STUDIES OF THE PHARMACODYNAMICS AND MODES OF ACTION OF ANTHELMINTIC DRUGS

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The aim of this work is to extend existing knowledge both with respect to the mode of action of anthelmintics and the biochemical and physiological mechanisms which may be disrupted by drug action. The helminth species examined include nematodes, *Ascaris suum, Ascaridia galli* and *Trichuris ovis* and cestodes, *Moniezia, T. hydatigena, T. taeniaeformis* and *Echinoccocus granulosus;* the anthelmintics studied were methyridine, diethylcarbamazine, pyrantel, morantel, tetramisole, levamisole, dichlorvos, vincofos, cambendazole and mebendazole. The helminth characteristics selected for most intensive study are (a) the occurrence and properties of helminth cholinesterase and (b) the uptake of glucose. The breadth of the study was limited by the availability of fresh material and not all combinations of helminth and drug were investigated.

The histochemical localisation of cholinesterase activity in whole mounts and sections of tapeworms using thiocholine esters revealed a complex network of tegumental receptors feeding a nervous system with efferents to suckers, rostellum and hook muscles. It is suggested that tapeworms have reflex arcs involving these structures allowing them to maintain their position in the host intestine in spite of peristaltic action. These arcs are susceptible to anticholinesterase anthelmintics. Other cholinesterase activity is associated with the scolex, cirrus, genital pore and sometimes the tegument.

High cholinesterase specific activities against acetylthiocholine were measured in *Echinococcus* scoleces and tapeworms, but lower levels in nematodes. Differential centrifugation of homogenates was used to

study their occurrence in the tissue and facilitate further characterisation. However, the enzyme was widely distributed in these species although somewhat higher in the particulate fractions. Activity was increased little, if any, by attempts to solubilise it with the detergent, Triton X-100. Cholinesterase in some fractions particularly from *T. ovis*, had a high temperature optimum around 60°C, but never showed the phenomenon of autoinhibition by substrate at concentrations up to 10^{-2}M. Cholinesterase in species of worm with high levels of enzyme was more sensitive to eserine inhibition than those with lower levels.

In studies of glucose uptake from the medium by *Ascaris* and two tapeworms, it was confirmed that transport into *Ascaris* was strongly inhibited by certain benzimidazole anthelmintics. Transport into *Ascaris*, but not the cestodes, was also discovered to be sensitive to local anaesthetics such as procaine or lignocaine. Uptake into tapeworms was inhibited by the absence of sodium ions, phlorizin, iodoacetate and dinitrophenol. It was less inhibited by benzimidazoles and not at all by organophosphate anthelmintics, but was sensitive to phenolic drugs such as hexachlorophene and nitroxynil.

In the dog and sheep, a number of anthelmintic drugs administered intravenously showed predominantly nicotinic effects on blood pressure and respiration supporting the cholinergic action of these drugs. Although sheep red-cell cholinesterase is more sensitive to inhibition than that of all helminths tested, the oral route of administration of anthelmintics remains safe for the host and effective against intestinal parasitic worms.

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CHAPTER 1 THE SIGNIFICANCE OF HELMINTH INFECTIONS AND THEIR CONTROL

1.1

1. THE SIGNIFICANCE OF HELMINTH INFECTIONS AND THEIR CONTROL

1.1 THE GLOBAL SIGNIFICANCE OF HELMINTH DISEASE

Because of the need for brevity only a few generalisations can be made concerning the overall relation of helminth disease to world health and livestock production.

In man, the major problems are undoubtedly located in tropical and sub-tropical areas where climatic, ecological, sociological and other factors combine to favour persistance and spread of parasite helminths, and in large areas of the world almost all the indigenous populations may be infected with such serious and debilitating conditions as bilharziasis (schistosomiasis) and hookworm disease (Wilson and Schild, 1959). The topic has been dealt with dramatically in the classic treatise 'This Wormy World' (Stoll, 1947). Some relevant data are given in Table 1.1

TABLE 1.1. SHOWING THE EXTENT OF SOME HELMINTH INFECTION IN MAN *

Infections	Number of infections (x 10^6)	References
Total number of infections	2250	Stoll (1947)
Number of people infected	800	Ditto
Ascaris lumbricoides	644	Ditto
Hookworm	456.8	Ditto
Trichuris trichiura	355	Ditto
Enterobius vermicularis	208.8	Ditto
Schistosoma	114	Ditto
Filariae	304	Ditto
Schistosoma japonicum (China)	33	Slack & Nineham(1968)
S. haematobium and *S. Mansoni* (Egypt)	12	Ditto
S. haematobium (West and Central AFrica)	8.5	Ditto
S. Mansoni (Brazil)	4.0	Ditto

* Total world population 2166 x 10^{-6}

In contrast to man, the main helminth problems associated with livestock production appear in temperate regions and are related particularly to development of highly stocked grasslands and intensive poultry and pig production units. The global economic loss arising from mortalities, inhibited production and associated with development and application of control measures, is a matter for conjecture - but must easily amount to billions of dollars annually.

1.2 CHEMOTHERAPY AND ANTHELMINTICS

As in the case of infection by all small or microscopic pathogenic organisms, the main line of therapeutic defence lies in utilising the selective toxicity of suitable drugs against the pathogen.

The term selective toxicity was introduced by Albert (1977) to embrace the use of chemical substances to destroy or inhibit an unwanted species (the uneconomic species) while, at the same time, causing little or no harm to a closely associated desirable species (the economic species).

In medicine, the economic species may be man or some other desired animal - whereas the uneconomic species may be a virus, bacterium, fungus, protozoan, helminth parasite or neoplastic cell. In this context the use of drugs with the object of causing maximal injury to the pathogen or neoplasm and minimal harm to the host is described as chemotherapy. Anthelmintics are drugs used for the chemotherapy of helminth infections and their role in disease control can scarcely be overestimated.

1.3. HELMINTH CONTROL BY DRUGS

Drugs play many roles in parasite control and the topic has been recently comprehensively reviewed by Campbell (1977). The most dramatic illustrations relate to protozoa. As examples, it is now regarded virtually axiomatic that an effective coccidiostat is indispensable for the successful intensive production of chickens; the role of antiplasmodial in the chemoprophylaxis of malaria (6×10^6 cases and $2 - 3 \times 10^6$ deaths annually) is widely recognised; visceral

leishmaniasis was regarded 95 per cent fatal before the introduction of antimonial drugs and about 95 per cent non-fatal afterwards (Campbell, 1977). With respect to nematodes, mass treatment with piperazine in ascariasis control programmes by a number of countries including Japan, Taiwan and Korea have resulted in a dramatic fall in prevalence, for example, in Japan from 43 per cent in 1953 to 5 per cent in 1964 (W.H.O., 1965). In filarial control, the use of diethylcarbamazine for reducing the reservoir of microfilariae in human populations, and thus preventing vector transmission is showing considerable promise of success (Wilson, 1968; Sasa, 1968; Desowitz, 1971).

1.4 DEVELOPMENT OF NEW ANTHELMINTICS

Screening techniques using the natural helminth flora of small laboratory animals such as rats and mice for testing large numbers of candidate compounds have been comprehensively reviewed by Standen (1963). Prior to the development of such screen, the discovery of new anthelmintics was largely empirical and few satisfactory anthelmintics existed. Since the mid 1950's however, the wide scale adoption of the laboratory screening technique has resulted in ever increasing flow of effective anthelmintic drugs. Concurrent with these developments, we have seen quickening interest in the basic mode of action of these substances with a view both to establishing a more rational and precise means of development of new compounds and in addition, to learning more of the basic physiological mechanisms which may be effected by the drug. The last named aspect follows the classical goal of general pharmacology to develop the use of the drugs as biological research tools.

1.5 SIGNIFICANCE OF THE PRESENT RESEARCH

The present thesis is concerned with mode of action with respect to the pharmacodynamics of anthelmintics themselves and the effects on glucose uptake and cholinesterase (both biochemically and histochemically) in a variety of parasitic helminths.

1.5

The overall goal is thus to increase the scope of existing knowledge to more species of helminths and more anthelmintic drugs, thus increasing our understanding of the basic actions of this important class of drugs on the organisms they are selected to affect.

CHAPTER 2 MODE OF ACTION OF ANTHELMINTICS

2.6 HETEROCYCLIC COMPOUNDS, BEPHENIUM AND THENIUM

(a) Methyridine

(b) Piperazine (diethylenediamine)

(c) Diethylcarbamazine

(d) Bephenium and thenium

2.7 BISPHENOLS

(a) Hexachlorophone

(b) Dichlorophen

(c) Bithionol

(d) Bithionol sulphoxide

2.8 SALICYLANILIDES

(a) Niclosamide

(b) Oxyclozanide

(c) Clioxanide

2.9 PHENOTHIAZINE AND XANTHONES

(a) Phenothiazine

(b) Xanthones

2.10 BENZIMIDAZOLES

(a) Thiabendazole

(b) Cambendazole

(c) Menbendazole

(d) Fenbendazole

(e) Oxybendazole

2.11 IMIDAZOTHIAZOLE DERIVATIVES

(a) Tetramisole

2.12 CYCLIC AMIDINES

2.0

CHAPTER 2 MODE OF ACTION OF ANTHELMINTICS

2.0 INTRODUCTORY REMARKS

Many thousands of anthelmintics have been developed and tested at the clinical level and although the chief advances have occurred only during the last two decades, a considerable body of information has accumulated concerning their mode of action. In the present review, an attempt has been made to summarise most of the relevant material and the plan has been adopted by which the anthelmintics are dealt with by grouping. The arrangement is largely on the basis of chemical similarities, although in some cases (such as the naturally occurring alkaloids) it is based on a similarity of biological actions. Again with the exception of natural compounds, the groups are dealt with in order of increasing complexity in chemical strucutre (see index previous page) beginning with metals and other ions and concluding with complex multi-ring structures as the benzimidazoles and imidazothiazoles.

The topic is receiving increasing attention in the literature and there are numbers of excellent modern reviews (Brown, 1961; Mansour, 1964; Keeling, 1968; Lämmler, 1968; Gibson, 1969; Sanderson, 1970; Mc Farland, 1972; Sanderson, 1973; Pappas and Read, 1975; Taylor, 1975; Campbell, 1977).

2.1 NATURALLY OCCURRING CYCLIC COMPOUNDS

Most of the compounds in this group are ancient remedies and are therapeutically,now virtually obsolete. Many of them, however, have clearly defined biological actions and are of great interest from a theoretical viewpoint. Moreover, in some instances the active moiety have been used as basis for the development of a new series of compounds.

(a) Arecoline. Arecoline is a pyridine derivative, the methyl ester of N-methyl guvacine. Although it is a cyclic compound the placement of its active groups and the inter-atomic distances are reminiscent of acetylcholine (Wilson and Gisvold, 1962) and it shares this neurohormones

TABLE 2.1. ARECOLINE, BIOLOGICAL EFFECTS ON VARIOUS HELMINTH PREPARATIONS

References	Biological test system	Significant findings
Rebello *et al.* (1928 a,b)	Tissue-bath preparations of segments of *T. pisiformis* & *Dipylidium caninum*	Arecoline proved a powerful depressant of the musculature of both tapeworms.
Batham (1946)	Tissue-bath preparations of *T. pisiformis* and *T. hydatigena*	-Do-
Baldwin and Moyle (1947)	Tissue-bath preparations of an exposed strip of dorsal musculature from *Ascaris*	Paralysed by low doses (6.4×10^{-6}M). High doses (6.3×10^{-3}M) cause stimulant effect similar to those caused by acetylcholine and nicotine.
Baldwin (1948)	Tissue-bath preparations of anterior and middle of *Ascaris suum*	A high concentration (6.4×10^{-3}molar) paralysed anterior portion. No effect on middle portion.
Chance and Mansour (1949)	Tissue-bath preparations of *Fasciola hepatica*	Powerful depressant of rythmical movements reversed by ampehetamine. Lower threshold of dilution 6.4×10^{-7}.
Duguid and Heathcoate, (1950)	Tissue-bath preparations portions of *Moniezia* and segments of *T. saginata*	Arecoline proved the most powerful of some 50 chemicals tested. Effects reversed by strychnine.
Forbes (1965)	Tissue-bath preparation Scolex, neck, body-regions and exposed portions of *T. hydatigena, T. ovis, T. taeniaeformis* and *T. pisiformis*	Powerful depressant of all species except *T. ovis* mimicked by nicotine and other nicotine like drugs. Effects reversed by strychnine but no other antagonists found. Exhibits tachyphylaxis. Lower threshold 10^{-5}M.
Forbes (1965)	*Schistosoma mansoni in vitro*	Worms paralysed by very small doses of arecoline.
Barker *et al* (1966)	-Do-	-Do- mimicked by carbachol and acetylcholine (both nicotine-like drugs).

TABLE 2.2.

References	Biological test system	Significant findings
Feldberg *et al.* (1934)	Adrenal medulla of the cat	Arecoline produces strong secretory action resistant to small doses of atropine. An action of the 'nicotine type'
Feldberg and Vartianen (1935)	Superior cervical ganglion of the cat	Acetylcholine, arecoline and nicotine stimulate the ganglion cells in small doses and paralyse their responses in high doses.
Euler and Domej (1945)	Nictitating membrane and gastrocnemius of cat	Arecoline shown to possess nicotinic action
Batham (1946)	Dog	Injections of arecoline produce purgation but no worm removal, anthelmintic action related to direct action on worm.
Forbes (1961)	Colon of unanaesthetised dog	Effective enema having powerful stimulant action on rectal muscle.
Forbes (1963)	Blood-pressure, respiration gut-movements anaesthetised dogs.	Arecoline rapidly detoxicated in the liver. Powerful muscarine like actions. Toxicity related to route of absorption.

biological actions, having both nicotinic and muscarinic actions (Table 2.1). It is an ancient remedy derived from the areca or betel nut and at the present time has a limited use as a taeniafuge in man and the dog. For hydatid control purposes in New Zealand, arecoline has an important role in diagnosis, for examination of the faeces following arecoline purging is the sole means of determining infection. The pharmacological actions and effects of arecoline on helminth preparations and hosts are recorded in Tables 2.1 and 2.2. *In vitro* the drug is an extremely powerful depressant of tapeworm movement and this effect appears to be related to its nicotine-like properties. Other nicotine like drugs, such as nicotine, carbachol and acetylcholine (after eserine) also have a depressant effect on tapeworm, but purely muscarine-like substances, like pilocarpine, are without action. The effect of arecoline is reversible for when the bathing medium is refreshed the worms almost invariably resume rhythmical activity. The anthelmintic action of arecoline is achieved by powerful nicotinic effect on worms coupled with its muscarine effect on host intestines to increase gut movement and secretion and produce purgation and so remove the narcotised worms (Batham, 1946).

(b) Aspidium oleoresin (extract of male fern, felix mas , felicin and phlorogluconol compounds). Aspidium, oleoresin was formally the major drug for treatment of tapeworm infection and eradicates *Taenia saginata, T. solium, Diphyllobothrium latum* in more than 80 per cent of patients (Meyers *et al.* 1976). However it is now regarded as an absolete, somewhat toxic remedy for tapeworm infection in man (Standen, 1963; Keeling, 1968) and the dog (Link, 1965). Extract of *felix mas* caused stimulation followed by depression of isolated segments of *Dipylidium caninum* and *T. serrata* Rebello *et al.* 1928) and an alcohol-soluble fraction and an emulsion of the whole extract of *felix mas* was employed in *in vitro* tissue-bath experiments in which the movement of *Moniezia expansa* were recorded kymographically. Large amounts (1:1000 and 1:5000) of both extracts caused an immediate large rise in tone followed rapidly by a fall with complete cessation of rhythmic movements. Lesser amounts (1:10,000 to 1:1000,000) has less marked effects and lowest concentration to have any effect was about 1: 250,000 (Duguid and Heathcote, 1950a).

One active constituent is filicin and others are phloroglucocinol compounds-including aspidium, flavaspidic acid and desaspadine and all are phenolic in character (Keeling, 1968).

Three notable anticestodal compounds-niclosamide, dichlorophen and desapidine are respectively chloro-nitro and methoxy-substituted derivatives of phenol and have some structural similarity to dinitrophenol(DNP)- a well-known uncoupler of aerobic oxidative phosphorylation in mammalian mitochondria (Bueding, 1969). Their structural characteristics prompted investigation into their action on phosphorylation pathways and there is indirect evidence suggesting that they may interfere with tapeworm phosphorylation pathways (Mattila and Takki, 1966; Strufe and Gönnert, 1967; Sanderson, 1973). More direct and detailed studies on the effects of the anthelmintics on helminth phosphorylation processes indicated that *Hymenolepis diminuta* has an electron transport system like that of pig *Ascaris* : fumarate reduction under anaerobic conditions being linked with reduced nicotinamide adenine nucleotide (NADH) oxidation and phosphorylation (Kmetec and Bueding, 1961).

Some classical uncouplers in aerobic tissues also interfere with the process in both *Ascaris* and *H. diminuta*, indicating that the helminth phosphorylation processes are linked to electron transport-although the final electron acceptor is fumarate rather than oxygen.

Desaspidin (in common with dichlorophen and niclosamide) reduces the incorporation of ^{32}P into ATP by intact *H. diminuta in vitro*, at concentrations of from $10^{-4}M$ to $10^{-6}M$ and there is evidence that this inhibition occurs at the level of the mitochondrial $^{32}P_1$ - ATP exchange reactions (Scheibel *et al.*1968; Sanderson, 1973). The three taeniacides inhibit the mitochondrial incorporation of ^{32}P into ATP in pig *Ascaris* mitochondria (Saz and Lescure, 1968; Saz, 1971[a]) thus demonstrating the similarity between nematode and cestode mitochondrial systems. The reason the drugs are not effective against *Ascaris in vivo* may be accounted for by their inability to permeate the intact nematode (Sanderson , 1973).

(c) Chenopodium (ascaridol) The oil is an extract of the American wormseed *Chenopodium ambrosoides* containing 60 -80 per cent of the active principle, ascaridol which is a single ringed cyclic compound with no notable structural features. It is extremely irritant to the skin and mucous membranes and produces marked gastrointestinal and neurological symptoms. Rico (1926) first demonstrated a paralysant action on anterior portion of pig *Ascaris* and Baldwin (1948) found that an emulsion of oil of chenopodium (concentration 1:5000) paralysed in 20-30 minutes a tissue-bath preparation of *A. suis*. Duguid and Heathcote (1950a) showed that the oil has a strong stimulant action on the musculature of *Moniezia* in all strengths down to 1: 500,000 Prolonged exposure to strong solutions resulted in death of the muscle.

Beyond these studies, the mode of action does not appear to have been examined systematically - but the broad scope of biological actions. (affecting, host, *Ascaris* and *Moniezia*) suggest a non-specific action probably related to its irritant properties. The structural formula reveals no features to suggest a chemical basis for biological actions (del Castillo, 1969).

(d) Kamala Kamala is a powdery substance comprising the hairs and glands covering the fruits of *Mallotus phillipinensis* an oriental tree.
It is strongly irritant to the alimentary canal and is regarded as only partially effective, toxic and obsolete. A filtered saturated solution has a paralysant effect on the rhythmical activity of tissue-bath preparations of *Fasciola hepatica* at a concentration 1:5000 and this effect was reversed by amphetamine (Chance and Mansour, 1949). Its anthelmintic action may be related to its non-specific irritant and purgative properties.

(e) Kainic Acid (2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine).
Kainic acid is the anthelmintic principle from the dried red alga *Digenear simplex* (Merck Index, 1959). The structural formula was elucidated by Nitta *et al.*(1958) and the acid is reported to have ten times the anthelmintic potency of santonin and to be without side effects. The stereoisomer, allokainic acid is only weakly anthelmintic, if at all (Standen, 1963). The stereospecificity points to a specific action on

receptor sites in the nematode and as it has structural features reminiscent of those of arecoline and arecaidine, pharmacodynamic studies with kainic acid might well reveal some interesting structure action relationships, particularly as kainic acid is reported to accelerate the motility of *Ascaris* in low doses and suppress the motility in high doses (Anon, 1968).

(f) Pelletierine (Pomegranate) This ancient remedy has been used as a taeniafuge from the earliest historical times and its medical properties are alluded to in the Ebers papyrus of 1500 B.C. In the crude form it comprises the rootbark of the pomegranate tree *Punica granatum*. Pelletierine tannate is a mixture of the tannates of several alkaloids from pomegranate and the extract and the tannates are regarded as somewhat toxic are now obsolete. L-pelletierine has a structure closely resembling that of coniine the poisonous alkaloid from hemlock. Coniine has well-defined nicotine-like properties in the vertebrate. Both alkaloids resemble closely the modern synthetic pyridine anthelmintic, methyridine. Pelletierine sulphate caused slight stimulation followed by paralysis of tissue-bath preparations of *T. serrata (=T. pisiformis)* and *Dipylidium* (Rebello *et al.* 1928b) and the alkaloid has a paralysant action on segments of *Moniezia* acting in dilutions as high as 2.5×10^{-6}. Two other alkaloids of *Punica granatum*, methyl isopelletierine and pseudo-pelletierine sulphate proved somewhat weaker depressants (Duguid and Heathcote, 1950a). The high degree of potency indicates a specific action on specialised receptors.

Mode of action. Pelletierine coniine and methyridine resembles acetylcholine in structure - at least to the extent that each contains an $N\text{-}C\text{-}CH_2\text{-}CH_2$ configuration. The last 2-named substances are known to have nicotine-like properties and pelletierine possess an action on tapeworm identical to that of arecoline. Moreover, methyridine (Forbes, unpublished) nicotine itself and acetylcholine (after eserine) all produce (Paasonen and Vartiainen 1958; Forbes, 1965) a depressant action on tapeworm and this effect of arecoline is regarded as the basis of its anthelmintic action.

In view of the above evidence it appears reasonable to assume that pelletierine exerts its effect by a paralysant action similar to that of acrecoline.

(g) Nicotine Tobacco leaves have some action in expelling tapeworms and nicotine sulphate has been employed as a taeniacide chiefly in ruminants. Combined with copper sulphate the salt has enjoyed considerable popularity as a cheap partially effective remedy for nematodes in ruminants (Link, 1965).

The alkaloid is however, of paramount significance as the pharmacological tool for it was on the basis of its biological actions that Dale (1914) proposed his classical exposition on the nicotine-like actions of acetylcholine. Nicotine alkaloid (1:200,000) caused a spastic contraction of pig *Ascaris* (Rico, 1926) and at concentrations of 500µg/ml or higher it paralysed fragments of *Ascaris* with the cuticle intact, but its effect at concentrations of 0.1 µg to 1000 µg/ml on exposed preparations was to cause a strong increase in tone. This effect was exactly the same as that of acetylcholine at a concentration of 0.1 µg per ml (Baldwin and Moyle, 1949; Baldwin, 1943). Stimulant effects were observed also by Norton and de Beer (1957) and Natoff (1969) who employed muscle strips of *Ascaris* prepared so as to permit access of water-soluble drugs to the muscle tissue (Michelson, 1973). Nicotine is a weak inhibitor of movements of *Moniezia expansa* and has no effects at concentrations below about 50 µg per ml (Duguid and Heathcote, 1950a). An intact preparation containing about 12 proglottides of *Taenia taeniaeformis* was paralysed by nicotine base and chloride at concentrations of from 1 to 10 µg/ml and a denervated preparation which lacked an intact tegument was paralysed by somewhat higher concentrations (Paasonen and Vartiainen, 1958). Nicotine alkaloid stimulated the tone of *T. hydatigena* and *T. taeniaeformis* in somewhat low doses (2.5 µg/ml) and in progressively higher doses (5 - 300 µg/ml) produced stimulation then paralysis, and eventually complete paralysis. Scoleces, neck, body portions and gravid segments were all affected in much the same fashion but an exposed preparation consisting of one half of a body portion, with the tegument not intact was paralysed by doses as low as 2.5 µg/ml (Forbes, 1965). Amongst the flukes, *F. hepatica*, appears quite susceptible to the paralysant actions of nicotine being affected by concentrations down to 5 µg/ml - an action reversed by amphetamine (Chance and Mansour, 1949) but paradoxically, nicotine was ineffective in concentrations of up to 1 x 10^{-2}M on the movement of intact

Schistosoma mansoni, even though the worms were quite susceptible to other nicotinic drugs such as arecoline (5×10^{-6}M) and carbachol (1×10^{-4}M) (Barker *et al.* 1966).

(h) Santonin

Santonin, a terpene, is an essential vegetable oil extracted from the plant *Artenisa cina*, the Levant wormseed, and other related species. It is an ancient remedy and has been used extensively for *Ascaris* in man (Wilson and Schild, 1959) but it is only partially effective prone to toxicity and is now obsolete (del Castillo, 1969). The anthelmintic is virtually inactive against tissue-bath preparations of *Moniezia* even in concentrations as high as 1:4000 (Duguid and Heathcote, 1950a). Concentrations as high as 0.1 per cent do not kill *Ascaris* (Lamson and Brown, 1936) but concentrations down to 1:100,000 paralyse the anterior preparation of the worm but to inhibit the intermediate preparation the dose must be increased ten-fold. This finding suggests that santonin exerts a paralysing effect on the nerve centres in the anterior part of the worm (Baldwin, 1943; del Castillo, 1969; Goodwin, 1958) without much affecting the peripheral neuromusculature.

Baldwin employed santonin as a prototype substance for a pioneering study of structure - action relations using an *in vitro* preparation of the anterior portion of *Ascaris*.

Taking santonin as a starting point Baldwin investigated the prospects of anthelmintic activity related to unsaturated ketones and by lactones. Benzylidene acetone (an aromatic ketone) may be derived from santonin and this compound and a series of related ketones were shown to possess significant effects on *Ascaris* and attempts were made to increase this by chemical manipulation of the molecule. Activity appeared to be mainly related to the ketonic group but despite numerous structural modifications no compound was found approaching santonin in potency.

(i) Proteolytic Enzymes

Gastrointestinal helminths are able to withstand the action of digestive enzymes. Ascarids produce an antienzyme 'ascarase', which has anti-trypsin and anti-pepsin activity, and protects the helminth from

damage. However, although intestinal helminths are highly resistant to digestive enzymes they are susceptible to the lytic action of several proteolytic enzymes synthesized by tropical plants. Pineapple juice contains bromelin. Paypaya contains the enzyme papain. Ficin is derived from the tree *Ficus dilaria*, which acts on *Trichocephalus*, *Ascaris* and hookworm. It is labile and must be freshly prepared before use (del Castillo, 1969).

2.2 METALS AND OTHER IONS

(a) Antimony (Sb)

Antimony resembles arsenic both chemically and biologically and it has been used by the ancients both as a medicine and cosmetic. Tartar emetic and antimony potassium tartrate were prepared in the sixteenth century and antimony compounds were introduced as an alternative to arsenicals early in the present century.

Despite the enormous expenditure on scientific effort antimonials can still be considered the most effective substances for the treatment of human schistosomiasis and tartar emetic which was introduced in 1918 as a specific for the treatment of schistosomiasis (Standen, 1963) remains a cheap and relatively effective remedy (Lämmler, 1968). However, the drugs must be given by repeated parenteral injections and side-effects commonly accompany their use - so that the therapy of schistosomiasis remains probably the most formidable unsolved proglems in helminth chemotherapy.

Mode of action. As with arsenic, biological activity appears to reside chiefly with the trivalent form and pentavalent antimonials have no schistosomicidal activity *in vitro* (Lee and Chung, 1935) nor do they reduce the rates of glycolysis or of phosphofructokinase activity (Mansour and Bueding, 1954).

Initially, it was assumed that the anthelmintic action of trivalent antimonials mirrored that of arsenic (ie) inactivation of SH enzymes (Findlay, 1950). However, in contrast to arsenicals antimonials do not affect glucokinase of *S. mansoni* (Mansour and Bueding, 1954)

but do appear to reduce the utilization of carbohydrate by schistosomes (Bueding, 1950) probably by interfering with a single step in the glycolysis of the schistosome - the phosphorylation of fructose-6-phosphate (F-6-P) by adenosine triphosphate (ATP) to fructose 1, 6-diphosphate (FDP) - a reaction catalysed by phosphofructokinase (Bueding and Mansour, 1956) an enzyme inhibited in schistosomes from antimony treated rats (Bueding and Mansour, 1957) F-6-P+ ATP——> FDP + ADP.

Glycolysis is regarded as the major, if not the only source of energy to worms and it appears that the schistosomicidal actions result from inhibition of phosphofructokinase (Bueding and Swartzwelder, 1957, Bueding, 1959) Mammalian phosphofructokinase is much less sensitive to the inhibitory effects of antimonials than the enzyme catalysing the same reaction in schistosomes (Saz and Bueding, 1966). Huang, *et al.* (1962) identified glutamic pyruvic transaminase (GPT) and glutomic-oxal-acetic transaminase (GOT) in *S. japonicum* and *F. hepatica* (Conolly and Downey, 1968) and trivalent antimonials were inhibitory to both transaminases but not to those of mouse liver.

(b) Arsenic (As)

Arsenic was known as a therapeutic agent to the ancient Greeks and Romans. It has been a classical poison and the history of folklore of arsenic prompted intensive studies by the early pharmacologists (Harvey, 1975). Arsenious oxide (AS_2O_3) was shown in 1893 to be effective for trypanosomiasis (but somewhat toxic) and it was soon superseded by sodium arsanilate (atoxyl). Ehlich modified the structure by the addition of different side-chains to the benzene-ring - firstly to produce dioxydiamido-arsenolbenzol (arsphenamine) and then 3,3-diamino-4,4-dihydroxyarsenobenzene-methylenesulfoxylate sodium (neoarsphenamine) whose activities extend to trypanosomes, spirochaets of relapsing fever, syphilis and spirillosis of poultry. The advent of neoarsphenamine was long regarded as the greatest achievement in chemotherapy and it remained the drug of choice for syphilis until 1945 when it was replaced by the more active and less toxic penicillin. Arsenic compounds have found wide application in therapy. They are important ectoparasiticides tonics and growth promotants, but their value as anthelmintics are in general, limited by their narrow chemotherapeutic ratio and their

2.2

somewhat low degree of efficiacy.

Lead arsenate is a cheap, effective remedy in ruminants for *Moniezia* and *Helictometra (Thysaniezia)* and has some activity for *Haemonchus contortus*. Copper aceto-arsenite and copper methyl-arsenate and arsenates of lead, tin, zinc and copper and calcium have been used and taeniacides in various ruminants and poultry (Keeling, 1968). The trivalent arsenoxide, melarsen - a triazine derivate, was developed in 1938 by Friedheim, for the treatment of schistosomiasis, but it has now been superseded. (Slack and Nineham, 1968).

A series of arsenophenyl compounds have shown microfilaricidal action *in vitro* Hawking (1940) and the most promising member, p-arsenobenzamide was prepared as a soluble thioglycollate and designated thiacetarsamide or carparsolate sodium (Hawking 1963). It is effective against adults and microfilariae of *Dirofilaria immitis* (Link, 1965) and *Wuchereria bancrofti* (Hawking, 1963). Dichlorophenarsanine hydrochloride (USP) is another organic arsenical effective against *Dirofilaria* and melarsonyl, a water soluble derivative of an organic compound of arsenic containing the melanine nucleus, has been used for the treatment of *Wuchereria* and *Onchocerca volvulus* (Rollo, 1975). When radioactive sodium arsenite was injected into cotton rats infected with *Litomisoides* a high concentration was found in the adult worms (Lawton *et al*.1945, cited by Hawking, 1963).

Mode of actions of Arsenicals. The most important organic arsenicals are derivitives of benzine arsonic acid ($C_6H_5AS{:}O(OH)_2$) and the presence or absence of various substitutes on the benzene ring determines not only the solubility of the drug but also its ability to penetrate cell membranes both of parasitic organisms and the host. Regardless of whether an arsenical is introduced into the body as trivalent or pentavalent form all the major toxic or chemotherapeutic actions may be attributed to the trivalent species.

The major effect on both host and parasite results from inhibition of enzymes containing sulphydryl (SH) groups and it appears the metal does

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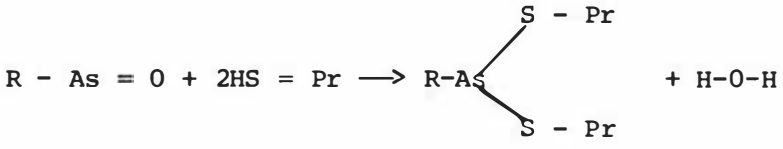
not combine significantly with any other chemical group. The general scheme of the reaction between an arsenoxide or arsenite and the SH of protein is as follows:



Where R is any group and Pr is protein and inactivation of essential SH enzymes is regarded as the first step in cell damage and pyruvate oxidase and many other enzymes are susceptible.

The selectivity of organic arsenicals for parasite (particularly trypanosomes) rather than host, may arise from the following:
(a) arsenoxides may be able to penetrate into the parasite more readily than into mammalian tissue cells.
(b) the SH enzymes of the susceptible parasite may be more sensitive or rate limiting than those of non-susceptible parasites or the host. Arsenic - sensitive trypanosomes have very simple metabolic schemes almost without shunts or convergent pathways, so that inhibition at a single point may prevent nearly all energy production or high energy phosphorylation. Moreover, they have a high energy demand, and metabolism probably needs to proceed nearly at capacity.
(c) mammalian tissue may oxidise the intracellular arsenical to the inactive arsonic acid more readily than does the parasite. Some trypansomes are virtually devoid of aerobic metabolic systems and thus lack the necessary means of oxidising the trivalent form.

The development of resistance appears to be related to a decrease in permeability to the candidate arsenical, and does not necessarily extend to other arsenicals, but may even confer resistance to a non-arsenicals. Moreover, the arsenate ion is capable of uncoupling phosphorylation through the formation of unstable arsenate esters *in lieu* of certain phosphate esters that one normally oxidised to high-energy phosphate donors (Harvey, 1975).

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(c) Cadmium

Cadmium carbonate, bromide and oxide and cadmium salts or aromatic or aliphatic sulphonic acids are reported to have activity against various nematodes and cestodes (Keeling, 1968). However, cadmium salts are somewhat toxic and do not appear to have become firmly established in therapy.

Mode of action. The anthelmintic action is probably related to cadmium's ability to combine with SH group and thus inhibit sulphydryl enzymes.

(d) Copper

Copper sulphate has long been employed as a cheap partially effective remedy for abomasal nematodes and tapeworms infections in ruminants and its anthelmintic action may depend on the pH of the environment for it appears that simple copper salts are only lethal to parasites in acid medium (Whitlock, cited by Link, 1965). Copper carbonate has taeniacidal actions (Keeling, 1968) and the use of copper salts of arsenic has been mentioned above.

Mode of anthelmintic may be related to ability of the cupric ion (Cu^{++}) to inhibit succinic dehydrogenase - an enzyme essential for energy metabolism in various helminths (Chaper 5). The succinic dehydrogenase of *Ascaris* appears more sensitive to inhibition than is the enzyme catalysing the same process in the mollusc *Biomphalania glabrata* (Cheng unpublished, cited by Campbell, 1977). Moreover, Cu^{++} will retard the normal development of *S. mansoni* in *B. glabrata* when infected snails are exposed to 60 ppm of Cu (as $CuSO_4$) for 20 hours. Campbell (1977) considers that further studies on the chemotherapeutic properties of copper should be carried out.

(e) Fluorides

Sodium fluoride (USP) was widely employed in dry ground feed for the control of pig *Ascaris* in the U.S. The drug is also partially effective against *Ascarops* and *Physocephalus*. It has a narrow margin of safety and has been superseded by more modern drugs.(Link, 1965).

2.2

Mode of action The principal pharmacological activity of the fluoride ion consists of inhibition of cellular enzymes, decreased oxygen consumption and decreased lactic acid production in muscles of the host and its anthelmintic action may be related to similar effects in the worm (Hammond, 1965).

(f) Lead

Apart from the arsenate and possibly d-n-butyllead acetate (Keeling, 1968) lead has no role in helminth therapy. Any anthelmintic action probably results from its affinity for SH groups (Hammond, 1965).

(g) Tin

Various organic tin compounds have shown promise as taeniacides, particularly in poultry (Keeling, 1968) and stannous oxide was claimed effective in urinary schistosomiasis (Deschiens, 1951) but no activity could be demonstrated against *S. mansoni* in mice (Standen, 1963).

2.3 ORGANOPHOSPHORUS COMPOUNDS

Organic esters of phosphoric acid were shown in 1937 to be powerful inhibitors of cholinesterase. They were initially developed as insecticides and later as highly toxic nerve gases for potential use in war fare. Since the demise of the organochlorines they have become paramount against arthropods both in agriculture and animal husbandry (Slack and Nineham, 1968) and they have a small therapeutic role in opthalmology and for myaesthenia gravis (Crossland, 1970) but are important anthelmintics and several are in current veterinary use particularly for Strongyloidea; these include haloxon, trichlorphon (metrifonate), dichlorvos, vincofos, napthalophos and crufomate. Dichlorvos and vincofos have activity against both nematodes and cestodes (del Castillo, 1969; Sanderson, 1973; Mc Farland, 1972). Trichlorphon and dichlorvos have been used clinically in man for nematodiasis (Mc Farland, 1972) Trichlorphon (metrifornate) is regarded effective for the treatment of human urinary schistosomiasis caused by *S. haematobium* but lacks activity in animals and human subjects infected with *S. mansoni* (references in Bueding *et al*.1972). The margin of safety, may however, be too narrow for the widescale adoption of OP's for human therapy.

Mode of action appears to be related chiefly to inhibition of helminth cholinesterase and to varying sensitivities of enzymes between host and parasite. The topic is discussed in detail in Chapter 4 where experimental findings are reported. There is evidence, however, that glycogenic activity in *F. hepatics* and *S. mansoni* may be inhibited by eserine and that it has been suggested that the action of metrifonate (trichlorphon) may be related to inhibition of carbohydrate metabolism (Ehlich, 1966; Standen, 1971).

2.4 SIMPLE ALIPHATIC HALOGENATED HYDROCARBONS

As a group these anthelmintics may be regarded as biologically somewhat nonspecific, in that they affect a range of tissues including both host and parasite. They have a narrow margin of safety and in many ways resemble vapourous anaesthetics and in common with them they have a propensity for liver damage and sensitising the myocardium to catecholamine (Link, 1965).

(a) Carbon Disulphide (CS_2). This noxious substance has the simplest chemical structure of the group and about the same acute toxicity as chloroform and produces general anaesthesia when inhaled. It is also strongly irritant and has been used chiefly for removal of *Gastrophilus* from the horse. It also has action against *Ascaris* in the horse. Its mode of action is almost certainly related to its general depressant effect on nervous tissue.

(b) Carbon Tetrachloride (CCl_4) This hydrocarbon is a volatile colourless liquid and is very similar chemically to chloroform ($CHCl_3$). It was introduced as an anaesthetic at the time of discovery of chloroform but was subsequently discarded because of toxicity. In 1921, Hall introduced carbon tetrachloride for therapy after discovering it almost 100 per cent effective for removing hookworm from dogs. It was universally used until the discovery of the less toxic tetrachlorethylene, and later n-butylchloride (Link, 1965). Its present day role is as an inexpensive, effective, but somewhat toxic remedy for *Fasciola* in ruminants (Lämmler, 1968; Watkins, 1958; Kendall and Parfitt, 1962). Carbon tetrachloride passes through the stomach unchanged and is partially absorbed in the small intestine. It is highly lipophylic and large amounts of fat in the intestine increase

the absorptive capacity and toxicity. Applied locally, CCl_4 is an irritant and rubifacient and after oral ingestion produces a feeling of warmth in the stomach and stimulates peristalsis. It depresses the central nervous system, is directly toxic to the heart and may cause cardiac arrhythmias and marked hypotension. It is selectively toxic to hepatic and renal cells and disrupts numerous enzymes systems both of the biochemical and cytological levels (Swinyard, 1975). Alexander (1969) is of the view that the fasciocidal action may result from liver damage rather than from a direct specific effect on the fluke itself. However, Duguid and Heathcote (1950a) found CCl_4 caused stimulation then depression of tissue-bath preparations of *Moniezia*. Similar findings were obtained with *F. hepatica* (Chance and Mansour, 1949). In view of the chemical's wide spectrum of noxious actions, including central nervous depression, general protosplasmic toxicity, irritation to tissues and known action on stimulation of peristalsis, it appears probable that there are direct effects on the worms.

(c) Tetrachlorethylene ($Cl_2C=CCL_2$)

This halogenated unsaturated hydrocarbon was introduced by Hall and Schillinger (1925) as a substitute for the more toxic carbon tetrachloride. Tetrachlorethylene resemble CCl_4 in its physical properties but is only one fifth as soluble in water, but very soluble in fat and in the absence of fat in the intestine, it not absorbed to any appreciable extent. This probably accounts for its relatively low toxicity. Tetrachlorethylene has long been regarded the standard treatment for hookworm in man and it is considerably more effective against *Necator americanus* than against *Ancylostoma duodenale*. It also has appreciable activity for the small intestinal flukes, *Heterophyes heterophyes* and *Metagonimus yokogawi* and the large intestinal flukes *Fasciolopsis buski, Echinostoma Spp.* and *Gastrodiscoides hominis*. Although little appears to be absorbed, $Cl_2C = CCl_2$ often causes central nervous effects similar to those of chloroform. Adult hookworm removed from treated patients after purgation are motile and it is assumed the worms are paralysed sufficently to release their attachment to the intestinal wall. Tetrachlorethylene was shown by Rogers (1944) to have a stimulant effect on *Nipponstrongylus muris*, and there appears to be an exaggeration of normal movement

since it caused the parasite to leave the intestinal mucosa (Chance and Mansour, 1949; Gibson, 1971). Brown (1936) found 100 per cent of worms were killed by a 2 hour exposure to the drug and Baldwin (1943) showed it about equipotent with carbontetrachloride in causing paralysis of both the anterior and middle portions of *Ascaris*. The drug has stimulant and lethal effects on tissue-bath preparation and *Fasciola*. (Chance and Mansour, 1949).

In the absence of direct experimental evidence it has been suggested that tetrachloethylene, the same as other halogensted hydrocarbons, acts by becoming dissolved in lipid deposits existing in the muscle cells, particularly - the area of innervation processes (Mueller, 1929) thus interfering with muscle function (del Castillo, 1969).

(d) *Hexachlorethane* was introduced in 1928 as a remedy for *F. hepatica* but subsequently it has proved to have toxic effects and variable anthelminthic actions.

(e) *Difluorotetrachlorethane* is a volatile liquid which is probably a mixture of 2 isomers, 1,1 and 1,2 difluorotetrachlorethane. The mixture was introduced for *Fasciola* om ruminants but its margin and safety is insufficient and it has been discarded.

(f) *N - butyl-chloride* (CH_3 $(CH_2)_C$ *Cl)*. Kudicke and Weise (1926) in an examination of a number of halogenated derivatives of lower hydrocarbons found that the anthelmintic activities were universally related to the solubilities of the compounds. In general the bromo were more active than the chloro analogues. Butyl chloride was found by Wright and Schaffer (1932) in their study of chlorinated alkyl hydrocarbons to be highly active against hookworms and ascarids of dogs (Whitney and Whitney, 1954) but the substance has found limited use in veterinary practice. Its action is undoubtedly related to its lipid soluble and halogenated properties.

2.5 SINGLE RINGED CARBOCYLIC COMPOUNDS

Oxidation and other substitutions in the benzene ring including methylation and halogenation and amalgamation of the carbocylic rings gives rise to many valuable therapeutants - phenols, resorcinols,

cresols, xylenes, chlorocresols, bis-phenols, napthalenes and so on. However, in general the mode of anthelmintic action of such compounds does not appear to have been clearly defined and many of them appear somewhat non-specific and affect a variety of organism. For example, hexachlorophene acts on fluke and tapeworm and is an effective skin disinfectant Hexylresorcinol affects roundworms, tapeworms and flukes is used as an antiseptic and has previously been used as a urinary disinfectant.

(a) *Toluene (methyl benzene)* is the simplest member and is useful against roundworms in dogs and cats and *Ascaris* and *Gastrophilus* in the horse (Link, 1965) but it is not used in man, probably because it is somewhat irritant and toxic.

(b) *Hexachloroparaxylene* or Hetol of Hoechst is an example of anthelmintic derived by chlorination of xylene (dimethylbenzene). It is active against mature *F. hepatica* but not against the juvenile forms. In high doses it has shown activity against *Dicrocoelium dendriticum* (Lämmler,1968). However in *Fasciola* treatment the margin of safety appears much lower than that of carbon tetrachloride (Boray and Happich, 1968) and the drug may offer no advantage over the older remedy. Hexachloroparazylene has in laboratory animals high activity and a favourable therapeutic range against *Opisthorchis filineus* and *Clonorchis (= Opisthorchis) sinensis*. In man, it showed promise of being effective against *O. filineus, C. sinensis* (Lämmler, 1968) and *O. viverrini* (Harinasuta, *et al.* 1966) However recently it is reported to have caused renal tubular damage, centrilobular necrosis and sudden death in experimental animals and consequently it has been withdrawn from further clinical trials (Marsden and Schultz, 1969).

(c) *Hexylresorcinol* is a dihydric phenol and an example of one of the first phenolic compounds introduced into helminth therapy. In man it has a broad spectrum, including *Ascaris, Trichuris, Ancylostoma, Necator, Enterobius, Hymenolepsis, Fasciolopsis* and *Taenia* (Meyers,*et al.* 1968). It is only partially effective against some of the worms, but on the grounds of its known safety, broad spectrum and low cost it may well prove a useful drug for some time yet. It is not

used extensively in veterinary practice - possibly because of its irritant properties. Single oral therapeutic doses removed nearly 100 per cent of *Ascaris* and 85 per cent of *Ancylostoma* hookworms from dogs (Link, 1965).

The drug is poorly absorbed and relatively nontoxic. The mucin secreted by the intestinal mucosa is believed to protect the intestine from the caustic action of the phenol.

Lamson and Ward (1932) reported hexylresorcinol killed 100 per cent of experimental worms following a 2 months exposure, thymol required 30 minutes to produce the same effect. They were of the opinion that hexylresocinol acts primarily on the nematode cuticle since the worms that had been killed showed cuticular blistering. It is also reported to change the permeability of the body wall to phosphorus containing compounds which escape from the worms and furthermore, to paralyse muscle (del Castillo 1965) Baldwin (1943) tested hexylresorcinol on anterior and body portions of *Ascaris* and both portions in from 20-30 minutes. It was the most potent of some 12 anthelmintics tested and from 2-5 times as active as thymol and β. napthol, which were about equal in activity. Moreover, Baldwin (1943) found that the worms were completely paralysed long before blistering could be detected and he concluded that the primary site of action was not the cuticle. Baldwin and Moyle (1949) perfused body-preparations and *Ascaris* by means of fine cannulae tied between the gut and the muscle masses. In high concentrations (1:1000) hexylresorcinol caused a very strong contraction and arrest of rhymical movements of *Moniezia* and after 20 minutes exposure at concentrations of 1:10,000 the cells were almost invariably killed. Lower concentrations (1:1,000,000) produced variable combinations of stimulant and depressant actions and the lower limit of activity was 1:2,000,000 (Duguid and Heathcote, 1950a). Hexylresorcinol is a phenolic compound and as such it might be expected to affect oxidative phosphorylation (Sections 2.1(b) and 5.3.2 (e)

(d) Disophenol (2, 6-diiodo-4-nitrophenol). A narrow spectrum anthelmintic and the mode of action has been studied, particularly in the context of the phenolic structure and consequent chemical relation to dinitrophenol (Sanderson, 1973). Prichard (1973) found that disophenol inhibits the fumarate reductase system of thiabendazole susceptible and resistant *H. contortus*.

(e) *Nitroxynil (4-cyano-2-iodo-6-nitrophenol)* Closely resembles disophenol and is a useful fasciolicide especially for acute infections. Corbett and Goose (1971) showed that nitroxynil stimulates oxygen uptake by *F. hepatica in vitro* and uncouples oxidative phosphorylation in liver mitrochondria. The topic is dealt with more fully in Chapter 5 section 3.3.

(f) *Bitoscanate*. This drug (Jonit of Hoechst) is a phenylisothiocyanate which proved effective for hookworm in man. It has a low margin of safety and almost certainly relied on its phenolic structure for its modes of anthelmintic action (Mc Farland, 1972).

2.6 HETEROCYCLIC COMPOUNDS, BEPHENIUM AND THENIUM

Compounds in this group share chemical features of acetylcholine as well as its biological actions.

(a) *Methyridine* is a pyridine derivative with the distinction of being about the first injectable drug effective against nematodes. It is widely distributed and acts against lungworm and abomasal dwelling helminths. It has a narrow margin of safety and cross-reacts with diethylcarbamazine. It has a tertiary nitrogen atom and shares CH_2-CH_2-O configuration with acetylcholine. Its pharmacological actions are discussed more fully in Chapter 3 (Table 3.1). It is highly likely that it achieves its action by disrupting nerve and muscle co-ordination of the helminth - probably by stimulating ganglion like structures (Coles, 1974).

(b) *Piperazine*. This simple cyclic compound has been the subject to considerable research with respect to its basic mode of action. Aspects of its biological actions are summarized in Table 2.3. The drug produces a gradual reversible paralysis and blocks the stimulatory effects of acetylcholine on *Ascaris* muscle. It is believed to activate inhibitory receptors (del Castillo, 1964d) and it causes flaccid paralysis which is antagonised by methyridine (Fiakpui, 1967). Its basic mode of action appears to be related to activation of hyperpolarising neuromuscular receptors (del Castillo, 1969). It also has an inhibitory effect on succinate formation but this appears secondary to paralysis.

(c) *Diethylcarbamazine*. This filaricidal drug is active against *Wuchereria bancrofti, Brugia malayi, Loa loa* of man (Rollo, 1975) and *Dirofilaria immitis* of the dog. Its pharmacology is reviewed in Chapter 3 (Table 3.2). There is no doubt that the drug possesses

TABLE 2.3 PIPERAZINE. BIOLOGICAL ACTIONS ESPECIALLY IN RELATION TO MODE OF ANTHELMINTIC ACTION

References	Significant findings
Goodwin and Standen (1954)	Pig *Ascaris* immersed in ringer containing piperazine citrate (1: 500 or higher) slowly became immobilized in 6-18 hours. Worms were not killed. Worms voided from treated patients recovered normal activity in a few hours.
Standen,(1955)	Piperazine citrate, adipate and phosphate are equally active *in vitro* against *Ascaris lumbricoides* from the pig. Narcosis occurs in 24 hours in worms exposed to drug dilutions of 1:200 - 1:560 and recovery occurred in less than 3 hours, even if exposed to piperazine for 48 hours.
Brown *et al.* (1956)	Human *Ascaris* placed in a piperazine solution become paralysed over a long period.
Norton and de Beer (1957)	Onset of piperazine paralysis in intact *Ascaris* is related to drug concentration. Piperazine blocks the stimulatory effects of acetylcholine on *Ascaris* muscle, but not electrical stimulation and the anthelmintic action probably results from a curare-like neuromuscular block.
Bueding *et al.* (1959)	Piperazine reduced the production of succinate by *A. lumbricoides*. This effect was reversible. There was a close parallelism between the concentrations of piperazine which paralysed the worm and those which inhibited the formation of succinate.
Miyagawa,(1961)	Piperazine about 10^{-2}M lowers histamine content of intact *Ascaris in vitro*.
Bueding (1962)	The lack of succinate production in *Ascaris* seems to be a result, rather than a cause, of the piperazine-induced paralysis and results from lowered energy transformation in the flaccid muscle.
Goodwin and Vaughan Williams, (1963)	Coaxial stimulation in the region of the circumpharangeal nerve ganglia caused inhibition of both spontaneous and electrically-stimulated contraction of the body of *Ascaris*. Piperazine shifted the strength-duration curve of electrical threshold, to the right implying nervous on neuromuscular blockade.
del Castillo *et al.* (1964d)	The activation of the inhibitory receptors by the drug increases the permeability of the syncytial membrane to chloride ions.

References	Significant findings
Fiakpui (1967)	Piperazine caused flaccid paralysis of the free-living nematode *Caenorhabditis briggsae* which antagonised partially by methyridine and acetylcholine.
del Castillo (1969)	Contraction of somatic muscle cells of *Ascaris* is elicited by rhythmic repetitive action potentials which are nicotinic in origin and which occurs at frequencies of up to 12 per second. The nerve cord has a modulatory role both excitatory (mediated by acetylcholine) and inhibitory (possible mediated by gama-aminobutyric acid). Piperazine (10^{-5} and 10^{-3} w/v) increases resting potential of muscle cells from 30 mV to 45 mV and have a depolarizing effect on the entire cell but the inhibitory effect appears to be related to activation of hyperpolarizing neuromuscular receptors resulting in increase of permeability of the membrane to chloride ions. Piperazine reduces succinate formation in *Ascaris* tissue but this seems to result from lowered energy requirements in flaccid muscle.
Natoff (1969)	Piperazine considerably weaker antagonist than atropine on the receptors responding to acetylcholine, dimethylphenypiperazium (DMPP) and pyridine-2-aldoxine in *Ascaris* muscle.
Sanderson, (1970)	Review Neuromuscular System. Causes reversible paralysis, resembles an inhibitory transmitter, inhibits *Ascaris* cholinesterase and lowers histamine content of intact *Ascaris*.

nicotine-like properties and that its actions are closely associated with its effects on nicotine receptors in the helminth parasites.

(d) *Bephenium and thenium* are closely related compounds both possessing the $-N.CH_2.CH_2.O-$ configuration in common with acetylcholine. Unlike methyridine they are poorly absorbed from the gut because they possess a quaternary nitrogen atom. In the exposed neuromuscular preparation of *Ascaris* these substances produce contracture and bephenium is five times more active than acetylcholine. This may be because the phenyl group attached to the ether oxygen atom allows greater lipid solubility and an increase in nicotine-like action (Broome, 1962). It is likely that the anthelmintic actions of these substances are related to effects on helminth nicotine-like receptors.

2.7 BISPHENOLS

These substances consist of two substituted phenolic rings connected through a methylene or other bridge. They almost certainly owe their anthelmintic activity to their ability to uncouple oxidative phosphosylation.

(a) *Hexachlorophene:* This is a chlorinated bisphenolic compound which has a broad spectrum of biological activity and a narrow margin of safety. It has had a limited degree of use for immature *Fasciola* infections. Being a phenol it might be expected to uncouple oxidative phosphorylation and in this way to affect glucose uptake (see Chapter 5.3.3).

(b) *Dichlorophen.* Another chlorinated bisphenol, again exhibits a broad spectrum of biological activity and has had limited use as taeniacide in the dog and ruminants. Its action almost certainly arises from the phenolic structure.

(c) *Bithionol.* A chlorinated bisphenol separated by a sulphur bridge which has been useful against *Clonorchis* and *Opisthorchis* in man.

(d) *Bithionol sulphoxide.* Another chlorinated bisphenol separated by a sulphoxide bridge which shares the anthelmintic spectrum with bithionol and both substances are almost certainly active by the virtue of their phenolic structures.

2.8 SALICYLANILIDES

These substances consist of two carbocylic rings connected by a - CO-NH- group. They represent an important group of taeniacides and antitrematode drugs. In all instances at least one of the carboxylic rings has a hydroxyl group attached and the salicyl.anilides can therefore be described as phenols.

(a) Nichosamide. (N-(2-chloro-4-nitrophenyl)-5-chlorosalicylamide) can be described as a chlorinated nitrophenol. It has an important role as a broad-spectrum taeniacide with a wide margin of safety and is used extensively in the dog, cat, ruminant, horse and man. Its mode of action of anthelmintic is fully reviewed in Chapter 5.3.3.

(b) Oxyclozanide (5-bromosalicylic acid-L-bromanilide). This is an example of a salicylanilide with bromide substituents as well as chlorine. It is an effective fasciolicide with a good margin of safety. Mode of action is almost certainly related to its phenolic structure, which is no doubt enhanced by halogenation.

(c) Clioxanide (2-acetoxy-4-chloro-3,5-diiodobenzanilide). This was the first salicylonide introduced for the treatment of Fascioliasi It has iodine-chlorine substituents but lacks the hydroxy phenolic strucutre. However, following introduction it was shown that the compound must be hydrolysed within the body to be free phenolic derivative which carries the anthelmintic activity. This finding proves that anthelmintic activity rests.with the phenolic structure.

Numerous other salicylanalides have been introduced as fasciolicides. All have similar properties with respect to spectrum, toxicity and mode of action.

2.9 PHENOTHIAZINE AND XANTHONES

These substances are grouped as both possess a 3-ringed structure with a heterocyclic middle ring containing a sulphur atom. With phenothiazine the second atom in the middle ring is a nitrogen and with the xanthones, the second atom is a carbon.

2.9

(a) *Phenothiazine (thiodiphenylamine)* is of great historic interest. The nucleus yields many important drugs - particularly in the nature of tranquillisers. Phenothiazine enjoyed a premium position as a broad spectrum anthelmintic over a period of some 20 years prior to the advent of thiabendazole. Despite its extensive use, the mode of action has never been satisfactorily defined. The drug concentrates in worm parasites and in some way inhibits muscular activity so that the worms are voided while still living. It apparently has a weak anticholinesterase activity and as its action is potentiated by organophosphorus compound and it is possible that cholinesterase inhibition is the basis of its effectiveness (Broome, 1962).

(b) *Xanthones.* Lucanthone and hycanthone are examples of xanthones used for the treatment of human schistosomiasis. The structure action relationships of these substances is reviewed by Gönnert and Kölling (1962) and despite extensive studies the exact mode of action of these substances has not been defined.

2.10 BENZIMIDAZOLES

Thiabendazole, the first member of this series, was introduced in the late 1950's and its advent heralded a new era in anthelmintic development. It held the premier position as a broad spectrum anthelmintic, particularly in ruminants, until the introduction of other drugs, notably tetramisole and pyrantel. Within the last few years there has been a new surge of development with the introduction of several new benzimidazoles which appear to possess unique properties particularly in their breadth of spectrum which now extends through nematodes (including lungworm) trematodes and cestodes. Aspects of pharmacology are summaries in Table 2.4

(a) *Thiabendazole.* This substance possesses the benzimidazole ring which has no remarkable features from a pharmacological viewpoint. Aspects concerning its mode of action are summarised in Table 2.4. As well as being a broad spectrum anthelmintic, the drug has powerful larvicidal properties (Standen, 1963) and it is also ovicidal. It is a powerful inhibitor of fumarate reductase (Prichard, 1970) and

TABLE 2.4 THIABENDAZOLE AND BENZIMIDAZOLES

References	Significant findings
Standen, (1963)	Thiabendazole has larvicidal properties *in vitro* at dilutions of 10^{-5}µg/ml. It is also claimed that the drug inhibits the production of eggs by the worms.
Robinson *et al*, (1965)	Thiabendazole does not possess atropine-like, adrenergic blocking, or ganglionic blocking properties.
Prichard, (1970)	Thiabendazole inhibited fumarate reductase in adult *Haemonchus contortus* and the enzyme probably functions in NADH.
Malkin and Comacho (1972)	Thiabendazole and cambendazole inhibited the fumarate reductase in sensitive strain of *H. contortus* and cambendazole to a lesser degree, in a thiabendazole-resistance strain.
Van den Bossche (1972)	Mebendazole was found to inhibit exogenous glucose uptake by *Ascaris suum*.
Prichard (1973)	Thiabendazole inhibited the fumarate reductase system of the susceptible strain of *H. contorus* but no effect could be observed in the tolerant strain with a concentration of thiabendazole of 2×10^{-3}M. Cambendazole inhibited the system in both strains but less susceptible in thiabendazole tolerance strain. Mebendazole was not found to effect the fumarate reductase system in either strain.
Van den Bossche and De Nollin (1973)	Glucose uptake by *Ascaris suum* was inhibited by mebendazole.
Colglazier *et al.* (1975)	The experimentally produced cambendazole resistance strain of *H. contortus* was cross resistant to thibendazole, mebendazole and oxybendazole but not to levamisole
Heath *et al.* (1975)	Oral administration of mebendazole to a rate of 1 g/kg feed (approximately 50 mg/kg body weight/day) for 14 days killed mature and immature cysticeri of Taenia *pisiformis* in rabbit. It has a lethal effect on larval cestodes.
Rollo (1975)	Extremely potent larvicidal activity 10 pg/ml. Ovicidal 1 ppm. Antipyretic, analgesic and anti-inflamatory and fungicidal.
Romanowski *et al.* (1975)	Cambendazole inhibited fumarate reductase 20-40% (varying with drug concentration) in the cambendazole-thiabendazole resistant strain of *H. contortus* more than in the cambendazole-resistance strain. It showed that cambendazole was a more potent inhibitor than thiabendazole.

thiabendazole resistance strains helminth have arisen under field conditions.

(b) *Cambendazole* is an example of benzimidazole which has a carbamate function in position 5 - in common with mebendazole, parbendazole and oxybendazole. On the basis of its structure (Van den Bossche, 1972) postulated that like mebendazole, it would inhibit glucose uptake. The pharmacology of this substance is discussed in full in Chapter 5, Section 5.6

(c) *Mebendazole*. The pharmacology of this anthelmintic has been fully reviewed by Van den Bossche, (1972) and Van den Bossche and De Nollin (1973). The drug has broad spectrum of activity extending from gut-dwelling nematodes to lungworm and tapeworm. It is considered to act by interferring with glucose uptake and thus disrupting the worm's carbohydrate metabolism and energy sources (Chapter 5.3.1).

(d) *Fenbendazole*. This substance has been recently introduced and is apparently active against the histotrophic phases of *Ostertagia* in cattle. The substance appears to upset glucose absorption in *A. suum* and also interferes with the breakdown of glycogen to glucose. In addition the substance may have a neurotoxic effect (Düvell, 1977)

(e) *Oxybendazole*. This substance has only just been introduced and it possesses a remarkable spectrum being active against gut-dwelling nematodes, lungworms, *Fasciola* and cestodes. Its mode of action has been discussed by Campbell (1977) who implied that although oxyhendazole possesses a carbamate function its mode of action may be related to inhibition of fumarate reductase.

2.11 IMIDAZOTHIAZOLE DERIVATIVES

(a) *Tetramisole (2-3,5,6- tetrahydro-6 phenyl-imidazo (2,1-b thiazole))*. The discovery of this potent broad spectrum anthelmintic was reported in 1966 (Thienpont *et al*.1966) and its biological actions with respect to pharmacological effects on host and worms are summarised in Chapter 3, Table 3.4. The substance consists of mixture of D and L-isomers and subsequently it was shown that the anthelmintic action resides in the L-isomer. It is in this form (levamisole) that

the substance is now available.

It was initially considered that the mode of action arose from the substance's ability to inhibit fumarate reductase (Van den Bossche and Janssen, 1967). However, later work has suggested that the drug may exert its anthelmintic action by acting as a ganglion stimulant within helminths, thus interfering with the normal motor activity. This finding is consistent with the earlier reports of Forbes (1972b) who showed that levamisole possesses distinct nicotine-like properties in the dog. The present studies (Chapter 3) show that the drug possesses similar properties in sheep.

2.12 CYCLIC AMIDINES

Investigation of large numbers of cyclic amidines (Mc Farland, 1972) led to the introduction of pyrantel and subsequently morantel. The biological actions of these substances are reviewed in Chapter 3, Table 3.3. It should be pointed out that pyrantel closely resembles in chemical structure the substance thiazathienol, which was the precursor of the imidazothiazole series and tetramisole and levamisole. Pyrantel shares the biological properties of tetramisole and is claimed to act as a neuromuscular blocking agent in helminths. In our view, pyrantel is a similar substance to levamisole but is less readily absorbed and consequently its spectrum is limited to gut-dwelling helminths. The effects, when given intravenously in dog and sheep, exactly mirror those of tetramisole (Chapter 3). Morantel is the methyl ester of pyrantel and it appears to be in all ways similar to the parent compound (Chapter 3).

CHAPTER 3 PHARMACODYNAMIC STUDIES RELATED TO ANTHELMINTIC ACTION AND THEIR EFFECTS ON HOST CHOLINESTERASE

3.0

3.2.3 Results

(a) Red cell and plasma cholinesterase activities

(b) Inhibition of cholinesterase in vitro

(c) Inhibition of cholinesterase in vivo

3.2.4 Discussion

(a) Red cell and plasma cholinesterase

(b) Inhibition of cholinesterase in vitro

(c) Inhibition of cholinesterase in vivo

3.

3. PHARMACODYNAMIC STUDIES RELATED TO ANTHELMINTIC ACTION AND THEIR EFFECTS ON HOST CHOLINESTERASE.

3.0 INTRODUCTORY REMARKS

The term pharmacodynamics refers to the action of drugs on living organisms, where the object is to enhance or inhibit the subjects normal physiological mechanisms, in such a way as to alleviate symptoms or produce desirable effects. In the context of the use of anthelmintics, the pharmacodynamics is of most significance with respect to the toxicity and side-effects concurrent with drug use. However, there is a second connotation and this refers to interpretations of the basic actions of the drug which may be deduced from laboratory studies on vertebrates. It must be borne in mind that most existing pharmacological knowledge arises from vertebrates studies - although the reverse situation sometimes applies in studies of fundamental mechanisms. For example, most of our basic knowledge of transmission along nerve trunks arises from the use of the giant squid axon as an experiment model. The present work is divided into two areas, the first concerned with the actions of methyridine, pyrantel, morantel, diethylcarbamazine, tetramisole and levamisole in the dog and sheep (3.1); the second with the actions of eserine, pyrantel and levamisole on blood cholinesterase of sheep (3.2.).

The work on pharmacological action of anthelmintics has been developed in conjunction with pharmacological teaching and a portion of the work, particularly related to the findings with the dog, has been previously published (Forbes 1971, 1972a,b). The present study includes unpublished work undertaken with the chief supervisor. The candidate undertook the original development of the neuromuscular preparation from the gastrocnemius of the sheep.

3.1 PHARMACOLOGICAL STUDIES WITH METHYRIDINE, DIETHYLCARBAMAZINE, PYRANTEL, MORANTEL, TETRAMISOLE AND LEVAMISOLE.

A synopsis of the literature related to the various anthelmintics are

TABLE 3.1 METHYRIDINE. BIOLOGICAL ACTIONS ESPECIALLY IN RELATION TO MODE OF ANTHELMINTIC ACTION

References	Preparations used and significant findings
Broome (1961)	*In vitro* experiments with intact nematodes *(Ostertagia, Nematospiroides dubius, Heterakis spumosa, Nippostrongylus muris* and *Ascaris)* methyridine penetrates cuticle and produces "irreversible" paralysis, probably due to neuromuscular blockade of the decamethonium type. Drug widely distributed and distribution related to pH and the basic properties of the drug. Paralysing doses have no influence on oxygen uptake.
Broome (1962)	Methyridine and acetylcholine produce contracture of exposed *Ascaris* muscle but the effect of methyridine is not reversible. Both methyridine's and acetylcholine's effect are blockaded by d-tubocurarine and piperazine. Methyridine regarded as a depolarising drug on both worms and hosts, but the worms may be more sensitive.
Fiakpui (1967)	Using the free-living nematode *Caenorhabditis briggsae,* methyridine was shown to cause a contracted paralysis which was not reversible and was antagonised by piperazine.
Sanderson (1969)	At concentrations which paralyse *Nippostrongylus brasiliensis* glucose uptake was not affected.
Mc Farland (1972)	Methyridine a weak neuromuscular blocking agent and a weak inhibitor of cholinesterase.
del Castillo (1969)	Confirmed decamethonium type blocking action of methyridine.
Coles (1974)	The presence of levamisole at the receptor sites blocks the action of methyridine and it appears the two substances may act at the same site.

TABLE 3.2 DIETHYLCARBAMIZINE. BIOLOGICAL ACTIONS ESPECIALLY IN RELATION TO MODE OF ANTHELMINTIC ACTION

References	Preparations used and significant findings
Gonzalez-Barranco et al. (1962)	At 10µg/ml increases motility of *Onchocerca volvulus* microfilariae and adults but eventually paralyses the former.
Harned et al. (1948)	High therapeutic ratio. Intravenously in dogs, causes rise in blood pressure.
Thevathasan (1970)	Diethylcarbamazine produces an eosinophilia in adrenalectomised - splenectomised rats.
Forbes (1972a)	In the dog doses of diethylcarbamazine citrate of 2.5 to 10 mg/Kmg caused a precipitous hypertension and a sharp increase in rate and depth of respiration. These effects are identical to those of low doses of nicotine and are anatogonised by hexamethonium. The hypertensive effects were selectively inhibited by phentolamine (1mg/Kgm).
Abaitey and Parratt (1976)	Drug has marked effects on sympathetic ganglia of anaesthelized cats.
Abaitey and Parratt (1977)	Diethycarbamazine was examined on guinea-pig isolated ileum, rabbit duodenum, chick oesophagus, rat portal vein and pig coronary artery. The drug contracted all gut muscles and its action was antagonised by hexamethonium and atropine. Diethylcarbamazine possesses nicotine-like properties.

given in Tables 3.1, 3.2, 3.3 and 3.4, respectively.

3.1.1 Introduction

(a) Methyridine.

Anthelmintic aspects of the use of this drug have been reviewed in Chapter 2 and various biological actions are outlined in Table 3.1 In brief, methyridine is a narrow spectrum anthelmintic with a low margin of safety. It is basic substance which ionises at body pH and its wide distribution in the body is related to the proportion of non-ionised species (Broome, 1962). It has a low margin of safety and is known to cross-react with diethylcarbamazine, so that the two drugs given together have additive effects or one may potentiate the other, so that toxicity is exacerbated. This inter-reaction suggests both substances act on the same receptor within the body. There are reports of pharmacodynamic studies with small laboratory animals (Eyre, 1970) but no reports of similar studies in large animals.

(b) Diethylcarbamizine (Table 3.2).

Anthelmintic use of this drug has been discussed in Chapter 2. Following its introduction in about 1945, the drug has retained its pre-eminent role in the control of filariasis and must still be regarded as a most important therapeutant in tropical medicine. Aspects of its pharmacology are reviewed in Table 3.2. The early work of Harned *et al.* (1948) showed a very wide margin of safety and only very mild pharmacodynamic actions. Forbes (1972a) reported the actions of diethylcarbamizine in dogs and showed that a somewhat high dose intravenously caused reactions typical of those of low doses of nicotine. These reactions were blocked by ganglion-blocking agents and hypertensive effects were selectively inhibited by the alpha adrenergic blocking agent, phentolamine. These findings fit exactly the assumption that diethylcarbamazine elicits its responses by affecting nicotine-like receptors within the body.

(c) Pyrantel and morantel.

