activity It should be noted that the sedimentation coefficients (S) assigned to the *O. volvulus* AChEs were calculated based on their migration distances through the sucrose gradient relative to those of the standard proteins. The values are therefore approximations only

The existence of different molecular forms of acetylcholinesterase was also demonstrated by the gel filtration chromatography on Sephacryl S-300 (Figs. 9 and 10; 15 and 16) and PAGE under non-denaturing conditions (Figs. 17 and 18).

Fig. 11: Sucrose Gradient Centrifugation of AChE of Adult *O. volvulus*

The 60,000 x g supernatant fractions used were obtained from adult worms extracted in (A) 50 mM Tris-HCl buffer, pH 7.5 (low-salt buffer); (B) same buffer containing 1% Triton X-100 (detergent buffer) For each experiment, 0.2 ml of the extract was layered on the 5-20% sucrose gradient prepared in the same extraction buffer. The arrows show the positions of protein markers which were also run alongside the extracts. From right to left: Catalase (11.4S) and *E. coli* ß-galactosidase (16S). Acetylcholinesterase activity together with those of catalase and ß-galactosidase were determined in each fraction as described in the Materials and Method.

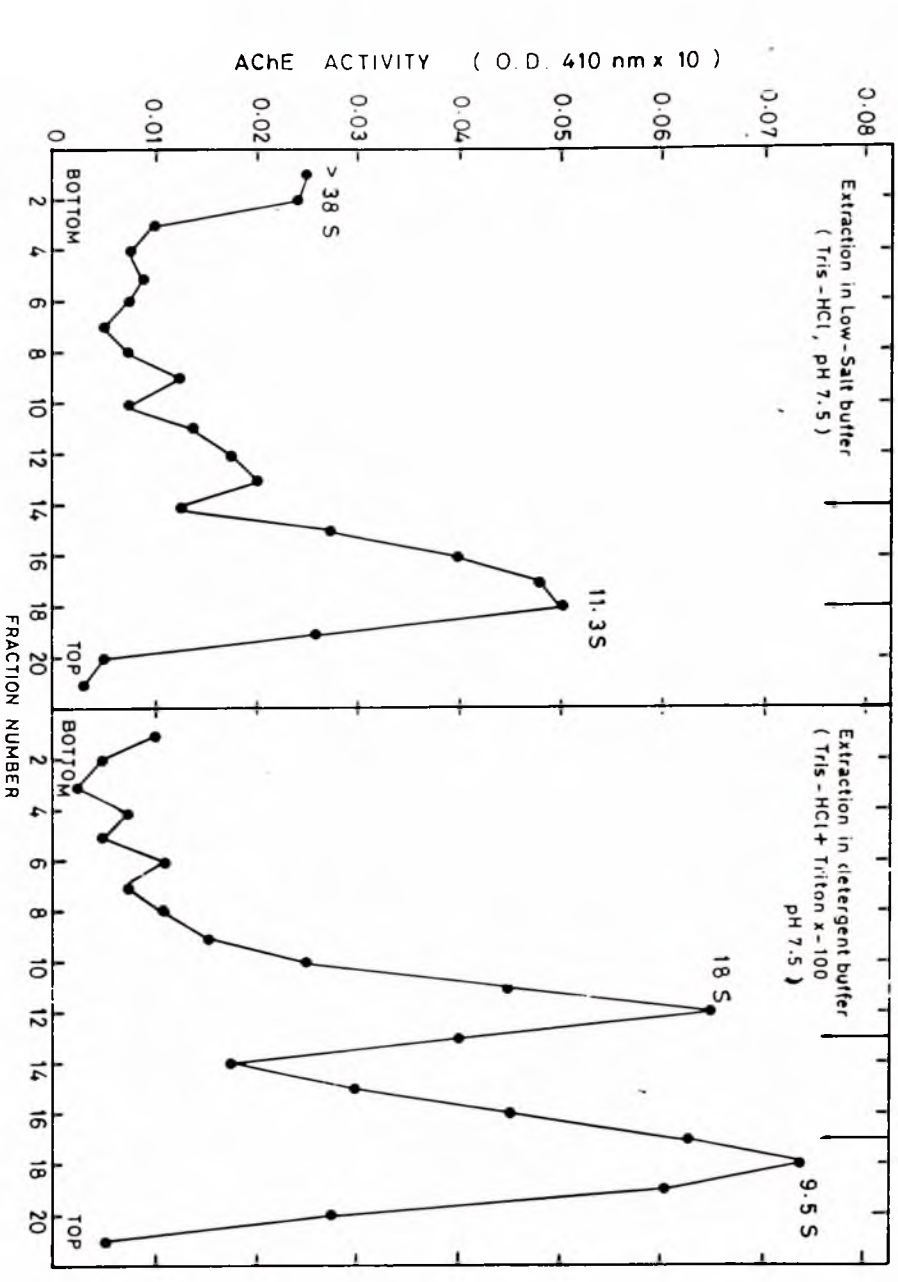


Fig. 11 SUCROSE GRADIENT CENTRIFUGATION OF AChE OF ADULT O. VOLVULUS

3.4 Molecular Weight Determinations

The molecular weight of the native AChE enzymes was determined by gel filtration on Sephacryl S-300 and by PAGE under non-denaturing conditions. Elution profiles of the various AChE species were compared with those of the protein markers run under identical conditions (Figs. 15 and 16) The molecular weights of the various AChE species were obtained by interpolation from a standard curve (Fig. 12). The molecular weights as estimated from the standard curve are: >600 KD and 400KD for the enzymes in the low-salt buffer extract (Fig. 15); and 525 KD and 375 KD for those in the detergent buffer extract (Fig. 16)

PAGE under nondenaturing conditions and staining specifically for acetylcholinesterase activity, and by Coomassie blue staining also revealed several AChE species in the low-salt buffer extract with molecular weight corresponding to 500 KD, 280 KD, 220 KD and 58 KD (Fig. 18) while 582 KD, 355 KD, 290 KD and 67 KD were obtained for the AChE species in the detergent buffer extract (Fig. 17) The molecular weights obtained were deduced from standard curves (Figs. 13 and 14).

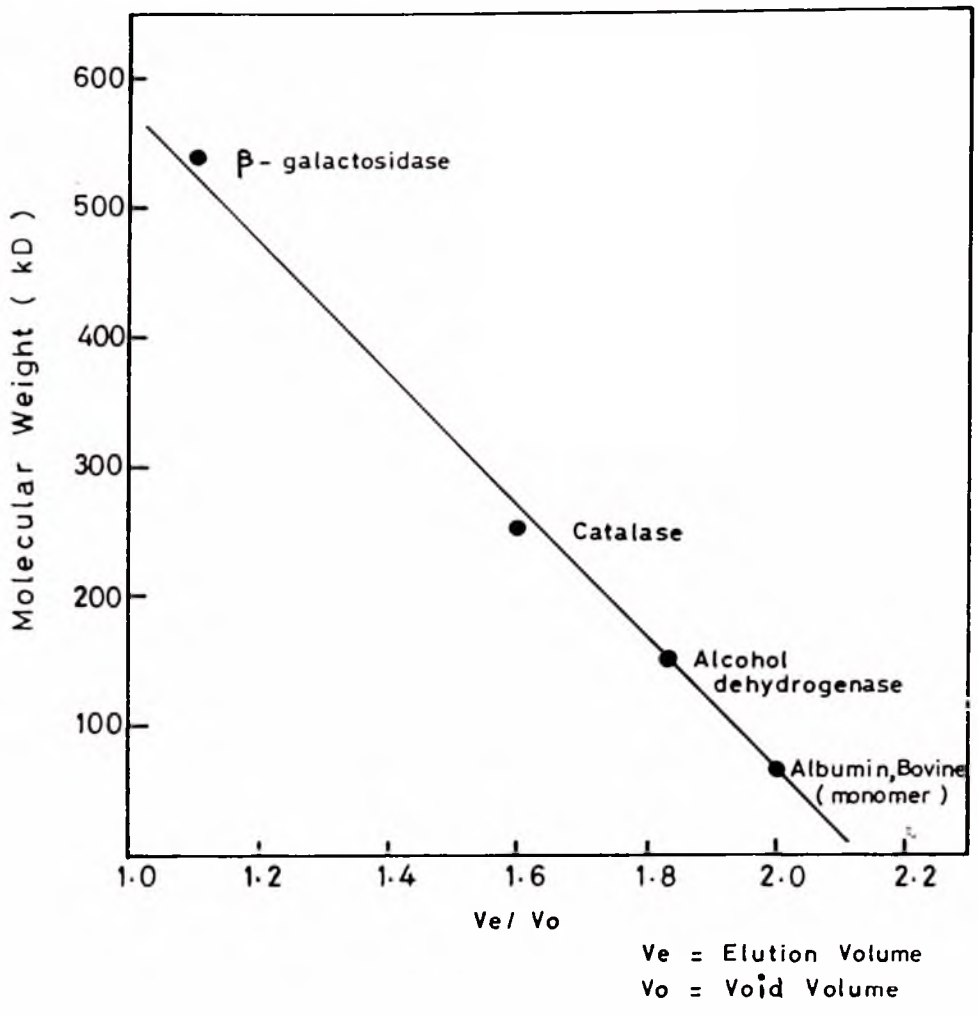


Fig. 12 CALIBRATION CURVE FOR GEL FILTRATION CHROMATOGRAPHY ON SEPHACRYL S - 300 .

The column was calibrated with the markers as described in the materials and method. Albumin (monomer) and Alcohol dehydrogenase were detected individually at 280 nm. β-galactosidase and catalase were assayed, as described previously in test.

