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Hymenolepis diminuta : MONOSACCHARIDE ABSORPTION BY THE CYSTICERCOID

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PhD Thesis

1985

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ABSTRACT

The cysticercoid of *Hymenolepis diminuta* was shown to absorb a limited number of monosaccharides by mediated transport. The monosaccharides transported were: glucose, galactose, mannose, 1-deoxyglucose, 2-deoxyglucose, 2-deoxygalactose, 3-O-methylglucose and α-methylglucoside. Other similar monosaccharides i.e. glucose analogues, were not absorbed and it was suggested that they do not possess the minimal structural requirements of actively absorbed compounds. Uptake studies on the recently excysted adult illustrated that it, too, absorbed the same limited range of monosaccharides as the cysticercoid.

Extensive inhibition studies were performed, which demonstrated that all of the above mentioned monosaccharides were competitive inhibitors. The K_i value calculated for each hexose was very similar to its own K_t value for the uptake locus. From these data it was concluded that all the hexoses transported were absorbed *via* the same locus in cysticercoids and recently excysted adults. Hexose absorption in all cases involved a negligible diffusion component and glucose and galactose uptake was also inhibited by phlorizin, several metabolic inhibitors, but not ouabain. The absorption of glucose and galactose by cysticercoids and recently excysted adults was predominantly Na^+-sensitive, although 70% of control uptake in cysticercoids and 30% in recently excysted adults took place in Na^+-free media. It was assumed that this Na^+-insensitive uptake was a function of the uptake locus and therefore probably also

occurs in the absorption of the other transported hexoses not tested. The possibilty of two monosaccharide uptake loci in the cysticercoid and recently excysted adult- one Na^+-sensitive, the other Na^+-insensitive- was discussed.

This study has highlighted differences in the uptake specificities and characteristics of the adult and cysticercoid monosaccharide absorption loci. The recently excysted adult was shown to posses 'intermediate' characteristics between those of the cysticercoid and 10-day-old adult worm. The differences between adult and cysticercoid monosaccharide uptake loci are thought to reflect both the difference in carbohydrate requirement of cysticercoids and adults and also the availability of absorbable carbohydrate in their environments.

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PREAMBLE

There is a wealth of literature on carbohydrate uptake in adult *Hymenolepis diminuta* and some information on adults and metacestodes of other species. However, there have been few such studies on the cysticercoid of *H. diminuta.* The reasons for this include the relative difficulty, compared with the adult worm, of producing standardised cysticercoids and, historically, an overwhelming interest shown by parasitologists in adult tapeworms. The adult and cysticercoid possess the same genome which expresses adult characteristics in the rat intestine and cysticercoid characteristics in the haemocoel of the intermediate host. Associated with the obvious differences in morphology between adult and cysticercoid, there are a number of differences in the biochemistry and physiology of the two stages. In this study, the differences in the functional physiology of the tegument and its carbohydrate uptake system between adult and cysticercoid have been investigated.

Chapters 1 and 2 are a review of aspects of the biology of *H.diminuta*. Chapter 1 contains an account of the life cycle of *H. diminuta* from egg to mature adult with comments on certain features of the biology of the various stages. The developmental morphology and composition of the cysticercoid is described with details of the factors affecting growth and maintenance *in vivo* and *in vitro*. Chapter 2 discusses the relationships between *H. diminuta* and the intermediate host.

A brief explanation of the various types of transport is provided in Chapter 3 with special reference to the Cestoda. The mechanisms and hypotheses of transport are discussed together with the kinetics and graphical transformations most frequently used to illustrate these processes. A brief discussion of the features of carbohydrate transport in *H. diminuta* is also included.

Chapter 4 contains information on general methodology, the reasons for a particular technique, the choice of salines, intermediate hosts etc. The results of the preliminary studies are given and the evolution of the final technique is described.

The uptake studies are the subject of Chapter 5, which discusses the results, and their relevance, of the experiments performed on the cysticercoid.

The effect of various inhibitors on carbohydrate transport is the subject of Chapter 6 and, from the results, a basic outline of the specificity of the carbohydrate transport system in the cysticercoid of *H. diminuta* is proposed.

Finally, a general discussion brings together the salient features of the carbohydrate transport systems of the cysticercoid of *H. diminuta* with the information already known and that acquired during this study.

CHAPTER 1

LIFE CYCLE AND MAINTENANCE OF *Hymenolepis diminuta*

A. ESTABLISHMENT OF ADULT *Hymenolepis diminuta*

Following the ingestion of a viable cysticercoid by a rat, a series of events occur, resulting in the liberation of the young adult worm. The cysticercoid first experiences a rise in temperature from approximately 26 to 37°C, followed by a drop in pH of the surrounding media, as it enters the rat stomach. The gastric juices have an approximate pH of 2, and it is here, in the presence of pepsin, that the outer protein layers of the cysticercoid surrounding the presumptive scolex are thought to be gradually digested (for cysticercoid structure see later). Older cysticercoids, having thicker walls, take longer to digest, but this does not significantly affect the overall timescale of the infection process (Goodchild and Harrison, 1961). As the partly digested cysticercoid passes into the duodenum it is exposed to pancreatic juice (where trypsin continues the protein digestion) and bile, at a pH of about 6.7. The precise action of bile on the cysticercoid is unknown, but it has been suggested to have three main effects: it alters the permeability of membranes, it initiates motility and it is thought to act synergistically with host digestive enzymes (Rothman,1959). The result is to activate the presumptive scolex which then begins to move up the 'anterior canal' in a series of very active wriggling movements. The young worm eventually frees itself from the cyst tissue and will then preferentially locate and attach in the first quarter of the small intestine. The entire excystment process can be

easily performed in vitro, where it is possible to excyst cysticercoids using bile and trypsin without a pepsin prime (Richards, K.S.,pers. comm.). In bile alone, some individuals excyst successfully, but the majority of presumptive scoleces are activated without being able to break out of the inner capsule. The excystment process takes between 20 - 40 min in vitro and approximately 3 - 4 h in vivo (Goodchild and Harrison,1961).

Once established in the small intestine, a number of factors affect the location, growth and maintenance of adult H. diminuta:

1. LOCATION

Current opinion suggests that the location of the worm is commensurate with its digestive processes and with those of its host (Ulmer,1971; Holmes,1973; Crompton,1973). The worm locates in a position offering ample concentrations of low molecular weight organic nutrients which it can absorb, and this optimum site is known to alter with the feeding regime of the host. The preferred site of a worm may vary according to the species of host, but it is defined as that location where worms exhibit maximum growth and fecundity (Mettrick and Dunkley,1969). There is a strong suggestion that H. diminuta actively selects its preferred site. Transplants of young adults or scoleces to different sites subsequently re-locate to the preferred site (Hopkins,1970). These data indicate the ability of the cestode to detect an adverse site, recognise the

direction of the preferred site and migrate to it.

Although the location of the preferred sites for *H. diminuta* is known, the factors which determine it are not.

The location of adult worms has been shown to be age-dependent. During the initial 24 h post-infection, the preferred location of adults changes from the first to the second quarter of the small intestine, with 68% located in the second quarter after five days (Goodchild and Harrison,1961). It was also noted that 4- and 5-day-old worms were generally clumped together in an area 25 - 28cm from the stomach. By 16 - 18 days post-infection, adults have moved to the uppermost fifth of the intestine, but as they increase in size and become patent they can be found as far as half way down the small intestine (Bråten and Hopkins,1969; Cannon and Mettrick,1970).

Superimposed on age-dependent site selection is the phenomenon of circadian migration. Reviews by Hopkins (1970), Bailey (1971), Mettrick (1971) and Mettrick and Podesta (1974) point to the general conclusion that adults move anteriorly and posteriorly in the intestine daily, apparently in response to host feeding regimes and/or nutritional gradients. In addition to the migration of worms, there is also daily contraction and extension of the parasite. Alteration of the feeding pattern of the host clearly affects this movement and has led to the conclusion that it occurs in response to exogenous stimuli.

Mettrick and Podesta (1974) have shown that circadian migration (together with the choice of long-term preferred site) is the response of the parasite to changing carbohydrate gradients in the intestine. Previous studies indicating a relationship between vagal activation of gastrointestinal secretion and a migrational response of adult
H. diminuta (Mettrick and Cho,1981a,1981b,1982; Cho and Mettrick, 1982) provided circumstantial evidence that a major factor in the worm response was the changing levels of luminal 5-hydroxytryptamine (5HT or serotonin). Following host feeding there was an increase in luminal 5HT levels with a concomitant increase in worm tissue 5HT levels and this was accompanied by an anterior migration of the worms. The strong inhibiting action of this normal pattern of migration by both methyltryptamine and, to a lesser extent, magnesium sulphate suggests that 5HT, rather than other possible endogenous secretions under vagal control, is directly involved in the worm migratory response (Mettrick and Cho,1982).
5-hydroxytryptamine is thought to act in the following ways: it is a neurotransmitter and therefore stimulates motility and it mobilises glycogen reserves thus providing the energy for migration. The gradients of 5HT in the lumen act as a guide to the direction of migration and, as a result, the worm also moves up the glucose gradient. Finally, it was suggested that 5HT is therefore an indirect stimulant of glucose transport (Mettrick and Cho,1981b). Another factor which appears important in the migratory response of H. diminuta is the presence of the strobila. Hopkins and Allen (1979) determined

this by removing the strobila of worms, 14 days after infection. Using single worm infections they found that on day 17 worms (or scoleces) were found in a more posterior position. They found that this posterior movement was relatively slow (taking about 48 h) and suggested that the strobila normally received 'adverse' stimuli from its environment which generated a signal to the scolex resulting in anteriad migration. However, the nature of the stimuli received by the strobila and the signal received by the scolex are unknown.

Other factors affecting the location of H. diminuta include high density of infection, which extends the preferred site, resulting in worms being located over a larger area. Circadian movement still occurs in heavy infections, but not in starved hosts (Mettrick and Cho,1981b). Also the immune response of the host may cause an alteration in site. Hindsbo, Andreassen and Hesselberg (1974) showed that if the immune response of a rat was strong enough to cause destrobilisation, the scoleces will move forwards into the duodenum from the jejunum.

2. INTRASPECIFIC COMPETITION

When present in high density, adult worms exhibit a 'crowding effect'. This describes an inverse relationship between parasite population density and parasite size and fecundity. It results in a decrease in weight of individuals, the number of proglottides shed per parasite per day, the number of eggs per proglottis and the mean daily egg output per

parasite (Read and Kilejian,1969; Bailey,1972; Read,1959; Roberts,1966,1983; Roberts and Mong,1968; Befus,1975). Goodchild and Harrison (1961) showed that this effect could not be demonstrated in large infections (100 +) of worms less than 5-days-old, but postulated that the worm might still be too small (average length = 14mm) to compete intensively for space and nutrients. It is apparent from the literature that the crowding effect is most obvious in the pre-patent period when, due to size and nutrient requirement, competition between parasites will be at its peak. However, although high densities produce stunted worms in laboratory rats, in the wild there is evidence to suggest that a small number of adults will develop normally while large numbers will be stunted (Avery, 1969; Wisniewski, Szymanik and Bazanska,1958).

The cause of the crowding effect is still unclear and is probably the result of a combination of factors. Information highlighting the similarity between cestodes in hosts maintained on sub-optimal diets, and those worms under the influence of crowding, has led to the suggestion of competition for available carbohydrate as a cause for reduced fitness (Roberts,1966; Roberts and Mong,1968). Keymer, Crompton and Singhvi (1983) reinforced the idea of mixed causality and provided data to show that the crowding effect cannot be explained simply in terms of inter-worm competition for carbohydrate. Other similarities between the effect of the immune response and that of crowding suggest an immunologically mediated cause (Befus,1975; Hesselberg and Andreassen,1975). Kennedy (1983) suggested that at high densities, immunological

factors may be more important in inducing the symptoms of crowding, while at medium densities competition may prove the more important. However, the number of possible factors involved in this effect could easily account for the variation in symptoms exhibited by individual cestodes in wild populations. In summary, intraspecific competition results in reduced fecundity and is, therefore, an important factor to be considered in the maintenance of adult H. diminuta.

3. INTERSPECIFIC COMPETITION

The rat small intestine is the habitat of several other adult cestodes, trematodes, acanthocephalans and nematodes, all of which compete for their preferred sites and nutrients. H. diminuta has been the subject of many studies involving interspecific competition, providing ample data. The acanthocephalan, Moniliformis dubius, when established in rats, will cause a subsequent infection of H. diminuta to 'prefer' a more posterior site than usual. Also, when H. diminuta is the established infection, a subsequent infection of M. dubius causes the tapeworms to move posteriorly (Holmes,1961,1962). Therefore, the presence of M. dubius prevents normal site location and attachment in H. diminuta. Concurrent infections of H. diminuta and M. dubius in rats produced retarded growth and decreased egg production in H. diminuta,as a result of the worm being prevented from occupying its 'normal' site (Holmes,1961,1962). The immune response induced by an infection of Trichinella spiralis (Nematoda) in mice caused loss of H. diminuta (Behnke, Bland and Wakelin,1977). The growth of H. diminuta in rats was also stunted in rats previously or

concurrently infected with T. spiralis, and this stunting became more pronounced when cestodes were administered closer to the time when the gut becomes inflamed and T. spiralis is expelled. This stunting of H. diminuta was also related to the density of T. spiralis infection (Christie,Wakelin and Wilson,1979), and higher nematode densities resulted in destrobilisation and reduced fecundity (Silver, Dick and Welch, 1980).

The effect of other species of Hymenolepis on site selection in H. diminuta is also of importance. In a study on the intestinal parasites of the shrew (Lewis,1966), five cyclophyllideans (including three species of Hymenolepis) all had a different preferred site, which was the same in the presence or absence of the other parasites. However, interaction between different parasite species can result in site displacement and sometimes in competitive exclusion. Hair and Holmes (1975), in their study on intestinal worms of the lesser scaup (a diving duck of Genus Aythya), found that out of thirty-three species of Hymenolepis, only two appeared to be influenced by interspecific interactions. The general implication therefore, is that cestodes do not compete with one another for a preferred site. Site dislocation is more common, however, between cestodes and non-cestode intestinal worms. The importance of interspecific competition in the wild is unknown but, as the overall effect is not beneficial, it is an important consideration in the maintenance of adult H. diminuta in the laboratory.

4. HOST DIET

The diet of the rat host has been shown to be important for the normal growth and reproduction of H. diminuta. In particular, it was noted that both the quantity and the quality of the food eaten by the host influenced the adult worm (Read,1959; Read and Rothman,1957; Read and Simmons,1963; Roberts,1966,1980; Dunkley and Mettrick,1969). H. diminuta is known to require carbohydrate for growth and reproduction, and this is probably also true for other cestodes. However, certain carbohydrates are less effective in maintaining growth when supplied as the only carbohydrate in the diet of the host. Dunkley and Mettrick (1969) found that cestode size, measured by weight, length and the number of proglottides, was under the influence of host dietary components. A variation in fecundity was also noted. A diet containing high concentrations (75%+) of starch proved to be one that produced worms with maximum fecundity. Glucose, sucrose and fructose in the diet of the host also produced 'normal' worms, but with a lower fecundity than obtained with starch. Rats fed a diet containing little or no carbohydrate, lost many of their already established worms, and those worms which were able to survive were stunted and unable to produce eggs. Also, those hosts receiving low starch or sucrose diets also produced stunted worms with fewer proglottides. A recent study by Keymer, Crompton and Singhvi (1983) showed that mannose, fed as the only carbohydrate to infected rats, produced the heaviest worms (approximately 46mg). In comparison, glucose, galactose and fructose produced worms averaging 18mg when fed as the sole carbohydrate.

In the above study, it was confirmed that H. diminuta was unable to metabolise fructose in vitro and that fructose as sole carbohydrate in the diet of the host did not support normal growth and reproduction (Read and Rothman,1957b; Keymer et al.,1983). The remaining three monosacharides (glucose, galactose and mannose) supported growth, but to varying degrees, and it was suggested that this was in relation to their respective affinities for transport loci on the intestinal mucosa of the rat. For example, glucose is taken up rapidly and actively by the rat (Herman,1974), while mannose is taken up slowly by a passive process, thus enabling H·diminuta to compete effectively for this carbohydrate. The effect of other dietary components on the growth and fecundity of H. diminuta is less well known. Vitamin deficient diets have reduced egg production in some instances and this also occurs when bile is absent or present in low amounts (Beck,1952; Roberts,1980). However, altering the lipid and protein content of the host diet appears to have no effect upon cestodes. Amino acids have a range of effects on H. diminuta growth when included or excluded from the hosts diet (Roberts,1980).