Pyrantel was first introduced and given wide acceptance as a drench in ruminants. Toxicity problems associated with absorption by the lung appear to have caused the manufacturers to introduce the methyl derivative (Morantel) in its place. In essence, the two substances

TABLE 3.3 PYRANTEL. BIOLOGICAL ACTIONS ESPECIALLY IN RELATION TO MODE OF ANTHELMINTIC ACTION

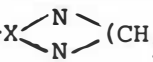
References	Preparations used and significant findings
Lynch and Nelson (1959)	Screened in mice, 2-(thenylthio-2-imidazoline) showed nematocidal activity. In sheep, however, shows little activity-decomposing by hydrolysis to 2-thenylthiol and 2-imidazoline. Methyl substituted for R results in increase in potency.
Pfizer International (1967)	ID50 (bovine erythrocyte cholinesterase)= 9.4 x 10^{-5}M. Local anaesthetic properties. Amine end of the molecule considered analogous to the choline portion of acetylcholine and that anticholinesterase activity and related neuromuscular blocking, ganglion stimulant and spasmogenic results from competitive action with acetylcholine.
Aubrey *et al.* (1970)	(1) Rat phrenic-nerve diaphragm (2) chick semi-spinalis muscle (3) toad rectus-abdominus (4) contraction of the soleus and tibilalis-anterior muscles of the anaesthetised cat. Pyrantel caused blockade of nerve stimulation greater than that of decamethonium deepened by physostigmine and surmounted by tetanic stimulation.
Eyre (1970)	(1) Guinea-pig isolated ileum (2) cat nictitating membrane (3) rat isolated phrenic-diaphagm (4) chick isolated biventor nerve muscle preparation (5) chick isolated semispinalis muscle (6) peroneal nerve digital extensor muscles, respiration and carotid blood pressure of the rabbit. (7) cholinesterase activity. Pyrantel caused - (1) sustained contraction spontaneous spikes when washed from the bath blocked by hexamethonium and atropine (2) contraction and hypertension (antagonised by hexamethonium) and transient dyspnoea (3) augmented direct and indirect twitch response (10^{-6} and 10^{-5}molar) partial neuromuscular block (10^{-5}) unaffected by tetanus, potassium or eserine. Molar EC/50 value is 1.2 x 10^{-4} (4) (a) increased twitch response (10^{-6} and 10^{-5}molar) and reversal

of tubocurare (b) reversible neuromuscular block and contraction of the muscle indicative of depolarisation (5) contraction inhibited by tubocurarine. Marked tachyphylaxis. (6) Immediate brief hypotension and apnoea followed by a more sustained pressure response anatagonised by hexamethonium (7) acetylcholinesterase of sheep erythrocytes pI50=4-5. Butyrylcholinesterase of horse plasma pI50=4.0.

O'Brien (1970) Pyrantel has half the local anaesthetic activity of lignocaine in toad nerve muscle preparations. Depolarising nerve block similar to decamethonium. Myocardial depression and vasomotor collapse when given into the lungs. Oral LD50 is 175mg/Kgm (mice) 170mg/Kgm (rats).
Pyrantel shows depolarising activity in the chick biventor cervicis muscle (a mixed muscle containing both slow and twitch fibres) but not in toad rectus. Similar, but not identical to decamethonium. Ganglion stimulant when tested on the cat nictitating membrane.

Forbes (1971) Anaesthetised dogs. Carotid blood pressure, respiration and electrocardiogram. Precipitous hypotension and hyperpnoea completely anatagonised by hexamethonium. E.C.G. showed marked tachycardia and bizarre arythmias reminiscent of adrenalin. Actions are nicotine-like.

Forbes (1972b) Anaesthetised dogs. Transient drop in blood pressure followed by pronounced hypertension together with respiratory stimulation anatagonised by hexamethonium. Phentolamine mesylate nullifies hypertension but does not affect respiration. Actions are nicotine-like.

Mc Farland (1972) Pyrantel pamoate very slowly absorbed so as to be effective against *Enterobius* in the lower bowel. Structure action relations with the protocol substance Aryl-X  (CH$_2$)n revealed 2 thienyl the most active aryl group and the tetrahydropyrimidine ring system (n=3) as generally superior to the imidazoline system (n=2).

appear to have the same biological actions so that the information shown in Table 3.3 can be expected to apply also to morantel. Pyrantel is an excellent anthelmintic with the wide margin of safety provided it does not enter the lungs. It is widely used in human medicine particularly for *Ascaris* and hookworm. Pharmacological studies in small laboratory animals have been carried out by Aubry *et al.*(1970) and Eyre (1970) and O'Brien (1970) reported pharmacological studies carried out by the manufacturers (Pfizer International). Forbes (1971, 1972b) studied the effect of the drug in anaesthesitised dogs and reported that its effects almost exactly mirrored those of diethylcarbamazine and levamisole acting as nicotine-like drugs.

(d) Tetramisole (Table 3.4)

Tetramisole is a remarkable anthelmintic both in respect to its broad spectrum, ranging from gut nematodes to *Angiostrongylus* in the brain and its wide distribution in the body. Early clinical observations indicated that the drug has cholinergic properties (Theinpont 1966; O'Brien, 1966; Forbes, 1966; Camera and Bueding, 1966) and the early work of the Belgian school (Van den Bossche and Janssen, 1967) indicated that the drug may act by inhibiting helminth fumarate reductase. However, the latest work (Coles, 1974; Coles *et al.* 1974) indicated that inhibition of carbohydrate metabolism may be only secondary to paralysis caused by tetramisole's action on the worm's nervous system. The work of Forbes (1972b) using the anaesthetised dog, has clearly indicated the ganglion-stimulant or nicotine-like properties of the drug and its actions are essentially the same as those mentioned for the three previous anthelmintics.

Levamisole is the L-isomer of tetramisole and as most of the anthelmintic activity resides in this isomer, it is now usual for tetramisole to be marketed in the form of levamisole. There are no previous reports of studies of the pharmacology of this substance in sheep.

TABLE 3.4 SHOWING BIOLOGICAL ACTIONS OF TETRAMISOLE ESPECIALLY IN RELATION TO ITS MODE OF ANTHELMINTIC ACTION

References	Preparations Used and Significant Findings
Thienpont, *et al.* (1966)	Sheep - Active by mouth or by injection. Worms often alive when excreted. Overdosage produced transient side-effects including hyperpnoea, salivation and lachrymation. Concentrations of 10µg/ml quickly paralyse adults of several species of nematodes
Walley (1966)	Sheep - Overdosage in sheep caused salivation, muscle tremors, nervous irritation, lip-licking and disturbance of gait.
Forsyth (1966)	Sheep - As for Walley (1966). Symptoms regarded as reminiscent of organophosphorus poisoning.
Kaemmera and Budden (1966)	With rats, cats and dogs pharmacological experiments showed small doses caused pressor effects in the dog but a depressor effect in the cat and rat. Larger doses depression in the cat. Repeated administration caused tachyphylaxis. In cattle and sheep tetramisole caused lachrymation, salivation, increased gut activity, chronic muscular spasms. Cattle show spastic tail erection characteristic of central nervous stimulation.
Van den Bossche and Janssen (1967)	Using homogenates of *Ascaris* incubated in anaerobic conditions DL-tetramisole was shown to be a potent inhibitor of fumarate reductase. Activity largely resides with the L-isomer.
Van den Bossche and Janssen (1969)	Homogenates of *Ascaris suum, Ascaridia galli, Toxocara cati, Dictyocaulus viviparous, Taenia taeniaeformis, T. pisiformis, Dipylidium caninum,* rat liver and pigeon breast muscle were studied. In nematodes, tetramisole reduced succinate, NAD and ATP levels and increased fumarate $NADH_2$ and inorganic phosphate levels. L-isomer the most potent. Fumarate reductase inhibited stereospecifically in nematodes, succinate dehydrogenase unaffected in other organisms tested.
Van den Bossche *et al.* (1969)	Third stage larvae of *Haemonchus contortus* can derive some energy from aerobic metabolism but under anaerobic conditions they may meet some of their energy requirements by $NADHH_2$-coupled fumarate-succinate system which is tetramisole sensitive.
Eyre (1970)	(1) Guinea-pig isolated ileum, (2) cat nictitating membrane, (3) rat isolated phrenic-nerve diaphragm, (4) chick isolated biventer nerve-muscle preparation, (5) chick isolated semi-spinalis muscle, (6) Peroneal nerve-digital extensor muscles, (7) respiration and carotid blood pressure of the rabbit (8) cholinesterase activity whole blood.

References	Preparations Used and Significant Findings
	Tetramisole cause : (1) irregular spontaneous activity abolished by atropine, (2) strong contraction of the membrane and a small brief fall in blood pressure followed by secondary rise, brief hyperventilation. Not blocked by hexamethonium. Dibenamine reversed the pressor response of tetramisole and partly inhibited action on nictitating membrane. (3) tetramisole augmented twitch responses both indirectly and directly in small doses and caused partial neuromuscular block in higher doses. (4) increased twitch and reversed tubocurarine, higher doses caused blockade. (5) produced contraction inhibited by tubocurare (6) enhanced neurotransmission (7) tetramisole caused sharp transitory fall in the pressure. Partially antagonised by atropine.
Sanderson (1970)	Tetramisole appears specifically to inhibit fumarate-succinate system, the laevo-isomer is the more potent. Tapeworm and mammalian succinate dehydrogenase are much less sensitive to tetramisole.
Denham (1970)	Tetramisole exhibits a reversible paralysing effect on exsheathed third stage larvae of *Ostertagea circumcincta* and caused some inhibition of growth in culture. (b) dorsal-nerve muscle strip. Produces rapid sustained contraction followed by paralysis. Mimicked by DMPP (dimethylphenyl-piperazinium). Laevo-isomer twice as active as the racemic form and 25 times more active than the dextro-isomer. Partially blocked by hexamethonium but irreversible.
Forbes (1972b)	Anaethetised dogs - Levamisol caused preciptors pressor effect and respiratory stimulation - antagonised by hexamethonium. Hypertension antagonised or reversed by phenotolamine. Effects attributable to sympathetic ganglion stimulation and adrenal discharge. Tetramisole possesses nicotine-like properties in common with pyrantel, morantel, diethylcarbamazine and arecoline.
Van Nueten (1972)	(a) Isolated mammalian and avian tissues. Strips of rabbit duodenum, guinea pig ileum, rat stomach and *vas deferens* of rat and guinea pig. Guinea pig ileum and *vas deferens* stimulated transmurally. Phrenic-nerve rat diaphragm and biventer cervicis muscle of chick. (b) *Ascaris* dorsal nerve-muscle strip and intact worms. L & DL tetramisole produces a reversible ganglion stimulating effect on mammalian tissues, but are devoid of antispasmodic activity or anti-cholinergic or andrenergic blocking actions caused contraction of *Ascaris* muscle and intact *Ascaris* partly inhibited by hexamethonium.
Van den Bossche (1972)	Particles (R_2) containing succinoxidase were isolated from muscle strips of *Ascaris suum*. Tetramisole shown to be a non-competitive inhibitor of fumarate reductase as measured by the anaerobic oxidation of NADH in the presence of fumerate . Both L and D tetramisoles inhibit the malate-induced -32p incorporation into organic phosphate in mitochondria isolated from *Ascaris muscle*.

References	Preparations Used and Significant Findings
Coles (1974)	Mecamylamine and pempidene when injected into *Ascaris* block the contractions caused by injected levamisole supporting Van Neuten's (1972) suggestion that levamisole acts as a ganglion stimulant. The anthelmintic exhibits tachyphylaxis and its *in vitro* paralysant action on *Nippostrongylus braziliensis* is reversible. Inhibition of fumurate reductase is unlikely to play a part in anthelmintic action
Coles *et al.* (1974)	Paralysis of *Ascaris* by levamisole is reversible and there is antagonism between levamisole and pyrantel or methyridine, suggesting that all the drugs act at the same receptor site.
Wang and Saz (1974)	L-tretramisole at low concentrations (4.2×10^{-7}M) completely stops motility of adult *Litomosoides carinii* , *Dipetalonema viteae* and *Brugia pahangi* within 1.5 minutes of exposure.
Rew and Saz (1977)	Microfilariae of *Brugia pahangi* are immobilized by levamisole and in aerobic conditions there is decreased glucose utilization and a shift towards homolactate fermentation. The metabolic effects are probably secondary to paralysis.

3.1.2 Materials and Methods

Dogs of various ages, breeds and sex were used. They were anaesthetised with pentobarbitone sodium or alternatively, anaesthesia was induced with thiamylal sodium and maintained by infusion with chloralose. Sheep of various ages ranging from 20 - 40 kg were employed and anaesthesia was the same as for dogs. The use of chloralose favoured better reflex activity in the anaesthetised animals and maintained higher levels of blood-pressure. However, it should be pointed out that we have found the use of chloral or chloralose in association with catecholamines (or drugs which stimulate release of endogenous catecholamines such as nicotinic drugs) may be hazardous. On occasions we have experienced sudden death in experimental animals, after injection of, for example, adrenaline. The syndrome is one of the sudden and complete cessation of cardiac output, with a fall in blood pressure to zero. These effects undoubtly result from ventricular fibrillation produced by the catecholamine acting on the myocardium sensitised by chloralose. Blood pressure was recorded from a mercury manometer connected to the carotid artery, respiration from a tambour connected with a T-shaped tracheal cannula, and gut movement from a water-filled balloon inserted in the duodenum 20 cm behind the pylorus. On occasions electrocardiograph recordings were made in the conventional fashion.

In addition, a neuromuscular preparation using the gastrocnemius muscle of the sheep was developed for research and teaching purposes and this is the first report of the use of the animal for these purposes. The skin from the region of the stifle to below the hock was reflected and the biceps femoris muscle dissected to reveal the sciatic nerve. The tibialis nerve from the sciatic nerve supplying the gastrocnemius muscle was isolated for electrical stimulation and close arterial injections were made by retrograde perfusion of a side branch of the femoral artery. The muscle tendon was attached by a strong ligature to weights suspended by use of a pulley and recordings were made on a smoked drum from another ligature attached to a lever by means of a pulley system. Methyridine was used in the form of Promintic (I.C.I) and diethylcarbamazine citrate as a 40 per cent solution (Boots Drug

3.1.2

Company), both prepared for injection in cattle. Pyrantel, morantel and tetramisole were used as watery solutions of pure drugs.

3.1.3 Results

(a) Methyridine

The effect of methyridine (10 mg/kg) on the blood pressure, respiration, intestinal movements of a dog anaesthesitised with pentobarbitone sodium is shown in Fig 3.1. It can be seen that there is a sharp,brief fall in blood pressure followed by a precipitous secondary rise and a gradual return to normal over a period of a few minutes. There is a temporary period of apnoea lasting a minute or so followed by a prolonged period of hyperpnoea corresponding roughly to the period of hypertension. There is also initially a sharp intestinal spasm lasting about the same period as the apnoea followed by increased intestinal movements.

Administration of hexamethonium (1 mg/kg) eliminated the hypotension, the hyperpnoea and reduced the intestinal movements. The alpha-adrenergic blocking agent phentolamine (1 mg/kg) was given and this reduced blood-pressure slightly. When methyridine was given after the blocking agent, the hypertensive and intestinal effects were abolished but the hypotensive effect and action to cause apnoea followed by hyperpnoea remained more or less unchanged (Fig 3.2A).

For comparison, a small dose of nicotine alkaloid (2.5 µg/kg)was given following a dose of phentolamine (Fig 3.2B). It can be seen that the result almost exactly matches the effect of methyridine after phentolamine (Fig 3.2.A) Subsequently, atropine (0.1 mg/kg) was given and the procedure with methyridine and nicotine repeated. Following this treatment it was found that the small hypotensive phase was abolished but the effects on respiration were unaltered.

(b) Diethylcarbamazine

The actions of this drug in the dog have been published previously (Forbes, 1972a). The responses are dose-related, doses of 2.5 mg/kg producing a modest rise in blood pressure and respiratory stimulation (Fig 3.3.A). Four times this dose produced a precipitous biphasic hypertension and respiratory stimulation (Fig 3.3.B). All these effects are blocked by hexamethonium and the hypertensive phase alone is abolished or sometimes

Fig 3.1 Showing the effects of methyridine (10mg/Kg) on the blood-pressure (upper tracing) respiration (middle tracing) and duodenal movements (lower tracing) of a dog anaesthetised with pentobarbitone sodium.

Fig 3.2 Dog and recording as for Fig 3.1.
(A) Methyridine (10mg/kg) given after phentolamine (1mg/kg).
(B) Nicotine (2.5µg/kg) given after phentolamine (1mg/kg).

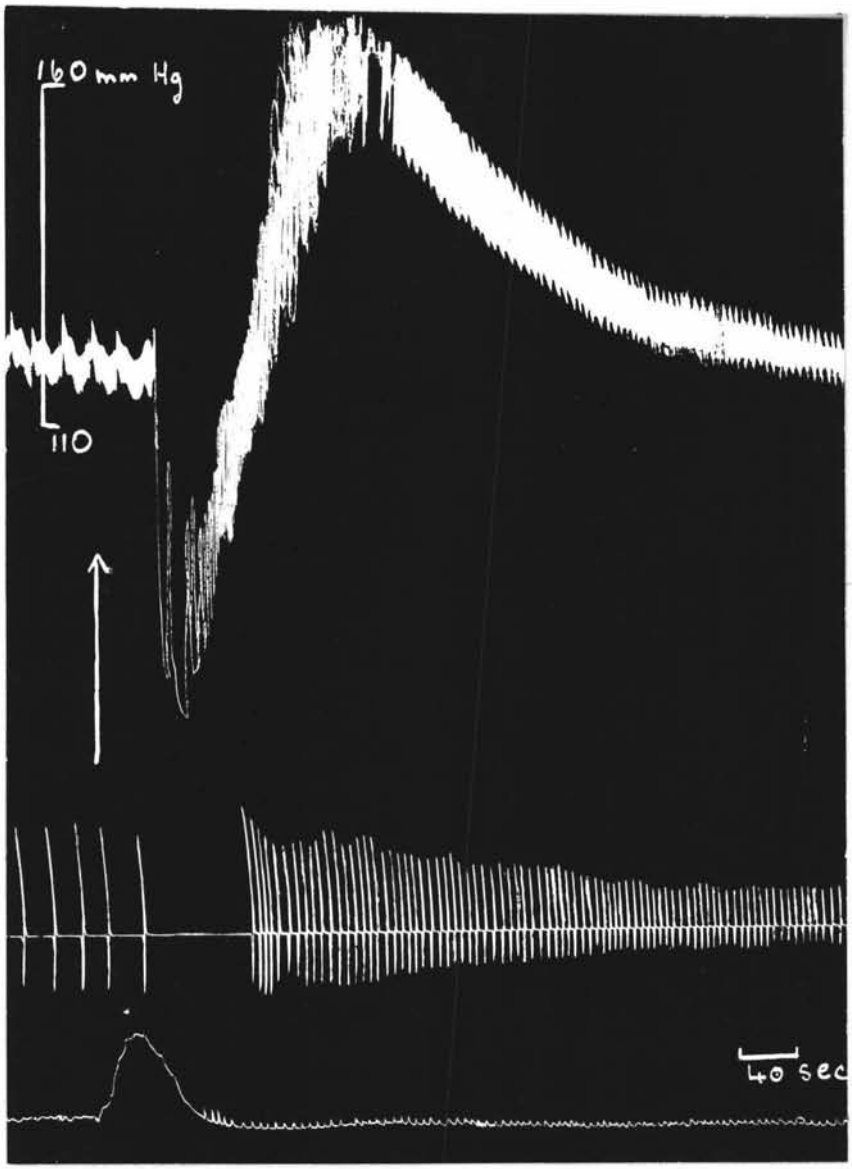


Fig 3.1

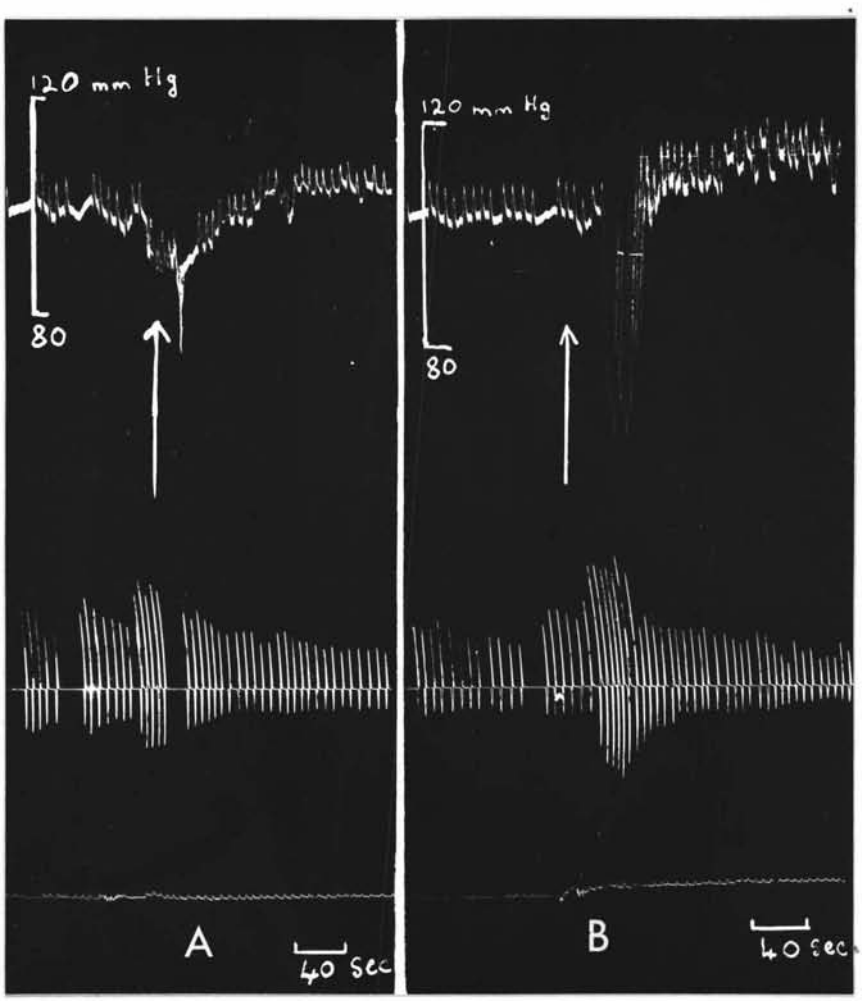
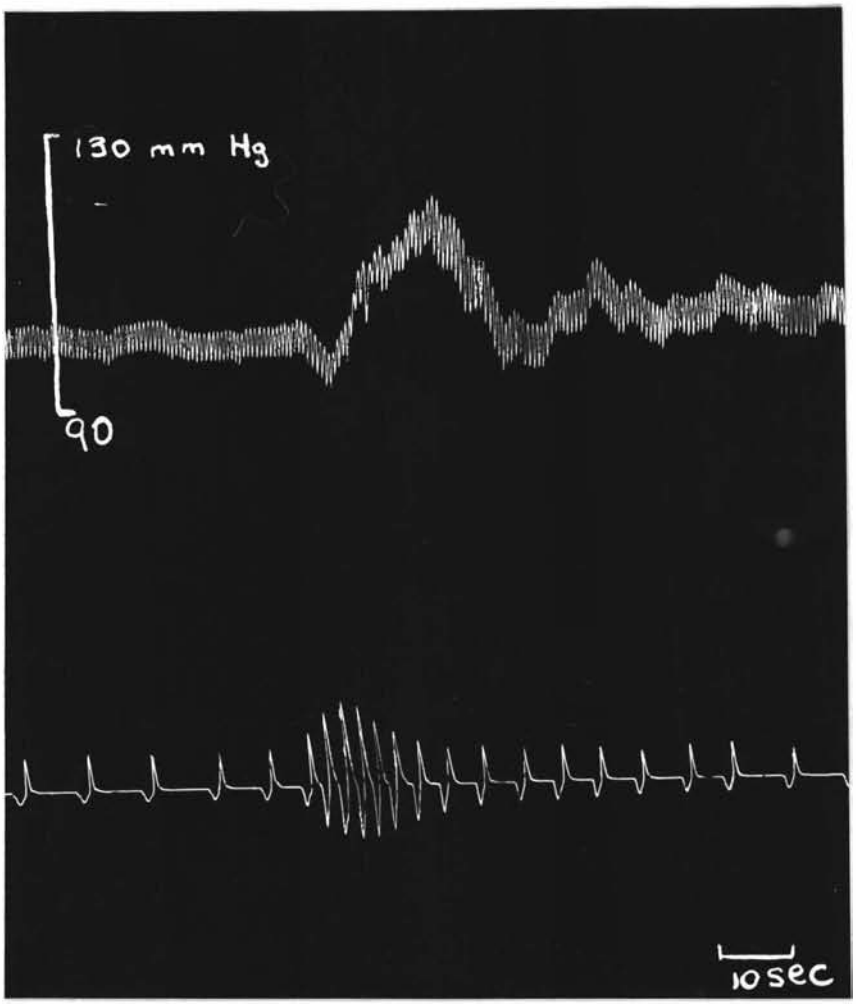


Fig 3.2

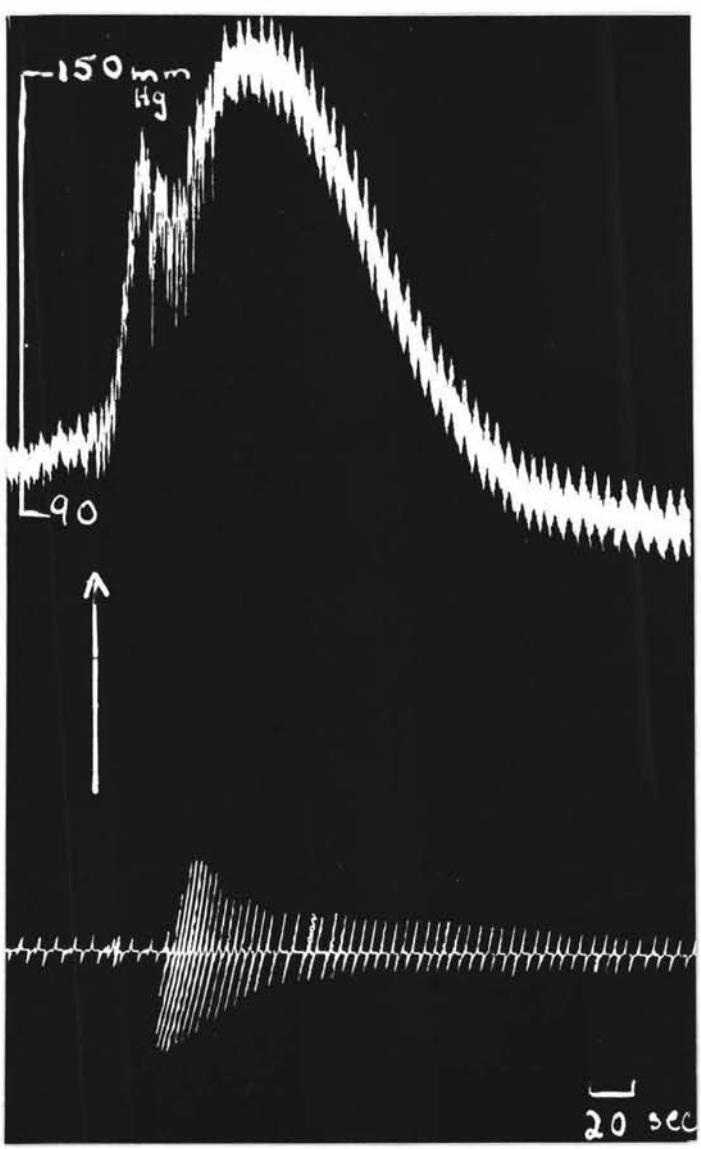
Fig 3.3 Showing the effect of diethylcarbamazine on blood pressure (upper tracing) and respiration (lower tracing) of dog anaesthetised with pentobarbitone sodium.

(A) Dose, 2.5mg/kg diethylcarbamazine.

(B) Dose, 10mg/kg diethylcarbamazine.



A



B

Fig 3.4 Showing the effect of pyrantel (2mg/kg) on the blood pressure (upper tracing) and respiration (lower tracing) of a sheep anaesthetised with chloralose.

Fig 3.5 Showing the effect of morantel (2mg/kg) on the blood pressure (upper tracing) and respiration (lower tracing) of a dog anaesthetised with chloralose.

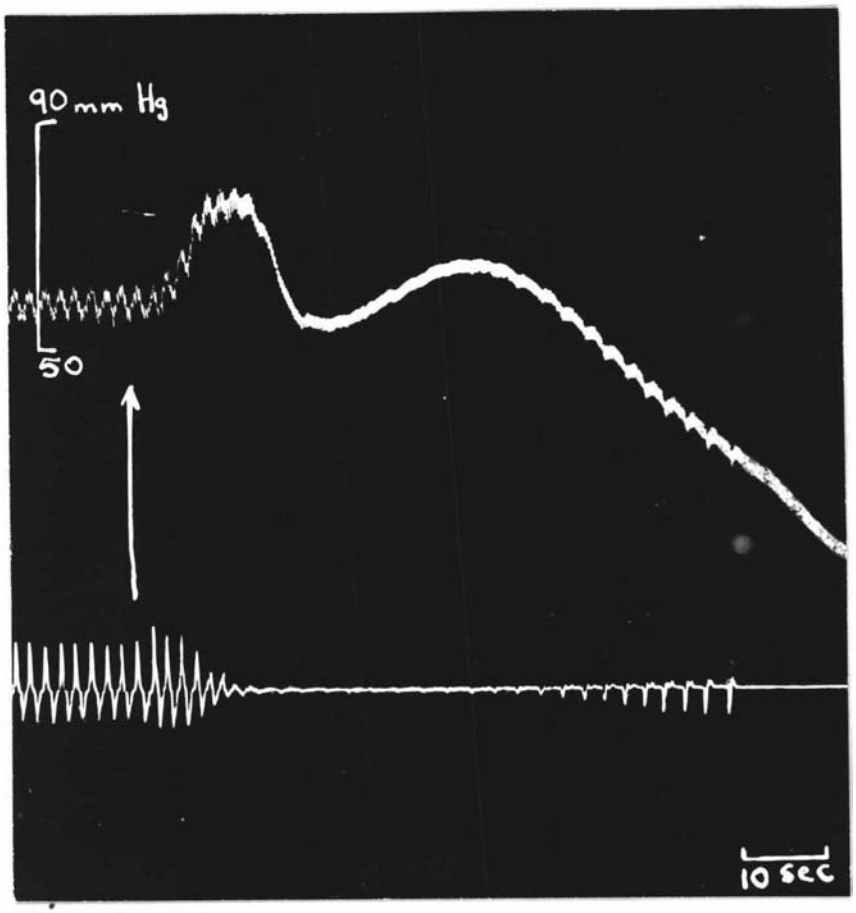


Fig 3.4

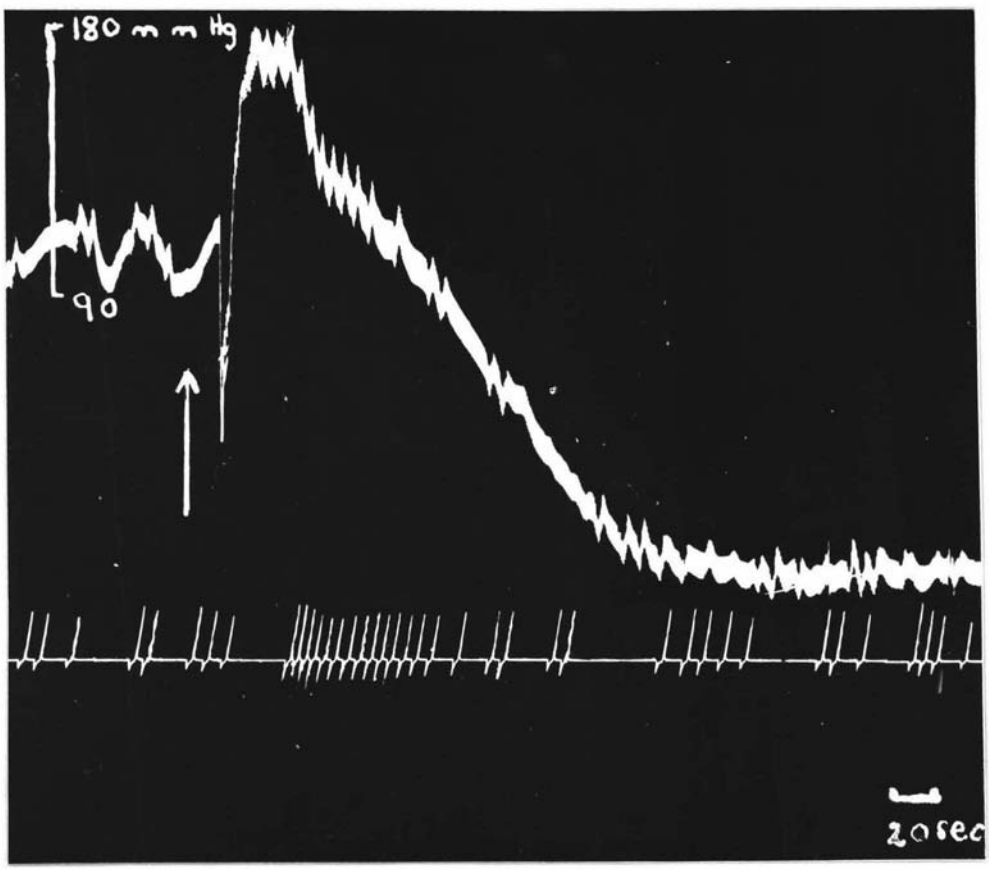


Fig 3.5

reversed by prior administration of the alpha-adrenergic blocking agent, phentolamine (Forbes, 1972a). In the sheep, the drug produces similar effects except that the respiratory action is to cause apnoea, rather than stimulation.

(c) Pyrantel and morantel

Pharmacological actions of pyrantel in the dog have been reported previously (Forbes, 1972). The drug gave exactly the same response as did diethylcarbamazine. The action was blocked by a hexamethonium and the hypertensive phase subject to blockade by phentolamine.

In the sheep anaesthetised with chloralose, the effects of 2 mg/kg pyrantel are demonstrated in Fig 3.4. This dose proved fatal. It can be seen that there is a primary rise in blood pressure followed by a fall, a secondary rise and eventual complete loss of vascular tone. Respiration, rather than being stimulated, was depressed.

The effect of morantel (2 mg/kg) in a dog anaesthetised with chloralose is shown in Fig 3.5. This information has been published previously (Forbes, 1972b). The effects are the same as those of pyrantel. There is a sharp fall in blood pressure followed by a rise concurrent with respiratory stimulation. All these effects are blocked by hexamethonium and the hypertensive phase is obliterated or reversed by the use of phentolamine.

The effect of morantel in the sheep has not been reported previously. The response to a dose of 2 mg/kg is shown in Fig 3.6. It can be seen that there is precipitous rise in blood pressure, then a secondary fall followed by another rise and gradual falling away of pressure. As with pyrantel in the sheep, there is a period of apnoea following the injection and this is in direct contrast to the findings in the dog.

(d) Tetramisole and levamisole

The effect of intravenous levamisole in the dog has been reported previously (Forbes, 1970b). Injection of the drug was followed by brief hypertensive stage then a precipitous hypertension which was often biphasic in nature. The respiration was stimulated with the typical

Fig 3.6 Showing the effects of morantel (2mg/kg) on blood pressure (lower tracing) and respiration (upper tracing) of a sheep anaesthetised with chloralose.

Fig 3.7 Showing the effects of levamisole on the blood pressure (upper tracing) and respiration (lower tracing) of a sheep anaesthetised with chloralose.

(A) A dose of 5mg/kg levamisole.
(B) As for (A) given 10 minutes after (A).
(C) As for (A) given 5 minutes after (B).

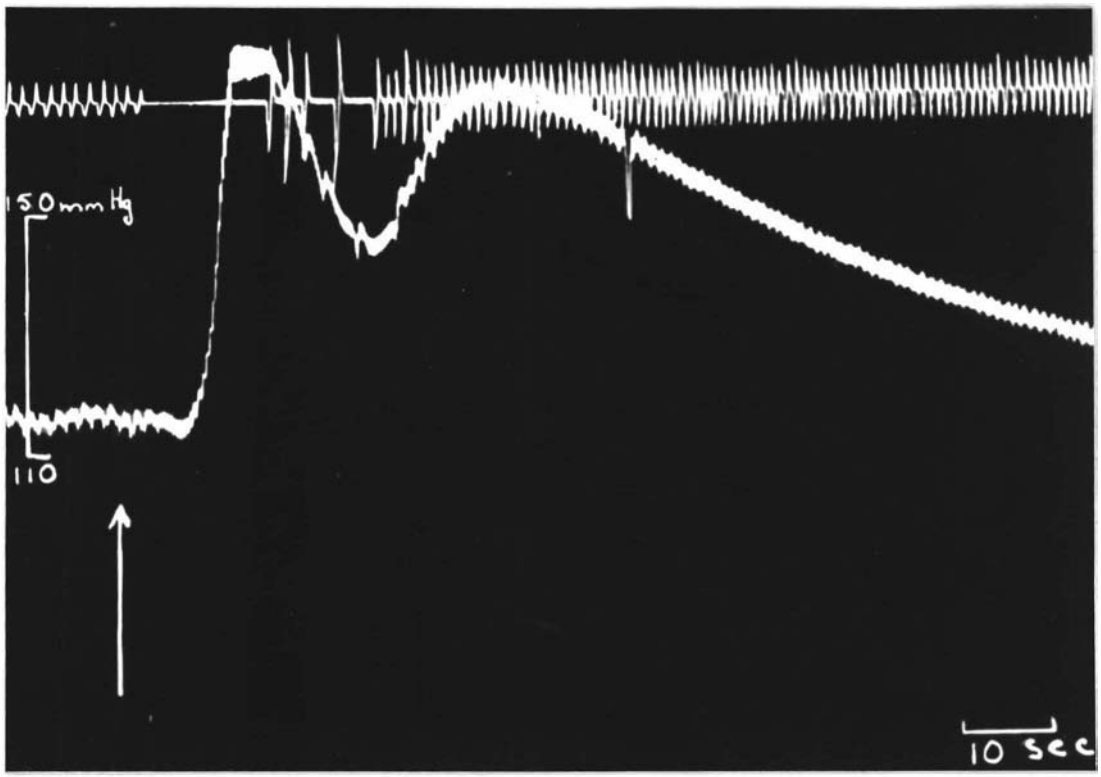


Fig 3.6

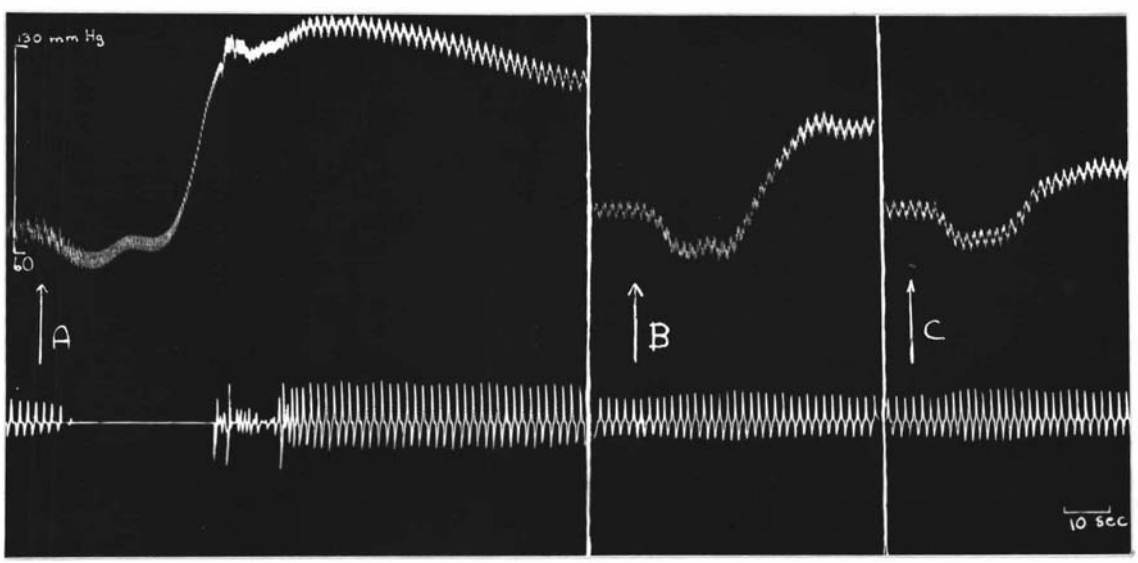


Fig 3.7

3.1.3

nicotine-like response.

Again sheep gave a distinct response with this anthelmintic. Low doses (in the order of 0.5 mg/kg) gave a brief hypotension but as the dose was increased a secondary hypertensive stage dominated the reaction. A typical response to 5 mg/kg is shown in Fig 3.7A. Here there was a slight hypotension which lasted some thirty seconds, followed by a precipitous hypertension. Again, as previously observed with nicotine-like drugs, in the sheep the administration was immediately followed by a period of apnoea.

Tachyphylaxis (the development of acute tolerance) is a recognised feature of the action of tetramisole both in the vertebrate and the helminth parasite. Fig 3.7A,B and C illustrates this phenomenon with levamisole as far as the nicotinic responses in the sheep are concerned. Fig 3.7A illustrated the typical results of a 5 mg dose and B, the same dose repeated 10 minutes later. It can be seen that there is a marked reduction in response over the 10 minute interval and an even further reduction when the dose is repeated again 5 minutes later at C. This is a typical example of the tachyphylatic response so characteristic of nicotine-like drugs.

The sheep gastrocnemius preparation was used to examine the effect of the levamisole given by close arterial injection. The drug produced a temporary enhancement of the twitch response followed by depression and again this is a typical nicotine-like effect.

3.1.4 Discussion

The pharmacodynamics of methyridine in the dog have not been reported previously. The fact that the reactions are obliterated by the prior administration of the ganglion blocking agent, hexamethonium, indicates that methyridine is exerting its effect on ganglia. The use of the adrenergic blocking agent, phentolamine, has shown that the hypertensive phase results from endogenous catecholamine release. This could be expected to be from sympathetic nerve endings following sympathetic ganglion stimulation and from the adrenal medulla,

following stimulation of that organ. The reaction of methyridine after phentolamine, is almost exactly mirrored by low doses of nicotine, suggesting again, that the hypotension results from ganglion stimulation and it seems most likely that methyridine stimulates the parasympathetic ganglia within the cardiac musculature. The same explanation would also be employed for the effects on respiration, where stimulation of parasympathetic ganglia within the lung might be expected to cause broncho-constriction and the short period of apnoea. These contentions are further supported by findings that both effects are blockaded by atropine.

In relation to diethylcarbamazine, there is a slight hypotensive phase in lower doses, but at the high dose (10 mg/kg) the effects are purely hypertensive. In the tracing shown (Fig 3.3B) the hypertensive phase is biphasic. The first peak undoubtedly results from sympathetic ganglion stimulation with consequent release of catacholamine from the postganglionic nerve endings to cause generalised vasoconstriction in the visceral region. The second peak almost certainly arose from the release of catacholamine from the adrenal medulla and its subsequent distribution by the circulation causes further vasoconstriction.

In the dog, pyrantel, morantel and tetramisole all produced similar effects. Previous studies have shown that these undoubtedly resulted from the effects on nicotinic receptors. In the sheep the vascular effects are the same as those in the dog but the respiratory effects are distinctly different and in our experience the pattern of response to nicotine-like drugs between the dog and sheep have been quite consistent. Almost invariably, nicotine-like drugs produce respiratory stimulation in the dog whereas almost invariably they produce apnoea in sheep. The reason for the failure to stimulate respiration is unknown, but preliminary experiments (unpublished) indicate that apnoea may arise from paralysis of the respiratory muscles. It is interesting to note that nicotine-like drugs have been recommended as respiratory stimulants for use in overcoming respiratory depression during anaesthesia. The recommendations have undoubtedly arisen from

3.1.4

experiments using animals other than the sheep. It is obvious that the use of the drug for this purpose is contra-indicated, certainly for sheep and possibly in other ruminants and herbivores as well.

3.2 EFFECTS OF ANTHELMINTHICS ON SHEEP BLOOD CHOLINESTERASE

3.2.1 Introduction

Some organic compounds used as anthelmintics in livestock can cause toxic symptoms in the host animal. This effect, common in the case of the organophosphates and extending to other agents such as insecticides, is thought to be largely due to the inhibition of cholinesterase activity and consequent accumulation of acetylcholine.

Although there is a poor correlation between toxic reactions and inhibition of cholinesterases (see section 3.2.1.b) the loss in activity of blood cholinesterase continues to be used as an index of anticholinesterase toxicity because of the ease of sampling and measurement.

The present work consists of a small investigation of the blood cholinesterase activities of three species, the effect of anticholinesterases including anthelmintics on these *in vitro*, and the time course of sheep erythrocyte (RBC) cholinesterase following administration of anticholinesterases *in vivo* by injection or by oral drenching.

(a) Cholinesterases of vertebrate blood

The blood of vertebrates usually contains at least two enzymes capable of hydrolysing acetylcholine, both of which are sensitive in some degree to cholinesterase inhibitors. However, there is considerable species variation in the relative amounts, sensitivities and substrate specificities of these enzymes, so that the expected response to any inhibitor depends on the species and conditions of assay.

In most vertebrates, erythrocytes contain 'true' acetylcholinesterase which hydrolyses acetylcholine and acetyl-β-methyl choline and plasma contains 'pseudo' cholinesterase which hydrolyses acetylcholine, benzoylcholine and butyrylcholine. (See section 4.1 in the next chaper) However, exceptions to this general rule exist. Rabbit plasma shows high activity towards acetyl-β-methylcholine and low activity towards

3.2.1

either butyrylcholine or benzoylcholine suggesting the presence of a greater amount of the true than pseudocholinesterase in rabbit plasma (nabb and Whitfield, 1967). Furthermore, rat plasma has both true and pseudocholinesterase (Nabb and Whitfield 1967). Although high rates of butyrylcholine hydrolysis by mammalian true cholinesterases are rare (Davison, 1953; Koelle, 1953; Whittaker, 1951). chicken plasma (Mendel *et al.* 1943; Earl and Thompson, 1952) avian plasma (Augustinsson 1971) and arthropod acetylcholinesterases are also known which hydrolyse both acetyl-β-methylcholine and butyrylcholine (Smallman and Wolfe, 1956; Casida, 1955).

Ruminants, the most common recipients of treatment by anticholinesterase anthelmintics, represent another special case in that they have little or not plasma pseudocholinesterase (Mendel *et al.* 1943; Gunter, 1946; Stowe 1955; Augustinsson, 1957). Sheep plasma contains no cholinesterase (Radeleff and Woodard, 1956; Lee and Hodsden, 1963), and 96% of the cholinesterase activity of whole blood of the bovine is due to erythrocyte cholinesterase (Robbins *et al.* 1958), while bovine plasma is inactive toward choline esters (Augustinsson, 1948). Therefore, whole blood samples could be used for measurement of bovine red cell cholinesterase (Robbins *et al.* 1958).

(b) Toxicity and blood cholinesterase levels

Although the measurement of blood cholinesterase activity is commonly carried out in order to assess the toxicity of drugs that inhibit cholinesterase, there is poor correlation between the blood cholinesterase activity and the severity of the symptoms.

In livestock suspected of organophosphorus poisoning, it is possible for animals to demonstrate severe symptoms-while cholinesterase levels remain relatively high but, particularly in acute organophosphorous poisoning, post mortem lesions are never outstanding and never pathognomonic; in many cases the findings are entirely negative. (Jolly, 1957; Radaleff and Woodard, 1957). Smith (1970) found no post mortem lesions in acutely poisoned ewes and lambs even though

the cholinesterase level was inhibited by 72 per cent.

It is generally considered that whole blood cholinesterase levels of less than 50 per cent are clinically significant (Fleisher *et al.* 1955). On the other hand, in the cat inhibition of up to 50 per cent of acetylcholinesterase had no effect (Heath, 1961). The degree of enzyme inhibition produced by quinuronium in therapeutic doses was 60 to 70 per cent, which was sufficient to produce acute acetylcholinesterase poisoning (Hawkings and Mendel, 1947; Nachmahnsohn and Feld, 1947). Both Cox and Baker (1958) and Haufe (1965) stated that poisoning could only be said to occur when inactivation of erythrocyte cholinesterase by more than 75 per cent of the normal was demonstrated along with a history and typical symptoms of organophosphorus poisoning. Trials of organophosphorus compounds carried out on grazing sheep by Solly (1971a) showed that, while no symptoms were apparent when cholinesterase levels were inhibited by 20 per cent, symptoms of organophosphorus poisoning developed after 4 days of grazing when the RBC cholinesterase was inhibited by 80 per cent, although none of the sheep died.

Toxicity symptoms depend on the time of exposure as well as the concentration of drug. In humans, Kaye (1967) stated that there were no toxic symptoms when blood cholinesterase levels were lowered to 0.2 'pH units' with gradual exposure to organophosphorus compounds, but the same blood chloinesterase levels decreased and toxic symptoms appeared when subjects were suddenly exposed to organophosphate. He also added that death can occur at 0.1 'pH units' of cholinesterase although people have been known to survive with much lower levels.

The rapidity with which toxic symptoms follow exposure depends on whether the compound can inhibit acetylcholinesterase directly or whether the compound must first be transformed in the body to an active form. Heath (1961) stated that the degree of inhibition by organophosphates varies with route of administration and whether it is fat soluble or water soluble, ionized or non-ionized; the

nonionized and fat soluble drugs act more quickly than the ionized and water soluble drugs.

The recovery of cholinesterase activity depends on the organophosphate involved and may take up to three months. (Medical Panel, quoted by Steyn, 1966). Recovery of blood cholinesterase activity after exposure of an animal to an organophosphate is slower for RBC enzymes than plasma enzymes (Solly, 1971b). In the dichlorvos-poisoned dog, acetylcholinesterase took at least three weeks to return to pre-treatment levels and serum cholinesterase took from eight days to over three weeks (Snow, 1971). The rapid initial return of plasma cholinesterase might be due to the fact that the liver is stimulated to increase its synthesis of cholinesterase in response to the lowered serum levels. However, RBC cholinesterase is firmly bound to the cell-membrane (Augustinsson, 1948; Ellman *et al.* 1961; Solly, 1971) therefore, the synthesis of new RBC enzyme is probably associated with the formation of new cells.

3.2.2 Materials and Methods

(a) Sample preparation

The animals used in this study were of mixed breed and sex.

Blood samples were taken into heparinised vacutainers by venepuncture of the cephalic vein in the foreleg of the dog or jugular vein of the horse and sheep. In experiments when anticholinesterases were administered to sheep, samples were taken through an indwelling polyethylene cannula in the jugular vein and both samples and cannula were heparinised immediately. Blood samples were chilled in ice at once and separation of plasma and red cells by centrifugation at 3000 rpm for 10 minutes carried out without delay - always within 1 hour. Plasma was removed and kept in an ice bath before analysis of cholinesterase activity. The white blood cells were aspirated from the red cells with a polythene tube attached to a syringe, and 1 ml of the red cells were transferred to a graduated conical centrifuge tube with about 3 ml of normal saline. The tube was stoppered and centrifuged at 3000 rmp for 10 minutes, the saline solution was sucked out and fresh saline was added into the tube up to the 4 ml mark.

3.2.2

The cells were suspended by gentle agitation with a small glass rod, and centrifuged as before. This process was repeated three times. The saline from the final wash was removed and the cells were haemolysed by adding distilled water up to a total volume of 10 ml (10 per cent dilution of red cells). The haemolysate was allowed to stand for at least 10 minutes before titration.

(b) Determination of blood cholinesterase

Several methods have been developed for the determination of blood cholinesterase enzymes. The method of continuous titration of the acetic acid liberated from the acetylcholine during the hydrolysis by the enzyme was introduced by Stedman *et al.* (1932) and modified by Glick (1937) and other research workers. Automatic titration of the acid produced by the enzyme activity has been used by Jorgenson (1959), and Nabb and Whitfield (1967) devised an automated 'pH-stat' method. The present method is based on the Nabb and Whitfield (1967) method, which does not use buffers or indicators and is rapid and simple to run.

(c) Reagents and apparatus

Acetylcholine chloride, 0.11M (2.0 g im 100 ml water)
Normal saline (9 g sodium chloride in 1 L water)
Sodium hydroxide, 0.01 N (by dilution of May and Bakers 'Volucon' 1 N sodium hydroxide).
The cholinesterase determinations were performed with apparatus consisting of a Beckman Expandomatic SS-2 pH meter; a 5 ml syringe-burette with a scale-expander (1 division equivalent to 0.005 ml) and a microtitration assembly thermostated by circulating water. In order to get a thorough and even mixing of blood enzyme, substrate and titrant NaOH solution in the sample beaker, a magnetic stirrer was used during titration. The assay of the cholinesterase activity was carried out at pH 8.0 at 37°C in a total volume of 5.0 ± 0.1 ml.

(d) RBC and whole-blood cholinesterase measurement

0.5 ml of the 10 per cent haemolysate or 10 per cent dilution of blood of dog, horse and sheep or 2 ml of sheep blood were pipetted into the titration vessel. Either eserine, levamisole or pyrantel of appropriate

concentration was added in a volume of 0.1 to 0.5 ml, followed by enough 0.9 per cent saline solution to bring the total volume to 4.8 ml. Initially, 1 or 2 drops of 0.5 N NaOH solution were added as a preliminary titration to bring the pH to almost 8.0, and 0.01N NaOH solution was added from the burette syringe to maintain a pH of exactly 8.0. The total volume of NaOH added in the preliminary titration was kept to approximately 0.1 ml to avoid significant decrease in the enzyme concentration which can change the rate of reaction. After the instrument was stabilized at pH 8.0, 0.1 ml of 0.11 M acetylcholine chloride solution was added to the solution from a microsyringe. The time was noted and 0.01 N NaOH solution was added to keep the needle of the pH meter at 8.0 $\pm$ 0.01. Burette syringe readings were recorded every 2 minutes for 10 minutes; a constant rate of reaction was obtained in the latter part of this period.

(e) Plasma cholinesterase measurement

0.5 ml of plasma was added to the reaction vessel with saline to bring the total volume to 4.3 ml, and the pH was adjusted to exactly 8.0 by preliminary titration as described for RBC. After the instrument was stabilized, 0.6 ml of 0.11 M acetylcholine chloride solution was added into the mixture and titration carried out in the same way as for the RBC enzyme.

Although the non-enzymatic hydrolysis of acetylcholine under the conditions of the assay was negligible, a small and reproducible acid-generation by plasma and RBC from dog and horse (not exceeding 0.05 micromoles/ml/min) was observed in the absence of substrate and was substracted from the catalysed rates measured above. This effect was absent in samples of sheep blood even when 2 ml sample were used and no correction was necessary.

Cholinesterase activities were calculated according to the equation: Micromoles of acetylcholine hydrolysed per minute per millilitre of plasma or red cells or whole blood.

$$= \frac{A \times N \times 1{,}000}{B \times C}$$

Where A is the titration volume in ml of NaOH delivered by the syringe micro-burette; N is the molarity of titrant; B is the time of titration in minutes; and C is the sample volume in ml.

(f) Maintenance of sheep for the in vivo study of anticholinesterases

0.3 ml of xylazine hydrochloride (2 per cent w/v 'Rompun' Bayer, Germany) was injected intramuscularly into a sheep for sedation. The side of the neck of the sheep was shaved and swabbed with alcohol. After injection of a subcutaneous local anaesthetic (2 per cent xylocaine), the jugular vein of the sheep was punctured with a 10-gauge hypodermic needle through which a polythene tube of approximately 2 mm diameter (Portex Ltd) was introduced. A horizontal mattress suture was made over the catheter at the puncture site, and surgical adhesive tape was applied around the catheter. The catheter was fixed to the skin immediately anterior to the puncture and caudal to ear, then led back along the dorsum and connected to an infusion pump.

Heparinized (15 I.U/ml) sterile 0.9per cent saline was infused continuously at 1 cm^3 hour to maintain catheter patency. Blood samples were withdrawn via a three-way tap at the infusion pump and the catheter was flushed with heparinised saline immediately after each withdrawal of blood. The sheep was kept in a metabolic crate and fed a maintenance diet of chaffed hay and concentrates, with water *ad libitum*.

After the sheep was adapted to these conditions for a few days, eserine salicylate (1 mg/kg) solution was injected subcutaneously into the flank of the sheep. Blood samples were taken through a three-way tap at the infusion pump before treatment and at 0.3, 0.5, 1.0, 24 and 48 hour-periods after injection.

A few days after the eserine injection, the same sheep was drenched with levamisole solution (Nilverm; ICI New Zealand Ltd.) at double the therapeutic dose (30 mg/kg). Blood samples were collected as before through three way taps at the infusion pump before treatment and at 0.3, 0.5, 1.0, 1.5, 2.0, 3.0, 24.0, 48.0, and 96.0 hours after drenching.

3.2.3

3.2.3 Results

(a) Red cell and plasma cholinesterase activities

The results obtained for the rates of hydrolysis of acetylcholine chloride by RBC, plasma and whole blood from dog, sheep and horse are presented in Table 3.5

The cholinesterase activities of dog RBC (1.93 μ moles/ml/min) and dog plasma (1.91 μmoles/ml/min) were similar. The cholinesterase activity of sheep whole blood (1.20 μmoles/ml/min) was about half of the activity of RBC (2.42 μmoles/ml/min) and no enzyme activity was detected in sheep plasma.

However the enzyme activity from horse plasma (4.23 μmoles/ml/min) was about twice the activity of horse RBC (2.67 μmoles/ml/min). The horse had the highest cholinesterase activity both in RBC and plasma of the three species tested.

(b) Inhibition of cholinesterase in vitro

The percentage inhibition of sheep RBC cholinesterase and the concentrations of eserine, pyrantel and levamisole producing 50 per cent inhibition (I_{50}) are presented in Table 3.6. The method for estimation of I_{50} from the data is described in detail in Chapter 4.

The values of I_{50} for eserine, pyrantel and levamisole were 2.17×10^{-7}M; 2.41×10^{-6}M and 1.41×10^{-5}M respectively. As in previous studies, eserine was the most potent, inhibiting at approximately 100-fold lower concentrations than pyrantel or levamisole. The latter two inhibitors were found to be of similar potency in inhibiting cholinesterase from the RBC of sheep.

(c) Inhibition of cholinesterase in vivo

The time course of whole blood cholinesterase activity following subcutaneous injection of eserine salicylate into sheep are presented in Table 3.7. The whole blood cholinesterase activity was decreased to 64.3 per cent as early as 18 minutes after treatment and the maximum depression to 35.7 per cent was seen at 30 minutes and 1 hour. The

TABLE 3.5

RATE OF ACETYLCHOLINE HYDROLYSIS BY BLOOD CHOLINESTERASE OF DOG, SHEEP AND HORSE

SPECIES	SAMPLE	μ MOLES/ML/MINUTE*
DOG	RBC	1.93 ± 0.105
	PLASMA	1.91 ± 0.045
SHEEP	RBC	2.42 ± 0.377
	WHOLE BLOOD	1.20 ± 0.095
	PLASMA	0.0
HORSE	RBC	2.67 ± 0.259
	PLASMA	4.23 ± 0.045

* Each figure represents the mean of 6 experiments followed by ± S.D. except for sheep plasma in which only 3 determinations were made.

TABLE 3.6

EFFECT OF INHIBITORS ON THE HYDROLYSIS OF CHOLINESTERASE FROM RBC OF SHEEP

NAME	MOLAR CONCENTRATION	% INHIBITION*	I_{50}(M)
ESERINE	$1x10^{-7}$	23.0	
	$2x10^{-7}$	45.8	$2.17x10^{-7}$
	$3x10^{-7}$	68.8	($2.15x10^{-7}$ - $2.94x10^{-7}$)
	$4x10^{-7}$	79.0	
	$5x10^{-7}$	91.8	
PYRANTEL	$1x10^{-5}$	19.4	
	$2x10^{-5}$	39.9	$2.41x10^{-5}$
	$3x10^{-5}$	60.4	($1.94x10^{-5}$ - $2.75x10^{-5}$)
	$4x10^{-5}$	70.6	
LEVAMISOLE	$5x10^{-6}$	21.2	
	$1x10^{-5}$	43.3	$1.41x10^{-5}$
	$2x10^{-5}$	52.7	($0.35x10^{-5}$ - $7.92x10^{-5}$)
	$4x10^{-5}$	66.1	

* Each figure represents the mean of 4 experiments using acetylcholine chloride by the automated "pH-Stat" method at 37 C.
Figures in parentheses show 95% confidence limits for each I_{50}.
The I_{50} was calculated from a Probit transformation of the % inhibition to give an approximately linear "dose-probit response line" which was fitted by the method of least squares.

TABLE 3.7

THE EFFECT OF ESERINE SALICYLATE BY SUBCUTANEOUS INJECTION(1MG/KG) ON WHOLE BLOOD CHOLINESTERASE ACTIVITY IN SHEEP

TIME AFTER TREATMENT (HOUR)	CHOLINESTERASE ACTIVITY* (% OF PRETREATMENT VALUE)
0.3	64.3
0.5	35.7
1.0	35.7
24.0	96.4
48.0	96.4

* Each figure represents the mean of 4 experiments.

TABLE 3.8

THE EFFECT OF LEVAMISOLE BY MOUTH(30MG/KG)† ON RBC CHOLINESTERASE ACTIVITY IN SHEEP

TIME AFTER TREATMENT (HOUR)	CHOLINESTERASE ACTIVITY* (% OF PRETREATMENT VALUE)
0.3	100.0
0.5	100.0
1.0	93.1
1.5	89.7
2.0	89.7
3.0	86.2
24.0	93.1
48.0	93.1
96.0	93.1

† Double therapeutic dose.
* Each figure represents the mean of 4 experiments.

cholinesterase activity returned to pretreatement levels one day later.

The effect of the levamisole drench on sheep RBC cholinesterase is shown in Table 3.8
There was a very slight decrease in cholinesterase activity starting from one hour after treatment which was still apparent four days later.

3.2.4 Discussion

(a) Red cell and plasma cholinesterase activities

Cholinesterases in many tissues appear to be present in large excess of need and, even within a species and tissue type, much variation in total activity is found. It is thus difficult to define the normal value of blood cholinesterases since wide variations exist between individuals in a normal group (Michel, 1954). Fancher *et al.* (1971) reported considerable daily variation in pre-treatment values of both RBC acetylcholinesterase and plasma cholinesterase in dogs. Stedman *et al. (1932)* stated that the cholinesterase activity of the serum from individual horses varies considerably. Thus, it is not surprising that the values found for blood cholinesterases in the present work on small numbers of animals differ from some reports in the literature. The cholinesterase activity of RBC and plasma of dog found in the present work is similar to that reported by Fleisher *et al.*(1955) and Nabb and Whitfield (1967). However, the cholinesterase of the same species demonstrated by Hazelwood and Heath (1976) was higher. This might be due to use of a different method, utilising different incubation conditions. The cholinesterase of RBC and plasma of dogs found here were more or less in agreement with the results of Myers and Mendel, reviewed in the literature by Wills (1972).

It has been reported that there is no cholinesterase activity or a very low activity in the plasma of sheep (See section 3.2.1). This is confirmed in the present work. The range of cholinesterase in horse plasma was reported by Stedman *et al.*(1932) (after conversion to micromoles substrate hydrolysed per millilitre per minute) as 3.48 - 6.75. The result obtained in the present work was within their range

and agreed with the result (3.9 µmoles/ml/min) reported by Becker (1965). The cholinesterase activity of horse RBC was reported as 1.5 times greater than RBC of dog (Callahan and Kruchenberg, 1967), and was similar to the result of the present study.

(b) Inhibition of cholinesterases in vitro.

The I_{50} value for eserine salicylate (7.94×10^{-6}M) obtained by Eyre (1966) was greater than the result (2.17×10^{-7}M) from the present study on the cholinesterase from RBC of sheep. However, Eyre (1970) later reported the I_{50} of eserine on cholinesterase of sheep RBC as 3.98×10^{-7}M, which agrees better with the present result. The values of I_{50} for eserine on the cholinesterase from homogenates of different worms tested in Chapter 4 ranged from 2.67×10^{-7}M to 7.84×10^{-7}M except in *Ascaris suum* which was 4.56×10^{-6}M. The sensitivity of sheep RBC cholinesterase is thus very similar to those from homogenates of different worms, with the exception of *Ascaris suis*. The value of I_{50} for pyrantel on the cholinesterase from RBC of sheep (2.41×10^{-5}M) in the present study was found to agree with the result (3.16×10^{-5}M) obtained for the same tissue by Eyre (1970). This inhibitor is thus about five to twenty times more potent against sheep RBC cholinesterase than that of the worms tested in Chapter 4 (I_{50} range $1.70 - 7.45 \times 10^{-4}$M). Levamisole was also a five to ten-fold more potent inhibitor of sheep RBC cholinesterase (I_{50} 1.41×10^{-5}M) than of different worms tested (I_{50} range 7.86×10^{-5} to 2.04×10^{-4}M) except for *Trichuris ovis*. (I_{50} 7.43×10^{-5}) where it was only three times as potent. It would seem from this comparison that the blood cholinesterase of the host animal in this case (sheep) is more susceptible to anticholinesterases than that of the parasites, when the drug is added to the tissue under the same conditions.

(c) Inhibition of cholinesterase in vivo

Cholinesterase activity of blood from sheep was depressed as early as five minutes after the subcutaneous injection of eserine. Eyre (1966) has investigated the effect of a subcutaneous injection of eserine on the whole blood cholinesterase activity in sheep. The present study showed that 64.3 per cent of whole blood cholinesterase activity was

found eighteen minutes after the subcutaneous injection of eserine into sheep. This generally agreed with the result (79 per cent after five minutes) obtained by Eyre, (1966). The cholinesterase activity was decreased to 35.7 per cent after half an hour and one hour of treatment. These results also agreed with those of Eyre (1966) which showed 37 per cent of residual cholinesterase during this period. The cholinesterase activity was found to return almost to its pre-treatment level (96.4 per cent) one day after the injection of eserine. This also agreed with Eyre (1966) who found 95 per cent to 99 per cent at one day after eserine treatment.

The cholinesterase activity of RBC from sheep was not affected significantly by a levamisole drench. The therapeutic dose of Nilverm for sheep is 15 mg/kg live-weight and the LD_{50} is 80 mg/kg. However Walley (1966b) in the United Kingdom has reported that one out of twenty sheep survived doses of 80 mg/kg. The dose used in this present experiment was double the recommended dose (30 mg/kg) but is still small when compared to the toxic dose. It may be concluded that the concentration of drug to which the sheep blood is exposed by this route of administration is relatively low.

CHAPTER 4 HELMINTH CHOLINESTERASE AND THE INFLUENCE OF INHIBITORS AND ANTHELMINTICS

4.0

4.5 DISCUSSION

4.5.1 Subcellular distribution of cholinesterase

4.5.2 Treatment with Triton X-100

4.5.3 Effect of temperature on cholinesterase

4.5.4 Effect of substrate concentration

4.5.5 Kinetic parameters of cholinesterase

4.5.6 Total cholinesterase activity of worm homogenates

4.5.7 The effect of inhibitors on helminth cholinesterases

a. Eserine

b. Levamisole

c. Pyrantel and morantel

d. Organophosphorus compounds

4.0

4. HELMINTH CHOLINESTERASES AND THE INFLUENCE OF INHIBITORS AND ANTHELMINTICS.

4.0 Introductory remarks

The existence of an enzyme which would bring about the hydrolysis of acetylcholine with 'flashlike suddenness' was predicted by Dale in 1914 some fifteen years before he and Dudley were able to show that acetylcholine is a normal constituent of animal tissues. He made his prediction on the basis of the evanescent effect of acetylcholine when injected into an animal and wrote "in the blood at body temperature it seems not improbable that an esterase contributes to the removal of the active ester from the circulation and restoration of the original condition of sensitiveness" (Silver, 1974). Loewi and Navratil (1926b) provided experimental support for this view showing eserine prolonged the effects of acetylcholine, but it was not until (1932) that Stedman *et al.* prepared the first crude extract of cholinesterase from horse serum and three years later Stedman and Stedman (1935) found the enzyme in cat brain. Since those days the acetylcholine/cholinesterase system has come to be recognised as ubiquitous throughout the animal kingdom and is now known to be of paramount significance in neuromuscular physiology, particularly in relation to muscle activity. Anticholinesterases are substances which inhibit cholinesterase and in this way allow accumulation of endogenous acetylcholine by preserving it from enzymatic destruction. These substances are of enormous significance because of their use as insecticides and anthelmintics and because of their potential toxicity.

There is much evidence to show that many parasitic helminths require a functional neuromuscular system in order to maintain their positions in their habitats and that disruption of this system will bring about dislodgement and consequent death (del Castillo, 1964). Many anthelmintics act in this way - the best known being the organophosphorus compounds, which inhibit helminth cholinesterase and so allow accumulation of acetylcholine with consequent interference with normal neuromuscular activity. Many other substances exhibit anticholinesterase properties including such as p-rosaniline, chloral and the phenothiazines.

4.0

Cholinesterases differ between species and even at different sites within the same animal and this difference may apply particularly to enzyme's susceptibility to inhibition. For example, Lee and Hodsden (1963) demonstrated that the reaction of haloxon with sheep erythrocyte cholinesterase is reversible while its reaction with *Haemonchus contortus* enzyme is irreversible. Moreover, in the case of napthalophos the I_{50} for the worm enzyme is more than 1000 times lower than for erythrocyte acetylcholinesterase. In the present studies, homogenates of *A. suum* (head and muscle)*Ascaridia galli*, *Trichuris ovis*, *Echinococcus granulosus*, *Taenia ovis* and *T. hydatigena*, and subcellular fractions of *Moniezia*, *T. ovis* and *T. hydatigena* and dog-liver where examined for cholinesterase activity and the enzymes characterised with respect to such as sub-cellular distribution effects of Triton X-100, temperature and substrate concentration. In addition the effects of eserine and the anthelmintics levamisole, pyrantel, morantel, dichlorvos and vincofos examined and the molar concentrations causing 50 per cent enzyme inhibition (I_{50}) were determined. There are no previous reports related to almost all of this work.

4.1 CHOLINESTERASES OF VERTEBRATES

Cholinesterases are enzymes that catalyze the hydrolysis of acetylcholine and related esters, and the available evidence indicates that it is not a single enzyme but rather a family. They can be further characterized as true cholinesterase and pseudocholinesterases (Mendal and Rudney, 1943), and can be classified in terms of tissue location, physiological function and substrate specificity (Michel, 1954). Acetyl, true, specific, erythrocyte, group I, or acetocholinesterase occurs in neurones, at the neuromuscular junction, in erythrocytes of many species, brain and other tissues, and in parasitic helminths. Its known physiological role is to bring about hydrolysis of acetylcholine released in the process of cholinergic transmission. Butyryl, pseudo, nonspecific, plasma, or group II cholinesterase is present in various types of glial or satellite cells of the central and peripheral nervous systems, also in plasma, liver and other organs and tissues. It also occurs in parasitic helminths. It's physiological role is not known.

4.1

The distinction between acetylcholinesterase and butyrylcholinesterase is made practically on the pharmacological effects of the anticholinesterase agents and on substrate specificity. Acetylcholinesterase hydrolyses acetylcholine at a greater velocity or rate than any other choline ester (Adams, 1949). It hydrolyses acetyl-β-methylcholine (methacholine), but not benzoylcholine (Koelle and Gilman, 1949) and is selectively inhibited by low concentrations of several bis-quaternary ammonium bases and by other inhibitors. By contrast, butytylcholinesterase exhibits a maximal rate of velocity of hydrolysis with butyrylcholine as substrate (Adams and Whittaker, 1949). It hydrolyses benzoylcholine but not acetyl-β-methylcholine (Koelle and Gilman, 1949) and is more sensitive than acetylcholinesterase to inhibition by several organophosphates such as diisopropylphosphorofluoride (DFP) mipafox, as well as some quaternary ammonium compounds.