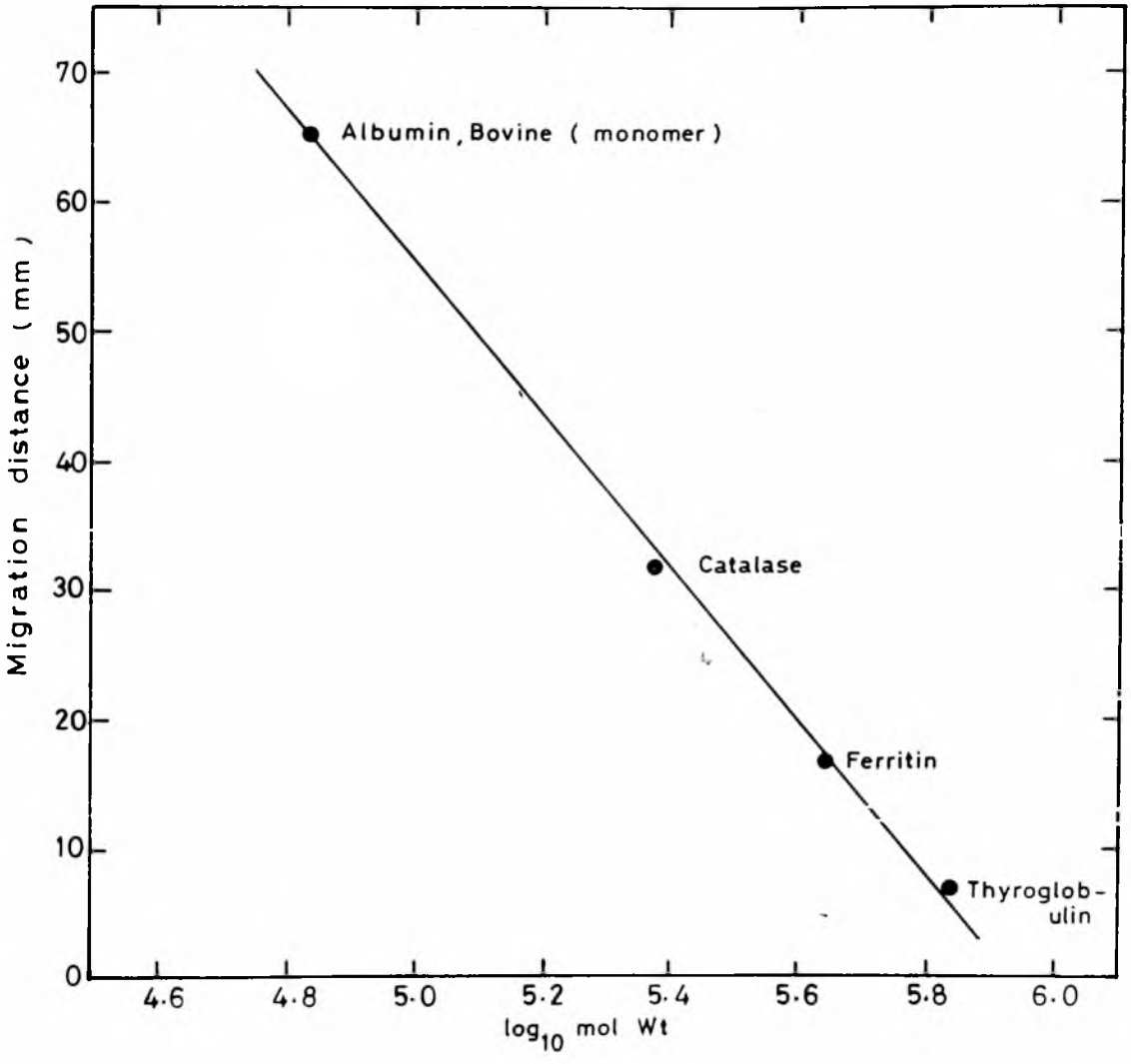


Fig. 13 CALIBRATION CURVE FOR GRADIENTS OF POLYACRYLAMIDE - GELS (Buffer : 50 mM Tris-Hcl; Triton x-100 . pH 7.5) .

15 µl of the protein markers were loaded into the gels. After electrophoresis the gels were stained in Coomassie blue and destained, as described previously. The distance moved by each marker was measured.

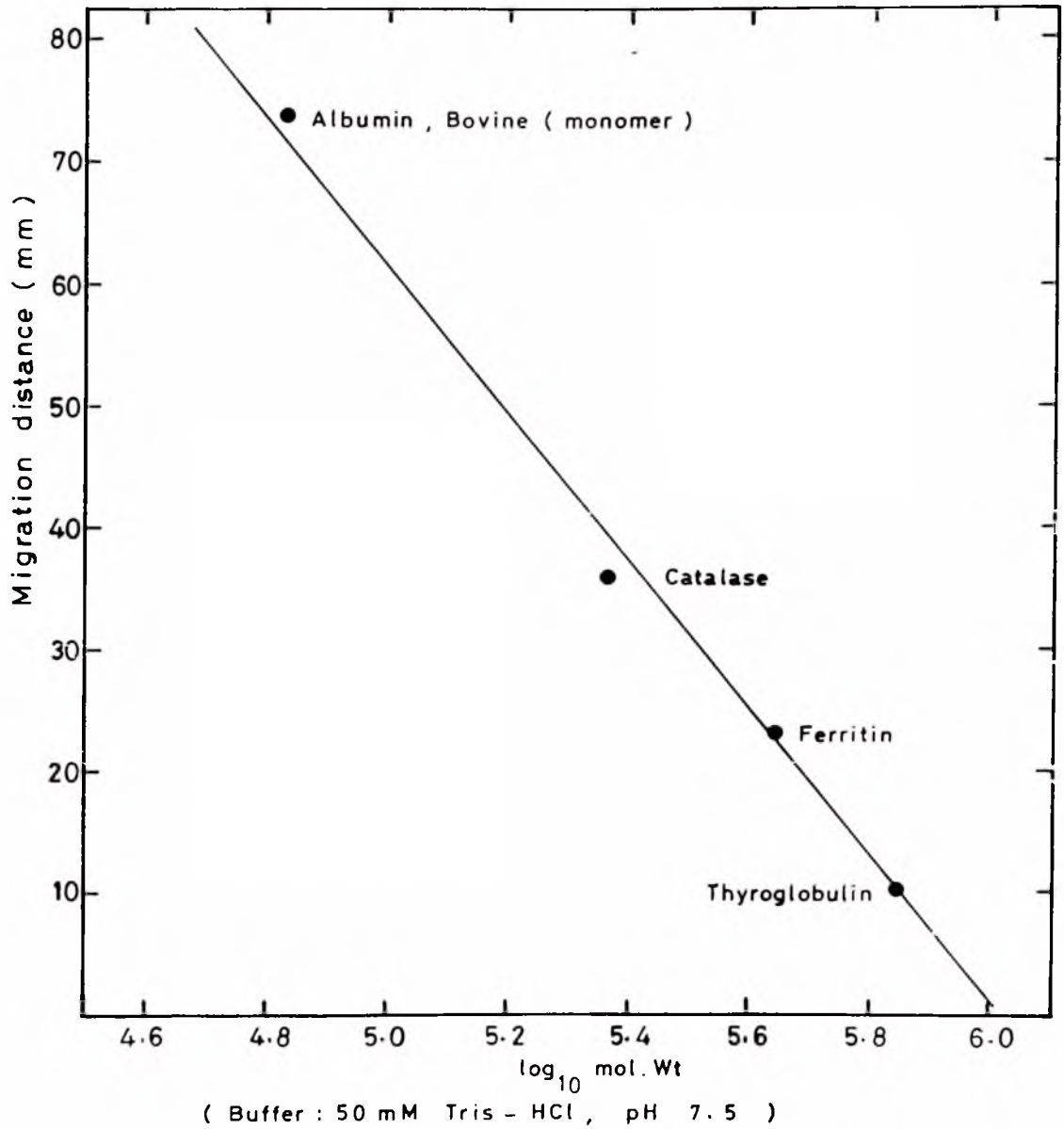


Fig. 14 CALIBRATION CURVE FOR GRADIENTS OF POLYACRYLAMIDE - GELS .

Procedure is the same as in Figure 13.

Fig. 15: S-300 Profile of AChE Extract
(Buffer: 50mM Tris-HCl, pH 7.5)

Fig. 16: S-300 Profile of AChE Extract
(Buffer: 50 mM Tris-HCl, Triton X-100, pH 7.5)

The 60,000 x g supernatant fraction of the low-salt buffer or the detergent buffer, containing approximately 2 - 4.5 mg protein was applied onto a Sephacryl S-300 superfine column (1.5 x 60 cm). Further treatments are found in the Materials and Methods. The protein markers were also run alongside the extracts. These are (arrowed) left to right : *E. coli* β-galactosidase (540 KD) catalase (250 KD). Alcohol dehydrogenase (150 KD) and bovine serum albumin (67 KD).

FIG.15

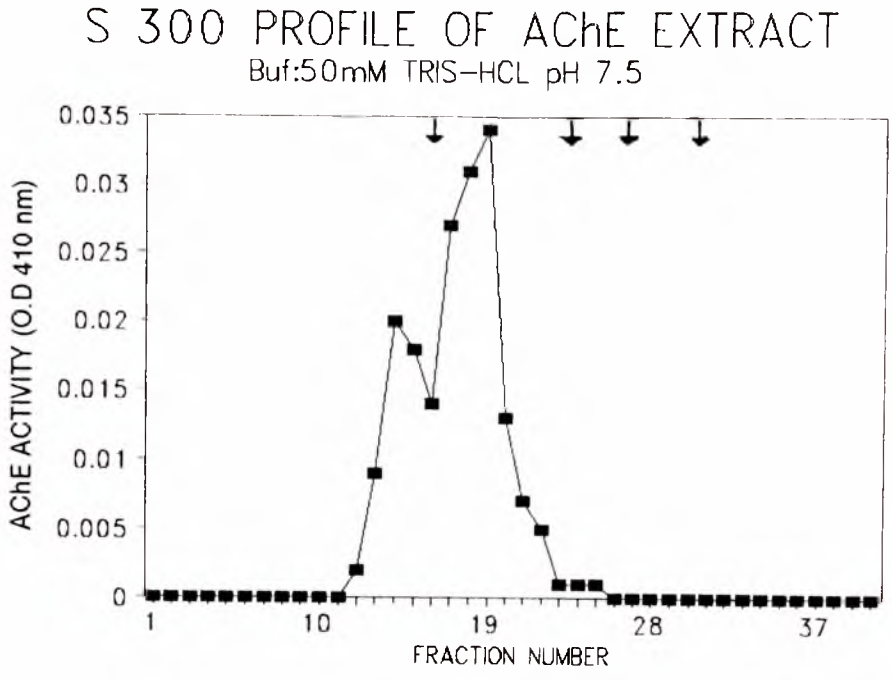


FIG. 16

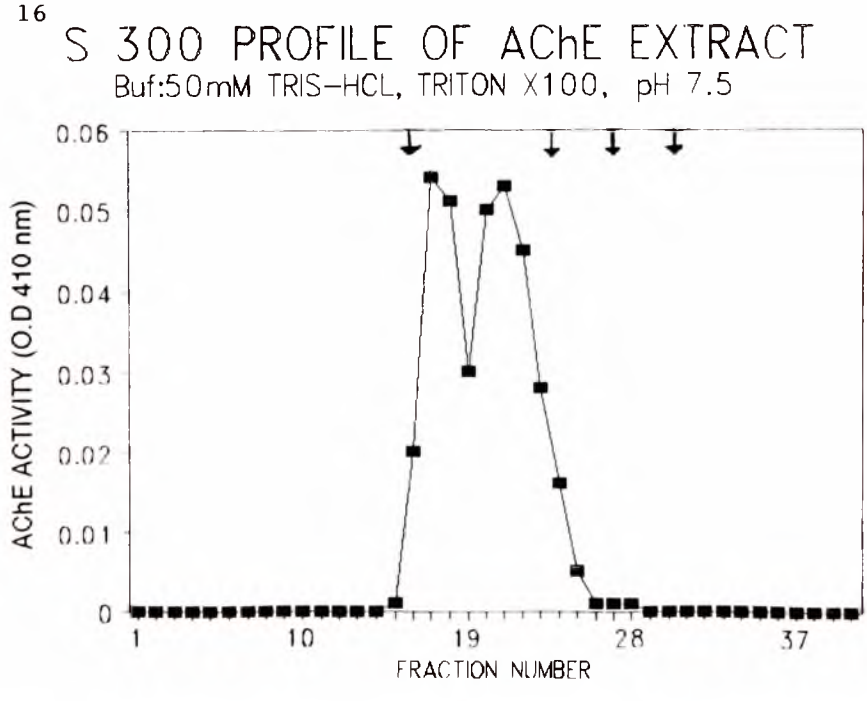


Fig. 17
PAGE under nondenaturing conditions of crude fractions of the detergent buffer extract of *O. volvulus* AChE.