An important consideration when analysing results from experiments with varying host diet, is the concomitant variations induced within the rat hosts. Dunkley and Mettrick (1969) noted a wide range of worm sizes in different rats fed identical diets and suggested that this may reflect differences in host nutrition. Read and Rothman (1957a) also suggested

that observed differences in worm size in rats fed different carbohydrate diets, could be the indirect result of availability arising from the rapidity with which individual carbohydrates are removed from the intestinal lumen by the host. Alterations in the physical conditions of the gut may also occur, in response to altered diet and these may give rise to changes in gut bacterial flora.

In summary, therefore, unsuitable diets produced stunted adults and extremely unsuitable ones will result in destrobilisation. These effects are generally reversible, but immediately reduce egg production, prolong the generation time and therefore reduce the reproductive potential of the cestode. Host diet is therefore an important consideration in the maintenance of *H. diminuta* particularly in terms of carbohydrate quality.

B. STRUCTURE AND DEVELOPMENT OF *Hymenolepis diminuta* EGGS

A newly established adult worm, located at its preferred site, with access to ample nutrients and experiencing minimal competition, will grow rapidly, attaining a mean length of 14mm in five days (Goodchild and Harrison,1961; present study). In the present study, an average dry weight of 0.05g, with a strobila length of 15-17cm was recorded for 10-day-old worms in male Wistar rats (averaging 60g) containing infections of 30

worms. Under these conditions the pre-patent period varied from 13 - 15 days, after which gravid proglottides detached from the strobila and disintegrated within the intestine to release the eggs which were detected in the faeces. The eggs of H. diminuta are viable for up to 6 months in the faeces, and are infective immediately to a wide variety of 'natural' and 'artificial' intermediate hosts, including flour and grain beetles, fleas, cockroaches and locusts (Burt,1981). Although eggs are fully embryonated when voided in the faeces, they may have to survive for some time before being ingested by a suitable host, and the membranes surrounding the embryo may have a function related to this need. These membranes and capsule, as well as being protective, must also be subject to removal, either physical or physiological, when in the gut of the intermediate host, to allow the escape of the hexacanth.

Mature adult H. diminuta have been known to release 10,000 eggs per day (Hager,1941) for which an unusual somatic anatomy is required to meet the demands of this production of biomass. The serial repetition of reproductive potential is the solution adopted by many tapeworms to overcome the statistical improbability of completion of a complex life-cycle by one embryo. In H. diminuta the reproductive organs are surrounded by a parenchyma specialised for storage of lipid and carbohydrate, and this, in turn, is surrounded by the tegument which is specialised for absorption. The large part of the somatic tissue is syncytial, with relatively few different cell types. All these features obviously enhance the ability of exponential tissue growth, a vital aspect of the reproductive capacity.

The development and structure of H. diminuta eggs has been extensively studied by Rybicka (1972,1973), Voge and Berntzen (1961), Berntzen and Voge (1965), Lethbridge (1971a,b,1972,1976) and Moczon (1972). The egg consists of the embryo surrounded by a thin membrane and this, in turn, is surrounded by inner and outer envelopes. The outer envelope is thought to synthesise the tough protective egg-shell, while the inner envelope secretes the thick internal embryophore which protects the developing oncosphere. A diagram of the structure of H. diminuta egg is shown in Fig.1.1. As the egg develops in the gravid uterus of the adult, the outer envelope disintegrates to be replaced by the egg shell while another membrane, the oncospheral membrane, separates the oncosphere from the inner border of the embryophore (Rybicka,1972). The oncosphere itself develops concomitantly with the formation of the envelopes and becomes obvious with the presence of three pairs of keratinised hooks (Gallagher,1964; Pence,1967) and a pair oflarge penetration glands. These structures are subsequently used by the hatched oncosphere to penetrate through the intestinal tissues of the intermediate host.

The processes of egg hatching and penetration of the oncosphere of H. diminuta and other Hymenolepis species have been extensively studied and reviewed by Lethbridge (1971,1972) Holmes and Fairweather (1980) and Rothman (1959) and these provide the most complete in vivo and in vitro studies on

FIGURE 1.1
SCHEMATIC DIAGRAM OF THE STRUCTURE OF A MATURE *Hymenolepis diminuta* EGG.

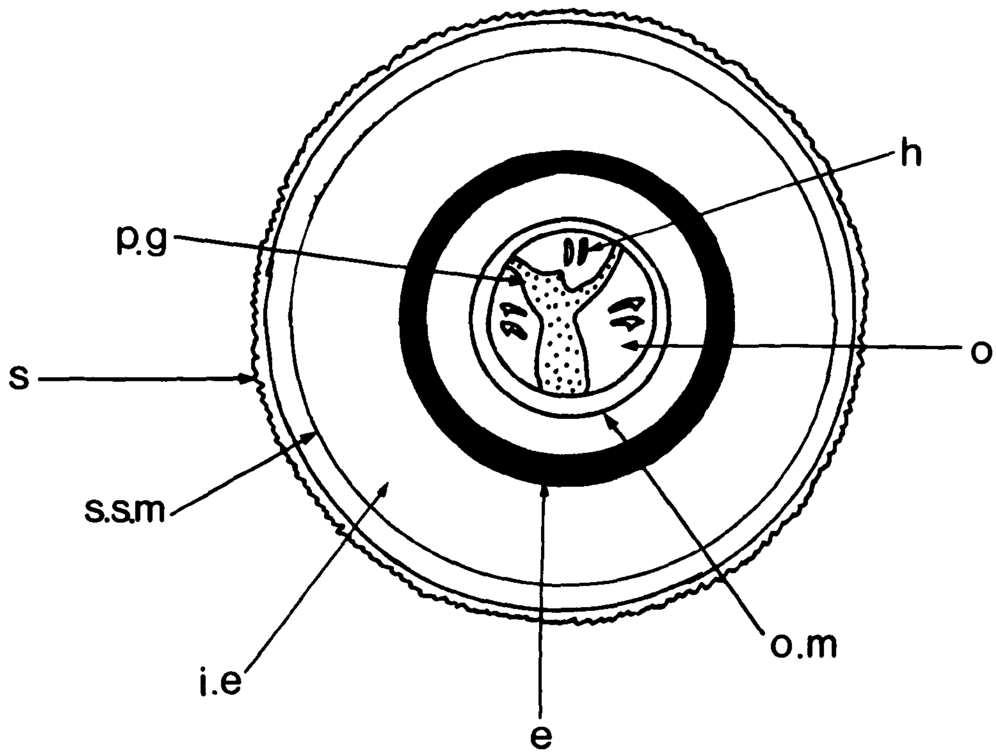
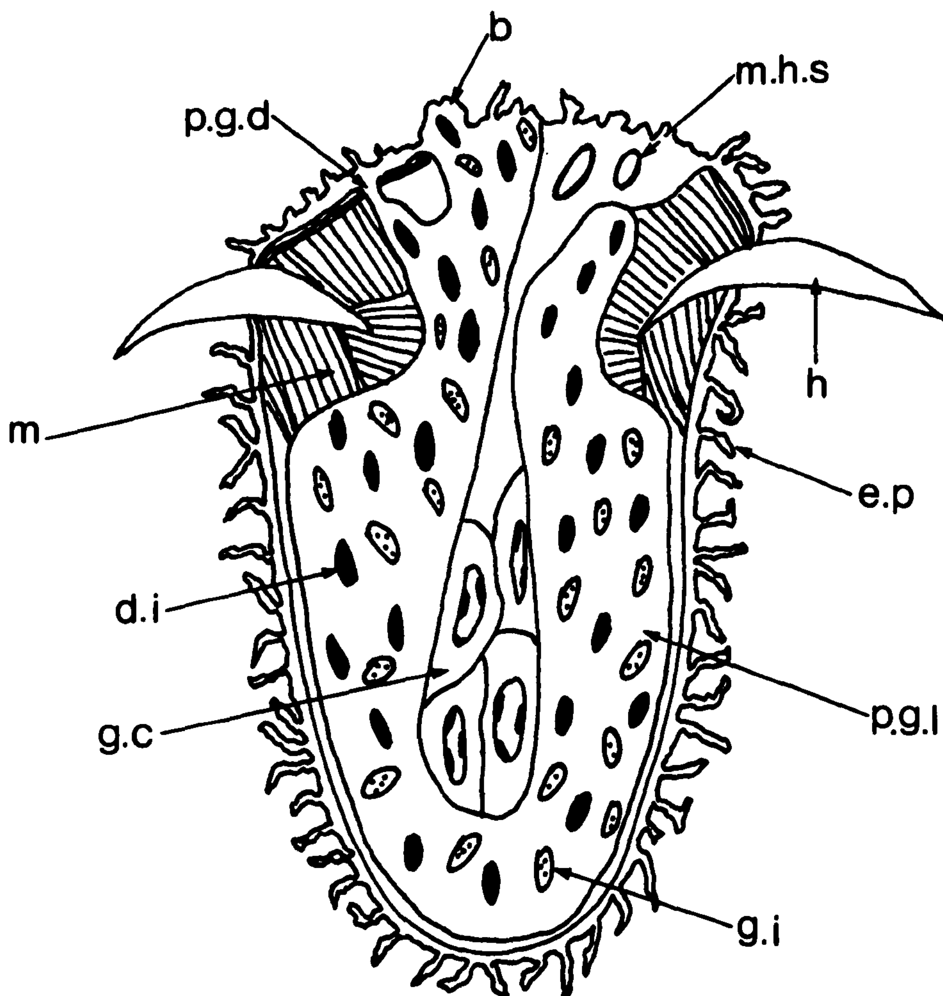


FIGURE 1.2
SCHEMATIC DIAGRAM OF THE INVASIVE ONCOSPHERE OF *Hymenolepis diminuta*.



N.B Diagrams not to scale

KEY TO FIGURES 1.1 AND 1.2

FIGURE 1.1

s = shell

s.s.m = sub-shell membrane

i.e = inner envelope

o.m = oncospheral membrane

e = embryophore

o = oncosphere

h = hook

p.g = penetration gland

FIGURE 1.2

m.h.s = median hook shaft

h = hook

e.p = epithelial process

p.g.l = penetration gland lobe

g.i = granular inclusions

g.c = germinal cells

d.i = dense inclusion

m = muscle

p.g.d = penetration gland duct

b = bleb: the contents of the penetration gland form blebs at the surface which increase in size and are eventually shed.

egg hatching in any tapeworm group. It is generally agreed that three events occur which precede hatching: the physical rupture of the egg shell, the activation of the oncosphere before its release from the remaining envelopes and the enzymatic destruction or weakening of the inner envelope and embryophore, to facilitate the escape of the oncosphere.

1. EGG-SHELL RUPTURE

Early workers on *H. diminuta* used a system of mirrors to observe the ingestion of *H. diminuta* eggs by an arthropod host, and they noted that mastication of ingested food resulted in fracture of the egg-shells (Reid, Allaman and Fitch,1951). This observation led to the successful development of an *in vitro* hatching system in which egg shells were mechanically ruptured and the remaining embryonic layers dispersed with digestive enzymes (Voge and Berntzen,1961; Berntzen and Voge,1965). Lethbridge (1971) showed that the egg shell was, in fact, unaffected by host digestive enzymes, and that eggs did not hatch in the midgut of adult or larval *Tenebrio molitor* unless the egg shell had been ruptured by the host mandibles. Intact eggs passed through the gut and were still viable in the faeces. There is therefore strong evidence that physical rupture of the egg shell is the first step in hatching that facilitates oncosphere activation and exposes the remaining embryonic envelopes to the digestive juices of the host. This

could restrict possible intermediate hosts to those with mouthparts capable of rupturing the egg-shell without injuring the oncosphere.

2. ONCOSPHERE ACTIVATION

It has been observed that oncospheres begin to move almost immediately after the egg shell has been broken (Voge and Berntzen,1961; Berntzen and Voge,1965). However, this activity may be the result of mechanical stimulation and it is the concomitant alteration in osmolarity of the medium surrounding the oncosphere that is thought to be the most important factor in inducing motility. Ogren, Ogren and Skarnulis (1969) showed that H. diminuta oncospheres became active within 1 min of being "deshelled" in distilled water, but motility was delayed for up to 8 min in 0.3M NaCl solution.

The physiological basis for activation has not been determined, but in H. diminuta it is clear that neither bile salts nor organic additives are required to initiate motility.

3. LIBERATION OF THE ONCOSPHERE

As soon as the egg-shell has been ruptured, an immediate and obvious swelling of the inner envelope occurs. Once the oncosphere has been deshelled, the inner envelope and embryophore become more transparent (when observed under the light microscope) as they are gradually digested. Lethbridge

(1971) used starved Tenebrio molitor which he allowed to feed on proglottides for 1 min. After this time they were immediately dissected and the gut and its contents placed, undiluted, onto a microscope slide for examination. He found that the liberation of the oncosphere took approximately 20 min, with digestion of the inner envelope taking 4 - 6 min, and the embryophore up to 15 min (Lethbridge,1971). Experimenting with dead eggs, Lethbridge also concluded that this process was passive - not aided by the oncosphere - as it followed the same sequence and time period as with living eggs (Lethbridge,1980). Some information on the factors essential for liberation has been gathered with in vitro experiments employing insect extracts, although results have been variable. Voge and Berntzen (1961) dissolved the inner envelope with extract of whole Dermestes vulpinus, but extracts of whole Tenebrio molitor were without effect. Lethbridge (1972) found that T. molitor extract, prepared from excised midguts, rapidly digested both the inner envelope and the embryophore and, by varying the concentration or pH of the extract, it was possible to modify the rate of removal of these structures. Lethbridge (1972) also showed that T. molitor gut extract separated into two proteolytic components when subject to electrophoresis, and that both components were able to dissolve the envelopes. In a study on four Hymenolepis species, in a variety of media, Berntzen and Voge (1965) showed that trypsin was essential for hatching in all species except H. microstoma, and H. nana also required the addition of sodium bicarbonate (a 1M solution used to adjust the pH from 3 to approximately 7.5) and a 95% N_2; 5% CO_2 gas phase. H. diminuta, appeared to require amylase

as well as trypsin for optimal hatching, but this has been disputed by Lethbridge (1972), who suggested contaminating trypsin and chymotrypsin in the amylase was sufficient to induce hatching. At present, it is thought that mechanical rupture of the egg-shell by the intermediate host mouthparts, followed by dissolution of the membranes surrounding the oncosphere by host enzymes and induced oncosphere motility, is the sequence of events which occurs in the in vivo hatching process.

A degree of host specificity could theoretically be related to hatching. If an arthropod is unable to weaken and disrupt the membranes surrounding the oncosphere, due to lack of appropriate enzymes, then it could not serve as an intermediate host. However, there is no evidence to suggest this, and insects that possess biting mouthparts are generally equipped with a wide complement of digestive enzymes (House, 1974). Lethbridge (1980) also pointed out that, as the actual stimulation of hatching in hymenolepids is unclear, and seems imprecise, it is unlikely to be a very significant factor in host specificity.

4. PENETRATION OF THE INTERMEDIATE HOST GUT

The ingested H. diminuta egg, as described above, hatches in the intermediate host gut, and the oncosphere has now to penetrate host tissues to gain access to the site where it will develop into the cysticercoid - the haemocoel. As a result of detailed studies by Rybicka (1966) and studies on the oncospheres of other species (Collin,1968,1969:

Lethbridge and Gijsbers,1974) a clear picture of the structure of oncospheres is available (Fig.1.2). A thin epithelium overlies the general musculature and the complex system of muscles used to operate the 3 pairs of hooks. A pair of large penetration glands open into the surface syncytium and a small core of cells, flanked by the penetration gland lobes, are thought to be involved in the subsequent development of the cysticercoid.