Acetylcholinesterase is inhibited in the presence of high concentrations of acetylcholine and the enzyme decomposes acetylcholine at a higher rate than butyrylcholine which is hydrolysed at a very low rate, or not at all (Augustinsson, 1957). Subsequent investigations have shown that true cholinesterase of human erythrocytes, horse erythocytes and pigeon brain (Adams, 1949; Mounter and Whittaker, 1950; Whittaker, 1949) is most sensitive or active towards those choline and noncholine esters whose structure approaches most closely that of acetylcholine. Pseudocholinesterase exhibits maximum action to butyrylcholine in dog, horse, cat, man, duck, squirrel, ferret and pigeon (Myers, 1953). Butyrylcholine is hydrolysed more rapidly by the serum enzyme than is acetylcholine (Stedman *et al.* 1932). Horse serum hydrolyses both acetyl and butyrylcholine, but the latter substrate is attacked more rapidly than the former. This enzyme was clearly different from the liver-esterase from the pig and the cat, since these are without action on esters of choline (Stedman *et al.* 1932). Horse serum, according to Mendel *et al.* (1943) contains chiefly a non-specific cholinesterase. Human plasma, like horse serum contains a non-specific cholinesterase (Bulbring and Chou, 1947). Chicken serum hydrolysed both acetyl-β-methylcholine and butyrylcholine (Earl and Thompson, 1952). True cholinesterase of chicken brain, in contrast to pseudocholinesterase

of serum is inhibited by excess substrate (Myers, 1953). Some anomalies to this classification exist: chicken's true and pseudcholinesterases exhibit maximum activity to propionylcholine rather than acetylcholine and butyrylcholine. Certain true cholinesterases exhibited approximately equal activities to both acetylcholine and butyrylcholine. (Augustinsson, 1949).

4.2 CHOLINESTERASES OF INVERTEBRATES - PARTICULARLY PARASITIC HELMINTHS

Relatively little information is available concerning cholinesterases of parasites, when compared with the tissue cholinesterases of different species of vertebrates. The available material is summarized in Table 4.1. The presence of cholinesterase activities in cestodes, trematodes and nematodes was demonstrated by the early experiments of Bacq and Oury (1937) Pennoit-DeCooman (1940) Artemov and Lure (1941) and Pennoit-DeCooman and van Grembergen (1942). However, the methods used by these authors were later criticized as being insufficient to prove the presence or absence of a true acetylcholinesterase. Bueding (1952) first definitely identified specific acetylcholinesterase in *S. mansoni* and *Ascaris* muscle, using the manometric method of Nachmansohn and Rothenberg (1945). He found the substrate acetylcholine was hydrolysed at a high rate, whereas butyrylcholine was resistant to hydrolysis. The optimum substrate concentration for the hydrolysis by a particulate suspension of *S. mansoni* was 4×10^{-2}M and enzyme activity of *Ascaris* muscle homogenate was about fifteen times less than that of *S. mansoni* homogenate. Later Schwabe (1959) demonstrated cholinesterase activity in scoleces and brood capsules of *E. granulosus*. The work of Chance and Mansour (1953) and Sekardi and Ehrlich (1962) suggested that *F. hepatica* possess acetylcholinesterase but no pseudocholinesterase. However, Melinkhova (1970) concluded that the fluke has both enzymes and the activity of the true enzyme was about twice that of pseudocholinesterase. Melinkhova (1970) also reported that *T. muris* displayed higher activities of acetylcholinesterase and butyrylcholinesterases than did *A. suum*, *F.hepatica* and *Gnathostoma spumsosa*. However Krvavica *et al.* (1967) and Krvavica *et al.* (1971) demonstrated both

4.1. A

TABLE 4.1. A CHOLINESTERASE ACTIVITIES IN NEMATODES

Order and family	Species	Stage	Enzyme	Activity	Reference
Strongyloidea					
Strongylidae	*Oesophagostomum dentatum*	Adult	Total ChE	.00080a	Hart and Lee (1966)
	O.columbianum	Adult	Total ChE	.00267a	Hart and Lee (1966)
	O. venulosum	Adult	Total ChE	.01406a	Hart and Lee (1966)
	Chabertia Ovina	Adult	Total ChE	.00118a	Hart and Lee (1966)
Ancylostomidae	*Bunostomum trigonocephalum*	Adult	Total ChE	.03001a	Hart and Lee (1966)
Trichostrongylidae	*Haemonchus contortus*	L_3 infective	AChE	.00093a	Lee and Hodsen (1963)
		Adult	AChE	.00059a	
		Adult	AChE	.00065a	Hart and Lee (1966)
	Nippostrongylus brasiliensis	Egg	AChE	.00105a	Sanderson and Ogilvie (1971)
		L_3 Infective	AChE	.00265a	Sanderson and Ogilvie (1971)
		L_3 in lung	AChE	.00473a	Sanderson and Ogilvie (1971)
		L_3 moulting	AChE	.00848a	Sanderson and Ogilvie (1971)
		L_4	AChE	.01265a	Sanderson and Ogilvie (1971)
		L_5 immature	AChE	.01134a	Sanderson and Ogilvie (1971)
		Adult (Mixed sex)	AChE	.01593a	Sanderson (1969)
		Adult (female)	AChE	.01580a	Sanderson (1969)
		Adult (male)	AChE	.01730a	Sanderson (1969)
Metastrongylidae	*Dictyocaulus viviparus*	Adult	Total ChE	.01629a	Hart and Lee (1966)
		Adult	Total ChE	.00963a	Polyakova (1967)

Order & family	Species	Stage	Enzyme		Reference
Filaroidea	*Litomosoides carinii*	Adult	Total ChE	.04640b	Bueding (1952)
Ascaroidea	*Ascaris lumbricoides (suum)*	Adult (muscle)	Total ChE	.00125b	Bueding (1952)
		Adult	Total ChE	.00032a	Hart and Lee (1966)
		Adult (muscle)	Total ChE	.02616c	Knowles and Casida (1966)
		Adult	Total ChE	.00040a	Polyakova (1967)
		Adult	Total ChE	.00002c	Melikhova (1970)
		Adult (mixed sex)	Total ChE	.09340c	Hutchinson and Probert (1972)
		Adult (female)	Total ChE	.02510c	Hutchinson and Probert (1972)
		Adult (male)	Total ChE	.03200c	Hutchinson and Probert (1972)
	Ascaridia galli	Adult	Total ChE	.00047a	Polyakova (1967)
Trichuroidea	*Trichuris muris*	Adult	Total ChE	.00095c	Melikhova (1970)
	T. ovis	Adult	Total ChE	.00070a	Hart and Lee (1966)

TABLE 4.1 B CHOLINESTERASE ACTIVITIES IN CESTODES

Order & family	Species	Stage	Enzyme	Activity	Reference
Pseudophyllidea	*Diphyllobothrium latum*	Adult	Total ChE	.01463b	Pylkko (1956a)
Taeniidae	*Taenia taeniaeformis*	Adult	Total ChE	.00290a	Eranko *et al* (1968)
	T. saginata	Adult	Total ChE	.01473b	Pylkko (1956b)
	Echinococcus granulosus	Scolex and brood capsules	Total ChE	16.190b	Schwabe *et al* (1961)

4.1. C

TABLE 4.1 C CHOLINESTERASE ACTIVITIES IN TREMATODES

Order & family	Species	Stage	Enzyme	Activity	Reference
Fasciolidae	*Fasciola hepatica*	Adult	AChE	.00750a	Chance and Monsour (1953)
		Adult	Total ChE	.00070c	Melikhova (1970)
Schistosomatidae	*Schistosoma mansoni*	Adult (male)	Total ChE	.01820b	Bueding (1952)
		Adult (female)	Total ChE	.01840b	Bueding (1952)
		Adult (mixed sex)	Total ChE	.01840b	Bueding (1952)

Total ChE = Total cholinesterase

AChE = Acetyl cholinesterase

a = u Moles Ach hydryolysed/mg wet weight/minute

b = u Moles Ach hydrolysed/mg dry weight/minute

c = u Moles Ach hydrolysed/mg protein/minute

4.2

biochemically and histochemically that acetylcholinesterase and nonspecific cholinesterase are present in *F. hepatica*. In 1963, Lee and Hodsden demonstrated that the cholinesterase activity in *H. contortus* was inhibited by eserine and organophosphorus anthelmintics and the effect of substrate concentration and temperature in activity. The effect of pH on cholinesterase activity of *M. expansa* was studied by Winsborrow (1971). Cholinesterases of *M. expansa* and *M. benedini* by Polyakova (1966) of *D. latum* by Pylkko (1956a,b) of *Ascaris* and *A. galli* by Palyakova (1966). The acetylcholinesterase and butyrylcholinesterase activity of *E. granulosus* scoleces and brood capsules have been determined by Schwabe (1959) and of *T. taeniaeformis* by Eranko *et al.* (1968). Haites *et al.* (1972) showed that cholinesterase activity in *F. hepatica* and *A. suum*. Gear and Fripp (1974) compared the acetylcholinesterase kinetic values of *S. mansoni*, *S. haemotobium*, *S. bovis* and *S. mattheei*.

The cholinesterase activities of the nematodes species were summarised by Sanderson (1969) and Hutchinson and Probert (1972). Apart from these summaries, the Russian workers Polyakova (1966) have reported on the cholinesterase activities in *Ascaris*, *A. galli* and *D. filaria* (Melikhova 1970), the cholinesterase activities of *A. suum*, *T. muris* and *G. spumosa* (Shishov and Chuprov, 1971). Langer *et al.* (1972) reported the specific activites and Km values of the cholinesterase of *A. suum* and *H. contortus* using various substrates and inhibitors, including tetramisole.

In our present study, the cholinesterase activities of *A. suum* (muscle and head portion) *E. granulosus* (brood capsules and scoleces and cyst fluid) *T. ovis* and *T. hydatigena* were determined in addition to inhibition by eserine, pyrantel, levamisole, morantel, vincofos and dichlorvos. The potency of the various inhibitors was expressed in terms of their I_{50} values, which are defined as the molar concentration of inhibitor reducing cholinesterase activity to one half under standard conditions. However, since plots of percentage inhibition against log molar concentration are non-linear, the I_{50} values were calculated from a probit-transformation of percentage inhibition. There has been no

4.2

available data on these above determinations except for *A.suum* with eserine (Bueding, 1952; Knowles and Casida, 1966; Hutchinson and Probert, 1972) and dichlorvos (Knowles and Casida, 1966).

4.3

4.3 MATERIALS AND METHODS

4.3.1 Helminth sources

Adult *A. suum*, were obtained after stripping small intestines of pigs killed at the Kiwi Bacon Company, Longburn, New Zealand. They were washed several times in 0.9% saline at 37° C and transferred to the laboratory in vacuum flasks. Specimens of Moniezia were obtained from the intestines and *Echinococcus* cysts from the liver and lungs of sheep freshly killed at the Longburn Freezing Works. *Ascaridia galli* were obtained by the following procedure. Chicken intestines were collected from a poultry farm near Palmerston North, New Zealand, and the adult worms were washed and stored in o.9% saline in the refrigerator. Female worms were selected and the eggs teased from uteri. These ova were incubated at 37° C in a mixture of one part 2% formalin in 75 parts of water (Hansen *et al*, 1954 and 1956). The media was shaken daily and examined periodically for the development of the infective larvae - usually at about 2 weeks. An estimation of viability was made from counts of eggs containing motile larvae. Two weeks old white-leghorn chickens fed low protein poultry feed and water *ad libitum*, were infected by squirting 0.5 ml suspension of 500 viable eggs in water into the chicken crop through a glass pipette with a rubber squeezer. The *A. galli* reached the adult stage after 5 weeks. At that time the chickens were killed and the gut opened. The adult worms were thoroughly washed on 0.9% saline and blotted dry with paper-towels.

Adult *T. ovis* worms were obtained from dogs infected with *Cysticercus ovis* in a lamb carcass kindly supplied by the courtesy of Dr D.D. Heath, Wallaceville Animal Research Centre, Wellington, New Zealand. Portions of the carcass and infected heart muscle were fed to three adult dogs aged about three years and seventeen puppies aged from six weeks to two months. The animals were of mixed sex and breed. Larval *T. hydatigena* (*Cysticercus tenuicollis* cysts) were obtained from the viscera of lamb freshly slaughtered at the Longburn Freezing Works, New Zealand. The scoleces were removed from the cysts, severed about one centimeter behind the rostellum and packed into gelatin capsules (size 000) so that each capsule contained five larvae.

4.3

These were force-fed to twenty-two puppies aged between two and six weeks, each receiving one capsule on two consecutive days. As required for experimental work the animals were destroyed by intraperitoneal injections of pentobarbitone sodium (Euthatal). Intestines were removed, slit longitudinally, the worms recovered and washed in saline and used immediately for experiments. At the time of the experiment the worms age ranged from two to seven months. In the laboratory the worm materials were washed in saline, blotted dry with paper towels and either used immediately for experiments or wrapped in small groups in plastic, put in plastic containers and frozen, stored at -18° C until required.

4.3.2 Homogenation and sub-cellular fractionation

Moniezia (both fresh and frozen) and fresh *T. ovis* and dog-liver were used as sources of tissue for homogenation and sub-cellular fractionation in studies related to effects of the detergent Triton X-100, temperature and substrate concentrations on cholinesterase activity. The following tissue preparations were made: whole-tissue homogenates and nuclear, mitochondrial, microsomal and cytosol fractions. Centrifugation was at the following speeds and periods: 1000g for 10 minutes, 12000g for 20 minutes, 40,000g for 60 minutes.

Sub-cellular tissue fractions were prepared in the following manner. Fresh *M. expansa* were blotted dry and 15 gram samples cut into small pieces. These were placed in a glass Potter-Elvehjem homogenizer together with 25 ml volumes of ice-cold phosphate buffer (pH 7.8) containing 0.25 M sucrose. (Rappaport *et al*, 1959). The material was homogenised in ice for 3 minutes at full speed. Frozen *M. expansa* were thawed on paper towels at room temperature. Five gram samples were weighed and cut into small pieces. Each sample was homogenised in 12 mls of ice-cold buffer/sucrose mixture. The procedure was similar for fresh *T. ovis* except that 1.5 gram samples were used and these were homogenised with 14 ml of the buffer/sucrose mixture. The dog-liver homogenate was made from a mixture of 3 grams liver and 14 ml of buffer. Sub-cellular fractionation was carried out by differential centrifugation using a method modified from that of Knowles and Arurkar (1969). Preliminary work was required to define the experimental

4.3.2

parameters, particularly in relation to dilution factors required to establish a satisfactory rate of hydrolysis. For these reasons it is necessary to report the procedure for each tissue in some detail. With the fresh *M. expansa*, the following was found to be satisfactory. Samples of homogenates (6 ml) as prepared above, were centrifuged at 1000 g for 10 minutes using Beckman cellulose nitrate centrifuge tubes (0.5 x 2.5 inches) in a 39 sw rotor of an ultracentrifuge (Beckman model L). The precipitate was resuspended in 10 ml of ice-cold buffer/sucrose to yield the nuclear fraction which also contained cell debris and unbroken cells. The supernatant was made up to 6 ml with ice-cold buffer/sucrose and centrifuged at 12,000 g for 20 minutes and the resultant precipitate resuspended in 3 ml of buffer to give the mitochondrial fraction. The 12,000 g supernatant was made up to 6 ml and centrifuged at 40,000 g for 60 minutes. The precipitate was then washed and resuspended in 1.5 ml of buffer/sucrose to yield the microsomal fraction. The remaining 40,000 g supernatant was considered free of subcellular organelles, and designated the soluble fraction or cytosol.

For Triton X-100 effects, the various fractions (nuclear, mitochondrial and microsomal) were resuspended in ice-cold buffer/sucrose containing 1% Triton X-100 (Mair & Co. Ltd. Auckland New Zealand). The homogenate and cytosol fractions were treated with Triton X-100 at a final concentration of 1%. The various homogenates and fractions with and without 1% Triton X-100 were incubated and shaken for 1 hour at 37° C prior to assay. The homogenate solutions were diluted four fold with their respective buffer solutions just before assaying for cholinesterase enzyme activity. The frozen *M. expansa* preparation of subcellular fractions was the same as that for the fresh material except for the volume of buffer used for resuspending the precipitated materials. The 1000 g precipitated nuclear fraction was resuspended in 1.4 ml of buffer; the 12,000 g precipitated mitochondrial fraction was resuspended in 5 ml of buffer and 40,000 g precipitated microsomal fraction was resuspended in 2.4 ml. The treatment with Triton X-100 1%, of homogenate and fractions was the same in treated as fresh *Moniezia*. The homogenates with and without Triton X-100 were diluted two fold with their respective buffers just before assaying for

4.3.2

cholinesterase enzyme activity. In studies of the effects of temperature and substrate concentration, the homogenate was diluted four fold and the fractions diluted two fold with buffer just before assaying for enzyme activity. In preparing the fresh *T. ovis* material 1.5 gram worm pieces were homogenised with 14 ml of buffer and the subcellular fractions were prepared as for *Moniezia* with slight modifications with respect to the volume of buffer used to resuspend the precipitated materials. The 100 g nuclear fraction was resuspended in 5 ml of buffer; the 12,000 g mitochondrial fraction in 2.5 ml of buffer and 40,000 g microsomal fraction in 1.5 ml. The treatment with Triton X-100 was as with *Moniezia*. The homogenates without and with 1% Triton X-100 were diluted ten fold and all the fractions were diluted five fold with their respective buffers just before the cholinesterase enzyme assay was carried out. In studies of the effects of temperature the homogenate was diluted forty fold and the fractions diluted twenty fold with buffer just before assaying for the cholinesterase activity. For studies of substrate concentration effects solution was diluted five fold with ice-cold phosphate buffer before assay. With fresh dog liver, the 100 g nuclear fraction was resuspended in 3.3 ml of buffer; the 12,000 mitochondrial fraction in 3.3 ml and 40,000 g microsomal fraction in 1.5 ml. For study of the effects of temperature and substrate concentration the homogenate was diluted forty fold and the fractions diluted twenty fold with buffer just before assay.

4.3.3 Homogenation for inhibitor experiments

Hydatid fluid was withdrawn from cysts with a syringe equipped with a large bore hypodermic needle and the brood capsules and scoleces washed free of the germinal membrane using copious quantities of saline. This material was washed several times and excess fluid decanted. Both fluid and solid material were either used in a fresh condition or frozen at -18° C until required. Preliminary experiments were done to select amounts of helminth material, of suitable potency to meet the parameters of the experiments. Homogenates of frozen *Ascaris* were made after thawing in the shade at room temperature. As the worms became soft they were blotted dry with paper towels, in order to

4.3.3

remove excess water and moisture. The worms were then cut longitudinally, the cuticle stripped off and all the viscera, intestines and sex organs taken out and discarded. The muscle portion of the worm was cut into small pieces and weighed. The head portion was prepared by cutting 2.5 cm behind the anterior tip and this portion in turn was stripped of cuticle. Samples weighing 88 mg of muscle and 400 mg of the head portion were placed in 5 ml volume Omnimixer cuvettes together with approximately 3 ml of ice-cold 0.1M phosphate buffer (pH 8.0). The material was homogenised at full speed for 2 minutes using the micro-attachment and the cuvette was kept cold by submerging it in ice flakes in a plastic bucket. The homogenate was then diluted to 10 ml with cold phosphate buffer, mixed thoroughly and then used directly for assay of cholinesterase. Homogenate of *Trichuris ovis* worms were made in a similar fashion except that 200 mg samples of the worms were used. When using *A. galli*, 400 mg samples of worm was required and with *T. ovis* and *T. hydatigena*, 200 mg samples proved satisfactory. Brood capsules and scoleces of *E. granulosus* were used at a concentration of 40 mg per 10 ml and cyst fluid in the volume of 5 ml fluid to 5 ml buffer. Cyst membrane was used at a ratio of 400 mg/10 ml, but the last named homogenate showed no cholinesterase activity.

4.3.4 Drug sources

The cholinesterase inhibitors tested on different homogenates and cell fractions were; eserine salicylate (Macfarlan Smith Ltd., Edinburgh); pyrantel tartrate, morantel tartrate and levamisole hydrochloride (courtesy of Cyanamid Canada Ltd., Toronto); dichlorvos and vincofos (courtesy of Shell Biological Science Research Centre, California, U.S.A.).

4.3.5 Reagents and their preparation

Phosphate buffers contained monobasic sodium phosphate and dibasic sodium phosphate of 0.1 M pH were adjusted to pH 8.0 and pH 7.0. The substrate was acetylthiocholine iodide, (Sigma Chemical Company, St. Louis, U.S.A.). A 0.075 M (21.67 mg/ml) solution was used and

4.3.4

it retained its potency for up to 15 days when refrigerated. The colouring agent comprised 0.01M 5:5-dithiobis-2-nitrobenzoic acid (DTNB) (Sigma Chemical Company, St. Louis, U.S.A.) equal to 39.6 mg DTNB in 10 ml, 0.1 M phosphate buffer (pH 7.0) sodium bicarbonate (15 mgs) was added to DTNB mixture. The solution was more stable in buffer of pH 7.0 rather than pH 8.0, but for the present experiments was freshly prepared every few days.

4.3.6 Cholinesterase assay

The enzyme assays were done the same day as the cell fractionation. Acetylcholinesterase activity was measured by the colorimetic determination of Ellman *et al.* (1961) with slight modifications, using a Unicam spectrophotometer (model S P 500 Series 2) with an external recorder (model S P 20 Series) and thermoregulated cuvette-holder. The procedure involved essentially the hydrolysis of the substrate acethythiocholine by cholinesterase to acetate and thiocholine. The thio moiety is then coupled with DTNB to give a coloured compound the absorbance of which can be measured on spectrophotometer and compared with a standard.

The procedure was as follows:

(a) A 0.4 ml sample of homogenate or subcellular fraction was added to a 3 ml cuvette containing 2.5 ml 0.1M phosphate buffer. (pH 8.0).

(b) One hundred µl of the DTNB reagent was added with a Gibson pipette and mixed with a current of air introduced through a fine capillary pipette.

(c) The cuvettes were placed in the thermostated cell compartments (37°C) of the spectrophotometer which in turn was fitted with an external recorder.

(d) After incubating the buffer, worm material and DTNB at 37°C for 5 minutes in the photocell, 20 µl of the substrate (acetylthiocholine iodide) was added by means of micropipette, and the cuvette contents remixed with a current of air. After mixing, the photometer required 30 to 40 seconds to become stabilised and the rate of change of absorbance at 412nm calculated from the chart record over the next 5 minutes.

4.3.6

In dealing with the worm homogenates a blank, consisting of worm homogenate, DTNB and buffer without substrate was required to correct for release of thiol material from the homogenised cells. To study the effects of inhibitors and anthelmintics, homogenates of high activity were pre-incubated for five minutes with the drugs at concentrations from 10^{-7}M to 10^{-2}M. Test drugs were added in volumes of 0.1 ml and the final volume, before substrate was 3 ml. The homogenates and fractions were kept in glass-tubes in ice throughout the assaying period. Temperature effect on cholinesterase activity was studied over a range from 10° C to 70° C and the temperature was maintained during a five minutes preincubation period and as well as during the five minutes test period. In the temperature experiment it was necessary to employ two blanks at each temperature examined. A "no-substrate" blank consisting of fractionated homogenate, DTNB, and a "no-enzyme" blank containing substrate, to correct for the increased non-enzymatic hydrolysis of higher temperatures.

For the substrate study enzyme activity was measured at 37° C in a final volume of 3 ml reaction mixture which was preincubated for five minutes before the addition of the substrate. Substrate concentrations were examined over a range 10^{-6}M to 10^{-2}M acetylthiocholine iodide. Two blanks were employed. A "no-substrate" blank consisting of fractionated homogenates, DTNB and buffer to correct for the non specific reaction and "no-enzyme" blank containing the substrate - to correct the spontaneous hydrolysis.

The standard curve for cholinesterase assay was carried out in the following manner. A 25 mg/100 ml solution of glutathione (Reduced form; Sigma Chemical Company) was used to construct a standard curve representing a range of 0.05 ml to 0.2 ml of glutathione (Fig 4.1) It was found that 0.1 ml of solution (0.08135 uMoles SH) when added to the assay cuvette gave a deflection of 60 units on the recorder paper. Consequently a 1 unit deflection on the recorder chart was equivalent to the liberation of 0.00135 uMoles of thiol group (SH). Enzyme rate can be calculated as follows:

$$R = \frac{A \times F}{C \times 5}$$

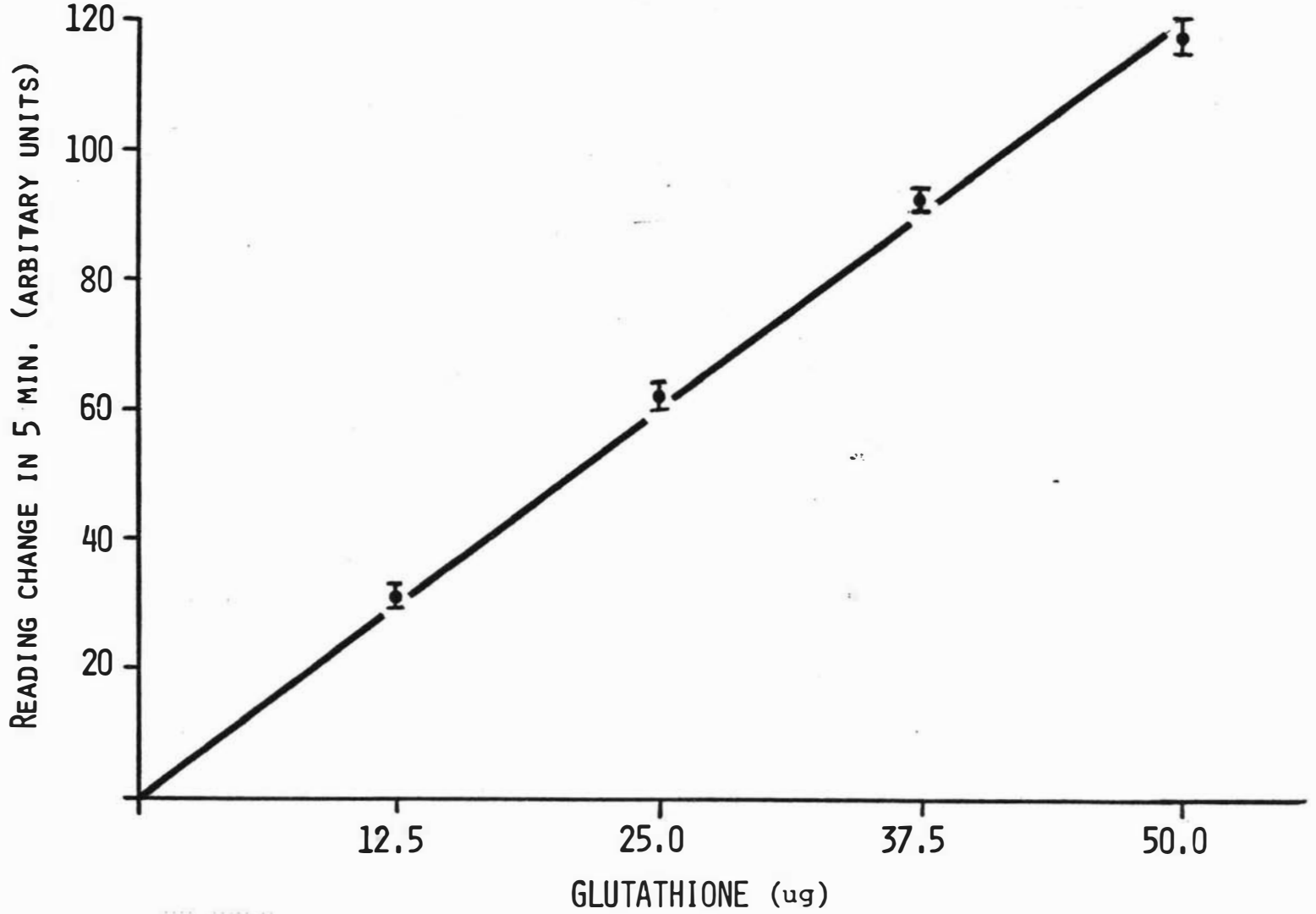


FIG. 4.1 COMPOSITE STANDARD CURVE (MEAN ±S.E. OF FIVE CONSECUTIVE ASSAYS) FOR CHOLINESTERASE ASSAY.

4.3.6

Where R = rates in uMoles substrate hydrolysed per minute per mg of tissue.

A = scale unit changes in 5 minutes on recorder.

C = original concentration of tissue (mg/ml).

F = uMoles - SH per scale unit change.

4.3.7 Protein assay.

The specific activity of cholinesterase is related to the absolute level of protein in the tissue sample. Protein assay was done on the day following the experiment and the material was stored at -18 C. Specific activity was determined for homogenates and fractions in fractionation experiments and in the inhibition experiments activity was estimated on a wet-weight basis.

The protein concentration of each homogenate and fractions was determined colourimetrically by the method of Lowry *et al.* (1951) as described by Kabat and Mayer (1961) using purified bovine serum albumin (Sigma Chemical Company, St. Louis, U.S.A.) as the standard (Figure 4.2)and measured on the Unicam spectrophotometer (model S P 500 Series 2). Both standards and unknown samples were assayed in triplicate using 200 µl samples of the helminth materials which were diluted 10 to 60 fold with *Moniezia*, 20 to 120 fold with *T. ovis* and dog liver - using phosphate buffer (pH 7.8) containing 0.25 M sucrose.

4.4 RESULTS

Moniezia(both fresh and frozen)*T. ovis* and dog liver (fresh) were used as sources of tissue for homogenation and subcellular fraction studies related to the effects of the detergent Triton X-100, temperature and substrate concentration. In these experiments (4.4.1) the total protein content of the samples was determined and the cholinesterase specific activity expressed in terms of substrate hydrolysis per milligram of protein per minute.

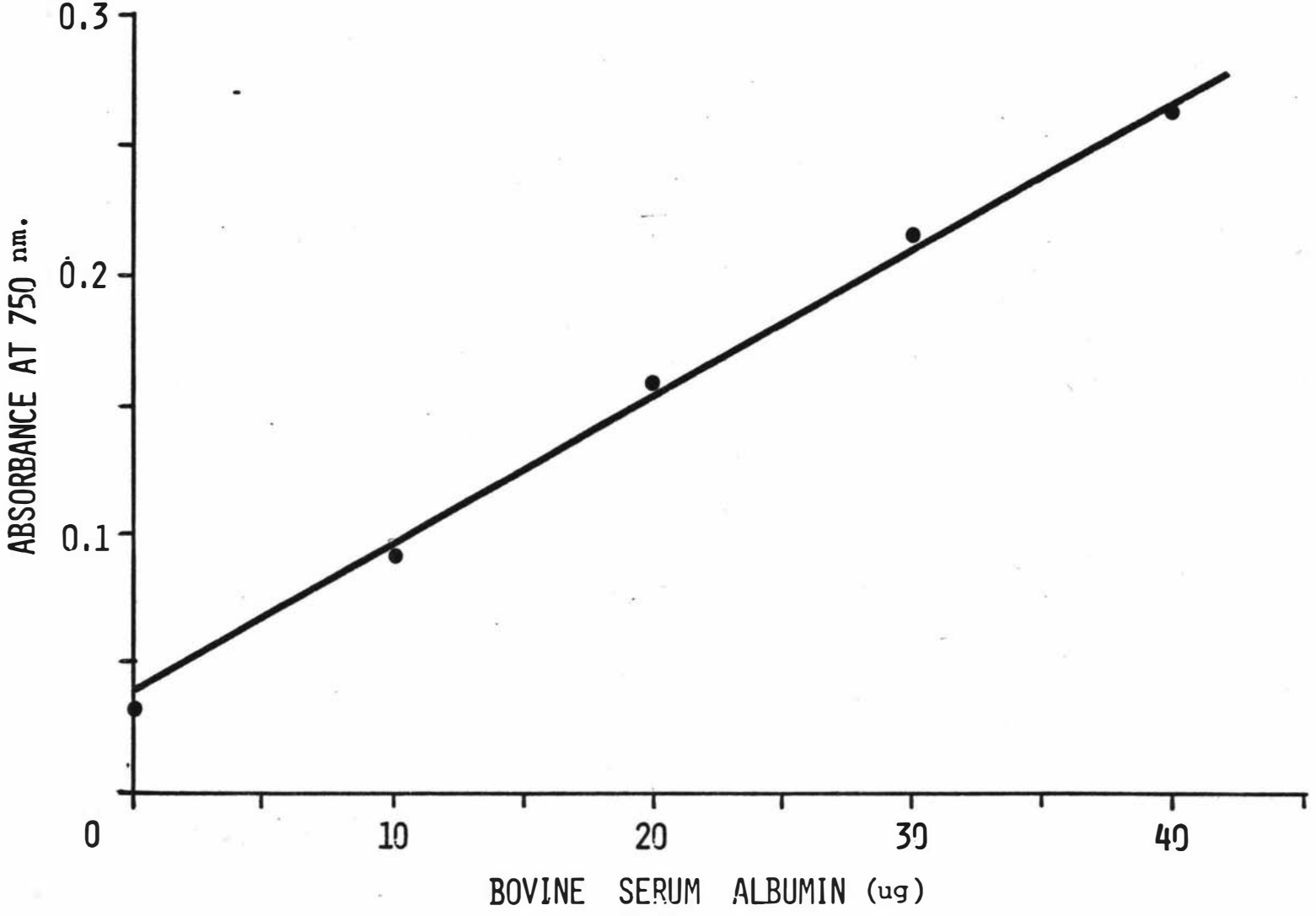


FIG. 4.2 COMPOSITE STANDARD CURVE (MEAN OF FIVE CONSECUTIVE ASSAYS) FOR PROTEIN ASSAY.

4.4.1

A second series of experiments (4.4.2), using homogenates only, were concerned chiefly with the influence of inhibitors - particularly anthelmintics. In this work the absolute rate of enzyme activities are expressed in terms of substrate hydrolysis per milligram wet weight of worm per minute.

4.4.1

(a) Subcellular distribution and solubility in Triton X-100

With fresh *Moniezia* the specific activity of homogenate and subcellular fractions, both in the presence and absence of Triton, is shown in Table 4.2. These data are derived from the original material recorded in Table 4.3. The information has also been plotted to show the relationship between specific activity and percentage distribution of protein (Fig. 4.3) as in the procedure of de Duve *et al.* (1955). The highest activity occurred in the mitochondrial and microsomal fractions and the lowest in the cytosol, the descending values of activity being as follows: mitochondria 13.5, microsomes 10.5, homogenate 6.3, nuclei 4.6 and cytosol 2.8 with activity expressed as nanomoles acetylthiocholine hydrolysed per milligram of protein per minute. A statistical analysis of the effect of Triton on the rate of specific activity of both homogenate and subcellular fractions of fresh *Moniezia* indicated that there is no significant differences between the treated and untreated materials.

With frozen *Moniezia* the data are again derived from the information summarised in Table 4.3 and this material is presented in Table 4.4 and Fig. 4.4. The distribution of activity is different from that of fresh material. The highest activity occurred in the microsomal fraction rather than the mitochondrial, which now showed the lowest activity of all the fractions having activity only a little higher than that of homogenate. The values in descending order are: microsomes 15.5, nuclei 12.7, cytosol 7.7, mitochondria 7.1 and homogenate 6.8. Cholinesterase specific activities are plotted against protein distribution in Fig. 4.4 and an application of the *t* test to the data in Table 4.4 indicates a significant effect of Triton in increasing enzyme activity in the homogenate and all four subcellular fractions ($P < 0.01 - < 0.05$).

TABLE 4.2

EFFECT OF 1% TRITON X-100 ON THE SPECIFIC ACTIVITY OF CHOLINESTERASE FROM HOMOGENATES AND FRACTIONS OF FRESH *MONIEZIA*

EXPERIMENT NUMBER	TRITON ADDED	NANOMOLES ACETYLTHIOCHOLINE IODIDE/MG PROTEIN/MINUTE				
		HOMOGENATE	NUCLEI	MITOCHONDRIA	MICROSOMES	CYTOSOL
1	(-)	7.59	6.60	13.14	10.69	4.08
	(+)	6.66	8.52	15.36	10.39	3.18
2	(-)	7.11	3.89	13.50	9.39	2.04
	(+)	5.49	4.53	11.79	9.79	2.90
3	(-)	5.48	3.48	12.07	11.25	2.60
	(+)	5.60	4.87	12.23	12.32	2.43

4	(-)	5.24	4.94	16.13	11.18	2.93
	(+)	6.24	4.52	18.75	12.83	3.19
5	(-)	6.28	4.03	12.48	9.82	2.30
	(+)	6.47	4.23	15.77	10.25	2.39
Mean	(-)	6.34	4.59	13.46	10.47	2.79
	(+)	6.09	5.33	14.70	11.11	2.82
Significance by t.test P value (n.s $<$ .05)		n.s	n.s	n.s	n.s	n.s	n.s

TABLE 4.3

SUBCELLULAR DISTRIBUTION OF CHOLINESTERASE ENZYME (ChE) AND PROTEIN FROM FRESH AND FROZEN MONIEZIA AND FRESH TAENIA OVIS

SPECIES	TRITON X-100	COMPONENT	ABSOLUTE VALUE * 100%	PERCENTAGE VALUE IN FRACTIONS				RECOVERY% ± S.E.
				NUCLEAR	MITOCHONDRIA	MICROSOMES	CYTOSOL	
MONIEZIA (FRESH)	-	Protein	74.3	56.0 ± 3.2	5.3 ± 0.5	4.9 ± 0.2	34.2 ± 1.8	100.4 ± 4.5
		ChE	0.465	49.8 ± 3.8	13.8 ± 0.4	10.1 ± 0.8	18.4 ± 0.4	92.1 ± 4.1
	+	Protein	75.2	54.0 ± 3.7	5.6 ± 0.4	4.6 ± 0.3	32.7 ± 2.0	96.9 ± 4.1
		ChE	0.447	54.7 ± 3.0	16.3 ± 1.3	10.1 ± 1.0	18.1 ± 1.0	99.2 ± 4.6

		Protein	64.6	5.1 ± 0.6	34.0 ± 2.3	6.1 ± 0.7	44.9 ± 0.5	90.1 ± 2.3
	−							
MONIEZIA		ChE	0.453	9.4 ± 1.3	35.5 ± 2.3	13.8 ± 1.2	44.9 ± 6.2	103.6 ± 2.3
(FROZEN)		Protein	60.9	4.3 ± 0.2	35.7 ± 2.3	7.7 ± 0.7	44.9 ± 2.0	92.6 ± 4.0
	+							
		ChE	0.603	9.2 ± 0.9	33.3 ± 2.6	16.7 ± 1.3	41.4 ± 4.9	100.6 ± 3.2
		Protein	49.5	27.9 ± 1.9	14.9 ± 0.9	4.7 ± 0.5	49.5 ± 1.6	97.0 ± 2.6
	−							
TAENIA		ChE	2.574	27.2 ± 1.9	22.4 ± 1.5	9.1 ± 0.5	34.9 ± 1.8	93.6 ± 3.3
OVIS								
(FRESH)		Protein	47.7	28.4 ± 1.5	16.4 ± 0.8	4.9 ± 0.4	50.4 ± 2.0	100.1 ± 2.3
	+							
		ChE	2.667	28.9 ± 1.9	23.6 ± 1.0	9.8 ± 0.8	40.0 ± 4.6	102.3 ± 6.7

* Units are protein - mg/ml; cholinesterase - n moles acetylthiocholine/ml/minute.

4.3

FIG, 4.3

THE SUBCELLULAR DISTRIBUTION OF CHOLINESTERASE ENZYME IN FRESH MONIEZIA

(A) WITHOUT 1% TRITON X-100.

(B) WITH 1% TRITON X-100.

SPECIFIC ACTIVITY = NANOMOLES ACETYLTHIOCHOLINE-IODIDE HYDROLIZED/MG.PROTEIN/MIN.

N = NUCLEAR FRACTION.

M = MITOCHONDRIAL FRACTION.

P = MICROSOMAL FRACTION.

S = CYTOSOL FRACTION.

4.3

FIG. 4.3

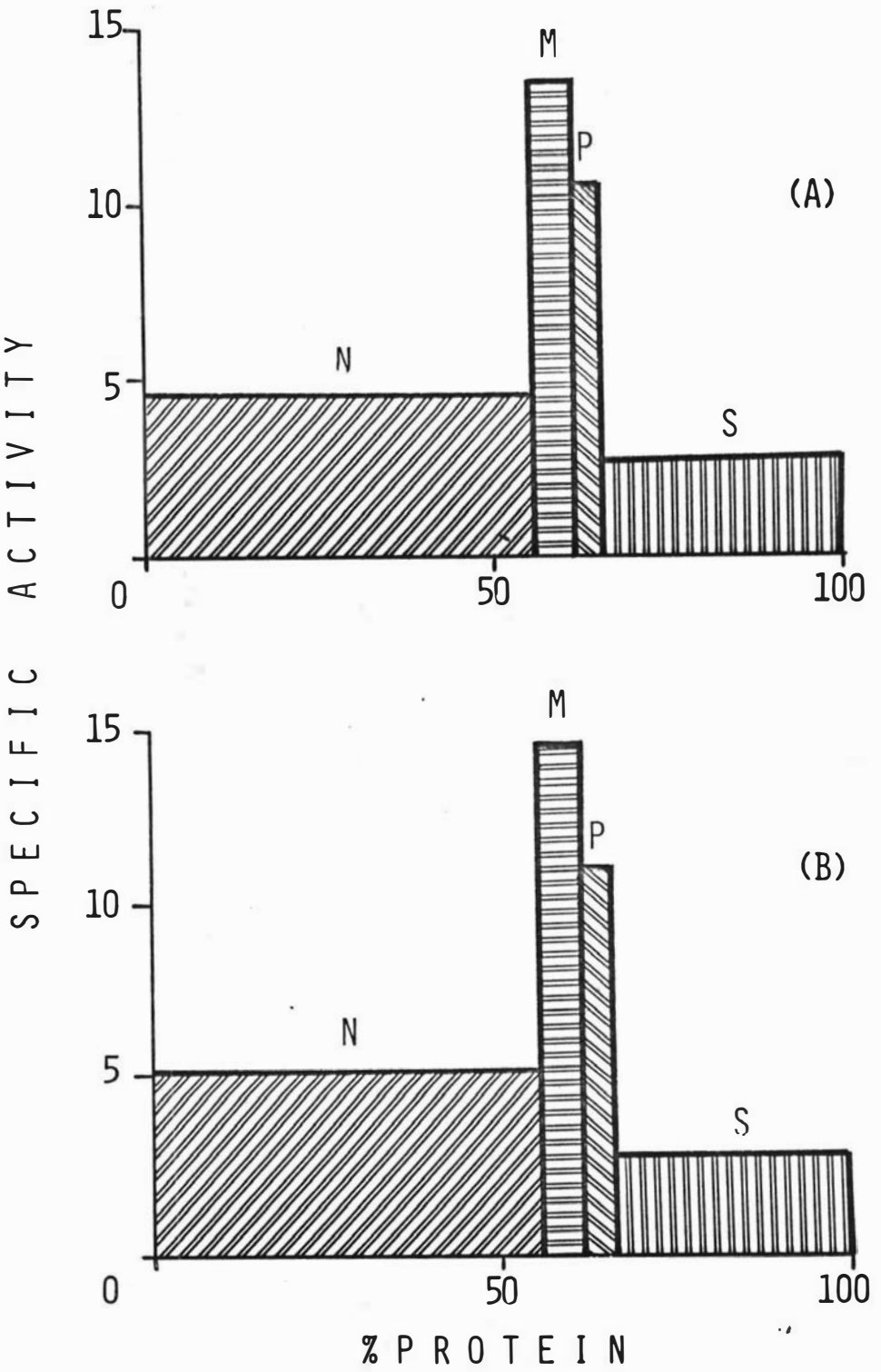

15
10
5
0
M
P
N
S
(A)
50
100
SPECIFIC ACTIVITY
15
10
5
0
M
P
N
S
(B)
50
100
% PROTEIN

TABLE 4.4

EFFECT OF 1% TRITON X-100 ON THE SPECIFIC ACTIVITY OF CHOLINESTERASE FROM HOMOGENATES AND FRACTIONS OF FROZEN MONIEZIA

EXPERIMENT NUMBER	TRITON ADDED	NANOMOLES ACETYLTHIOCHOLINE IODIDE/MG PROTEIN/MINUTE				
		HOMOGENATE	NUCLEI	MITOCHONDRIA	MICROSOMES	CYTOSOL
1	(-)	7.65	15.94	7.26	16.80	8.07
	(+)	8.68	19.16	8.11	21.93	11.72
2	(-)	7.73	14.97	9.14	20.18	11.02
	(+)	12.70	20.36	10.50	25.34	12.00
3	(-)	4.94	10.59	4.86	15.54	3.25
	(+)	7.45	19.90	7.52	25.40	5.30

4	(-)	4.71	6.87	5.23	10.92	3.33
	(+)	6.47	11.13	6.92	12.92	4.75
5	(-)	8.84	14.92	8.99	13.84	12.71
	(+)	10.31	18.35	10.53	17.99	14.17
Mean	(-)	6.78	12.66	7.10	15.45	7.68
	(+)	9.12	17.78	8.72	20.71	9.59
Significance by t.test P value		$<.05$	$<.02$	$<.01$	$<.02$	$<.02$

FIG. 4.4

THE SUBCELLULAR DISTRIBUTION OF CHOLINESTERASE ENZYME IN FROZEN *MONIEZIA*

(A) WITHOUT 1% TRITON X-100.

(B) WITH 1% TRITON X-100.

SPECIFIC ACTIVITY = NANOMOLES ACETYLTHIOCHOLINE-IODIDE HYDROLIZED/MG.PROTEIN/MIN.

N = NUCLEAR FRACTION.

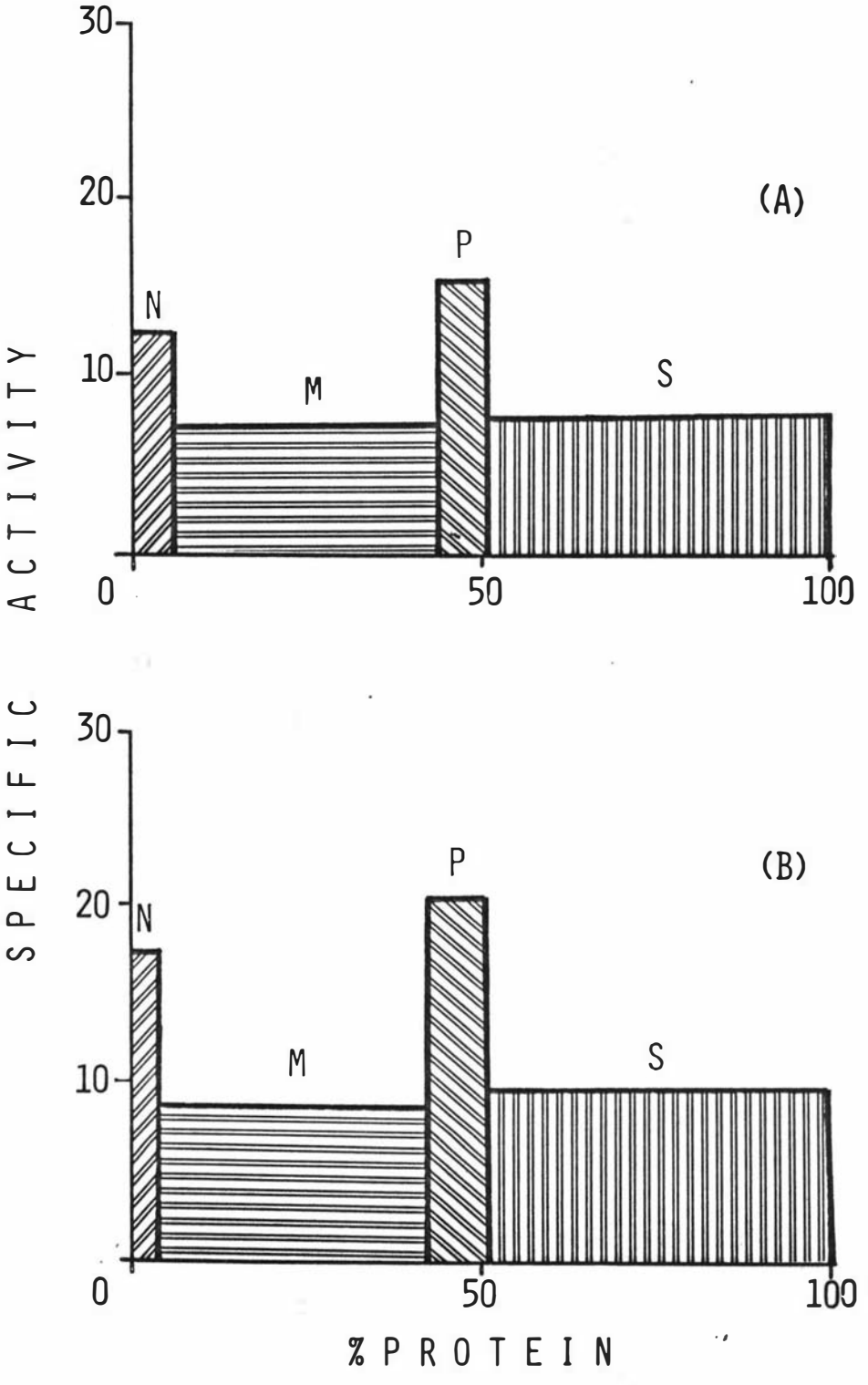
M = MITOCHONDRIAL FRACTION.

P = MICROSOMAL FRACTION.

S = CYTOSOL FRACTION.

4.4

FIG. 4.4


30
20
10
0
50
100
(A)
N
M
P
S
SPECIFIC ACTIVITY
30
20
10
0
50
100
(B)
N
M
P
S
% PROTEIN

4.4.1

An interesting feature of the work was the finding that with and without treatment with Triton, no significant differences occurred in the cholinesterase specific activities between fresh *Moniezia* and that frozen at -18^0C for eighteen months. However, the distribution of the enzyme activity of the subcellular fraction of the fresh and frozen worms were found to be significantly different ($P < 0.01$, < 0.001, < 0.02 and < 0.05) for nuclei, mitochondria, microsomes and cytosol respectivity.

The data for *T. ovis* is presented in Table 4.5 and Fig. 4.5 and is derived from basic data presented in Table 4.3. It is readily apparent that this worm has a much higher specific activity than does *Moniezia* (51 for *T. ovis* homogenate as compared with 6.3 for fresh *Moniezia)*. However the distribution of enzyme activity in homogenate and subcellular fractions was similar to that in fresh *Moniezia* except that activity was somewhat higher in the microsomal fraction, rather than in the mitochondria. The values in descending order are: microsomes 99.9, mitochondria 76.5, homogenate 51.1, nuclei 49.7 and cytosol 35.3. Thus the overall enzyme activity for all fractions was some six to twelve times higher than for similar fractions of *Moniezia* . The effect of Triton on both homogenate and all other fractions was to significantly increase enzyme specific activity ($P < 0.01$).

4.4.1

(b) Temperature and specific activity

It was found that the specific activity of homogenate and subcellular fractions of frozen *Moniezia* increased with temperature to a sharp peak at 50^0C. At 60^0C, homogenate, microsomes, cytosol and mitochondria showed about half maximum activity (Table 4.6, Fig. 4.6). Complete inactivation of the enzymes took place at 70^0C for all fractions. Below 60^oC the microsomal fraction was most sensitive to decreasing temperature and the cytosol, least sensitive. The homogenate and cytosol fractions showed the same temperature sensitivity from 30^0C to 70^0C, but at low temperatures (10^0C and 20^0C) the cytosol enzyme activity declined less rapidly than the homogenate activity.

TABLE 4.5

EFFECT OF 1% TRITON X-100 ON THE SPECIFIC ACTIVITY OF CHOLINESTERASE FROM HOMOGENATES AND FRACTIONS OF FRESH *TAENIA OVIS*

EXPERIMENT NUMBER	TRITON ADDED	NANOMOLES ACETYLTHIOCHOLINE IODIDE/MG PROTEIN/MINUTE				
		HOMOGENATES	NUCLEI	MITOCHONDRIA	MICROSOMES	CYTOSOL
1	(-)	56.86	51.79	76.92	105.17	42.66
	(+)	60.56	56.25	77.95	110.94	47.65
2	(-)	53.73	54.13	85.76	100.75	34.57
	(+)	55.82	57.54	88.39	113.42	37.72
3	(-)	56.25	50.68	84.89	107.28	33.36
	(+)	62.37	57.45	86.71	122.12	35.78

4	(-)	43.06	44.16	66.72	108.98	33.28
	(+)	45.46	50.94	69.44	121.62	36.86
5	(-)	45.37	47.69	68.00	77.46	32.59
	(+)	49.89	53.48	70.21	85.87	35.65
Mean	(-)	51.06	49.69	76.46	99.93	35.29
	(+)	54.82	55.13	78.54	110.80	38.73
Significance by t.test P value		$<.01$	$<.01$	$<.01$	$<.01$	$<.01$

4.5

FIG. 4.5

THE SUBCELLULAR DISTRIBUTION OF CHOLINESTERASE ENZYME IN TAENIA OVIS.

(A) WITHOUT 1% TRITON X-100.

(B) WITH 1% TRITON X-100.

SPECIFIC ACTIVITY = NANOMOLES ACETYLTHIOCHOLINE-IODIDE HYDROLIZED/MG.PROTEIN/MIN.

N = NUCLEAR FRACTION.

M = MITOCHONDRIAL FRACTION.

P = MICROSOMAL FRACTION.

S = CYTOSOL FRACTION.

4.5

FIG. 4.5

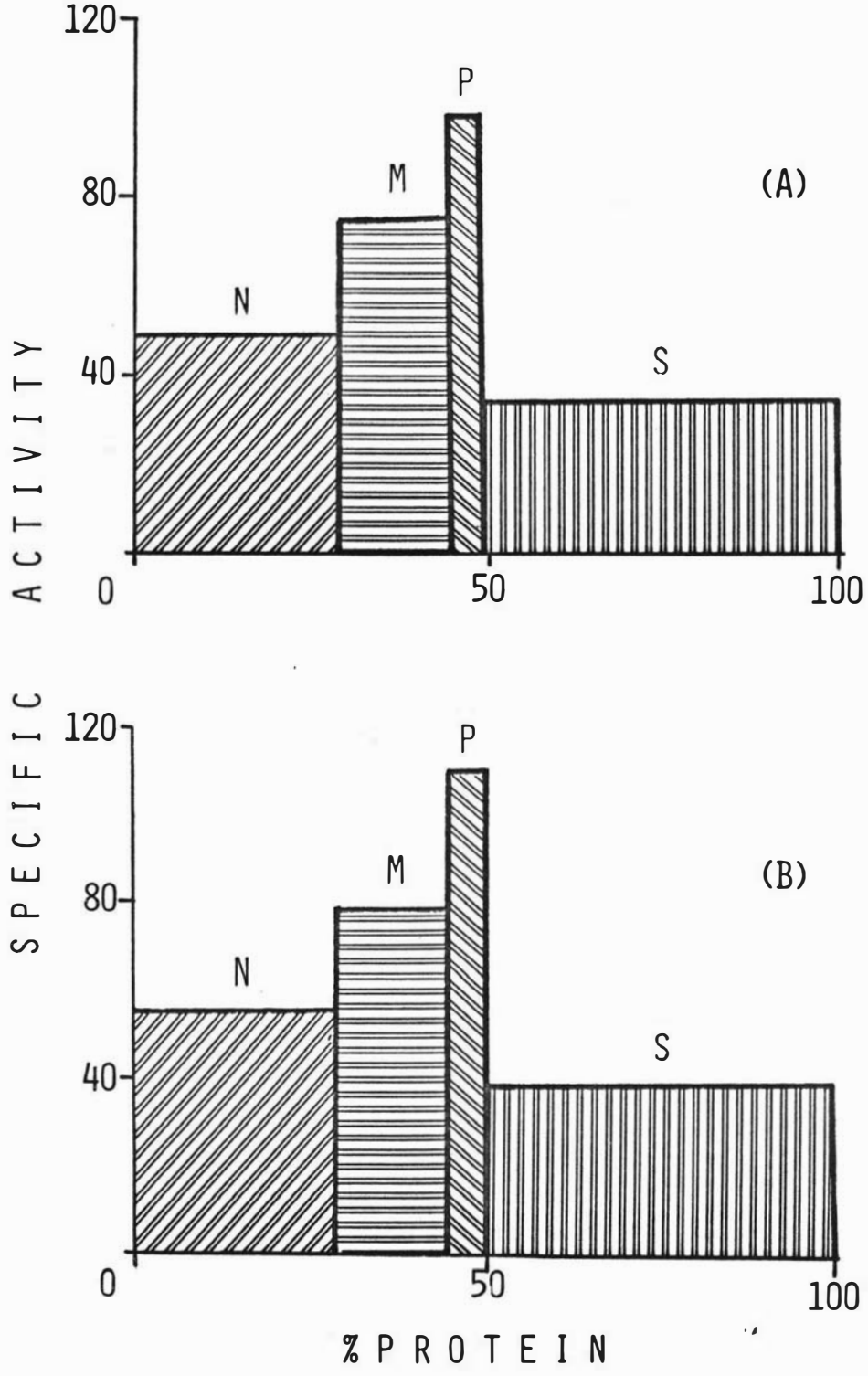


TABLE 4.6

INFLUENCE OF TEMPERATURE ON THE CHOLINESTERASE SPECIFIC ACTIVITY OF HOMOGENATE AND SUBCELLULAR FRACTIONS OF FROZEN *MONIEZIA*

BUFFER - Phosphate 0.1M pH 8.0
SUBSTRATE - Acetylthiocholine iodide 0.075M
INCUBATION TIME - 5 minutes

TEMPERATURE C	CHOLINESTERASE SPECIFIC ACTIVITY*			
	HOMOGENATE	MITOCHONDRIA	MICROSOMES	CYTOSOL
10	1.1 (9.2)	0.0 (0.0)	0.9 (3.9)	1.8 (15.7)
20	2.4 (20.2)	1.2 (7.6)	3.0 (13.2)	3.3 (28.7)
30	6.3 (52.9)	5.1 (32.3)	9.4 (41.2)	5.2 (45.2)
40	8.6 (72.2)	8.7 (55.1)	20.2 (88.6)	8.7 (75.7)
50	11.9 (100)	15.8 (100)	22.8 (100)	11.5 (100)
60	7.3 (61.3)	7.5 (47.5)	13.3 (58.3)	8.2 (71.3)
70	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

* Units are nanomoles acetylthiocholine iodide hydrolysed/mg protein/minute.
Data shown are mean values of three experiments.
Figures in parentheses are % of maximun activity.

4.6

FIG 4.6

ACTIVITY OF CHOLINESTERASE ENZYME OF FROZEN MONIEZIA IN RELATION TO TEMPERATURE

SPECIFIC ACTIVITY = NANOMOLES ACETYLTHIOCHOLINE IODIDE HYDROLIZED/MG.PROTEIN/MIN.


= HOMOGENATE.

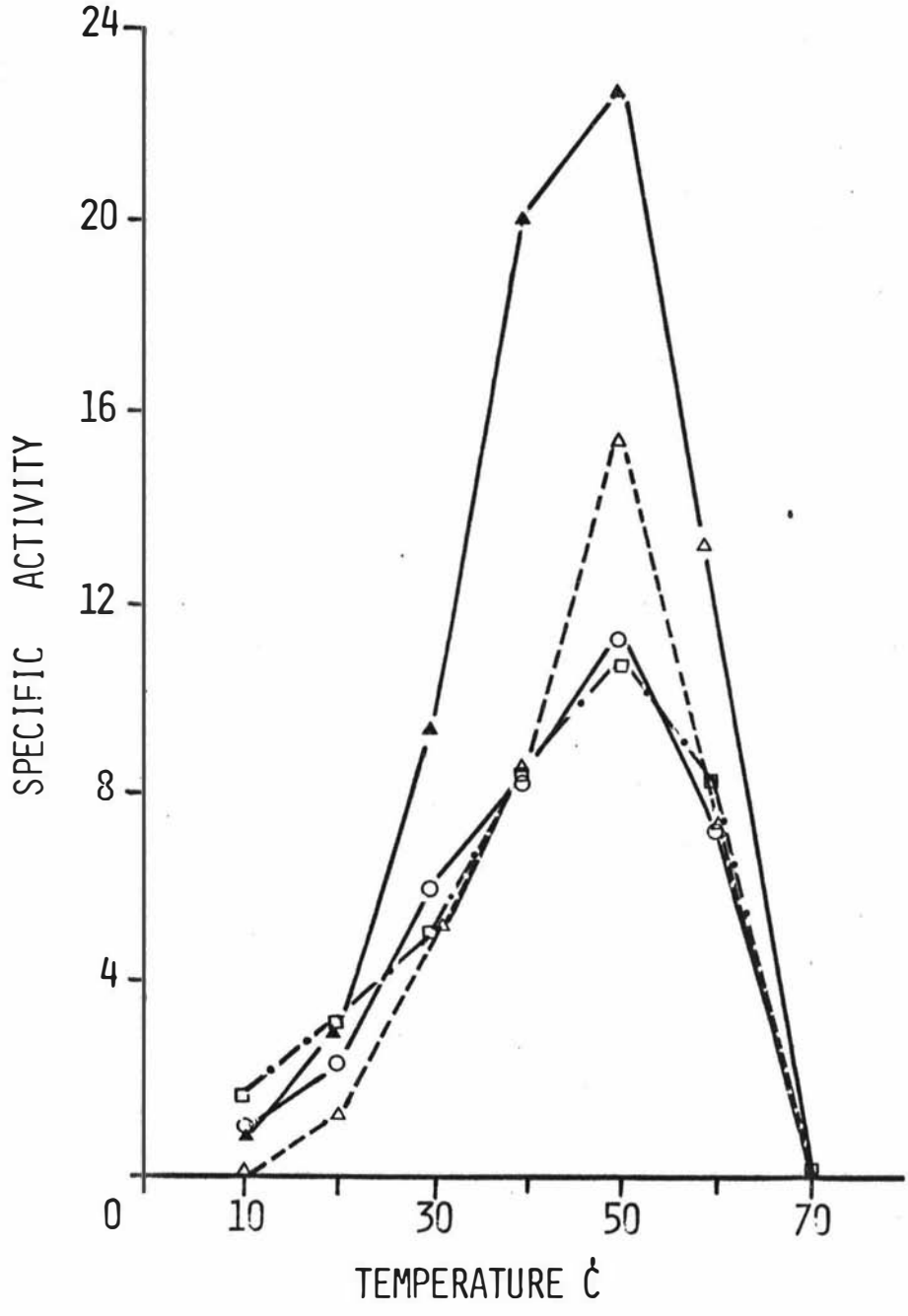

= MITOCHONDRIAL FRACTION.


= MICROSOMAL FRACTION.


= CYTOSOL FRACTION.

4.6

FIG 4.6



4.4.1

With fresh *T. ovis* the specific activity of the homogenate and all four cell fractions increased with increasing temperature to a maximum activity of 60°C and a sharp fall at 70°C (Table 4.7, Fig 4.7). The mitochondrial and microsomal fractions were most sensitive to increasing temperature reacting in a similar fashion to the microsomal fraction of *Moniezia* . At low temperatures, the homogenate, nuclear and cytosol fractions all behaved similarly. The nuclear fraction showed appreciably more activity than cytosol and homogenate over the range 30°C to 60°C.

The temperature peaks for specific activity of homogenate and the four fractions of dog liver differed from the two cestodes. Enzyme activity increased with temperature, over the range 10°C to 40°C and homogenate, nuclear and microsomal fractions showed their maximum activity at 40°C, but in contrast mitochondrial and cytosol fractions showed peak activity at 60°C. Even at 70°C for five minutes, the enzyme activity of the microsomal fraction was higher than the peak for the homogenate, nuclear and cytosol fractions. The enzyme activity of the mitochondrial fraction at 70°C showed almost the peak activity of the nuclear fraction and the enzyme activity of the mitochondrial fraction at 70°C retained the same enzyme activity as at 40°C. The nuclear fraction was much more sensitive to temperature than the homogenate and cytosol fraction. The microsomal fraction proved the most active in all three types of tissue although the optimum temperature varied between 40°C (liver) and 60°C (*T. ovis*). The mitochondrial fraction was the second most active in *Moniezia* and liver, but was as active as the microsomal fraction in *T. ovis*. The nuclear fraction in *T. ovis* and liver was the third most active. In *Moniezia* , the specific activity and optimum temperature were about the same, for homogenate and cytosol. At 70°C *Moniezia* fractions were inactivated but *T. ovis* fractions retained some activity, whereas liver enzymes were highly active (Table 4.8, Fig. 4.8).

4.4.1

(c) Substrate concentration and specific activity

Table 4.9 summarises for *Moniezia* the cholinesterase activities of homogenates and fractions incubated at different concentrations of acetylthiocholine iodide. Plots of rate of enzyme hydrolysis against

TABLE 4.7

INFLUENCE OF TEMPERATURE ON THE CHOLINESTERASE SPECIFIC ACTIVITY OF HOMOGENATE AND SUBCELLULAR FRACTIONS OF FRESH *TAENIAE OVIS*

BUFFER - Phosphate 0.1M pH 8.0
SUBSTRATE - Acetylthiocholine iodide 0.075M
INCUBATION TIME - 5 minutes

TEMPERATURE C	CHOLINESTERASE SPECIFIC ACTIVITY*									
	HOMOGENATE		NUCLEI		MITOCHONDRIA		MICROSOMES		CYTOSOL	
10	17.3	(24.8)	17.4	(18.6)	42.9	(21.0)	44.8	(22.2)	15.2	(19.6)
20	27.5	(39.5)	24.8	(26.6)	58.5	(28.6)	57.4	(28.5)	21.4	(27.5)
30	32.8	(47.1)	44.6	(47.8)	86.2	(42.1)	79.1	(39.2)	27.8	(35.8)
40	48.2	(69.2)	60.5	(64.8)	119.1	(58.2)	118.1	(58.6)	37.7	(48.5)
50	66.3	(95.1)	89.0	(95.3)	177.2	(86.6)	150.9	(74.9)	57.9	(74.5)
60	69.7	(100)	93.4	(100)	204.6	(100)	201.6	(100)	77.7	(100)
70	6.6	(9.5)	10.0	(10.7)	0.0	(0.0)	15.3	(7.6)	7.0	(9.0)

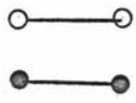
* Units are nanomoles acetylthiocholine iodide hydrolysed/mg protein/minute.
Data shown are mean values of two experiments.
Figures in parentheses are % of maximum activity.

4.7

FIG 4.7

ACTIVITY OF CHOLINESTERASE ENZYME OF *TAENIA OVIS* IN RELATION TO TEMPERATURE

SPECIFIC ACTIVITY = NANOMOLES ACETYLTHIOCHOLINE IODIDE HYDROLIZED/MG.PROTEIN/MIN.



= HOMOGENATE.

= NUCLEAR FRACTION.

= MITOCHONDRIAL FRACTION.

= MICROSOMAL FRACTION.

= CYTOSOL FRACTION.

FIG 4.7

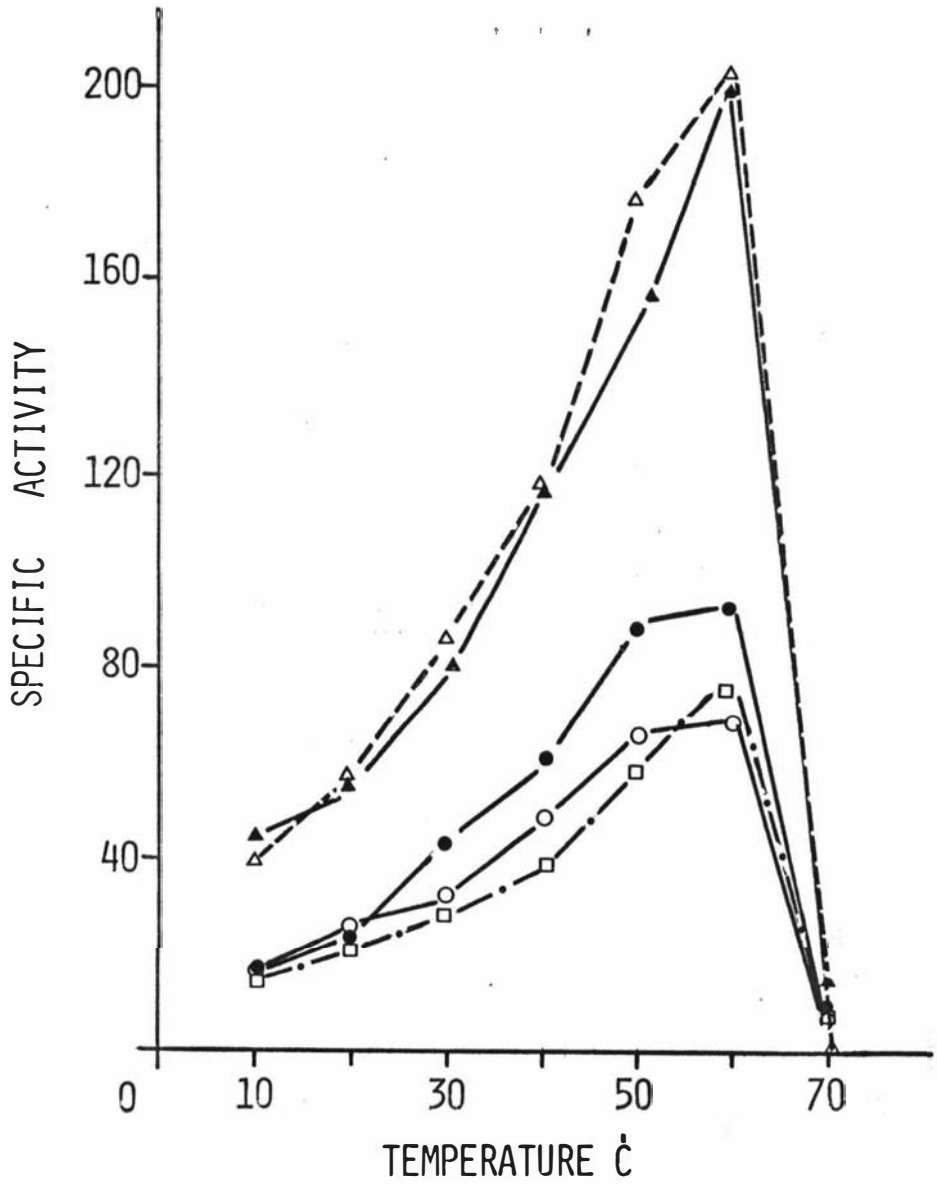

SPECIFIC ACTIVITY
200
160
120
80
40
0
10
30
50
70
TEMPERATURE C

TABLE 4.8

INFLUENCE OF TEMPERATURE ON THE CHOLINESTERASE SPECIFIC ACTIVITY OF HOMOGENATE AND SUBCELLULAR FRACTIONS OF DOG LIVER

BUFFER - Phosphate 0.1M pH 8.0
SUBSTRATE - Acetylthiocholine iodide 0.075M
INCUBATION TIME - 5 minute

TEMPERATURE C	CHOLINESTERASE SPECIFIC ACTIVITY*									
	HOMOGENATE		NUCLEI		MITOCHONDRIA		MICROSOMES		CYTOSOL	
10	17.6	(35.6)	21.1	(29.2)	17.3	(17.2)	48.8	(29.7)	8.2	(17.2)
20	26.3	(53.2)	30.0	(41.5)	27.7	(27.6)	74.4	(45.3)	11.5	(24.1)
30	34.0	(68.8)	44.5	(61.5)	41.5	(41.3)	125.8	(76.6)	16.5	(34.6)
40	49.4	(100)	72.3	(100)	69.2	(68.9)	164.3	(100)	26.3	(55.1)
50	46.1	(93.3)	66.7	(92.3)	86.5	(86.2)	154.0	(93.7)	32.9	(69.0)
60	38.4	(77.7)	47.8	(66.1)	100.4	(100)	92.4	(56.2)	47.7	(100)
70	17.6	(35.6)	31.1	(43.0)	69.2	(68.9)	77.0	(46.9)	19.8	(41.5)

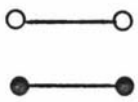
* Units are nanomoles acetylthiocholine iodide hydrolysed/mg protein/minute.
Figures in parentheses are % of maximum activity.