Fig. 18
PAGE under nondenaturing conditions of crude fractions of the low-salt buffer extract of *O. volvulus* AChE.

Samples were electrophoresed in Tris-borate, buffer pH 8.6 through 3 20% polyacrylamide linear gradient gels in the absence of denaturing and reducing agents.

e Fig. 17 A-E:

A. Markers: Thyroglobulin (669 KD), ferritin (440 KD), catalase (232 KD) and bovine serum albumin (67 KD).

B. Coomasie blue staining of the total extract.

C. Coomasie blue staining of the 60,000 x g supernatant fraction.

D. Specific AChE activity staining of the total extract.

E. Specific AChE activity staining of 60,000 x g supernatant fraction.

Fig. 18 F-J:

F. Specific AChE staining of the total extract.

G. Specific AChE staining of the 60,000 x g supernatant fraction.

H. Coomasie blue staining of total extract.

I. Coomasie blue staining of 60,000 x g supernatant fraction.

J Markers (as in Fig. 17 A).

The arrows (Fig.18) show AChE bands after the gels (F-G) were restained in Coomassie blue.

e The traced copy is presented. Cracks developed in the gel on drying for photography

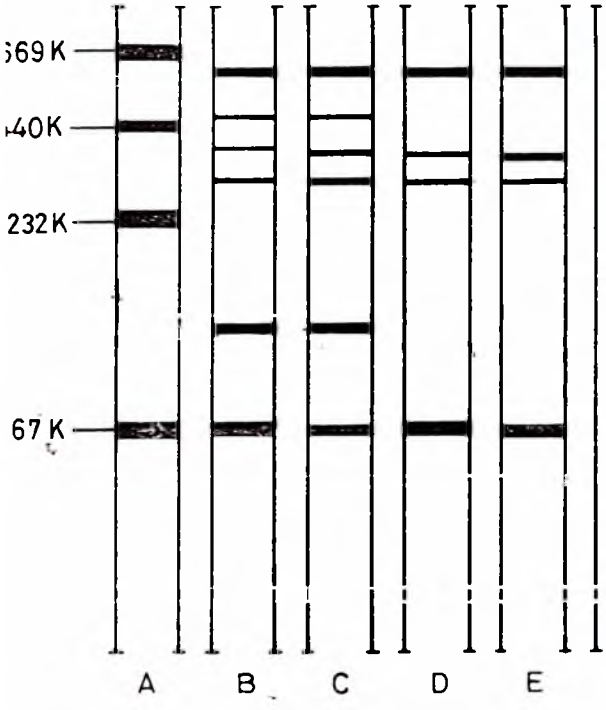


Fig. 17 PAGE under nondenaturing conditions of crude fractions of the detergent buffer extract of O. volvulus AChE.

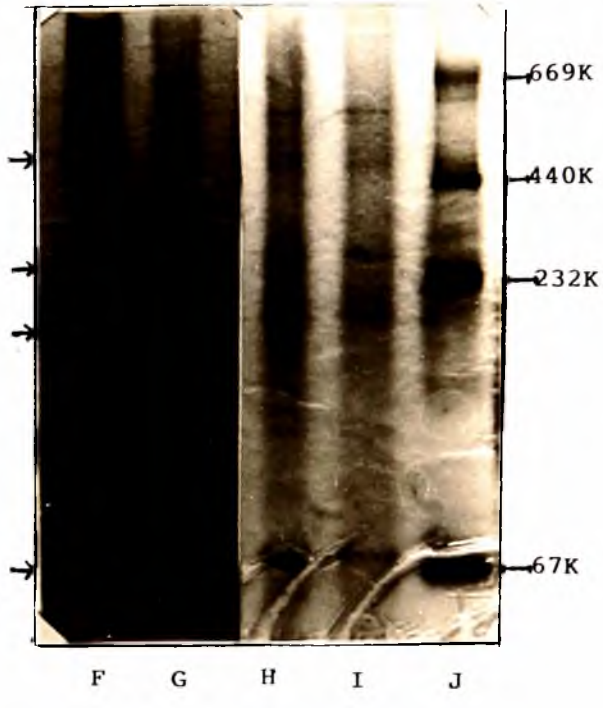


Fig. 18 PAGE under nondenaturing conditions of crude fractions of the low-salt buffer extract of O. volvulus AChE.

3.5 Linearity of *O. volvulus* AChE Activity with Time and Amount of Enzyme

Formation of thiocholine by the enzymic hydrolysis of acetylthiocholine was found to proceed linearly with time for up to about 20 minutes (Fig. 19).

Similarly, the rate of hydrolysis of the substrate was found to be directly proportional to the amount of enzyme present in the assay mixture (Fig. 20).

3.6 Effect of Ionic Strength on AChE Activity

Various concentrations of phosphate buffer, pH 8.0 were prepared to investigate their effect on the onchocercal AChE activity There was an increase in the enzyme activity with increase in ionic strength up to 0.1 M (Fig. 21) after which it levelled off, until at 0.3 M when decrease in enzyme activity was observed.

3.7 Effect of Temperature on AChE of *O. volvulus*

Figure 22 shows temperature effect on the onchocercal AChE activity. Activity increased with temperature up to about 38°C beyond which there was a progressive decrease, till at 70°C when no enzyme activity was observed (Fig. 22A).

In Figure 22B, the enzyme fraction was preincubated at various temperatures for 10 minutes after which the residual enzyme activity was determined at 25°C. The results show that there was gradual decrease in enzyme activity from 15°C to 40°C beyond which a drastic decrease in enzyme activity was observed.

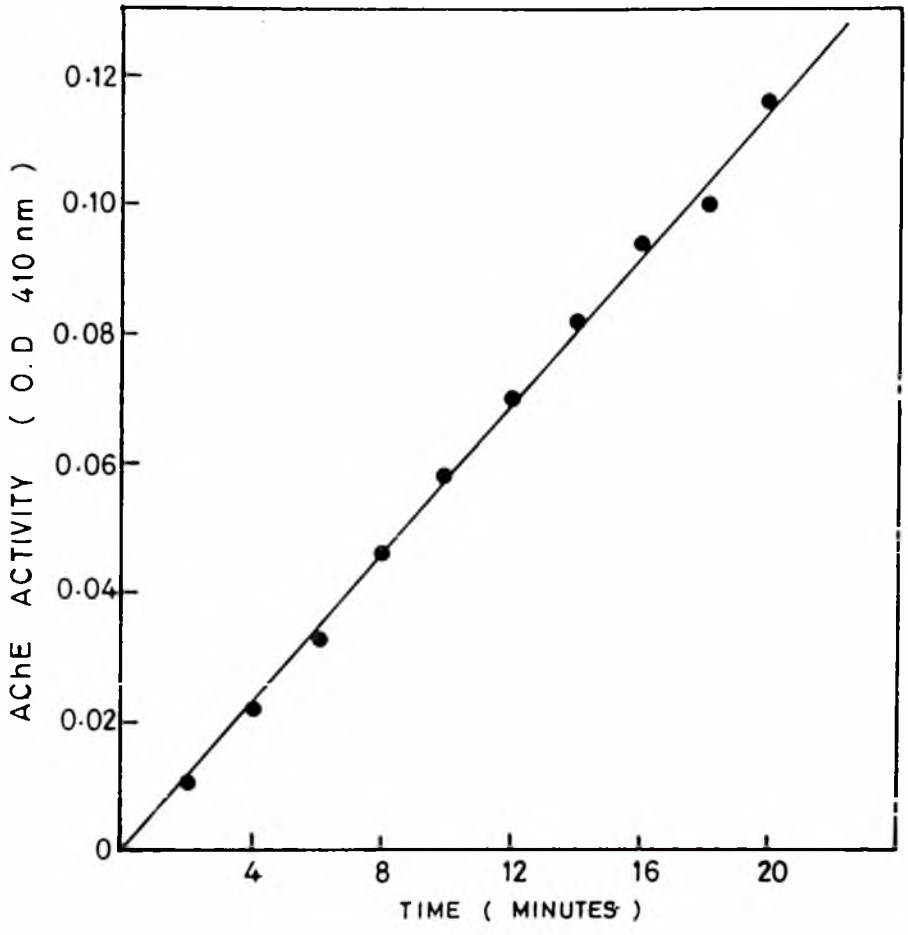


Fig. 19 TIME - COURSE REACTION OF RELEASE OF THIOCHOLINE FROM ACETYLTHIOCHOLINE BY AChE OF O. VOLVULUS .

The reaction mixture was made up of 100 mM phosphate buffer, pH 8.0; 0.47 mM acetylthiocholine and 0.3 mM DTNB solution. These were incubated at 20 ^{0}C for 10 minutes before the 60,000 x g supernatant fraction of the solubilized enzyme was added. Formation of thiocholine was monitored at 2 minutes interval for 20 minutes.