The motility of the newly-hatched oncosphere is characterised by a pattern of hook movements that result from the antagonistic interaction of the somatic and hook muscles. Although no motor neurones or similar structures have been detected in any species, acetylcholinesterase has been detected in H. diminuta oncospheres, but no discrete staining pattern was found to suggest specific nervous pathways. The pattern of hook movement is a very characteristic rowing movement, which is continually repeated. This movement is particularly suited to the formation of initial attachments of the oncosphere to the intestinal epithelium (Miyazato, Furukawa, Inoue, Niwa, Inoue and Shimoda,1977), and also for burrowing. The rate of hook movement has also been shown to increase with a rise in temperature, and the energy for this continuous vigorous movement comes from the large glycogen reserves of the oncospheres, known to be present before penetration (Rybicka,1966). Anderson and Lethbridge (1975) investigated

the influence of external carbohydrate on movement in *H.diminuta* oncospheres. They found that body movement was independent of the level of glucose in the surrounding medium and concluded that oncospheres relied solely on their endogenous reserves. After approximately 2 h, hook movements became irregular, and after 4 h movement ceased reflecting, in their view, a state of total depletion of energy reserves. It was concluded that penetration must therefore occur before the energy reserves of the parasite were depleted.

In addition to the use of hooks in penetration, the penetration 'glands' are thought to play an active role. These U-shaped glands have canals opening into the surface syncytium between the median and lateral hooks on each side of the body. The secretion appears to be aided by the vigorous muscular contractions of the oncosphere during burrowing. Lethbridge and Gijsbers (1974) used the neutral red affinity of the penetration gland to determine the rate of expulsion of their product from oncospheres of *H. diminuta*. After incubating oncospheres in saline for nearly 2½ h, there was a dramatic reduction in the area of stainable materials in the gland, and this was equivalent to that observed in oncospheres that had penetrated the midgut of adult *Tenebrio molitor* in the same time. The nature of the secretion released by the penetration glands remains unknown although they are PAS positive. Several possible functions have been suggested, including facilitation of the adhesion of the oncosphere to the intestinal mucosa (Pence,1970; Miyazato,*et al*.,1977), lubrication of its passage

through the tissues (Lethbridge and Gijsbers,1974) and/or protection of the oncosphere from the digestive enzymes of the intermediate host (Moczon, 1977a). The secretions may also have a lytic function, a view supported by several workers (Silverman and Maneely,1955; Lethbridge,1971a Heath,1971) on the basis of histological changes observed in the tissues adjacent to the migrating oncosphere. Miyazato et al.(1977), working on Hymenolepis nana, noted that, although the oncosphere hooks ruptured the epithelial cells (which subsequently disintegrated), some pathological changes that might have been the result of penetration gland secretion, were noted in these cells prior to their rupture.

Another important factor in oncosphere penetration is the structure of the intermediate host gut wall. Voge and Graiwer (1964) showed that, although eggs hatched in the midguts of both adult and larval Tenebrio molitor, they seemed unable to penetrate the gut wall of larvae, while oncospheres injected directly into the haemocoel of larvae developed normally. These authors suggested that failure to penetrate was due to the thick peritrophic membrane that lines the gut, and the closely packed epithelial cells. Lethbridge (1971) found that oncospheres could penetrate the peritrophic membrane, but failed to penetrate a continuous muscle layer found in the larvae. In adult T. molitor, this muscle layer has regular areas of thinner muscle through which the oncospheres can successfully migrate. Lethbridge (1971) also showed that H.diminuta oncospheres injected into the haemocoel of locusts developed normally, but that they were usually unable to

penetrate the thick gut wall of the insect. Thus, host gut structure has a role in restricting the range of possible intermediate hosts.

The vigorous action of the oncospheral hooks, and the possible aid of penetration gland secretions, allows the oncosphere to migrate rapidly through the intestinal tissues and into the haemocoel. This process takes from 30 - 120 min in H. diminuta (Lethbridge,1971a) and the rate is thought to be directly dependent upon the available energy reserves. The oncosphere, therefore, has a maximum of 3 h to penetrate, after which time there is insufficient energy for further movement, and the oncosphere dies.

Once in the haemocoel, the oncosphere is bathed in the haemolymph - a rich organic fluid plasma in which nucleated cells (haemocytes) are suspended. This medium provides a suitable environment for the subsequent metamorphosis of the oncosphere to the cysticercoid. A more detailed study of the environment of the cysticercoid and the pathophysiology of infection in Tenebrio molitor (the intermediate host used in this study) is given in Chapter 2.

The oncosphere undergoes a metamorphosis which involves the redifferentiation of a number of cells and structures. It is accompanied by an increase in size, with most of the growth resulting from division of only a few precursor cells (Freeman, 1973; Ogren,1968; Ubelaker,1983). The majority of tissues are reabsorbed during metamorphosis, but the hooks and tegument are

not. The functional anterior of the oncosphere (i.e. where the hooks are located) becomes the functional posterior end of the cysticercoid, and metamorphosis is considered complete when the scolex and neck region of the presumptive adult are formed and can be excysted.

C. DEVELOPMENT OF THE CYSTICERCOID

There have been many studies of the morphology and development of cysticercoids and of particular interest to students of H. diminuta are those of:
Ubelaker (1980), Rybicka (1973), Cooper, Allison and Ubelaker (1975), Allison, Ubelaker and Cooper (1972) and Ubelaker, Cooper and Allison (1970).

It is generally agreed that metacestode development can be divided into two types; 'primitive', which is charcterised by completion of development without the formation of an initial internal cavity and 'neoteric', characterised by the formation of an initial internal cavity or primitive lacuna. H. diminuta belongs to the latter group and its development follows 'pattern one' as defined by Ubelaker (1983). Pattern one describes the condition where the primitive lacuna is incorporated into the cysticercoid and provides a region for withdrawal of an exogenously developed scolex (pattern two represents those scoleces which develop within the primitive lacuna, Ubelaker,1983).

The following section describes aspects of the morphology,

development and physiology of the cysticercoid of H. diminuta necessary for an understanding of the experimental work and analysis of the results.

Development of the cysticercoid, although continuous, is usually divided for descriptive purposes into stages with respect to time at a specified temperature. The classification of Voge and Heyneman (1957), based on light microscope observations, is most often taken as reference and will be used in this work. More recently, several electron microscope studies on the morphology of H. diminuta cysticercoids have revealed further details of structure (Allison et al.,1972; Ubelaker et al.,1970; Cooper et al.,1975; Richards and Arme,1984a,1984b; Lumsden, Voge and Sogandares-Bergal,1982).

Stage 1 is identifiable 48 h after successful penetration of the oncosphere (at 28°C), when the cysticercoid appears as an ovoid body c.100μm in diameter, and with a few circular muscles. It possesses a rudimentary syncytial tegument, connected to an underlying nucleated cytoplasmic layer by connections through the musculature. This basic arrangement of the surface syncytium formed in the oncosphere remains with few alterations both in the cysticercoid and in the adult.

Stage 2 is characterised by the appearance of a cavity, the primitive lacuna, at about 72 h, and this increases the overall size of the cysticercoid. This cavity is towards the posterior of the cysticercoid while rapid growth is occurring at the anterior end resulting in the beginnings of scolex formation.

Stage 3 - The majority of the cell types remain undifferentiated until about 4 days after infection, when rapid cellular proliferation is observed resulting in further growth and elongation of the cysticercoid. This stage is, perhaps, the most active stage in terms of growth, with elongation of the primitive lacuna and the clear demarcation of the body into three regions; the scolex, mid-body and cercomer. The primitive lacuna is surrounded by a continuous sheath of epithelial cells and both circular and longitudinal muscles are present in the outer regions of the cysticercoid. There is further differentiation of the scolex and neck, with early development of the suckers and rostellum. Late stage 3 usually exhibits signs of imminent withdrawal, with a concentration of contractile fibres anterior to the lacuna and posterior to the scolex.

Stage 4 is characterised by withdrawl of the scolex, which occurs rapidly about 6 - 7 days after infection. In metacestodes of Hymenolepis microstoma, stage 4 is completed in less than two min (Caley,1974) and is accompanied by rhythmical muscular contractions which extend from the scolex to the posterior end of the cysticercoid. The presumptive scolex, bearing microtriches, is withdrawn into the space previously occupied by the primitive lacuna, which is compressed up and around the scolex and becomes cup-shaped.

Finally, Stage 5 represents 'maturation' with the completion of the microthrix border on the presumptive scolex. The scolex then remains without further development until it excysts in the rat intestine. The cysticercoid continues to grow in size after the onset of stage 5, mainly as a result of the deposition of collagen-like material in the mid-body region.

D. FACTORS AFFECTING DEVELOPMENT OF THE CYSTICERCOID

1. TEMPERATURE

Temperature plays an important role in the growth and development of cysticercoids (Voge,1958; Voge and Heyneman, 1957,1958; Voge and Turner,1956; Parmeter,1972). The original work of Voge and Turner (1956), on *H. diminuta* infections in *Tribolium confusum*, established the importance of developmental temperature. It was found that infective cysticercoids could develop normally between 15-31°C, with abnormal development and reduction in body size a common occurrence at> 31°C. Cercomer length was shown to vary remarkably with temperature, sometimes up to 2mm at 20°C, but no more than 0.5mm, or absent altogether, at 31°C. These authors came to the conclusion that cysticercoids grown at 31°C exhibited incomplete development and/or lack of the outermost membranes, with a marked reduction in cercomer size. It was also shown that development to stage 5 did not coincide with infectivity, with a further two days needed before successful excystment at

$31^{o}C$. Normal growth ceased to occur at $38.5^{o}C$, the upper temperature limit, and the cysticercoid was shown to be most sensitive to temperature between 2 - 6 (especially 3 - 5) days post-infection i.e. stage 3 - 5, at $26^{o}C$, the period of most active growth. Parmeter (1972), working on H.diminuta in Tribolium confusum, found that high temperatures experienced by cysticercoids affected their subsequent fitness as adults. Some cysticercoids exposed to temperatures of $37\text{-}40^{o}C$ for a week (after initial growth at lower temperatures), developed into adults without gross morphological abnormalities, but showing reduced numbers of gravid proglottides and a decrease in net weight. Parmeter also studied the development of cysticercoids in Tribolium confusum maintained at three different temperatures ($30^{o}C$, $34^{o}C$ & $38^{o}C$) and four different humidities (RH 0, 30, 70 & 90%). Low humidities (0 & 30%) reduced the temperature thresholds at which structural abnormalities occurred, but otherwise cysticercoids developed as expected. Exposure of beetles to temperatures of $4^{o}C$ and $10^{o}C$ had no effect on mature cysticercoids, but arrested the growth of immature ones, and freezing stage 5 cysticercoids in vitro (in Krebs Ringer - Tris saline) for short periods (< 10 min) had no effect on their ability to excyst and infect the definitive host (present study). An interesting finding was that cysticercoids from hosts fed diets of pure sugar during the total period of cysticercoid development, showed a reduction in abnormalities when under high temperature stress. The sugar was presented to the intermediate host Tribolium confusum both in aqueous and dry form, with the larger decrease in abnormalities associated with

a diet of aqueous sugar (Voge,1959). Generally, however, the effect of high temperature stress on the cells and tissues of the cysticercoid resulted in delayed development, asymmetrical growth and gross abnormalities. Not surprisingly, the inner capsule and its contents appeared the most resistant to temperature stress. This is the presumptive scolex of the adult, the structure for which the cysticercoid exists in order to convey it to the definitive host. Although there is little work on low temperature stress, the main effect appears to be arrested growth in immature cysticercoids. Thus, the cysticercoid of H. diminuta grows normally, but at varying rates, over an approximate range of 15 - 35°C - the likely extremes of temperature experienced by the intermediate host in the wild.

2. DENSITY OF INFECTION

The number of cysticercoids per beetle has been shown to be related to the extent of host feeding, and this in turn is related to the ambient temperature (Dunkley and Mettrick,1971, working with Tribolium confusum). Most workers routinely starve the intermediate host for several days before infecting, and this has the effect of increasing the number of cysticercoids per beetle, as a direct result of increased feeding due to hunger. However, Dunkley and Mettrick (1971) found that higher than average burdens were obtained when they increased the ambient temperature to between 20 - 35°C, increased the period of starvation (up to 8 days) and infected beetles between 0 and 15 days old.The density of infection is

likely to affect the development of the cysticercoid in a number of ways: a. there could be significant intraspecific competition for available nutrients and preferred sites. b. high densities may affect the normal growth and development of cysticercoids resulting in the occurrence of abnormalities and loss of viability and c. heavy infections could seriously affect the mortality and fecundity of the intermediate host, thus reducing the likelihood of successful completion of the life cycle.

In heavily parasitised individuals, the effect of crowding results in smaller cysticercoids with shorter 'tails' - probably the result of reduced nutrient availability. Cysticercoids normally locate on the haemocoel side of the mid-gut, i.e. the penetrating oncosphere, once in the haemocoel, does not appear to move far from the point of entry. However, in heavy infections, cysticercoids have been found in the thorax region of the haemocoel and throughout the abdominal region. Macdonald and Wilson (1964) found that the size of cysticercoids in Tribolium confusum was dependent upon the site of their development (irrespective of density of infection) with cysticercoids developing in the pro-thorax being smaller than those from the abdomen. However, as cysticercoids are located in a circulating nutrient-rich medium, location is probably not of major importance. Location in the gut is likely to affect hatched oncospheres, however, since they may find themselves having to penetrate through the thicker intestinal tissue of the hind-gut, having been swept through the mid-gut in a reduced time due to increased ingestion of

food. As with temperature stress, the effect of a high density of infection is to increase the proportion of abnormal cysticercoids present (Dunkley and Mettrick,1971), but these workers found that viability of the normal but smaller cysticercoids was not affected by density of infection. The most common abnormality in this study appeared to be arrested growth of cysticercoids at stage 2 or earlier as proportionately large numbers of these were found in heavy infections. 'Heavy infections' were defined by Dunkley and Mettrick as 20+ in Tribolium confusum and infections of 200-250+ in Tenebrio molitor.

The work of Keymer (1980,1982)and Keymer and Anderson (1979) explored the effects of the density of infection upon the intermediate host (Tribolium confusum) in detail, and is discussed in Chapter 2.

Interspecific competition in the development of H. diminuta cysticercoids is therefore an area of study that awaits further investigation.

3. THE EFFECT OF AGE AND SEX OF THE INTERMEDIATE HOST

The age of an intermediate host could affect its subsequent infection with H. diminuta in several ways. It is well known that, when infecting beetles in the laboratory, younger beetles are easier to infect and harbour larger infections (Dunkley and Mettrick,1971). The reasons for this may include reduced feeding in older beetles, a thicker gut wall, causing reduced penetration of oncospheres, and possible

age-related physiological changes in the host. For example, the composition of haemolymph may alter with age of beetle and become less favourable for cysticercoid development, but a host immune response appears not to be important (see Chapter 2). The age of the intermediate host (Tribolium confusum) was shown by Kelly, O'Brien and Katz (1967) to affect the incidence and size of the cysticercoids. Young female beetles contained more and larger cysticercoids than older beetles, whereas males had the greater burden (and larger cysticercoids) during mid-life. Females less than 5 weeks old, and males more than 23 weeks old, had the heaviest infections. The sex of the intermediate host also affects development of the cysticercoids. Possible effects include a higher infectivity in one sex over the other, differences in haemolymph composition with sex, producing differential development, and a possible ecological advantage of one sex over the other in terms of maximum age attained. In the majority of the literature the effect of both age and sex are investigated together. Soltice, Arai and Scheinberg (1971) reported the apparently faster development of cysticercoids in female beetles. Haemolymph composition is known to differ considerably between male and female Tenebrio molitor, largely as a result of egg production. There are several significant differences in haemolymph amino acids and protein concentrations between the sexes and these differences, together with those brought about by infection, are discussed in Chapter 2 (Hurd and Arme,1984a,b).