4.8

FIG 4.8

ACTIVITY OF CHOLINESTERASE ENZYME OF DOG LIVER IN RELATION TO TEMPERATURE

SPECIFIC ACTIVITY = NANOMOLES ACETYLTHIOCHOLINE IODIDE HYDROLIZED/MG.PROTEIN/ MINUTE.



= HOMOGENATE.

= NUCLEAR FRACTION.

= MITOCHONDRIAL FRACTION.

= MICROSOMAL FRACTION.

= CYTOSOL FRACTION.

4.8

FIG 4.8

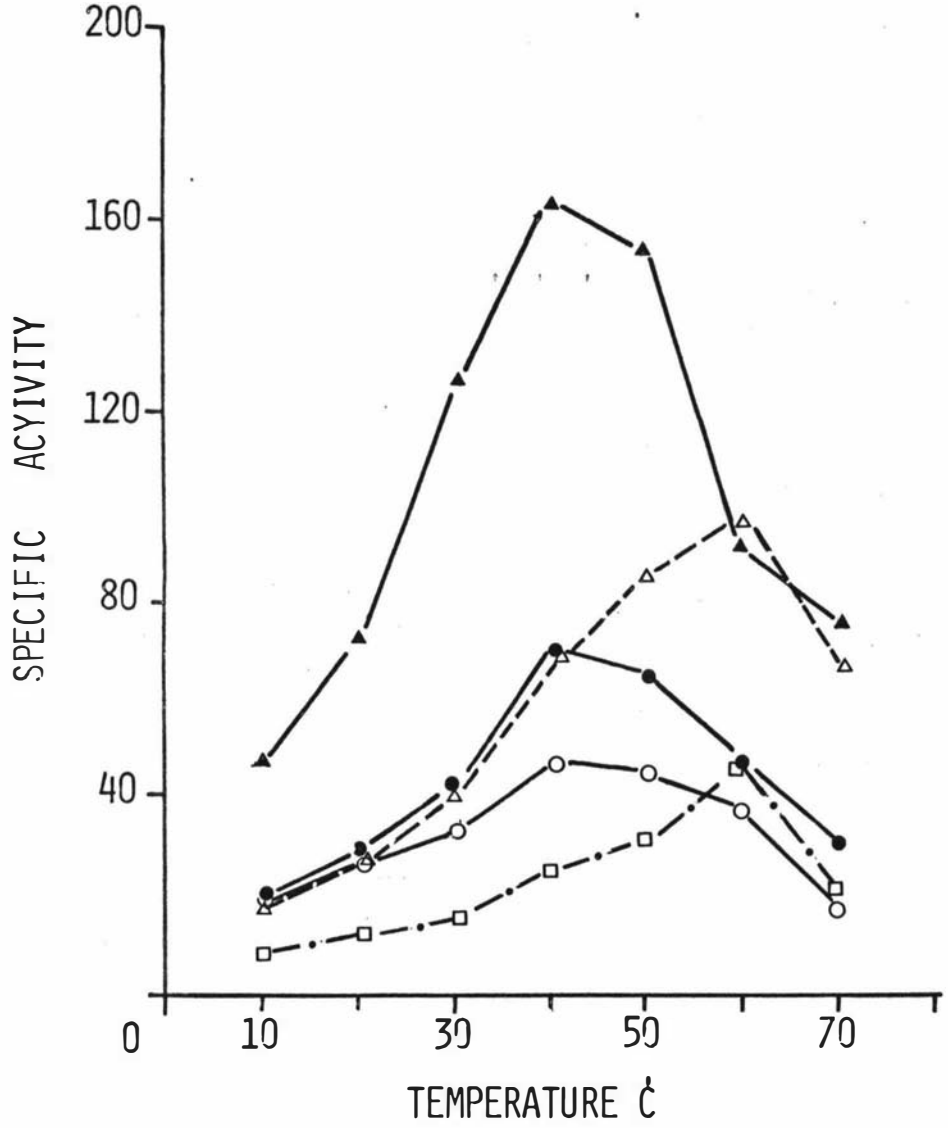

200
160
120
80
40
0
10
30
50
70
SPECIFIC ACYIVITY
TEMPERATURE C

TABLE 4.9

INFLUENCE OF SUBSTRATE ON THE CHOLINESTERASE SPECIFIC ACTIVITY OF HOMOGENATE AND FRACTIONS OF FROZEN MONIEZIA

BUFFER - Phosphate 0.1M pH 8.0
SUBSTRATE - Acetylthiocholine iodide
TEMPERATURE - Incubation at 37C for 5 minutes

SUBSTRATE CONCENTRATION M	CHOLINESTERASE SPECIFIC ACTIVITY*			
	HOMOGENATE	MITOCHONDRIA	MICROSOMES	CYTOSOL
10^{-6}	0.0	0.0	0.0	0.0
$5x10^{-6}$	0.42	0.51	0.0	0.0
10^{-5}	0.63	0.76	0.0	0.80
$5x10^{-5}$	1.25	1.78	4.39	2.66
10^{-4}	2.50	2.80	6.82	3.99
$5x10^{-4}$	9.79	12.48	16.57	7.44
10^{-3}	10.63	16.30	18.03	10.10
$5x10^{-3}$	18.75	20.63	21.93	18.62
10^{-2}	22.71	23.18	27.78	21.79

* Units are nanomoles acetylthiocholine iodide hydrolysed/mg protein/minute.

4.9

FIG. 4.9

ACTIVITY OF CHOLINESTERASE ENZYME OF MONIEZIA (FROZEN) IN RELATION TO SUBSTRATE CONCENTRATION.

SPECIFIC ACTIVITY = NANOMOLES ACETYLTHIOCHOLINE IODIDE HYDROLIZED/MG.PROTEIN/ MINUTE.

 = HOMOGENATE.

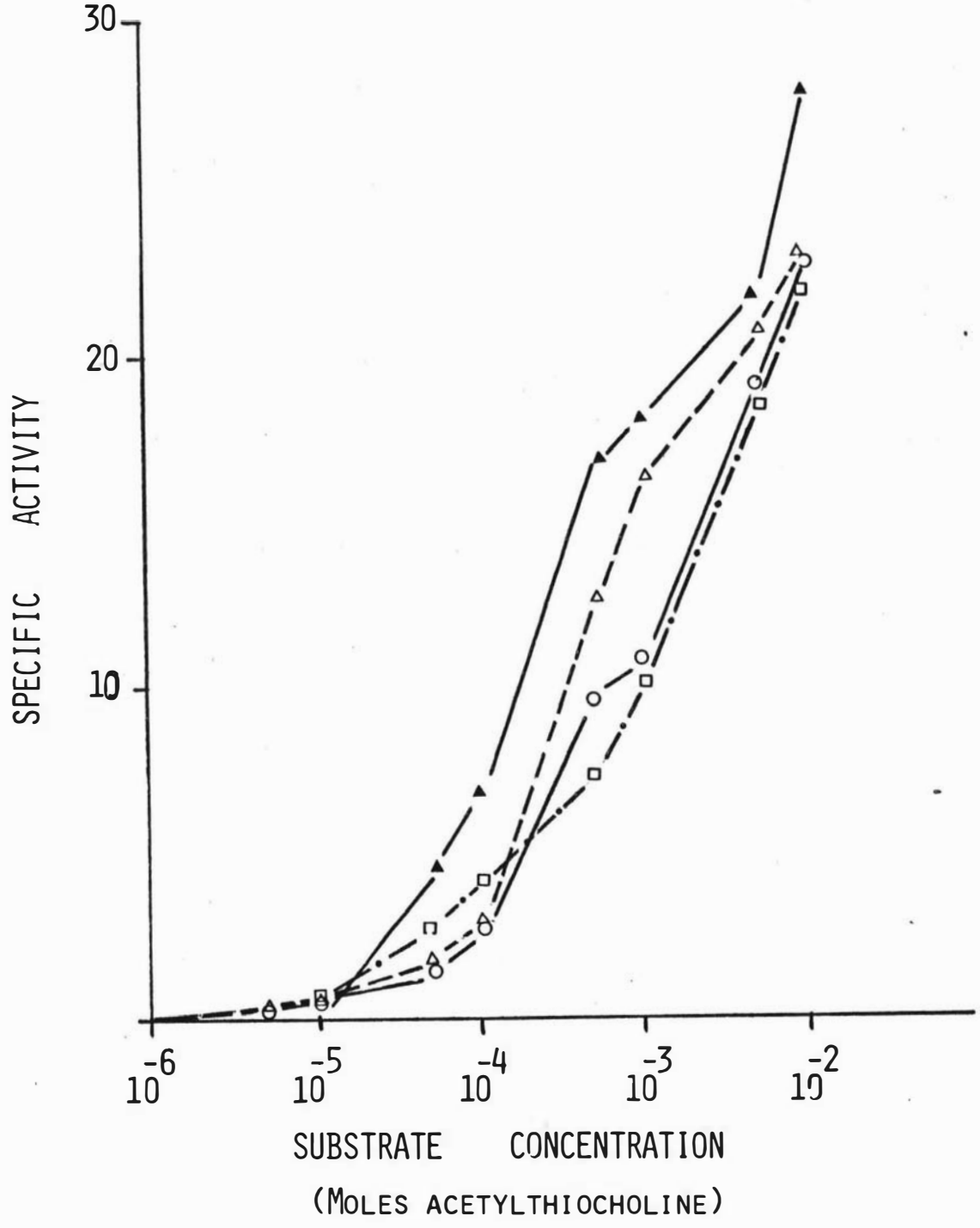
 = MITOCHONDRIAL FRACTION.

 = MICROSOMAL FRACTION.

 = CYTOSOL FRACTION.

4.9

FIG 4.9


30
20
10
SPECIFIC ACTIVITY
10^-6
10^-5
10^-4
10^-3
10^-2
SUBSTRATE CONCENTRATION
(MOLES ACETYLTHIOCHOLINE)

substrate concentration are shown in Fig. 4.9. It can be seen that, increasing substrate concentrations from 10^{-6}M to 10^{-2}M was accompanied by increased cholinesterase specific activity of homogenate and all subcellular fractions. Consequently cholinesterase activity in *Moniezia* does not show autoinhibition by acetylcholine in concentrations as high as 10^{-2}M.

The results of the influence of substrate concentration on cholinesterase specific activity of homogenate and the four cell fractions of *T. ovis* are shown in Table 4.10 and Fig. 4.10. *T. ovis* showed an appreciably higher overall rate of hydrolysis than *Moniezia* , but the effects of substrate concentrations are the same. The experiments with dog liver, Table 4.11 and Fig. 4.11, gave similar results.

Determination of the Michaelis-Menten constant, (Km) and maximum rate of hydrolysis (Vmax)

The kinetics of the hydrolysis of acetylthiocholine iodide by cholinesterase from homogenate and fractions of frozen *Moniezia* , *T. ovis* and dog liver were studied at pH 8.0 and 37°C. The results were plotted according to the double reciprocal method of Lineweaver-Burk and the Km and Vmax values determined as shown for homogenate and fractions of frozen *Moniezia* (Fig. 4.12) for *T. ovis* (Fig. 4.13) and for dog liver (Fig. 4.14).

The Km values for homogenate and mitochondrial enzymes of *Moniezia* were 10.28 x 10^{-4}M and 8.43 x 10^{-4}M respectively. These Km values were about four to eight times higher than the Km values of microsomes and cytosol, 2.12 x 10^{-4}M and 1.48 x 10^{-4}M, respectively (Fig. 4.12). For *T. ovis* the Km values for homogenate, 2.86 x 10^{-4}M; nuclei, 3.54 x 10^{-4}M, 3.54 x 10^{-4}M; mitochondria, 2.14 x 10^{-4}M; microsomes, 2.21 x 10^{-4}M and cytosol 2.33 x 10^{-4}M were all of similar magnitude (Fig 4.13). For dog liver, the Km values for the homogenate, 1.96 x 10^{-4}M; nuclei, 1.78 x 10^{-4}M; mitochondria, 1.73 x 10^{-4}M; microsomes, 1.80 x 10^{-4}M and cytosol 1.37 x 10^{-4}M, (Fig.4.14) similar in magnitude, as was the situation with *T. ovis*.

TABLE 4.10

INFLUENCE OF SUBSTRATE ON THE CHOLINESTERASE SPECIFIC ACTIVITY OF HOMOGENATE AND FRACTIONS OF FRESH *TAENIAE OVIS*

BUFFER - Phosphate 0.1M pH 8.0
SUBSTRATE - Acetylthiocholine iodide
TEMPERATURE - Incubation at 37C for 5 minutes

SUBSTRATE CONCENTRATION M	CHOLINESTERASE SPECIFIC ACTIVITY*				
	HOMOGENATE	NUCLEI	MITOCHONDRIA	MICROSOMES	CYTOSOL
10^{-6}	0.0	0.0	0.0	0.0	0.0
$5x10^{-6}$	1.17	0.62	2.28	2.39	0.68
10^{-5}	3.04	1.87	5.78	5.82	2.41
$5x10^{-5}$	10.31	8.73	27.07	26.50	9.64
10^{-4}	19.66	19.02	46.18	45.58	17.20
$5x10^{-4}$	43.82	45.22	108.63	100.13	35.09
10^{-3}	51.77	58.00	127.28	112.34	41.95
$5x10^{-3}$	58.81	65.49	137.78	122.13	54.63
10^{-2}	69.05	73.28	179.49	152.30	72.21

* Units are nanomoles acetylthiocholine iodide hydrolysed/mg protein/minute.
Data shown are mean values of two experiments.

4.10

FIG 4.10

ACTIVITY OF CHOLINESTERASE ENZYME OF TAENIA OVIS IN RELATION TO SUBSTRATE CONCENTRATION.

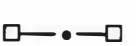
SPECIFIC ACTIVITY = NANOMOLES ACETYLTHIOCHOLINE IODIDE HYDROLIZED/MG.PROTEIN/ MINUTE.

 = HOMOGENATE.

 = NUCLEAR FRACTION.

 = MITOCHONDRIAL FRACTION.

 = MICROSOMAL FRACTION.

 = CYTOSOL FRACTION.

4.10

FIG 4.10

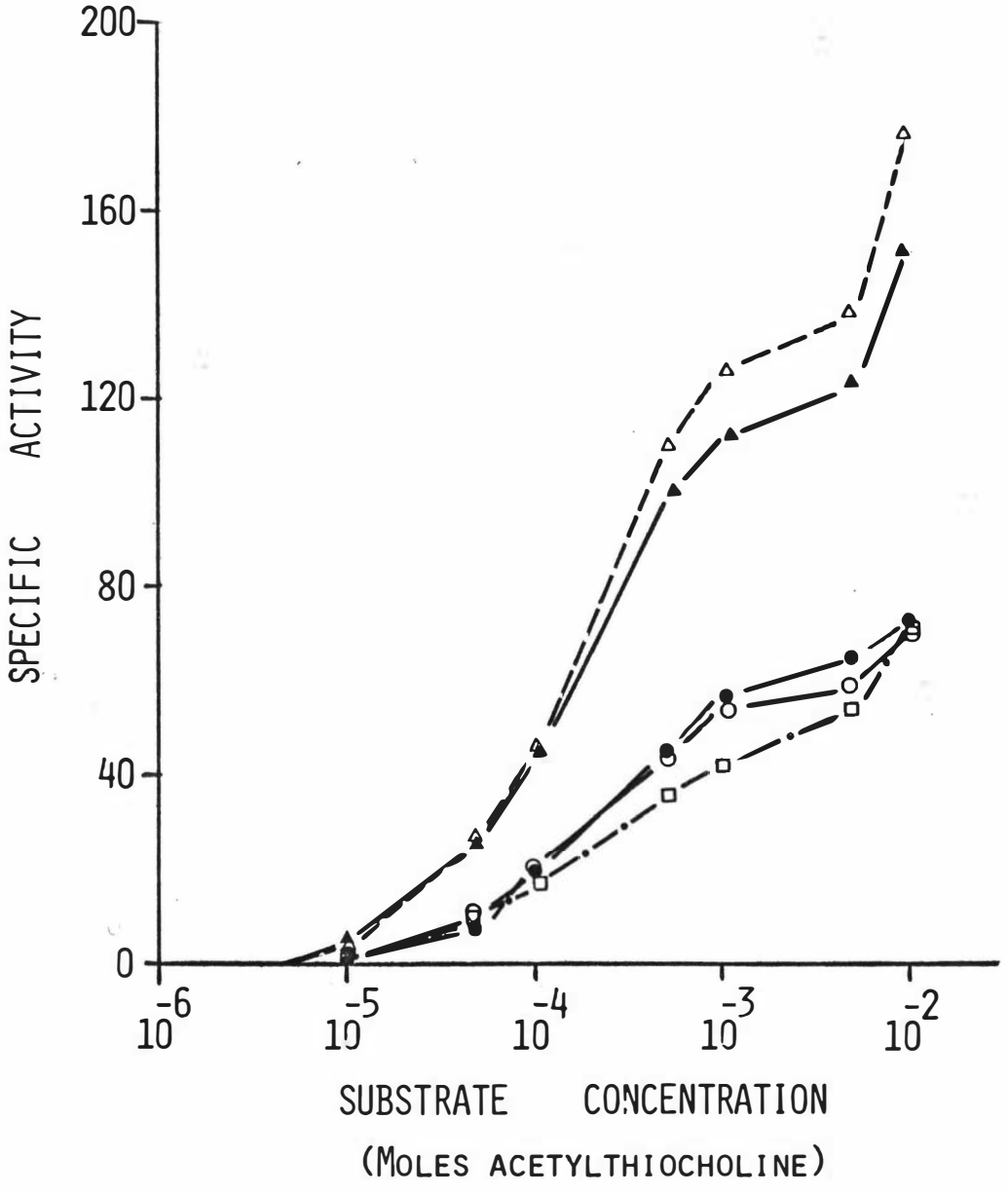

200
160
120
80
40
0
SPECIFIC ACTIVITY
10^-6
10^-5
10^-4
10^-3
10^-2
SUBSTRATE CONCENTRATION
(MOLES ACETYLTHIOCHOLINE)

TABLE 4.11

INFLUENCE OF SUBSTRATE ON THE CHOLINESTERASE SPECIFIC ACTIVITY OF HOMOGENATES AND FRACTIONS OF DOG LIVER

BUFFER - Phosphate 0.1M pH 8.0
SUBSTRATE - Acetylthiocholine iodide
TEMPERATURE - Incubation at 37C for 5 minutes

SUBSTRATE CONCENTRATION M	CHOLINESTERASE SPECIFIC ACTIVITY*				
	HOMOGENATE	NUCLEI	MITOCHONDRIA	MICROSOMES	CYTOSOL
10^{-6}	0.0	0.0	0.0	0.0	0.0
$5x10^{-6}$	2.20	1.11	0.0	0.0	0.0
10^{-5}	7.68	4.45	6.92	2.57	1.65
$5x10^{-5}$	14.27	28.91	20.77	43.63	9.88
10^{-4}	25.24	35.59	31.15	71.86	16.46
$5x10^{-4}$	43.90	71.17	51.92	128.33	24.70
10^{-3}	59.27	83.40	76.15	153.99	36.22
$5x10^{-3}$	104.27	141.23	193.85	266.92	80.67
10^{-2}	127.32	239.09	263.08	349.05	125.12

* Units are nanomoles acetylthiocholine iodide hydrolysed/mg protein/minute.

4.11

FIG 4.11

ACTIVITY OF CHOLINESTERASE ENZYME OF DOG LIVER IN RELATION TO SUBSTRATE CONCENTRATION.

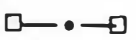
SPECIFIC ACTIVITY = NANOMOLES ACETYLTHIOCHOLINE IODIDE HYDROLIZED/MG.PROTEIN/ MINUTE.

 = HOMOGENATE.

 = NUCLEAR FRACTION.

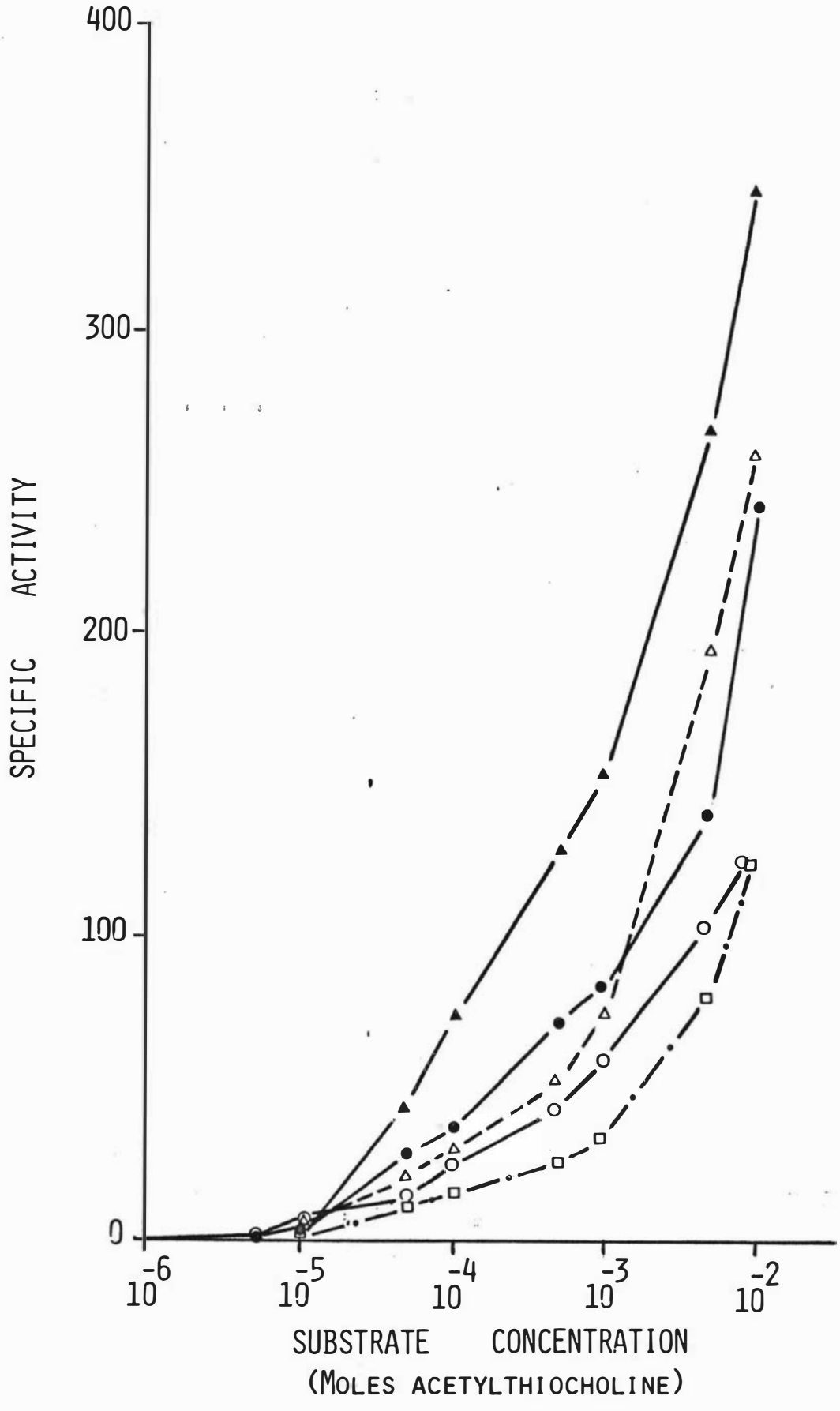
 = MITOCHONDRIAL FRACTION.

 = MICROSOMAL FRACTION.

 = CYTOSOL FRACTION.

4.11

FIG 4.11


400
300
200
100
0
SPECIFIC ACTIVITY
10-6
10-5
10-4
10-3
10-2
SUBSTRATE CONCENTRATION
(MOLES ACETYLTHIOCHOLINE)

4.12

FIG 4.12

LINEWEAVER BURK PLOT FOR THE HYDROLYSIS OF ACETYLTHIOCHOLINE IODIDE BY CHOLINESTERASE FROM HOMOGENATE AND FRACTIONS OF MONIEZIA

BUFFER - PHOSPHATE 0.1 M pH 8.0

TEMPERATURE - INCUBATED AT 37C FOR 5 MIN.

= HOMOGENATE.

= MITOCHONDRIAL FRACTION.

= MICROSOMAL FRACTION.

= CYTOSOL FRACTION.

V = SPECIFIC ACTIVITY = n MOL. ACETYLTHIOCHOLINE IODIDE HYDROLYZED/MG.PROTEIN/MIN.

S = SUBSTRATE CONCENTRATION (mM).

K_m FOR CHOLINESTERASE ACTIVITY OF HOMOGENATE AND FRACTIONS OF MONIEZIA

PREPARATION	V_{max} 10^{-3}	K_m 10^{-4}M
HOMOGENATE	27.0	10.28
MITOCHONDRIA	29.4	8.43
MICROSOME	21.7	2.12
CYTOSOL	10.2	1.48

4.12

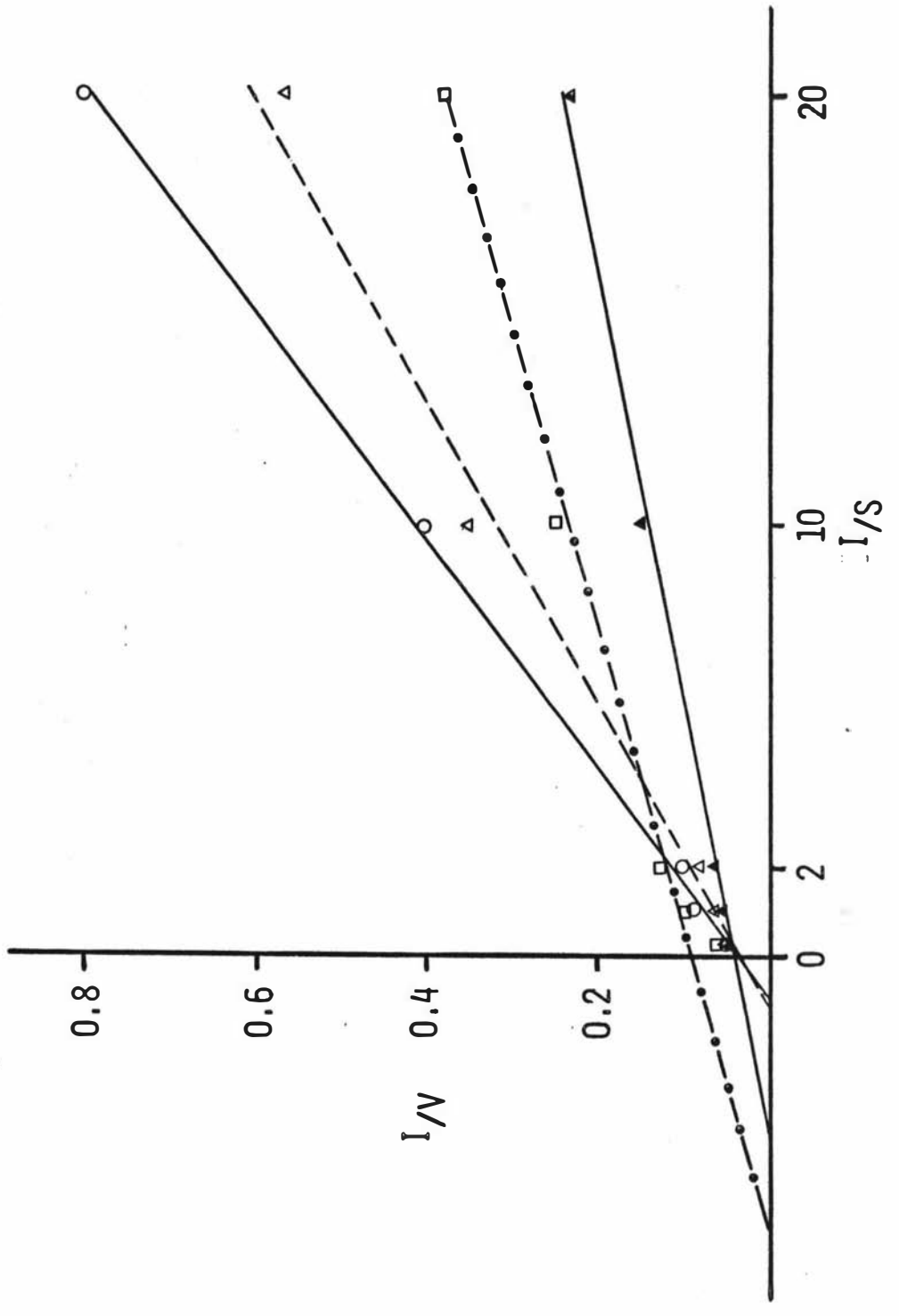


FIG 4.12

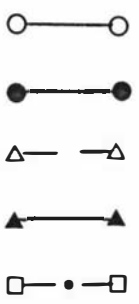
4.13

FIG 4.13

LINEWEAVER BURK PLOT FOR THE HYDROLYSIS OF ACETYLTHIOCHOLINE IODIDE BY CHOLINESTERASE FROM HOMOGENATE AND FRACTIONS OF TAENIA OVIS

BUFFER - PHOSPHATE 0.1M pH 8.0

TEMPERATURE - INCUBATED AT 37°C FOR 5 MIN.



= HOMOGENATE.

= NUCLEAR FRACTION.

= MITOCHONDRIAL FRACTION.

= MICROSOMAL FRACTION.

= CYTOSOL FRACTION.

V = SPECIFIC ACTIVITY = n MOL. ACETYLTHIOCHOLINE IODIDE HYDROLYZED/MG. PROTEIN/MIN.

S = SUBSTRATE CONCENTRATION (mM).

K_m FOR CHOLINESTERASE ACTIVITY OF HOMOGENATE AND FRACTIONS OF TAENIA OVIS

PREPARATION	V_{max} 10^{-3}	K_m 10^{-4}M
HOMOGENATE	71.4	2.86
NUCLEAR	76.9	3.54
MITOCHONDRIA	142.9	2.14
MICROSOME	142.9	2.21
CYTOSOL	55.6	2.33

4.13

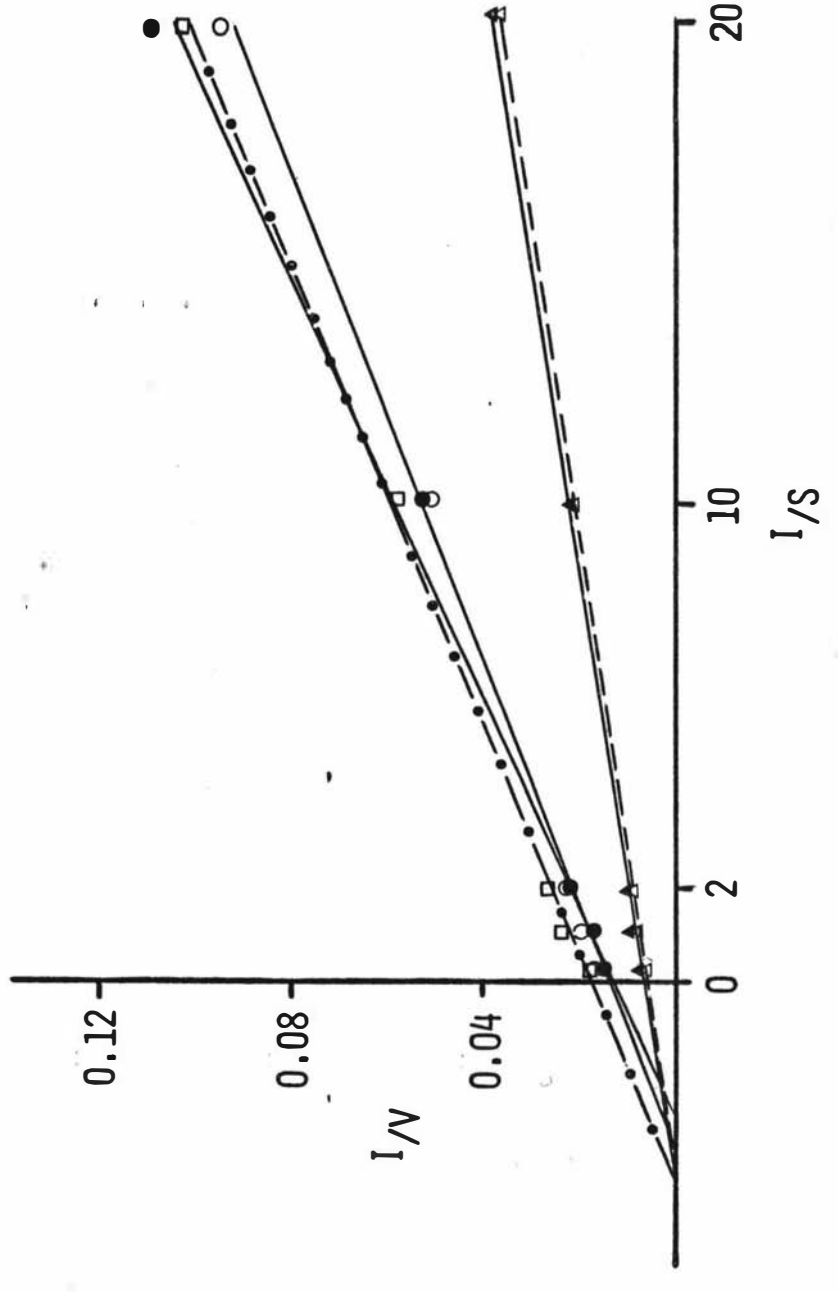


FIG 4.13

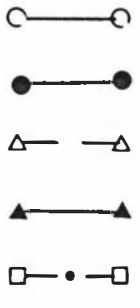
4.14

FIG 4.14

LINEWEAVER BURK PLOT FOR THE HYDROLYSIS OF ACETYLTHIOCHOLINE IODIDE BY CHOLINESTERASE FROM HOMOGENATE AND FRACTIONS OF DOG LIVER

BUFFER - PHOSPHATE 0.1M pH 8.0

TEMPERATURE - INCUBATED AT 37°C FOR 5 MIN.



= HOMOGENATE.

= NUCLEAR FRACTION.

= MITOCHONDRIAL FRACTION.

= MICROSOMAL FRACTION.

= CYTOSOL FRACTION.

V = SPECIFIC ACTIVITY = nMOL.ACETYLTHIOCHOLINE IODIDE HYDROLYZED/MG.PROTEIN/MIN.

S = SUBSTRATE CONCENTRATION (mM).

K_m FOR CHOLINESTERASE ACTIVITY OF HOMOGENATE AND FRACTIONS OF DOG LIVER

PREPARATION	V_{max} 10^{-3}	K_m 10^{-4}M
HOMOGENATE	71.4	1.96
NUCLEAR	111.1	1.78
MITOCHONDRIA	90.9	1.73
MICROSOME	200.0	1.80
CYTOSOL	37.0	1.37

4.14

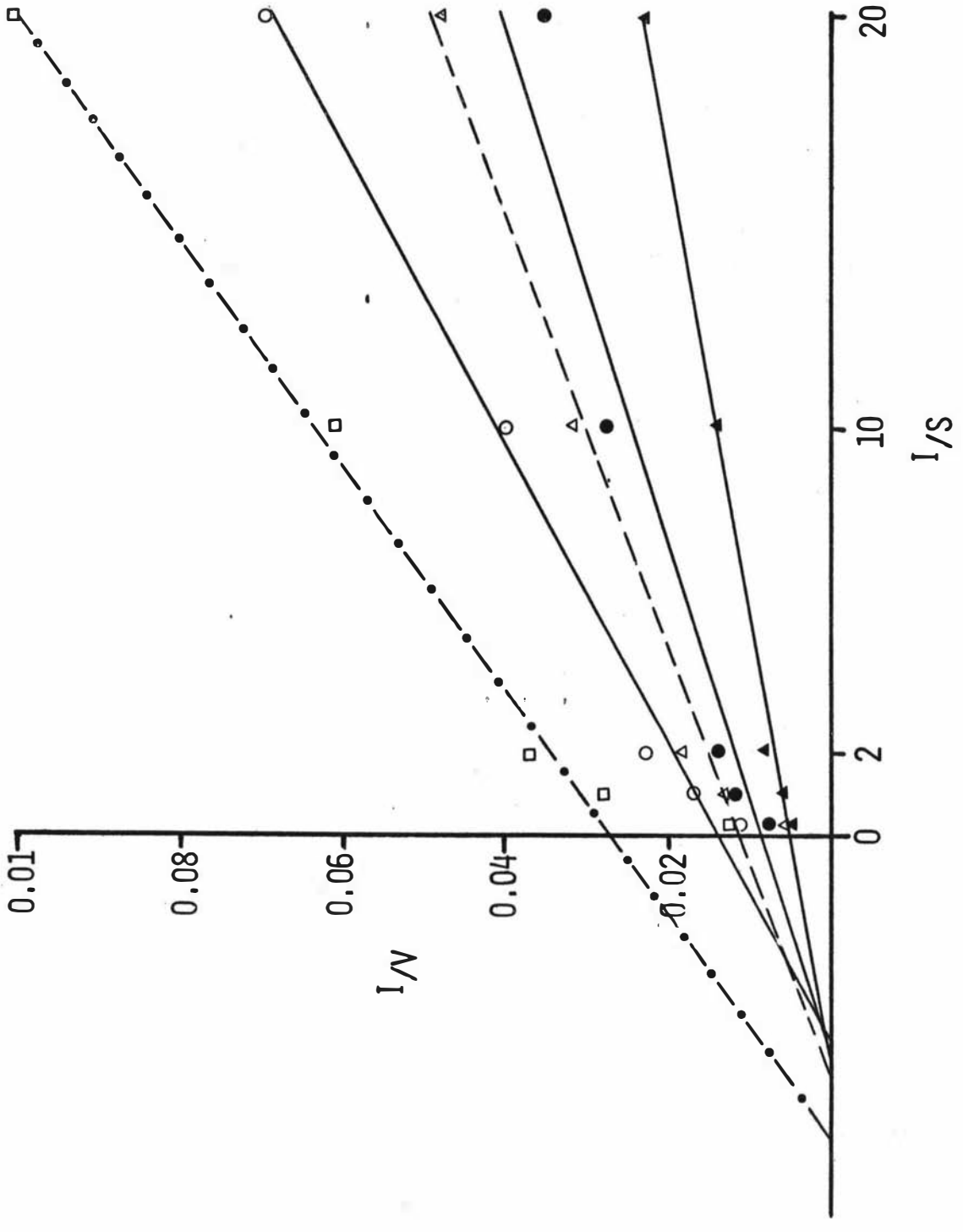


FIG 4.14

To enable comparison of Km values, the above information is summarised in (Table 4.12).

It should be noted that Km values for all kinetic studies were calculated from data using substrate concentrations in the range of 5×10^{-5}M to 5×10^{-3}M. In some cases there were deviations from linearity of the Lineweaver-Burk plot at higher substrate concentrations such that the enzyme was apparently not saturated even at 10^{-2}M: such points were excluded in the present analysis.

4.4.2

(a) Cholinesterase activity of helminth homogenates

The cholinesterase activity of various species and tissues is shown in Table 4.13. Among the nematode materials, the head portion of *A. suum* showed the greatest rate of cholinesterase activity (0.76 n mol/mg/min) which is more than three times that shown by the muscle portion of the same worm (0.24 n mol/mg/min). The hydrolysis rate of homogenate from entire *A. galli* (0.56 n mol/mg/min) falls into an intermediate position. It is clear however, that *Trichuris* has the highest rate of cholinesterase activity of the nematodes examined.

Among the tapeworm material, a strikingly high rate of 13.47 n mol/mg/min is displayed by brood capsules and scoleces of *Echinococcus* whereas the cyst fluid had a high degree of activity and no activity was detected from the cyst membrane. The rate for *T. ovis* (3.69 n mol/mg/min) appeared about sixty per cent higher than that of *T. hydatigena* (2.25 n mol/mg/min).

4.4.2

(b) Inhibition of helminth cholinesterase

In the following section the inhibition effect of various drugs is assessed from their 'I_{50}' values. This value is defined as the molar concentration of the antagonist which reduces by 50 per cent the activity of the test tissue as compared with the drug-free controls. This value was calculated from a Probit transformation of the percentage inhibition to give an approximately linear 'dose-probit

TABLE 4.12

MICHAELIS CONSTANTS OF CHOLINESTERASE OF HOMOGENATES AND FRACTIONS OF *MONIEZIA*, *TAENIA OVIS* AND DOG LIVER

PREPARATION	K_m (10^{-4} M)		
	MONIEZIA	*T. OVIS*	DOG LIVER
HOMOGENATE	10.28	2.86	1.96
NUCLEI	—	3.54	1.78
MITOCHONDRIA	8.43	2.14	1.73
MICROSOMES	2.12	2.21	1.80
CYTOSOL	1.48	2.33	1.37

TABLE 4.13

RATE OF SUBSTRATE HYDROLYSIS BY CHOLINESTERASE IN HOMOGENATES OF VARIOUS WORMS

Enzyme source	Hydrolysis rate (n mol/mg wet weight/min)		
	Mean	S.E.	No.of experiments
Ascaris suum (head portion)	0.76500	0.01800	22
Ascaris suum (muscle)	0.24560	0.01000	25
Ascaridia galli	0.56389	0.02492	18
Trichuris ovis	1.09960	0.01957	25
Echinococcus (brood capsules & scoleces)	13.47867	0.11191	15
Echinococcus (cyst fluid) *	0.15600	0.00141	18
Echinococcus (cyst menbrane)	0.00000	—	3
Taenia ovis	3.69364	0.08158	77
Taenia hydatigena	2.25685	0.03536	73

* Hydrolysis rate expressed in u Moles/ml/minute.
Substrate concentration 0.075 Moles of acetylthiocholine in 0.1 M phosphate buffer pH 8.0 at 37C.

response line' which was then fitted by the method of least squares. The data are reported in both tabular and graphic forms in Figs. 4.15 to 4.22. The overall information with respect to all the inhibitors and worm tissues employed is summarised in Table 4.14.

4.4.2

(c) Inhibition of Ascaris, Ascaridia and Trichuris

Results with eserine, pyrantel and levamisole for head and muscle of *Ascaris* are presented in Fig. 4.15 and 4.16 and it can be seen that eserine is the most powerful inhibitor, with an I_{50} value of 4.56×10^{-6}M for both homogenates. The two anthelmintics also exhibted inhibitory effects and their I_{50} values (1.70×10^{-4} M and 2.04×10^{-4}M) were essentially the same when tested on the head portion. Moreover, pyrantel appears only slightly less active on muscle cholinesterase than on that from the head region but with respect to levamisole, the muscle enzyme appears about four times more resistant to inhibition.

Using homogenates made from complete *Ascaridia* worms, eserine was again the most potent of the three drugs tested, having its I_{50} in the 10^{-7}M range. Levamisole proved the second most potent ($I_{50} = 2.2 \times 10^{-4}$M) and pyrantel the least. This order of potency is the reverse of that for *Ascaris* . However the range of I_{50} for the anthelmintics, is quite small, amounting at the most, to a four-fold difference (Fig. 4.17).

Trichuris cholinesterase (Fig. 4.18) was also highly susceptable to eserine but levamisole proved to be the second most potent inhibitor with an I_{50} about five times less than that of pyrantel.

4.4.2

(d) Inhibition of Echinococcus

The effect of the three inhibitors on the materials derived from the *Echinococcus* are shown in Figs 4.19 and 4.20. Eserine was again the most potent inhibitor, the differences in I_{50} between fluid and solid material was only three fold, and the ranges overlapped. With capsules and scoleces, pyrantel and levamisole had virtually identical effects

TABLE 4.14

IN VITRO INHIBITION OF CHOLINESTERASE IN HOMOGENATES OF VARIOUS WORMS BY DRUGS

DRUGS	I_{50} (M)							
	A. suum (head)	*A. suum (muscle)*	*A. galli*	*Trichuris ovis*	*Echinococcus (brood capsu. & scoleces)*	*Echinococcus (cyst fluid)*	*T. ovis*	*T. hydatigena*
Eserine	4.56×10^{-6}	4.56×10^{-6}	7.84×10^{-7}	4.61×10^{-7}	2.67×10^{-7}	5.65×10^{-7}	2.68×10^{-7}	4.61×10^{-7}
Levamisole	2.04×10^{-4}	7.85×10^{-4}	2.20×10^{-4}	7.43×10^{-5}	3.82×10^{-4}	6.13×10^{-4}	7.86×10^{-4}	5.70×10^{-4}
Pyrantel	1.70×10^{-4}	2.14×10^{-4}	7.45×10^{-4}	2.48×10^{-4}	3.01×10^{-4}	3.47×10^{-4}	-	3.82×10^{-4}
Morantel							2.33×10^{-4}	2.07×10^{-4}
Dichlorvos							3.94×10^{-5}	1.15×10^{-4}
Vincofos							1.86×10^{-5}	3.19×10^{-4}

I_{50} is the concentration given 50% inhibition of cholinesterase, derived from observation with at least four concentrations of inhibitor over a 100-fold range. Each figure represents the response over several dose levels with 3 to 7 experiments at each level. Probit transformation of the % inhibition gave an approximately linear "dose-probit response line" which was fitted by the method of least squares. Incubation with drug at 37 C for 5 minutes before adding acetylthiocholine iodide.

FIG. 4.15

EFFECT OF INHIBITORS ON THE HYDROLYSIS OF CHOLINESTERASE FROM *ASCARIS SUIS* (HEAD PORTION)

NAME	MOLAR CONCENTRATION	% INHIBITION*	I_{50} (M)
ESERINE	10^{-7}	6.4	
	10^{-6}	19.2	4.56×10^{-6}
	10^{-5}	66.5	$(2.20 \times 10^{-6} - 9.46 \times 10^{-6})$
	10^{-4}	91.0	
PYRANTEL	10^{-5}	0.0	1.70×10^{-4}
	10^{-4}	40.0	$(1.10 \times 10^{-4} - 2.84 \times 10^{-4})$
	10^{-3}	80.0	
LEVAMISOLE	10^{-5}	0.0	
	10^{-4}	36.0	2.04×10^{-4}
	10^{-3}	78.7	$(1.19 \times 10^{-4} - 3.80 \times 10^{-4})$

* Each figure represents the mean of 3 to 7 experiments. Preincubation with drugs at 37° C was for 5 minutes before adding acetylthiocholine iodide.
Figures in parentheses show 95% confidence limits for each I_{50}.
The I_{50} was calculated from a Probit transformation of the % inhibition to give an approximately linear "dose-probit response line" which was fitted by the method of least squares.

FIG 4.15

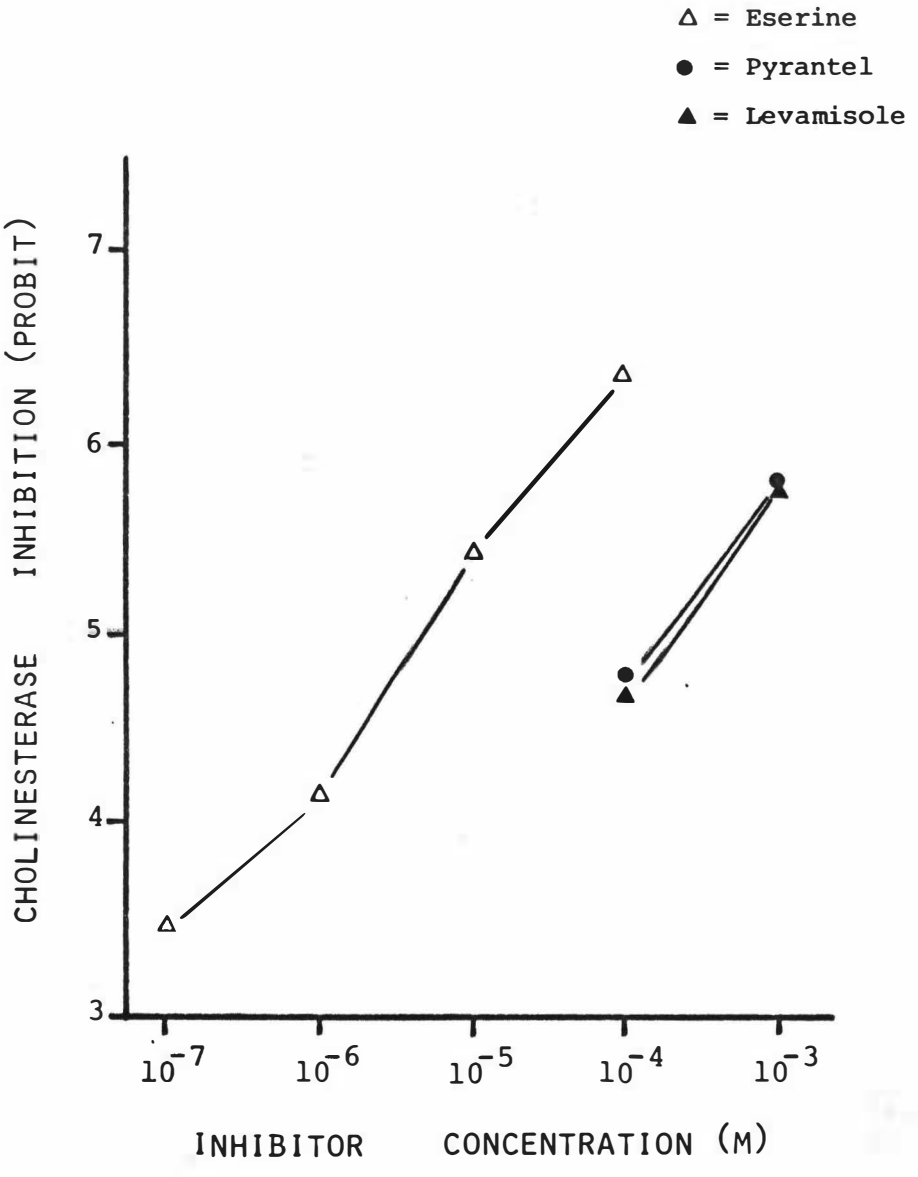

Δ = Eserine
● = Pyrantel
▲ = Levamisole
CHOLINESTERASE INHIBITION (PROBIT)
7
6
5
4
3
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
INHIBITOR CONCENTRATION (M)

FIG. 4.16

EFFECT OF INHIBITORS ON THE HYDROLYSIS OF CHOLINESTERASE FROM ASCARIS SUIS (MUSCLE)

NAME	MOLAR CONCENTRATION	% INHIBITION*	I_{50}(M)
ESERINE	10^{-7}	11.9	
	10^{-6}	31.2	4.56×10^{-6}
	10^{-5}	57.6	$(0.63 \times 10^{-6} - 448.4 \times 10^{-6})$
	10^{-4}	84.6	
PYRANTEL	10^{-6}	0.0	
	10^{-5}	9.1	2.14×10^{-4}
	10^{-4}	38.0	$(1.38 \times 10^{-4} - 3.37 \times 10^{-4})$
	10^{-3}	74.4	
LEVAMISOLE	10^{-6}	8.4	
	10^{-5}	14.8	7.85×10^{-4}
	10^{-4}	32.7	$(2.49 \times 10^{-4} - 197.2 \times 10^{-4})$
	10^{-3}	53.3	

* Each figure represents the mean of 3 to 7 experiments. Preincubation with drugs at 37° C was for 5 minutes before adding acetylthiocholine iodide.
Figures in parentheses show 95% confidence limits for each I_{50}.
The I_{50} was calculated from a Probit transformation of the % inhibition to give an approximately linear "dose-probit response line" which was fitted by the method of least squares.

FIG 4.16

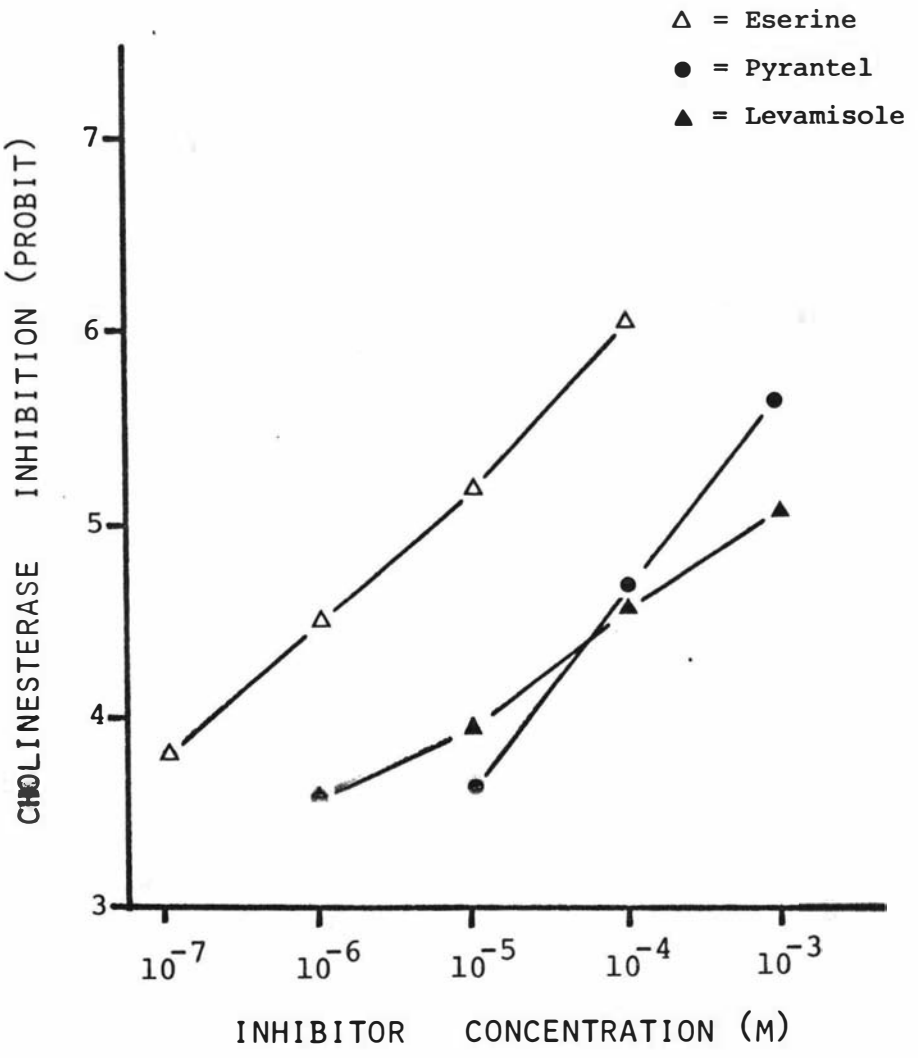

Δ = Eserine
● = Pyrantel
▲ = Levamisole
CHOLINESTERASE INHIBITION (PROBIT)
7
6
5
4
3
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
INHIBITOR CONCENTRATION (M)

FIG. 4.17

EFFECT OF INHIBITORS ON THE HYDROLYSIS OF CHOLINESTERASE FROM HOMOGENATE OF ASCARIDIA GALLI

NAME	MOLAR CONCENTRATION	% INHIBITION*	I_{50}(M)
ESERINE	10^{-7}	16.8	
	10^{-6}	59.6	7.84×10^{-7}
	10^{-5}	85.6	$(1.82 \times 10^{-7} - 31.40 \times 10^{-7})$
	10^{-4}	93.3	
PYRANTEL	10^{-6}	0.0	
	10^{-5}	8.4	7.45×10^{-4}
	10^{-4}	16.0	$(1.37 \times 10^{-7} - 57.9 \times 10^{-7})$
	10^{-3}	59.7	
LEVAMISOLE	10^{-6}	1.1	
	10^{-5}	10.0	2.20×10^{-4}
	10^{-4}	32.5	$(0.32 \times 10^{-4} - 18.40 \times 10^{-4})$
	10^{-3}	76.8	

* Each figure represents the mean of 3 to 7 experiments. Preincubation with drugs at 37 C was for 5 minutes before adding acetylthiocholine iodide.
Figures in parentheses show 95% confidence limits for each I_{50}.
The I_{50} was calculated from a Probit transformation of the % inhibition to give an approximately linear "dose-probit response line" which was fitted by the method of least squares.

FIG 4.17

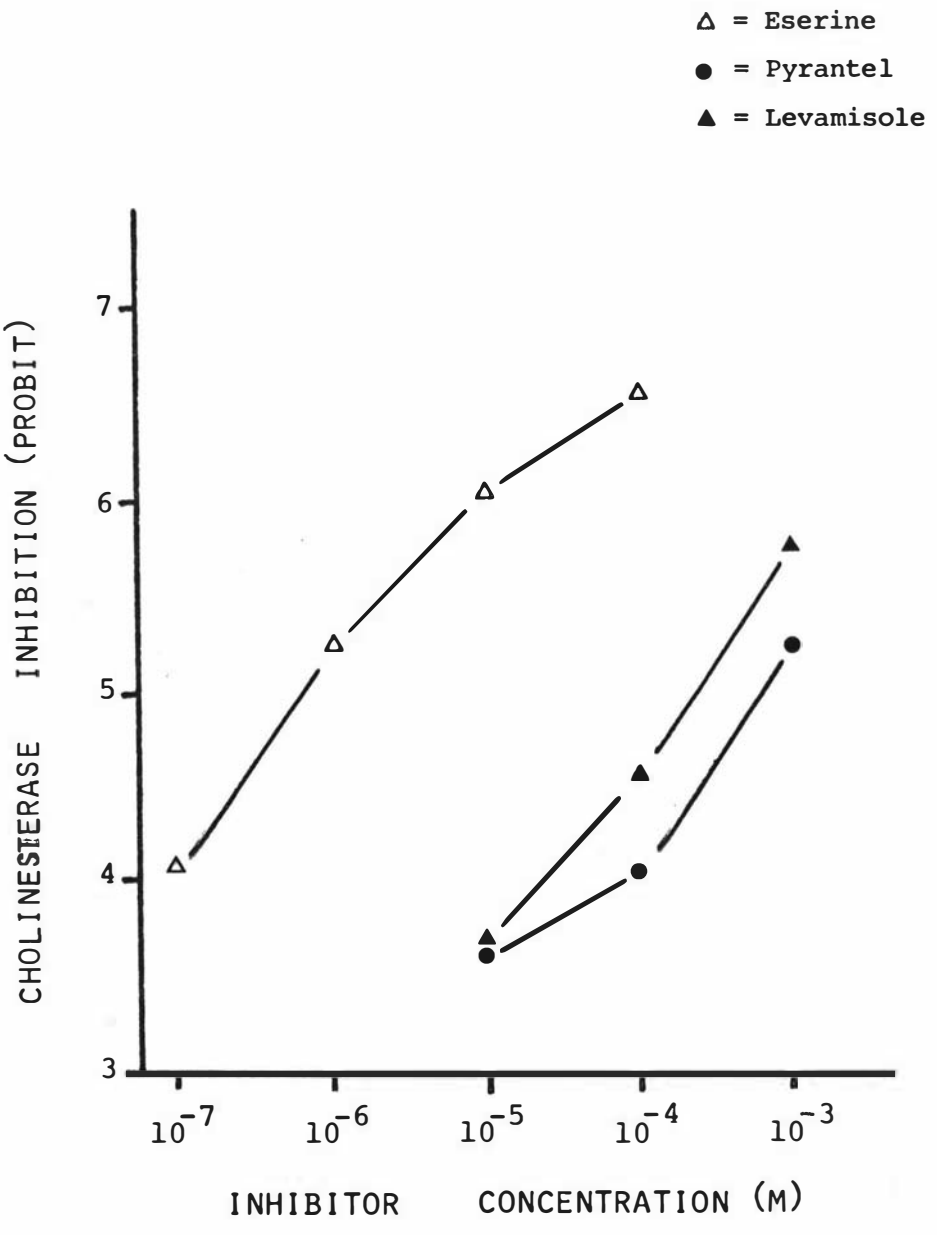

△ = Eserine
● = Pyrantel
▲ = Levamisole
CHOLINESTERASE INHIBITION (PROBIT)
7
6
5
4
3
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
INHIBITOR CONCENTRATION (M)

FIG. 4.18

EFFECT OF INHIBITORS ON THE HYDROLYSIS OF CHOLINESTERASE FROM HOMOGENATE OF TRICHURIS OVIS.

NAME	MOLAR CONCENTRATION	% INHIBITION*	I_{50}(M)
ESERINE	10^{-7}	24.5	4.61×10^{-7} (0.32×10^{-7} - 49.90×10^{-7})
	10^{-6}	76.2	
	10^{-5}	86.6	
	10^{-4}	99.6	
PYRANTEL	10^{-6}	0.0	2.48×10^{-4} (0.11×10^{-4} - 153.0×10^{-4})
	10^{-5}	7.0	
	10^{-4}	34.5	
	10^{-3}	69.1	
LEVAMISOLE	10^{-6}	0.0	7.43×10^{-5} (3.22×10^{-5} - 17.10×10^{-5})
	10^{-5}	13.2	
	10^{-4}	53.2	
	10^{-3}	93.1	

Figures in parentheses show 95% confidence limits for each I_{50}. The I_{50} was calculated from a Probit transformation of the % inhibition to give an approximately linear "dose-probit response line" which was fitted by the method of least squares.

* Each figure represents the mean of 3 to 6 experiments. Preincubation with drugs at 37°C was for 5 minutes before adding acetylthiocholine iodide.

FIG 4.18

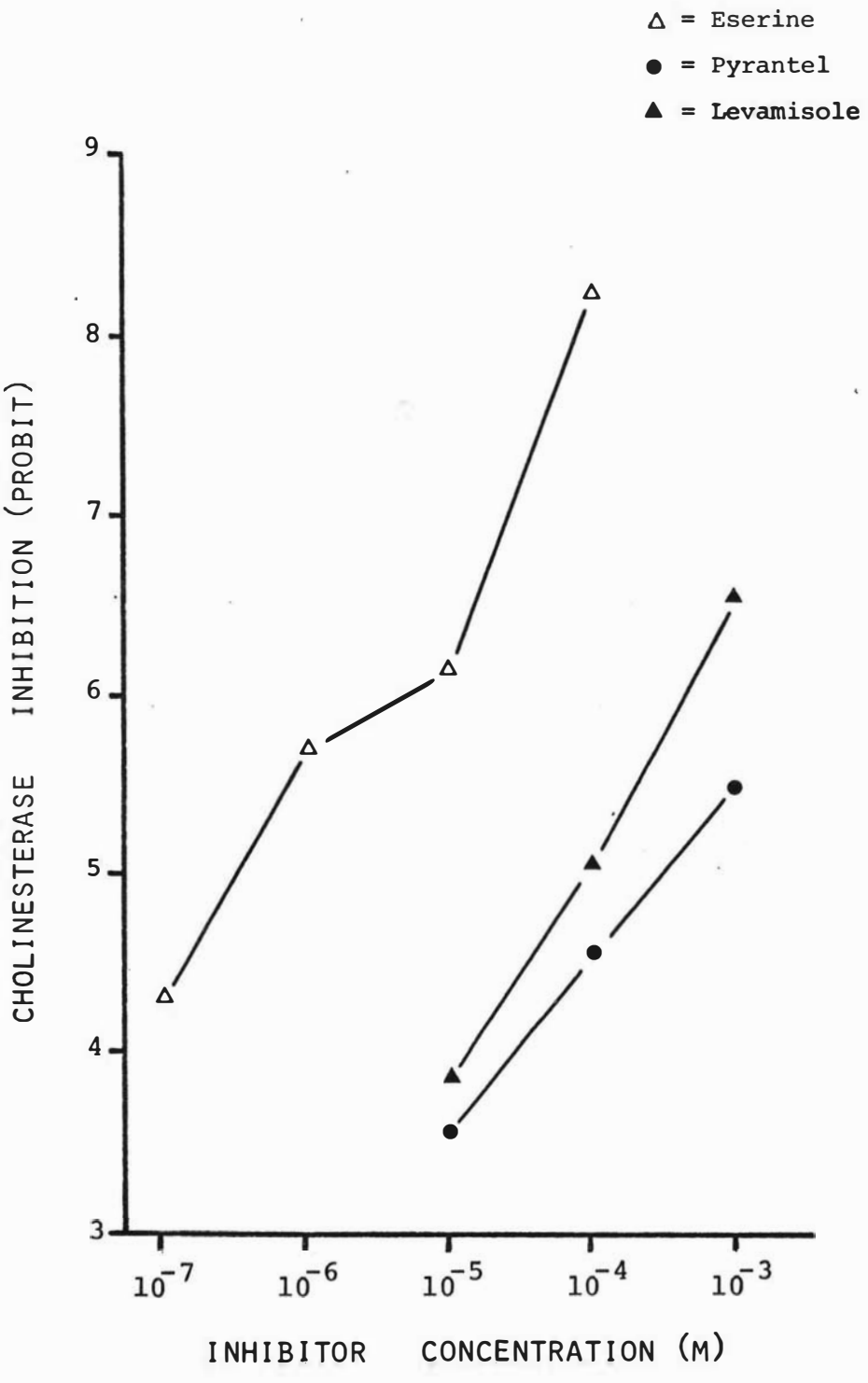

△ = Eserine
● = Pyrantel
▲ = Levamisole
CHOLINESTERASE INHIBITION (PROBIT)
9
8
7
6
5
4
3
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
INHIBITOR CONCENTRATION (M)

FIG. 4.19

EFFECT OF INHIBITIORS ON THE HYDROLYSIS OF CHOLINESTERASE FROM *ECHINOCOCCUS* (BROOD CAPSULES AND SCOLECES)

NAME	MOLAR CONCENTRATION	% INHIBITION*	I_{50}(M)
ESERINE	10^{-7}	21.5	2.67×10^{-7} ($3.02\times10^{-}$ - 1.27×10^{-4})
	10^{-6}	84.2	
	10^{-5}	93.3	
	10^{-4}	95.4	
PYRANTEL	10^{-6}	0.0	3.01×10^{-4} (2.45×10^{-4} - 3.71×10^{-4})
	10^{-5}	4.2	
	10^{-4}	28.7	
	10^{-3}	72.9	
LEVAMISOLE	10^{-6}	0.0	3.82×10^{-4} (0.72×10^{-4} - 18.74×10^{-4})
	10^{-5}	3.3	
	10^{-4}	27.0	
	10^{-3}	79.3	
	10^{-2}	88.6	

* Each figure represents the mean of 3 to 4 experiments. Preincubation with drugs at 37° C was for 5 minutes before adding acetylthiocholine iodide.
Figures in parentheses show 95% confidence limits for each I_{50}.
The I_{50} was calculated from a Probit transformation of the % inhibition to give an approximately linear "dose-probit response line" which was fitted by the method of least squares.

FIG 4.19

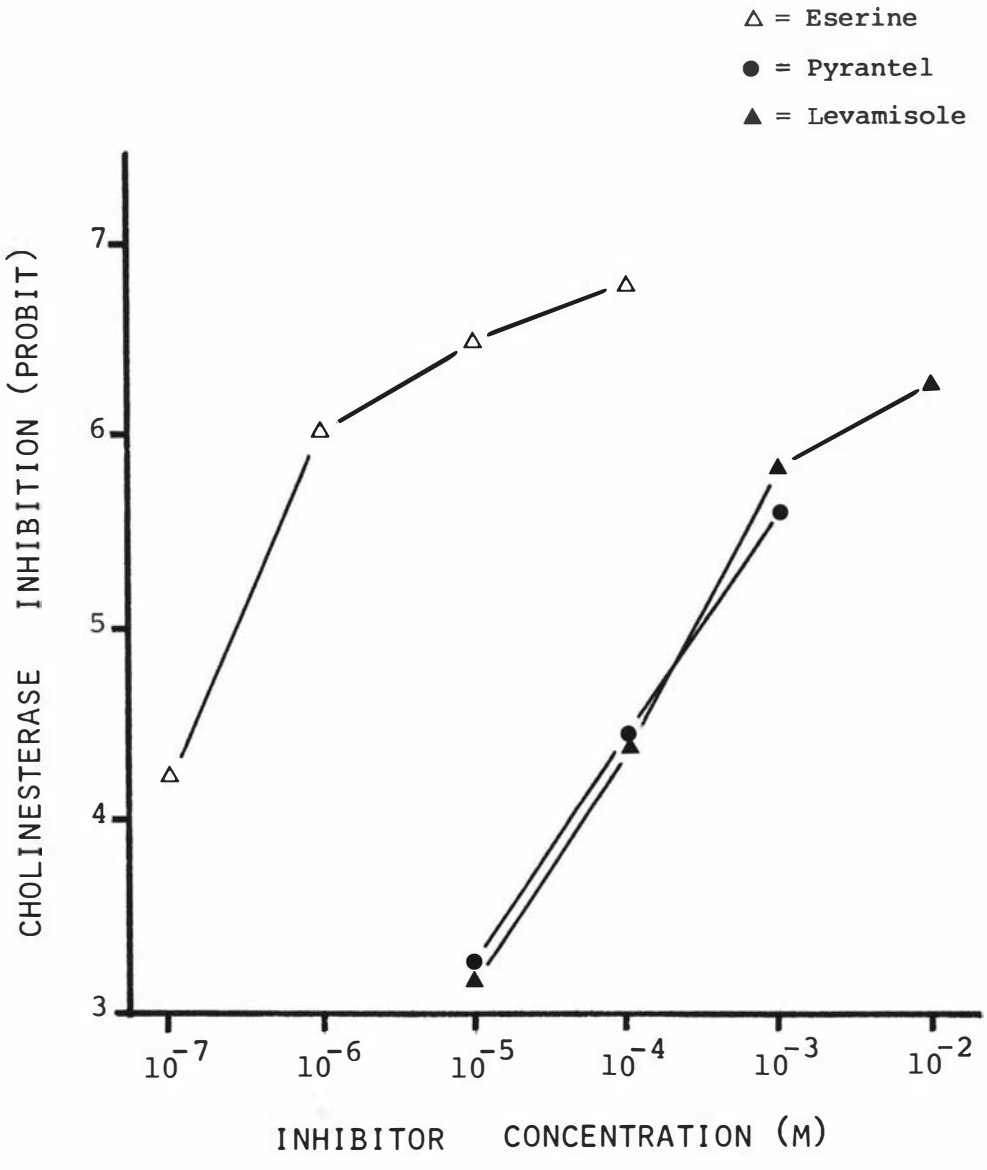

△ = Eserine
● = Pyrantel
▲ = Levamisole
CHOLINESTERASE INHIBITION (PROBIT)
7
6
5
4
3
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
INHIBITOR CONCENTRATION (M)

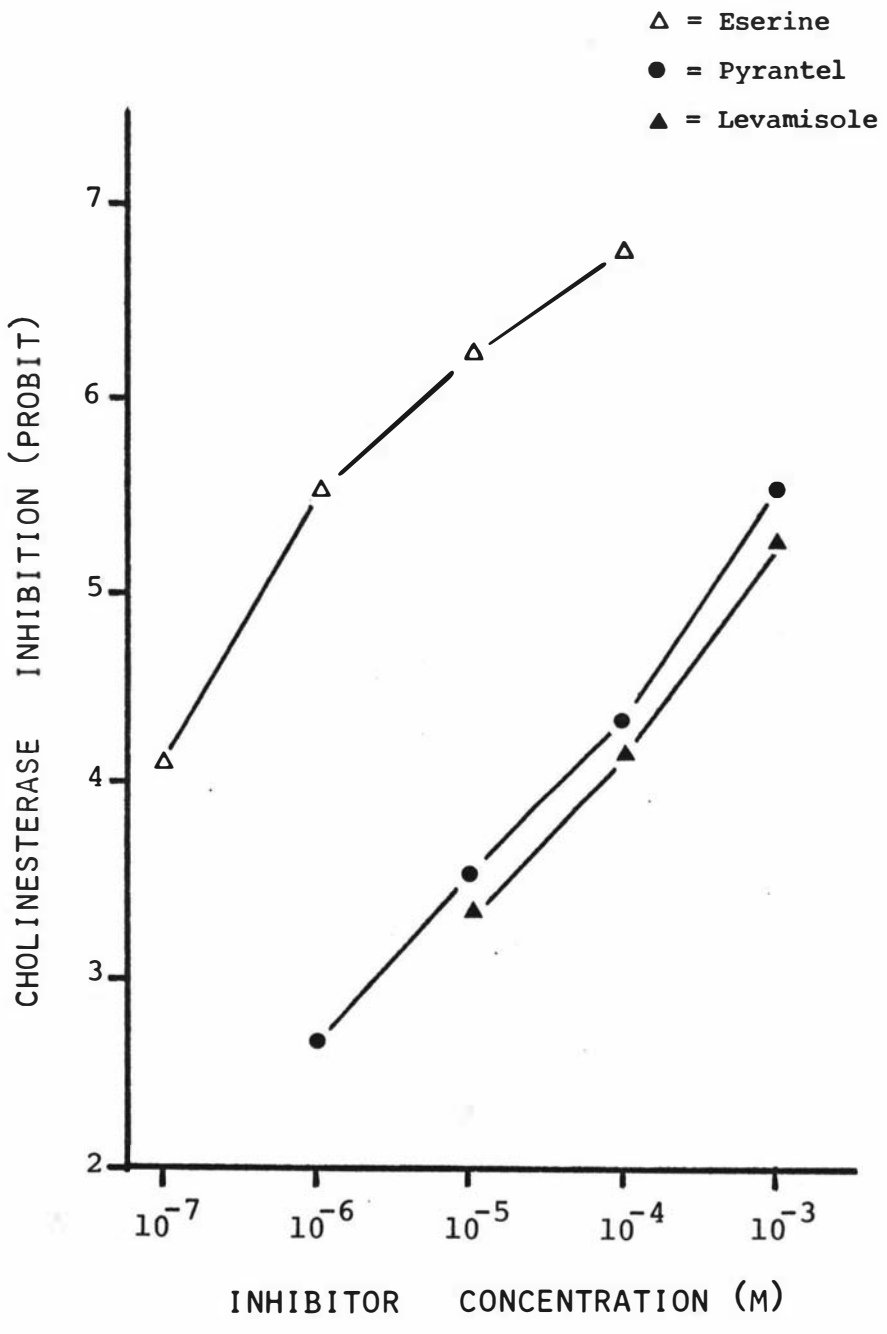
FIG. 4.20

EFFECT OF INHIBITORS ON THE HYDROLYSIS OF CHOLINESTERASE FROM *ECHINOCOCCUS* (CYST FLUID)

NAME	MOLAR CONCENTRATION	% INHIBITION*	I_{50}(M)
ESERINE	10^{-7}	18.3	5.65×10^{-7} (1.03×10^{-7} - 27.8×10^{-7})
	10^{-6}	69.9	
	10^{-5}	88.9	
	10^{-4}	96.0	
PYRANTEL	10^{-6}	1.0	3.47×10^{-4} (1.32×10^{-4} - 9.59×10^{-4})
	10^{-5}	7.6	
	10^{-4}	24.4	
	10^{-3}	70.8	
LEVAMISOLE	10^{-6}	0.0	6.13×10^{-4} (3.66×10^{-4} - 10.4×10^{-4})
	10^{-5}	4.6	
	10^{-4}	19.5	
	10^{-3}	59.9	

* Each figure represents the mean of 3 to 7 experiments. Preincubation with drugs at 37° C was for 5 minutes before adding acetylthiocholine iodide.
Figures in parentheses show 95 % confidence limits for each I_{50}.
The I_{50} was calculated from a Probit transformation of the % inhibition to give an approximately linear "dose-probit response line" which was fitted by the method of least squares.