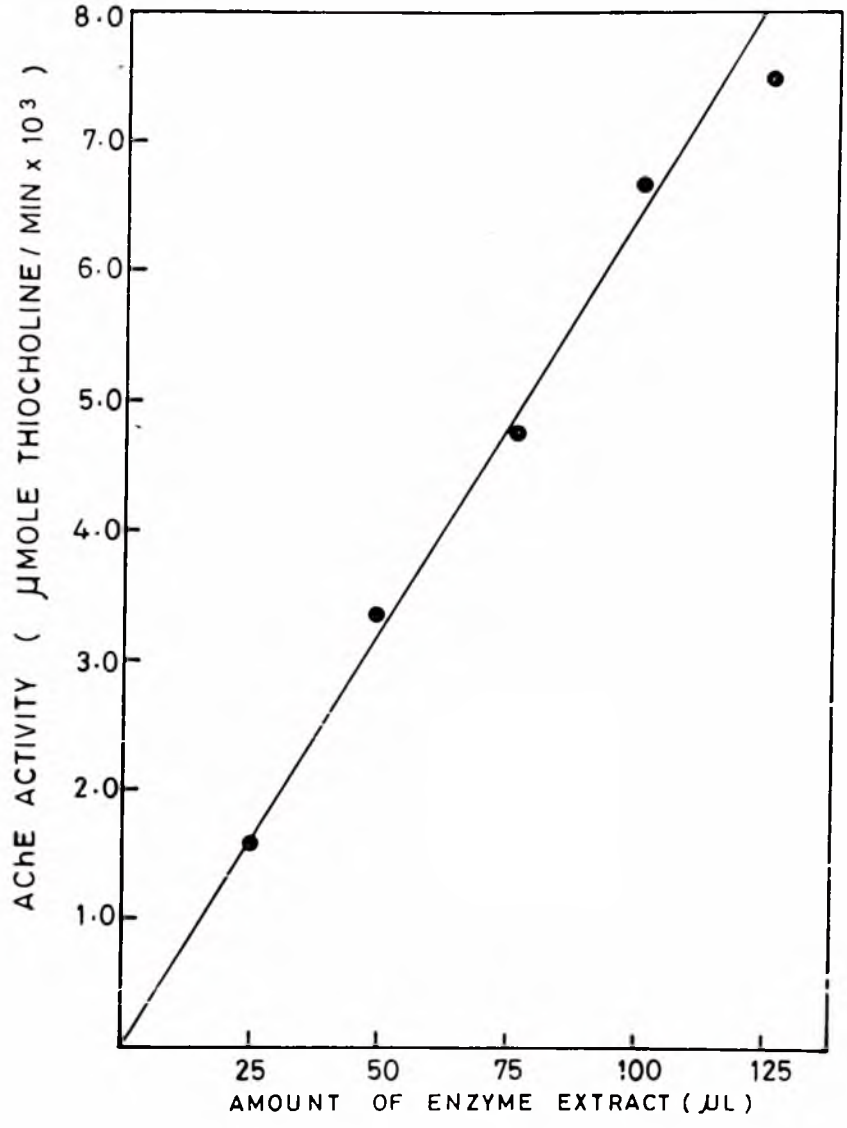
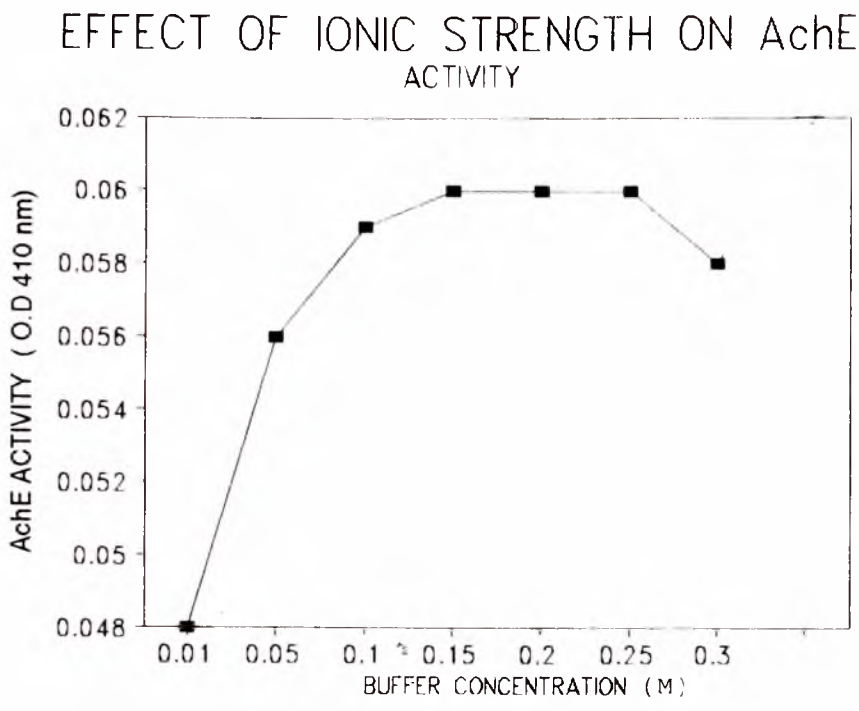


Fig. 20 FORMATION OF THIOCHOLINE AS A FUNCTION OF AMOUNT OF ENZYME

Different volumes of the 60,000 x g supernatant fraction of the Triton X-100 treated enzyme (1.7 mg/ml protein) were added to the reaction mixture as indicated in section 2.2.11. Subsequent treatment is as detailed in the standard assay.

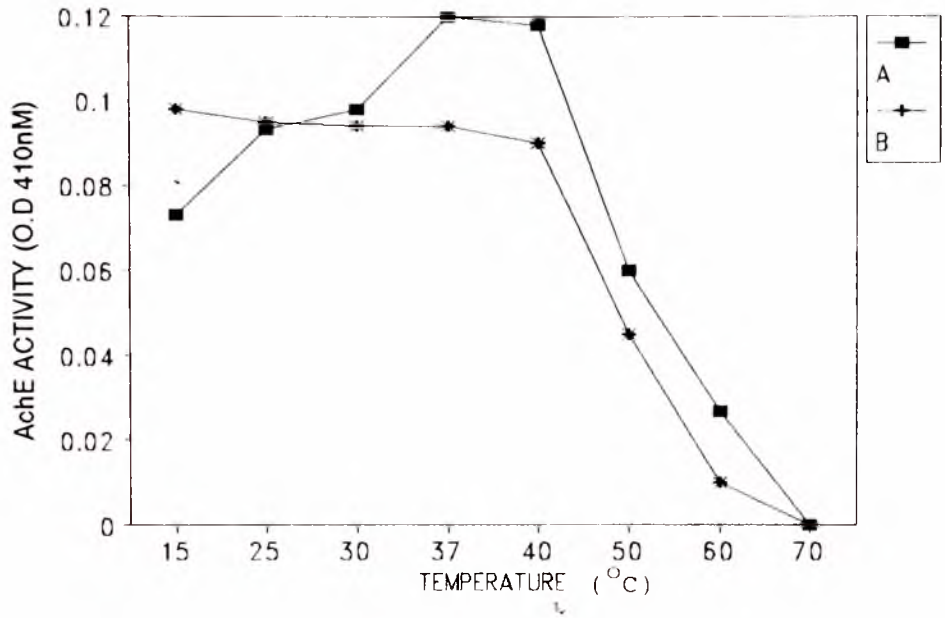
FIG. 21



Various concentrations of phosphate buffer, pH 8.0 were prepared as described in the materials and method. Determination of enzyme activity is also described in the method section. The 60,000 x g supernatant fraction of the solubilized enzyme was utilized.

FIG. 22

EFFECT OF TEMPERATURE ON ACTIVITY OF AchE of O.VOLVULUS



In (A) enzymatic reaction was performed at the various temperatures inidcated in section 2.2.14. In (B) the enzyme was preincubated at each temperature for 10 minutes and the residual enzyme activity was determined under the standard assay conditions. In each case, 60,000 x g supernatant of the solubilized enzyme fraction was used.

3.8 Effect of Storage on AChE Activity

The stability of the *O. volvulus* AChE at extreme cold conditions was investigated by keeping the enzyme extract(s) at -71°C and thawing monthly to determine the residual AChE activity The results (Table 5) show that there was no marked decrease in AChE activity for the five months storage period. Six percent (6%) decrease in activity relative to the control (that is, AChE activity before the enzyme was first kept at -71°C) was observed for the AChE extract prepared in the detergent buffer. For the enzyme prepared in low-salt buffer, 5% decrease in activity was observed.

Table 5: Effect of Storage (X°C) on *O. volvulus* AChE Activity

	AChE Activity (%)	
Time (months)	Extract Prepared in 50 mM Tris-HCl, pH 7.5	Extract Prepared in 50 mM Tris-HCl, pH 7.5, containing 1%(v/v) Triton X-100
Control	100	100
1	102	100
2	98	96
3	99	97
4	96	95
5	95	94

The 60,000 x g supernatant fraction of the detergent buffer enzyme or the low-salt buffer enzyme was kept frozen at -71°C. At a month interval, the enzyme was quickly thawed and the AChE activity was determined as described in the standard assay (section 2.2.1.1) The results are given as the activity (%) relative to that of the control time (i.e AChE activity determined before the enzyme was first frozen).

3.9 Effect of Buffers on AChE of *O. volvulus*

Figure 23 shows the results observed with the three buffer systems. The pH-activity curves in Tris-HCl and Veronal buffers were parabolic showing a maximum at pH 8.0 (Fig. 23) The optimum was broad in these buffers. The activity of the onchocercal AChE was influenced by the buffering species. The enzyme exhibited greatest activity in the phosphate buffer. This was followed closely by the Veronal buffer The Tris-HCl buffer produced the least activity The phosphate buffer (Sorensen's) could not be used beyond pH 8.0. Beyond this pH, the ionic species present namely $HPO4^{2-}$ and $H_2PO_4^-$ lose their buffering capacities.

3.10 Substrate Specificity of AChE of *O. volvulus*

The enzymic activity of the Sephacryl S-300 fractions of *O. volvulus* AChE as a function of acetylthiocholine concentration is shown in Figure 24. All the fractions examined showed Michaelis-Menten kinetics with acetylthiocholine. Substrate concentrations beyond 1×10^{-2} M were inhibitory Enzyme Peak 3 was not used in this experiment. The yield obtained in the purification was rather low. The kinetic parameters are shown in Table 7 Similar experiment could not be carried out with the S-butyrylthiocholine for comparison because of inadequacy of parasite material.