Mankau (1977) investigated sex as a factor influencing the infection of various *Tribolium* species with *H. diminuta*. She used *T. confusum*, *T. castaneum* and *T. brevicornis* and infected both males and females to determine the effect of host sex, and the degree of host specificity. The results indicated that the females of *T. confusum* and *T. castaneum* had higher infection rates and larger numbers of cysticercoids than the corresponding males. No difference was found between males and females in *T. brevicornis* although overall (in all three species) the percentage of females infected, compared to males, was significantly greater. Host species and strain has not been thoroughly investigated as a factor in cysticercoid development, but any differences resulting in altered physiology and haemolymph composition are likely to affect cysticercoids. Rau (1979) investigated the frequency distribution of cysticercoids in natural populations of *Tenebrio obscurus* and *T. molitor*, and found that in both species and both sexes it conformed to a negative binomial. However, there were more cysticercoids and a higher mean intensity of infection in *T. obscurus* than in *T. molitor*. There was no difference in intensity of infection between the sexes, but larvae were always found to be more lightly infected than adults.(for reasons stated earlier).

E. CYSTICERCOID GROWTH REQUIREMENTS

The nutrient-rich, stable environment of the intermediate host haemocoel renders it an ideal habitat for growth and development of the cysticercoid. The nutritional requirements of the cysticercoid during growth must be met by utilisation of host haemolymph constituents, but what are these requirements? Several studies on the absorption of low molecular weight organic nutrients across the tegument of the cysticercoid have clearly illustrated the presence of specific membrane transport systems, with a range of amino acids and sugars being absorbed (Arme, Middleton and Scott,1973; Arme and Coates,1971; Jeffs,1984; Jeffs and Arme,1984,1985a,b; this study).

Uptake studies are discussed in detail in Chapter 5. However, the development of a successful *in vitro* culture technique has provided valuable data concerning the nutritional requirements of the cysticercoid.

1. In vitro-CULTURE OF CYSTICERCOIDS

Initial attempts at *in vitro* cultivation of adult *H. diminuta* by Schiller, Read and Rothman (1959), were very successful. By 1961, Berntzen had succeeded in growing mature adult worms from cysticercoids *in vitro* and later Schiller (1965) simplified the method of adult *in vitro* cultivation. It was Graham and Berntzen (1970) who first established a method of monoxenic cultivation of *H. diminuta* cysticercoids with rat fibroblasts. By inoculating a monolayer fibroblast culture

(from 18-day-old rat embryos) with oncospheres, adding various media, altering the gas phase and incubating at 30°C, they successfully grew cysticercoids and established those conditions that produced maximum growth. They also found that a glucose concentration in the medium of up to 3mg/ml accelerated the rate of cysticercoid maturation, but concentrations of 4 and 5 mg/ml inhibited growth and maturation. Cultures required to be maintained at a pH of 7 - 7.4 and there appeared to be no correlation between the degree of maturation and the percentage of O_2 or N_2 in the gas phase; CO_2 however, was essential at a minimum concentration of 10%. Although the cysticercoids exhibited the same pattern of development as those _in vivo_ (Voge and Heyneman,1957), growth was much slower, parasites taking nearly four times as long to reach maturation. An interesting observation was that the presence of eggs, or egg shell debris, inhibited cysticercoid growth and maturation, and the extent of inhibition was directly related to the amount of shell debris in the culture (Graham and Berntzen,1970). However, Voge (1975) succeeded in culturing _H. diminuta_ cysticercoids axenically, having first been successful with _H. citelli_ cysticercoids (Voge and Green,1975). Oncospheres of _H.diminuta,_ hatched _in vitro_, were grown to fully developed metacestodes infective to rats. It was found that the requirements for _in vitro_ cultivation of _H. diminuta_ differed from those for _H. citelli_, with respect to optimum temperature and concentration of L-cysteine in the growth medium (with _H. diminuta_ needing less L-cysteine). The optimum temperature for culture was 28°C, with 25°C apparently inhibiting

growth and giving rise to structural abnormalities. These abnormalities are a common feature of _in vitro_ culture of all hymenolepid cysticercoids to date, although the reasons for them are unknown. Similarly, individuals within a culture do not develop in synchrony with many different developmental stages present in the same culture flask. Originally, the gas phase was air, although Voge and Green (1975) investigated the effect of different gas phases on culture in more detail. In a study on the synergistic growth-promoting effect of L-cysteine and nitrogen on cysticercoid growth _in vitro_, they concluded that "it was not the level of CO_2, but rather the excessive amount of O_2 which is crticial and which delays development". Thus a gas phase of 100% N_2 or 95% N_2/ 5% CO_2 or 95% N_2/5% O_2 produced a marked increase in speed of development (when L-cysteine is present) when compared with controls grown under air. A modified Landreau's medium was used for cultivating cysticercoids (originally developed for culturing cockroach cells) with a total of 16 amino acids, 6 salts, 2 sugars (glucose and trehalose), 5 organic acids, yeast extract, lactalbumen hydrolysate and non-activated foetal calf serum (with antibiotics). Growth of cysticercoids proceeded as expected at 28°C, with stage 5 cysticercoids obvious at day 14. However, several additional days were needed, after scolex withdrawal, before the cysticercoids were infective to rats. Voge (1975) also noted the motility of the various growth stages of cysticercoids. Slow contractions along the neck and mid-body of pre-withdrawal stages provided considerable changes in shape, as well as temporary folds in the body wall. Withdrawn cysticercoids also moved with slow wave-like

contractions whenever the culture medium was disturbed. However, Voge stated that this movement had not been previously noted in cysticercoids dissected from beetles, probably because of the sudden transfer to a completely different environment. In this study slow but positive movement of cysticercoids dissected from beetles was noted throughout, even when in saline for periods of 2 h or more.

Therefore, it is now possible to complete the life cycle of H. diminuta in vitro, and current studies on the composition of the cysticercoid environment in vivo, together with the effects of the parasite on its environment, may produce a clearer picture of the requirements of this organism (Hurd and Arme,1984a,b).

F. SOME BIOCHEMICAL PROPERTIES OF *Hymenolepis diminuta* CYSTICERCOIDS

Following on from the initial structural and developmental studies on the cysticercoid, interest was turned to its biochemistry. Prescott and Voge (1959) described the synthesis of RNA in the wall of the cysticercoid, indicating capacity for protein synthesis, and this has been confirmed by Jeffs and Arme (1984) who demonstrated the incorporation of four different amino acids into protein by stage 5 cysticercoids. Bogitsch (1967) illustrated the presence of acid phosphatase activity in the' intermediate cell layers' of the cysticercoid of H. diminuta. The two enzymes present exhibited optimal

activity at pH 5.0 and 6.9, and he postulated that the former enzyme was associated with lysosomes and involved in excystation, although this has never been investigated. However, the optimum pH for phosphatase activity varied according to the location of the enzyme. Walkey and Fairbairn (1973) detected lactic dehydrogenase (LDH) and observed markedly different kinetics between metacestode LDH and the adult enzyme. Carter and Fairbairn (1975) reported two isozymes of pyruvate kinase in oncospheres and cysticercoids, and three additional ones present in juvenile worms and adults. This, they postulated, provided evidence for the adaptive control of metabolism. For example, phosphoenol pyruvate can either be anabolised to glycogen or catabolised by, for example, pyruvate kinase to pyruvate, or phosphenolpyruvate carboxykinase to oxaloacetic acid. If it is catabolised, enough must be used in order to satisfy the energy requirements of the worm, but uncontrolled catabolism would recycle the carbon at the expense of ATP. Therefore, regulation of pyruvate kinase activity is important in controlling this, and the presence of different isozymes at different stages of the development of H. diminuta suggests the adaptive control of metabolism. The work of Moczon has given some valuable information about the range and types of enzymes present in H. diminuta cysticercoids (1973a,b; 1977a,b,c and d). Oxidoreductases were investigated histochemically and provided evidence of glucose metabolism through the pentose cycle

enzymes and degradation of hexoses through the Embden-Meyerhoff pathway. Histochemical tests for phosphatases demonstrated the presence of non-specific acid and alkaline phosphatases and Ca^+ and Mg^+ dependent ATPases, in some or all tissues, although tests for glucose-6-phosphatase and fructose 1,6 diphosphatase were negative. Alkaline phosphatase was located in the tegument of the presumptive scolex only (Moczon,1973b) and this, he postulated suggested that only the scolex was active in absorption. However, a controlled experiment in Chapter 5 shows clearly that the cysticercoid tegument is active in absorption (present study). Enzymes for the synthesis and phosphorolytic degradation of glycogen were also detected with the greatest activity around the scolex, inner capsule and the cercomer. This corresponds with the distribution of glycogen in the cysticercoid (Voge and Heyneman,1957; Moczon,1977c). Many other enzymes have been detected in cysticercoids (at least 22 altogether), and their activity has largely been located within the cysticercoid as opposed to the surface. Mitochondrial enzymes have also been detected within the presumptive scolex, although this contrasts with adult worms that apparently lack mitochondria in the scolex region (Allison et al.,1972). A source of excretory nitrogen in cysticercoids was found to be L-glutamate (Moczon,1977e). In a study by Goodchild and Wells (1957), a wide range of amino acids were detected in hydrolysates of parts of, and whole, cysticercoids. The only identifiable polysaccharide appears to be glycogen, which was first located by Heyneman and Voge (1957) in the'tail', the scolex

musculature and the tegument of the scolex. The amount of glycogen increased with development, but was found in reduced quantitites in those cysticercoids grown under temperature stress. The level of glycogen however remained constant in very old individuals (greater than 80 days) (Heyneman and Voge,1957).

G. COMPARISON OF ADULT AND CYSTICERCOID TEGUMENT

1.STRUCTURE

In order to understand the uptake mechanisms across the tegument of H. diminuta cysticercoids, a knowledge of the structure, properties and other known functions of the tegument is desirable. There is a wealth of literature describing the adult tegument but relatively little on the cysticercoid. In this section, a resumé of the known features of adult and cysticercoid tegument is presented. The present knowledge of the morphology of adults and cysticercoids is the subject of several good, recent reviews (Lumsden and Murphy,1980; Lumsden and Specian,1980; Ubelaker,1980; Lumsden, Voge and Sogandares-Bergal,1982).

The tegument of tapeworms has been of major interest in parasitological research over the past 30 years. The host parasite interface, defined by Read, Rothman and Simmons (1963) as "the region of chemical juxtaposition of regulatory mechanisms of both host and parasite, involved in determining the nature and extent of integration and thus the outcome of

the relationship, is important in defining the physiochemical environment and therefore in regulating other chemical processes occurring with the parasite beyond the tegument". For both metacestodes and adults this interface is generally delimited by the glycocalyx overlying the surface membrane of a protoplasmic syncytium.

The basic plan of the tegument is the same for adults and cysticercoids of H. diminuta - a distal cytoplasm supported by a layer of connective tissue (the basement membrane) and joined by cytoplasmic processes to nucleated cell bodies (tegumentary cytons) which are in the cortical parenchyma.

The most important feature of the distal cytoplasm is the brush border, consisting of many digitiform processes. It is here that an obvious difference between adult and cysticercoid occurs. Adults possess microtriches, which characteristically have pointed tips comprising an electron-dense matrix of closely packed fibrils. The shaft support of the microthrix is cylindrical, made up of a core of microfilaments, and the tip is separated from this shaft by a multilaminate baseplate. The whole microthrix is covered by the surface plasma membrane. The shape and number of the microtriches of adult H. diminuta are known to vary according to location, with those on the strobila tending to have shorter shafts and longer tips. In the cysticercoid the digitiform processes are known as microvilli. They are slender, vertically orientated and cylindrical. They are also covered by the surface plasma membrane and consist of a core of microfilaments. However,

they do not have the electron-dense tip associated with adult microtriches. Microvilli in H. diminuta cysticercoids are sometimes branched (Ubelaker et al.,1970) and may end in an apical bulblike swelling.

The surface plasma membrane present in adults and cysticercoids is c.12nm thick and trilaminate.Freeze-fracture studies have shown this surface plasmalemma to be particulate and thus conforming to the fluid mosaic model for biological membranes proposed by Singer and Nicholson (1972).

Covering this outer plasma membrane is a thin carbohydrate-rich coating, the glycocalyx (Lee,1966,1972; Lumsden,1975a,b). It is polyionic and usually manifests itself under the electron microscope, as the fuzzy coat over the plasma membrane.

The core of microfilaments, present in both microtriches and microvilli, closely resembles the actin component of vertebrate epithelial brush border (Tilney and Mooseker,1971), with extensions of the microfilaments from the microvilli in H. diminuta cysticercoids, creating a structure, similar to a terminal web, in the distal cytoplasm (Ubelaker et al.,1970). However, the distal cytoplasm of both adult and cysticercoid is very similar. It contains many membrane-bound vesicles and granules of various sizes and structures. The majority of the vesicles arise from the Golgi apparatus of the tegumentary cytons and are transported to the distal cytoplasm via the internuncial canals. Many functions have been attributed to

these inclusions, including secretion, formation of microvilli/microtriches/glycocalyx and formation of the basal lamina (Oaks and Lumsden,1971; Lumsden, Oaks and Mueller,1974; Lumsden et al.,1982). Mitochondria are found, usually, in the basal region of the distal cytoplasm, but not in the scolex region of adults. Endoplasmic reticulum, Golgi and ribosomes are found in the distal cytoplasm during early development of the cysticercoid, but not in mature cysticercoids. Also, the plasma membrane at the base of the distal cytoplasm is continuous with the membrane of the cytoplasmic processes and the tegumentary cytons.

The tegumentary basal lamina is very similar to the glycosaminoglycan-composed basal lamina of more conventional epithelial tissues and is probably produced in the distal cytoplasm (Lumsden et al.,1982). In the cysticercoid, there is a layer of collagen-like fibrils adjacent to the basal lamina which according to Lumsden et al.(1982) appears more like oxytalin fibrils of vertebrate connective tissue than collagen. However, this collagen lacks hydroxyproline and varies in structure from true collagens. A fibrous connective tissue component the basement lamella is also present in adults, and is thought to be elaborated by the underlying musculature (Lumsden et al.,1982). Similarly, the production of basement lamella fibrils has been attributed to myocytons in the cortex of cysticercoids (Lumsden et al.,1982). Therefore, the basal lamina is elaborated by the distal cytoplasm, while the connective tissue component of the basement lamella is elaborated by the muscles/myocytons. This would coincide with

the pattern of development, with differentiation of the distal cytoplasm, and therefore the basal lamina, occurring before that of the musculature (and therefore the fibrillar component of the basement lamella).

An unusual feature of *H. diminuta* cysticercoids is the presence of 'hairy' processes as described initially by Voge (1960). These are actually bundles of connective tissue filaments perpendicular to the basement membrane and in contact with the outer zone of distal cytoplasm just beneath the surface plasma membrane. Voge (1960) describes them as lying within the distal cytoplasm but electron microscopy has shown them to be enclosed in extracellular space caused by infoldings of the external plasma membrane (Lumsden *et al.*,1982). Their function has been suggested to be supportive (Lumsden *etal.*, 1982).

Thus, in general morphology the tegument of the cysticercoid and adult *H. diminuta* is similar. The basic plan of a syncytial surface tegument with cell bodies located away from the surface and connected to it *via* cytoplasmic connections, is a structure which persists from oncosphere to cysticercoid to adult.

2. BIOCHEMICAL ASPECTS OF THE TEGUMENT

The majority of work on the biochemical properties and composition of the cysticercoid of H. diminuta has been histochemical. These data are valuable in suggesting possible composition and function of tegumentary components, but are not a substitute for data obtained from the now well-developed biochemical techniques for these analyses. A comparison of the surface biochemistry of the adult and cysticercoid of H. diminuta is therefore difficult. The glycocalyx of metacestodes has been the subject of much research, mainly using electron microscopical and cytochemical techniques. The carbohydrate content of the metacestode glycocalyx has been established for Taenia crassiceps (Trimble and Lumsden,1975), Cysticercus cellulosae (Sosa,1977) and Spirometra mansonoides (Oaks and Mueller,1981) and it is known that glycocalyces usually contain both acidic and neutral glycans (Lumsden,1975). Thakur, Schwabe and Koussa (1971) determined the presence of glucose, glucosamine, galactosamine and 14 amino acids in the glycocalyx of Taenia hydatigena, while Friedman, Weinstein, Davidson and Mueller (1980), using PAGE analysis, illustrated the presence of 20 major polypeptides (MW 12,000 - 200,000) in isolated membranes of Spirometra mansonoides plerocercoids. Similar work on isolated membranes of Mesocestoides corti tetrathyridia has also shown the presence of glycopeptides/glycoproteins of approximately 110, 70, 60, 40, 18 and 12 kilodaltons (Lumsden et al.,1982). The majority of metacestode surface glycans are elaborated by the parasites and are not adsorbed from the host, as demonstrated

autoradiographically by Trimble and Lumsden (1975), using tritiated galactose labelled glycoproteins in *Taenia crassiceps*. The function of the glycocalyx in metacestodes is unknown, but suggestions include the provision of absorptive sites, a contribution to the hydration of the surface membrane and electrolyte concentration in the immediate external environment and an influence over the immunogenicity of the parasite and its interactions with host cells.