FIG 4.20


△ = Eserine
● = Pyrantel
▲ = Levamisole
CHOLINESTERASE INHIBITION (PROBIT)
7
6
5
4
3
2
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
INHIBITOR CONCENTRATION (M)

4.4.2

but with fluid, levamisole appears less potent although the ranges overlapped and essentially the I_{50}'s may be regarded as identical.

4.4.2

(e) Inhibition of T. ovis worms

In experiments with *T. ovis* and *T. hydatigena* , the range of inhibitors was extended to include the organophosphorus taeniacides, dichlorvos and vincofos. The results for *T. ovis* are presented in Fig. 4.21. Eserine again proved the most potent inhibitor with its I_{50} in the 10^{-7}M range and levamisole was the weakest drug tested. Morantel's potency lay between levamisole and that of dichlorvos and vincofos. In terms of inhibitory potency the order of the drugs was eserine >> and dichlorvos > morantel and levamisole.

4.4.2

(f) Inhibition of T. hydatigena worms.

Results with *T. hydatigena* are presented in Fig. 4.22. Eserine again proved the most effective inhibitor exhibiting a potency similar to that recorded with all other tissues. All the anthelmintics exhibited similar I_{50} values and as can be seen from the graph (Fig. 22) all the ranges overlapped.

4.4.2

(g) The effect of Dichlorvos and Vincofos on T. ovis and T. hydatigena

To enable easy comparison, the data previously recorded for *T. ovis* (Fig. 4.21) and *T. hydatigena* (Fig. 4.22) are summarised in Fig. 4.23. It can be seen that the two organophosphorus compounds are more potent inhibitors of *T. ovis* cholinesterase than that of *T. hydatigena*.

4.4.2

(h) Effect of eserine on homogenate and subcellular fractions of T. ovis

Subcellular fractions from homogenates of *T. ovis* were prepared as for Section 4.3.2and the effects of eserine examined over a range of

FIG. 4.21

EFFECT OF INHIBITORS ON THE HYDROLYSIS OF CHOLINESTERASE FROM HOMOGENATE OF *TAENIA OVIS*

NAME	MOLAR CONCENTRATION	% INHIBITION*	I_{50}(M)
ESERINE	10^{-7}	21.3	2.68×10^{-7} (0.25×10^{-7} - 25.00×10^{-7})
	10^{-6}	83.1	
	10^{-5}	96.0	
	10^{-4}	97.3	
MORANTEL	10^{-5}	6.5	2.33×10^{-4} (1.03×10^{-4} - 5.28×10^{-4})
	10^{-4}	38.7	
	10^{-3}	79.2	
	10^{-2}	93.7	
LEVAMISOLE	10^{-6}	0.0	7.86×10^{-4} (4.03×10^{-4} - 15.70×10^{-4})
	10^{-5}	4.4	
	10^{-4}	16.1	
	10^{-3}	56.4	
DICHLORVOS	10^{-5}	17.2	3.94×10^{-5} (2.24×10^{-5} - 6.89×10^{-5})
	5×10^{-5}	50.3	
	10^{-4}	79.4	
	10^{-3}	98.6	
	10^{-2}	100.0	
VINCOFOS	10^{-5}	38.0	1.86×10^{-5} (0.70×10^{-5} - 3.25×10^{-5})
	5×10^{-5}	74.4	
	10^{-4}	81.9	
	10^{-3}	95.6	
	10^{-2}	100.0	

* Each figure represents the mean of 3 to 7 experiments. Preincubation with drugs at 37 C was for 5 minutes before adding acetylthiocholine iodide.
Figures in parentheses show 95% confidence limits for each I_{50}.
The I_{50} was calculated from a Probit transformation of the % inhibition to give an approximately linear "dose-probit response line" which was fitted by the method of least squares.

FIG 4.21

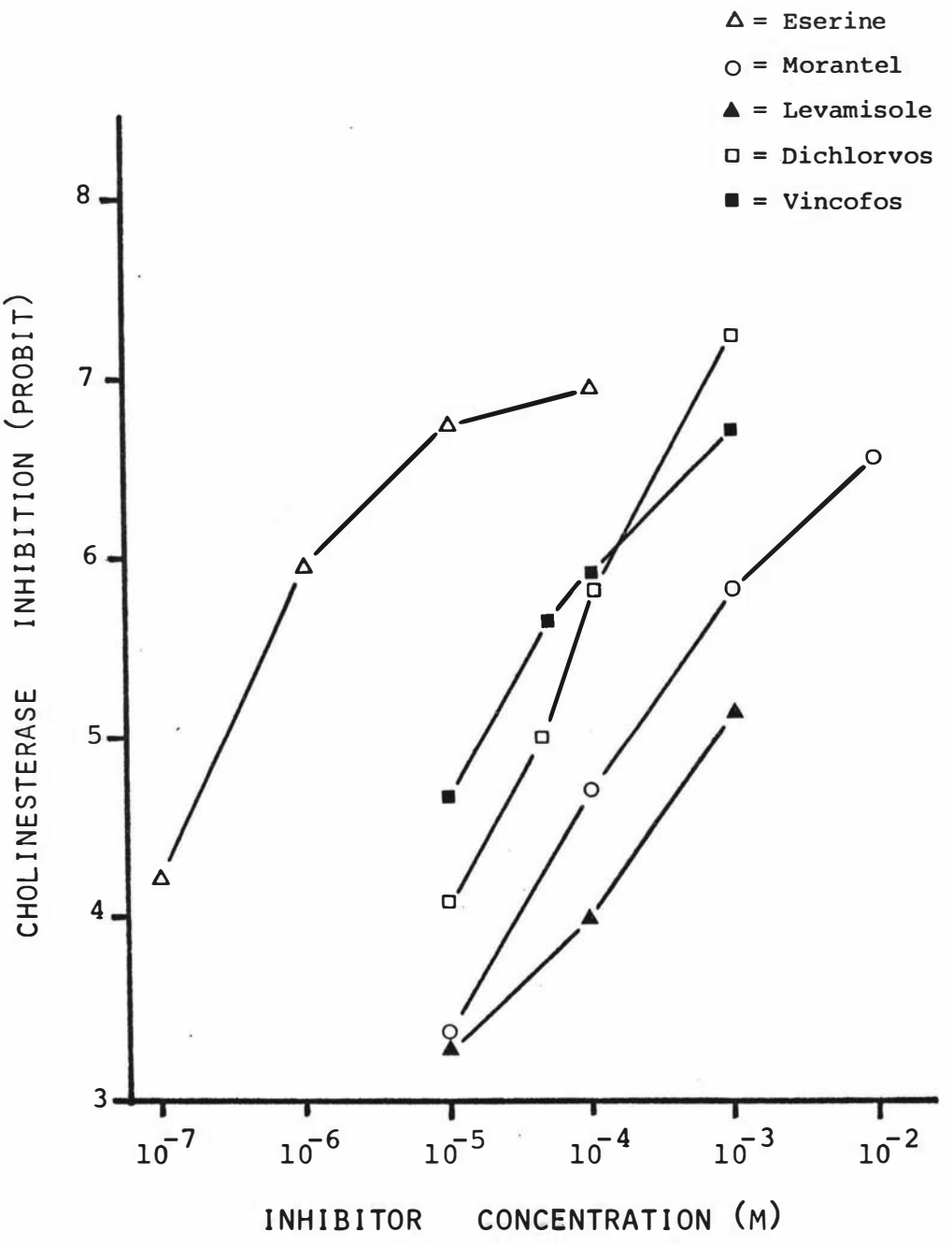

△ = Eserine
○ = Morantel
▲ = Levamisole
□ = Dichlorvos
■ = Vincofos
CHOLINESTERASE INHIBITION (PROBIT)
8
7
6
5
4
3
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²
INHIBITOR CONCENTRATION (M)

FIG. 4.22

EFFECT OF INHIBITORS ON THE HYDROLYSIS OF CHOLINESTERASE FROM HOMOGENATE OF *TAENIA HYDATIGENA*

NAME	MOLAR CONCENTRATION	% INHIBITION*	I_{50}(M)
ESERINE	10^{-7}	10.2	4.61×10^{-7} (0.18×10^{-7} - 5.06×10^{-7})
	10^{-6}	65.2	
	10^{-5}	92.4	
	10^{-4}	97.0	
PYRANTEL	10^{-6}	0.0	3.82×10^{-4} (1.93×10^{-4} - 5.06×10^{-4})
	10^{-5}	3.9	
	10^{-4}	25.8	
	10^{-3}	68.0	
MORANTEL	10^{-5}	8.2	2.07×10^{-4} (0.97×10^{-4} - 4.34×10^{-4})
	10^{-4}	39.8	
	10^{-3}	80.7	
	10^{-2}	93.6	
LEVAMISOLE	10^{-5}	0.0	5.70×10^{-4} (0.18×10^{-4} - 361.2×10^{-4})
	10^{-4}	11.2	
	10^{-3}	57.0	
	10^{-2}	95.5	
DICHLORVOS	10^{-5}	6.7	1.15×10^{-4} (0.42×10^{-4} - 4.36×10^{-4})
	10^{-4}	39.0	
	5×10^{-4}	87.7	
	10^{-3}	93.1	
	10^{-2}	97.1	
VINCOFOS	10^{-5}	7.7	3.19×10^{-4} (1.52×10^{-4} - 6.60×10^{-4})
	10^{-4}	31.0	
	5×10^{-4}	59.7	
	10^{-3}	70.6	
	10^{-2}	90.3	

Data in parentheses show range of I_{50}.
* Each figure represents the mean of 3 to 7 experiments.

FIG 4.22

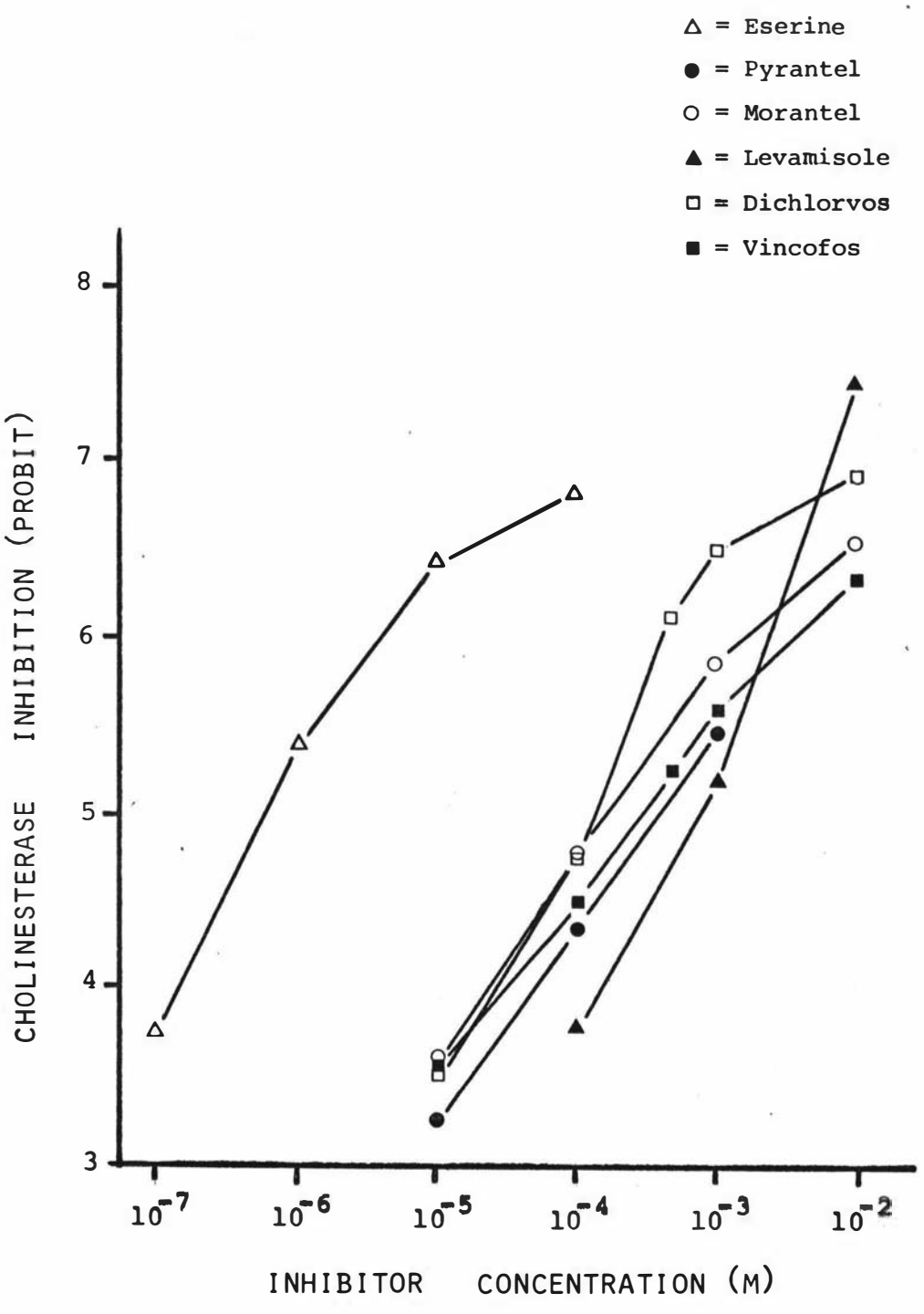

△ = Eserine
● = Pyrantel
○ = Morantel
▲ = Levamisole
□ = Dichlorvos
■ = Vincofos
CHOLINESTERASE INHIBITION (PROBIT)
8
7
6
5
4
3
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²
INHIBITOR CONCENTRATION (M)

FIG. 4.23

INHIBITION(I_{50}) BY DICHLORVOS (OPEN SQUARES) AND VINCOFOS (FULL SQUARES) OF THE CHOLINESTERASE ACTIVITY TOWARDS HOMOGENATES OF T.OVIS (BROKEN LINES) AND T.HYDATIGENA (CONTINUOUS LINES).

HELMINTH	DICHLORVOS I_{50}(M)	VINCOFOS I_{50}(M)
T.OVIS	3.94×10^{-5} (2.24×10^{-5} - 6.85×10^{-5})	1.86×10^{-5} (0.70×10^{-5} - 3.25×10^{-5})
T.HYDATIGENA	1.15×10^{-4} (0.42×10^{-4} - 4.36×10^{-4})	3.19×10^{-4} (1.52×10^{-4} - 6.60×10^{-4})

Figures in parentheses show 95% confidence limits for each I_{50}.
Probit transformation of the % inhibition of inhibitor concentrations ranging from 10^{-5} M to 10^{-2} M, gave an approximately linear "dose-probit response line". The line was fitted by the method of least squares and the I_{50} estimated from the equation for the line.
Results were calculated from 3 experiments involving 10^{-2} M dichlorvos in T.hydatigena, and from 7 experiments involving the remaining work.
Preincubation with inhibitors at 37°C was for 5 minutes prior to the addition of substrate.

FIG 4.23

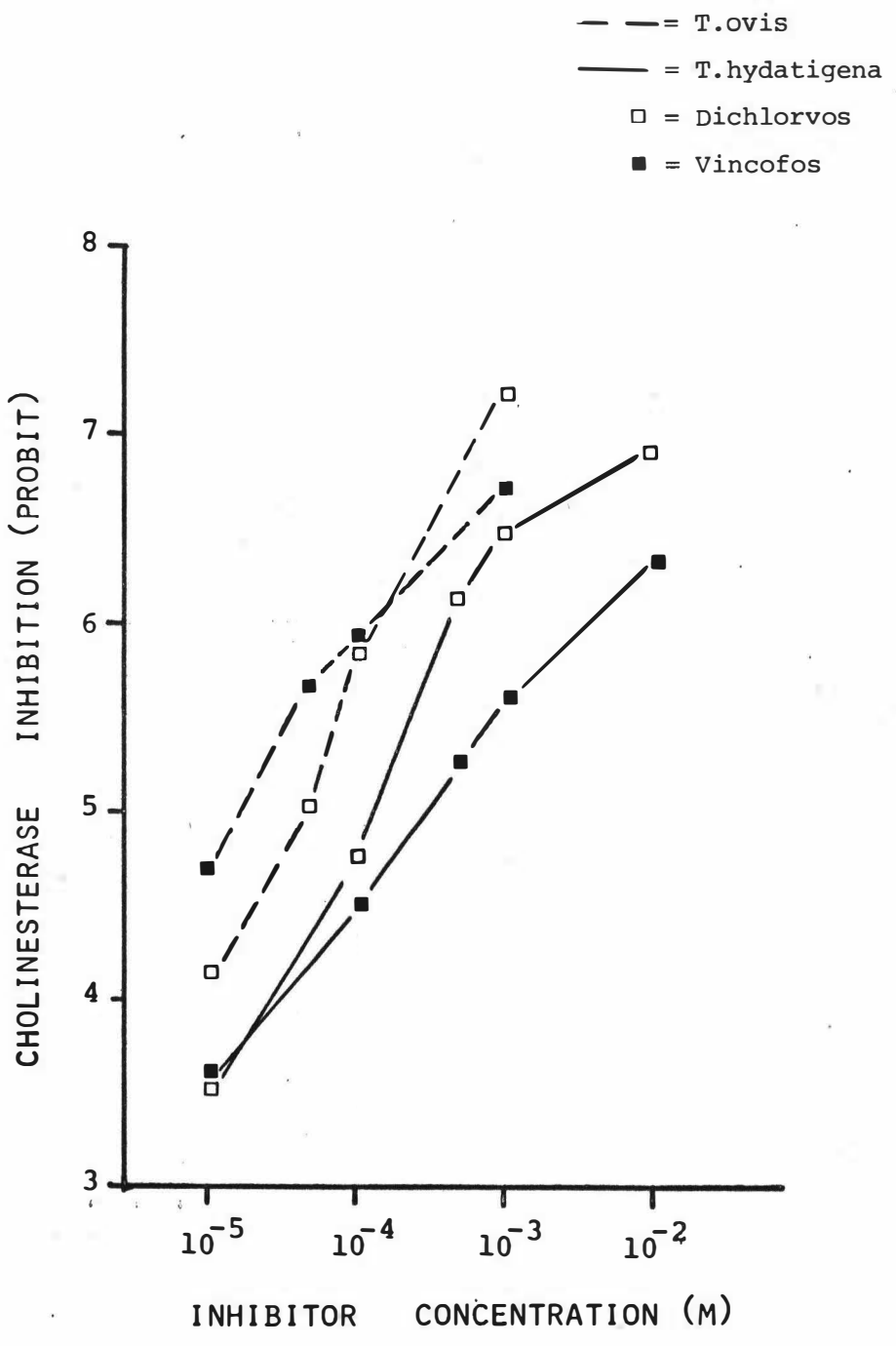

— —= T.ovis
——— = T.hydatigena
□ = Dichlorvos
■ = Vincofos
CHOLINESTERASE INHIBITION (PROBIT)
8
7
6
5
4
3
10^{-5}
10^{-4}
10^{-3}
10^{-2}
INHIBITOR CONCENTRATION (M)

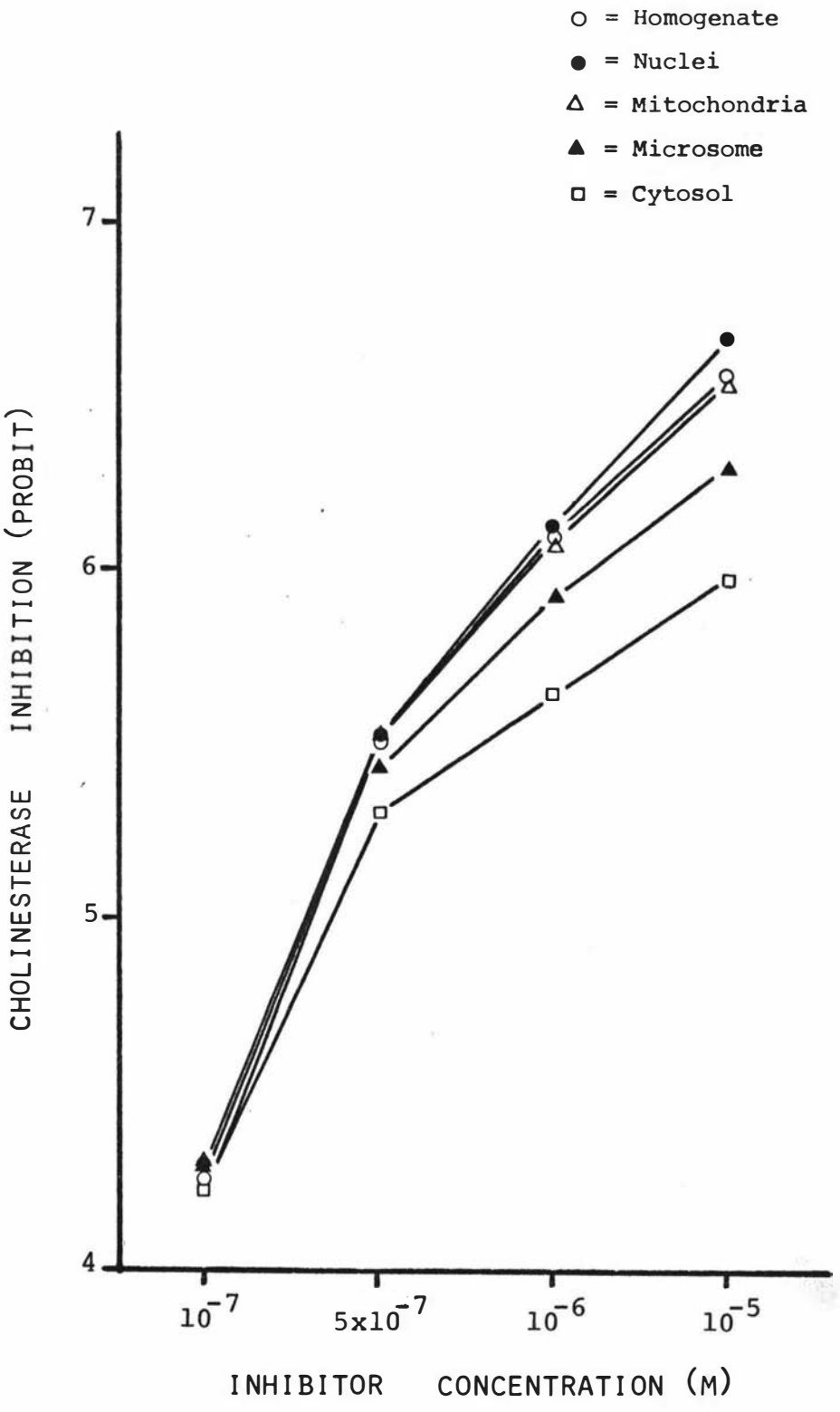
FIG. 4.24

EFFECT OF ESERINE SALICYLATE ON THE HYDROLYSIS OF CHOLINESTERASE FROM HOMOGENATE AND FRACTIONS OF *TAENIA OVIS*

PREPARATION	% INHIBITION* MOLAR CONCENTRATION 10^{-7}	5×10^{-7}	10^{-6}	10^{-5}	I_{50}
HOMOGENATE	22.1	69.5	86.6	94.2	2.50×10^{-7} (0.58×10^{-7} - 12.42×10^{-7})
NUCLEI	24.0	70.6	87.0	95.0	2.29×10^{-7} (0.62×10^{-7} - 10.51×10^{-7})
MITOCHONDRIA	24.1	70.4	86.3	93.6	2.30×10^{-7} (0.44×10^{-7} - 12.25×10^{-7})
MICROSOME	22.6	66.7	82.2	90.3	2.80×10^{-7} (0.39×10^{-7} - 16.58×10^{-7})
CYTOSOL	21.5	62.2	74.5	83.7	3.84×10^{-7} (0.31×10^{-7} - 30.28×10^{-7})

Figures in parentheses show 95% confidence limit for each I_{50}.
The I_{50} was calculated from a Probit transformation of the % inhibition to give an approximately linear "dose-probit response line" which was fitted by the method of least squares.
* Each figure represents the mean of three experiments. Incubation with eserine at 37° C for 5 minutes before adding acetylthiocholine iodide.

FIG 4.24


O = Homogenate
● = Nuclei
Δ = Mitochondria
▲ = Microsome
□ = Cytosol
CHOLINESTERASE INHIBITION (PROBIT)
7
6
5
4
10⁻⁷
5x10⁻⁷
10⁻⁶
10⁻⁵
INHIBITOR CONCENTRATION (M)

4.4.2

concentrations 10^{-7}M - 10^{-5}M. The findings are reported in Fig. 4.24. The eserine gave I_{50} values for homogenate (2.50 x 10^{-7}M) nuclei (2.29 x 10^{-7}M) mitochondria (2.30 x 10^{-7}M) microsomes (2.80 x 10^{-7}M) and cytosol (3.84 x 10^{-7}M). It can be seen from these data and from the graph (Fig. 4.24) that there is very little difference in eserine I_{50} values with homogenate and all cell fractions - particularly at the lowest 2 or 3 concentrations and in addition all the ranges overlap significantly.

4.5 DISCUSSION

4.5.1 Subcellular distribution of cholinesterase

In order to understand throughly the possible functions of cholinesterase in tapeworms, more information on the subcellular distribution of this enzyme is required, since location and function are related. It was also felt that such distribution studies of cholinesterase might cast some light on the mode of action of drugs on these parasites.

Cholinesterases in various organisms are known to differ in activity as well as in subcellular distribution. The present study with fresh *M. expansa* reveals the presence of a high activity of particulate cholinesterase within the mitochondrial fraction as well as the microsomal fraction. In *T. ovis* a high activity of cholinesterase was also found in the microsomal and mitochondrial fractions. Similar overall activities of cholinesterase have been reported by Bueding (1952) working with *Schistosoma mansoni* who also found highest activity in the whole homogenate and in a particulate fraction sedimenting at 18,000 rpm in one hour. Working with fractions of *Ascaris suum*, Knowles and Casida (1966) and Hutchinson and Probert (1972) also found most of the cholinesterase activity in particulate fractions. These findings suggest a distribution similar to that found with *M. expansa* and *T. ovis* in the present work.

By contrast, the high activity found in mitochondria from fresh *M. expansa* contrasts with the low activity of mitochondria from human rectus - abdominis muscle (Smith *et al.* 1963) lobster leg nerve and house fly head fractions (Soeda *et al.* 1975). However it has been pointed out by Smallman and Wolfe (1956) that the distribution of cholinesterase between particulate and soluble fractions of insect heads is dependant on pH, becoming soluble in neutral homogenates, but remaining with the particulate fraction at lower pH. In contrast to fresh tissue, the mitochondrial fraction of frozen *M. expansa* showed the lowest cholinesterase specific activity of this tissue. The difference between the mitochondrial fractions of fresh and frozen *M. expansa* is undoubtedly

due partly to differences in the sedimentation properties of particles produced on homogenisation. The frozen tissue yielded a large amount of visible flocculant material on homogenisation which remained in suspension at low-speed centrifugation but sedimented with the mitochondrial fraction. This material, absent from homogenates of fresh tissue, may well have low cholinesterase activity and so dilute the 'mitochondrial' specific activity of the frozen tissue. Furthermore, the cholinesterase activity in the nuclear fraction was high in this frozen *Moniezia* , when compared to fresh *Moniezia* and *T. ovis*, and may have acquired some of the activity sedimenting with the mitochondrial fraction from fresh tissue. The process of freezing and storing at -18°C also resulted in an increase in the total and specific activity of cholinesterase in the soluble fraction of *Moniezia* suggesting that particulate enzyme had been solubilised by the freezing process.

It should be noted that the cholinesterase specific activities of homogenates of fresh and frozen *Moniezia* showed no significant difference so that if *Moniezia* is stored for 18 months at -18°C, it retains its original cholinesterase activity. The freeze-dried homogenates of four species of schistosoma stored at -16°C have also been reported to retain cholinesterase activity for at least 2 years and that of the intact organisms stored at -30°C remained constant for at least several months (Gear and Fripp, 1974). The robustness of some cholinesterases is further underlined by the findings of Keilin and Wang (1947) who found that a 42 year-old sample of horse blood which was collected aseptically and preserved in the dark at room temperature retained up to 85% of its original cholinesterase activity.

The present work suggests that, although the total cholinesterase activity cf *Moniezia* is relatively stable to freezing and storage, frozen tissue cannot be used to study the subcellular distribution of the enzyme.

4.5.2 Treatment with Triton X-100

The nonionic detergent, Triton X-100, has proved to be an excellent agent for solubilizing a variety of enzymes. Acid phosphatase,

ribonuclease, deoxyribonuclease, cathespin and B-glucuronidase activities were released quantitively from preparations of rat liver exposed to 0.1 - 0.25% (v/v) Triton X-100 (Wattiaux and de Duve, 1956; Shibko *et al*, 1965). Acid phosphatase of lysosomal and microsomal fractions of *F. hepatica* was solubilized when these two fractions were treated with 0.5% Triton X-100 and then incubated at 37°C for one hour (Lwin, 1974). Cholinesterases in some tissues are also solubilised by Triton X-100, such as the acetylcholinesterase from Torpedo electroplax (Eldefrawi *et al*. 1972).

Often this solubilisation effect leads to an increase in enzyme activity. For example, the specific activities of cholinesterases of mitochondrial, microsomal and soluble fractions of adult *A. suum* were increased when treated with 1% Triton X-100 (Hutchinson and Probert, 1972) probably due to conversion of membrane-bound enzyme to a form with an increased catalytic activity. On the other hand, the acetyl-cholinesterase activity of both the soluble and particulate fractions of lobster nerve was inhibited after treatment with Triton X-100 (Soeda *et al*. 1975) although this treatment consisted of storage rather than short-term incubation of the fractions.

In the present work, the cholinesterase activity of homogenates and all subcellular fractions of *T. ovis* was increased slightly but significantly by Triton treatment, and no marked inhibition by the detergent was apparent. By contrast, the treatment was without effect on the fractions isolated from fresh *Moniezia* , although all fractions of frozen material were stimulated by the Triton incubation. It may be concluded that the effect of Triton X-100 treatment in increasing the cholinesterase activity of subcellular fractions from the species examined is small and variable. The discrepancy between this work and that on *A. suum* by (Hutchinson and Probert, 1972) who found up to 3-fold increases in activity with Triton treatment may be ascribed to the species difference.

4.5.3 Effect of temperature on cholinesterase

Most enzymes demonstrate an increase in catalytic activity with rising temperature up to an optimum between 30°C and 40°C, decreasing at higher temperatures as the enzyme is denatured. Exceptions exist, such as some ribonucleases which can withstand temperatures up to 100°C. With respect to cholinesterases, the temperature optimum appears to vary considerably with the tissue of origin.

Early studies on the temperature effect on cholinesterase were carried out by Abderhalden and Paffrath in 1925. They demonstrated that the cholinesterase in press-juice of the small intestine of the pig was unaffected by heating at 55-58°C for 5 minutes, suggesting a relative resistance to heat denaturation. Similarly, the acetylcholinesterase from *Bungarus fasciatus* venom, although showing its optimum temperature at 40°C, still showed approximately 85% of its maximum activity at 50°C. (Kumar and Elliott, 1975b). In helminth tissue, the cholinesterase activity of *Fasciola hepatica* homogenate was reported to be constant between 30°C and 60°C (Probert and Durrani, 1977). On the other hand Lee and Hodson (1963) showed that the optimum temperature of cholinesterase of *H. contortus* was as low as 39°C, while that of *S. mansoni* homogenate was reported as 37°C, the enzyme being inactive at temperatures greater than 62°C. (Gear and Fripp, 1974). In view of this variability, it seemed logical to study the resistance of cholinesterase in tapeworms to elevated temperatures since this data are not available in the literature.

A high temperature optimum was obtained from homogenates and subcellular fractions of tapeworm tissues. The cholinesterase from the homogenate and three fractions of *Moniezia* showed temperature optima of 50°C and the homogenate and all fractions of *T. ovis* showed optima at 60°C. It appears that the cholinesterase from fractionated homogenate of *T. ovis* is more heat stable than the cholinesterase of *Moniezia* . All fractions were, however, inactive at 70°C. The study of similar fractions of dog liver, included for comparison, showed that, while homogenate, nuclear and microsomal fractions showed their expected optimum temperature at 40°C, the mitochondrial and cytosol fractions showed maxima at 60°C like the *T. ovis* fractions.

Although it seems at first sight that tapeworm's cholinesterase activities especially in *T. ovis*, are relatively resistant to heat, the same resistance in some fractions of liver suggest that the technique used in the present work was affecting the results. The effect of high temperatures on enzyme activity is time dependant and it is possible that, even at denaturing temperatures, not much activity was lost in the short period of incubation (5 minutes) used in this work. Alternatively, protection by some material present in the preparation might account for the rather different temperatures of denaturation which have been obtained by various workers, as well as the high optima reported here. Such protection can be afforded by substrate: partially purified preparations of fly head cholinesterase heated to 54° - 55°C in the presence of acetylcholine did not show loss of activity (Dauterman *et al.* 1962). Sodium choloride was similarly observed to protect against heat denaturation (Wolfe and Smallman, 1956). Possibly the medium in which fractions were incubated in the present work afforded some protection to denaturation.

4.5.4 Effect of substrate concentration

Cholinesterases from some sources and with some substrates are unusual in demonstrating maximum activity at one substrate concentration and decreased activity ('autoinhibition') at higher concentrations (Augustinsson, 1948). There are various explanations for the mechanism of autoinhibition. The inhibition may result from the binding of excess substrate to the acetylated enzyme, (Krupka and Laidler, 1961; Gear and Fripp, 1974). Brestkin *et al.* (1965 and 1966) gave their opinion that a decrease in the catalytic properties of the enzyme occurred as a result of a change in its structural conformation.

An even more unusual effect was observed by Hutchinson and Probert (1972) who found *two* peaks of maximum total cholinesterase activity of adult *A. suum* extracts against acetylcholine, at 10^{-5}M and 10^{-2}M. A similar observation of two peaks of maximum activity against acetylthiocholine iodide has been recorded with homogenates of *F. hepatica* (Probert and Durrani, 1977). They suggested that the two peaks were due to heterogeneity of the cholinesterases in this preparation.

4.5.4

The concentration at which autoinhibition occurs depends on the substrate. The autoinhibition effect was seen when acetylcholine iodide, acetylthiocholine iodide, acetyl-β-methylthiocholine chloride but not acetyl-β-methylcholine were hydrolysed by *B. fasciatus* venom acetylcholinesterase (Kumar and Elliott, 1975a). Thiocholine esters need higher concentrations before autoinhibition is seen: using homogenates of adult female *A. lumbricoides*, Knowles and Casida (1966) found 3×10^{-3}M) for acetylcholine iodide, propionylcholine p-toluenesulfonate and butyrylcholine p-toluenesulfonate; and 3×10^{-2}M. for acetylthiocholine iodide, propionylthiocholine iodide and butyrylthiocholine iodide. This may explain why no maximum was obtained in the present study of acetylthiocholine hydrolysis by homogenates and subcellular fractions of *Moniezia* , *T. ovis* and dog liver, where the enzyme activity increased with the increasing substrate concentrations up to 10^{-2}M.

The possibility that the cholinesterases studied here might be inhibited by very high substrate concentration in excess of 10^{-2}M remains untested. In this respect, it is significant that the cholinesterase activity of the tapeworm *T. taeniaeformis* showed an optimum substrate concentration at greater than 3×10^{-2}M acetylcholine chloride (Eranko *et al.* 1968) with inhibition at 1.2×10^{-1}M substrate. Gear and Fripp (1974) also reported a high substrate optimum of 4×10^{-2}M acetylcholine iodide for homogenates of *S. mansoni*.

Early workers suggested that exhibition of a substrate optimum, or maximum in the activity -pS curve, was a characterstic of 'true' cholinesterases (Augustinsson, 1957). In contrast, butyrylcholine hydrolysis by pseudocholinesterase shows no such autoinhibition (Adams and Whittaker, 1949). Such observations have been used to characterise other cholinesterases: Sanderson (1969) concluded that the enzyme present in *Nippostrongylus* appears to be a specific acetylcholine-easterase, since the a ctivity was reduced when the acetylcholine concentration was raised above an optimum of 2×10^{-2}M, and because butyrylcholine and triacetin were both hydrolysed at a slower rate than acetylcholine.

On the other hand, some observations suggest that even 'true' acetylcholinesterase activity can be increased by increasing the substrate concentrations higher than 10^{-2}M acetylcholine. The study by Spielholz and Van der Kloot (1973) has shown that the acetylcholinesterase of crayfish muscle was lacking in excess substrate inhibition. The acetylcholinesterase of lobster nerve, both soluble and particulate, increased with the increase in substrate concentration up to 10^{-2} M acetylthiocholine iodide (Soeda *et al.* 1975). Thus the observation that the cholinesterase in the present study shows no substrate inhibition at high concentration cannot be taken as proof that these are of the 'pseudo' cholinesterase type.

4.5.5 Kinetic parameters of cholinesterases

A review of the literature revealed no available data on the substrate specificity and kinetic parameters of cholinesterases from *Moniezia* or *T. ovis*. The present comparison of subcellular fractions of these organisms and mammalian liver revealed that there were some differences between the fractions of same species and also between the species.

Very little can be concluded from the variations in Vmax in Figs 4.12 - 4.14 since the molar concentration of enzyme in each fraction are not known and high Vmax fractions may merely contain more enzyme. The higher Vmax values obtained with *T. ovis* fractions, compared with those of *Moniezia* reflect the higher enzyme activity described in a previous chapter. On the other hand, a Km value is characteristic of an enzyme and independent of its concentration so that comparison of Km values is valid. There is little difference in Km values between the homogenate and fractions of *T. ovis* and it could be suggested that, even though a slight configurational difference in the enzyme is possible, there is no evidence from thesedata for the presence of more than one form of enzyme. The same conclusion could be reached from the data on homogenate and fractions of dog liver. However, there is large variation in Km values for *Moniezia* fractions. The Km values of homogenate and mitochondrial fraction are much higher than those of microsomal and cytosol fractions and it could be considered that there

is a different enzyme in the mitochondrial fraction with a lower affinity of the active sites for substrate.

It is well known, however, that the Mitchaelis-Menten constant is influenced by a number of factors, such as salt concentration ,pH, temperature and the physical state of the enzyme, (i.e.) whether bound or free. Kumar and Elliott (1975) showed the effect of pH and temperature on Vmax and Km values of acetylcholinesterase from *B. fasciatus* venom. At high homogenate concentrations the clumping of the cell membranes occurred, and this would tend to reduce the number of active sites of particulate enzymes available for the enzyme-substrate reaction. This phenomenon could explain the variation in Km values between fractions observed here. In this case dilution of the mitochondrial fraction to minimise membrane interactions may have yielded a lower Km value, as suggested by Gear and Fripp (1974) for another system.

4.5.6 Total cholinesterase activity of worm homogenates

The value of 0.246 n moles/mg/min for the activity of *A. suum* muscle agrees with that of Bueding (1952) who found a mean activity equivalent to 0.208 n moles/mg/min in *A. lumbricoides* muscle (assuming dry weight to be one sixth of wet weight). The value reported by Hart and Lee (1966) for *A. suum* is slightly higher at 0.32 n moles/mg/min as it that of Polyakova (1967) at 0.40 n moles/mg/min, but the data published by Knowles and Casida (1966) Melikhova (1970) and Hutchin and Probert (1972) on this species cannot be converted into comparable units. In the present study, the cholinesterase activity in the homogenate of head portions of pig *Ascaris* showed higher enzyme activities than muscle homogenates. This is confirmed by the work of Lee (1962) and Knowles and Casida (1966) and is explained on the basis of the nerve ring in the head portion. Znidaric (1967) has shown histochemically that the anterior portion of the worm which contains the senses and the nerve ring around the oesophagus also contains higher enzyme activity.

4.5.6

The mean cholinesterase activity of *A. galli* (0.56 n moles/mg wet weight/min) in the present study is only slightly higher than that of 0.47 units reported by Polyakova (1967), and the latter lies within our range of observations on this species.

In the case of *Trichuris ovis*, the present mean value of 1.09 units is somewhat higher than the 0.70 units found by Hart and Lee (1966) using acelylcholine perchlorate as substrate. The discrepancy might be due to the technique of blotting the surface moisture from the thawed worms, which influences wet weight.

The fresh-weight activity of our present study of brood capsules and scoleces of *E. granulosus* was 13.48 n mol/mg fresh weight/min, which is appreciably different from the value of 16.19 n mol/mg *dry* weight/min derived from the data of Schwabe *et al.* (1961) on the same tissues from bovine cysts. However, the conversion factor for dry weight to wet weight is not available for this tissue, so that an exact comparison cannot be made. Schwabe comments that these activities are high because brood capsules and scoleces contain contractile elements for motor activity and thus possessed cholinesterase. The high cholinesterase activity in cyst fluid of *E. granulosus* can be explained on the basis of the presence of germinal cells.

The present work constitutes the first report of cholinesterase activities in the two tape worms, *T. ovis* and *T.hydatigena*, and it is of interest to compare the values found (3.7 and 2.3 n moles acetylthiocholine/mg wet weight/minute respectively) with those reported for other species. Using a manometric method based on CO_2 evolution, Pylkko (1956b) has reported values for the tapeworm *D. latum* whose mean converts to 2.45 n moles acetylcholine/mg wet weight/min, assuming the dry weight of this worm is one sixth of the wet weight. The cholinesterase activity of the tape worm of the cat, *T. taeniaeformis* has been measured by Eranko *et al.* (1968) against acetylcholine chloride and acetyl-β-methylcholine chloride and when converted to units comparable with the present work, this gave activities in the range

2.60 - 3.35 n mol/mg/minute. The present data are thus in good agreement with that of other species of tapeworm.

4.5.7 The effect of inhibitors on helminth cholinesterases

There are no reports available in the literature on the effects of inhibitors on the cholinesterases of *T. ovis* and *T. hydatigena*. Only a few workers have studied the inhibitory effect of eserine on cholinesterase from *Ascaris* (Bueding, 1952; Knowles and Casida, 1966; Hutchinson and Probert, 1972) dichlorvos on adult female *A. lumbricoides* sections (Knowles and Casida, 1966) and eserine on brood capsule and scoleces of *Echinococcus*, (Schwabe *et al.* 1961). In our present study, the percentage inhibition and I_{50} of eserine, pyrantel, morantel, levamisole, dichlorvos and vincofos on the cholinesterases of *T. ovis*, *T. hydatigena*, brood capsules and scoleces and cyst fluid from *Echinoccus*, muscle and head portions from *A. suum*, *A. galli* and *Trichuris ovis* are reported.

4.5.7.

(a) Eserine

The I_{50} for eserine action on the homogenates of the worms tested ranged from 7.84×10^{-7}M in *A. galli* to 2.67×10^{-7}M in brood capsules and scoleces of *Echinococcus* except for homogenates of *A.suum* which were much higher at 4.56×10^{-6}M for both head and muscle portions. Thus the cholinesterase from *A,suum* was found to be less sensitive to eserine than homogenates of other worms. Bueding (1952) has pointed out that *A. lumbricoides has* less cholinesterase activity than the *Schistosomes* and the *Filariae* and that this explained why eserine does not sensitize *Ascaris* muscle to the effect of low concentrations of acetylcholine *in vitro*, as reported by Baldwin and Moyle (1949). The relatively low sensitivity of *Ascaris* cholinesterase to eserine has been reported previously. Knowles and Casida (1966) found an eserine I_{50} in *A. lumbricoides* of 3.94×10^{-6}M, and added that enzymatic hydrolysis of choline and thiocholine esters by *Ascaris* esterases was relatively resistant to inhibition by eserine. Hutchinson and Probert (1972) reported 76% inhibition of cholinesterase in a soluble extract of *A. suum* muscle by 10^{-5}M eserine, which is higher than that found in the present study (57.6%). The discrepancy might be due

to the preincubation period of 30 minutes at 37°C in their work which was reduced to 5 minutes in our study.

Lee and Hodsden (1963) have shown that cholinesterase from the parasite nematode, *H. contortus* is also relatively resistant to inhibition by eserine compared with cholinesterase from sheep erythrocytes and guinea pig erythrocytes and plasma. The percentage inhibition by eserine at 10^{-6}M, 10^{-5}M and 10^{-4}M in homogenates of *H. contortus* (Lee and Hodsden, 1963) were similar to the nematode parasites of *A. suum* (muscle and head portion) and *A. galli* reported in the present study.

Eranko *et al.* (1968) have studied the cholinesterase activity in the tape worm, *T. taeniaeformis* both biochemically and histochemically. From their data on the inhibition by eserine salicylate of the cholinesterase from the worm homogenate, the approximate level for 50% inhibition was 10^{-6}M against 0.03M acetyl-β-methylcholine and 10^{-8}M against 0.12 M butyrylcholine. Our mean values of I_{50} for eserine on the cholinesterase from tape worm homogenates of *T. ovis* 2.68 x 10^{-7}M); *T. hydatigena* (4.61 x 10^{-7}M), brood capsules and scoleces of *Echinococcus* (2.67 x 10^{-7}M), and cyst fluid of the latter (5.65 x 10^{-7}M) lay between their results. This is reasonable since acetylthiocholine iodide, which is hydrolysed by both acetylcholinesterase and butytylcholinesterase, was used as substrate. Much lower inhibitions of homogenates of hydatid scoleces and brood capsules have been reported by Schwabe *et al.* (1961) namely 32% and 86% at 10^{-3}M and 2 x 10^{-3}M eserine respectively. Although these authors comment themselves that this inhibition is much lower than those reported for cholinesterases of other tissues, there is no apparent reason for this discrepancy with the present results and those of others.

Comparison of the data in this work with published reports must be guarded, especially when different techniques and substrates are used. To quote one extreme example, Babers and Pratt, (1951) reported that the I_{50} for eserine on the cholinesterase from adult house fly head

was 2.3 x 10^{-6}M after measuring acetylcholine bromide hydrolysis by the Ammon (1933) technique. However, Soeda *et al.* (1975) had reported that the I_{50} for eserine of the cholinesterase from the same tissue (i.e. house fly head) microsomes and soluble fractions were 8.2 x 10^{-11}M and 8.4 x 10^{-11}M respectively with acetylthiocholine, using Ellman's method. These very different sensitivities to eserine could be due to the difference in substrate used, the different method, or the difference in preincubation period with the inhibitor (30 mins by Soeda *et al.* and 2 mins by Babers and Pratt).

4.5.7

(b) Levamisole

Levamisole, the laevorotatory isomer of tetramisole (2,3,5,6-tetrahydro-6-phenylimidazo (2,1-b) thiazole (Hcl) is known to be a broad spectrum anthelmintic. Some of the mechanisms of actions are direct action on nerve ganglia, the inhibition of fumarate reductase in nematode preparations (Van den Bossche and Janssen 1967, 1969; Van den Bossche, 1972) and inhibitions of cholinesterase activity (Eyre, 1970; Langer *et al.* 1972; Vertinskaya *et al.* 1972). L-tetramisole was at least 100 times less active as a cholinesterase inhibitor than eserine and is devoid of anticholinesterase effect on the intestinal tissues of rabbit (Van Belle, unpublished results quoted by Van Nueten, 1972).

The values of I_{50} for levamisole on the cholinesterase from the worm homogenates tested varied between 7.86 x 10^{-4}M in *T. ovis* and 2.04 x 10^{-4}M in *A. suum* (head portion) except for *Trichuris ovis* in which it was about ten times (7.43 x 10^{-5}M) more sensitive than the other worms tested. Vertinskaya *et al.* (1972) reported that, 72.2% of the cholinesterase from *A. galli* was inhibited by 4 x 10^{-3}M of tetramisole. This compares with 76.8% inhibition of the cholinesterase from the same species by 10^{-3}M levamisole in our study. However, Vertinskaya and co workers added that there was 26.8% inhibition in their first experiment, so the variation in their results was very wide. Langer *et al.* (1972) pointed out that tetramisole and piperazine were generally more effective cholinesterase inhibitors than thiabendazole or parbendazole, but there were no data quoted in the paper.

4.5.7

4.5.7

(c) Pyrantel and morantel

Pyrantel and morantel, act as depolarizing neuromuscular blocking agents in both vertebrates (Eyre, 1970; Aubry *et al*,1970) and helminths (Aubry *et al*.1970) are known to be broad spectrum anthelmintics. Pyrantel also inhibits the cholinesterase activity in blood: the I_{50} for pyrantel on acetylcholinesterase activity of sheep erythrocyte (I_{50} 3.16 x 10^{-5}M) and on the butyrylcholinesterase of horse plasma (I_{50} 10^{-4}M) has been reported by Eyre (1970). The I_{50} values for pyrantel inhibition of cholinesterase in cyst fluid and homogenates of different worms tested in the present work were slightly higher, varying between 7.45 x 10^{-4} in *A. galli* and 1.70 x 10^{-4}M in *A. suum* (head portion). Similar results were shown by morantel against the cholinesterase from homogenates of *T. ovis* (I_{50} 2.33 x 10^{-4}M) and *T. hydatigena* (I_{50} 2.07 x 10^{-4}M). It was seen that morantel (I_{50} 2.07 x 10^{-4}M) was a slightly more potent cholinesterase inhibitor than pyrantel (I_{50} 3.82 x 10^{-4}M) in the same species (*T. hydatigena)*.

4.5.7

(d) Organophosorus compounds

Organophosphorus compounds known to be the inhibitors of cholinesterase also exhibit a toxic action on worms. Lee and Hodsden (1963) have demonstrated the cholinesterase inhibition by haloxon, trichlorphon, paraoxon and other compounds using enzyme sources from parasites (*H. contortus* and *Trichuris ovis*) and blood of sheep and guinea-pig. Cholinesterases of nine parasitic nematodes from sheep and pig have been shown by Hart and Lee (1966) to be inhibited by the organophosphate anthelmintic, haloxon. Effects of organophosphates including dichlorvos, trichlorfon and haloxon on *A. lumbricoides* from pig have been examined by Knowles and Casida (1966). Inhibition of cholinesterase in four species of *Schistosomes* and in mouse brain by dichlorvos and trichlorfon) have been reported by Bueding *et al*. (1972).

4.5.7

In our present studies, the percentage inhibition of cholinesterases of T. *ovis* and *T. hydatigena* by dichlorvos and vincofos with other inhibitors are as shown in Fig. 4.21 and Fig.4.22 respectively and I_{50} values for the two organophosphates are shown in Fig. 4.23. The inhibition by dichlorvos of adult female *A. lumbricoides* anterior section using acetylthiocholine iodide as substrate (I_{50} 2.51 x 10^{-7}M) reported by Knowles and Casida (1966) is lower than these results using homogenates of *T. ovis* and *T. hydatigena*(I_{50} values ranging from 1.9 x 10^{-5}M to 1.2 x 10^{-4}M). The I_{50} values for the same inhibitor of the cholinesterase enzyme of *S. mansoni* and *S. haematobium*(mixed sex) were between 7.5 x 10^{-7}M and 3 x 10^{-6}M (Bueding *et al.* 1972). The higher inhibitory potency of dichlorvos shown in their results may be due either to the species difference or the difference in pre-incubation period with inhibitors. The preincubation time of worm homogenate with inhibitor before addition of substrate was 20 minutes (Knowles and Casida, 1966) and 90 minutes (Bueding *et al.*1972), compared with 5 minutes in the present work.

It is noteworthy that both organophosphates proved to be much more potent anticholinesterases in *T. ovis* than *T. hydatigena* : dichlorvos was about seven times and vincofos about eleven times more effective against the former tapeworm.

A preliminary report of the histochemical and biochemical studies with the cholinesterase of *Moniezia* and *T. ovis* from the present work has been published in abstract form (Soe, Forbes and Greenway, 1977) a copy of this abstract is attached (Addendum A).

ADDENDUM A

HISTOCHEMICAL AND BIOCHEMICAL STUDIES WITH CHOLINESTERASE OF *Moniezia expansa* AND *Taenia ovis*

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Histochemical studies with acetylthiocholine and butyrylthiocholine revealed cholinesterase in the teguments, nerves, ganglia, genital pores and cirrus sacs of *Moniezia* and *Taenia ovis* and also in the interproglottid glands of *Moniezia*. Enzyme activity was inhibited by eserine (10^{-5}M) and the organophosphorus anthelmintics dichlorvos (10^{-3}M) and vincofos (10^{-3}M).

The Specific Cholinesterase Activity (S.C.A) for homogenates of freshly collected *Moniezia* was 6.3 nanomoles of acetylthiocholine hydrolysed per minute per milligram of protein. No loss of cholinesterase potency occurred in worms frozen at -18°C for 21 months.

The S.C.A. (51.1) for fresh *T. ovis* was strikingly higher than that of *Moniezia* and there was no significant difference in rate of hydrolysis (on a wet weight basis) from fresh worms and those frozen for 6 months.

Fractions of fresh *Moniezia* showed S.C.A.'s of, 4.6 for nuclei, 13.5 for mitochondria, 105 for microsomes and 2.8 for cytosol. Pretreatment with Triton X-100 did not significantly alter cholinesterase activity. With frozen material however, the S.C.A's of homogenate and the 4 fractions were all significantly increased by Triton and the distribution of activity between the fractions was altered from that of fresh material (nuclei 12.7, mitochondria 7.1, microsomes 15.5, cytosol 7.7.

The S.C.A.'s of homogenates and fractions of frozen *Moniezia* and fresh *T. ovis* increased with substrate concentrations over the range examined (i.e.) 10^{-6} to 10^{-2}M acetylthiocholine.

CHAPTER 5 HELMINTH GLUCOSE UPTAKE AND THE INFLUENCE OF INHIBITORS AND ANTHELMINTICS

5.0 INTRODUCTORY REMARKS

A considerable volume of experimental evidence exists - involving a variety of nematodes, cestodes and trematodes - which firmly established glucose as an essential metabolite for helminth energy production and indicates that uptake mechanisms, similar to those existing amongst higher animals, are probably universal amongst the more primitive organisms. The prospects that inhibition of glucose uptake may be a primary mode of anthelmintic action is fully discussed in Chapter 2. and with particular reference to mebendazole (2.10c) and phenols, particularly niclosamide (2.8a).

In the present studies a small amount of work was done using the nematode, *Ascaris suum* and it was clearly shown that glucose uptake is inhibited by the benzimidazole, cambendazole and by salts of the local anaesthetic procaine (procaine hydrochloride and procaine penicillin G).

Almost all the present work, however, was with *Taenia hydatigena* and *Taenia ovis* using recognised inhibitors as well as a variety of anthelmintic substances. There appear to be no earlier reports of the use of these worms in similar experimental studies. Moreover, the influence of many of the anthelmintics has not been investigated previously.

5.1 MEMBRANE PERMEABILITY AND TRANSPORT MECHANISMS

A major characteristic of the metazoan organism is its sequestration by biological membranes into various body-compartments - which exist both at the cellular and sub-cellular levels. A common feature of all these membranes is their lipid character and for life to exist, essential nutrients and their metabolites must permeate the lipid-barriers in the processes of absorption, distribution, biotransformation and excretion. This extensive topic is of basic significance in general pharmacology and is comprehensively reviewed by Czaky (1969) and Binns (1964). In essence, the following list indicates the various ways by which substances can pass across a membrane:

(A) Diffusion
- (a) through lipid
- (b) through pores

(B) Carrier-facilitated transport
- (a) facilitated diffusion
- (b) exchange diffusion
- (c) active transport

(C) Vesiculation , pinocytosis and phagocytosis.

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Diffusion through lipid is paramount significance with respect to drugs and other foreign substances, such as terpenes and alkaloids, ingested in foodstuff. The process involves the perfusion of non-ionised species down a concentration gradient and the rate is closely correlated to lipid-solubility. Diffusion through pores is the mechanism by which hydrophilic substances such as water and small water soluble particles like hydrated Na^+, K^+, and urea, with a particle radius of less than 4 Angstroms, can pass through pores by osmotic water flow.

Carrier-facilitated transport mechanisms are involved with passage of many polar (hydrophilic) molecules, such as carbohydrates, which are too large to pass through pores and too lipid insoluble to pass by diffusion. Such substances combine temporarily with a carrier in the membrane, which facilitates transport. Carrier-sites are substrate specific and competitive-type cross inhibition usually occurs between substances of similar chemical composition. Moreover, the carrier-sites exhibit saturation. The carrier may merely enhance diffusion down a concentration gradient (facilitated diffusion) or the carrier may combine with a molecule on the outside; and release it on the inside, while picking up a molecule of similar substance and in exchange, transporting it to the outside (exchange diffusion). On the other hand, the transport may proceed against a concentration gradient, a process requiring the steady expenditure of energy by the cell (active transport). These systems are often described as biological pumps. Two components are involved - a carrier facilitated transfer and a mechanism for providing energy.

Active transport mechanisms are widely distributed in the living world and are regarded as one of the fundamental functions of the cells. As a general rule, cells contain a high concentration of potassium and a low concentration of sodium whereas in the extracellular fluid, the ionic ratio is reversed. The membrane is freely permeable to both Na^+ and K^+ ions and the asymmetric distribution is maintained by the continuous pumping of sodium outward and potassium inward. When the pyrophosphate linkage of ATP is hydrolysed, energy is released (at least 6,000 calories/mole) and the hydrolysis is catalysed by many nonspecific phosphatases as well as more specific ATPases. Skou (1927) isolated from crab nerve an ATPase which was inactive unless sodium and

potassium were added to the medium and such sodium-potassium activated ATPases were later found in almost every cell membrane. The enzyme ('membrane' or 'pump' ATP) occurs together with the pump mechanisms and both systems require sodium and potassium, utilize ATP and are inhibited by digitalis and other cardiac glycosides.

Vesiculation, pincytosis and phagocytosis are processes readily observed in amoebae, white blood cells and cancer cells but it is possible that every cell is capable of engulfing large solute particles such as bacteria.

5.2 VERTEBRATE GLUCOSE TRANSPORT - EFFECTS OF SODIUM IONS, PHLORIZIN, IODOACETATE AND DINITROPHENOL (DNP)

In the vertebrate, sugars are transferred actively in the intestine, the proximal tubules of kidney and the choroid plexus and the energy required for transport is derived from cellular metabolism (either aerobic or anaerobic) the immediate source being adenosine triphosphate (ATP).

Sodium is essential for the active transport of many nonelectrolytes (including sugars) and many biological pumps function only in the presence of a definite concentration of the ion in the medium, as examples, the transport of glucose, amino acids, purines and pyrimidines in the intestines, kidney and central nervous system are all sodium-dependant. The exact role of sodium is not fully understood but it has been suggested that active transport is tied closely to the sodium-potassium pump and that the cardiac steroids (such as digitalis and ouabain) inhibit the mechanism by preventing the access of potassium ion to membrane ATPase.

Phlorizin is a plant glycoside obtained from the bark of the apple tree. It is a very effective and selective inhibitor of glucose transfer in many different species of animals - including vertebrates and parasitic helminths. Its action was first demonstrated by Nakazawa (1922) who showed it an active inhibitor of intestinal glucose absorption and to be the most potent of several structural analogues (including phloretin). It is metabolic poison (Neil, 1960) and attaches to the aldose carrier with high affinity - thus competitively inhibiting the attachment and subsequent transport of all aldose sugars

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such as glucose, galactose and xylose. Because the carrier is inactivated, both active transport and facilitated diffusion are inhibited at the membrane site (Lotspeidr and Woronkow, 1958). Jervis *et al* (1956) showed the glycosyl moiety to be more effective inhibitor of intestinal transfer than phlorhizin itself and Newey *et al*. (1963) postulated that the glycosyl configuration and its position of attachment to the phlorizin group are relevant structural features. The kinetic studies of Alvarado and Crane (1962) suggested that phlorizin behaves as a competitive inhibitor to the active transfer glucose in the hamster intestine. Czaky and Ho (1966) also showed that phlorizin inhibited glucose influx into the small intestine of rat from media containing salts of both sodium and potassium.

Iodoacetate is an example of nonspecific irreversible enzyme inhibitor. It reacts with sulphydryl(SH) groups of enzymes including phosphatases and in addition may tie-up SH groups of membrane proteins. It inhibits glucose uptake from the gut (Wilbrandt and Laszt, 1933) and reabsorption from the proximal tubules of the frog kidney (Walker and Hudson, 1937) and has become well-known as an inhibitor in experimental glucose studies (Conn and Stumpf, 1972).

Dinitrophenol (DNP) Inhibition of the production of ATP may be nonspecific or specific. The former is caused by the practically anything which depresses cellular metabolism: low temperatures, lack of oxygen, cyanide (in the case of tissue with vigorous oxidative metabolism) and a variety of enzyme inhibitors. More specific, are those inhibitors which interfere with the production of ATP by uncoupling oxidative phosphorylation. This function is a general characteristic of phenols (Chapter 2, Section 5. d.) and DNP and salicylates are classical examples.

5.3 HELMINTH GLUCOSE TRANSPORT: INTRODUCTORY ANATOMICAL CONSIDERATIONS AND THE INFLUENCE OF CARBON DIOXIDE AND OXYGEN.

The overall topic of membrane transport in helminth parasites is the subject of exhaustive and comprehensive modern review (Pappas and Read, 1975) and for the present purposes significant information on glucose transport has been summarised: for nematodes (Table, 5.1) cestodes (Table 5.2) and trematodes (Table 5.3).

TABLE 5.1.

GLUCOSE UPTAKE BY PARASITIC NEMATODES

Species	Source	Experimental Findings
Ascaris lumbricoides	Cavier & Savel (1952)	The cuticle of *Ascaris* shown to be impermeable to sugar. Using ^{14}C-glucose. Intestine shown to absorb glucose freely against a concentration gradient. Cuticular absorption only slight and the major route by way of the gut.
Ascaris suum	Beames (1971)	Used sac of mid-gut of *Ascaris*. Transport of 3-0-methyl glucose from luminal solution to the pseudocoelomic fluid occurred in 95% N_2 + 5% CO_2 when glucose was present. Transport was CO_2-dependent and 95% 0_2 + 5% CO_2 or 99% N_2 markedly decreased the movement of sugar.
"	Van den Bossche (1972)	Mebendazole inhibited glucose uptake or transport leading to glycogen depletion and ultimately to decreased generation of A.T.P
"	Van den Bossche & De Nollin (1973)	
Ascaridia galli	Weatherly *et al.* (1963)	Glucose uptake through cuticle of *A. galli* was demonstrated but it was quantitiatively insignificantly.
Trichuris vulpis	Bueding *et al.* (1961)	Concentrations of diathiazanine above 10^{-6}M inhibited glucose uptake.
Syphacia muris	Van den Bossche *et al.*(1972)	Endogenous glucose utilization was not changed by shifting aerobic to anaerobic conditions.
Mermis nigrescens (larvae)	Colombo & Semenzal	Phlorizin and phloretin inhibited intestinal sugar transport through unrelated mechanisms and they two compete mutually.
"	Rutherford & Webster (1974)	Measured the transcuticular uptake of ^{14}C glucose. There is no experimental evidence to suggest that the nematode tegument has a role in active transport of glucose.
Litomosoides carinii	Bueding (1949)	The worm has a high rate of aerobic and anaerobic glucose metabolism. No postanaerobic increase of the oxygen uptake noted. Respiration of the filaria was completely inhibited by cyanide ($2x10^{-4}$M). Oxidative metabolism is essential for filaria worm.

TABLE 5.2

GLUCOSE UPTAKE BY PARASITIC CESTODES

Worm Species	Source	Experimental Findings
Hymenolepis diminuta	Laurie (1957)	Consumes exogenous glucose by anaerobic fermentation of glucose and galactose. Phlorizin is a non-competitive inhibitor of glucose fermentation.
"	Phifer (1960a)	Phlorizin inhibited uptake of isotopically labelled glucose and its action was neither competitive nor noncompetitive. Dinitrophenol (10^{-5} to $5x10^{-3}$M) decreased the rate of glucose absorption.
"	Phifer (1960b)	Iodoacetate ($3x10^{-4}$ - 10^{-3}M) inhibited glucose uptake during a 30 minute incubation period but dinitrophenol failed to inhibit glucose uptake in a 60 second period even after pre-incubation in the inhibitor. Dinitrophenol did not act on the surface of the worm.
"	Phifer (1960c)	Preincubation in either unlabelled glucose or galactose prior to incubation in C^{14} glucose, gave a higher rate of uptake than when preincubation in the sugar was omitted. Preincubation in fructose had no accelerating action.
"	Fairbairn *et al.*(1961)	*H. diminuta* is more effective in exogenous glucose utilization when CO_2 is present than when it is absent.
"	Lee *et al.* (1963)	The cardiac glycoside, strophanthin G has no inhibitory effect on glucose uptake.
"	Scheibel & Saz (1966)	The citric acid cycle is not operative as an energy yielding pathway in *H.diminuta*. CO_2 is required for energy production to transport glucose into the worm and for the synthesis of glycogen.
"	Read (1966)	5 per cent of CO_2 stimulated glucose uptake. Air had no significant effect. 20 per cent CO_2 lessened glucose uptake. In 2 hour incubation period, glucose uptake was dependent on the concentration of glucose up to about 10 mM.

TABLE 5.2 (cont'd)

"	Strufe & Gonnert (1967)	Niclosamide and dichlorophen inhibited glucose uptake and influenced the enzyme systems for carbohydrate metabolism.
"	Scheibel *et al.* (1968)	The presence of an anaerobic phosphorylation site associated with an electron transport system was demonstrated in this worm. Uncouplers of oxidative phosphorylation and anticestodal drugs, (chlorsalicylamide, dichlorphen and dinitrophenol inhibited the anaerobic incorporation of ^{32}P into ATP. Removal of CO_2 produced a marked decrease in both ^{32}P incorporation and ATP levels.
"	Dike & Read (1971)	^{14}C-glucose absorption was inhibited 95-100% in a Na^+-free medium. Ouabain had no effect on glucose uptake.
"	Pappas *et al.* (1974)	Glucose absorption was by active transport. Glucose was accumulated against a concentration gradient.
"	Read *et al.* (1974)	Glucose uptake was Na^+ dependent. K^+, tris or choline in the absence of Na^+ decreased glucose influx.
"	McCracken & Lumsden (1975)	Phlorizin was found to be competitive inhibitor of active ^3H-glucose transport. Phloretin had no significant effect on the initial rate of glucose absorption.
Hymenolepsis nana	Read & Rothman (1958)	Used a Manometric method. Metabolised glucose and galactose but did not metabolise fructose, mannose, maltose, trehalose, lactose or sucrose.
Hymenolepis microstoma	Pappas & Freeman (1975)	^{14}C-glucose influx was Na^+ dependent and phlorizin partially inhibited glucose uptake. Phloretin was a mixed inhibitor of glucose uptake. K^+, tris or choline in absence of Na^+ in medium decreased glucose influx.
Taenia crassiceps	Pappas *et al.* (1973)	Glucose absorption was carrier-mediated transport, sodium dependent and inhibited by phlorizin.
Taenia taeniae-formis	Von Brand *et al.* (1964)	Adult glucose uptake was completely Na^+ dependent

TABLE 5.2. cont'd.

Taenia taeniaeformis	Von Brand & Gibbs (1966)	10-fold increase or omission of K^+, Ca^+, Mg^+ and phosphate in the presence of Na^+ ion had no significant effect on glucose uptake by either adult or larvae. Na^+ ion had a marked influence on glucose uptake and this was blocked in the absence of Na^+ ions by both adult and larvae.
Calliobothrium verticillatum	Read (1957)	Determinations were made of exogenous carbohydrate utilization and acid production by *C. verticillatum* and *Lacistorhynchus tenuis*. The two worms fermented glucose and galactose but did not ferment or remove from the medium, mannose, sucrose, fructose, & etc.
"	Laurie (1961)	Cestodes from elasmobranch fishes absorbed glucose and galactose. Several glycosides and other phenolic derivatives exerted a predominantly inhibitory effect on carbohydrate absorption by the worms. Benzene sulfonic acid accelerated the rate of glucose uptake by *C. verticillatum*.
"	Fisher & Read (1971)	Phlorizin 10^{-4}M produced competitive inhibition of ^{14}C-glucose transport but phloretin 10^{-4}M for 2 minute incubation had no effect. Ouabain inhibited the glucose transport which was Na^+ dependent.
"	Pappas & Read (1972)	Replacement of Na^+ by K^+, choline or tris almost complete inhibition of glucose uptake. Li^+ produced a small stimulation of glucose influx.