However hydrolysis of acetylthiocholine relative to butyrylthiocholine by *O. volvulus* AChE was compared, using a single concentration of the substrates. This was done for

both the crude and Sephacryl S-300 enzyme fractions. The observed rate of hydrolysis of acetylthiocholine by the crude enzyme was approximately eight times faster than that of butyrylthiocholine (Fig. 26) With the Sephacryl S-300 enzyme fractions the rate was even higher (Fig. 26; Table 8)

Table 6: Kinetic Parameters of the Sephacryl S-300 Fractions of AChE of *O volvulus*

AChE Species	Vmax	Km(mM)
Enzyme Peak 1	2.5×10^{-3}	0.28
Enzyme Peak 2	2.9×10^{-3}	0.22
Enzyme Peak 4	1.9×10^{-3}	0.34

The values of Km and Vmax at pH 8.0 and 25°C were estimated from figure 25. Vmax is expressed in µmole of thiocholine min^{-1}

Table 7: Substrate Specificity of AChE Enzyme Isolated from Adult *O. volvulus*

		Relative Activity (%)			
Substrate	Conc. (mM)	Peak 1	Peak 2	Peak 3	Peak 4
Acetylthio-choline	0.47	100	100	100	100
Butyrylthio-choline	0.47	11.6	11.0	13	12

The reaction was performed in 100 mM Tris-HCl, buffer pH 8.0 containing 50 mM $MgCl_2$. The rate of hydrolysis of acetylthiocholine was taken as 100%. The peaks 1, 2, 3, 4 refer to various AChE species fractionated after Sephacryl S-300 gel chromatography (section 3.2)

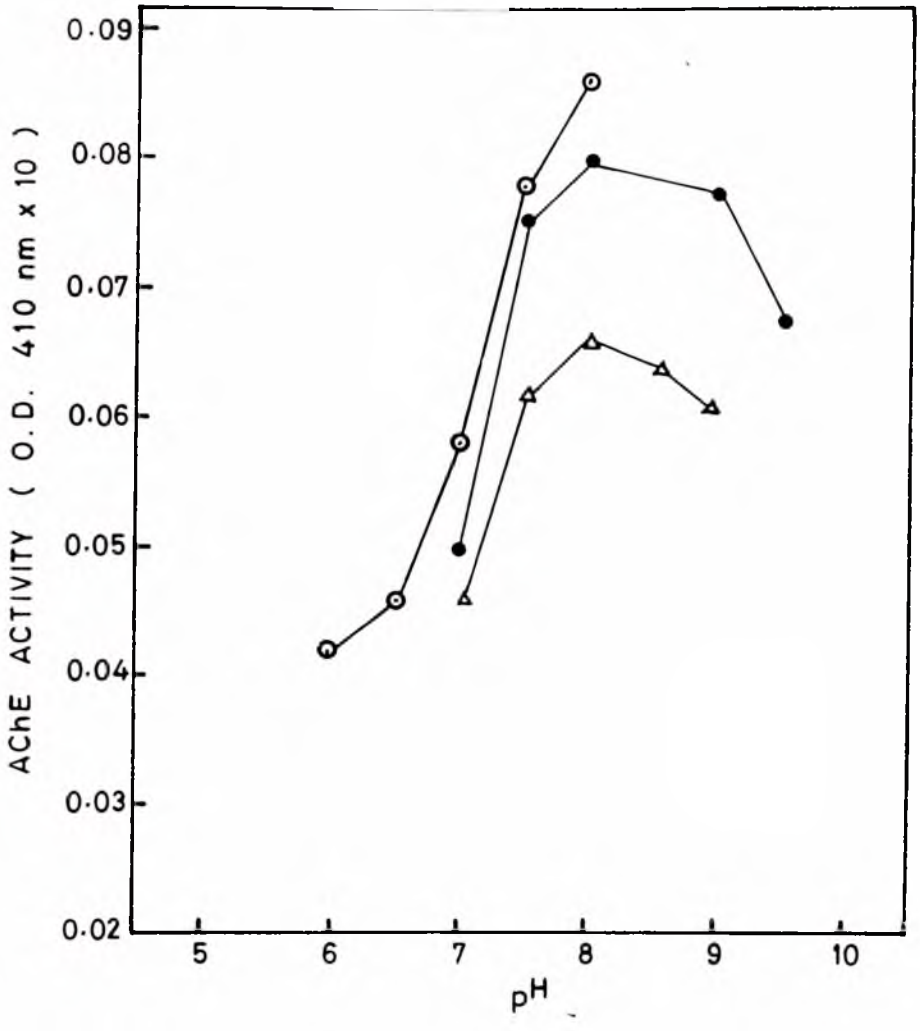


Fig. 23 EFFECT OF BUFFERS ON ACTIVITY OF AChE OF O. VOLVULUS

⊙——⊙ PHOSPHATE BUFFER

●——● VERONAL BUFFER

△——△ TRIS – HCL BUFFER

The 60,000 x g supernatant fraction of the detergent-treated enzyme extract was preincubated in the buffer for 15 minutes, before acetylthiocholine was added. The amount of thiocholine formed was determined as described in the standard assay.

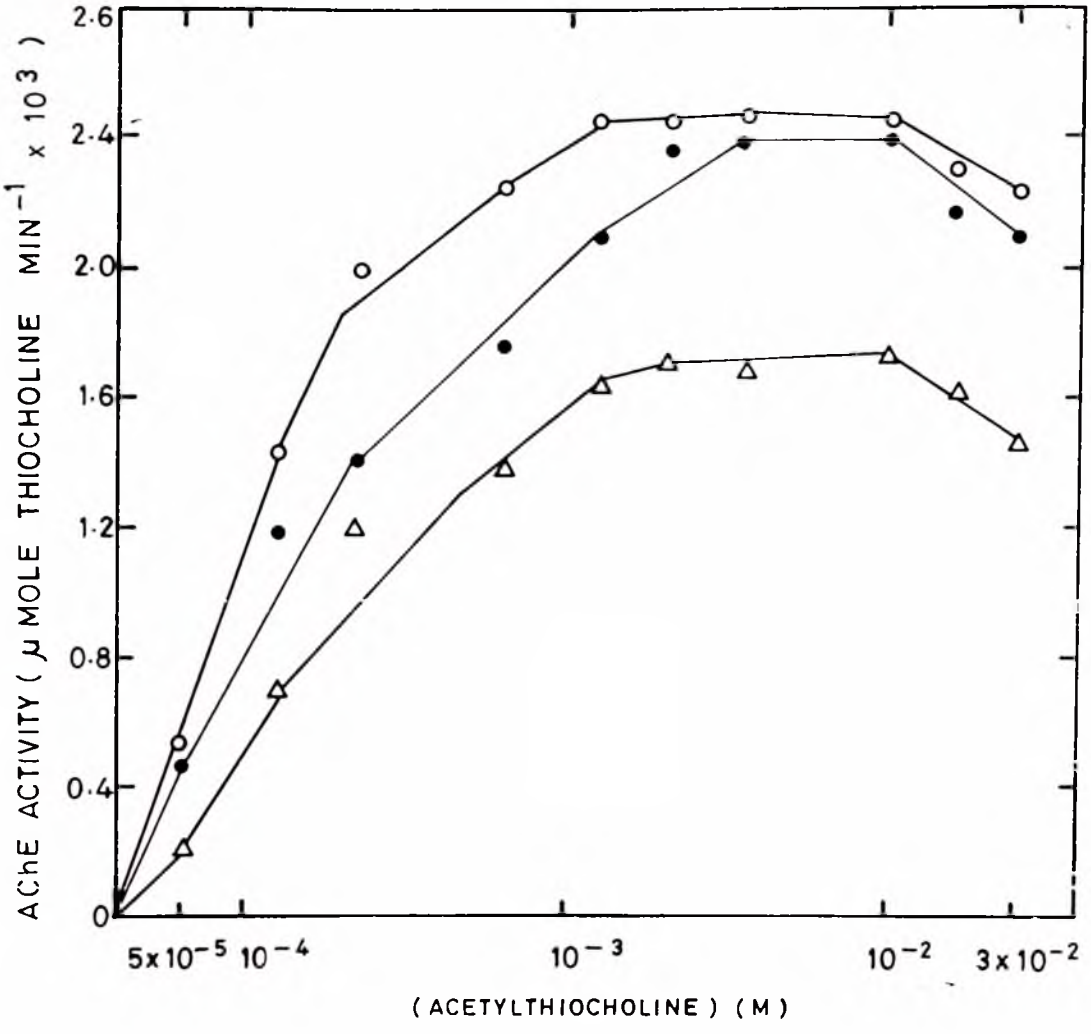


Fig. 24 THE EFFECT OF SUBSTRATE CONCENTRATION ON THE ACTIVITY OF VARIOUS MOLECULAR FORMS OF O. VOLVULUS AChE: (Δ) Peak 4 (●) Peak 1 (o) Peak 2 AChE ACTIVITY WAS DETERMINED BY THE COLORIMETRIC METHOD AT SUBSTRATE CONCENTRATIONS RANGING FROM 5 x 10^{-5} M TO 3 x 10^{-2} M.

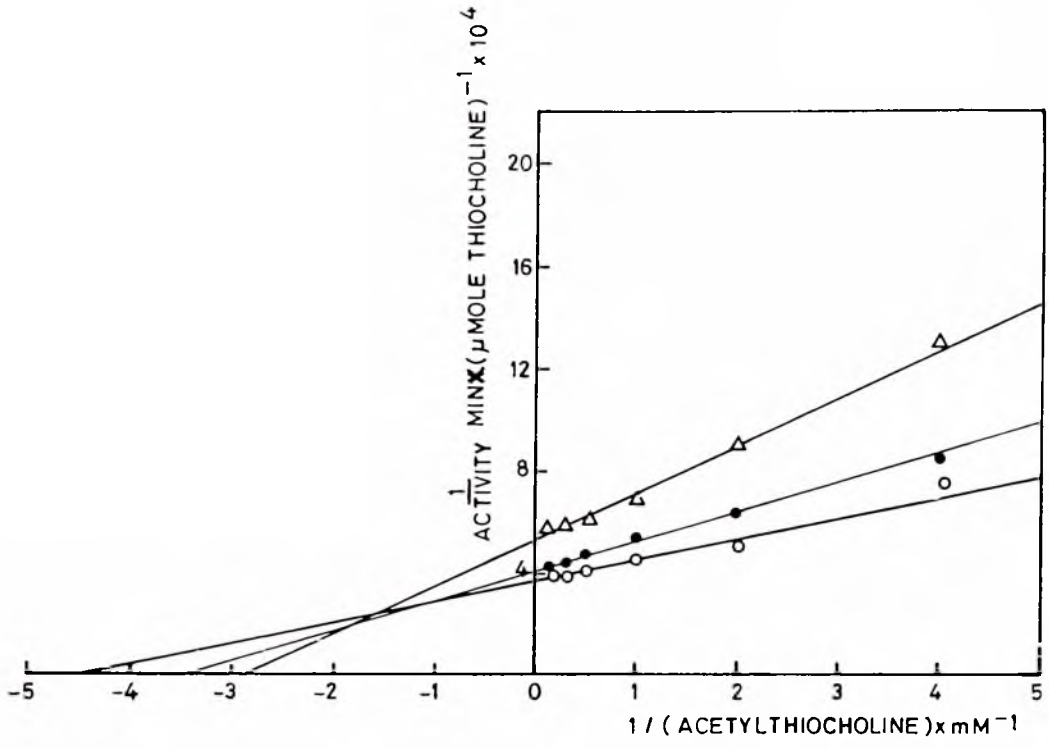


Fig. 25 LINEWEAVER - BURK PLOT OF ACTIVITY AGAINST SUBSTRATE CONCENTRATION .

AChE activity was determined by the colorimetric method at substrate concentrations ranging from 5×10^{-5}M to 3×10^{-2}M.