An investigation into the contents of tegumentary vesicles has shown that some contain carbohydrates while others stain for acidic moieties. The work on both *Mesocestodes corti* and *Spirometra mansonoides* has shown that a number of polypeptides are common to both the brush border and the tegumentary vesicles (Friedman *et al*.,1980; Lumsden *et al*.,1982). This has given support to the view that some of these vesicles contribute to the formation of microvilli, glycocalyx etc.

Studies on the enzymatic activity of the cysticercoid tegument are few, but the work of Moczon (see previous section) provides some useful information. In contrast,there is considerably more information on the biochemistry of the tegument of adult *H. diminuta*. Knowles and Oaks (1979) were the first to successfully develop a technique for the separation of the brush border from the underlying cytoplasm, for the purposes of biochemical study. Adult worms were incubated in a dilute solution of a non-ionic detergent to remove the tegument, which was then subjected to solutions of high and low

osmolarity, and the brush border fraction was collected by differential centrifugation. The purity of this fraction was then verified by electron microscopy, lectin binding and enzymatic studies (Knowles and Oaks,1979). Further analysis led to the definition of at least thirty polypeptides in H. diminuta brush border, using SDS polyacrylamide gradients. Both phosphorylated proteins and glycoproteins are found in H. diminuta brush border membrane, as well as membrane-bound enzymes (Knowles and Oaks,1979; Gamble and Pappas,1980, 1981a,1981b; Pappas,1980c,1981,1983b). Their location, however, is less well known, although at least seven polypeptides are orientated externally (Knowles and Oaks,1979). Also, membrane-bound enzymes of H. diminuta can be solubilised in detergent without dissolution of the membrane,suggesting that there are probably not deeply embedded therein (Pappas,1980c). The difficulties and potentials of using detergents to solubilize membrane proteins of the H. diminuta brush border has been discussed by Pappas (1980a,1980b).

Membrane-bound enzymes are an intrinsic feature of cestode surfaces and have been examined in detail in adult H. diminuta. The surface activity of alkaline phosphohydrolase has been demonstrated by cytochemistry (Lumsden et al.,1968; Dike and Read,1971a), product analysis of the incubation medium (Arme and Read,1970; Dike and Read,1971a) and by measuring the effects of hydrolysis on mediated transport (Dike and Read,1971a,1971b; Uglem, Pappas and Read,1974; Pappas and Read,1974). A review by Pappas (1980a) describes and analyses these studies. Multiple phosphohydrolase activity has been

suggested to be present in the surface membrane of H. diminuta (Pappas and Read,1974; Kuo,1979). However, preliminary analysis of substrate and inhibitor specificities suggested the occurrence of only a single enzyme or if multiple enzymes were present, they possessed very broad and overlapping specificities which cannot currently be differentiated (Pappas,1982b). Available data show that nucleotides (including AMP) are also hydrolysed at H. diminuta surface (Lumsden et al.,1968; Dike and Read,1971a; Pappas and Read,1974; Kuo,1979). Only type I phosphodiesterase, which hydrolyses phosphodiester bonds, has been detected in H. diminuta (Gamble and Pappas,1981a). Other membrane-bound enzymes detected in H. diminuta include RNase and ATPase. The RNase has been shown to be of worm origin, cannot readily be removed and also displays kinetics different from that of the rat host enzyme (Pappas et al.,1973a; Gamble and Pappas,1981b). ATPase activity has also been demonstrated by Lumsden et al.(1981b) and Pappas (1981).

H. EXCYSTATION OF CYSTICERCOIDS

The cysticercoid, once matured, remains in the intermediate host haemocoel until the beetle is eaten by a rat. In the laboratory, infection of the definitive host is readily effected and details are given below. Once the cysticercoid enters the stomach it is subject to the combined effects of increased temperature (from 26 - 37°C), a drop in pH to between 1.5 and 2.5, and the digestive effects of a pepsin/HC1 mixture. Goodchild and Harrison (1961) administered 50

cysticercoids every hour for 3 h to rats, and 1 h later examined the stomach and intestine to determine the location of cysticercoids and their condition. Two groups were located in the stomach, one of which could still be recognised as cysticercoids although much of the outer layers had been digested away, while the other group had had most of the outer layers digested but the inner capsule was still intact. The third group were in the small intestine where 4-h-old worms were found in scrapings of the duodenum. In no instance were excysted worms found in the stomach, or intact cysticercoids found in the small intestine. Therefore, the period in the stomach serves to digest the majority of cystic tissue, but leaving the presumptive adult and its capsule intact. The small intestine, however, provides another change in pH from the acid stomach (pH 1.5 - 2.5 to a more neutral duodenum pH c.6+). Here the 'cysticercoids', or what remains of them, are exposed to a wide range of digestive enzymes, and also bile. Pancreatic proteases continue digestion and remove any cystic tissue that remains, while the effect of bile is less well known. However, it is thought that bile is essential for activation of the presumptive scolex and its subsequent excystment. The whole process, in vivo, takes between 2 - 4 h, after which the young adult locates and begins to grow and develop.

An interesting feature of the newly excysted worm is that it is not affected by trypsin. The study of how H. diminuta survives in an environment full of hydrolytic enzymes without being digested has largely been the work of Pappas and his

colleagues. They have shown that, in the presence of intact H. diminuta, trypsin is inactivated. However, Schroeder, Pappas and Means (1981) were unable to demonstrate the presence of an inhibiting agent, although they stated that inactivation was effected by a small change in conformation of the enzyme which causes partial inactivation. The original work of Pappas and Read (1972a,b) did not demonstrate the presence of substances secreted into the incubation media, but Uglem and Just (1983) have claimed that the presence of proteins released by H. diminuta may inhibit trypsin activity. These proteins could be antienzymes or non-specific macromolecules that interfere with trypsin activity or even assay systems. H. diminuta was also shown to be capable of adjusting the pH of its environment to 5.0 by excreting organic acids. This capability protects the worm from trypsin digestion because trypsin activity is minimal at this pH. In vivo the intestine of infected rats is more acidic (Mettrick,1971) as organic acids, products of metabolism, are excreted and serve to create a protective environment for the worm.

CHAPTER 2

THE RELATIONSHIP BETWEEN *Hymenolepis diminuta* CYSTICERCOIDS AND ADULT *Tenebrio molitor*

A. INTRODUCTION

The relationship between adult tapeworms and their definitive hosts has been extensively investigated, e.g. the *H. diminuta*/rat association (Arme,1975; Mettrick,1980,1982; Arme, Bridges and Hoole,1983) and there is also a substantial literature on metacestodes and their vertebrate hosts (see Arme, Bridges and Hoole,1983). However, in contrast, invertebrate host/metacestode relationships have largely been neglected (Freeman,1983), although some information on *H. diminuta*/*T. molitor* and *H. diminuta* /*T. confusum* is now available (Keymer,1980, 1982; Hurd and Arme,1984a,b).

B. THE ENVIRONMENT OF THE CYSTICERCOID

The environment of the cysticercoid will be discussed in terms of location, previous reports on chemical composition and further data regarding chemical composition resulting from this study.

1. LOCATION

Oncospheres of *H. diminuta*, liberated from eggs in the gut of the intermediate host, burrow through the mid-gut wall and into the haemocoel. Once they have penetrated the gut, oncospheres are generally found in the thorax and abdominal regions of the haemocoel, these areas providing ample space for growth and differentiation of the cysticercoid. Observation of dissected insects shows that parasites are found in association with the serosal surface of the gut and also with areas of fat body. In all cases the 'head' of the cysticercoid appears attached to these surfaces , while the "tail" floats freely in the haemolymph (personal observation). In a study by MacDonald and Wilson (1964), it was found that cysticercoids removed from the thorax of *Tribolium confusum* were significantly smaller than those from the abdominal regions, perhaps a result of their having a smaller space for growth. Also, larger infections produced smaller cysticercoids in *T. molitor* infections (personal observation) and it is only in these larger infections that cysticercoids are found to be located in the thoracic regions of the haemocoel.

Oncospheres locate, metamorphose and grow into mature cysticercoids bathed in the haemolymph which consists of a fluid plasma in which nucleated cells (the haemocytes) are suspended. It resembles both blood and lymph in vertebrates, but in insects it is usually colourless and does not carry pigment-combined oxygen.

2. HAEMOLYMPH

The haemolymph contains several types of haemocytes of which four are found in most insects; the prohaemocytes, the plasmatocytes, granular haemocytes and coagulocytes (Jones, 1962,1964). However, the confusing terminology of insect haemocytes has masked the true number of different haemocytes present, and, indeed, there may be only one type, which has been described in all its physiological states (Jones,1970). The classification of Rowley and Ratcliffe (1981) is generally taken as the 'standard' for haemocyte terminology.

The number of haemocytes present can fluctuate considerably over short periods because not all cells are free in the circulation, many of them adhering to the surface of tissues etc. Also, as the total haemolymph volume changes, so the the number of cells in a unit volume will change. Therefore the type and number of haemocytes needs to be defined not only by species, but also physiological state (e.g. before moulting) and possible infection.

The main function of haemocytes is phagocytosis of foreign particles. Foreign bodies too large to be phagocytosed e.g. metazoan parasites, are generally encapsulated by haemocytes resulting in death of the parasite. Encapsulation does not always occur, however, and parasites may evade, or fail to induce, a response in their insect host. Haemocytes also have a role in wound healing and possibly storage and intermediate metabolism.

Approximately 85% of the haemolymph is water (Florkin and Jeuniaux,1974), although this percentage varies during the life cycle and according to environmental conditions experienced by the insect. Inorganic constituents include sodium, chloride, calcium and magnesium, but also trace elements such as copper, iron and zinc in some insects.

Insect haemolymph is characterised by its high concentration of amino acids, but it also contains proteins and sugars. The haemolymph has many functions including transport of materials around the body, and excretory products to the Malpighian tubules. It can store substances e.g. trehalose, water and amino acids, and act as a buffer, enabling CO_2 to be transported from the tissues to the tracheae *via* the haemolymph. Haemolymph thus provides a nutrient-rich, stable environment, highly suitable for growth and development of cysticercoids. It is, however, relevant to this study to investigate the chemical composition of *Tenebrio molitor* haemolymph and the effect of *H. diminuta* upon it.

a. Carbohydrate composition of the haemolymph

The cysticercoid located in the haemocoel is exposed to a range of low-molecular weight organic nutrients that the parasite has the ability to absorb (Arme, Middleton and Scott, 1973; Arme and Coates,1971,1973; Jeffs,1984; Jeffs and Arme, 1984; 1985a,b).

Data on the carbohydrate composition of insect haemolymph are available, but, as with amino acid composition, there is

great variability between species and within orders. Wyatt and Kalf (1957) first discovered the presence of a non-reducing disaccharide, trehalose, in high concentration in haemolymph of the cecropia silk-moth, and since then, its presence, and the presence of the enzyme trehalase, has been recorded in many insects (Candy and Kilby,1961; Clegg and Evans,1961; Sacktor and Wormser-Shavit,1966; Weins and Gilbert,1967). Trehalase was shown to catalyse the formation of glucose from trehalose. The rapid conversion of glucose absorbed across the gut to trehalose effectively maintains a concentration gradient of glucose across the gut, thus aiding glucose absorption.

Trehalose is also found in most insect tissues. Unlike vertebrates, there is no evidence to suggest a homeostatic regulation of haemolymph sugars, although Friedman (1968) demonstrated that fat-body synthesis of trehalose was subject to end-product inhibition, and Steele (1961,1963) also showed that extracts of corpora allata could influence trehalose concentration, through its action on the fat body. However, the variability induced by alteration of food, season and stage of the life cycle, suggests that precise regulation in the vertebrate sense, does not occur.

Bedford (1977) studied the concentrations of carbohydrates in 21 species of insect and other arthropods, prompted by the fact that reducing sugars, especially glucose, were shown to occur in relatively low concentrations in insects, as opposed to most other active organisms, in which glucose plays a central role in metabolism and energy production (Wigglesworth, 1972). The dependence of adult H. diminuta on carbohydrate has

been well documented (Read,1959; Read and Rothman,1957a,b). It might therefore be expected that there would be a similar requirement for carbohydrate by the cysticercoid. If trehalose is the only available carbohydrate, then it is possible that the cysticercoid has the ability to absorb disaccharides, or to hydrolyse them and absorb the resulting monosaccharides. In order to answer these questions it is necessary to know the carbohydrate composition of Tenebrio molitor haemolymph.

The only published work on carbohydrate quality and quantity in Tenebrio molitor, gave a figure of 122mg/100ml total sugar (Marcuzzi,1955). Since then, however, more accurate and reliable techniques have become available for the detection of sugars, eliminating interference by other haemolymph components. Because of the paucity of information concerning T. molitor haemolymph sugar concentrations, an investigation was undertaken to determine the type and quantity of haemoymph sugar in adult Tenebrio molitor. Two types of analysis were performed on haemolymph samples to determine their carbohydrate content. A glucose assay was initially used and the results obtained were recorded, but suspicion was aroused when the variation between samples from individual beetles was shown to be very small. Possible explantions for this included the insensitivity of the technique for small samples (i.e. <10μl), and, in particular, the lack of sensitivity at the lower end of its detection range. The work of Bedford (1977) not only suggested that this lack of variation was unlikely to be a true representation of glucose concentrations, but it also highlighted the use of gas liquid

chromatography (GLC) in identifying and quantifying carbohydrate from the haemolymph of individual insects. Subsequent studies on haemolymph were therefore carried out using GLC techniques. Both methods are fully described in Chapter 4.

A total of 300 adult T. molitor were used to determine carbohydrate content in haemolymph. Forty non-infected adults, 20 males and 20 females were analysed by the glucose oxidase assay method and the results shown in Table 2.1. Females have more glucose, and a larger variation between individuals than males. 260 adults were analysed by GLC, of which 132 were non-infected and the results are shown in Table 2.1.

Therefore, in this study, a mean value for glucose concentration in the haemolymph of non-infected Tenebrio molitor was 0.1mM (males) and 0.3mM (females). Mean trehalose concentrations were considerably greater: 2.87mM (males) and 3.02mM (females).

b. Amino acid composition of haemolymph

Amino acids are characteristically present in high concentration in endopterygote insect haemolymph, with large amounts of proline and glutamic acid predominating (Duchateau and Florkin,1958; Sutcliffe,1963). The precise amino acid composition varies according to species and developmental

TABLE 2.1

THE CARBOHYDRATE CONTENT OF *Tenebrio molitor* HAEMOLYMPH

TYPE OF ANALYSIS	SEX (NO.)	MEAN GLUCOSE CONTENT	MEAN TREHALOSE CONTENT
Glucose assay	male NI 20	0.1mM ± 0.08	-
	female NI 20	0.3mM ± 0.1	-
G.L.C	male NI 60	0.1mM ± 0.04	2.87mM ± 0.1
	female NI 72	0.3mM ± 0.09	3.02mM ± 0.3
"	male I 60	0.9mM ± 0.1	2.98mM ± 0.1
	female I 72	1.2mM ± 0.08	3.65mM ± 0.2

NI = non-infected

I = infected

G.L.C = gas liquid chromatography

Each mean is given ± standard error.

stage, is also related to those amino acids absorbed from food. Tyrosine accumulates before moulting and subsequently decreases sharply as it is used in tanning and melanisation of the new cuticle (Barrett,1974). The haemolymph free amino acids have been analysed in several insects (for example; Barrett,1974; Barrett and Laifook,1976; Bosquet,1977; Collett,1976b; Evans and Crossley,1974; Horie and Watanabe,1983; Kulkarni,1970; Levenbrook,1966; Mack, Samuels and Vanderberg,1979; Prabhu and Nayar,1971; Terra, De Bianchi and Gambarini,1973; Woodring and Blakeney,1980). Bradfisch and Punzo (1977) first analysed the free amino acid content of Tenebrio molitor haemolymph, and noted a gradual increase in total free amino acids from larval to adult stage.