TABLE 5.3

GLUCOSE UPTAKE BY PARASITIC TREMATODES AND PROTOZOA

Species	Source	Experimental Findings
Fasciola hepatica	Mansour (1959)	Glucose uptake of flukes ligated between anterior and posterior sucker, was almost identical with that of the control. (110-195 µM/g. wet wt/6 hr.). Neither glucose absorption from the medium, nor the excretion of metabolites were carried out through the gut.
"	Metzger & Duwell (1973)	Glucose absorption was inhibited by oxycloxinide (zanil) and DDBA.
"	Isseroff & Read (1974)	^{14}C-glucose absorption was Na^+ insenitive and not inhibited by phlorizin and ouabain. Sugar absorption was not effected by the absence of Na^+.
Fasciola gigantica	Abdel-Fattah & Al-Barwari (1974)	Exogenous glucose uptake (31µM/g. wet wt./hr.) was primarily through the tegument. Glucose uptake rate did not depend on the level of carbohydrate reserves.
"	Al-Barwari & Abdel-Fattah (1974)	Glucose absorption was by active transport. Phlorizin and iodoacetate inhibited glucose uptake. $4x10^{-3}$M phlorizin decreased the glucose absorption by about 95%.
Haematolocus medioplexus	Burton (1962)	Demonstrated the incorporation of ^{14}C-glucose from a saline medium into glycogen by histochemistry.
Haematolocus medioplexus and *Gorgoderina trematodes*	Parkening & Johnson (1969)	Demonstrated auto-radiographically the incorporation of tritiated glucose by the teguments of these two trematodes *in vitro*

TABLE 5.3 cont'd

Species	Source	Experimental findings
Schistosoma mansoni	Bueding (1950)	Phlorizin, phloretin, and ouabain inhibited glucose transport.
"	Bueding (1962)	Phloretin 8×10^{-5}M and 2×10^{-4}M inhibited the glucose uptake by 50% and 69% respectively
"	Kloetzel (1966)	Glucose and oxygen consumption reported under aerobic conditions.
"	Isseroff *et al.*(1972)	Radio-active glucose absorption was inhibited by Na^+-free medium and was insensitive to ouabain (0.02 - 2.0mM). High concentration of phlorizin (2.0mM) and iodoacetate (2.0mM) had no effect on glucose uptake.
"	Uglem & Read (1975)	Glucose uptake was Na^+ dependent and sensitive to phlorizin and ouabain. Both phlorizin and phloretin were effective against sugar transport, but the effects were not additive and not the same mechanism of action.
Schizotrypanum cruzi	Zeledon (1960)	Showed glucose uptake, using a manometric method.
Trypanosoma rhodesiense	Ryley (1962)	Cultured forms of the worm can utilize glucose, fructose, mannose. The respiration of the cultured form was sensitive to cyanide, although that of blood-stream forms was not.

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Nematodes possess potentially two absorptive areas - the body surface and the intestinal lumen. Glucose uptake was demonstrated through the tegument of *Ascaridia galli* but the route was considered quantitatively insignificant (Weatherly *et al.* 1963). The body surface of *A. lumbricoides* proved impermeable to soluble sugars (Cavier and Savel, 1952; Castro and Fairbairn, 1969) but there is evidence of cuticular uptake of C^{14} glucose in the larvae of *Mermis nigrescens* (Rutherford and Webster, 1974). On the other hand active transport of glucose and other monosaccharides by the gut epithelium, was readily demonstrated in *A. lumbricoides* (Castro and Fairbairn, 1968) and *A. suum* (Beames, 1971).

Cestodes lack a digestive tract and all nutrients must enter through the external body covering which is cellular in nature and is modified morphologically to resemble intestinal brush border of the mammal.

Trematodes, like nematodes, possess two potential absorptive surfaces but, in contrast to nematodes, it appears the tegument is the chief site of glucose absorption in *F. hepatica* (Mansour, 1959; Isseroff and Read, 1974; Hanna, 1976) *F. gigantica* (Abdel-Fattah and Al-Barwari, 1974) and *S. mansoni* (Bueding, 1950).

Carbon dioxide's role in parasite helminth metabolism has been recently extensively reviewed (Bryant, 1975). Bicarbonate is an important constituent in cultivation media and is an essential component for growth and maintenance. It is required for energy production along anaerobic respiration pathways and for gluconeogenesis and as examples its role has been demonstrated in the following nematodes, *Haemonchus contortus* Ward *et al.*1968) *A. suum* (Van den Bossche, 1969) *Trichinella spiralis* (Ward *et al.* 1969) *Dictyocaulus viviparus* (Vaatstra, 1969) *Syphacia muris* (Van den Bossche *et al.* 1971) *Nippostrongylus brasiliensis* (Saz *et al.* 1971) cestodes *H.diminuta* (Prescott and Campbell, 1965) *Echinococcus granulosus* (Agosin and Repetto, 1963) and *Moniezia expansa* (Bryant, 1972) trematodes, *F. hepatica* (Prichard and Schofield, 1968) *Entobdella bumpusi* (Hammen and Lum, 1962) and various acanthocephala (Beitinger and Hammen, 1971; and Graff, 1965). Read, (1967) demonstrated that glycogenesis and glucose uptake by *H. diminuta* and *H. citelli* are stimulated by five per cent carbon dioxide in the gas phase. With *Ascaris* and *H. diminuta*,

carbon dioxide was fixed into phosphoenolpyruvate (PEP) - an essential intermediate in the glycolytic cycle - and under anaerobic conditions at the electron transport level energy is generated in the form of ATP. (Bueding, 1969). The formation of C4-dicarboxylic acid is dependant on the fixation of CO_2 into PEP and there is a marked decrease in ATP levels when this is prevented by incubating *H. diminuta* in the absence of CO_2 (Scheibel *et al*.1968). Carbon dioxide is required for the incorporation of phosphate into ATP in the intact worm. Inhibition of a formation, in the absence of CO_2 , results in inhibition of anaerobic generation of ATP. Scheibel and Saz (1966) demonstrated that *H. diminuta* metabolises carbohydrates along a pathway similar to that found in *A. lumbricoides*. Tracer experiments with C^{14} glucose and $C^{14}O_2$ indicated strongly that the sugar is glycolysed to pyruvate. CO_2 is fixed to the 3-carbon acid and the resulting dicarboxylic acid reduced to succinate. The tricarboxylic acid cycle does not operate as an energy yielding pathway in *H. diminuta* and CO_2 is required for optional synthesis of glycogen and for energy metabolism, which in turn is needed for transport of glucose into the worm. Moreover, Saz (1969) found that the tricarboxylic acid cycle does not function as an energy-yielding pathway in *Ascaris* mitochondria, which operate anaerobically.

Increasing numbers of worms have been found to survive and metabolize carbohydrate for long periods under anaerobic conditions. Most intestinal dwelling parasites are in this category. *Heterakis gallinae* consumes oxygen at an appreciable rate (Glockin and Fairbairn, 1952) but at a low oxygen tension (7.6 mm or 0.2% O_2) glucose uptake was 34 per cent of that in air and high oxygen tensions (266-380 mm) were toxic - leading to death within 2 hours. Oxygen toxicity may result from the formation of hydrogen peroxide due to the presence of peroxidase.

Bueding and Charms (1952) reported that there was neither cytochrome C nor cytochrome oxidase in muscle or female reproductive organs of *Ascaris* and that oxygen is not an essential component in energy metabolism which is generated by way of anaerobic pathways. Oxygen taken up by the worm appears to play no physiological role in energy generating reactions. Saz (1971a) also pointed out that *Ascaris* mitochondria function anaerobically unlike mammalian mitochondria, which require oxygen in order to generate ATP by the electron transport

system. Read (1967) reported that oxygen is not a significant electron receptor and that it has no effect on glucose uptake and glucogenesis in *H. diminuta*. On the other hand, some blood-flukes *(S. mansoni)* contain cytochrome oxidase and consume oxygen (Bueding and Charms, 1952) although it can continue to survive and produce viable eggs when its respiratory enzymes are inhibited by cyanine dyes (Beuding 1950, Beuding *et al*. 1953).

Scheibel and Saz (1966) and Scheibel *et al*. (1968) using *H. diminuta*, cultured anaerobically, found:

(a) No evidence of cytochrome oxidase activity.

(b) No significant quantity of isotopic CO_2 formed from glucose 1-C^{14}, indicating the absence of both the tricarboxylic acid cycle and the pentose shunt mechanisms.

(c) Mitochondrial incorporation of P^{32} into ATP by means of an anaerobic electron-transport-associated exchange reaction. This evidence indicates that similarities exist between *H. diminuta* and *Ascaris* metabolism.

5.3.1 Nematode glucose transport

It appears that almost all nematode glucose absorption (Table 5.1) occurs through the intestinal epithelium although the published information relates only to a few species and most particularly to *Ascaris* (Section 5.3).

The uptake from intestinal sac preparations is a sodium-sensitive active process inhibited by phlorizin (Senhueza *et al*. 1968) and the active transcuticular transport of *Mermis nigrescens* larvae is inhibited by phloretin and DNP (Rutherford and Webster, 1974). In addition, intestinal absorption by these larvae was reduced by both phlorizin and its analogue phloretin and their actions were mutually antagonistic.

DNP inhibits the exchange of ^{32}P in added ATP of *Ascaris* mitochondria (Saz and Lescure, 1968; Saz, 1971b).

Dithiazanine, a cyanine dye, at 10^{-6}M inhibits anaerobic glucose uptake by *Trichuris vulpis* (Bueding, *et al*. 1961) and at 10^{-3}M inhibits transport by *Nippostrongylus braziliensis* (Sanderson, 1969).

Bephenium hydroxynapthoate ($2\text{-}5\times10^{-3}$M) inhibits glucose uptake and volatile fatty acid synthesis by pig *Ascaris* (Pushkarev, 1966;

5.3.1

Lazdinya and Pushkarev, 1966) and the taeniacide, nicolosamide, inhibits the exchange of ^{32}P into ATP in pig *Ascaris* mitochondria (Saz and Lescure, 1968).

The anthelmintic action of mebendazole may result from inhibition of glucose uptake (Van den Bossche, 1972; Van den Bossche and De Nollin, 1973) and in contrast to DNP the benzimidazole appears to interfere with the carrier mechanism, rather than with energy metabolism.

Rew and Saz (1977) reported that levamisole caused decreased glucose utilization of microfilariae and they added that it might be secondary to the immobilization of the worm rather than from a primary effect on carbohydrate metabolism.

5.3.2 Cestode glucose transport

Most of the relevant literature is summarised in Table 5.2. The great bulk of the work has been with *Hymenolepis* species, particularly *H. diminuta* - but there have been some studies with *H. nana* and *H. microstoma*. *Calliobothrium verticillatum* and other cestodes from elasmobranch fishes have been used but the only reports of work with *Taeniidae*, are those concerning *T. crassiceps* and *T. taeniaeformis* (= *Hydatigera taeniaeformis*) *T. hydatigena* and *T. ovis* have not been studied previously.

(a) Normal mechanisms. In *H. diminuta*, glucose was taken up by a sodium-dependant active transport mechanism and the process showed a saturation effect, and with glucose accumulating against an apparent concentration gradient (Phifer, 1960 a and b). In addition Pappas and Freeman (1975) showed that the absorption of glucose in *H. microstoma* was carrier mediated, saturable and inhibited by several monosaccharides and phlorizin. They held the view that glucose transport in *H. microstoma* did not differ from that of *H. diminuta*, *T. crassiceps* and *C. verticillatum*. While it appears that cestode species do not differ markedly in their ability to actively transport glucose, there are no data on the relative importance of the three mechanisms for glucose uptake.

5.3.2.(b)

(b) Sodium and miscellaneous substances. The first report of the *in vitro* sodium sensitivity of glucose uptake in cestodes (adult *T. taeniaeformis*) was that of von Brand *et al.*(1964) who, using sodium-free media, demonstrated glucose absorption 92 per cent inhibited under anaerobic conditions and completely inhibited or even reversed by glucose leakage, in the presence of oxygen. This work was confirmed and extended by von Brand and Gibbs (1966) who found that glucose uptake of larvae as well as adults was inhibited by lack of sodium and that other ions, namely Ca^{++}, Mg^{++}, K^{+} and PO_4^{-}, did not significantly effect glucose uptake in adult worms.

Similar sodium-dependence has been reported in *T. crassiceps* larvae (Pappas *et al.* 1973) *H. diminuta* (Dike and Read, 1971; Read *et al.*1974) *H. microstoma* (Pappas and Freeman, 1975) and *C verticillatum* (Fisher and Read, 1971; Pappas and Read, 1972). The mediated glucose uptake in the last-named three species was almost completely inhibited when sodium in the external medium was replaced by potassium, choline or tris buffer. The effect of sodium removal was reversible, in that replacement of the sodium restored normal glucose uptake in *H. diminuta* and *H. microstoma* (Read *et al.* 1974; Pappas and Freeman, 1975). In both species glucose transport was sodium-dependant and sodium-coupled. Moreover, the influx of sodium in *H. diminuta* and *C. verticillatum* was stimulated significantly by the presence of glucose in the external medium (Read *et al.*1974; Pappas and Read, 1972).

The absence of calcium, magnesium, potassium or phosphate in a medium with a normal sodium level, did not alter significantly the glucose uptake by either larvae or adult *T. taeniaeformis*, moreover, a tenfold increases in calcium or magnesium, a thirteenfold increase in potassium, and a thirty-three fold increase in phosphate did not significantly affect glucose consumption by the worm (Von Brand and 1966). Glucose uptake by *T. crassiceps* larvae did not occur in sodium-free medium in the presence of potassium, choline and tris buffer (Pappas *et al.* 1973)

(c) Phlorizin inhibition of glucose uptake has been demonstrated by: Laurie (1975, 1961) Phifer (1960a,c) Von Brand *et al.* (1964) Fisher and Read (1972) Pappas and Read (1972) Pappas *et al.*(1973) McCracken and Lumsden (1975) Pappas and Freeman (1975). Phifer (1960a)

showed that the uptake of glucose by *H. diminuta* was inhibited by phlorizin in only one minute. He considered that the site of action of the glycoside is at the worm surface. The data from a Lineweaver-Burk analysis however, could be interpreted as suggesting that phlorizin was neither competitive nor a noncompetitive inhibitor. This is in contrast to the situation in vertebrate where the glucoside is considered to be competitive.

Phloretin, the aglycone of phlorizin, inhibited uptake by *H. microstoma* (Pappas and Freeman, 1975) but not uptake by *H. diminuta* (McCracken and Lumsden, 1975) or *C. verticillatum* (Fisher and Read, 1971). McCracken and Lumsden (1975) suggested that two glycosides were not competing for the common carrier site. In *H. microstoma* phlorizin inhibition was characterized as partly competitive and it was suggested that the drug did not interact directly with the glucose transport system, but rather effected transport by binding at an another site. Moreover, the kinetics of phlorizin inhibition suggested a combination of competitive and noncompetitive inhibition (Pappas and Freeman, 1975). In *H. diminuta*,however, phlorizin inhibition was considered to be fully competitive and Pappas and Freeman (1975) were of the view that the concentration of phlorizin (0.21×10^{-3}M and 0.63×10^{-3}M) used by Phifer (1960a) was too high to allow distinction between competitive and noncompetitive inhibition.

(d) Iodoacetate. Relatively few studies have been reported on the influence of iodoacetate since Phifer (1960b) first demonstrated a significant inhibition of glucose uptake in *H. diminuta*. Concentrations of 3×10^{-4}M and 10^{-3}M iodoacetate were employed and in order to demonstrate effects during a short term incubation (60 seconds) preincubation for 15 minutes was required - suggesting iodoacetate does not act on a surface mechanism but probably affects the general metabolic activity of the worm (Phifer, 1960b).

Using *C. verticillatum*, however, Fisher and Read (1971) produced a 30 per cent inhibition and glucose transport with 0.5 mM sodium iodoacetate during a two-minute incubation and they considered the inhibition resulted from iodoacetate combining with the carrier system.

(e) Dinitrophenol (DNP) is a classic uncoupler of vertebrate oxidate phosphorylation and is assumed to inhibit parasite ATP formation so as to influence glucose absorption. At concentrations from 10^{-5}M to $5x10^{-3}$M, glucose absorption by *H. diminuta* is reduced during a 30 minute period (Phifer, 1960a) but DNP fails to inhibit the uptake during a one minute incubation, even after preincubation with the inhibitor for 15 minutes (Phifer, 1960b). These findings suggest that endogenous stores of ATP are sufficient to maintain glucose transport over a limited period and are considered to lend support to the view that the phenol acts primarily by interfering with energy metabolism (Phifer, 1960b).

Using the same tapeworm, Scheibel *et al*. (1968) DNP markedly inhibited the incorporation of ^{32}P into ATP, by inhibiting an electron transport linked mitochondrial ^{32}P-ATP exchange reaction. Similar findings have been made with *Ascaris* mitochondria (5.3.1)

(f) Phenolic anthelmintics and bunamidine. The fasciolocides, nitroxynil and hexachlorophene and the taeniacides, dichlorophen and niclosamide are all uncouplers of oxidative phosphorylation and inhibit the anaerobic incorporation of ^{32}P into ATP in *H. diminuta* (Schiebel *et al*. 1968). The first four-named are phenols and in addition, niclosamide is a salicylanilide. Bunamidine is closely related chemically to N,N-Diheptyl-4-pentyloxynapthamidine, an amidine, known to act in the same way as the above-named phenols (Scheibel *et al*.1968). Nitroxynil and hexachlorophene uncouple oxidative phosphorylation in rat-liver mitochondria (Van Miert and Groeneveld, 1969; Corbett and Goose, 1971) and in addition nitroxynil has a stimulatory effect on basal metabolism in the sheep (Anon, 1974). Pritchard (1973) found that closely related chemical disophenol inhibited oxidative phosphorylation in *H. contortus*.

Strufe (1963) reported that incubation with sublethal doses of niclosamide led to inhibition of glucose uptake and Euzeby (1967) reported that the drug paralysed *M. expansa, in vitro*. Strufe and Gonnert (1967) found that low doses of niclosamide and dichlorophen stimulate oxygen consumption of *H. diminuta* and higher doses inhibit glucose uptake.

5.3.3

The detailed studies of Scheibel *et al.*(1968) have shown that both drugs reduce the anaerobic turnover of ^{32}P labelled inorganic phosphate into ATP and it appears that these compounds act by inhibiting the incorporation of phosphate into ATP. Niclosamide, stimulated *M. benedeni, in vitro* (Khalilov, 1971) and had a scolicidal effect on protoscolices of *E. granulosus* (Sakamoto and Gemmel, 1975).

Biochemical studies with amidine, N, N-Diheptyl-4-pentyloxynaphthamidine reveal a mode of action identical to that of the phenols (Scheibel *et al.* 1968). The closely related amidine, bunamidine, in vertebrates causes irreversable neuromuscular blockage and inhibition of cholinesterase. A concentration of 1.07×10^{-5}M bunamidine causes rapid damage to the tegument of *T. hydatigena*. Double the concentration causes the dramatic stripping of the tegument and this damage was considered a possible mode of action of the taeniacide (Laws and Featherston, 1970).

5.3.3 Trematode glucose transport

Both flukes and schistosomes possess an alimentary canal and thus two potential absorptive surfaces. The trematode tegument is cellular and apparently modified for absorptive purposes (5.3).

In striking contrast to cestodes and nematodes uptake by *F. hepatica* is insensitive to sodium depletion and phlorizin and there is evidence for the presence of two distinct monosacchoride uptake systems (Isseroff and Read, 1974). On the other hand, with respect to *F. gigantica,* Al-Bawari and Abdel-Fattah (1974) reported phlorizin (4×10^{-3}M) and iodoacetate (10^{-2}M) almost completely inhibited glucose uptake.

Hexachlorophene is a phenolic uncoupler of oxidative phosphorylation (Scheibel, 1968) and stimulated ATPase activity of *F. hepatica* (Thorsell, 1967). Oxyclozanide, DDBA(2,6-dihydroxy-3,5-dichlorobenz-4-chloranilide) and a series of commercially available halogenated phenolic salicylanilides all interfered with succinic dehydrogenase of *F. hepatica* leading to a marked decrease in glucose consumption. The fluke enzyme was more sensitive than the similar enzyme of the rat liver (Metzger and Düwell, 1973).

5.4.3

In contrast to *Fasciola* glucose uptake in *S. mansoni* is sodium dependant (Isseroff *et al.* 1972; Uglem and Read, 1975) but insensitive to ouabain and phorizin (Isseroff *et al.*1972) over a 2-minute incubation period. However, 15 minute preincubations with ouabain do produce significant inhibition of glucose transport and it was considered that the mechanism is sodium-coupled and similar to that in cestodes (Uglem and Read, 1975). Glucose uptake was also inhibited by phloretin (Bueding, 1962).

5.4 MATERIALS AND METHODS

5.4.1 Helminth Sources, *A. suum, T. ovis, T. hydatigena* were obtained from abattoir material and laboratory infected animals as reported previously (4.3.1).

5.4.2 Drugs Sources. Drugs and chemicals employed were obtained from the following sources: Phlorizin (BDH England); lignocaine (Xylocaine 2% of Astra) dichlorvos and vincofos (courtesy of Shell Biological Science Research Centre, California, U.S.A.); hexachlorophene (BDH): nitroxynil (Trodax of May & Baker Ltd.); dinitrophenol (Kuhn il Drug Co. Inc. Seoul. Korea); dichlorophen (May & Baker) quinine hydrochloride (Sigma Chemical Co. St. Louis, U.S.A.); niclosamide (Bayer, Germany), Mebendazole (courtesy of Janssen Pharmaceutical, Belgium) bunamidine hydrochloride (Cooper, McDougall & Robertson (N.Z.) Ltd); and sodium iodoacetate (B.D.H.).

5.4.3 Preparation of Reagents.

Zinc sulphate solution, 5 per cent (w/v); 50 gm Zn $SO_4.7H_2O$ was dissolved in water and diluted to 1 litre. Barium hydroxide solution, approximately 0.3N; 50 gm of $Ba(OH)_2.8H_2O$ was dissolved in water and diluted to 1 litre. After standing for 2 days in a covered container, the solution was decanted. This solution was balanced against the zinc sulphate solution as follows: 10.0 ml of zinc sulphate were diluted with 25 ml of water, phenolphthalein indicater was added and the mixture titrated slowly with the barium hydroxide solution, using vigorous mixing, to a definite permanent pink colour. The stronger of the two reagents was diluted, if necessary, such that 10.0 ml of zinc sulphate solution requires 10.0 ± 0.1 ml of barium hydroxide solution for the end point. Dianisidine solution, 1 per cent (w/v) 50 mg dianisidine were dissolved in 5 ml water. Peroxidase solution: fifteen mgs peroxidase was dissolved in 15 ml glycerol buffer.

5.4.4

Glycerol buffer solution, pH 7.0 : 3.48 gm of anhydrous Na_2HPO_4 and 2.12 gm of KH_2PO_4 were dissolved in 600 ml of water, 400 ml of glycerol added and mixed thoroughly. Sulphuric acid, 6N: 200 ml of concentrated sulphuric acid were added to 1000 ml of distilled water with mixing. Stock standard glucose solution, 1 per cent (w/v) 1 gm dry reagent grade glucose was dissolved in 0.2% benzoic acid solution, made up to 100 ml and stored in a tightly stoppered bottle in the refrigerator. Working standard glucose solution; 10 ml of 1% glucose stock solution were diluted to 100 ml with 0.2 ml benzoic acid solution. Glucose oxidase reagent solution was prepared by adding 0.2 ml of dianisidine solution, 0.8 ml of Fermoczyme solution (Hughes and Hughes), 1.0 ml of Peroxidase solution and 18.0 ml of glycerol buffer. They were mixed and warmed to room temperature.

5.4.4 Experimental procedures

Ascaris worms were blotted dry with paper towels, and random samples of two worms were taken and weighed. They were transferred to 150 ml conical flasks containing 100 millilitres of glucose-salt mixture of Van den Bossche (1972) and comprising the following: 0.12M NaCl 0.001M $CaCl_2$, 0.001M $MgCl_2$, 0.005M KCL, 0.045M $NaHCO_3$, 0.005M potassium phosphate buffer (pH 7.4). Freshly prepared glucose was added to give a final concentration of 0.016M. Antibiotics were added to both controls and experimental flasks in order to retard bacteria growth during the incubation periods. These comprised streptomycin sulphate 0.1 mg per ml (Distrep-Vet, A/S Rosco-Denmark) and sodium penicillin G 2,000 units per ml, (Crystapen, crystalline benzylpenicillin, sodium salt, Glaxo Laboratories, N.Z.). On some occasions the procaine rather than the sodium salt of the penicillin was employed. Procaine hydrochloride was used in the form of injectable local anaesthetic prepared by McGraw Ethicals Ltd. New Zealand, and a pure sample of cambendazole (Lot. 149A028) was obtained through the courtesy of Bayer Pharmaceuticals Leverkusen, Germany. This drug is insoluble in water and for the purpose of experiments was dissolved in dimethyl-sulphoxide (DMSO) so as to give a final concentration of 0.1% DMSO in the incubating medium. Control worms were incubated in drug-free glucose medium containing the same quantity of DMSO.

To achieve anaerobic conditions, the media and flasks were flushed for several minutes with the mixture of 95% N_2 and 5% CO_2. The incubation was

5.4.5

carried out in a metabolic waterbath shaker at the temperature of 37° C and the glucose media changed and the flasks regased at 24-hour intervals. Experiments were run for three days in relation to the procaine experiments, and two days when the cambendazole effect was examined.

In the laboratory *T. ovis* and *T. hydatigena* worms were washed several times in saline at 37° C. Each worm was removed and divided into two portions. The site of section was at that part of the worm where the segmental length began to exceed its width. The portion anterior to the cut was designated immature (non-sexual) and was considered to contain no genital primodia or at most only a very rudimentary apparatus. That portion behind the section was designated mature (sexual) and could be expected to contain various combinations of reproductive organs - depending on the stage of maturity. Obviously gravid segments were removed from the mature portion. Both the mature and immature section were subdivided into smaller portions each about seven centimeters in length and weighing an average, 800 mgs. Two weighed portions were taken and placed in each 25 ml Warburg flasks containing 10 ml of glucose salt-medium of Von Brand and Gibbs (1966). Glucose was added to give a final concentration of 200 mg %. Antibiotics were added as outlined previously for *A. suum*. For the experiments a shaking Warburg waterbath was employed and its use enabled up to twentyeight flasks to be incubated simultaneously. The flasks were flushed with 95% N_2 and 5% CO_2 as in the *Ascaris* experiments. The experiments were conducted for two consecutive two hour-periods (totalling four hours). Media samples were taken at the end of the first and second two-hour period and the flasks were reflushed after the first sampling.

5.4.5 Assay methods

Glucose uptake was measured from depletions of levels in the incubating media. For this purpose the glucose oxidase method of Washko and Rice (1961) was used with slight modifications. The method is specific for the determination of glucose and relies on the catalytic oxidation of glucose by molecular oxygen to gluconic acid and hydrogen peroxide. The last named substance is formed in the presence of peroxidase and reacts with o-dianisidine to form a coloured material which is stoichiometrically related to the original amount of glucose. Colour intensity was measured using a Unicam Spectrophotometer (model S P 500 Series 2).

5.4.5

The procedure is as follows: A 0.1 ml sample from the worm incubation medium was taken and mixed with 1.5 ml of water and 0.2 ml of barium hydroxide solution. After mixing and standing at least 30 seconds, 0.2 ml of zinc sulphate solution was added, mixed thoroughly and allowed to stand for 2 minutes. The mixed solution was centrifuged at 3000 r.p.m. for 2 minutes to yield a 1:20 protein-free supernatant. Samples (0.2 ml) of supernatant were pipetted into test tubes and 1 ml of glucose oxidase reagent solution was added to each tube one at a time, in a timed sequence. Each tube was mixed immediately and placed in a 37° C water bath. After exactly 30 minutes, the tubes were removed one at a time and mixed with 5.0 ml of 6N sulphuric acid. In this manner, the incubation time for all tubes was kept constant. After 5 minutes, but within an hour, the absorbance of each tube was monitored at 540 nm in the spectrophotometer. The blank solutions were prepared in an identical manner, using 0.2 ml of water. The blank and test samples were assayed in duplicate. Glucose content was determined by reference to a standard glucose calibration curve (Fig. 5.1) and depletion rates from the medium expressed as mg. glucose consumed/gram wet weight of tissue/24 hours, for *A. suum* and µg/100 mg wet weight of worm/hour for *T. ovis* and *T. hydatigena*.

During the course of most experiments the worms usually retained their original colouration and appeared viable and active. However, some drugs, at higher concentrations, had an obviously detrimental effect. As examples, when DNP (10^{-4}M) was used, the worms stained yellowish green and became contracted; with bunamidine (10^{-3}M) they became flaccid and appeared lifeless; with hexachlorophene (10^{-3}M) they became rounded and bunched. With niclosamide and dichlorophen the worms appeared somewhat inactive and their viability was checked by removing them from the drug-containing medium and incubating them in a Warburg apparatus in 0.9% saline for half-an-hour. All the worm material examined consumed oxygen, indicating that the previous exposure to the drugs had not been lethal. All worms tested were active unless shown to be otherwise by footnote on the tables and results.

5.1

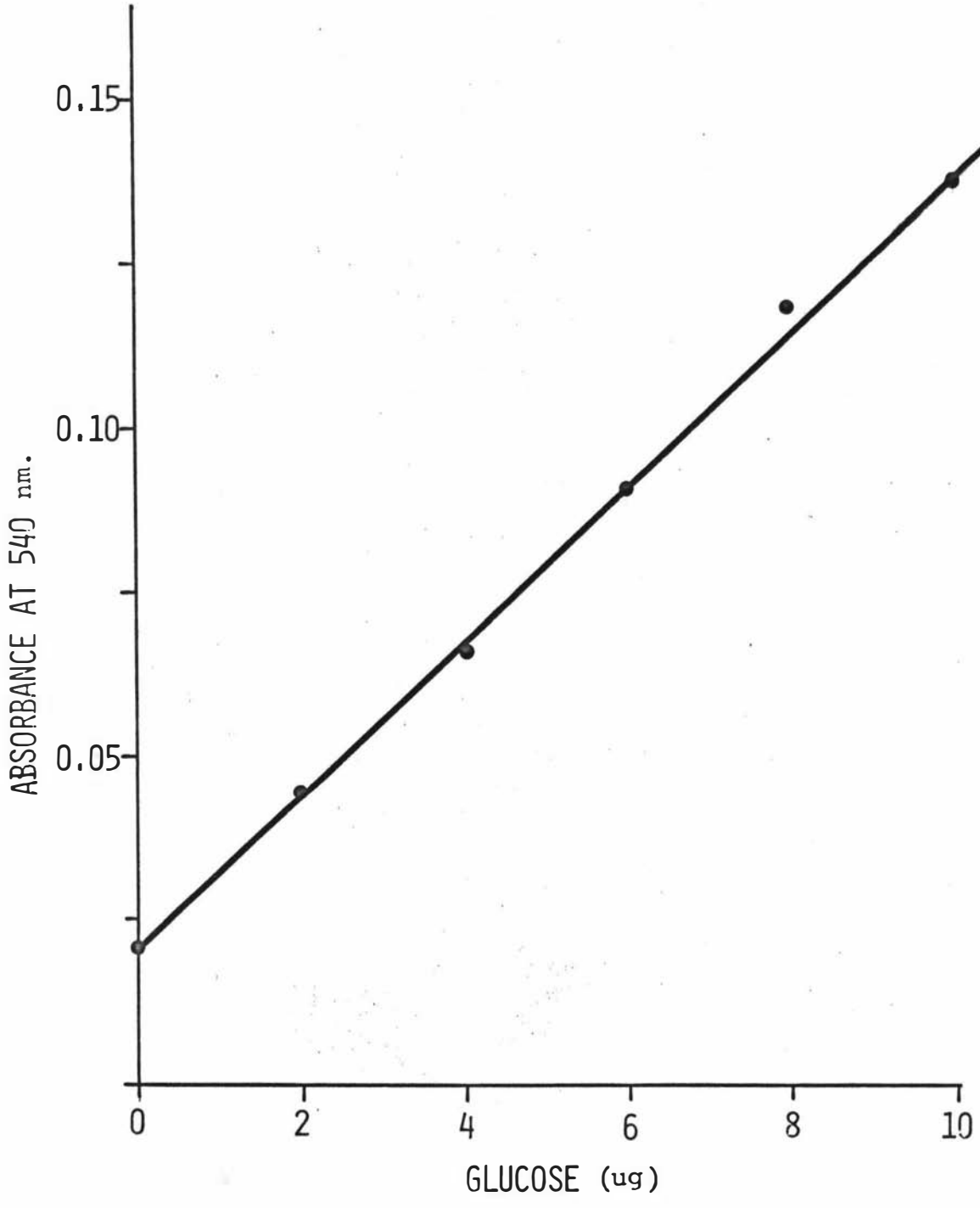


FIG. 5.1 COMPOSITE STANDARD CURVE (MEAN OF FIVE CONSECUTIVE ASSAYS) FOR GLUCOSE ASSAY.

5.5

5.5 RESULTS

5.5.1 General Comments

The bulk of the present studies were carried out with *T. hydatigena* and *T. ovis*. However, there was a small amount of preliminary work with *A. suum*, largely in order to establish the experimental technique and to examine the effect of cambendazole, a benzimidazole structurally analogous to mebendazole and which is postulated to act on glucose uptake in the same manner as the last named drug. In the initial experiments, the procaine salt of penicillin was employed for bacteristatic purposes. In the presence of this salt, great difficulty was experienced in demonstrating glucose uptake in the control flasks. Subsequently the sodium salt of penicillin G was substituted and it became immediately apparent that procaine may interfere with glucose uptake in *A. suum*. Further experiments were conducted with procaine penicillin and with procaine hydrochloride.

However, the bulk of the work has been with *T. hydatigena* and *T. ovis* neither of which have been examined previously. Much preliminary work was done to establish parameters in relation to such as comparing uptake between mature and immature portions of the tapeworms, between the first and second two-hour incubation periods and between *T. ovis* and *T. hydatigena*. Systematic studies were made of the effect of sodium deprivation, phlorizin, iodoacetate, dinitrophenol; several phenolic anthelmintics; organophosphorous compounds, quinidine and benzimidazoles.

5.5.2 *Ascaris* transport

The effects of procaine hydrochloride and procaine penicillin on glucose uptake as compared to drug-free controls, is reported in Table 5.4. Uptake by controls, over the three days, averaged about 15 mg per gram wet weight of worm for each 24-hour period. The uptake was almost identical for each of the three periods - indicating little deterioration of the helminth during the progress of the experiments. The results agree closely with the rate of glucose uptake reported for *Ascaris* in Belgium (Van den Bossche, 1972). At the drug-concentrations employed, the motility of *Ascaris* was not impaired and at quite low concentrations (6.8×10^{-4}M) the two procaine salts produced a similar and almost complete inhibition of glucose uptake,

TABLE 5.4

THE EFFECT OF PROCAINE HYDROCHLORIDE AND PROCAINE PENICILLIN ON THE EXOGENOUS GLUCOSE UPTAKE BY *ASCARIS SUUM*

INCUBATION MEDIA	GLUCOSE UPTAKE* MG/G WORM/24 HOURS					
	FIRST 24-HOUR	% INHIBITION	SECOND 24-HOUR	% INHIBITION	THIRD 24-HOUR	% INHIBITION
CONTROL	15.17 ± 1.11(8)	—	15.09 ± 1.01(8)	—	14.79 ± 1.36(8)	—
PROCAINE HYDROCHLORIDE +	1.17 ± 0.10(4)	92	0.05 ± 0.05(4)	99	2.86 ± 0.60(4)	81
PROCAINE PENICILLIN +	0.36 ± 0.24(4)	98	0.00 ± 0.00(4)	100	2.80 ± 0.25(4)	81

* Units are mg glucose consumed per g wet weight of worm per 24 hours.
Values are mean ± S.E. with number of determinations in parentheres.
\+ The final concentration of procaine as free base was 6.8×10^{-4} M.

5.5.2

particularly during the first two 24-hour periods. The almost identical effects of the equimolar concentrations, suggest that the two salts dissociate to the same extent and thus liberate for metabolism, similar amounts of the active moiety. The degree of inhibition fell slightly to 81 per cent during the third 24-hour test period, possibly as a result of the organism reinforcing its uptake mechanisms in the face of glucose deprivation.

The effect of cambendazole is reported in Table 5.5 and again, the concentrations employed did not effect the motility of the worms. The normal glucose uptake of control worms agreed closely with that reported in the previous experiments and there was only a slight difference in rate between the two 24-hour period. Inhibition of uptake by cambendazole, was dose related and almost complete inhibition occurred at the extremely low concentration of 1.6×10^{-7}M cambendazole. Moreover, in Table 5.5 the drug concentration has been shown in micrograms cambendazole per millilitre, in addition to the molar expression.

This has been done to enable the direct comparison between the effects of cambendazole in the present work and that of mebendazole reported by Van den Bossche (1972). Mebendazole appears to be less potent and to have a slower onset of action than cambendazole. For example, at the concentration of 0.0500 µg/ml (1.6×10^{-7}M) mebendazole causes only a 48 per cent inhibition during the first 24-hours, whereas cambendazole causes a 97 per cent inhibition. During the second and and third 24-hour periods the inhibition by mebendazole rose to 70 percentage and overall, the data suggest that a considerably higher concentration of mebendazole would be required to produce the same effect as cambendazole at 1.6×10^{-7}M.

TABLE 5.5

THE EFFECT OF CAMBENDAZOLE ON THE EXOGENOUS GLUCOSE UPTAKE BY *ASCARIS SUUM*

DRUG CONCENTRATION		GLUCOSE UPTAKE* MG/G WORM/24 HOUR			
UG/ML	(M)	FIRST 24-HOUR	% INHIBITION	SECOND 24-HOUR	% INHIBITION
0.0		14.82 ± 1.22(8)	—	13.81 ± 3.20(8)	—
0.0125	4×10^{-8}	9.14 ± 0.43(4)	38	4.30 ± 0.49(4)	69
0.0500	1.6×10^{-7}	0.44 ± 0.44(4)	97	1.27 ± 0.43(4)	91
0.2500	8.2×10^{-7}	0.35 ± 0.28(4)	98	0.97 ± 0.41(4)	93

* Units are mg glucose consumed per g wet weight of worm per 24 hours. Values are mean ± S.E. with number of determinations in parentheses.

5.5.3 Cestode Transport

(a) Worm portions, species, periods of incubation and DMSO

The rates of glucose uptake by mature and immature portions of *T. ovis* and *T. hydatigena* have been compared over a 4-hour period (Table 5.6). It can be seen that there is no significant differences in glucose uptake between the two portions of the worms in both *T. ovis* and *T. hydatigena*. In addition, glucose uptake between the two species during the two 2-hour periods are compared in Table 5.7. *T. hydatigena* exhibited a slightly higher rate of glucose uptake during the first 2-hour period ($P < 0.01$). During the second 2-hour period there was no significant difference between the two species, but over the total 4-hour period *T. hydatigena* exhibited a slightly higher rate of glucose uptake, although the difference was barely significant ($P < 0.02$). In addition, the rates of uptake have been compared between the periods of both mature and immature portions of *T. ovis* and *T. hydatigena* (Table 5.8). In the case of the first-named worm the uptake was the same for both portions during both periods, whereas *T. hydatigena* appeared to have a markedly higher rate during the first 2-hour period, (mature, $P < 0.02$ and immature $P < 0.001$), followed by a reduction in rate similar to that of *T. ovis* during the second period. The overall findings indicate that there are only small differences in glucose uptake between the two species, the two portions of the worms and the two incubation periods.

Dimethylsulphoxide was employed as a solvent for drugs which are insoluble in water. The concentration used was such as to give a final level of 0.1% in the incubation media. Previous experiments have shown that this concentration has no effect on glucose uptake by *Ascaris* (Van den Bossche, 1972) and for the present purposes, an experiment was conducted with *T. hydatigena*. The results are presented in Table 5.9 from which it can be seen that 0.1% DMSO has no influence on the rate of glucose uptake. A tenfold increase concentration, however, did result in a moderate interference with the rate of uptake. Considering these results it can be assumed that the solvent is no practical significance as far as our inhibition experiments are concerned.

TABLE 5.6

EXOGENOUS GLUCOSE UPTAKE OVER A 4-HOUR PERIOD BETWEEN THE MATURE AND IMMATURE PORTION OF *TAENIA OVIS* AND *TAENIA HYDATIGENA*

SPECIES	GLUCOSE UPTAKE* UG/100MG WORM/HOUR		P
	MATURE[1]	IMMATURE[2]	
TAENIA OVIS	344 (18)	369 (18)	n.s
TAENIA HYDATIGENA	392 (42)	412 (42)	n.s

* Units are ug glucose consumed per 100 mg wet weight of worm per hour.
Values are means followed by the numbers of determinations in parentheses.

1. Posterior portion contains genital primordia.
2. Anterior portion contains no genital primordia.

TABLE 5.7

EXOGENOUS GLUCOSE UPTAKE BY MATURE AND IMMATURE PORTIONS BETWEEN *TAENIA OVIS* AND *TAENIA HYDATIGENA*

PERIOD	GLUCOSE UPTAKE* µG/100MG WORM/HOUR		P
	TAENIA OVIS	*TAENIA HYDATIGENA*	
FIRST 2-HOUR	369 (18)	428 (42)	<0.01
SECOND 2-HOUR	344 (18)	375 (42)	n.s
TOTAL 4 HOURS	357 (36)	402 (84)	<0.02

* Units are ug glucose consumed per 100 mg wet weight of worm per hour.
Values are means followed by the numbers of determinations in parentheses.

TABLE 5.8

EXOGENOUS GLUCOSE UPTAKE BY 2 PORTIONS OF TAPEWORMS DURING TWO 2-HOUR INCUBATION PERIODS

SPECIES	WORM PORTION	GLUCOSE UPTAKE* µG/100MG WORM/HOUR		P
		FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD	
TAENIA OVIS	mature[1]	346 (9)	341 (9)	n.s
	immature[2]	391 (9)	347 (9)	n.s
TAENIA HYDATIGENA	mature	414 (21)	369 (21)	<0.02
	immature	443 (21)	381 (21)	<0.001

* Units are ug glucose consumed per 100 mg wet weight of worm per hour.
Values are means followed by the numbers of determinations in parentheses.

1. Posterior portion contains genital primordia.
2. Anterior portion contains no genital primordia.

TABLE 5.9

THE EFFECT OF DI-METHYL-SULPHOXIDE (DMSO) ON EXOGENOUS GLUCOSE UPTAKE BY *TAENIA HYDATIGENA***

DRUG CONCENTRATION	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
	FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
0	401 ± 13 (4)	389 ± 18 (4)
0.1%	408 ± 13 (4)	389 ± 14 (4)
1.0%	319 ± 24 (4)	337 ± 22 (4)

** Both immature and mature portions of the worm segments. (See text)
* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are means ± S.E. with number of determinations in parentheses.

5.5.3

5.5.3

(b) Sodium deprivation, phlorizin, iodoacetate and DNP

The above named substances have all been extensively employed in studies of fundamental mechanisms related to carbohydrate transport and in the present work their effects were examined using *T. ovis* and *T. hydatigena*. There appear to be no previous reports of similar studies with these tapeworms.

The detailed results of the experiments are recorded in Tables 5.11, 5.12, 5.13, 5.14 and 5.15, each of which records the way in which the actual experiment was undertaken. Thus the controls and test for each worm material procedure were derived from a pooled aliquot of helminths and in turn these data have been expressed in terms of percentage inhibition and summarised in Table 5.10. The detailed results of sodium deprivation are given in Tables 5.11 (*T. ovis*) and 5.12 (*T. hydatigena)* and it can be seen that in the absence of this ion, the uptake in both tapeworms is more than 90 per cent inhibited, during both incubation periods. The influence of phlorizin is recorded in detail in Tables 5.11 and 5.12, and it can be seen (Table 5.10) that the glycoside (4×10^{-4}M) almost completely inhibits glucose uptake in both species of tapeworms, during both incubation periods.

Iodoacetate, Table 5.13 was examined only with *T. hydatigena* where it proved ineffective at 10^{-5}M but almost completely effective at 10^{-4}M, again during both incubation periods.

Detailed results with DNP are reported in Tables 5.14 and 5.15. In the present of a 10^{-5}M solution the glucose uptake decreased about 75 per cent in *T. ovis* and 85 - 90 per cent in *T. hydatigena* and was almost completely inhibited by 10^{-4}M, DNP in both species. In the media containing 10^{-4}M DNP, the worm segments became stained a yellowish green, were contracted and 'bunched' and appeared to be dead. In these circumstances the inhibitory effect on glucose uptake might be considered to have resulted from a lethal or noxious effect on the worm, rather than a specific effect on the glucose uptake mechanism.

TABLE 5.10

SHOWING EFFECTS OF SODIUM DEPLETION, PHLORIZIN, IODOACETATE AND DNP ON GLUCOSE UPTAKE BY *TAENIA OVIS* AND *TAENIA HYDATIGENA*

SUBSTANCE OR MEDIUM	SUMMARISED FROM TABLES	PERCENTAGE INHIBITION OF GLUCOSE UPTAKE					
		TAENIA OVIS			*TAENIA HYDATIGENA*		
		FIRST 2 HOURS	SECOND 2 HOURS	TOTAL 4 HOURS	FIRST 2 HOURS	SECOND 2 HOURS	TOTAL 4 HOURS
SODIUM-FREE MEDIUM	5.11 & 5.12	93	95	94	92	90	91
PHLORIZIN 4×10^{-4} M	5.11 & 5.12	95	90	92	95	95	95
IODOACETATE							
10^{-5} M	5.13	-	-	-	0	0	0
10^{-4} M		-	-	-	93	96	94
DINITROPHENOL							
10^{-5} M	5.14 & 5.15	74	76	75	87	91	89
10^{-4} M		98	100	99	94	95	94

TABLE 5.11

THE EFFECT OF SODIUM-FREE MEDIA AND PHLORIZIN ON THE EXOGENOUS GLUCOSE UPTAKE BY *TAENIA OVIS***

INCUBATION MEDIA	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
	FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
CONTROL	436 ± 28.3 (4)	395 ± 38.7 (4)
SODIUM FREE	31 ± 9.3 (8)	20 ± 7.7 (8)
PHLORIZIN †	25 ± 5.4 (8)	43 ± 7.3 (8)

** Both immature and mature portions of the worm segments. (see text).
*Units are ug glucose consumed per 100 mg wet weight of worm per hour.
Values are means ± S.E. with number of determinations in parentheses.
† 4×10^{-4} M in incubation media.

TABLE 5.12

THE EFFECT OF SODIUM-FREE MEDIA AND PHLORIZIN ON THE EXOGENOUS GLUCOSE UPTAKE BY *TAENIA HYDATIGENA***

INCUBATION MEDIA	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
	FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
CONTROL	445 ± 44.8 (4)	360 ± 24.0 (4)
SODIUM FREE	35 ± 4.7 (8)	33 ± 5.2 (8)
PHLORIZIN†	20 ± 5.2 (8)	18 ± 3.5 (8)

** Both immature and mature portions of the worm segments. (see text)

* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are mean ± S.E. with number of determinations in parentheses.

† 4×10^{-4}M in incubation media.

TABLE 5.13

THE EFFECT OF BUNAMIDINE HYDROCHLORIDE, SODIUM-IODOACETATE AND PROCAINE HYDROCHLORIDE ON THE EXOGENOUS GLUCOSE UPTAKE BY *TAENIA HYDATIGENA***

DRUG	CONCENTRATION (M)	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
		FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
-	0	392 ± 32.9 (4)	356 ± 25.5 (4)
BUNAMIDINE HYDROCHLORIDE	10^{-5}	383 ± 33.0 (4)	294 ± 32.7 (4)
	10^{-3}	0 † (4)	0 † (4)
SODIUM IODOACETATE	10^{-5}	398 ± 6.7 (4)	369 ± 22.2 (4)
	10^{-4}	28 ± 9.9 (4)	17 ± 1.2 (4)
PROCAINE HYDROCHLORIDE	10^{-5}	394 ± 36.8 (4)	356 ± 26.6 (4)
	10^{-3}	420 ± 34.4 (4)	376 ± 37.2 (4)

** Both immature and mature portions of the worm segments. (see text)
* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are means ± S.E. with number of determinations in parentheses.
† Media became white and turbid and worm segments were flaccid and appeared lifeless.

TABLE 5.14

THE EFFECT OF DINITROPHENOL AND CAMBENDAZOLE ON EXOGENOUS GLUCOSE UPTAKE BY *TAENIA OVIS***

DRUG	CONCENTRATION (M)	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
		FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
CONTROL	0	210 ± 7 (4)	247 ± 23 (4)
DINITRO-PHENOL	10^{-5}	55 ± 17 (4)	61 ± 18 (4)
	10^{-4}	5 (4)†	2 (4)†
CAMBENDA-ZOLE	10^{-5}	85 ± 6 (4)	94 ± 17 (4)
	10^{-3}	65 ± 8 (4)	71 ± 12 (4)

** Both immature and mature portions of the worm segments. (see text)

* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are means ± S.E. with number of determinations in parentheses.

† Worm segments appeared stained with yellowish green and were contracted.

TABLE 5.15

THE EFFECT OF LIGNOCAINE, QUININE HYDROCHLORIDE AND DINITROPHENOL ON THE EXOGENOUS GLUCOSE UPTAKE BY *TAENIA HYDATIGENA***

DRUG	CONCENTRATION (M)	GLUCOSE UPTAKE* µG/100 MG WORM/HOUR	
		FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
-	0	416 ± 31.3 (4)	357 ± 10.7 (4)
LIGNOCAINE	10^{-4}	415 ± 9.5 (4)	351 ± 40.2 (4)
	10^{-3}	406 ± 26.8 (4)	356 ± 18.3 (4)
QUININE HYDROCHLORIDE	10^{-5}	424 ± 13.4 (4)	321 ± 20.9 (4)
	10^{-3}	336 ± 39.2 (4)	269 ± 35.5 (4)
DINITROPHENOL	10^{-5}	58 ± 12.0 (4)	35 ± 7.0 (4)
	10^{-4}	26 ± 7.0 (4)†	19 ± 7.0 (4)†

** Both immature and mature portions of the worm segments. (See text)

* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are means ± S.E. with number of determinations in parentheses.

† Worm segments appeared stained with yellowish green and were contracted.

5.5.3

(c) Procaine and Lignocaine

To enable a comparison with the studies previously reported with *A. suum* (5.5.2) experiments were conducted with the local anaesthetics procaine and lignocaine. Detailed results are presented in Tables 5.16, 5.13, 5.17, and 5.15 and summarised in Table 5.18. It can be clearly seen that even at the higher concentration tested, under the condition of the present experiments, the local anaesthetics had no influence on glucose uptake. This is in direct contrast to the findings with *Ascaris*.

(d) Nitroxynil, dichlorophen, hexachlorophene, niclosamide and bunamidine

As reported and discussed previously (Section 5.3.2f) these substances are all believed to exert an uncoupling effect on oxidative phosphorylation and in this way interfere with energy production mechanisms. The detailed results with nitroxynil are reported in Tables 5.19 and 5.20. The drug was tested at concentrations of 10^{-5}M and 10^{-3}M and the extent of percentage inhibition summarised in Table 5.21. At the lower concentration (10^{-5}M) glucose uptake was not significantly inhibited during the first 2-hour period in both species of tapeworm. However during the second 2-hour period (that is during the third and fourth hours of exposure) the rate of uptake was significantly retarded ($P < 0.001$). However, when the concentration of nitroxynil was increased to 10^{-3}M the inhibition was increased to between 80 to 95 per cent and the onset of inhibition extended over both 2-hour periods. The above results suggest that the effects of nitroxynil are related both to dose and to the length of exposure.

The detailed results with dichlorophen are presented in Table 5.22. The drug was tested at the concentrations ranging from 10^{-8}M to 10^{-4}M. There was no significant effect on glucose uptake at concentrations of 10^{-6}M or lower in both the first and second 2-hour incubation periods. At 10^{-5}M there was a 22 per cent inhibition during the first 2-hour period and this difference was statistically significant ($P < 0.001$). During the second 2-hour period the percentage inhibition increased to 68 and this increase was significant

TABLE 5.16

THE EFFECT OF BUNAMIDINE HYDROCHLORIDE, VINCOFOS AND PROCAINE HYDROCHLORIDE ON THE EXOGENOUS GLUCOSE UPTAKE BY *TAENIA OVIS***

DRUG	CONCENTRATION (M)	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
		FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
-	0	360 ± 28.7 (4)	307 ± 7.6 (4)
BUNAMIDINE	10^{-5}	433 ± 55.9 (4)	354 ± 24.9 (4)
	10^{-3}	0 (4)†	0 (4)†
VINCOFOS	10^{-5}	418 ± 12.0 (4)	343 ± 14.2 (4)
	10^{-3}	373 ± 27.0 (4)	360 ± 18.4 (4)
PROCAINE HYDROCHLORIDE	10^{-5}	422 ± 12.4 (4)	356 ± 32.5 (4)
	10^{-3}	423 ± 17.2 (4)	355 ± 29.2 (4)

** Both immature and mature portions of the worm segments. (See text)

* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are mean ± S.E. with number of determinations in parentheses.

† Media became white and turbid and worm segments were flaccid and appeared lifeless.

TABLE 5.17

THE EFFECT OF LIGNOCAINE, DICHLORVOS AND MEBENDAZOLE ON THE EXOGENOUS GLUCOSE UPTAKE BY *TAENIA OVIS***

DRUG	CONCENTRATION (M)	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
		FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
-	0	314 ± 34.4 (4)	336 ± 36.4 (4)
LIGNOCAINE	10^{-4}	295 ± 34.3 (4)	312 ± 32.7 (4)
	10^{-3}	292 ± 28.1 (4)	345 ± 8.8 (4)
DICHLORVOS	10^{-4}	315 ± 31.0 (4)	346 ± 26.6 (4)
	10^{-3}	345 ± 27.9 (4)	352 ± 32.4 (4)
MEBENDAZOLE	10^{-5}	300 ± 28.6 (4)	366 ± 37.4 (4)
	10^{-4}	341 ± 38.7 (4)	357 ± 25.8 (4)

** Both immature and mature portions of the worm segments. (see text)

* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are means ± S.E. with number of determinations in parentheses.

TABLE 5.18

SHOWING EFFECTS OF PROCAINE AND LIGNOCAINE ON GLUCOSE UPTAKE BY *TAENIA OVIS* AND *TAENIA HYDATIGENA*

SUBSTANCE	SUMMARISED FROM TABLES	PERCENTAGE INHIBITION OF GLUCOSE UPTAKE					
		TAENIA OVIS			TAENIA HYDATIGENA		
		FIRST 2 HOURS	SECOND 2 HOURS	TOTAL 4 HOURS	FIRST 2 HOURS	SECOND 2 HOURS	TOTAL 4 HOURS
PROCAINE							
10^{-5} M	5.16 & 5.13	0	0	0	0	0	0
10^{-3} M		0	0	0	0	0	0
LIGNOCAINE							
10^{-5} M	5.17 & 5.15	7	8	7	0	2	1
10^{-3} M		8	0	4	3	1	2

TABLE 5.19

THE EFFECT OF NITROXYNIL AND HEXACHLOROPHENE ON EXOGENOUS GLUCOSE UPTAKE BY *TAENIA OVIS***

DRUG	CONCENTRATION (M)	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
		FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
-	0	256 ± 21 (2)	239 ± 2 (2)
NITROXYNIL	10^{-5}	203 ± 77 (2)	160 ± 5 (2)
	10^{-3}	23 ± 12 (2)	30 ± 10 (2)
HEXACHLORO-PHENE	10^{-5}	42 ± 17 (2)	55 ± 5 (2)
	10^{-3}	35 ± 10 (2)†	35 ± 10 (2)†

** Both immature and mature portions of the worm segments. (see text)
* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are means ± S.E. with number of determinations in parentheses.
† Worm segments appeared rounded.

TABLE 5.20

THE EFFECT OF NITROXYNIL AND HEXACHLOROPHENE ON EXOGENOUS GLUCOSE UPTAKE BY *TAENIA HYDATIGENA***

DRUG	CONCENTRATION (M)	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
		FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
-	0	474 ± 22 (4)	437 ± 46 (4)
NITROXYNIL	10^{-5}	399 ± 15 (4)	155 ± 18 (4)
	10^{-3}	99 ± 32 (4)	23 ± 6 (4)
HEXACHLORO-PHENE	10^{-5}	249 ± 64 (4)	116 ± 47 (4)
	10^{-3}	116 ± 38 (4)†	116 ± 24 (4)†

** Both immature and mature portions of the worm segments. (see text)
* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are means ± S.E. with number of determinations in parentheses.
† Worm segments appeared rounded.

TABLE 5.21

SHOWING EFFECTS OF NITROXYNIL, DICHLOROPHEN, HEXACHLOROPHENE, NICLOSAMIDE AND BUNAMIDINE ON GLUCOSE UPTAKE BY *TAENIA OVIS* AND *TAENIA HYDATIGENA*

SUBSTANCE	SUMMARISED FROM TABLES	PERCENTAGE INHIBITION OF GLUCOSE UPTAKE					
		TAENIA OVIS			TAENIA HYDATIGENA		
		FIRST 2 HOURS	SECOND 2 HOURS	TOTAL 4 HOURS	FIRST 2 HOURS	SECOND 2 HOURS	TOTAL 4 HOURS
NITROXYNIL							
10^{-5} M	5.19 & 5.20	21	34	27	16	65	40
10^{-3} M		92	88	90	80	95	87
DICHLOROPHEN							
10^{-8} M		-	-	-	4	10	7
10^{-7} M		-	-	-	3	13	8
10^{-6} M	5.22	-	-	-	3	23	13
10^{-5} M		-	-	-	22	68	45
10^{-4} M		-	-	-	83	96	89

HEXACHLORO -PHENE							
10^{-5} M	5.19 & 5.20	84	77	80	48	74	61
10^{-4} M		87	86	86	76	74	75
NICLOSAMIDE							
10^{-8} M		14	20	17	13	1	7
10^{-7} M		34	44	39	45	69	57
10^{-6} M	5.23 & 5.24	97	99	98	81	95	88
10^{-5} M		98	100	99	92	96	94
10^{-4} M		99	100	99	95	98	96
BUNAMIDINE							
10^{-5} M	5.16 & 5.13	0	0	0	3	18	10
10^{-3} M		100	100	100	100	100	100

TABLE 5.22

THE EFFECT OF DICHLOROPHEN ON EXOGENOUS GLUCOSE UPTAKE BY *TAENIA HYDATIGENA***

DRUG CONCENTRATION (M)	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
	FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
0	436 ± 18.7 (4)	371 ± 27.8 (4)
10^{-8}	421 ± 27.0 (4)	337 ± 27.3 (4)
10^{-7}	426 ± 26.4 (4)	325 ± 32.2 (4)
10^{-6}	423 ± 9.2 (4)	286 ± 28.5 (4)
10^{-5}	343 ± 17.3 (4)	121 ± 22.1 (4)
10^{-4}	78 ± 10.0 (4)	18 ± 2.3 (4)

** Both immature and mature portions of the worm segments. (See text)

* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are means ± S.E. with number of determinations in parentheses.

($P < 0.001$). Consequently it appears that at this concentration the degree of inhibition is related to the length of exposure to the anthelmintic. A tenfold increase in concentration to 10^{-4}M dichlorophen, produced clear-cut inhibitory effects ranging from 83 to 96 per cent during the two 2-hour periods (Table 5.21).

Hexachlorophene, was tested on both tapeworms at concentrations of 10^{-5}M and 10^{-4}M (tables 5.19 and 5.20). At the lower concentration uptake was significantly inhibited during the first 2-hour period (48 per cent $P < 0.001$). In all the other instances the inhibition ranged from 74 to 87 per cent. Niclosamide was tested in concentrations ranging from 10^{-8}M to 10^{-4}M and detailed results are presented in Tables 5.23 and 5.24. The drugs had no significant effect at 10^{-8}M but at 10^{-7}M the rate of uptake was reduced to about half ($P < 0.001$). At 10^{-6}M and higher the degree of inhibition increased markedly to between 81 and 100 per cent.

Bunamidine was tested at the concentrations of 10^{-5}M and 10^{-3}M. (Tables 5.13 and 5.16). The lower concentration had no significant effect on uptake but the higher concentrations completely inhibited uptake (Table 4.21). Nonetheless, it should be recognised that at higher concentrations bunamidine had a non-specific lethal effect on the worms as they became immobilised and appeared lifeless.

(e) Organophosphorus compounds and quinine

The detailed results are presented in Tables 5.15, 5.16, 5.17, 5.25, 5.26 and 5.27 and summarised in Table5.28. Even in high concentrations (up to 10^{-3}M) dichlorvos and vincofos were no influence on glucose uptake in both *T. ovis* and *T. hydatigena*. In the case of quinine however, the concentration of 10^{-3}M produced an apparent reduction of about 20 per cent in *T. hydatigena,* but examined statistically this difference was found not to be significant. The drug has an even smaller effect on glucose uptake of *T. ovis*.

(f) Benzimidazoles

The effect of the benzimidazoles are shown in Tables 5.14, 5.17, and 5.25 and the material from these tables are summarised in Table 5.29. The glucose uptake of *T. ovis* was not significantly inhibited by concentrations

TABLE 5.23

THE EFFECT OF NICLOSAMIDE† ON EXOGENOUS GLUCOSE UPTAKE BY *TAENIA OVIS***

DRUG CONCENTRATION (M)	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
	FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
0	421 ± 21.5 (4)	390 ± 30.6 (4)
10^{-8}	360 ± 20.6 (3)	312 ± 10.5 (3)
10^{-7}	278 ± 32.3 (4)	221 ± 9.1 (4)
10^{-6}	16 ± 8.9 (5)	5 ± 3.3 (5)
10^{-5}	9 ± 7.4 (5)	0 (5)
10^{-4}	7 ± 5.5 (4)	0 (4)

** Both immature and mature portions of the worm segments. (see text).
* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are means ± S.E. with number of determinations in parentheses.
† Niclosamide-piperazine salt.

TABLE 5.24

THE EFFECT OF NICLOSAMIDE† ON EXOGENOUS GLUCOSE UPTAKE BY *TAENIA HYDATIGENA***

DRUG CONCENTRATION (M)	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
	FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
0	426 ± 18.3 (12)	384 ± 15.8 (12)
10^{-8}	372 ± 26.3 (4)	383 ± 29.3 (4)
10^{-7}	238 ± 29.8 (4)	121 ± 12.1 (4)
10^{-6}	81 ± 1.3 (4)	23 ± 4.2 (4)
10^{-5}	37 ± 5.8 (4)	18 ± 4.4 (4)
10^{-4}	35 ± 7.7 (4)	8 ± 1.4 (4)

** Both immature and mature portions of the worm segments. (see text)

* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are mean ± S.E. with number of determinations in parentheses.

† Niclosamide-piperazine salt.

TABLE 5.25

THE EFFECT OF DICHLORVOS, MEBENDAZOLE AND CAMBENDAZOLE ON THE EXOGENOUS GLUCOSE UPTAKE BY *TAENIA HYDATIGENA***

DRUG	CONCENTRATION (M)	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
		FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
-	0	414 ± 11.6 (12)	369 ± 11.9 (12)
DICHLORVOS	10^{-4}	417 ± 36.6 (4)	365 ± 21.3 (4)
	10^{-3}	405 ± 35.5 (4)	348 ± 18.5 (4)
MEBENDAZOLE	10^{-5}	284 ± 27.6 (8)	243 ± 24.9 (8)
	10^{-4}	293 ± 31.3 (7)	230 ± 16.3 (7)
CAMBENDAZOLE	10^{-5}	310 ± 24.5 (8)	263 ± 25.9 (8)
	10^{-4}	185 ± 15.3 (7)	161 ± 18.9 (7)

** Both immature and mature portions of the worm segments. (See text)

* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are mean ± S.E. with number of determinations in parentheses.

TABLE 5.26

THE EFFECT OF VINCOFOS ON EXOGENOUS GLUCOSE UPTAKE BY *TAENIA HYDATIGENA***

DRUG CONCENTRATION (M)	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
	FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
0	482 ± 21.3 (4)	402 ± 11.9 (4)
10^{-5}	514 ± 33.5 (4)	430 ± 38.7 (4)
10^{-3}	483 ± 44.7 (4)	444 ± 38.9 (4)

** Both immature and mature portions of the worm segments. (See text)

* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are means ± S.E. with number of determinations in parentheses.

TABLE 5.27

THE EFFECT OF QUININE HYDROCHLORIDE ON THE EXOGENOUS GLUCOSE UPTAKE BY *TAENIA OVIS***

DRUG CONCENTRATION (M)	GLUCOSE UPTAKE* µG/100MG WORM/HOUR	
	FIRST 2-HOUR PERIOD	SECOND 2-HOUR PERIOD
0	266 ± 26.1 (4)	260 ± 17.0 (4)
10^{-5}	322 ± 30.5 (2)	271 ± 16.5 (2)
10^{-3}	231 ± 39.5 (2)	245 ± 58.0 (2)

** Both immature and mature portions of the worm segments. (See text)
* Units are ug glucose consumed per 100 mg wet weight of worm per hour. Values are means ± S.E. with number of determinations in parentheses.

TABLE 5.28

SHOWING EFFECTS OF ORGANOPHOSPHORUS COMPOUNDS AND QUININE ON GLUCOSE UPTAKE BY TAENIA OVIS AND TAENIA HYDATIGENA

SUBSTANCE	SUMMARISED FROM TABLES	PERCENTAGE INHIBITION OF GLUCOSE UPTAKE					
		TAENIA OVIS			TAENIA HYDATIGENA		
		FIRST 2 HOURS	SECOND 2 HOURS	TOTAL 4 HOURS	FIRST 2 HOURS	SECOND 2 HOURS	TOTAL 4 HOURS
DICHLORVOS							
10^{-4} M	5.17 & 5.25	0	0	0	0	2	1
10^{-3} M		0	0	0	3	6	4
VINCOFOS							
10^{-5} M	5.16 & 5.26	0	0	0	0	0	0
10^{-3} M		0	0	0	0	0	0
QUININE							
10^{-5} M	5.25 & 5.15	0	0	0	0	11	5
10^{-3} M		14	3	8	20	25	22

TABLE 5.29

SHOWING EFFECTS OF BENZIMIDAZOLES ON GLUCOSE UPTAKE BY *TAENIA OVIS* AND *TAENIA HYDATIGENA*

SUBSTANCE	SUMMARISED FROM TABLES	PERCENTAGE INHIBITION OF GLUCOSE UPTAKE					
		TAENIA OVIS			TAENIA HYDATIGENA		
		FIRST 2 HOURS	SECOND 2 HOURS	TOTAL 4 HOURS	FIRST 2 HOURS	SECOND 2 HOURS	TOTAL 4 HOURS
MEBENDAZOLE							
10^{-5} M	5.17 & 5.25	5	0	2	32	33	33
10^{-4} M		0	0	0	30	38	34
CAMBENDAZOLE							
10^{-5}	5.14 & 5.25	60	62	61	26	29	27
10^{-4}		70	72	71	56	57	56

5.5.3

of 10^{-5}M and 10^{-4}M mebendazole. However, uptake of T. hydatigena was significantly inhibited by these two concentrations of the drug. Cambendazole proved a more powerful inhibitor and in contrast to the situation with mebendazole *T. ovis* appeared to the more sensitive worm, which was inhibited at the 60 to 70 per cent level with both 10^{-5}M and 10^{-4}M cambendazole. On the other hand, *T. hydatigena* was less effective and inhibition was more closely dose related - being about 27 per cent for the 10^{-5}M concentration and around 56 per cent for the 10^{-4}M concentration. Both sets of results exhibit statistically significant effects and the length of incubation appears to have no influence on the degree of inhibition.

5.6 DISCUSSION

With respect to *Ascaris* the chance discovery that procaine is a potent inhibitor of glucose transfer is of considerable interest. We have been unable to locate any references to work referring to this action of the local anaesthetic - either in vertebrates or in parasitic helminths. The observation may prove to be of considerable significance in relation to mode of local anaesthetic action and in this respect further studies are fully justified. One suggestion related to mode of action, indicates that procaine functions as a membrane stabilizer and in turn interferes with permeability of both potassium and sodium ions, and thus disrupts the transient permeability to sodium associated with the passage of the nerve action potential (NAP) (Ritchie and Greengard, 1966). An alternative suggestion is that of Ekenstram (1966) who postulated that the protein layer of the nerve cell membrane possesses receptor sites which bind with the local anaesthetic resulting in occlusion of the pores. Procaine appears to act on the internal surface of the nerve membranes including that of the giant squid axons (Narahashi *et al*, 1970; Narahashi and Frazier, 1975). In addition, procaine is recognised to inhibit cholinesterases derived from many different sources - as an example lobster nerve homogenates (Simpkins *et al*, 1972) and house fly head (Soeda *et al*, 1975). In view of the results reported later with organophosphorus compounds it would seem unlikely that the glucose transport is related to an acetylcholine-cholinesterases system. It is, however, known that active glucose uptake is sodium linked and it seems probable that the inhbition of uptake is related to the local anaesthetics potential to inhibit sodium pump mechanisms. Further studies in this area could well prove fruitful in both elucidating glucose transfer mechanisms as well as the fundamental mode of action of the local anaesthetic procaine.

Cambendazole and mebendazole are distinguished from other benzimidazoles in that both possess a carbamate function in the 5 position. Inhibition of glucose uptake was postulated as the primary mode of action of mebendazole and as cambendazole also possesses the carbamate moiety, Van den Bossche (1972) predicted that the last named benzimidazole would also interfere with glucose uptake. This suggestion has been

confirmed by the present study. Examination of the chemical structure of procaine indicates features reminiscent of the carbamate side-chain of the 2-benzimidazoles and it is possible that all these substances act as competitive inhibitors by combining with the glucose carrier. Clarification of these aspects must wait further investigation.

With tapeworms, the initial studies indicate that in general, anatomical regions of the worms and absolute rate of glucose uptake are not related, consequently, the results obtained from various portions may validly be combined for comparative purposes. The uptake is clearly sodium dependant in both *T. hydatigena* and *T. ovis*, so that these worms conform to the general pattern outlined for other cestodes in the introductory section (a small amount of transfer occurs in sodium-free media and this could possible be the reflection of a passive diffusion mechanism).

As in the situation with most other cestode previously examined (Section 5.3.2 c) phlorizin proved a potent inhibitor of glucose transfer in both species of tapeworm and in addition a higher concentration (10^{-4}M) of iodoacetate was clearly shown to interfere with the uptake mechanism of *T. hydatigena*.

The actions of dinitrophenol are fully in agreement with its known action as an uncouple of oxidative phosphorylation and in low concentrations it appears to be a potent inhibitor of glucose transport in both *T. hydatigena* and *T. ovis*. It can be assumed from the above observation that glucose uptake through the tegument of *T. hydatigena* and *T. ovis* conforms to the established pattern among other cestodes.

The findings with procaine clash with those related to intestinal absorption by *Ascaris* because under the conditions of the tapeworm experiments no procaine inhibition would be demonstrated. These results are however, equivocal to the extent that the total period over which tapeworm experiments were conducted was four hours and it is possible a more prolonged incubation period (such as the 24-hour period usual for *Ascaris* experiments) may reveal an inhibitory effect. Nonetheless, the failure of even high concentrations of both procaine and lignocaine (10^{-3}M) to show evidence of inhibition, suggest strongly that these substances

do not interfere with the uptake mechanism in the tapeworm. Further work should be carried out to confirm the findings, because a close comparative study between the effect of local anaesthetic on cestodes and nematodes may reveal valuable fundamental information on transfer mechanism operating between members of these two great phyla.