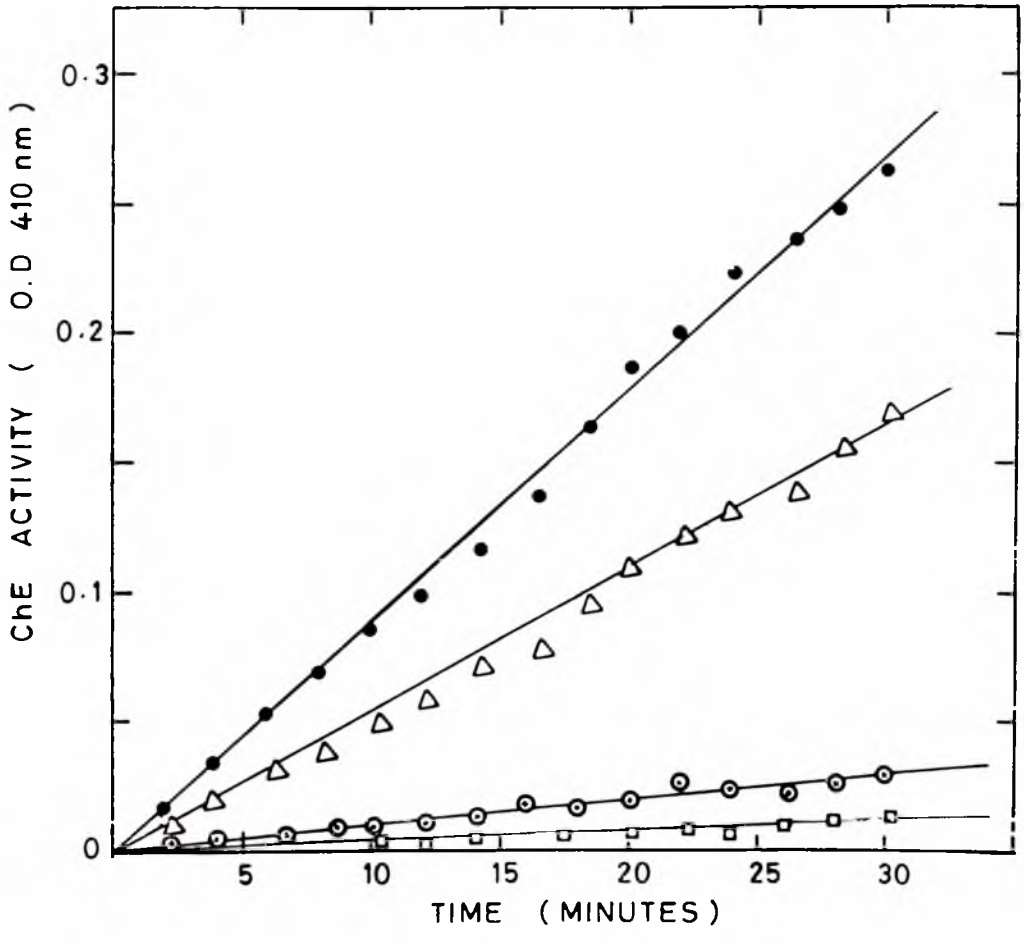


Fig. 26 Substrate Specificity of Adult O. volvulus AChE

Activity of the cholinesterases was determined by the colorimetric method with acetylthiocholine or butyrylthiocholine as substrates.

●——● , crude enzyme + acetylthiocholine

△——△ , purified enzyme + acetylthiocholine

◎——◎ , crude enzyme + butyrylthiocholine

□——□ , purified enzyme + butyrylthiocholine

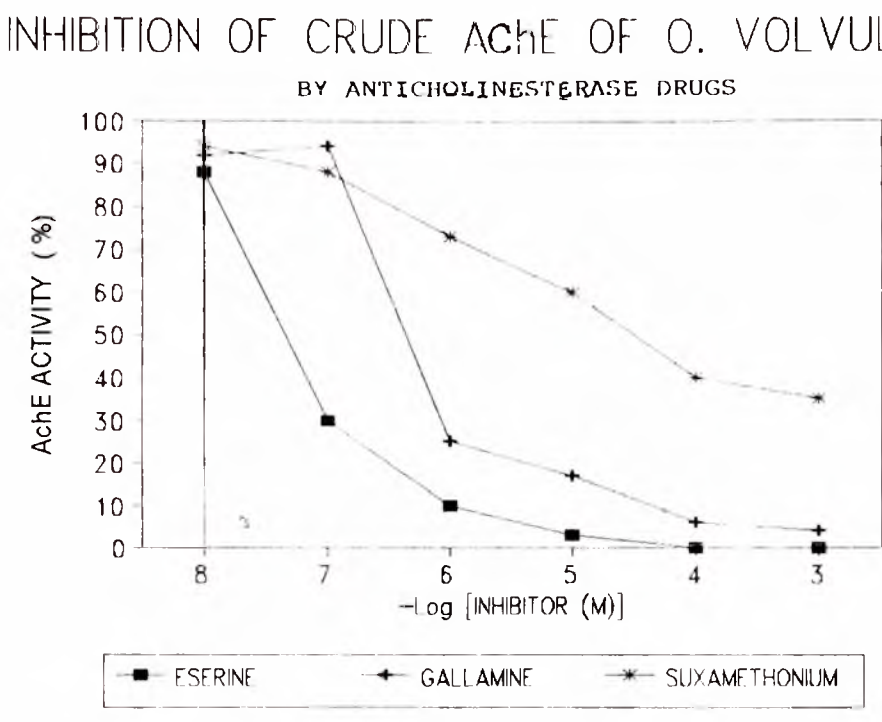
The 60,000 x g supernatant fraction of the Triton-treated enzyme and the Sephacryl S-300 enzyme Peak 2 were used for the study.

3.11 Sensitivity of Acetylcholinesterase of *O. volvulus* to Anticholinesterases

The anticholinesterase drug, eserine is a powerful inhibitor of cholinesterases; it enables these enzymes to be distinguished from other esterases. Figure 27 shows that the 60,000 x g supernatant enzyme fraction of solubilized extract of *O. volvulus* was almost completely inhibited by 10^{-5} M eserine. Gallamine produced remarkable inhibition, virtually inhibiting the enzyme at 10^{-3} M, whereas suxamethonium exhibited mild inhibition. The Sephacryl S-300 enzyme fractions were also totally inhibited by 10^{-5} M eserine (Fig. 28). Molar concentrations of these drugs that gave 50% inhibition over the control (i.e no inhibitor), IC_{50}, are shown in Table 8.

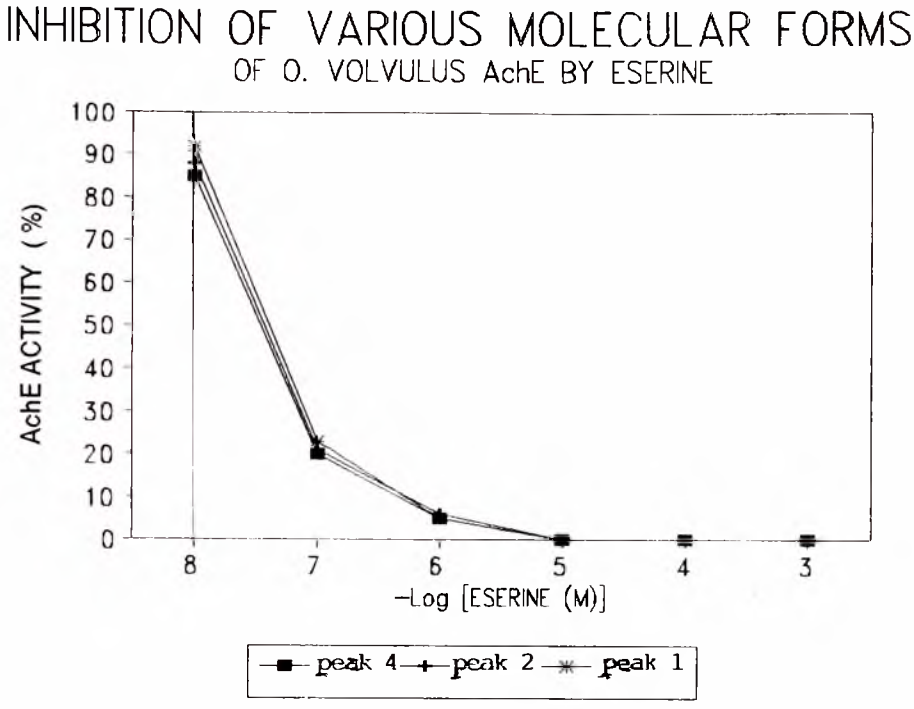
The inhibitory effect of eserine on crude cholinesterase activity was also tested, using butyrylthiocholine as substrate (Fig. 29) Results comparable with those of acetylthiocholine (Fig. 27) were observed. However, the IC_{50} value was 9.0×10^{-8} M, twice higher than that in the presence of acetylthiocholine, 5.5×10^{-8} M.

FIG. 27



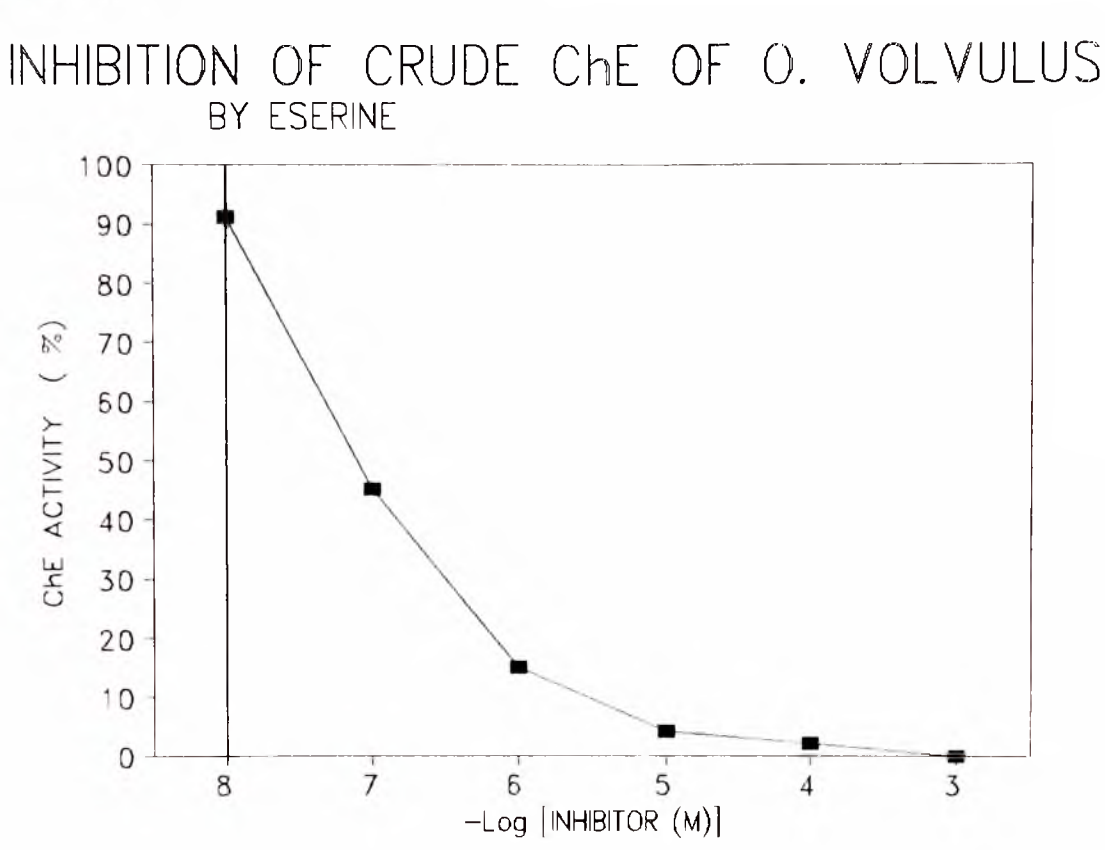
The crude 60,000 x g supernatant fraction of the solubilized enzyme was preincubated with each inibitor for 20 minutes. Subsequently, AChE activity was determined as detailed in materials and method.