A more recent detailed study by Hurd and Arme (1984a) investigated the amino acid composition of adult Tenebrio molitor haemolymph and the effect of infection with H. diminuta cysticercoids. Using an amino acid analyser a total of 16 amino acids were detected and quantified in both males and females. Proline was present in the highest concentration (up to 55mM in non-parasitised females and 58mM in males). Other abundant amino acids included histidine, lysine, alanine, and valine. The total concentration of amino acids ranged from 34 - 94 mM, with the highest occurring 15 days post-emergence. The osmotic pressure of T. molitor haemolymph was determined as 500mOsm, and it is apparent that the amino acids contribute significantly to this value (Hurd and Arme,1984a).

Cysticercoids of H. diminuta can absorb a variety of amino acids by specific membrane transport mechanisms (Jeffs, 1984;Jeffs and Arme , 1985a,b). Also leucine, phenylalanine, alanine and proline have all been shown to be incorporated into protein by the cysticercoid in vitro (Jeffs and Arme,1985b). Thus, it is probable that the environmental amino acids satisfy nutritional demands.

c. Haemolymph proteins

Electrophoretic and immunological methods have demonstrated a large number of different proteins in insect haemolymph (e.g Laufer,1960; Mjeni and Morrison,1973; Ramade and Le Bras,1973; Whitmore and Gilbert,1974). Twenty-one different proteins have been recorded in Locusta spp. and 19 in Drosophila spp. (McCormick and Scott,1966a,b; Chen and Levenbrook,1966). These vary within and between species and change during the life cycle (especially at ecdysis) and with the reproductive cycle of the female (Schmidt,1974). Many haemolymph proteins are conjugated with lipid and carbohydrate and there are, of course, many that function as enzymes. Reviews by Wang and Patton (1968) and Wyatt and Pan (1978) have detailed the methods used to detect haemolymph proteins as well as the types and concentrations found in a variety of insects.

The total haemolymph protein of infected and non-infected adult T. molitor were studied by Hurd and Arme (1984b). In non-infected beetles of both sexes, age-related variations in haemolymph protein concentration were observed although, at all

ages, protein concentration in females was significantly higher than in the males. Haemolymph protein varied from 62 - 82mg/ml in females and 36 - 54 mg/ml in males. Electrophoretic analysis revealed the presence of 13 bands, and investigation into the quantitative alteration of some of these bands is discussed on page 64.

The significance of haemolymph proteins in the biology of cysticercoids is unknown, although proteins are probably not used in nutrition. The role of exogenous protein in tapeworm nutrition is a matter of some controversy (Pappas,1983) and it is apparent that protein absorption is restricted to only a few species. In preliminary experiments on H. diminuta cysticercoids, Arme and Richards (unpublished observations) were unable to demonstrate the uptake of, either free or liposomally entrapped, ruthenium red and studies have shown adult H.diminuta to be impermeable to a variety of macromolecules (e.g. Lumsden, Threadgold, Oaks and Arme,1970).

d. Inorganic constituents and osmolarity of haemolymph

Haemolymph from non-infected adult T. molitor has been analysed for its cation content. Sodium, potassium, calcium and magnesium were detected and measured by atomic absorption spectrophotometry (Hurd, Brown and Arme,1982). Sodium was present in the greatest concentration, at approximately 150 mM, with potassium, calcium and magnesium at concentrations of c. 21mM, 26mM and 23mM respectively. The cation totals for

females were approximately 230mM and for males, 163mM. The total osmolarity of T. molitor haemolymph has been quoted as 500 mOSM from non-infected individuals (Hurd and Arme,1984a).

The osmolarity and ionic composition of the haemolymph give a broad description of the conditions under which the cysticercoids develop and grow. They are important in the design of in vitro media and can point to the suitability or otherwise of media used in in vitro work.

The above indicates that broad details of the composition of adult Tenebrio molitor haemolymph are now available, together with described effects of H. diminuta infection on its composition. These data further enable a better understanding of the relationship between cysticercoid and host haemolymph and, in particular the mechanisms and specificities of the absorption of nutrients by the cysticercoid.

C. ALTERATIONS IN HAEMOLYMPH COMPOSITION OF Tenebrio molitor EFFECTED BY Hymenolepis diminuta CYSTICERCOIDS

1. CARBOHYDRATE COMPOSITION

The carbohydrate composition of the haemolymph of 260 infected and non-infected beetles was analysed by gas liquid chromatography and the results are shown in Table 2.1. Infected beetles showed an increase in free glucose concentration over the controls. This was most pronounced

in 12-day-old beetles containing 9-day-old cysticercoids. Trehalose concentrations also increased with age of beetle/infection, but there was no significant difference in trehalose concentration between infected and non-infected beetles. Bedford (1977) suggested that glucose represented the metabolic energy source and trehalose the storage carbohydrate. This detectable glucose therefore represents glucose in excess to host immediate metabolic requirements, which would normally be converted to trehalose for storage. Two possible explanations for the presence of glucose suggest themselves. First, the enzymatic inhibition by the parasite of the above conversion. This is considered unlikely however, as the levels of trehalose increase with age, but partial inhibition or regulation could be possible. The second explanation involves the liberation of glucose by the cysticercoid as a result of hydrolysis of the disaccharide at the parasitic surface.

2. AMINO ACID COMPOSITION

Hurd and Arme (1984a) studied the effect of parasitism on the amino acid composition of T. molitor haemolymph. Haemolymph was collected from beetles, 9, 12,15 and 30 days post-emergence, and beetles were infected 3 days after emergence so that 9-day-old beetles had 6-day-old infections etc. Their study showed that the concentration of nine amino acids in infected females was significantly altered. Isoleucine, leucine, arginine, serine and threonine were increased, while tyrosine, phenlalanine, proline and alanine were decreased in concentration. Infected males were affected

to a lesser extent with only four amino acids altered; threonine, glycine, histidine and arginine. The explanations given for the above include competition between host and parasite for nutrients and the increased removal of amino acids for glucogenesis resulting from lack of host haemolymph carbohydrate (Gourd, Williams and Ramade,1983). However the greater effect on female beetles indicates that factors other than nutrient removal may be involved and the effect of parasitism on the total haemolymph protein suggests an overall link between all these effects, possibly at the endocrine level (Hurd and Arme,1984a,b).

3. HAEMOLYMPH PROTEIN

Hurd and Arme (1984b) compared the total haemolymph protein concentration in infected and non-infected individuals. They found no significant difference between 9- and 12-day-old beetles, but there was an increase in protein concentrations in infected females after 15 days. This difference (46.9% over and above control values) persisted in 20- and 30-day-old females. There was no such difference observed in males. Parasite density was also recorded, with no correlation between elevated protein concentrations and density of infection. Electrophoretic studies revealed the presence of 13 bands, of which two were elevated by 70% in parasitised females. The work of Harnish and White (1982), which identified two

female-specific proteins in T. molitor, allowed these 2 elevated bands to be identified as host vitellogenins. Therefore parasitism resulted in increased amounts of vitellogenin in female haemolymph, but the significance of this to the host and parasite is not yet known. However, it is thought the cysticercoid interacts with T. molitor endocrine system which controls vitellogenin production and their uptake by the ovaries (e.g. see Hegedorn and Kunkel,1979). Hurd and Arme (1984b) suggested that the presence of vitellogenin in the haemolymph was due to one of three reasons. Either there was increased vitellogenin synthesis and/or release by the fat body, a failure to absorb vitellogenin by developing oocytes or, resorption of oocytes. Recent studies (Hurd and Arme, personal comm.) using ^{14}C-leucine, which was injected directly into the haemocoel of adult T. molitor females, highlighted two important results; the fat body synthesis and secretion of vitellogenin was indeed reduced by infection, but so too was the sequestration of vitellogenin by the ovaries. This, therefore, appeared to explain the increase in haemolymph vitellogenin concentration observed, and electrophoresis revealed that the labelled protein was primarily vitellogenin. Since juvenile hormone is responsible for the synthesis and uptake of vitellogenins, the current hypothesis is that the cysticercoids of H. diminuta are producing an inhibitor of juvenile hormone production, or a substance which decreases the concentration of juvenile hormone (which could also account for the results recorded). Adult H. diminuta is known to synthesise farnesol, isomers of which are juvenile hormone mimics (Frayha

and Fairbairn,1969; Fioravanti and MacInnis,1977) and ecdysteroids have also been found in the cysticercoid, thus their possible role in host parasite interactions warrants further investigation.

4. CATION COMPOSITION OF HAEMOLYMPH

In a study on infected male and female *T. molitor* haemolymph cations, a significant difference in potassium concentration in males was recorded, with a large but insignificant difference in potassium in females (Hurd, Browne and Arme, 1982).

D. EFFECTS OF PARASITISM ON HOST POPULATION DYNAMICS AND EGG PRODUCTION.

Information concerning the effects of *H. diminuta* infections on factors such as the survival and fecundity of *Tenebrio molitor* is sparse. However, a series of experiments designed to measure the effects of *H. diminuta* infections on *Tribolium confusum* fitness, have been carried out (Keymer,1980, 1982; Keymer and Anderson,1979). By exposing groups of beetles

to several 'feedings' of eggs, Keymer found that a linear relationship between the number of exposures to eggs and the number of cysticercoids per host appeared. It was noted that cysticercoid size (measured as total length) decreased with increased parasite burden, although not in a linear fashion. Production of eggs by infected female beetles (taken as daily recordings) declined non-linearly with increasing cysticercoid burden. These results, therefore, suggested a direct effect of parasite presence and burden upon the fecundity of T. confusum. However, a linear relationship between observed beetle mortality and parasite burden was not always seen, due to the effects of over-dispersion. The main effect of large numbers of parasites apparently resulted from the damage caused to the gut wall by penetrating oncospheres. However, the reduction in host fecundity, because of its non-linear relationship with parasite burden, was thought to be attributed to increased biomass of parasites in the host (Keymer,1980). Some work on the egg production of female T. molitor with H. diminuta infections has been carried out (Hurd,personal comm.). No significant differences in the number of eggs laid in 30 days, or the number of oocytes retained was found. However, both oocyte volume and ovary dry weight appeared significantly lower in infected individuals after 30 days.

Schom, Novak and Evans (1981) studied infections in Tribolium confusum of Hymenolepis nana, H. diminuta, H. microstoma and H. citelli. They found that all cysticercoids caused some mortality. The effect was most

notable during the first 15 days of the infection and it was concluded that H. citelli was the most pathogenic of the species. The reason for this mortality is unknown, but Schom et al. postulated three explanations; In normal in vitro infection procedures for hymenolepid spp. the beetles are starved prior to infection, and this could seriously affect their fecundity. The degree of experimental parasite burden is perhaps greater than would be expected, even in a naturally over-dispersed population. Finally, sex of beetle also affected its survival prospects, with male beetles surviving nearly 2 days longer overall than females. These authors also noted the cysticercoid development was faster in the males than in the females, and female beetles were generally more heavily infected than males - this fact probably accounting for the differences in mortality. The data behind these three suggestions were also provided by the above authors who showed that total mortality was higher following starvation for 6 days prior to infection (73 - 93%) compared with starvation of only 1 day (51%). Those beetles which survived the experimental time period of 15 days, generally had low numbers of cysticercoids (< 14), compared to those that died earlier (> 20 ;Schom et al., 1981).

E. CELLULAR RESPONSE TO INFECTION

The study of encapsulation of parasites by invertebrate host haemocytes is one aspect of the study of the host-parasite relationship. Encapsulation by haemocytes involves congregation of cells around the parasite, causing the inner cells to become flattened. This inner layer of cells breaks down to form a continuous band of nucleated cytoplasm which eventually becomes connective tissue, impermeable to oxygen and most other substances, causing death of the parasite. However, some parasites in invertebrate hosts are able to survive and apparently possess methods of evading the effectiveness of the host response.

A particularly interesting feature of hymenolepid infections in *Tenebrio* and *Tribolium* spp. is that the parasites are not encapsulated and appear not to evoke a response by the host. Lethbridge(1971c) described a method of infecting the locust *Schistocerca gregaria* with cysticercoids of *H. diminuta* by inoculating hexacanths, hatched *in vitro,* directly into the haemocoel, thus avoiding the gut wall, which was a barrier to penetration. Lethbridge observed normal cysticercoid growth and a lack of host response in this unnatural host. However, *H. diminuta* cysticercoids can undoubtedly elicit a reaction in some insects, and *T. molitor* can respond to foreign material. Parasites which were not encapsulated presumably either evaded recognition by the host, or if recognised as "non-self", prevented encapsulation. During development of the 'normal' cysticercoid, few or no haemocytes are found attached to its surface (Rothman,1959;

Heyneman and Voge, 1971), but Ubelaker _et al._ (1970b) suggested that vesicles of parasite origin, found in the vicinity of host haemocytes, were responsible for producing a lytic substance which attracted haemocytes. The view that there is a limited cellular response to _H. diminuta_ cysticercoids by otherwise 'passive' hosts has recently been supported by Lackie (1981) and Richards and Arme (1985). Lackie (1981) re-assessed the possible reasons for the occasional cell debris noted in conjunction with the parasite surface, and concluded that occasional lysis of granulocytes did occur, but there was no flattening of cells (e.g. as in early capsule formation), neither was lysis sufficient to elicit a full response by host cells. Richards and Arme (1985) also noted cellular debris and haemocytes in association with (usually) the anterior end of the cysticercoid. They observed phagocytosis of microvilli, although no haemolymph precipitation or melanisation was recorded. Adhering haemocytes were most often found in batches of 'surface stressed' cysticercoids which produced large and small blebs (see Appendix B) when dissected in saline. Haemocytes are known to be attracted to negatively charged objects, and it was suggested that some 'fault' in the polyanion-rich glycocalyx surrounding the cysticercoid had altered the charge, making it more attractive to haemocytes (and directly or indirectly causing the observed blebs). This alteration could provide the first tier of recognition, resulting in the initiation of the cellular response. The reason for the limited response was suggested as simply a localisation of these imperfect areas at the anterior and thus they were insufficient to elicit full encapsulation. As the

glycocalyx is produced by the tegumentary cytons (Oaks and Lumsden,1971; Ubelaker,1980; Richards and Arme,1984b) an impairment in one or two cytons could alter the properties of a small area of the cysticercoid glycocalyx. Finally, the aggregation of haemocytes at the anterior end noted also by Ubelaker (1970b) was explained in terms of the immediate and rapid growth of this region after scolex withdrawal. Imperfections in this region could therefore result in it being different from the rest of the cysticercoid surface - implying biochemical differences over the cysticercoid surface. Thus, only impaired 'surface stressed' cysticercoids appeared subject to this limited cellular response of the host. Heyneman and Voge (1971) studied Tribolium confusum infected with H. diminuta, H. microstoma and H. citelli simultaneously. They found that all parasites developed normally in spite of the presence of the others. They also noted that all cysticercoids became coated with host cells, 1 - 3 layers deep, during days 3 - 5 of the infection. By day 6, however, the host cells had disappeared and no further encapsulation occurred. It was not until the work of Lackie (1976,1979,1981a,b,1982) that reasons for this 'lack of response' were investigated. In 1981, Lackie studied seven species of insect which were tested as hosts for H. diminuta. Only Tenebrio molitor larvae and Schistocerca gregaria were suitable hosts, with Periplaneta americana producing only c. 15% unencapsulated cysticercoids. Using transplantation of cysticercoids and tissue between the above host species , Lackie was able to show that haemocytes of Periplaneta americana recognise and encapsulate tissue from 4 of 6 donor species tested whereas the

haemocytes of *Schistocerca gregaria* recognise and encapsulate tissue from only 1 of 6 donor species. It was suggested that the parasite adsorbed some host component onto its surface, so that it was effectively camouflaged against the host immune system. The ability of *H. diminuta* cysticercoids to lyse small numbers of haemocytes was also noted - but rejected as a major protective mechanism. Camouflage with haemocyte debris was also ruled out, as time-course experiments showed that insufficient debris could accumulate in a short enough time to provide a 'protective layer'. The transplantation experiments indicated that the surfaces of tissues of those insect species in which *H. diminuta* is not encapsulated, have sufficiently similar properties not to stimulate encapsulation when transplanted *inter se*. Therefore it was concluded the parasite either possessed a surface which was inherently compatible with its host tissue surfaces or it was able to inhibit encapsulation.