The results obtained from the actions of the various phenolic anthelmintics and bunamidine might have been predicted on the basis of their known properties as uncouplers of oxidative phosphorylation both in vertebrates and *Hymenolepis*. However, the present studies are the first indicating that activity of the anthelmintics extends to *T. hydatigena* and *T. ovis*. The presence of true cholinesterase (acetylcholinesterase) in the teguments of many species of tapeworm has raised the intriguing questions as to its role (Chapter 4). As acetylcholine is of paramount significance in receptor permeability it is easy to postulate that cholinesterase in worm teguments may be involved with absorptive mechanism and to assign such a role to the enzyme. Nonetheless it should be remembered that the presence of the biologically active substance does not presuppose and an essential physiological role. For it is recognized that throughout evolution redundant mechanisms may be incorporated in the more advanced or younger forms of the animal.

Dichlorvos and vincofos are known to inhibit helminth cholinesterase and can be shown histochemically to inactivate the acetyl enzyme in cestode teguments (Chapter 6). Nonetheless these substances had no effect whatsoever on glucose uptake and it appears that therefore the enzyme has no role in carbohydrate transport.

Quinine is also recognised to possess anticholinesterase properties and likewise might be postulated to have no action on glucose uptake. Its effect in a high concentration, however, might possible be related to its well known ability to uncouple phosphorylated oxidation.

CHAPTER 6 HELMINTH CHOLINESTERASE: HISTOCHEMICAL STUDIES AND THE INFLUENCE OF INHIBITORS

6.0

CHAPTER 6 HELMINTH CHOLINESTERASE: HISTOCHEMICAL STUDIES AND THE INFLUENCE OF INHIBITORS

6.0 INTRODUCTORY REMARKS

It is a generally accepted pharmacological principle that drugs seldom, if ever, confer new biological properties on cells, but rather bring about their effect by modifying (enhancing or inhibiting) normal physiological mechanisms. Consequently, the detection of the presence of bioactive substances, such as enzymes and neurohormones and their localisation in relation to anatomical structures, such as nerve muscle or tegument forms an important basis for interpretation of drug action.

Various techniques may be employed - such as outlined in Chapter 4, where information was obtained on the sub-cellular localisation of cholinesterase. However, the anatomical structure of worms is such that the isolation for analysis of discrete organs such as nerves or ganglia is not possible. This is in contrast to the vertebrate where dissection of tissues such as portions of brain to enable assay of bioactive substances is feasible and an important technique in neurophysiology. To some extent this problem with helminths is overcome by the use of histochemical techniques. In general, these methods rely on a chemical reaction, which may be related to the presence of an enzyme or a bioactive substance, and which results in the production of staining or fluorescence. The methods related to cholinesterases and their inhibition have been used in the present work and these reveal cholinesterases and their localisation in species not previously studied and the effects of dichlorvos and vincofos as inhibitors.

6.1 HELMINTH CHOLINESTERASE HISTOCHEMISTRY

Biochemical studies of helminth cholinesterase have been reported previously (Chapter 4),

Pepler, (1958) making use in part of histochemical methods, demonstrated cholinesterase in the miracidia of *S. mansoni*. In the same year, Fennell and Pastor (1958) reported cholinesterase in *Tetrahymena pyriformis*. Lee (1962) investigated cholinesterase and other

esterases in pig *Ascaris* using three methods of histochemical staining; 5-bromoindoxylacetate method, the naphthol AS acetate-fast TR salt method of Pears (1960) and the acetylthiocholine method of Gomori (1957). Later, Lee *et al.* (1963) outlined the sites of cholinesterase activity in *H.taeniaeformis (T. taeniaeformis)* and *H. diminuta* and Lee and Tatchell demonstrated the sites of cholinesterase in horse tapeworm of *Anoplocephala perfoliata,* using Gomori's method. Forbes (1965) outlined the cholinesterase sites of *T. taeniaeformis, E. granulosus* and *M. expansa* by the Gerebtzoff (1959) method. In cercariae of *S. mansoni,* cholinesterase activity was shown by Lewet and Hopkins (1965) by the Gomori (1957) technique. Wilson (1965) demonstrated the distribution of acetylcholinesterase in the scolex of *Hymenolepis spp* and Bogitsh (1967), used histochemistry to visualise the nervous system of *Hymenolepis.* In addition the histochemical localization of acetylcholinesterase of *H. diminuta* was reported by Graff and Read (1967). Douglas (1966) used the thiocholine technique for *H. diminuta and S. mansoni.* Fripp (1967) revealed sites of cholinesterase activity in adult schistosomas, namely *S. haematobium; S. mansoni* and *S. rodhaini,* using Gomori's method and acetylthiocholine iodide for true cholinesterase and butyrylthiocholine iodide for pseudocholinesterase. He observed that the inhibitor, 62 C47, which is a specific inhibitor for mammalian acetylcholinesterase is only partially inhibitory to the acetylcholinesterase of parasites and that, cercariae and miracidia possess only true cholinesteras activity. In *F. hepatica* cholinesterase was located in the prostate (Boust *et al.* 1966) and intestines (Halton, 1969). Kralj (1967), using the staining method of Koelle and Friedenwald (1949) demonstrated the site of cholinesterase localization in *M. expansa, T. hydatigena* and *D. caninum.* Eserine sulphate was used for the selective determination of acetylcholinesterase and butyrylcholinesterase. Znidaric and Lui (1967) demonstrated the cholinesterase localization in *Strongylus edentatus; S. vulgaris; Metastrongylus elongatus* and *Choerostrongylus pudendotectus.* An investigation of localization of cholinesterase activity in *A. suum, Parascaris equorum, Neoascaris vitulorum* and *Oxyuris equi* was undertaken by Znidaric (1967) and Krvavica *et al.* (1967) and Krvavica *et al.* (1971) demonstrated

6.1

the localization of acetylcholinesterase and non-specific cholinesterase sites in *F. hepatica*. Panitz and Knapp (1967) studied the enzyme in the miracidia and Panitz and Knapp (1970) showed that in *F. hepatica* acetylcholinesterase is inhibited to a greater extent by paraoxon than is pseudocholinesterase. They concluded also that *Fasciola* has at least two cholinesterases.

Eranko *et al.* (1968), demonstrated both the biochemical and histochemical localization of cholinesterase in *T. taeniaeformis*. Iso-OMPA and BW62C47, which are inhibitors of butyrylcholinesterase and acetylcholinesterase respectively, in mammalian tissue and blood, did not inhibit the worm cholinesterase.

Histochemical identification of cholinesterases of *H. taeniaeformis*, *D. caninum* and *E. granulosus* were made by Shield (1969), using the method of Karnovsky and Roots (1964) with different inhibitors. He concluded that *E. granulosus* has no butyrylcholinesterase. Reznik (1970) showed the localization of nonspecific esterase and cholinesterase in *A. galli*. Ramisz and Szankowrka (1971) studied the cholinesterases in migrating larvae and encysted *Trichinella spiralis* using various cholinesterase inhibitors. He concluded that both enzymes were present in muscle fibres and in 60 to 70 per cent of *T. spiralis* capsules. Rothwell *et al.* (1973) demonstrated that the fourth stage larvae of Trichostrongylus species had the highest level of cholinesterase activity. DiConza and Basch (1975) demonstrated cholinesterase in sporocysts of *S. mansoni* and tested eserine (10^{-5}M) and other inhibitors. Wright and Awan (1976) developed improved histochemical methods to reveal the sites of cholinesterase in the region of the nematode nerve ring by using ultrasonic pretreatment and staining by Karnovsky and Roots (1964). They also used eserine, iso-OMPA and BW284C51 as inhibitors and found that iso-OMPA, a potent butytylcholinesterase inhibitor of mammalian tissues, had no apparent effect on the nematode esterase when both acetylthiocholine iodide and butyrylthiocholine iodide were substrates.

6.2 MATERIALS AND METHODS

6.2.1 Helminth sources

6.2.2

T. ovis and *T. hydatigena* were obtained from experimentally infected dogs and *Moniezia* from a local freezing works (Chapter 4.3.1)

6.2.2 Drugs and Reagents

The inhibitors and their final concentrations were eserine (10^{-5}M) (physostigmine salicylate) (Macfarlan Smith Ltd. Edinburgh); vincofos (10^{-3}M) (formula No. SD 13803 9-7-7-6 TKH); and dichlorvos (10^{-3}M) (formula No SD 1750 7-16-0-13 TKH). The last two chemicals were made available by courtesy of Shell Development Company Biological Sciences Research Centre, Modesto, California, U.S.A. Acetylthiocholine iodide (Sigma Chemical Company, St. Louis, U.S.A.) and butyrylthiocholine iodide (B.D.H. Chemical Ltd. Poole, England) were used as substrates.

6.2.3 Histochemical Techniques

Following collection the worms were immediately washed three or four times with 0.15M NaCl solution at 37°C, blotted on paper towels and fixed in cold (5°C) 10 per cent phosphate formalin buffer (4% formaldehyde) pH 7.0 for a period varying from 1 day to four weeks. There was no evidence that the enzyme activity became reduced over this period (Shield, 1969).

These worm segments intended for whole mounts were flattened and fixed between two glass-slides fastened by rubber rings at both ends. The flattened worm segments were taken from the fixative and washed briefly in distilled water before incubation. The unflattened worm segments, used for sections, were washed briefly in distilled water. They were put on a chuck supported in Ames O.C.T. compound and frozen on the freezing stage of an international model CTD cryostat. Frozen worm segments were cut at 30 to 40 μ at a temperature of -20°C to -25°C. The sections were placed in a tray of distilled water and the free-floating ones lifted out on albuminized glass slides and air dried.

The indoxylacetate and Karnovsky and Roots (1964) methods were used as described by Pearse (1960, 1972). For the last named method incubation was carried out at 37°C for 16 to 18 hours for whole mounts, and 1 to

6.2.3

3 hours for sections. The Gerebtzoff (1959) method was employed as described by the author. Whole mounts were incubated for 16 to 22 hours and sections 2 - 3½ hours.

In the inhibitor experiments the worm material was preincubated with the inhibitor for 1 hour (whole mounts) and 30 minutes (sections) prior to incubation with substrate. Controls which were lacking inhibitors in both incubation phases were always included in each staining run. No colour developed in the preincubation stage. Following incubation the tissues were washed with distilled water, dehydrated in 50, 75, 95 per cent and absolute alcohols, cleared in xylol and mounted in DPX, allowing one hour for the alcohol steps for whole mounts and a few seconds for sections.

Key to Lettering For Figures 6.1 to 6.14

A.N = apical nerve
Af.N = afferent nerve
Ax = axon
C = cirrus
CC = circular commissure
Co = calcareous corpuscles
CG = cervical ganglion
CS = cirrus sac
D = dendrites
ED = excretory duct
GP = genital pore
GPr = genital primordia
GC = ganglion cell
H = hook
HM = hook muscle
IG = interproglottid glands
LNT = lateral nerve trunk
NN = nerve network
PC = primordial cell
R = rostellum
RC = receptor cell
RH = rostellar hook
S = sucker
SD = spermatic duct
ST = sub-tegument
T = tegument
TN = transverse nerve
UL = uterine lining

6.3 RESULTS

The results are reported in two groups. The first concerned with normal cholinesterase staining without inhibitors (6.3.1) and the second where inhibitors were employed (6.3.2).

6.3.1 Normal Staining Reactions

(a) Techniques

The Gerebtzoff, Karnovsky and Roots and the indoxyl-acetate methods, were all investigated. The last named gave persistently poor results and was soon abandoned. The Karnovsky and Roots method has not been previously employed for parasitic helminth work and in the present study was used for staining whole mounts of *Moniezia* (Fig 6.2 A,B,C) whole mounts of *T. ovis* (Fig 6.4,A,B,C,D and Fig 6.6) and frozen sections of *T. ovis* (Fig 6.5 A,B). In our experience the method gave far less satisfactory results than the Gerebtzoff method previously employed. Unpublished work (Forbes 1965) had shown (Fig 6.1) that the cuticle of *T. taeniaeformis* consistently gave a positive reaction to acetylthiocholine (demonstrating the presence of acetylcholine-esterase (Fig 6.1B) and this is in agreement with the findings of Lee *et al.* (1963). With the Karnovsky and Roots method and acetylthiocholine however, although apparent staining of the tegument could be demonstrated on some occasions, as with *T. ovis* (Fig 6.4A,C and Fig 6.5A,B, on other occasions the structure failed to stain, as for example, with *Moniezia* (Fig 6.2A). On one occasion, the influence of eserine was tested on the hydrolysis of butytylthiocholine and inhibition demonstrated by the Karnovsky and Roots method (Fig 6.4E). It should be noted however, that Lee and Tatchell (1964) were unable to demonstrate cholinesterase in the cuticle of *Anoplocephala perfoliata* and Kralj (1967) - using the method of Koelle and Friedenwald (1969) - was unable to demonstrate acetylcholinesterase activity in the tegument of *T. hydatigena, Moniezia expansa* and *Dipylidium caninum*. Our overall impression is that staining reactions for illustrating sites of cholinesterase action can be capricious and vary between techniques and possibly also between laboratories and individual workers. The main contention arises from the apparent presence of acetylcholinesterase and the absence of pseudocholinesterase in the tegument of some species and the complete absence or only weak activity of both enzymes in other species. The

6.3.1

Gerebtzoff method was employed for most of the work, both for demonstrating normal patterns of cholinesterase distribution and the action of the various inhibitors. Both acetylthiocholine and butyrylthiocholine were employed as substrates. Acetylcholinesterase and pseudocholinesterase hydrolyse acetylthiocholine, but butyrylthiocholine is hydrolysed only by the nonspecific enzyme. The presence of a reaction when butyrylthiocholine is used as substrate can be taken as presumptive evidence of the presence of pseudocholinesterase.

6.3.1

(b) Reactions

Scoleces were studied in the form of whole mounts (Fig 6.1A; 6.4A,B; 6.10A; 6.13A) and frozen sections (Fig 6.7A,B,C,D). With whole mounts the Karnovsky and Roots method (Fig 6.4A,B) proved unsatisfactory for defining the deep structures of the scolex region. With acetylthiocholine as substrate, the tegument sometimes stained a dark brown, but this reaction was far from consistent (Fig 6.4A) and with butytylthiocholine as substrate there was little or no staining reaction (Fig 6.4B). By contrast, whole mounts of *T. taeniaeformis* treated by the Gerebtzoff method and using butyrylthiocholine as substrate, clearly outlined the nerves and ganglia of the scolex region (Fig 6.1A). In this cleared specimen, a strong cholinesterase response can be seen in the cerebral ganglion and circular commissure connecting the ganglia at the base of the rostellum. From the commissures arise the apical nerves which supply the rostellum and hooks and also the nerves joining circular nerve trunks which travel around the periphery of the suckers. Longitudinal nerves, which traverse the length of the strobila arise from the region of the cerebral ganglia and the circular commissures. In the present study with *T. hydatigena* the Gerebtzoff method failed to show cuticular staining of whole mounts when acetylthiocholine was used as a substrate (Fig 6.10A). However, the circular arrangement of the nerve trunks to the sucker can be clearly seen and a rich display of activity occurs in the vicinity of the hooks. Pretreatment with eserine 10^{-5}M (Fig 6.10D) enables finer details of the network to be visualised. The nerves running around the circumference of the sucker can be clearly seen, as well as the tiny nerve endings, undoubtly supplying sucker muscles.

6.3.1

Fig 6.13A illustrates the use of the Gerebtzoff method with butyrylthiocholine as substrate. Unlike the whole mounts shown in Fig 6.10 the scolex of *T. hydatigena* was not cleared, and it can be seen in this instance that there is very little visualization of the deeper structures of the scolex region, although there is clear cut evidence of pseudocholinesterase activity from the muscles of the hooks and in the nerves supplying the suckers. There is no evidence of activity in the tegumental region.

Frozen sections of scoleces of *T. hydatigena* were stained using the Gerebtzoff method (Fig 6.7). It can be seen (Fig 6.7A) that where acetylthiocholine is used as a substrate, there is an intense staining of the tegumental region. This section, also illustrates intense activity in the region of the rostellar hooks and the section has traversed the area of the cerebral ganglion and shows nerve trunks travelling from this region to the tegumental structures. The section also traverses the longitudinal nerve trunk and the neural connection between them. Transverse nerves can be seen in the region of the neck. By contrast, Fig 6.7B illustrates a similar region stained using butyrylthiocholine as substrate. The most striking difference appears in the somewhat faint reaction in the tegumental region. Reaction has occurred in all the nerves and commissures of the scolex and in the longitudinal nerve trunk. Clear connections can be seen between the cerebral ganglion and the hooks. These features are illustrated in more detail in Fig 6.7C, where connections between the cerebral ganglia and the hooks are clearly illustrated, as well as a fine network of nerves ramifying in the periphery and reaching into the tegument. The region of the base of the hook consistantly shows a high degree of staining and this is exemplified in Fig 6.7D.

Whole mounts of the body region illustrate important anatomical and histochemical features of significance in predicting function. For comparison between species, a whole mount of a 35 day-old *E. granulosus* has been included. The segment has been stained by the Gerebtzoff method using butyrylthiocholine (Fig 6.1C) and the photograph has been enlarged to illustrate the sites of pseudocholinesterase activity in

the region of the cirrus sac. It can be seen that there are discrete plaques of intense staining covering the surface of the sac. These might be considered to represent defined areas of pseudocholinesterase activity associated with structures analagous to motor-end plates of vertebrate muscle. The cirrus opening itself, appears surrounded by an area of an intense activity and the lateral nerve cord stains strongly and numerous transverse nerves can be seen arising from it. The result, using the Gerebtzoff method with mature segments of *Moniezia* is shown in Fig 6.2. As mentioned previously, the Karnovsky and Roots method showed no intense staining of the tegument, but there is an intense reaction around the genital pore as previously shown with *Echinococcus*. With butytylthiocholine as substrate, the staining reaction in the tegumental region appears slightly less intense, but again there is intense reaction in the vicinity of the genital pore and the longitudinal nerve trunks. Moreover, a nerve network within the segment is apparent, but there is no evidence of activity in the region of the interproglottid glands. The results obtained with the Gerebtzoff method and butyrylthiocholine are illustrated in Fig 6.2C. Again there is staining in the vicinity of the genital pore, the lateral nerve trunks are showing a clear reaction and there is an intense response in the region of the genital primordia. The technique reveals a complex nerve network arising from the longitudinal nerves and most interestingly, there is a clearcut discrete reaction association with the interproglottid glands which in turn appear to be connected by fine fibrils to the nerve network. The function of these glands has never been defined. Fig 6.3A,B show higher magnifications of the nerve network of *Moniezia*, stained by the Karnovsky and Roots method with acetylthiocholine as substrate. A neurone-like cell is apparent connected with the nerve network.

Illustrations of whole mounts of segments of *T. ovis* (Karnovsky and Roots) with acetylthiocholine as substrate are shown in Fig 6.4C. As mentioned previously, this mount shows intense activity in the tegumental region, in contrast to Fig 6.4D, showing the genital pore region of a mature segment, where it can be seen there is no evidence of pseudocholinesterase activity in the tegument. The cirrus organ does, however, stain quite clearly and there is some reaction associated

the spermatic duct and the longitudinal nerve cord. Fig 6.4E illustrates the influence of eserine on a *T. ovis* segment stained by the Karnovsky and Roots method using butyrylthiocholine as substrate. It can be seen that there is very definite inhibition of the pseudocholinesterase activity. Moreover, the resulting faint staining enables visualization of the cirrus sac and the spermatic duct. Higher magnifications of whole mounts of proglottids of *T. ovis* reveal finer details of the nerve structure within the segment (Fig 6.6). In Fig 6.6.A, the Karnovsky and Roots method and acetylthiocholine were employed. Fine feature of the nerve network can be seen, and there appears to be discrete patches of the cholinesterase activity associated with the calcareous corpuscles. When butytylthiocholine was used as substrate, the reaction of the corpuscles was less marked, but the network is visualized and in the present illustration, a nerve cell is revealed which appears to have one axon and several dendrites. The cell is staining intensely for pseudocholinesterase. Its general structure suggests it may be a stretch receptor. Whole mounts of *T. hydatigena* shown in(Fig 6.10A,B) failed to demonstrate a strong acetylcholinesterase activity in the tegument. The reasons for this are not known. Nonetheless, the longitudinal nerves stained strongly and transverse and lateral nerves can be seen proceeding across the body and to the periphery. The cirrus organ stained quite strongly (Fig 6.10C). With the Gerebtzoff method and butytylthiocholine the cirrus organ again stained intensely and there was intense staining in the longitudinal nerve trunk. But again the tegument failed to show evidence of cholinesterase activity (Fig. 6.13B).

Frozen section of the body portion, with the Karnovsky and Roots method and acetylthiocholine as substrate are illustrated for *T. ovis* (Fig 6.5A,B). In the peripheral region, it can be seen that there is a clearly defined staining of the tegument (Fig 6.5A). The lining of the excretory duct also shows a positive reaction and there is reaction in the lining of the uterus and in the developing ova. Fig 6.5B again illustrates strong staining reaction with acetylthiocholine in the tegument and there is a bilateral series of dark spots illustrating the transection of the circular nerves. The uterine

6.3.1

lining gives a reaction and there is a response amongst the developing ova. The Gerebtzoff method has been employed on frozen body sections of *T. hydatigena* using acetylthiocholine as substrate. A gravid section is shown in Fig 6.8A. Strong reaction can clearly be seen in the transverse section of the longitudinal nerve trunk; from it two major trunks transverse to join the opposite trunk. From the transverse trunk arise large numbers of fine nerves which can be traced into the parenchyma and into the tegument itself. The uterine lining shows a positive reaction, as do the primordial cells within the uterus itself. The tegument shows a positive reaction for acetylcholinesterase. Fig 6.8B, illustrates a similar reaction, but more clearly defines the relationship of nerves running from the central elements to the periphery and here it can be clearly seen that many nerves are afferent in character and arise from tiny receptor cells within the tegument itself. Again the cuticle has shown a positive reaction as has the nerve trunk and the uterine elements. Fig 6.8C,D illustrate the finer structure of the nerve arrangement in the tegumental region in somewhat higher magnification. It can be seen the nerve network is associated with the rich source of receptor cells in the tegument and possibly subtegumental region as well. The complete connection between these receptors and the cells of the longitudinal nerve cord is well illustrated in Fig 6.8E. In Fig 6.9, a close visualization of the structure of the longitudinal nerve trunk is presented. It can be seen that there is a powerful cholinesterase activity at the centre of the cord but the activity becomes less clearly defined in the transverse branches. In Fig 6.9B, butyrylthiocholine has been employed as substrate and this method has enabled another clear visualization of the tegumental receptors and their connection to the central elements of the nervous system. Similar findings can be made from Fig 6.9C taken from a slightly different portion of the worm segment. Fig 6.11A shows acetylcholinesterase activity in a longitudinal section of *T. hydatigena* and it can be seen again that the tegument is showing a strong positive reaction. Calcareous granules can be seen within the parenchyma but these are not showing strong reactions. Fragments of the longitudinal nerves can also be defined. This activity was completely inhibited by pretreatment with vincofos 10^{-3}M. Other examples of transverse frozen sections of *T. hydatigena* are illustrated in Fig 6.12A,B and C. All were treated by the Gerebtzoff method using

acetylthiocholine as substrate. Fig 6.12A, traverses the genital pore and cirrus organ and a strong reaction can be seen in the organ itself and in the spermatic duct leading to its base. In Fig 6.12B, sectioning the region of the longitudinal nerve cord, a strong reaction can be seen in the nerve cord and a great arboration of nerve trunks from the cord to the periphery. The excretory canal is showing a positive reaction. Fig 6.12C illustrates a fairly strong reaction in the tegument and again the tiny tegumental receptors can be defined. The section also traverses the cirrus organ which in turn is showing a strong reaction. Fig 6.14A shows a mature frozen section treated with butyrylthiocholine as substrate cut in a longitudinal plane. There is a weak cholinesterase response in the cuticle and in a few of the deeper nerve elements arising from the circular nerve trunk. The section shown in Fig 6.14B traverses a gravid segment, and there is no evidence of pseudocholinesterase in the cuticle itself, but there is a fairly large zone of staining probably representing subtegumental nerve endings. The lateral nerve trunk and transverse nerve trunk show a weak reaction.

6.3.2 Influence of inhibitors

The influence of eserine on the reaction of *T. ovis* with butyrylthiocholine, using the Karnovsky and Roots method has been illustrated in Fig 6.4E. It can be clearly seen that eserine (10^{-5}M) has a marked inhibitory effect on the activity of the enzyme. Another illustration of inhibition is seen in Fig 6.11B where it can be seen that pretreatment with vincofos (10^{-3}M) for 30 minutes prior to incubating the section with acetylthiocholine for 2 hours, has produced an almost complete inhibition of acetylcholinesterase activity.

Fig 6.10A-H illustrates the influence of various inhibitors on cholinesterase activity of scolex and body portions of whole mounts of *T. hydatigena* treated by the Gerebtzoff method using acetylthiocholine as substrate. The figures A,B and C illustrate normal reactions of the scolex, a body segment and the cirrus region, respectively. Eserine 10^{-5}M has a clear-cut inhibitory effect on both the scolex and the cirrus region (Fig 6.10D,E). The effect of dichlorvos (10^{-3}M) on the scolex and a mature segment are shown in Fig. 6.10F,G. It is clearly

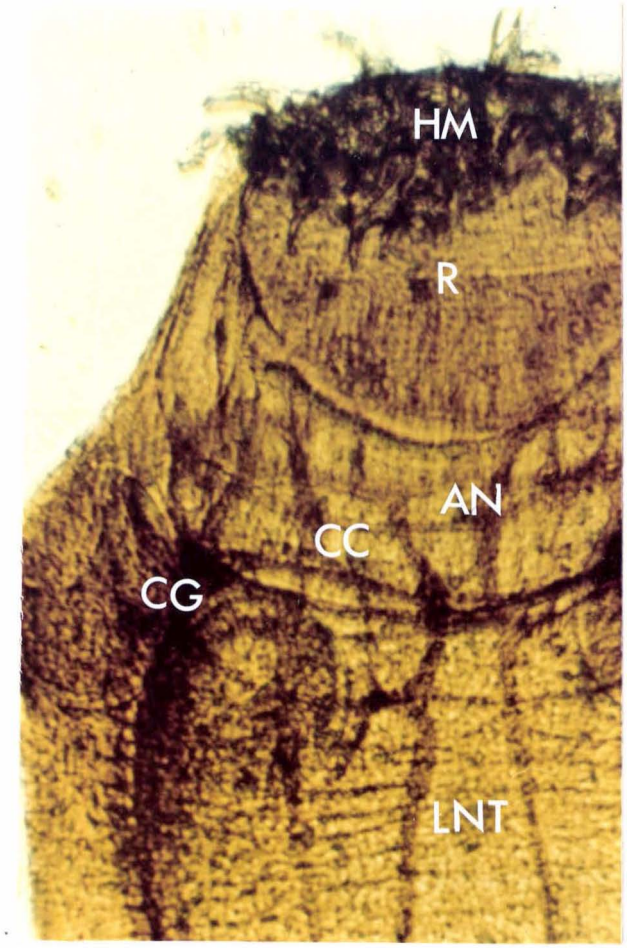
seen that this organophosphorus compound is a powerful inhibitor of acetylcholinesterase. Fig 6.10H illustrates the action of vincofos on the cirrus region of the gravid segment and should be compared with the untreated segment in Fig 6.10C. It can be seen that the normal acetylcholinesterase activity is appreciably inhibited by the vincofos.

Fig 6.12 illustrates the effect of inhibitor on frozen section of *T. hydatigena* using the Gerebtzoff method with acetylthiocholine as substrate. Fig 6.12A,B,C represent the control segments which have been discussed previously. Fig 6.12D shows the effect of preincubation with eserine at 10^{-5}M followed by an incubation for 4¼ hours with the substrate. It can be seen that this regime has brought about almost complete inactivation of cholinesterase activity. Similar findings occurred when segments were preincubated with dichlorvos (10^{-3}M) for 30 minutes and then incubated for a further 3 hours in substrate. The same procedure with vincofos, has been illustrated in Fig 6.12F and again it can be seen that there is appreciable inhibition of cholinesterase when this section is compared with the controls.

Butyrylthiocholine was employed as substrate with whole mounts of *T. hydatigena* using the Gerebtzoff method (Fig 6.13). Normal reactions (Fig 6.13A,B) have been discussed previously and represent a 16 hour incubation period in the butyrylthiocholine substrate. In the inhibition experiments, the segments were preincubated for 1 hour with inhibitor and for a further 16 hours with the inhibitor plus substrate. The effects are illustrated for eserine, Fig 6.13C, for dichlorvos Fig 6.13D and for vincofos, Fig 6.13E. In all instances it can be seen that the inhibitors have brought about a clear cut reduction in pseudocholinesterase activity.

The effect of inhibitors on pseudocholinesterase in frozen sections of *T. hydatigena* using the Gerebtzoff method and butytylthiocholine as substrate are illustrated in Fig 6.14. Fig A and B show respectively normal reaction to incubation of the longitudinal section and the transverse section (3½ hours). These staining reactions have been commented on previously. In these inhibitory experiments all

6.1 A



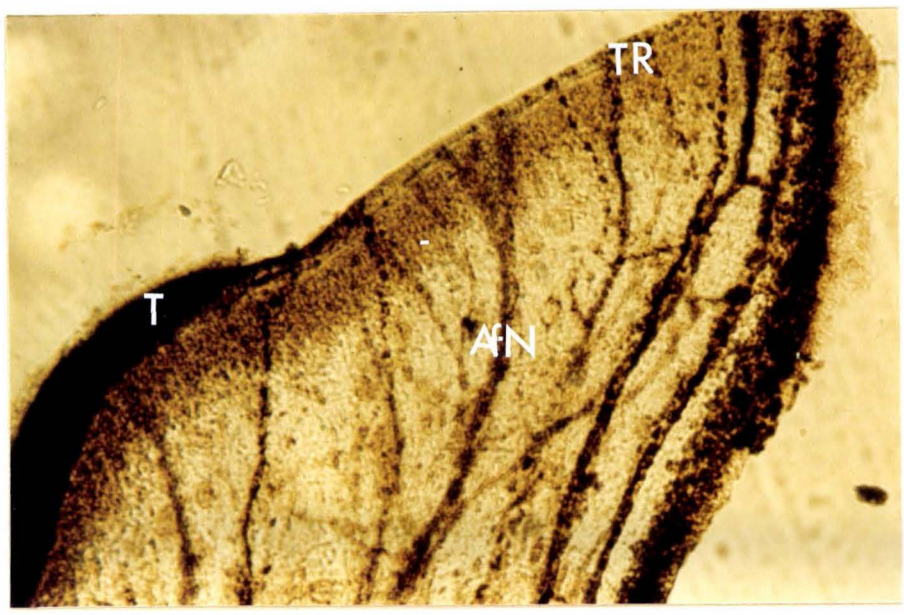
A

Fig 6.1 Showing sites of cholinesterase activity using the method of Gerebtzoff (1959).

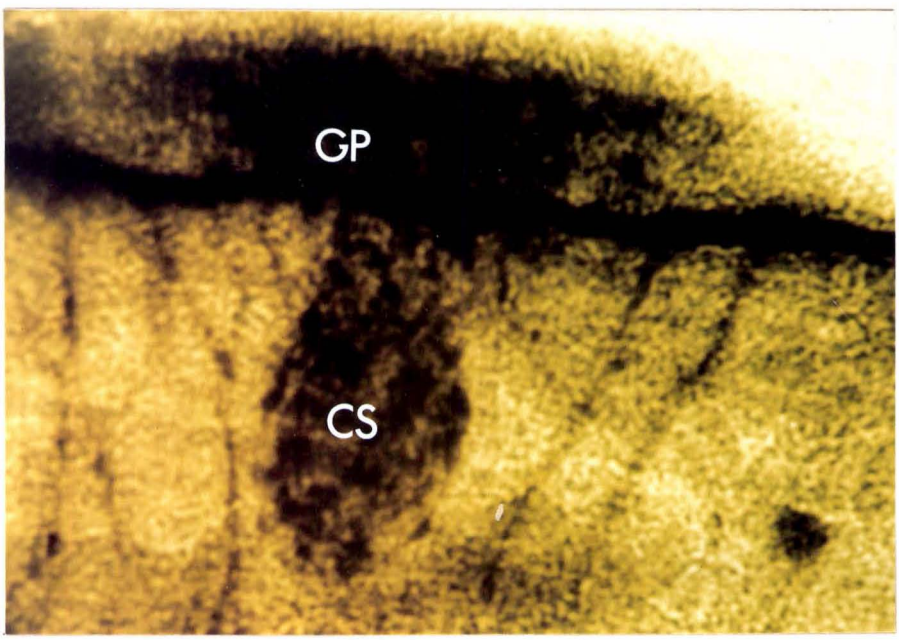
(A) Whole mounts of scolex of *T. taeniaeformis* (x 80). Butyrylthiocholine substrate.

(B) The section of a frozen mature segment of *T. taeniaeformis* (x 100). Acetylthiocholine substrate.

(C) Whole mount of 35-day-old *Echinococcus*. (x 600). Butytylthiocholine substrate.



B



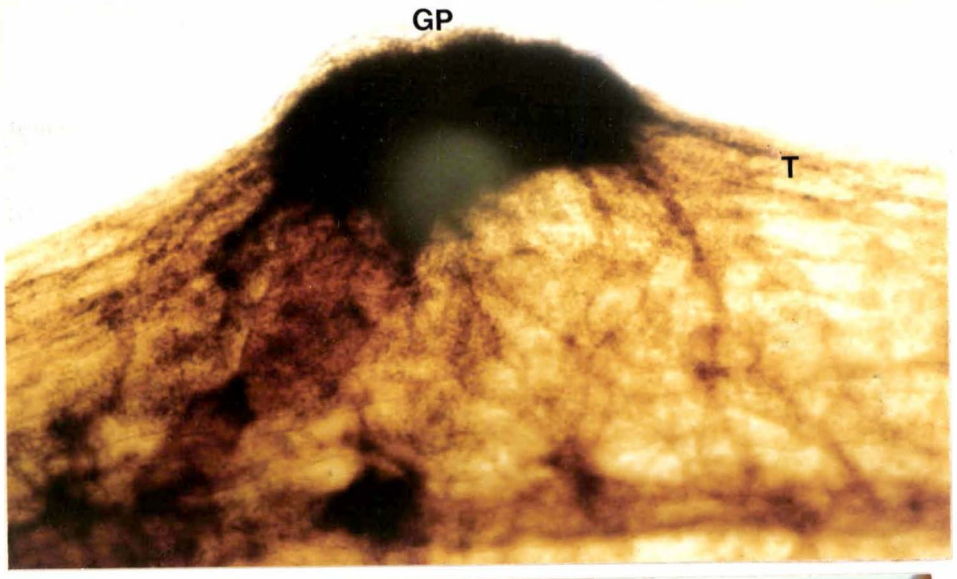
C

Fig 6.2 Showing sites of cholinesterase activity in whole mounts of *Moniezia*

(A) Mature segment (x 100). Karnovsky and Roots (1964) method. Acetylthiocholine substrate.

(B) Mature segment of *Moniezia* (x 100). Karnovsky and Roots method. Butyrylthiocholine substrate.

(C) Mature segment (x 65). Gerebtzoff method. Butyrylthiocholine substrate.



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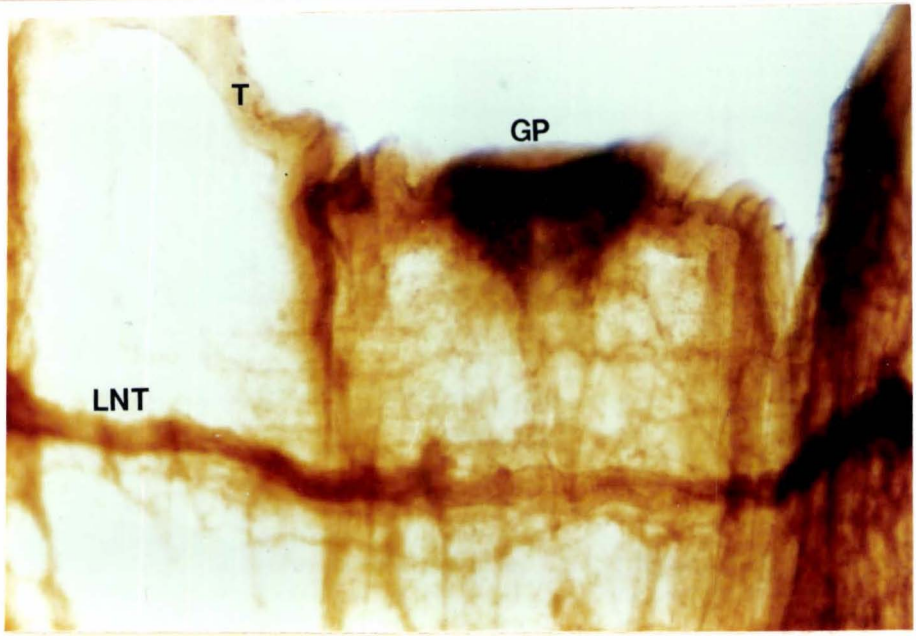

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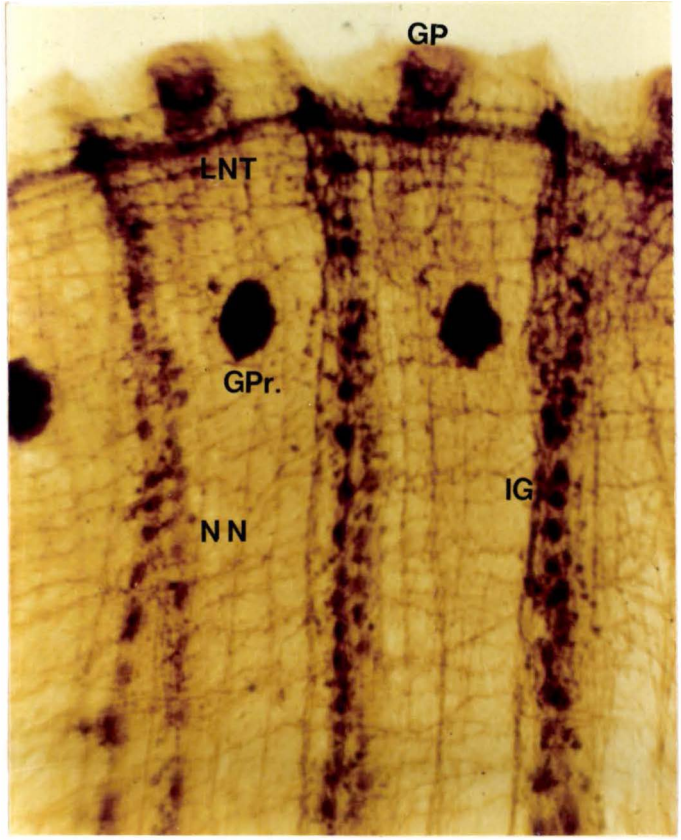
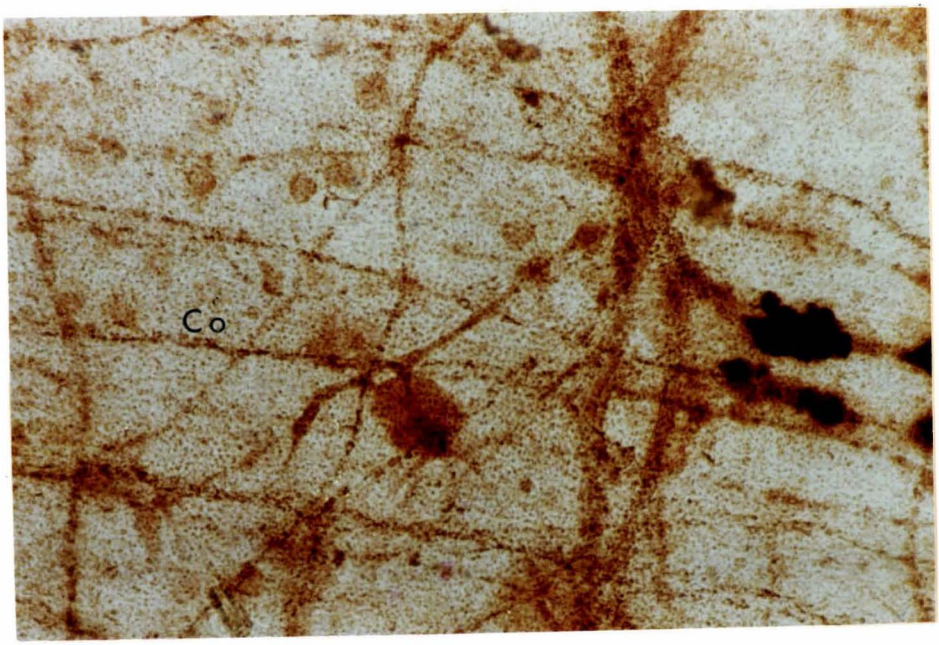


C



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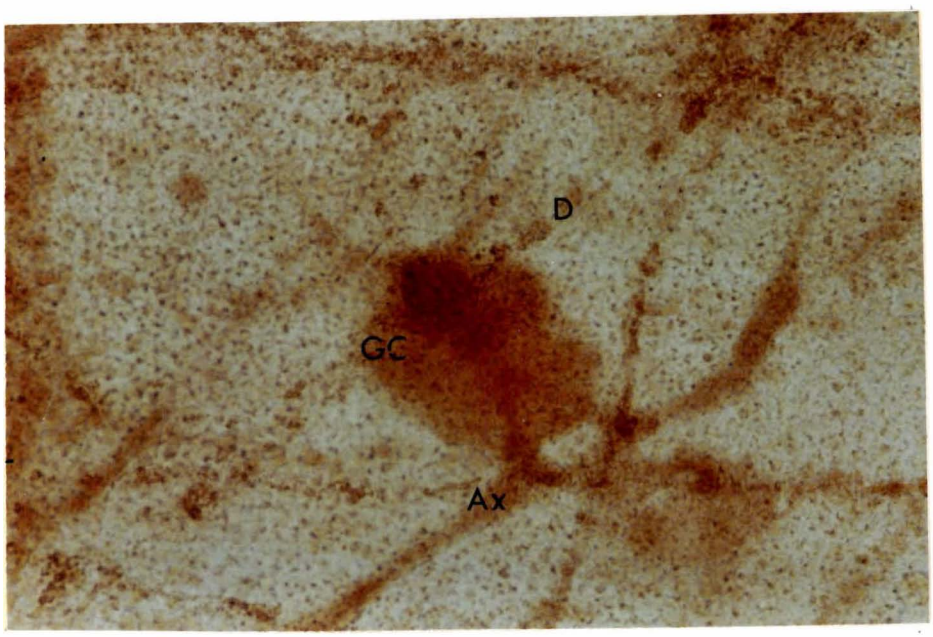


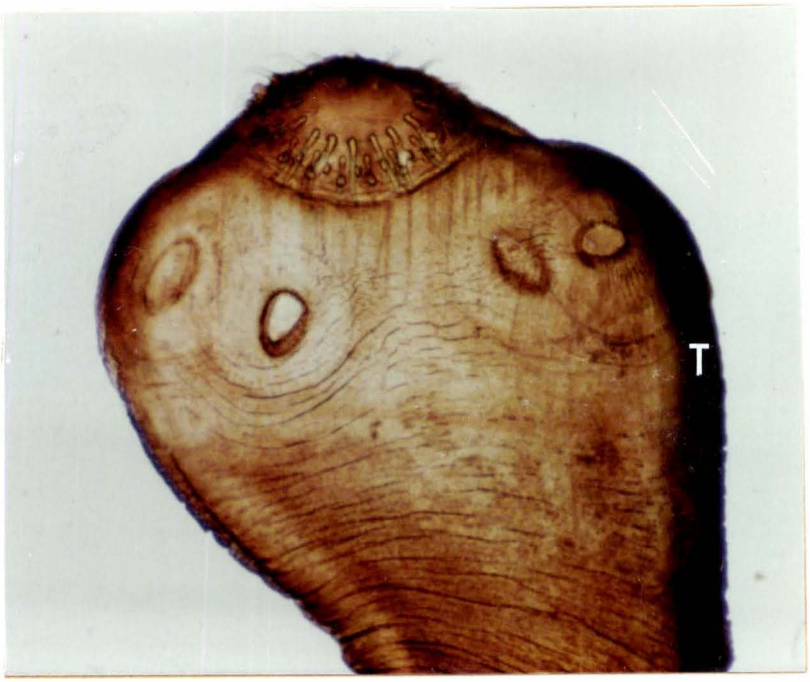
B

Fig 6.3 Showing sites of cholinesterase activity in whole mounts of *M. expansa* using the Karnovski and Roots method and acetylthiocholine as substrate

(A) Mature segment (x 260)

(B) As for (A) (x 620).

A



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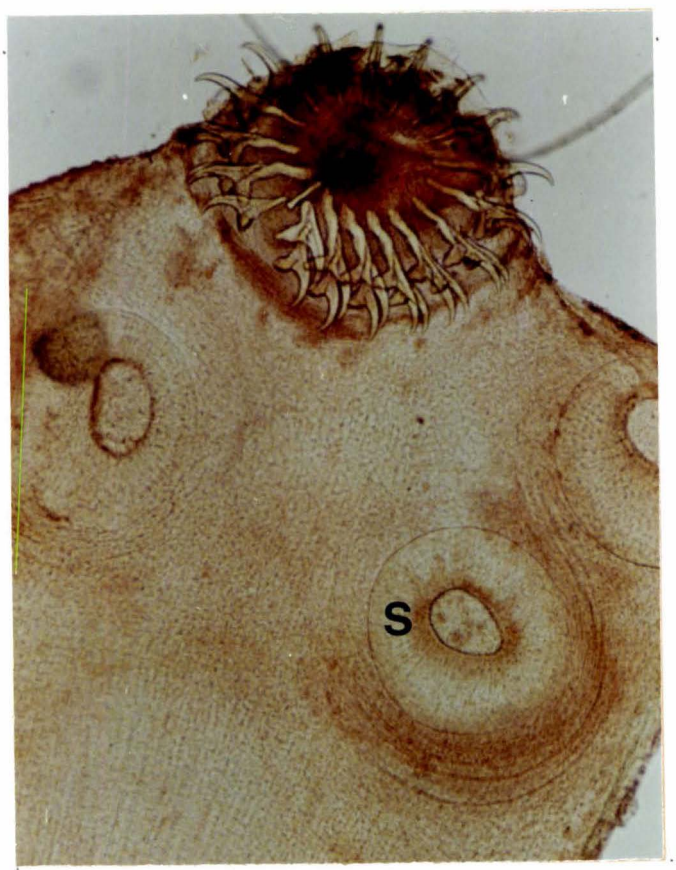


Fig 6.4 Showing cholinesterase activity in whole mounts of *T. ovis* and the inhibitory action of eserine.

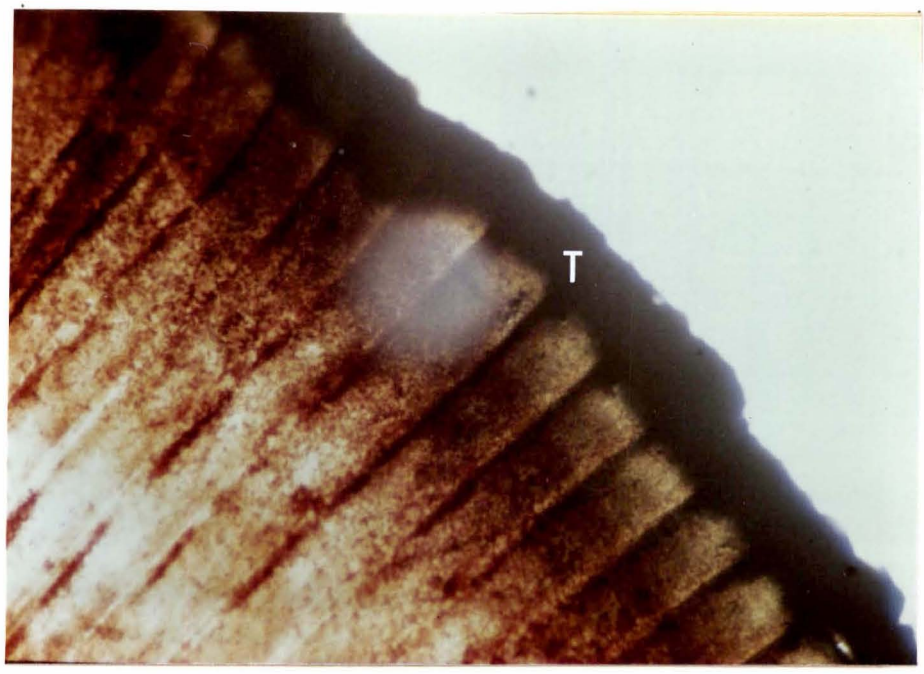
(A) Scolex (x 65) using Karnovsky and Roots method. Acetylthiocholine substrate.

(B) Scolex (x 100) using Karnovsky and Roots method. Butyrylthiocholine substrate.

(C) Immature segment (x 100) Karnovsky and Roots method. Acetylthiocholine substrate.

(D) Mature segment (x 65) Gerebtzoff method. Butyrylthiocholine substrate.

(E) Mature segment (x 100) incubated with eserine (10^{-5} M). Karnovsky and Roots method. Butyrylthiocholine substrate.



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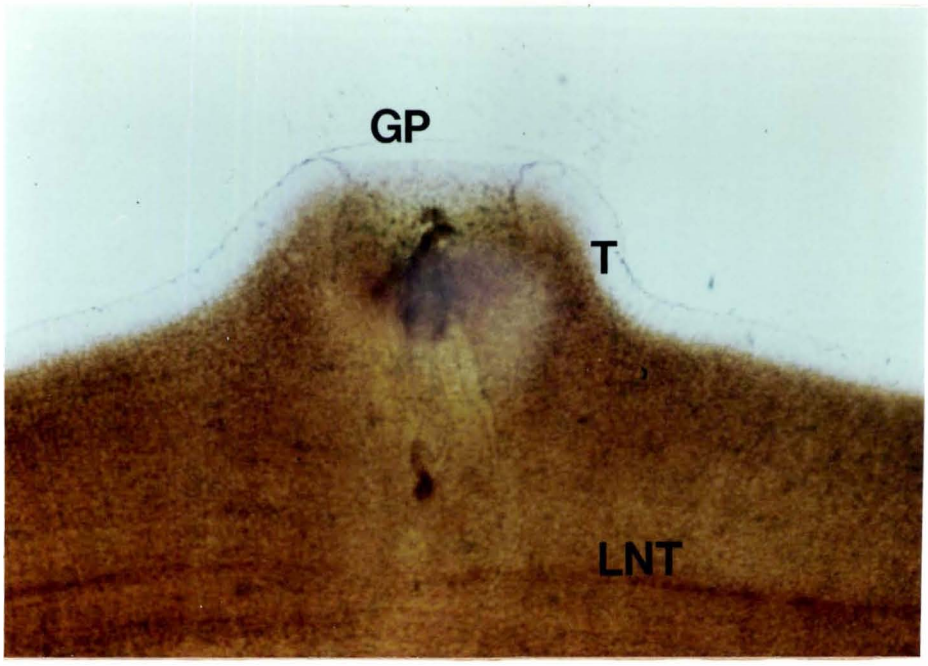

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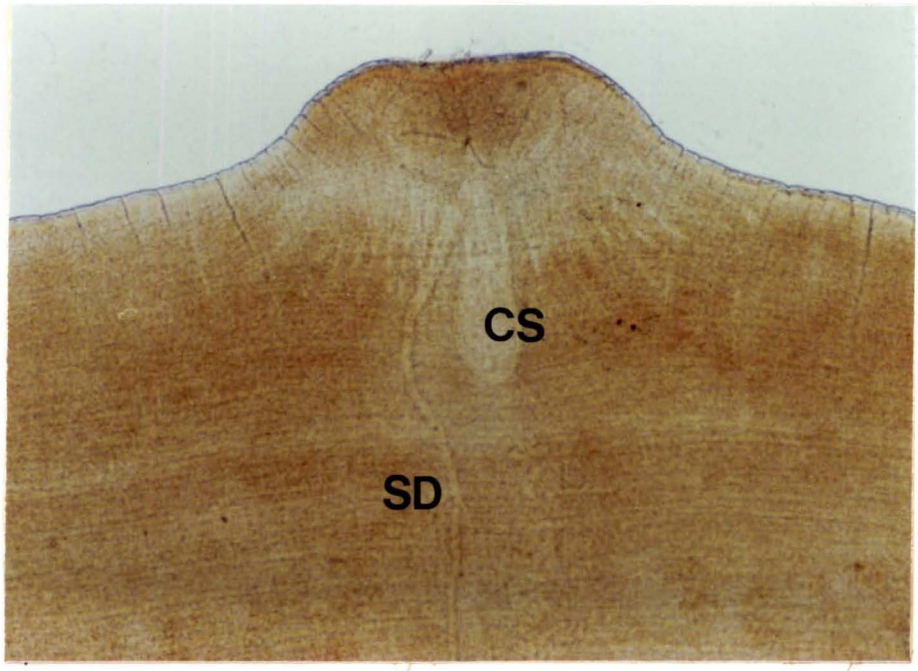

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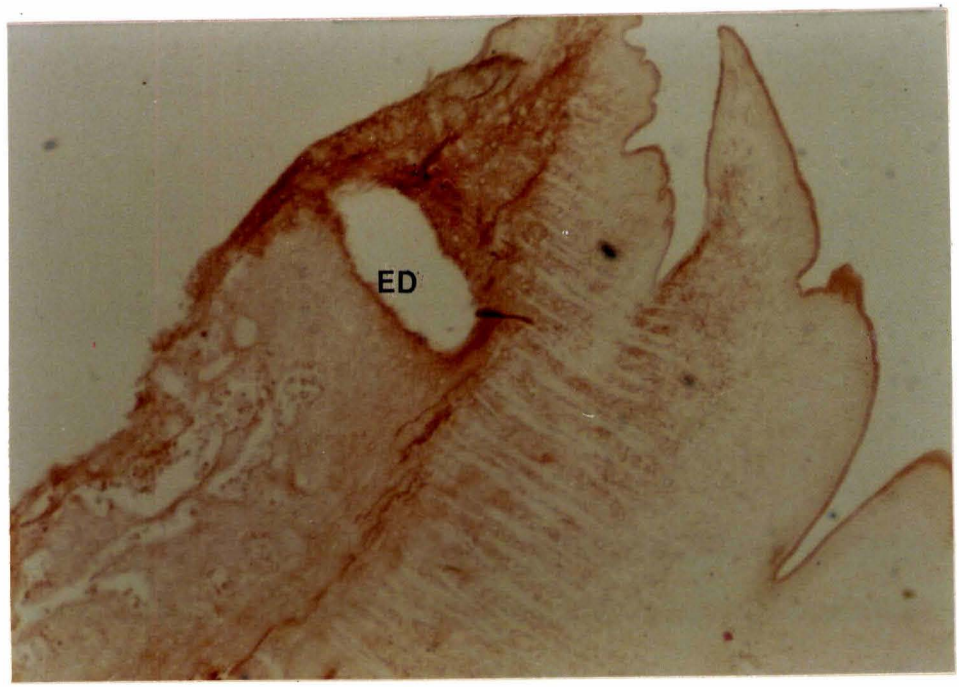
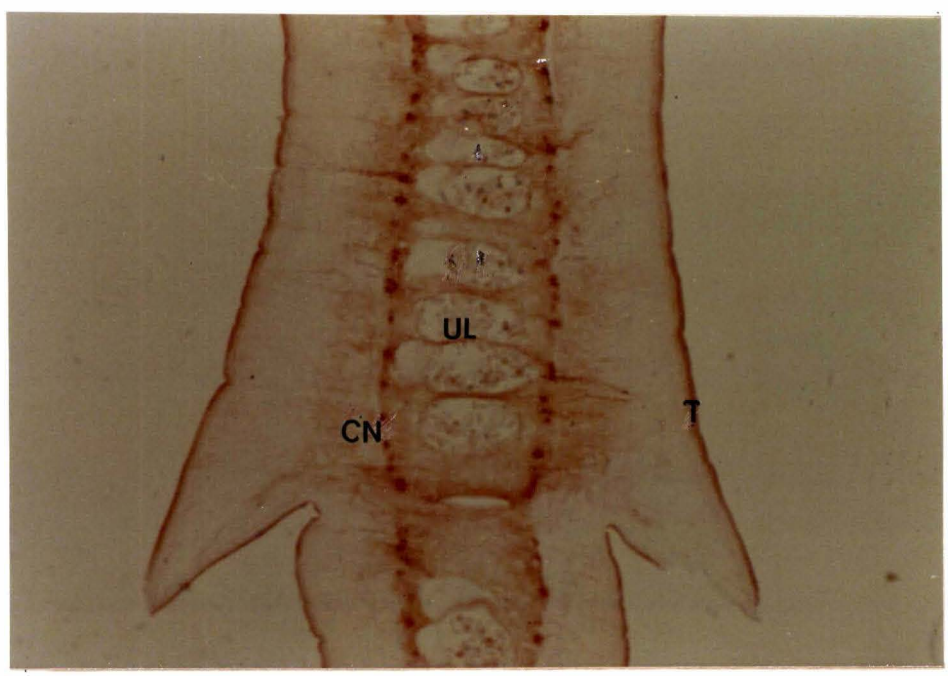
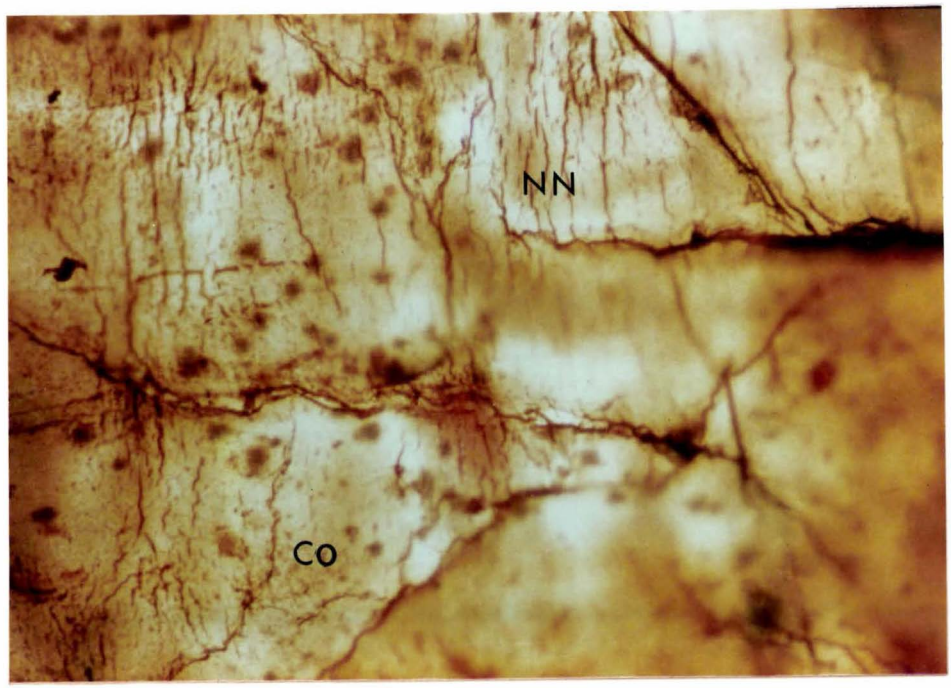


A

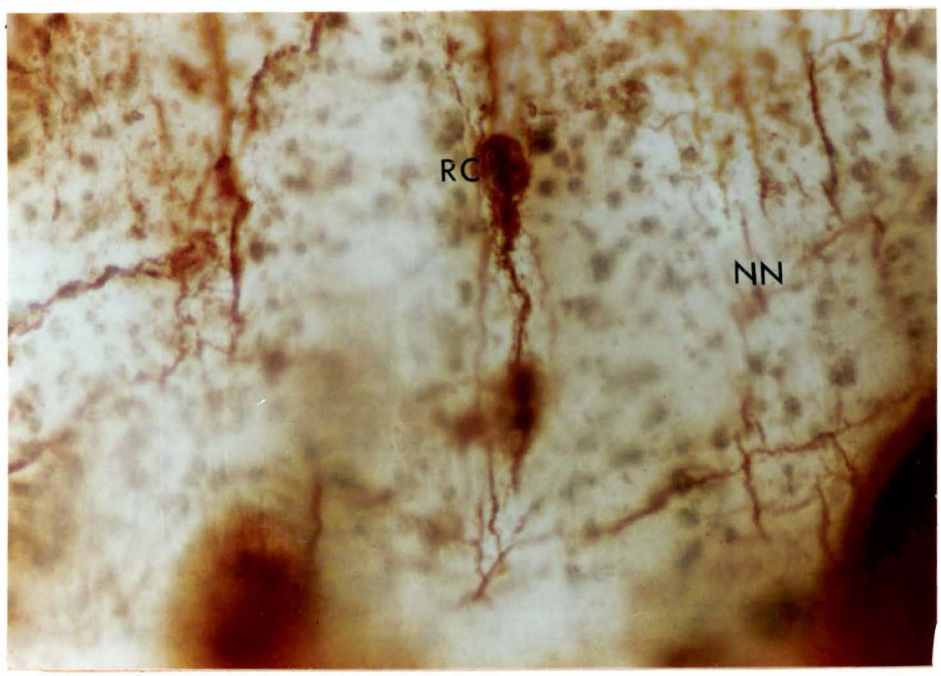


B

Fig 6.5 Showing cholinesterase activity in fresh frozen sections of *T. ovis* using the Karnovsky and Roots method and acetylthiocholine as substrate.
(A) Cut on the dorso-ventral plane, peripheral region (x 65).
(B) As for (A) intersegmental region (x 65).



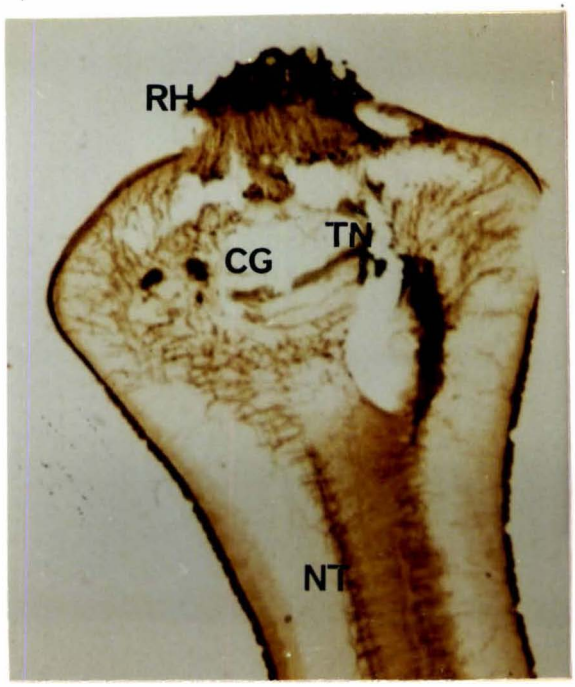
A



B

Fig 6.6 Showing cholinesterase activity in whole mounts of *T. ovis* by Karnovsky and Roots method.
(A) Sections (x 100) Acetylthiocholine substrate.
(B) Sections (x 250) Butyrylthiocholine substrate.

Fig 6.7 Showing sites of cholinesterase activity in frozen sections of the scolex of *T. hydatigena* using the Gerebtzoff method.
(A) Sagital section of scolex (x 100). Acetylthiocholine substrate.
(B) As for (A) (x 100). Butyrylthiocholine substrate.
(C) As for (B) (x 200).
(D) As for (C) (x 620).



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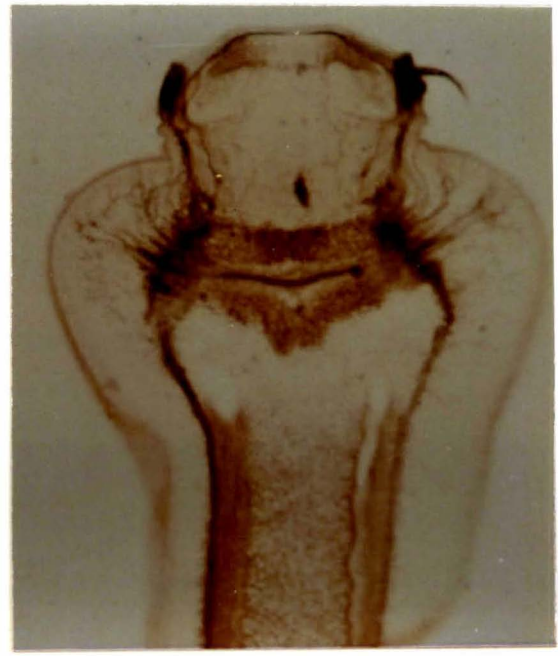

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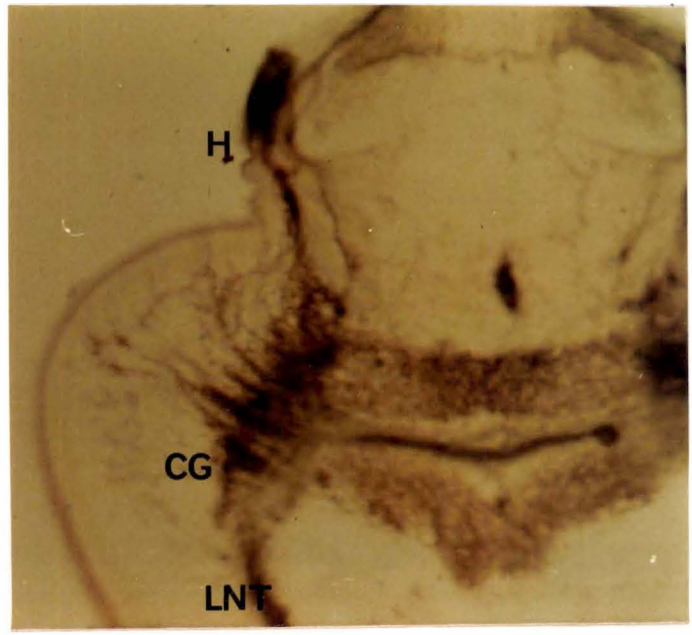

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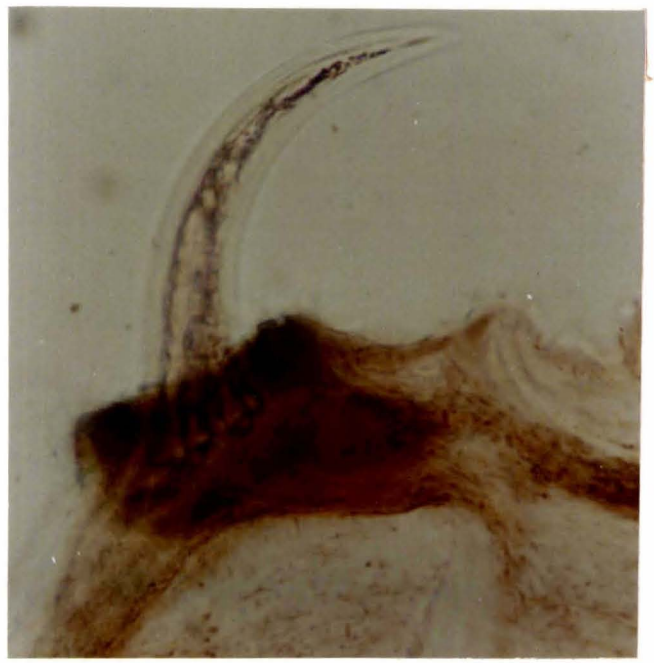
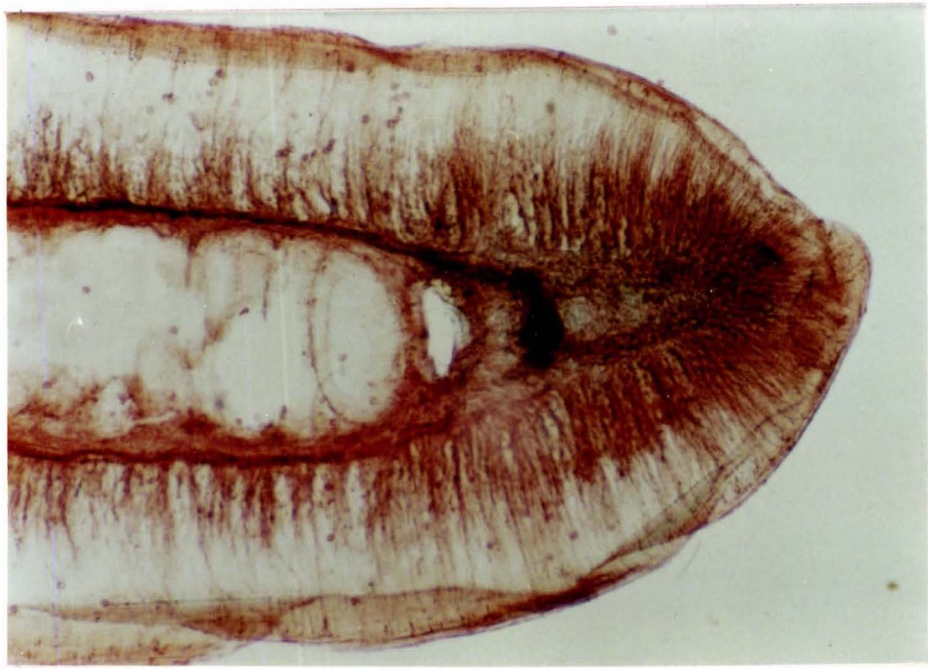


D

A



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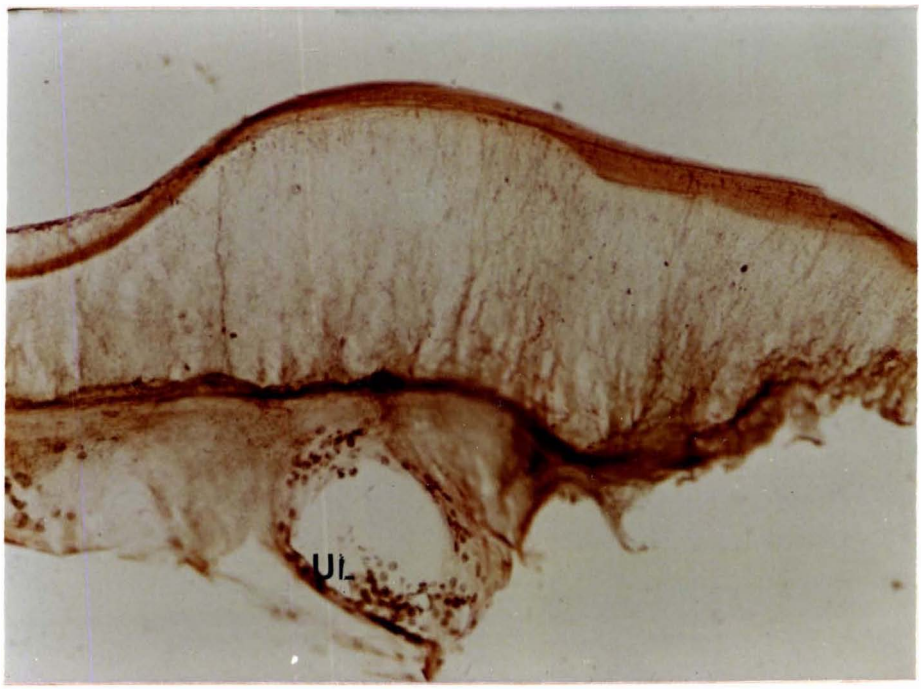
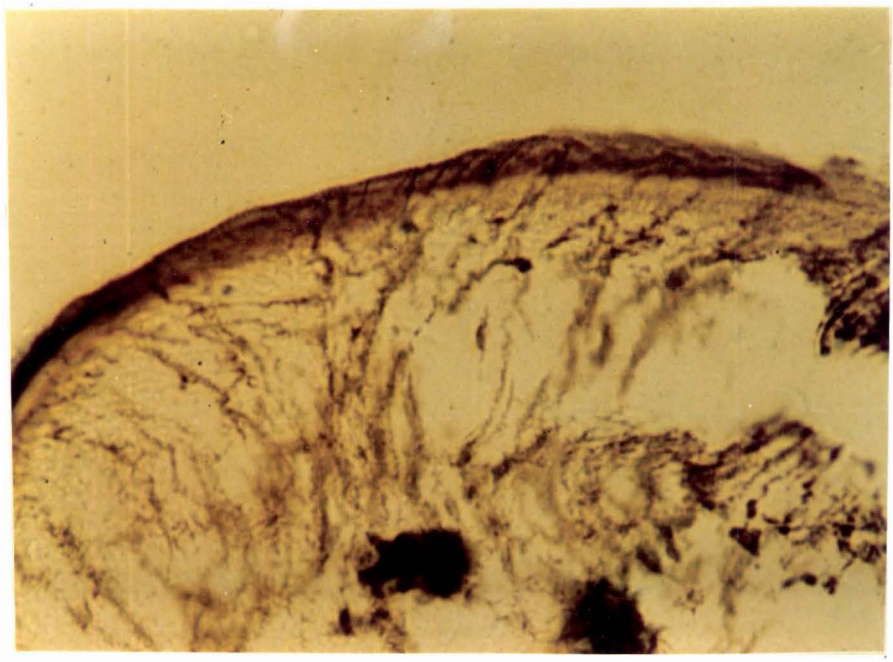


Fig 6.8 Showing cholinesterase activity in frozen transverse sections of *T. hydatigena* using the Gerebtzoff method and acetylthiocholine substrate.