FIG. 28



The Sephacryl S-300 enzymes were preincubated with the inhibitor for 20 minutes. The residual enzyme activity was determined as described in materials and method.

FIG. 29



The crude 60,000 x g supernatant fraction of the solubilized enzyme was preincubated with the inhibitor for 20 minutes. ChE activity was determined as described in materials and method.

Table 8: IC_{50} Values of Inhibitors on Acetylcholinesterase from Adult *O volvulus*

Inhibitor	(IC_{50} Values) (M)
Gallamine	5.6×10^{-7}
Eserine	5.5×10^{-8}
Suxamethonium	5.0×10^{-5}

The values show the IC_{50}, namely molar concentration of the drug that gave 50% inhibition relative to control (no inhibitor). The values were estimated from Figure 27 Final acetylthiocholine concentration in the assay mixture was 0.47 mM. The 60,000 x g supernatant fraction of the detergent buffer AChE extract was used in this experiment.

3.12 Effect of Metal Ions on Crude and Purified AChE of Adult *O. volvulus*

The effect of four divalent metal ions namely Ca^{2+}, Mg^{2+}, Co^{2+} and Mn^{2+} on the activity of the onchocercal AChE was investigated. Table 9 shows that all the metal ions exhibited some activating effect in the crude enzyme fraction, Mg^{2+} being the most effective. Mn^{2+} and Ca^{2+} produced moderate activating effect while Co^{2+} slightly lowered the enzyme activity at 0.01 M.

As a result of its pronounced stimulatory effect on the crude 60,000 x g supernatant fraction of the detergent-treated enzyme, the effect of Mg^{2+} on the Sephacryl S-300 enzyme extracts was studied Mg^{2+} also activated the purified AChE enzymes (Table 10). The magnitude of the activation on the various enzyme forms was comparable. In comparison with the 60,000 x g supernatant enzyme preparation, the activating effect of Mg^{2+} at 10^{-2} M on the purified enzymes was slightly lower

Table 9: Effect of Metal Ions on Dialysed Crude AChE of *O volvulus*

	Relative Activity (%)					
Cation Concentration (M)	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
Ca^{2+}	100	104	117	117	125	109
Mg^{2+}	100	114	110	169	174	145
Co^{2+}	100	91	127	116	85	ND
Mn^{2+}	100	106	123	120	114	128

The exhaustively dialysed 60,000 x g supernatant fraction of the detergent-treated AChE was preincubated in the presence of the metal ions for 15 minutes. This was followed by the addition of 0.47 mM acetylthiocholine iodide. Subsequent treatment is as described in the enzyme assay (section 2.2.1.1) The results are given as the activity (%), relative to that of the control incubation (i.e no metal ion present)

ND = Not determined.

Table 10: Activating Effect of Mg^{2+} on Purified AChE Species

	Relative Activity (%)		
Sephacryl S-300 AChE Fraction	0	10^{-2} M	5 x 10^{-2} M
Peak 1	100	147	161
Peak 2	100	145	154
Peak 3	100	150	163
Peak 4	100	144	157

The Sephacryl S-300 AChE fractions were each preincubated in the presence of the metal ion (Mg^{2+}) for 15 minutes. Similar treatment was followed as in Table 9. The results are expressed as detailed in Table 9.

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1 General Discussion

Acetylcholine is especially required for neuromuscular transmission (9 - 11, 102). The compound is present in large amounts in the nervous system of filarial worms (8). In these worms, acetylcholine is an excitatory neurotransmitter (11), whereas in some other helminths, it is an inhibitory neurotransmitter (9, 10). Once acetylcholine has initiated its desired response, it must be removed to allow for the next stimulus, or to prevent repeated and uncontrolled responses after a single stimulus. Cholinesterases hydrolyse acetylcholine and therefore remove its action from the synapses (102) These enzymes might be important in controlling motility of filarial worms.

Two types of cholinesterases are recognized, namely: acetylcholinesterase (AChE) and pseudocholinesterase, also called butyrylcholinesterase or cholinesterase (17). AChE is the predominant acetylcholine-decomposing enzyme of the nervous tissue, and erythrocytes of most species, whereas pseudocholinesterase resides chiefly in blood plasma.

In this study, attempts were made to purify and characterise AChE from adult *O. volvulus*.

For the majority of parasite species, enzyme assays and biochemical analysis have to be performed on homogenates and extract of whole organisms. This means that differences

between various tissues of the parasite are obscured. A low enzyme activity is normally encounted. This may be due to a low activity throughout the organism or may represent a high activity in some tissues and no activity in others (130).

In the present study, AChE activity in the partially purified enzyme extract(s) was relatively low This was compounded by the inability of the assay system employed (colorimetric) to detect relatively low concentrations of the enzyme activity This meant that relatively large volume of enzyme extract had to be used. As a result, some aspects of the work, especially the preliminary investigations, were made with the crude enzyme fraction, with the definitive ones being done with the purified AChE extract(s)

Several reports have indicated that AChE is a membrane bound enzyme (67 - 69). In *S. mansoni*, the enzyme was released from the membrane when Triton X-100 was incorporated into the extraction medium (69) The results from this study further support this assertion (Table 3) There was approximately two-fold increase in activity with the Triton X-100-contained enzyme extract. The association of the enzyme with the membrane might involve both hydrophobic and electrostatic forces. This can be deduced from the solubilization achieved with Triton X-100, a non-ionic detergent and also to a lesser degree by NaCl (Table 3). Triton X-100 will disrupt the non-polar forces whereas NaCl will break the electrostatic attraction

AChE extracted in the detergent buffer and the low-salt buffer was subjected to gel chromatography on Sephacryl S-300 column. As is evident from Table 4, A and B, the onchocercal AChE was not purified to a high degree. The yield also was relatively low The relatively low degree of purity achieved is not unexpected for a one-step purification with gel-filtration. This fractionation technique separates molecules based largely on their molecular size. Molecules with different enzyme activity but of roughly similar size will be eluted at the same time. This can result in poor resolution. The low degree of purity can be improved by combination of several fractionation techniques. Combination of separation methods such as ammonium sulphate fractionation, followed by ion-exchange and gel-filtration have been used successfully to purify AChE to a higher degree (16, 53, 85) In view of the relatively low activity of the onchocercal AChE in the worm, the use of these techniques to purify the enzyme may result in very low yield and even further cause enzyme dilution. The problem of low yield and further dilution of the onchocercal enzyme can be surmounted by employing affinity chromatography This technique exploits only the biological property of the molecules being separated. It can therefore handle low concentrations of solutions. Affinity chromatography has been used to purify AChE with incredible achievement both in terms of purity and yield (54, 81, 84). However due to lack of affinity adsorbent, it was not possible to use the separation technique to purify the onchocercal AChE. The partially

purified enzyme extract was then characterised to some extent.

With most enzymes, repeated freezing and thawing causes inactivation. AChE, however, is not significantly affected by these conditions (59, 69) The *O. volvulus* AChE is not very different from AChE of other species. Prolonged storage of the extract of *O. volvulus* AChE at -71°C and frequent thawing, did not produce appreciable loss of the enzyme activity (Table 5). Probably the secondary and tertiary conformations of the enzyme were not adversely distorted.

In contrast, AChE from *O. volvulus* appears to be unstable at relatively high temperatures (Fig. 22, A and B) This instability may be attributed to denaturation of the enzyme protein, although other factors such as destruction of some thermolabile component(s) which tend to stabilize the secondary and tertiary conformation of the enzyme cannot be ruled out. The sensitivity to inhibition at relatively high temperatures is not exclusive to the parasite enzyme; mammalian AChE is similarly unstable at these temperatures (52) Maximal activity at 37°C has been reported for the mammalian erythrocyte AChE (70) This value does not deviate much from the 38°C observed for the onchocercal enzyme (Fig. 22, A) Temperature effect on enzyme-catalysed reactions is time dependent. However, the time-dependency can be minimised when initial rate is measured.

In addition to temperature, the pH of the medium had effect on the onchocercal AChE activity. AChE from many

vertebrate sources is reported to be maximally active with a broad pH range - 7.5-9.0 (52) The pH profile observed for the *O. volvulus* AChE falls within this range (Fig. 23) The pH profile obtained can be employed to determine the amino acid residues present at the active site by using the relationship existing between pH and Vmax and pH and Km of an enzyme catalysed reaction (61) The former can be used to obtain the numerical value of the pka(s) of the amino acid(s) involved in the enzyme substrate complex which affect the activity while the latter gives amino acid residue(s) important for the affinity of the enzyme. In view of the relatively large quantity of enzyme extract likely to be used for these experiment(s), and judging from the low amount of AChE extact handled in this study, it was not favourable to employ these relationships to determine the pka(s) for the onchocercal AChE.

The optimum pH is in the region where α-amino groups are titrated. These amino acid residues may be implicated in the catalysis of the onchocercal enzyme. The titrated amino acid residues may also be due to either acidic groups or basic groups whose pKa(s) decrease(s) or increase(s) as a result of interaction with other ionizable groups in the protein.

The buffers employed in the study also affected AChE activity The apparent activation of the *O. volvulus* AChE by phosphate and Veronal buffers might be due to the peripheral site activation of the enzyme These buffers contain appreciable amount of Na^+ ion which could form a ternary

complex with the substrate and the peripheral anionic site of the enzyme. The enzyme might be activated as a result. Formation of a ternary complex by Na^+ ion, substrate and AChE from the electric eel has been reported (53)

The apparent existence of multiple molecular forms of AChE, using gel-filtration on Sephacryl S-300 (Figs. 15 and 16), sucrose gradient centrifugation (Fig. 11) and PAGE under non-denaturing conditions (Figs. 17 and 18), has been demonstrated for the onchocercal enzyme. The existence of distinguishable forms of AChE is not unique to the *O. volvulus* AChE. Several investigations, both in vertebrates and invertebrates, have confirmed the existence of AChE in multiple molecular species (16, 17, 53, 58, 62, 69). This indicates that the phenonmenon of multiple molecular forms is probably a ubiquitous property of this enzyme. The results from present study further show that these molecular forms differ from each other in their compartmentalization of enzyme activity as well as in their molecular size (Figs. 11, 15 and 16). Some molecular forms were extracted by low-ionic strength buffer, whilst other forms were solubilized by the addition of a detergent. In addition, higher molecular-sized onchocercal AChE species were obtained in low-salt buffer extraction medium (Fig. 11) These findings are in agreement with previous reports on *S. mansoni* AChE (69) and the mammalian brain and erthrocyte AChEs (53).