Other mechanisms of 'evading' the immune response of the host have been described in other species. Lackie (1975) described the production of a capsule by the parasite *Moniliformis dubius* in *Periplaneta americana*, This capsule gradually became loose, allowing the parasite within to grow and develop while haemocytes adhered to the outside. Some parasites occupy priviliged sites e.g. nerve ganglia (Salt,1970) and even *H. diminuta* cysticercoids are found 'unharmed' in the legs of *P. americana* where space precludes full encapsulation. Finally, many parasites can prevent capsule formation by moving and therefore disrupting haemocyte aggregation.

CHAPTER 3

THE MECHANISMS AND KINETICS OF MEMBRANE TRANSPORT

A. INTRODUCTION

The study of biological membranes and their function is an important theme in contemporary biology. The cell membrane has many functions, but of special relevance to this discussion is the control of the movement of molecules from outside to inside and *vice versa*. Membrane transport has, in the past, been viewed in two ways. The first has aimed at understanding the mechanisms involved and providing evidence for the theories associated with the phenomenon. The second involved characterising the kinetics and specificities of a particular system. In the latter, much data are available for vertebrate intestinal mucosa (eg.Crane,1970),erythrocyte membranes, (for example, Lefevre,1961; Stein,1967; Widdas,1954,55; Lieb and Stein,1970), epithelia of all kinds e.g. frog bladder (Skou,1965; Katz and Epstein,1968), muscle (Narahara and Ozand,1963), ascites tumour cells (Eddy,1965,1968), liver and kidney (Willis,1966,1968) bacteria (e.g. Kabak,1969; Kundig, Kundig, Anderson and Roseman,1966) and endoparasites (see Pappas and Read,1975; Pappas,1984). Within endoparasites, most data are available for tapeworms. Cestodes have no digestive tract and show no embryological evidence of ever having possessed one. Consequently all nutrients are absorbed trans-tegumentally. The structure of the tegument of

Hymenolepis diminuta cysticercoids has been described in Chapter one and bears a similarity to that of the adult tegument, which has been extensively studied both morphologically and biochemically. The prominent features of the tegument - the microtriches and microvilli have been credited with many functions, including protecting the tegument from attack by host cells and enzymes (Lee,1972), a means of attachment or anchorage to the host (Lee,1972) and a role in nutrition (Read,1962). They are thus, in many ways, analogous to the microvilli of the vertebrate intestine. For the most part, evidence for differences and similarities between adult and cysticercoid tegument is experimental and is discussed in detail later. However, inferences on the exact mechanisms involved are derived largely from transport work on other systems e.g. mammalian intestine, tumour and blood cells etc.

It is well established that the lipid layers of all membranes are a major factor in restricting their permeability. Generally, substances which are lipid soluble tend to move through the membrane more easily than water-soluble substances. Original models of membranes e.g. that of Davson and Danielli (1943) suggested that proteins were confined to two narrow bands on either side of the lipid bilayer. Polar side-chains of proteins were thought to interact with surrounding water molecules and with the polar regions of underlying lipids. However, it is now known that proteins can be orientated to expose both their hydrophobic and hydrophilic surfaces. The idea of proteins embedded in a lipid matrix was first suggested by Ponder (1948) and later expanded by Singer and Nicholson (1961) who used the term the 'fluid mosaic model' . This model

envisages proteins of various sizes partially or totally embedded in the membrane, and it has been suggested that some of these proteins are trans-membrane. Transmembrane proteins may be the 'carriers' for substances that are transported by mediated processes across the membrane. An important feature of membranes is the variation that can occur in their fluidity. Some may remain fluid, even below the transition temperature of the lipid, and there sometimes appear fluid areas in an otherwise 'rigid' membrane. These fluid areas contain proteins which override the natural tendency of the lipids to gel below their transition temperatures and therefore the molecules nearest the protein remain fluid at abnormally low temperatures. These current thoughts also shed light upon other properties of membranes e.g. the mobility of membrane components. Mobility is now known to be greatly influenced by fluidity and therefore the dramatic effect of temperature (and thus fluidity) on the activity of transport proteins can be explained. It must be emphasised, however, that the concept of a 'standard membrane' is invalid. For example, animal membranes tend to have less lipid than protein and the protein:lipid ratio varies considerably between membranes from different sources. Perhaps the membrane in which most variation is found, however, is the plasma membrane. This is not surprising as it forms the barrier between inside the cell and outside, and may therefore be particularly specialised for certain functions.

B. TYPES OF MEMBRANE TRANSPORT

Studies on the rates of passive movement of solute across membranes have provided significant information on the chemical and physical nature of these barriers. Quantitative analysis of permeability phenomena is complicated by the simultaenous movement of solute and solvent molecules, but methods have been devised which circumvent this and facilitate the quantitative comparison of the permeability characteristics of different membranes. There exist many types of transmembrane transport, which are readily distinguished by their characteristics. The most important include: Simple diffusion, Mediated uptake i.e. facilitated diffusion and active transport, Mediated efflux, Counterflow, Endocytosis and Mass flow (solvent drag).

1. SIMPLE DIFFUSION

The presence of a concentration gradient of solute molecules across a membrane can result in a net movement of solute molecules in the direction of the gradient. This is simple diffusion, described by Fick's law, which states that the rate of diffusion of a solute is directly related to the concentration difference on either side of the membrane, with solute always moving from a region of high concentration to an area of lower solute concentration.

In diffusion, the uptake rate of a solute is linear with respect to concentration over 'physiological ranges' and there is no inhibiting effect of structurally similar solutes or metabolic poisons. Diffusion has been shown to be temperature-dependent in erythrocytes and tumour cells (LeFevre,1957), and current explanations suggest that this is because solute molecules pass through the lipid hydrocarbon

region of the membrane, and it is this region which varies in fluidity with temperature (Barrett,1981). The degree of fluidity of a membrane will influence not only the transport systems but also the properties of membrane-bound enzymes, and it is an important factor to consider when studying endoparasites which encounter both homio- and poikilthermic hosts. Simple diffusion requires no energy expenditure on the part of the cell, rather, it is the kinetic energy of the molecules themselves which account for this phenomenon.
There are, however, only a few examples of simple diffusion providing the main route of nutrient entry in helminths (see Pappas and Read,1975). In adult H. diminuta, diffusion accounts for the entry of a limited number of nutrients and usually then only at high concentration. However, it is unlikely that the artificially high concentrations experienced by the parasite in vitro are ever experienced in vivo. Examples of substances entering adult H. diminuta by diffusion include glycerol (Pittman and Fisher,1972; Uglem, Pappas and Read,1974), acetate and palmitate (Arme and Read,1968; Chappell, Arme and Read,1969), some water-soluble vitamins, uracil, adenine and hypoxanthine (MacInnis, Fisher and Read, 1965), and amino acids such as proline, histadine, phenylalanine, tryptophan and tyrosine (Kilejian,1966a; Woodward and Read,1969).

2.MEDIATED UPTAKE

Mediated uptake differs from simple diffusion in a number of ways. Perhaps the most important characteristic is that saturation kinetics are observed, which in turn implies the presence of a carrier. In this study, the classification used by Pappas (1973) for the identification of parts of the carrier

shall be used. The carrier is also known as the locus and the precise point on the carrier where binding of the substrate occurs is known as the site. Thus for the transport of a particular substance, there may be one locus but it may have several sites (e.g. the uptake of uracil by adult H. diminuta; MacInnis et al.,1965). A locus is defined as the molecule which binds the substrate and transports it across the membrane to release it on the other side. It is probable that these carriers are proteinaceous, as a high degree of specificity is a characteristic of only a few biological molecules possessing the necessary variability in structure. The current view of proteins in membranes suggests that most carriers are large and span the membrane. Although the presence of loci have been demonstrated, they have not been fully identified or isolated in any system.

There are several theories as to the mechanism of carrier-mediated transport, although all agree on the presence of two steps as suggested by Stein (1967). The first involves binding of the substrate to the loci and the second its translocation across the membrane. Intrinsic proteins, which span the membrane have been implicated and transmembrane proteins in H. diminuta have been demonstrated by freeze fracture studies (Lumsden and Murphy,1980; Conder, Marchiondo, Williams and Anderson,1981). Several proteins involved in mediated transport have already been identified as spanning proteins (e.g.Kabak,1970), some of them with substantial glycopeptide segments which could stabilize them in a single orientation in a membrane. Such spanning proteins almost certainly provide a controlled pathway for permeants through the lipid barrier but there is no direct information on how this is achieved.

Loci, or binding sites on loci, show specificity for the compounds they transport and can therefore be inhibited by similar substances. Mediated systems generally show a high degree discrimination between, for example, D and L forms of a solute, but may also frequently transport closely related substances. These latter substances therefore compete with one another for the uptake site. Some inhibitors have the ability to attach to the uptake locus but are not able to be transported themselves. This is referred to as non-productive binding. Non-productive binding also occurs in the presence of substances capable of altering or blocking specific functional groups in proteins e.g. dinitrofluorobenzene which blocks NH_2 groups. In mediated uptake there is a finite number of loci and therefore the system has a defined capacity and can be saturated. As a result, uptake is not linear with respect to concentration of the substrate. The process of substrate binding, transport across the membrane and release of substrate on the other side all act to limit the rate of transport. An analogy can be made between transport and enzyme catalyzed reactions, where the rate of breakdown of the enzyme substrate complex also has a limiting effect. This comparison has led to the application of the Michaelis-Menten equation for use in transport studies, with the result that many terms and kinetic analyses between the two systems are similar. There are many important differences between the two systems, however, the substrate is transported and not altered in any way, and also carrier proteins, unlike many enzymes, have not yet been isolated or identified completely. Michaelis-Menten kinetics therefore allow the measurment of certain parameters,but do not reveal information about the actual mechanism of transport apart from the presence of an adsorption step.

Mediated transport can occur in either direction across a membrane, and the affinity of a substrate for its locus may alter, depending upon which direction the substrate is being transported. Mediated uptake therefore strictly represents the net result of simultaneous influx and efflux. In practice, it has proved difficult to separate influx from efflux and therefore measurements taken over short time periods ('initial rate studies') are regarded as representing the unidirectional movement of solute. Studies on the initial rate of uptake of a solute thus allow the kinetics to be accurately analysed by the Michaeli-Menten equation, as well as avoiding the complications resulting from diffusion associated with longer incubations. The initial rate period refers to the time over which uptake of a particular substrate is linear. This must be determined before any other uptake work is carried out. The deviation from linearity that often occurs after this initial time may be due to substrate efflux, substrate depletion,cell death and/or alterations in other physico-chemical parameters e.g. pH and O_2/CO_2 concentrations. Therefore, since the early work of Read _et al_. (1963) the use of initial rate studies has been widespread, ensuring that over a short period of time the amount of substrate accumulated within the 'cell' is small enough for efflux to be ignored in kinetic analysis.

There are two main types of mediated transport, facilitated diffusion and active transport. Both types possess similar properties with the important exception that active transport occurs against a concentration gradient. This therefore allows for the accumulation of solute inside the cell, and transport may continue even when the solute is in greater concentration inside the cell than inthe outside medium.

3. MEDIATED EFFLUX

Further studies on mediated uptake have highlighted the fact that under certain conditions mediated efflux (sometimes known as exchange diffusion) is established. This represents the exchange occurring when a tissue, which has been incubated in labelled solute until equilibrium is reached, is then transferred to unlabelled solute at the same concentration. A resulting mediated exchange occurs with no net transport of solute. Additional substrate may be added on either side of the membrane but the result is the same, a rapid unidirectional flux of labelled solute occurs in exchange for unlabelled solute. This is an important consideration during long term uptake experiments and it also clearly indicates that under conditions of zero net flux of substrate there is a rapid flow of molecules across the membrane in both directions.

4. COUNTERFLOW

Another phenomenon operating from inside to outside of the cell is counterflow or counter transport. It is , essentially, competitive mediated efflux. To demonstrate this a tissue is equilibrated with respect to one of two substrates which are trasnported by the same carrier (substrate A). When a second substrate (B) is added to the medium there will occur a net flux of substrate A into the medium. This is because initially substrate B will only compete with the flow from the outside to inside the cell, working with its concentration gradient until it too reaches equilibrium. The experimental evidence for counterflow and mediated efflux is therefore additional evidence for the presence of a mediated transport system.

5. ENDOCYTOSIS

Endocytosis has been described in the tegument of some adult pseudophyllideans, metacestodes, trematodes and acanthocephalans. Adults of Schistocephalus solidus and plerocercoids of Ligula intestinalis have both been shown to endocytose ruthenium red which traversed the tegument within 6 min (Hopkins, Law and Threadgold,1978; Threadgold and Hopkins, 1981). Also endocytosis has recently been demonstrated in the cysticercus tegument of Taenia crassiceps (Threadgold and Dunn, 1983; Dunn and Threadgold,1984). Macromolecules of host origin have been located in the bladder fluids of some cyclophyllidean metacestodes e.g. Echinococcus granulosus, T. crassiceps and the cysticercus of T. taeniaeformis (Coltorti and Varela-Diaz,1974, 1975; Hustead and Williams,1977a,b).

Adult H. diminuta was reported to absorb ferritin, colloidal thorium dioxide and colloidal carbon by "transmembranosis" (Rothman,1967), but these observations could not be confirmed in a later study (Lumsden, Threadgold, Oaks and Arme,1970).

6. MASS FLOW (SOLVENT DRAG)

Mass flow is a rather specialised form of entry and occurs in, for example, the vertebrate kidney, where a large solvent flow through a membrane also transports some solutes against their concentration gradients. The most important implication of this process is the transport of water brought about when transporting ions e.g. Na^+, which cause subsequent influx of

water due to osmotic forces. The work of Podesta and Mettrick (1975,1976) has provided evidence for the absorption of ions and water from the surrounding medium by adult H. diminuta. They also suggested that this mass flow was linked to the absorption of glucose, although this has not been confirmed.

C. THE KINETICS OF MEDIATED TRANSPORT

1. GENERAL

In carrier-mediated transport, the analogy between solute transport and enzyme catalysis provides methods for the kinetic analysis of data. Thus, as noted above, the carrier and substrate are analogus to the enzyme and tis substrate. In practice, as noted above, the process of transport is usually considered over a short time period so that data can be analysed without having to consider efflux, which can be regarded as negligible. Neame and Richards (1972) outlined the re-arrangement of the Michaelis-Menten equation that subsequently allows the calculation of the two most important kinetic parameters in transport - the K_t (the transport constant) and the V_{max}(the maximum velocity). This equation is given below;

$$V = \frac{V_{max} \cdot (S)}{K_t + (S)}$$

V is the rate of uptake of a solute concentration S, and V_{max} is the maximum rate of uptake of that solute. V_{max} is also a measure indirectly of the number of loci per unit biomass. K_t- the transport constant- is defined as the solute concentration at which the transport rate reaches half maximal velocity, and it is a constant for a given solute in the presence of a given carrier.

Also, the K_t represents the affinity of a substrate for its uptake site. Therefore, when measured under initial rate conditions a mediated transport process essentially follows Michaelis-Menten kinetics, and a plot of initial uptake rate (V) against substrate concentration (S) will give a rectangular hyperbola. The K_t and V_{max}, which characterise membrane transport, can be estimated from this plot but, in general, transformation of the data into a linear form and the subsequent algebraical or graphical fit of a straight line to the data is the most common procedure. There are several derivations of the Michaelis-Menten equation which produce a linear function, all of which can be plotted graphically. The derived equation of Lineweaver and Burk (1934) is given below;

$$1/V \doteq \frac{K_t}{V_{max}} \cdot 1/S + \frac{1}{V_{max}}$$

This is a double reciprocal graph represented by a plot of 1/V against 1/S, as shown in Figs.4.2/3(Chapter 4). Although this transformation is one of the most frequently used in the study of helminth membrane transport, it does have some drawbacks. The most important was highlighted by Dowd and Riggs (1965) who showed that this plot consistently over-emphasised the samllest and most variable values of V, thus giving unreliable estimates for the kinetic parameters. Other common transformations include Woolf (1932), Eadie (1942), Hofstee (1965) and Wilkinson (1961). However, all of these transformations have considerable bias under certain conditions (see Craig,1983, for a discussion of these conditions). For the purposes of comparison, however, the Lineweaver-Burk transformation is

used in this study as the majority of transport by H. diminuta has been analysed in this way.