(A) Gravid segment (x 65).
(B) As for (A) (x 100).
(C) Mature segment, cuticular region (x 250).
(D) As for (C) (x 620).
(E) As for (D) (x 85).



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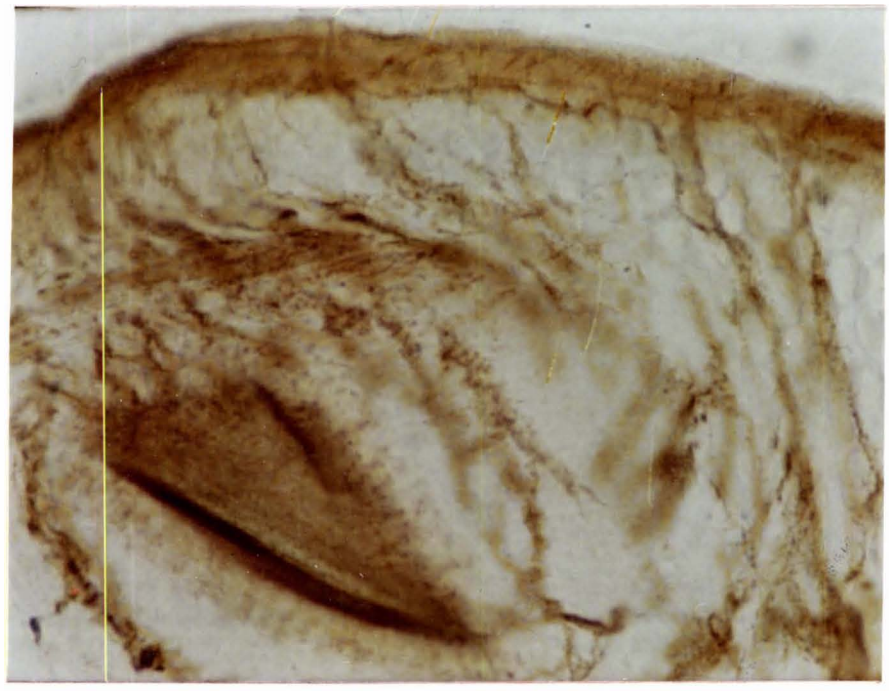

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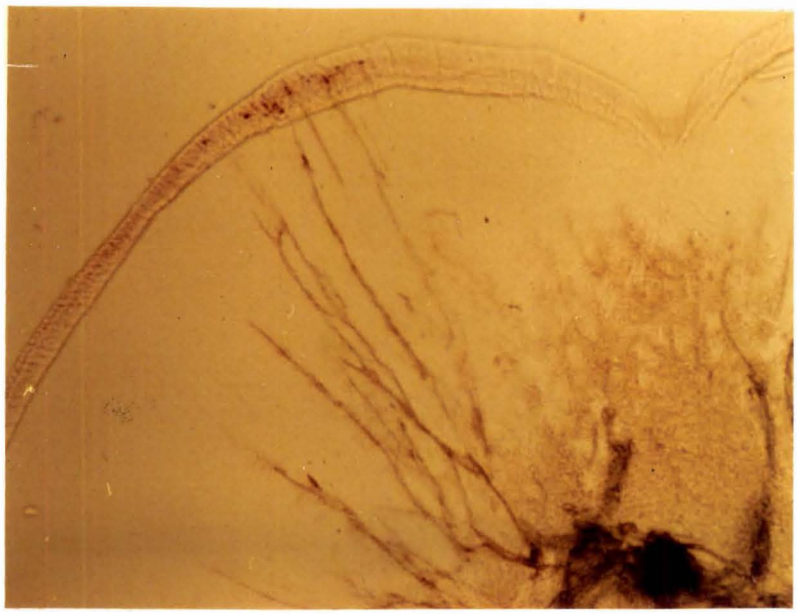

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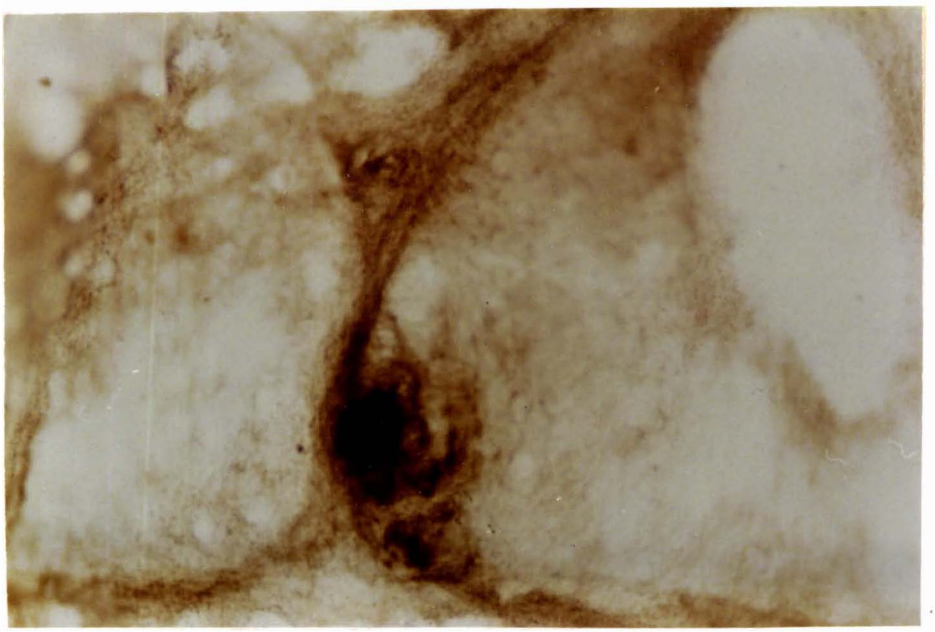

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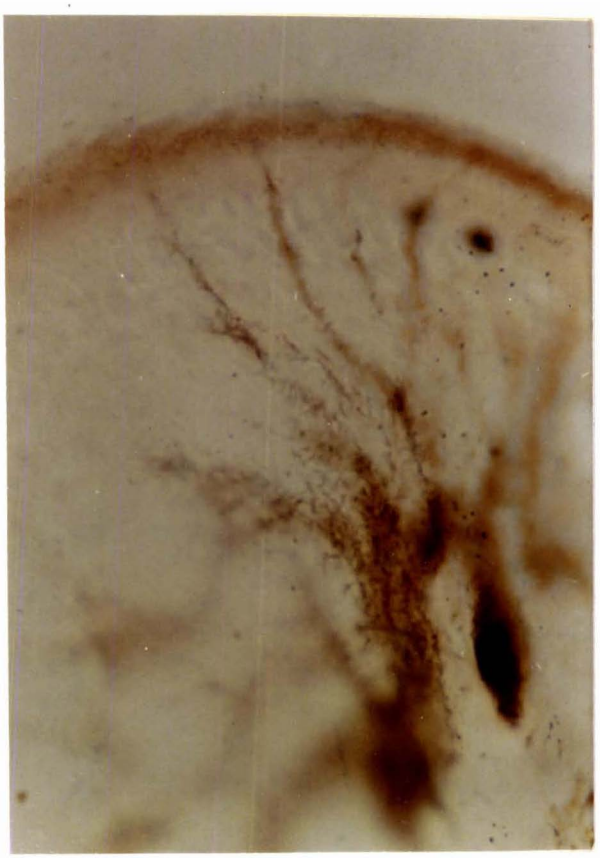


B

C

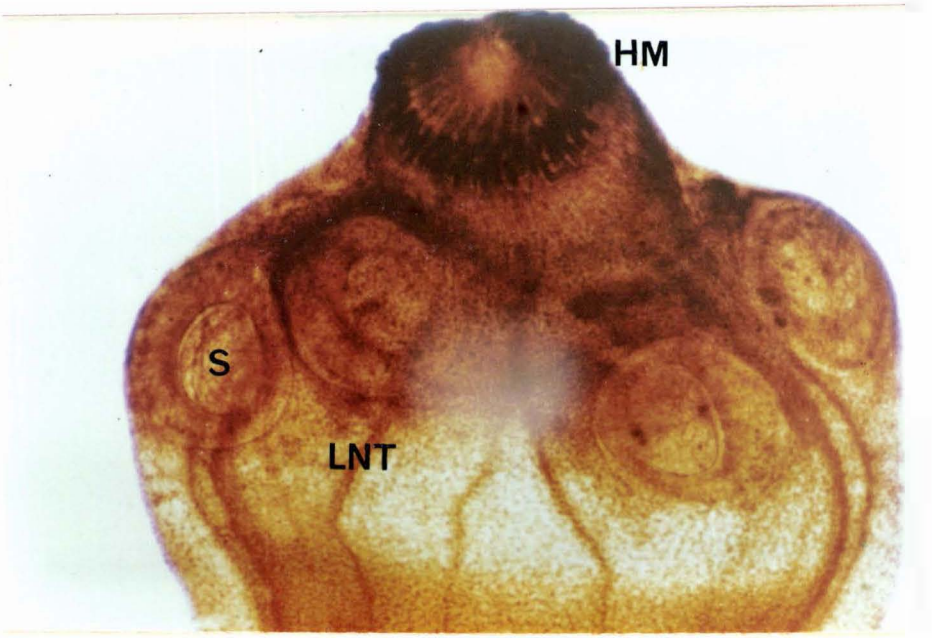
Fig 6.9 Showing cholinesterase activity in nerve tissue in frozen transverse section of mature *T. hydatigena*.

(A) Longitudinal nerve cord with transverse branches (x 250). Acetylthiocholine substrate.

(B) Peripheral afferent nerves from tegumental receptors going to longitudinal nerve trunk (x 620). Butyrylthiocholine substrate.

(C) As for (B).

A



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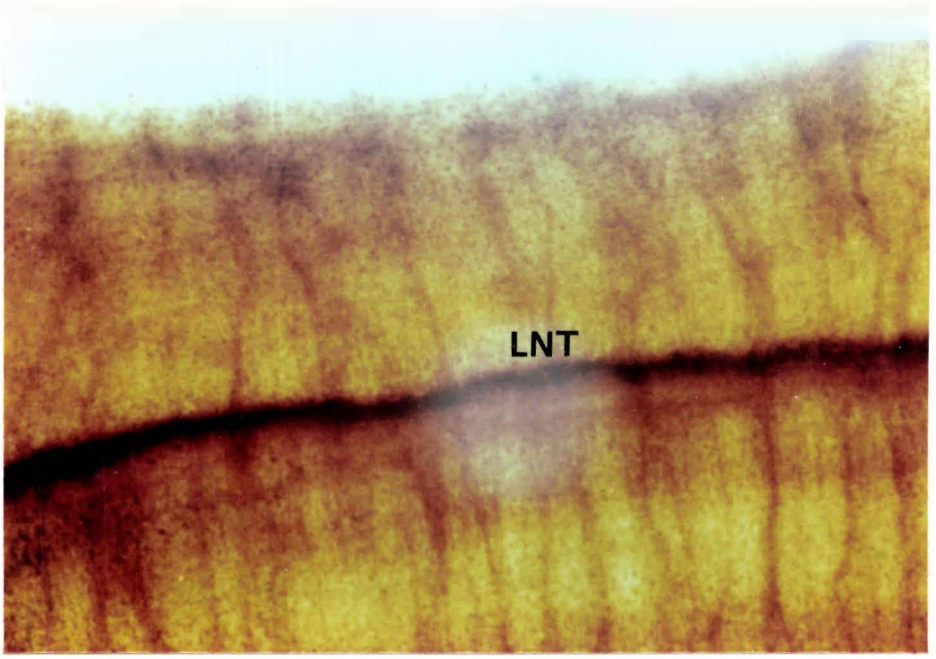

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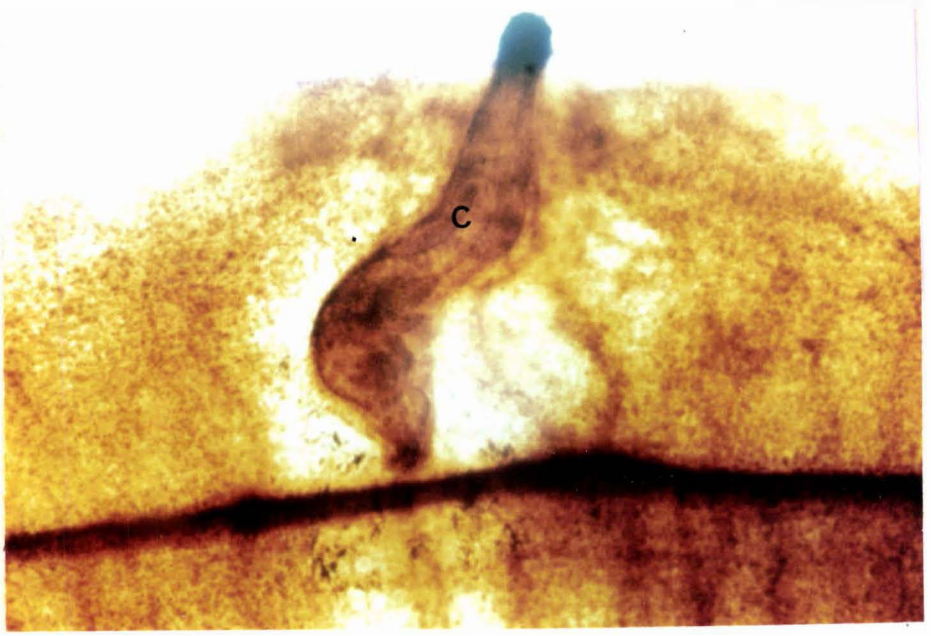

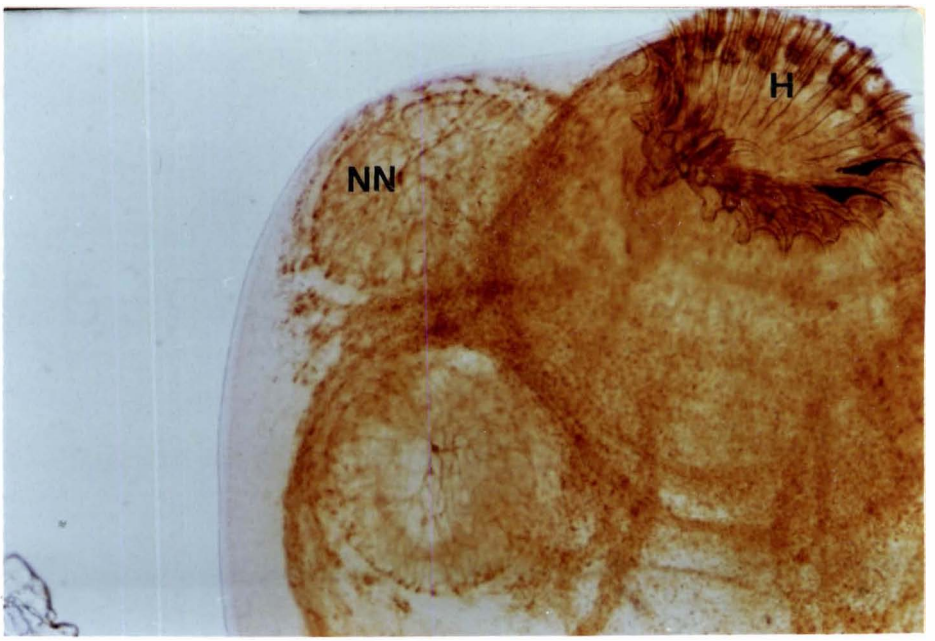

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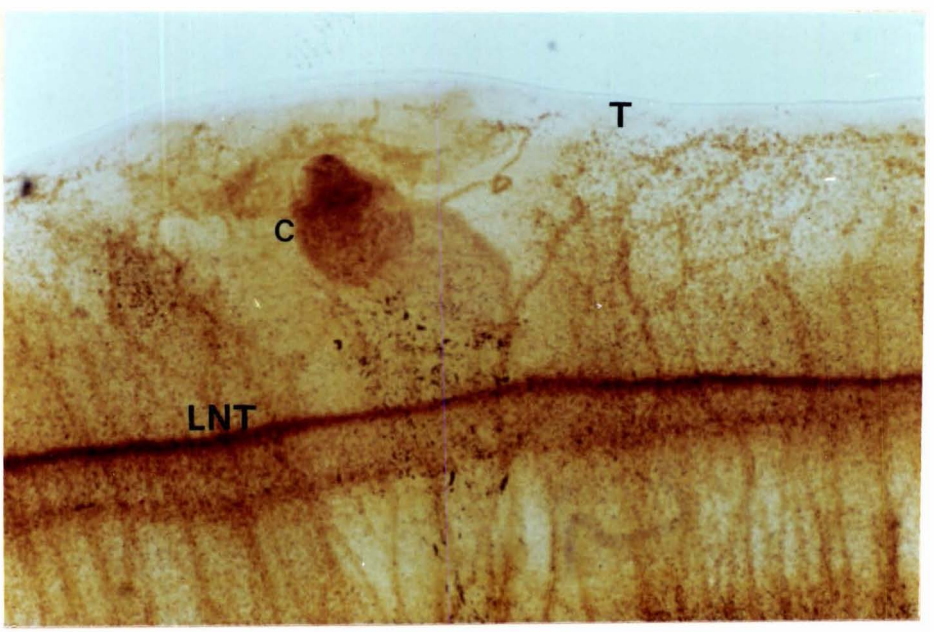
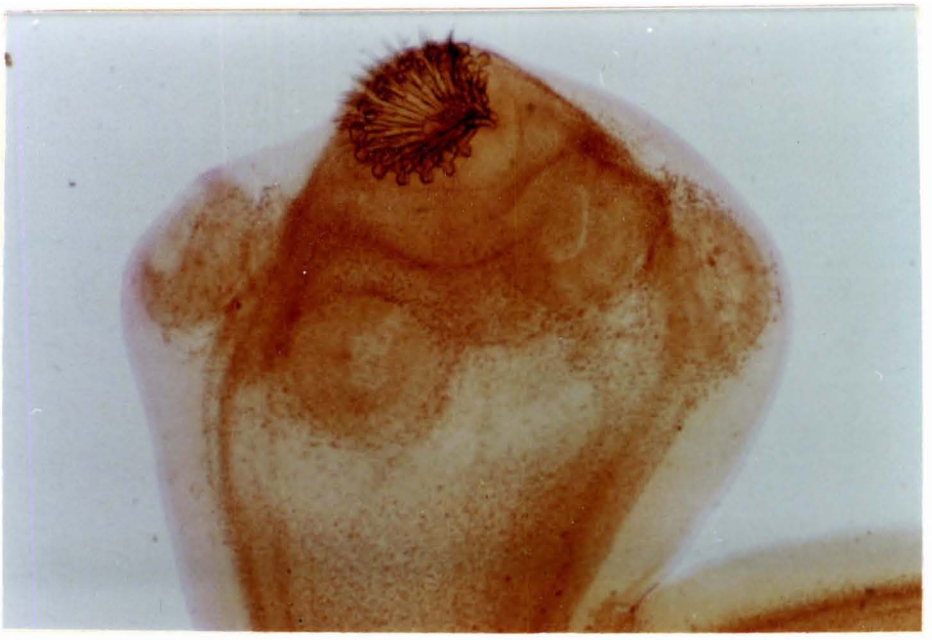


E

Fig 6.10 Showing cholinesterase activity in whole mounts of scoleces and segments of *T. hydatigena* treated by the Gerebtzoff method using acetylthiocholine as substrate and inhibition by eserine, dichlorvos and vincofos.

(A) Scolex region (x 65).
(B) Mature segment (x 100).
(C) Cirrus region (x 100).
(D) Scolex (x 65) showing effects of eserine (10^{-5}M).
(E) Cirrus region (x 65) showing effect of eserine (10^{-5}M).
(F) Scolex region (x 65) showing effect of dichlorvos (10^{-3}M).
(G) Mature segment of body region (x 65) as for (F).
(H) Cirrus region, gravid segment (x 65) showing effect of vincofos (10^{-3}M).

* Material was preincubated for 1 hour with the inhibitor alone and for a further 16 hours with both inhibitor and substrate.



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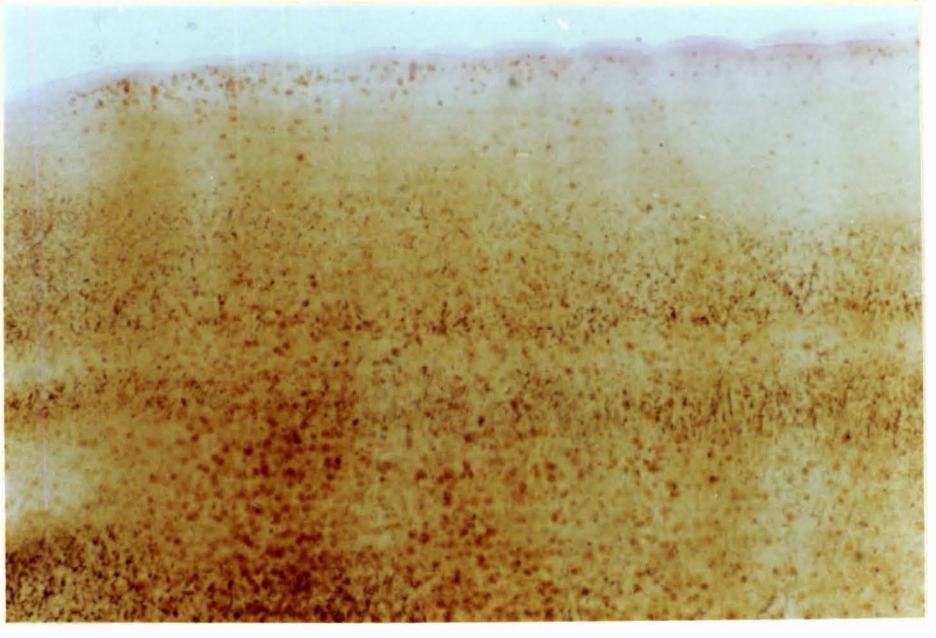

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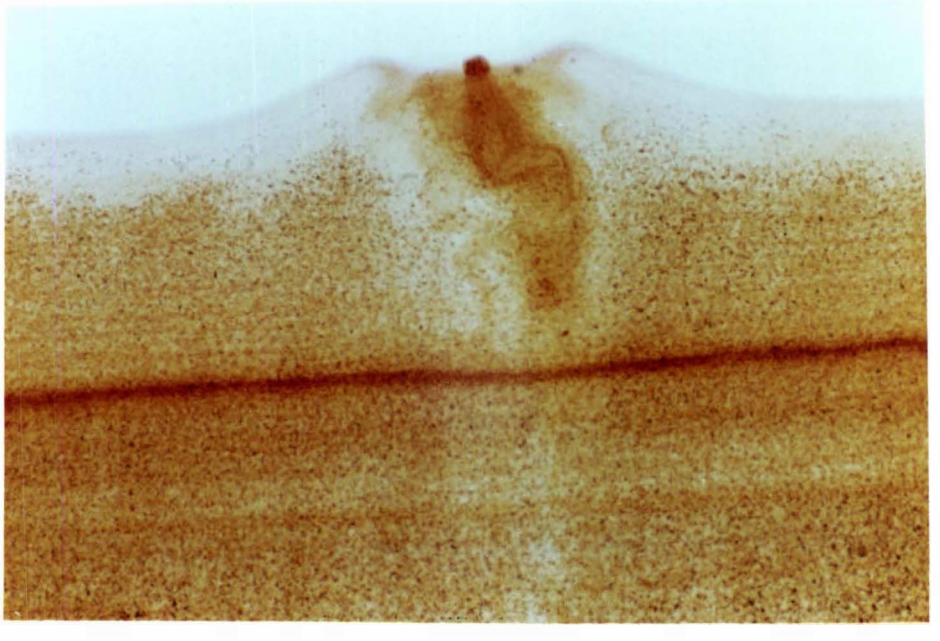

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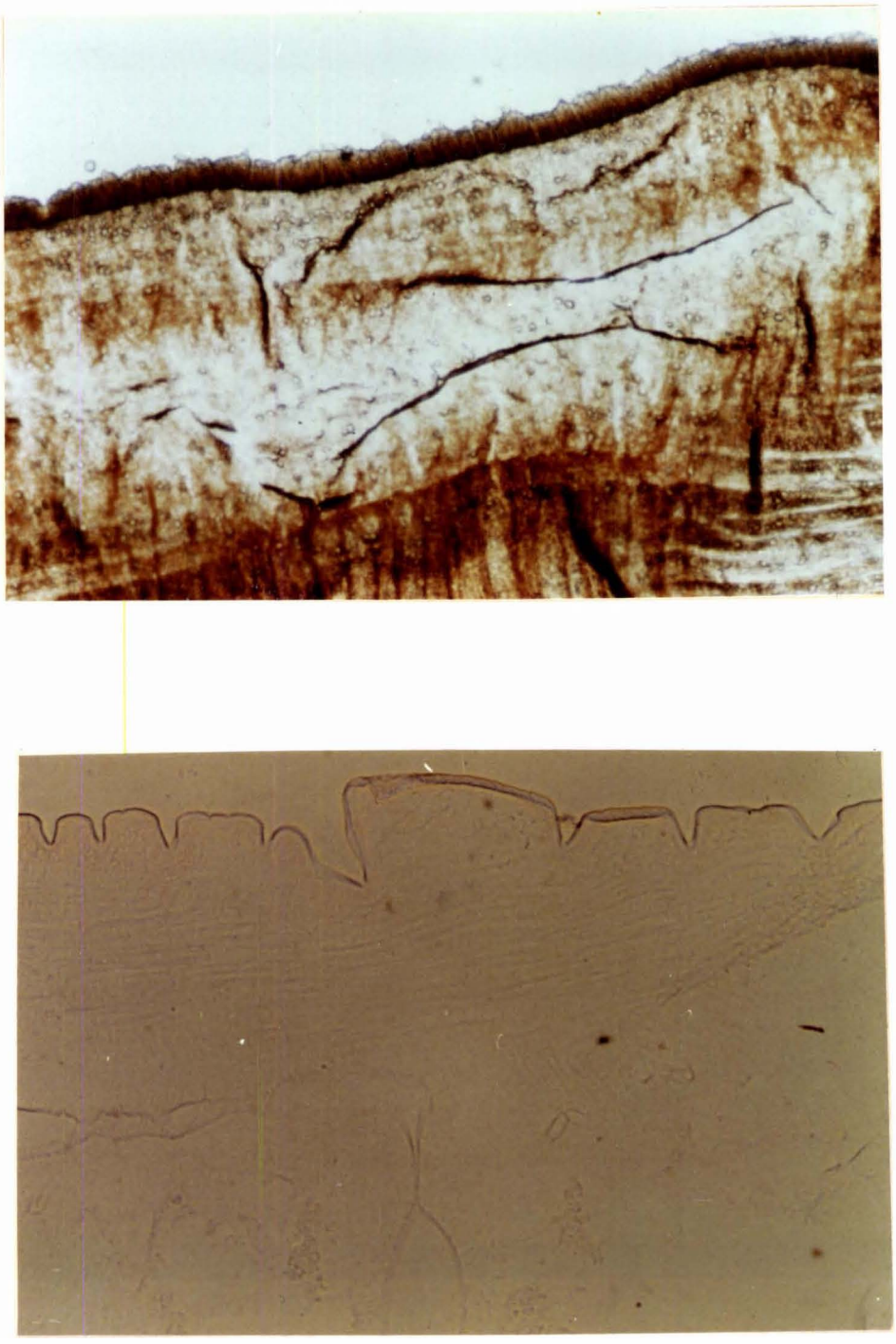
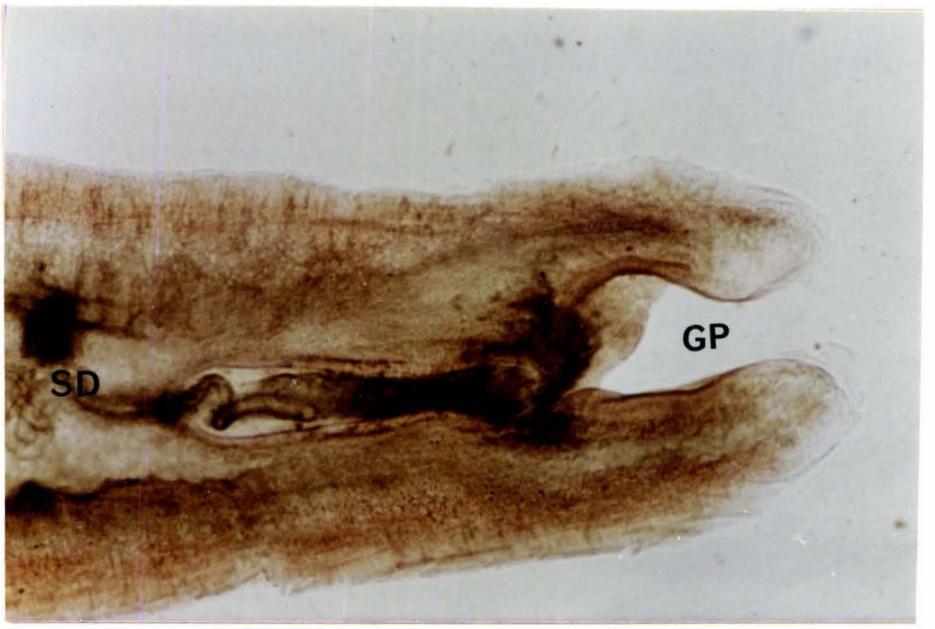


A

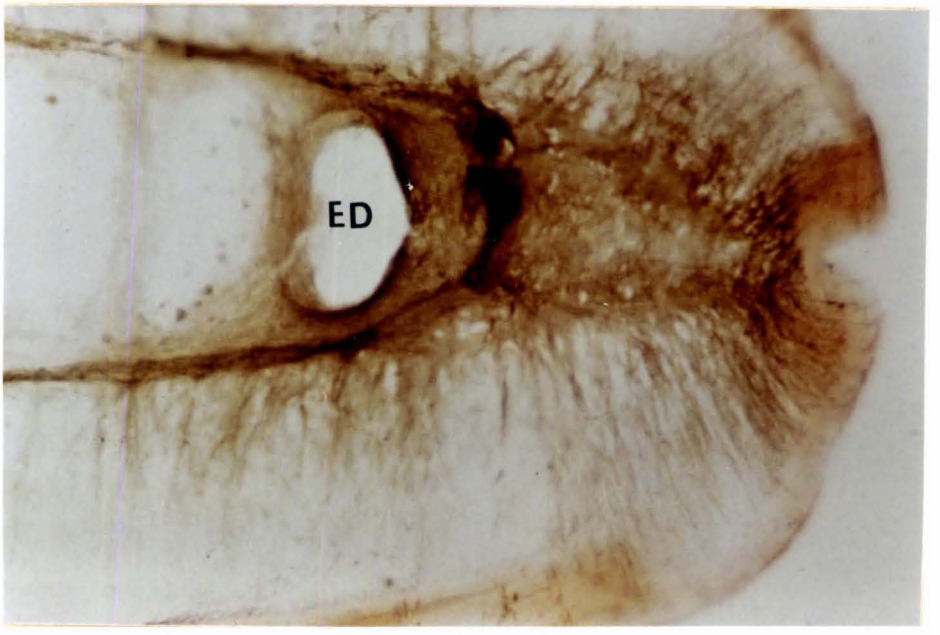
B

Fig 6.11 Showing normal acetylcholine activity in a longitudinal section of *T. hydatigena* using the Gerebtzoff method and the inhibition of the enzyme by vincofos.
(A) Mature longitudinal frozen section (x 65).
(B) As for (A) pretreated for 30 minutes with vincofos (10^{-3}M) and incubated with acetylthiocholine for 2 hours.

A



B



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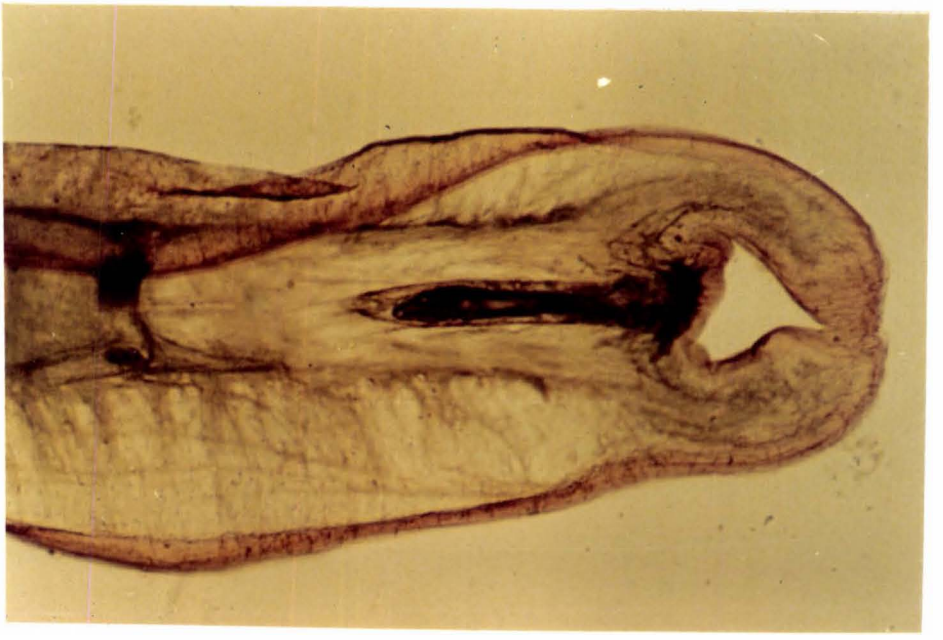
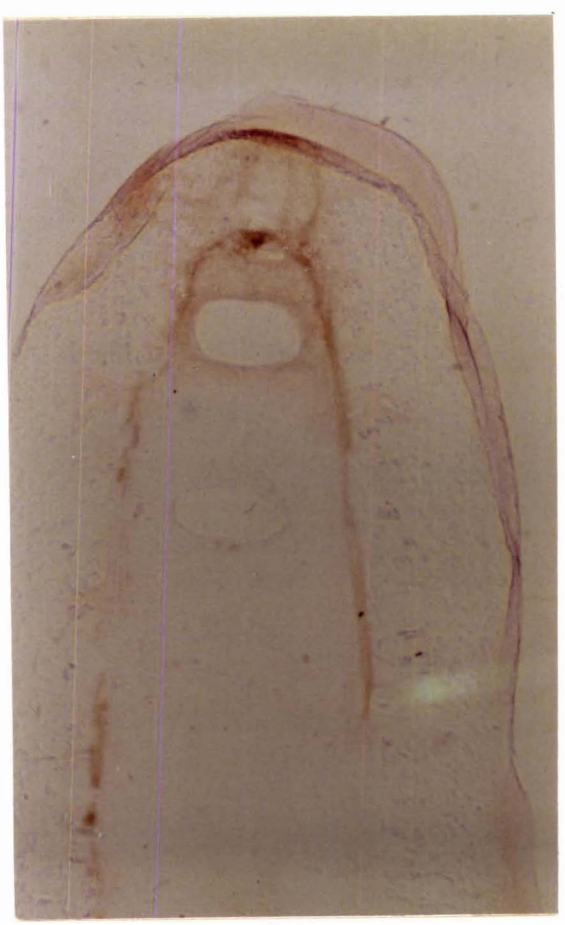
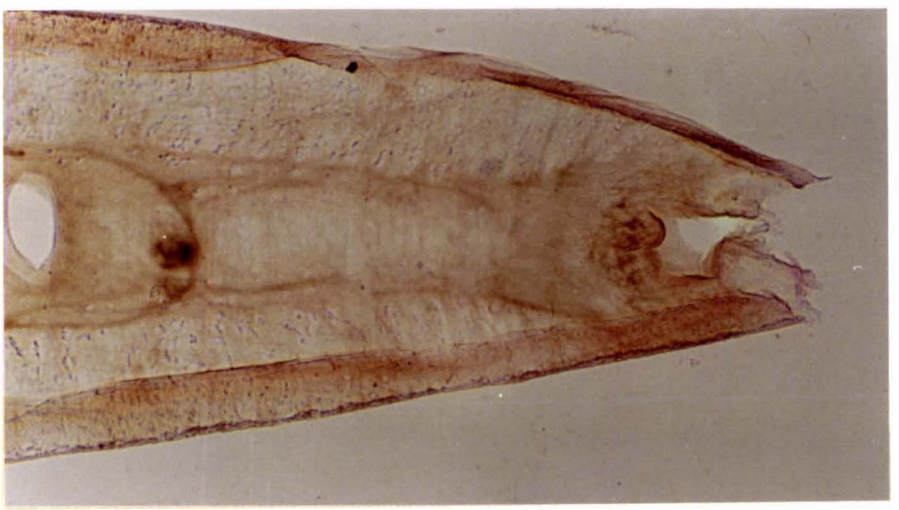




D

E



F

Fig 6.12 Showing cholinesterase activity in frozen transverse sections of *T. hydatigena* by the Gerebtzoff method using acetylthiocholine as substrate and the inhibition of enzyme activity by eserine, dichlorvos and vincofos.

(A) Mature frozen section, cirrus region (x 100) 2 hours incubation period.

(B) Gravid frozen section (x 100). Incubation 4½ hours.

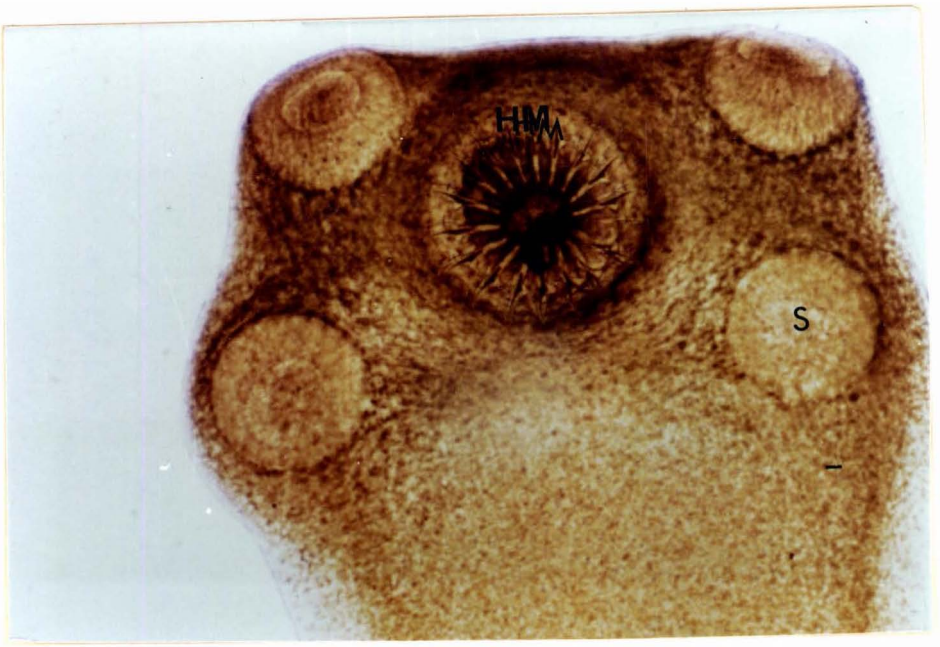
(C) Mature frozen section, cirrus region (x 65) 3 hours incubation period.

(D) Gravid frozen section (x 65). Preincubation 30 minutes with eserine (10^{-5}M) together with incubation with 4½ hours with substrate.

(E) Mature frozen section (x 65). Preincubation with dichlorvos (10^{-3}M) for 30 minutes prior to further 3 hours incubation with substrate.

(F) Gravid frozen section cirrus region (x 65) pretreated for 30 minutes with vincofos (10^{-3}M) prior to further 3 hours incubation in the presence of the substrate.

A



B

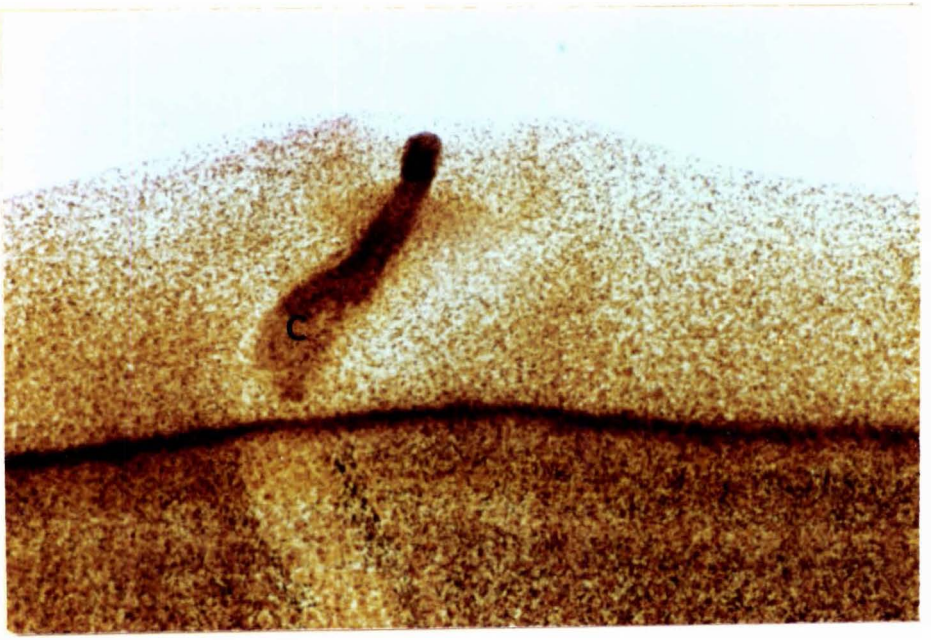
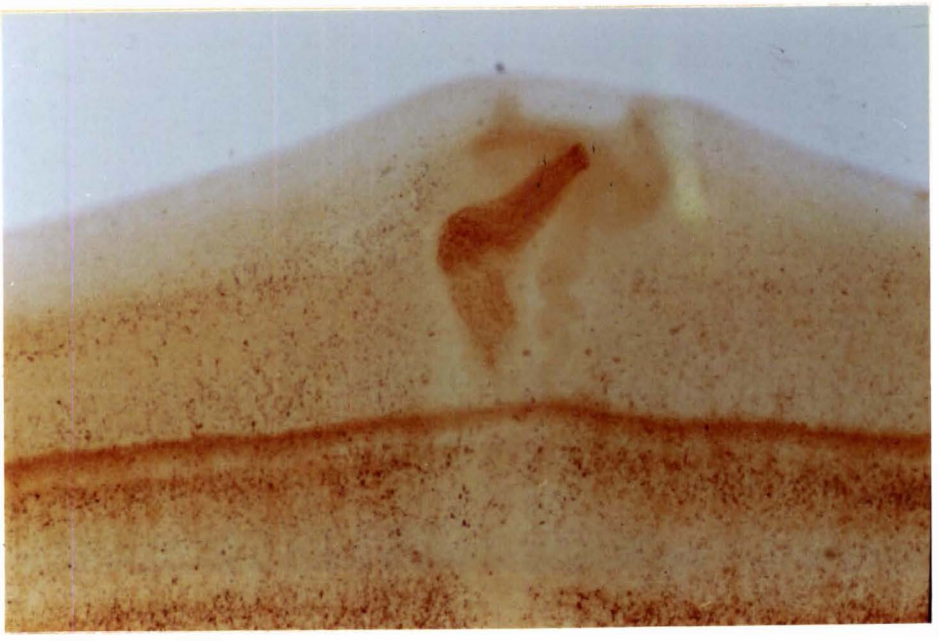


Fig 6.13 Showing cholinesterase activity in whole mounts of *T. hydatigena* using the Gerebtzoff method with butyrylthiocholine as substrate and the inhibitory effects of eserine, dichlorvos and vincofos.

(A) Scolex region (x 65).
(B) Cirrus region (x 65).
(C) Cirrus region (x 65). Inhibitor eserine (10^{-5}M).
(D) As for (C). Inhibitor dichlorvos (10^{-3}M).
(E) As for (D). Inhibitor vincofos (10^{-3}M).



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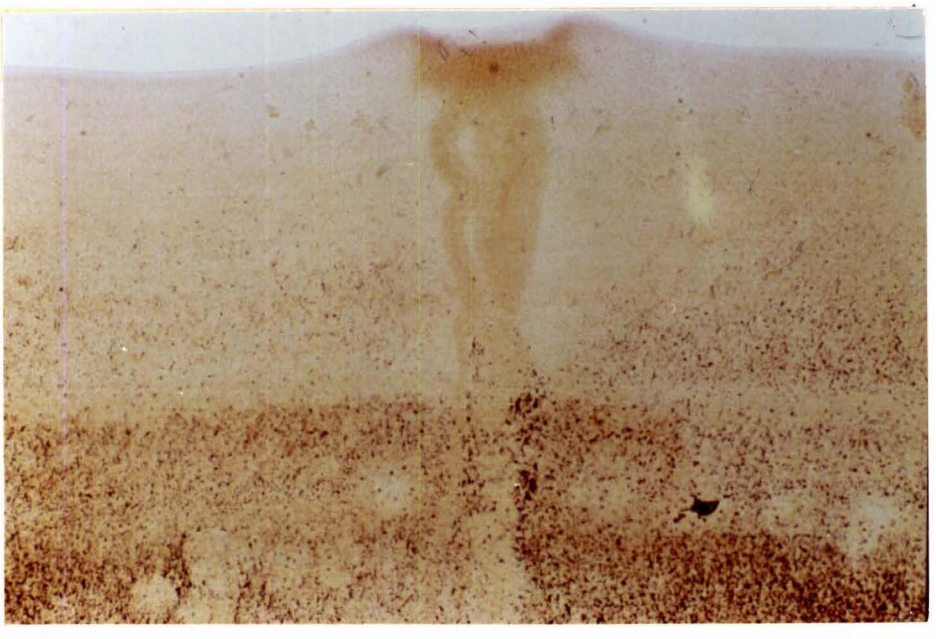

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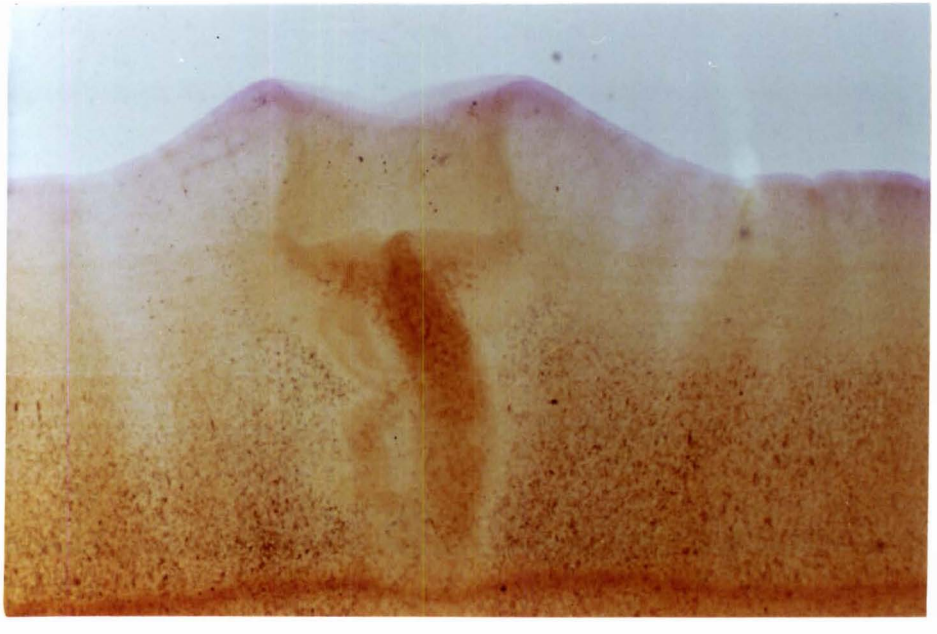

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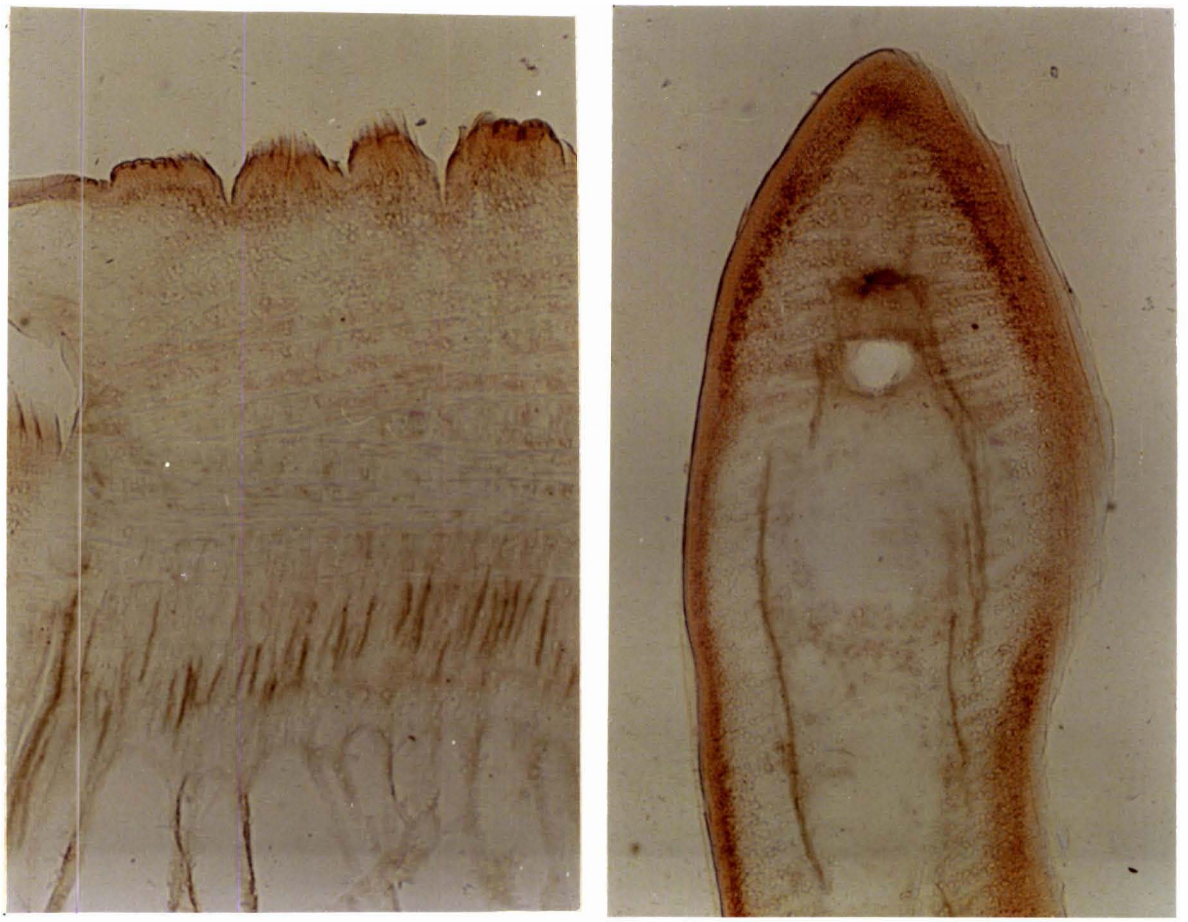
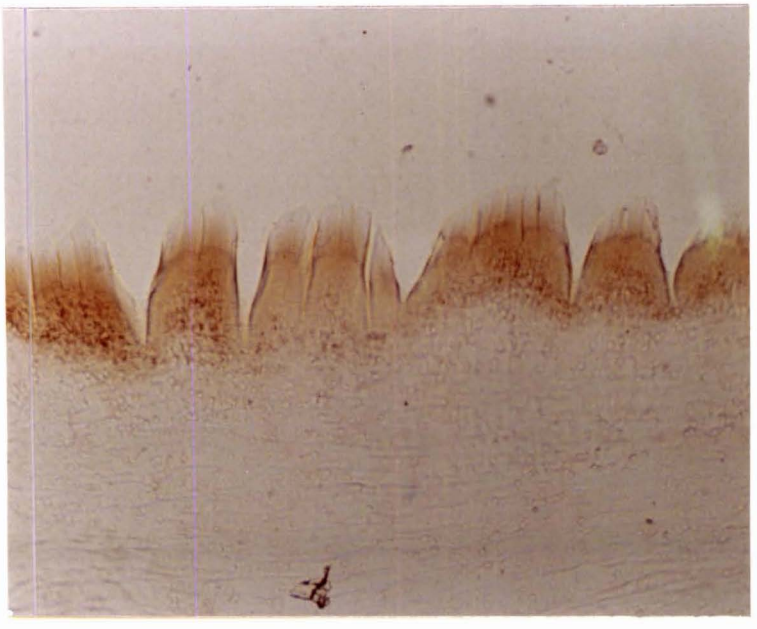


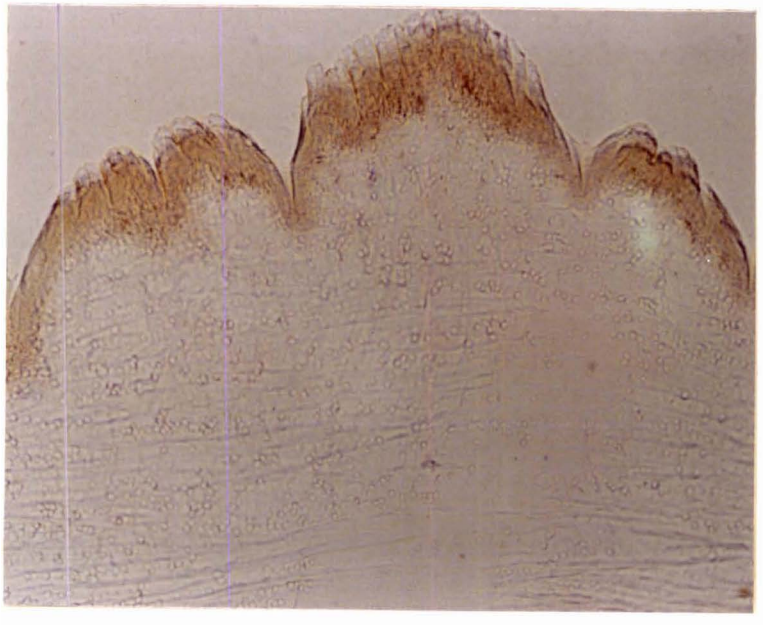
Fig 6.14 Showing sites of cholinesterase activity in frozen section of *T. hydatigena* using the Gerebtzoff method with butyrylthiocholine as substrate.

(A) Longitudinal section of mature segment (x 65). Incubation for 3½ hours.
(B) Transverse section of gravid segment (x 65). Incubation period for 2 hours.
(C) Longitudinal section (x 65). Incubation with eserine (10^{-5}M) for 3½ hours.
(D) Transverse section as for (C).
(E) Longitudinal section (x 65). Incubated with dichlorvos (10^{-3}M).
(F) Transverse section as for (E).
(G) Longitudinal section (X 65). Incubated with vincofos (10^{-3}M) and substrate for 3½ hours.
(H) Transverse section of gravid segment as for (G).



C

D



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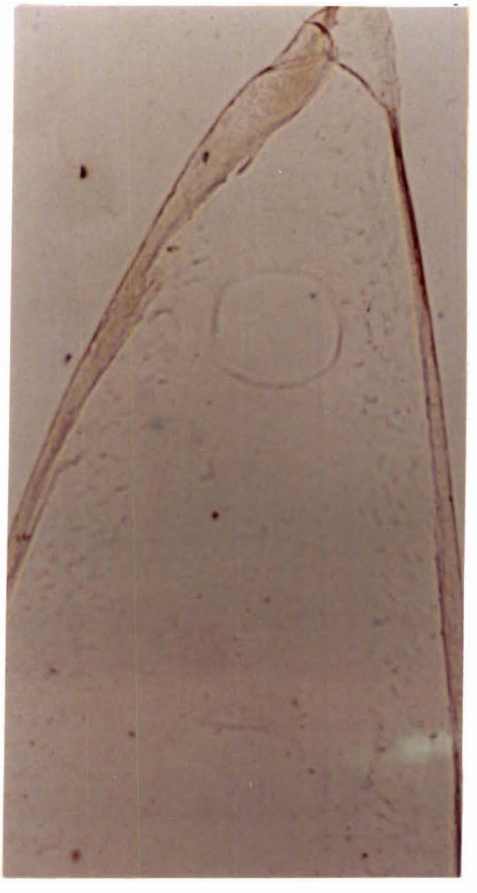

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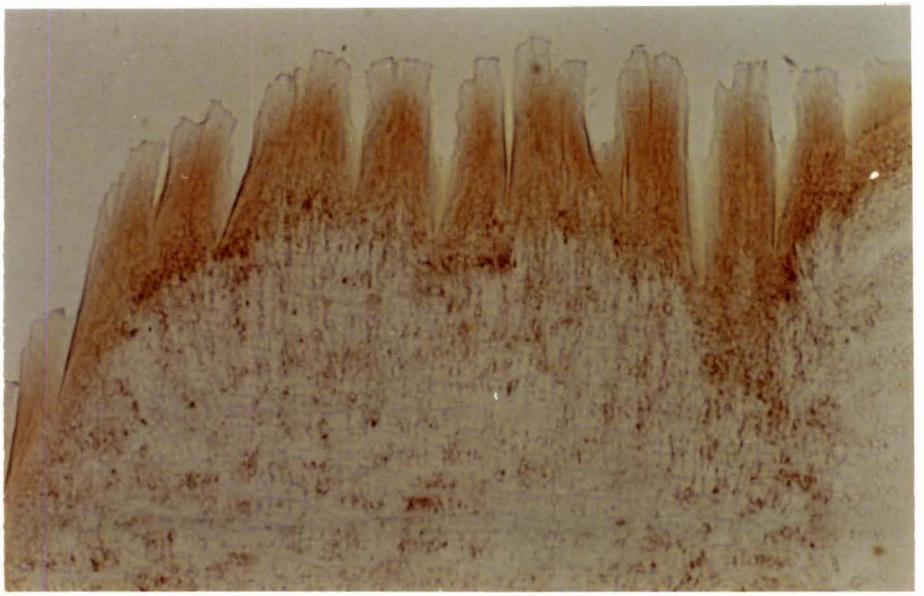
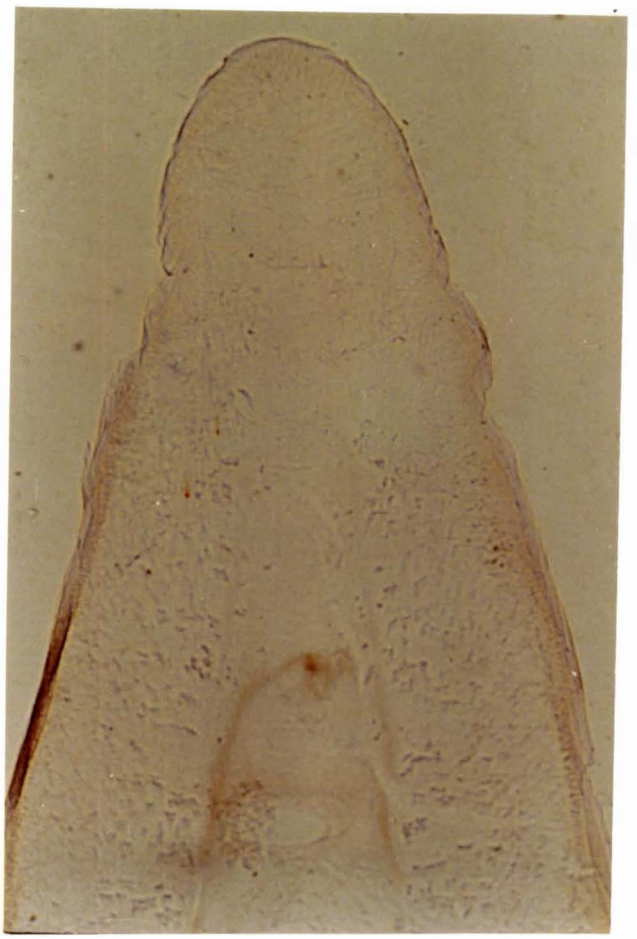


G



H

the frozen sections were preincubated with inhibitors for 30 minutes prior to incubation together with the substrate for further 3½ hours. The results for eserine are illustrated in Fig 6.14C,D; Dichlorvos, Fig 6.14E,F; and vincofos Fig 6.14G,H. It can be seen that with all the inhibitors there is almost complete inhibition of cholinesterase activity as far as the transverse sections are concerned. However the longitudinal section still appears to have the degree of activity in the subtegumental region and this may represent non-specific esterase activity.

6.4 DISCUSSION

6.4.1 Normal reactions

Although tapeworms are generally regarded as primitive creatures, histochemical studies reveal a complex nervous system.

The general layout of the nervous elements in the scoleces of *T. taeniaeformis*, *T. ovis* and *T. hydatigena* appeared to conform the general cyclophyllidiam pattern as described by Shield (1969). There are complex ramifications of the system. Commissures connect the two apical ganglia and the longitudinal nerve cords. There are efferent nerves to the suckers, the rostellum and the rostellar hooks and muscles and afferents supplied from the tegumental receptors proceeding to the central part of the nervous system. From the central area, longitudinal nerve trunks transverse the length of the strobila and in the body region the staining reveals relations between the longitudinal trunks and the circular trunks which number fifteen or more in each segment of *T. ovis*. Peripheral nerves supply the parenchymal organs and muscles. Afferent nerves arise from receptors in the tegument and subtegumental regions and travel to the nervous system. Moreover, there is a rich cholinesterase activity associated with the cirrus sac and the genital pore. Other sites of action include the uterine elements, excretory ducts and in the case of acetylcholinesterase only the tegument.

In view of the overall layout of the nervous system, it is appropriate to discuss the anatomical features in relation to function. It is

6.4.1

sometimes implied that the scolex (holdfast) functions as an anchoring organ whereby the worm attaches itself to the intestinal wall with the aid of its hooks and suckers and passively drapes the strobila back along the intestine. This might be visualised to be the situation with small worms (*Echinococcus)* where the bulk of the body may not be too disproportionate in relation to the size of the scolex. However, it could hardly be the situation with the large worms, particularly such as *T. saginata,* which may reach 10 metres in length and where body weight is massively disproportionate to the size of the scolex, which in turn may only measure a few millimetres in diameter. Yet it is known, that the functional integrity of the scolex is of fundamental significance in maintaining the worm *in situ.* As it would appear impossible for the scolex to anchor a passive mass of strobila, in the face of peristaltic movement, it seems highly likely that it acts rather as a co-ordinating centre, exerting its overall influence on the movement of the strobila in the face of peristalsis. In the scolex and in the strobila the nervous elements required for reflex arcs are present. *In vitro* as is well-known, the scolex will often maintain a searching and probing mission until it buries itself in available intestinal mucosa, at which time the movements will cease and the worm becomes quiescent. For this type of activity reflex arcs are obviously required. The animal must be able to respond to external stimuli and it would seem likely that the tegumental receptors function in response to touch and possibly pressure. The connections between receptors in the tegument of the scolex and the cerebral ganglia, are clearly defined, and between these structures and the suckers, rostellum and hooks and it is highly likely that the primitive searching and burrowing reflexes and co-ordination of hooks, rostellum and sucker movements, which achieve implantation, is brought about through the agency of reflex mechanisms. As cholinesterase is present in the nerve elements, these structures represent an obvious prime target for the influence of anthelmintic drugs, such as the anticholinesterases.

In addition to the concern of the cerebral structures in the affairs of the scolex itself, there are of course, abundant nerve connections

between the central structures and entire length of the strobila by virtue of the longitudinal nerve trunks. Nonetheless, muscular activity of individual proglottids is independent of central control and rhythmical movements, as evidenced by isolated segments, are quite independent of central connections. It must be emphasised that there is a rich nervous supply to the muscles and parenchyma within each segment as shown by the abundance of small nerves arising from the longitudinal and circular nerve trunks and the large numbers of circular nerves within each segment. Moreover, as far as *Moniezia* is concerned there is a complex nerve network. Observations on the spontaneous movements of isolated segments indicates the movements to be an alternation of longitudinal lengthening followed by transverse bunching which together can result in progression of the segment across a plane surface. This activity undoubtedly results from the alternative functioning of the circular and longitudinal muscles to cause respectively lengthening and then a thickening of the proglottid. del Castilla (1969) has given a dramatic description on the manner in which *Ascaris* coordinates its movements in order to brace itself against peristalsis and so maintain its position in the intestine. We believe that anatomical evidence is available to suggest that the tapeworm maintains its position by presenting coordinated waves of contraction and relaxation, probably with the active participation of both circular and longitudinal muscles, in the face of waves of peristalsis. Moreover, we suggest that the scolex acts as a central coordinating centre, which relays information concerning approaching peristaltic events to the remaining portion of the worm, so that it may prepare itself to withstand expulsion. In addition we believe reflex activity takes place in the body region in response to pressure exerted by the intestinal wall and we suggest that the overall response of the worm is to elongate, by constriction of circular muscle as the peristalsis wave passes over a region and expand the circumference of the worm by contraction of the longitudinal muscle as the peristaltic waves proceed. In this manner the musculature of the worm would be utilized to overcome propulsive forces of the intestine. It is obvious that precise muscular control would be required and we suggest that this is achieved through the integrated coordination of movement brought

about by the agency of abundant receptors, and the complex highly ramified nature of the nervous system.

The high degree of cholinesterase activity association with the cirrus, undoubtedly reflects the precise corpulatory function of that organ which is obviously reflex in character and almost certainly independent of central control.

The role of cholinesterase in the excretory duct and uterine elements is not known and it may possibly be related to muscular activity in those two organs. The presence of acetylcholinesterase in the tegument appears to be fairly well established although the enzyme does not appear to be present in all species of worm, nor is it necessarily demonstrated in all occasions when the worm is stained. It has also been suggested that the role of an enzyme in this position may be related to permeability control. But experiments, especially those using anticholinesterases to inhibit the enzyme, have been unable to confirm this observation. In the present study (Chapter 5) anticholinesterases did not influence glucose uptake.

Photographs at higher magnification, have demonstrated the presence of ganglion-like cells in the strobila, possibly receptors resembling stretch receptors of high animals. The presence of these structures support the overall picture of a complicated nervous system capable of much precise reflex activity.

From what has been said, it is obvious that the nervous system is of paramount significance to the well-being of the parasitic helminth and that the neural structures represent a prime target for drugs which may bring about disruption of movement and the consequent dislodgement of the worm.

6.4.2 Response to inhibitors

Experiments with inhibitors have demonstrated that eserine is a powerful inhibitor of both acetylcholinesterase and butyrylcholinesterase.

6.4.2

Inhibition occurs in both scolex region and in the strobila and cholinesterase activity in all organs and structures appears to be affected. The two organophosphorus compounds are much less powerful inhibitors giving a similar degree of inhibition at concentrations about a hundred times higher than that of eserine. The inhibitors are obviously more active for frozen sections rather than on whole mounts. This may be a reflection of the inability of the drugs to penetrate the tegument and other structures.

GENERAL DISCUSSION

The findings of the present studies have been discussed in some detail at the end of each chapter, and it is now necessary only to make some general comments.

A considerable volume of evidence (reviewed in Chapter 2) exists to suggest that almost all the modern anthelmintics (and many of the older drugs) act by interfering with motor activity of helminth parasites. This may be achieved through the drugs' action either to blockade or hyperactivate cholinergic receptor sites of the nicotinic type or by disruption of energy metabolism.

In essence it may be concluded the present work supports the contention that the normal turnover of acetylcholine is essential to helminth survival and that inhibition of the process is an important part of anthelmintic action. This conclusion arises from the observations that:

(a) The histochemical studies of cholinesterase (Chapter 6) reveal the presence of a well developed nervous system particularly well suited to maintain the helminth *in situ* by means of reflex arcs. Anticholinesterases would inhibit this enzyme and disrupt nervous function.

(b) High levels of cholinesterase were found chemically in many worms especially *Echinococcus* scoleces and brood capsules but also in other tapeworms notably, *T. ovis*. Levels in nematodes were considerably lower

(c) Centrifugal fractionation studies confirmed that the cholinesterase was distributed in several particulate as well as soluble forms which were similar in their response to Triton X-100 and cholinesterase inhibitors.

(d) A conventional anticholinesterase (eserine) and the anthelmintics tested, inhibited these cholinesterases in both chemical assays and histochemical studies.

(e) The pharmacodynamic studies in the dog and sheep indicate that methyridine, diethylcarbamazine, pyrantel, morantel, tetramisole and levamisole all possess nicotine-like properties and that the effects of these drugs in the sheep was to depress rather than stimulate respiration. This overall possession of nicotine-like properties has not been reported previously but it supports well

the findings that the drugs act as ganglion stimulants (levamisole and pyrantel) and neuromuscular blocking agents (methyridine) and points to the importance of cholinergic mechanisms in helminth neuromuscular activity. The work of Coles (1974) showing cross reactions between various drugs suggests a common receptor site.

With respect to disruption of energy metabolism, the present work is restricted to glucose uptake. It appears that in both *Ascaris* and tapeworms the glucose uptake mechanism are fairly conventional, in that they are sodium-dependant and inhibited by phlorizin, iodoacetate and dinitrophenol. However, there appears to be one important distinction in that nematode glucose transport is readily inhibited by low concentrations of local anaesthetics. In respect to the benzimidazoles the function to inhibit glucose uptake appears to reside in the carbamate moiety . In turn the carbamate structure is often associated with cholinergic and anticholinesterase activities (neostigmine and the carbamate insecticides). It might be postulated that acetylcholinesterase is functionally associated with glucose uptake although the present work with organophosphates indicates that this is not the case, at least with regard to tapeworms.

The development of new anthelmintics is progressing rapidly, and it appears that there are good opportunities for chemical manipulation to incorporate multiple functions in the drug molecule and so enable it to affect more than one biological mechanism. Another approach may be to use mixtures or combinations of anthelmintics, each of which is aimed at a separate target system in the helminth.

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