The type of cholinesterase in adult *O volvulus* is not certain yet. However from Figure 26, it appears that the

predominant acetylcholine-decomposing enzyme in adult *O. volvulus* is true AChE. Both the crude and the purified enzyme preparations showed preferential hydrolysis of acetylthiocholine, compared to butyrylthiocholine The crude enzyme hydrolysed acetylthiocholine approximatley eight times faster than it hydrolysed butyrylthiocholine (Fig. 26) The rate was even higher for the purified enzymes (Table 7) Ratios of seven and eight have been reported for the trematode, *S. mansoni* (54) and the nematode *S. dentatus* (55) respectively In some parasites, however, the rate of hydrolysis of acetylthiocholine is not quite different from that of butyrylthiocholine (56, 57) The Km found for the various molecular species of the onchocercal AChE (Fig. 25) was approximately ten times less than the values reported for the *S. mansoni* AChE (15, 54), and also roughly ten times higher than that of *C. elegans* AChE (58). However, the Km for the *O. volvulus* AChE is not very different from the Km reported for the electric eel AChE (59) and the fetal bovine serum AChE (66) Thus, while the onchocercal enzyme has higher affinity for acetylthiocholine compared to the *S. mansoni* AChE, it is lower with respect to the *C. elegans* AChE. Both the vertebrate AChE and the *O. volvulus* AChE appear to have roughly the same affinity for the substrate In addition, the various molecular species of the onchocercal AChE have approximately the same affinity for acetylthiocholine. The Km values were roughly the same (Table 6). Similar trend has been observed for the electric eel AChE (74).

Substrate inhibition further distinguishes AChE from pseudocholinesterase (60). Acetylthiocholine concentrations greater than 1 x 10^{-2} M were inhibitory to the onchocercal AChE (Fig. 24). Substrate inhibition of AChE has also been reported in other parasites (54 - 56) It has been proposed that substrate inhibition of AChE is a result of competition for the acylated enzyme by excess substrate molecules (61) Excess substrate molecule(s) may bind to the acylated enzyme and retard the deacylation process which is the rate-limiting step of the catalysis (71) This might account for the inhibition of the *O. vovlulus* AChE by high concentration of acetylthiocholine.

The apparent molecular weights of the onchocercal AChE species as estimated from gel-filtration on Sephacryl S-300 (Figs. 15 and 16) and by PAGE, under non-denaturing conditions, and staining specifically for AChE activity (Figs. 17 and 18) are within the range of values obtained for molecular forms of AChE isolated from other species (19, 53, 54, 69) The 400 KD found for the *O. volvulus* AChE Peak 4 (Fig. 15) resembles the molecular weight of 400 KD determined for AChE species from *N. americanus* on Sepharose 4B (106) A molecular weight of 450 KD has also been reported for the *S. mansoni* AChE on Sephacryl S-300 (69) The 500 KD revealed by PAGE under non-denaturing conditions in this study (Fig. 18) also resembles the 500 KD reported for the *S. mansoni* AChE on PAGE (54) A molecular weight of between 60-110 KD has been reported for the monomeric AChE from several species (53)

The higher molecular weight forms observed in this study (Figs. 15, 16, 17 and 18) are most probably an aggregate of several subunits and might contain collagenous tail as has been demonstrated for AChE of other species (17, 53, 69, 73). Discrepancies in the molecular weight revealed by the two fractionation techniques (Figs. 15 and 16, ; 17 and 18) for the same AChE extract could be attributed to some of the factors which govern these separation methods, which in turn affected their resolution (131). Molecules will migrate through gradients of polyacrylamide gels until the pore size becomes too small for them to move any further. Under these conditions the distance migrated depends on the size and shape of the molecules and not on its charge. Separation is therefore based largely on molecular size for both methods. However, gel-filtration is mostly affected by gel-flexibility, diffusion and adsorption to the gel material by compounds being separated. Especially adsorption becomes important when the separating gel contains extensive aliphatic chains, as has been found for Sephacryl (131). These factors retard effective resolution (131). Secondly, enzyme forms with different molecular size but roughly similar shapes will have approximately the same elution volume. This is especially the case for highly asymmetric molecules, like the eletric eel AChE (77). Proteolytic degradation of the onchocercal enzyme could not have brought about differences in the number of molecular weight forms since the extract(s) used were stored under similar conditions.

Although AChE is not a metalloprotein, certain divalent metal ions exert profound activating effect on the enzyme activity (87 - 89). AChE from electric organ of *Torpedo* is reported to be activated by divalent metal ions with Mn^{++} being the most activating (89). In the present

investigation, *O. volvulus* AChE was activated by Mg^{++} Mn^{++} and Ca^{++}, with Mg^{++} being the most activating (Table 9). However, Co^{++} appeared to cause enzyme inhibition at relatively high concentrations (Table 9). This is not very uncommon to the esterases. The inhibition of esterase activity of carboxy-peptidases by Co^{++} ion has been reported (101).

Three mechanisms have been put forward to account for the metal ion activation of enzymes (96). These are:

(i) Formation of a ternary complex,

(ii) Induction of favourable stereospecific conformation in the enzyme, and

(iii) Polarization effect due to interaction of the substrate with the metal ion.

These mechanisms which are not independent of each other could explain the activation effect observed with Mg^{++}, Mn^{++} and Ca^{++} ions on *O. volvulus* AChE. Co^{++} ion has a large association constant (98). Thus in addition to binding to the peripheral anionic site, Co^{++} might as well compete with the substrate at the catalytic site of the enzyme and cause inhibition.

AChE from *O. volvulus* exhibited immense sensitivity to inhibition by eserine (Figs. 27 and 28), the inhibition being comparable to those of mammalian erythrocyte AChE (52) and AChE from *S. mansoni* (54). Eserine forms at the active site a stable carbomoyl-enzyme complex which is not easily hydrolysed (101-102) while complexes formed by other anticholinesterases

can be hydrolysed at a relatively fast rate (102). This might account for the mild inhibition observed with suxamethonium (Fig. 27). Inhibitors like gallamine triethiodide exert biphasic response on AChE activity (53). The trend observed in the study appears to agree with this fact. The results (Fig. 27) showed relative insensitivity of the enzyme to the inhibitor at concentrations $\leq 10^{-7}$ M, while profound inhibition was observed thereafter.

4.2 Acetylcholinesterase As Possible Target in Control of Onchocerciasis

One approach to developing an approriate method to combat diseases caused by parasites is to study the biochemistry and/or physiology of the parasite and the host with a view to identifying parasite specific component(s) as target(s) for therapeutic intervention. Some of these components could be useful as probes for diagnostic purposes.

Metabolism associated with proliferative processes such as nucleic acid, protein and membrane synthesis are potentially suitable targets for therapeutic intervention. With helminths, such processes occur in specialised tissues. Inhibiting such processes may not affect the disease process as the parasite is not eliminated; the life cycle may, however, be interrupted. Potential targets associated with rapid growth and multiplication in helminths may therefore be less useful therapeutically. Suitable targets are likely to be muscular activity and neuromuscular coordination (11) Thus, enzymes which are involved in the control of

neuromuscular activity in these worms, like acetylcholinesterase, could be important areas of study.

In the present investigation, it has been found that the onchocercal AChE, in some respects, resembles the vertebrate AChE and AChE of other nematodes in its catalytic properties. Substrate specificity and sensitivity to inhibition by anticholinesterases together with the effects of pH and temperature on the onchocercal enzyme were not quite different from those of vertebrate AChE and AChE from other invertebrates.

In spite of the general inhibitory trend observed in this study with the anticholinesterases, it is possible that a more diligent investigation might reveal certain differences which could be of importance therapeutically. This is because it is known that helminth nervous system is anatomically different from that of the mammalian nervous system (11). This anatomical difference might explain the antiparasitic action of the drug, piperazine on nematodes (11). The compound interferes specifically with nematode nervous system by hyperpolarising the muscle membrane, thus preventing deplorisation and causing flaccid paralysis. The drug was later found to act directly as an agonist of GABA which is an inhibitory neurotransmitter in these worms (11). The GABA-ergic nerves in the host are protected by the blood-brain barrier in the central nervous system. The host nervous system is therefore not adversely affected.

In another example, the antiparasitic drug metrifonate inhibits specifically AChE of certain helminths (11, 121) The host AChE is again not adversely affected.

Other studies have revealed that AChE from certain helminths is an important antigen (19, 20, 118, 119) For example, antibodies raised in rabbits against the AChE of Schistosomula of *S. mansoni*, were found to cause complement-dependent cytotoxicity towards the intact worm (20). Moreover, AChE from certain nematodes is recognised as a specific antigen and has been implicated as possible target in the host immune response (20, 118, 119). It would be interesting to investigate the immunogenicity of the onchocercal AChE. If antibodies to the onchocercal enzyme should exhibit little or no cross-reactivity with the host AChE, the results could be of importance for possible vaccine production as well as the development of immunological probes for diagnostic purposes.

Also worth investigating is the carbohydrate nature of the *O. volvulus* AChE. Acetylcholinesterases are known to be glycoprotein (62 - 64). The carbohydrate portion differs for AChE from different species (65, 66). The carbohydrate moiety is thought to play a crucial role in the biology of the enzyme (17, 53, 69) AChE is a membrane-bound enzyme (67, 68) and in *S. mansoni* AChE, the attachment of the enzyme to the membrane involves the carbohydrate portion (69) Moreover, AChE from the electric organ of *Torpedo* is recognized by an anti-carbohydrate antibody (17). Hence, it is possible that study

of the carbohydrate moiety of the onchocercal AChE might provide suitable targets for interference by selective agents.

In conclusion, AChE from adult *O. volvulus* has been isolated, partially purified and characterised. Sephacryl S-300 fractionation showed two enzyme peaks each for the two media used to extract the enzyme. Moreover, these enzyme forms were characteristic of the extraction media employed. The enzyme was characterised as "true" AChE since it showed a marked preference for acetylthiocholine compared to butyrylthiocholine and since it was inhibited by high concentration of the former substrate In addition, it was inhibited by the general cholinesterases inhibitor, eserine

The results of the current study show that there are similarities between the onchocercal AChE and the isofunctional mammalian enzyme. This seems to suggest that the *O. volvulus* enzyme is unlikely to be an exploitable target in the therapy of onchocerciasis. However, in other helminths where a similar relationship has been found to exist, it has still been possible to exploit the parasite AChE in control measures. It is therefore possible that with diligent studies, unique features of the onchocercal AChE might be found that could be exploited as a target in onchocerciasis control measures.

Nevertheless, the results do increase our knowledge of the biochemistry and the neurophysiology of the *O volvulus*.

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