D. THE EFFECT OF THE UNSTIRRED WATER LAYER UPON UPTAKE

The unstirred water layer is defined as the extracellular fluid lying between and above the microtriches. To date, in the majority of absorption studies, the unstirred water layer has not been taken into consideration, and this is a potential source of error. The effects of the unstirred water layer have been shown to lead to an underestimation of the passive diffusion component, K_d, and to an overestimation of the Michaelis constant, K_t. Podesta (1977a) contended that more accurate estimates of the kinetic parameters were obtained if absorption experiments were carried out with stirred incubation media. The rates of uptake of glucose, galactose and alanine across the brush border membrane of adult H. diminuta have been shown to be dependent on the thickness of the unstirred layer, and, with the more water-insoluble compounds particularly, the fluxes can be overestimated (Podesta, Stallard, Evans, Luisser, Jackson and Mettrick,1977). Podesta and his co-workers have also found a hitherto undiscovered diffusion component in the uptake of methionine by adult H. diminuta, when stationary layer effects have been taken into account (Luisser, Podesta and Mettrick,1982). However, the work of Smithson, Millar, Jacobs and Gray (1980) on rat gut, and Murphy and Lumsden (1984) on H. diminuta, have indicated that the effect of the unstirred layer has been considerably overestimated. In particular, Murphy and Lumsden (1984) strongly critised the use of ^{14}C mannitol as a marker of extracellular space, as this was shown to be absorbed by adult H. diminuta. Podesta (1977a)

also used other non-absorbable markers obtaining similar results, but it was suggested that insufficient rinsing and blotting of worms after incubation gave these misleading data. Thus, using the precise rinsing and blotting procedures of Read *et al*.(1963) and many other workers since, Murphy and Lumsden were able to reduce the calculated magnitude of the unstirred layer considerably. Smithson *et al*.(1980) calculated the size of the diffusion barrier in vertebrate intestine using the dimensions calculated by previous workers. They discovered that the diffusion barrier would occupy half of the total luminal volume, and considerably more if all morphological convolutions (i.e. microvilli) were taken into account. Thus, if the rat intestinal unstirred layer occupies such a space and each adult *H. diminuta* has an additional 100μm, then very little remains for free solute movement. Murphy and Lumsden (1984) do not dispute the existence of this unstirred layer, but only its magnitude. A comment was also made on the movement of fluid between the microtriches of *H. diminuta*. The similarity between the microfilamentous core of microtriches and the actin core of intestinal microvilli suggest that microtriches may also have the motility shown in microvilli (Mooseker,1976). Thus, motile microtriches could stir the fluid adjacent to their surface. Finally, in several recent studies, unstirred layer effects have been investigated and in all cases there were no significant differences in results obtained from "stirred" and "unstirred" experiments (Craig,1983; Jeffs,1984 ; this study).

E. FEATURES OF CARBOHYDRATE TRANSPORT

It is clear that a number of features are common to nearly all carbohydrate transport systems studied. Uptake is predominantly active, especially for monosaccharides, and exhibits temperature dependence, stereospecificity and other characteristics associated with mediated transport.

Perhaps the most important feature of sugar transport is its association with sodium ions. This was observed long before even the structure of the plasma membrane had been determined, and early work on carbohydrate transport kinetics highlighted the importance of ionic composition of the media (e.g. Riklis and Quastel,1958; Csaky,1963; Csaky and Thale, 1960). Most types of Na^+-dependent transport are related to the movement of their co-substrate, which is against an electrochemical potential difference. The energy for this process is derived from the asymmetric distribution of Na^+ and K^+ across the cell membrane and this fact has shed light upon other active cation transport systems, which are abundant in animal cells. It must be noted that cations other than Na^+ play a role in coupled transport, although not to the same extent , especially in helminth systems. Much of the hexose absorption in *H. diminuta* is absoultely dependent on the presence of Na^+ (Dike and Read,1971; Read, Stewart and Pappas, 1974; Pappas, Uglem and Read,1974; Starling,1975), although Pittman and Fisher (1972) presented evidence for Na^+-independent transport of glycerol in adult *H. diminuta*, which was later confirmed by Uglem (1974). Other cestode transport agencies which appear to depend on the presence of $Na+$ in the bathing

medium include some amino acids, glycerol, uridine and thymidine (Podesta,1981a).

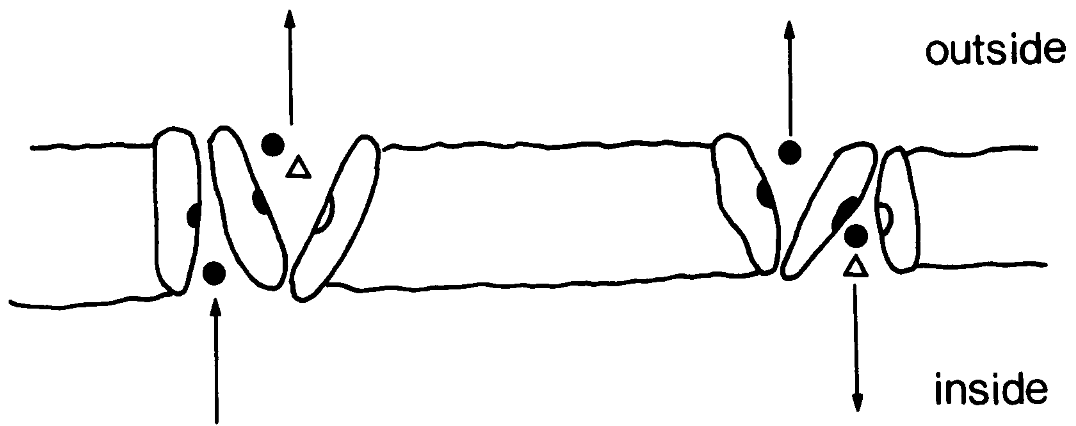
1. THEORIES OF Na^+-DEPENDENT TRANSPORT

There are two main theories of Na^+-dependent transport; the Na^+-gradient hypothesis and the theory of convective coupling.

The Na^+-gradient hypothesis (first suggested by Crane, 1960) assumes that a carrier is involved in a reversible reaction whose asymmetry is maintained by the asymmetric distribution of ions (Na^+ and K^+ between the cell and the extracellular fluid). Thus, efflux from the cell is influenced by local intracellular Na^+ concentration, just as entry of solute into the cell is influenced by the local extracellular Na^+ concentration. Solute can therefore be accumulated against a concentration difference simply because the intracellular Na^+ concentration is lower than that in the surrounding medium. The active extrusion of Na^+ *via* the Na^+-pump maintains a low intracellular Na^+ concentration and this establishes the source of potential energy which is used in the transport of various substrates. Therefore, the theory postulates an indirect link of solute transport with metabolic energy, as the latter is used in the Na^+-pump which maintains low intracellular Na^+ concentration. How is uptake of a substrate linked to the influx of sodium? Models have been used to describe the mechanics of this phenomenon (e.g. Crane,1960; Schultz and Curran,1970; Uglem,1976), but the model of Uglem (1976) is particularly relevant, as it refers to glucose uptake in adult *H. diminuta*. Membrane carrier proteins

are thought to consist of 4 or more protein subunits that bridge the membrane and provide fluid-filled channels through the membrane. A change in conformation, or spatial relationship of these subunits, could 'force' a molecule through the channel and out of the other side. Uglem proposed that two channels exist side by side, each consisting of 6 protein subunits and providing an influx and efflux channel (see Fig.4.1). Sodium and glucose molecules were thought to impinge on the active centre of the influx channel and, at the same time, Na^+ on the inside would bind to the active centre of the efflux channel. The carrier is asymmetric in that the active centres of the channels are structurally separate and that binding of Na^+ to the efflux channel active centre does not require glucose. The complementary alteration in the subunits which compels glucose and Na^+ through one channel is accompanied by another which ejects Na^+ from the cell. Uglem also noted that several predictions could be made from the model, which could be tested. Since the model requires the presence of Na^+ on both sides of the membrane, inhibition of Na^+-binding inside the cell should inhibit the coupled influx of Na^+ and glucose. Also the whole system should be electroneutral i.e. charge compensating transfers are taking place. In 1980, Uglem and Prior revised the model to include Cl^- fluxes and they suggested that Cl^- as well as Na^+ and glucose was attracted to the active centre of the influx channels, while Na^+ alone is attracted to the efflux channel. The operational features of this model appear to be consistent with the present data on adult <u>H. diminuta</u> carbohydrate

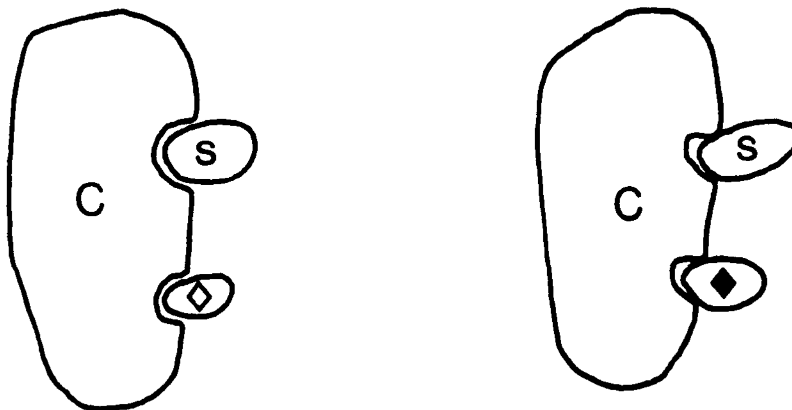
Figure 3.1



A DIAGRAM OF THE GLUCOSE TRANSPORT SYSTEM FOR *Hymenolepis diminuta* PROPOSED BY UGLEM (1977).

The carrier consists of six protein subunits forming two separate channels across the membrane. ● = Na^+, △ = glucose. The changes in the carrier which transport glucose and Na^+ through one channel are accompanied by complementary changes in the other channel which ejects Na^+ from the inside.

Figure 3.2



A DIAGRAM SHOWING THE PROPOSED METHOD OF K^+ INTERFERENCE WITH CARRIER FUNCTION.

C = carrier, S = substrate, ◇ = Na^+ and ◆ = K^+. The K^+-loaded carrier causes conformational changes resulting in a reduced affinity for the substrate (after Crane, Forstner and Eicholz,1965).

transport and accomodate all previous observations on this organism. However, there are features of the available data that are not consistent with the Na^+-gradient hypothesis, and this has prompted Podesta and his colleagues to speculate on another mechanism for Na^+-dependent transport, namely convective coupling, which was originally proposed by Naftalin and Curran, in 1974. Naftalin and his co-workers proposed a diffusive convective model for galactose transpot in rabbit ileum (Naftalin and Curran,1974; Naftalin and Holman, 1974,1975; Holman and Naftalin,1975; Simmons and Naftalin, 1976a,b). They noticed an increase in exit permeability of the brush border membrane to galactose as tissue Na^+ concentrations were increased, and came to the conclusion that galactose transport was correlated more closely with intracellular Na^+ concentrations (and Na^+-pump activity) than with the gradient of Na^+ across the brush border as predicted by the Na^+-gradient hypothesis. They proposed that Na^+ is pumped across the inward facing membrane into the lateral extracellular spaces, which separate the epithelial cells. This results in an osmotic gradient between the extracellular channels separating the cells and the fluid abthing the outward facing brush border membrane (i.e. the mucosal fluids). Water then flows from the mucosal fluid into the lateral extracellular channels. This coupling between water and solute transport across the brush border membrane could be achieved by convection i.e. solute entrained in a stream of fluid traversing the brush border through narrow channels, the ultimate driving force being the Na^+-pump located on the inward facing membrane of the epithelial cells. Therefore coupling between Na^+ and solute transport is achieved indirectly through convective transport or solvent drag across the brush border. The convective

coupling hypothesis requires that water crosses the brush border membrane, and in H. diminuta, all water transport across the syncytium must occur across the 'brush border'. However, the difficulty of establishing the validity of the convective coupling hypothesis as a mechanism for non-electrolyte transport by H. diminuta, is the paucity of information regarding water transport in this organism (Podesta,1980a).

2. FEATURES OF CARBOHYDRATE TRANSPORT BY H. diminuta

Glucose uptake by adult H. diminuta has been shown to be temperature-dependent, saturable, stereospecific and inhibited by structurally similar monosaccharides (Phifer,1960a,b). Although most uptake is active, there is a small diffusion component which is not inhibited by similar monosaccharides (McCracken and Lumsden,1974a). Glucose was also shown to be accumulated against a concentration gradient (Phifer, 1960b; Pappas et al.,1974). The first studies on carbohydrate uptake in H.diminuta were carried out by Phifer (1960a,b,c). To provide evidence for the energy requirements of uptake a number of metabolic inhibitors were used. Iodoacetate and 2,4-dinitrophenol required worms to be preincubated in them before an effect was noted and Phfier suggested that a period of time was required to target the appropriate system. Two important publications on the ion requirements of glucose, methionine and glycerol uptake in adult H. diminuta have been published (Pappas, Uglem and Read,1974; Uglem, Pappas and Read, 1974). Unusual results in the former study led to the first description of glucose efflux. It was noted that when worms were incubated in Na^+-free KRT containing glucose,there was

a decrease in glycogen levels but no increase in tissue glucose as it was transported in the direction of the prevailing Na^+-gradient i.e. out of the worms. The definitive paper on glucose and sodium fluxes in adult H. diminuta showed that by replacing Na^+ in the medium with K^+, $Tris^+$ or $choline^+$, a large decrease in glucose influx occurred (Read, Stewart and Pappas,1974). Using $Tris^+$ as a replacement cation, the glucose influx rate was significantly higher than when using K^+, lithium or $choline^+$. When K^+ was used as replacement cation, with Na^+ in suboptimal concentrations, there was no effect on the glucose uptake rate. The effect of Na^+-deletion upon glucose influx was shown to be totally reversible, but only after 10min or more. When observing glucose influx as a function of glucose concentration in media with Na^+ concentrations of 154, 50, 25 and 10mM, there appeared only a slight decrease inthe V_{max} with decreasing Na^+ concentrations. Thus glucose influx as a function of Na^+ concentration displayed Michaelis-Menten kinetics. Another feature of glucose and Na^+ influx was that they exhibited first order kinetics for at least 2min. Coupling coeffecients were inversely related to Na^+ concentration and independent of glucose concentration over a 50-fold range. In the absence of glucose, Na^+ influx occurred by a mediated process which was unaffected by phlorizin.

In 1977 Pappas and Hansen published data on chloride sensitive glucose transport in adult H. diminuta. It was noted that anions such as bicarbonate$^-$, lactate$^-$ and acetate$^-$ could not readily replace Cl^- in glucose transport. The effect of Cl^- deletion was readily reversible and glucose in the medium enhanced $^{36}Cl^-$ influx, but in Na^+-free media the presence of glucose had no effect. H. diminuta did however, accumulate glucose against a concentration gradient when

Cl^- was replaced by anions such as acetate$^-$, bicarbonate$^-$ and nitrate$^-$ although not to the same extent. The conclusion reached was that Cl^- influx was coupled to the influx of Na^+ and glucose, although it was not known if uptake occurred via the same carrier (as postulated for glucose and Na^+).

In 1978, Uglem and Love attempted to estimate the coupling coeffecients for glucose and sodium in H. diminuta. Using pulse-chase techniques with $^{22}Na^+$ and ^{3}H-glucose, estimates of efflux rates following uptake were obtained. The effect of Na^+ concentrations on efflux rates was also determined. It appeared that glucose efflux was by counter-transport. This suggested that a coupling coeffecient (i.e. Na^+ influx/glucose influx) might be underestimated because of this phenomenon. Therefore, coupling coeffecients calculated in Na^+ concentrations of 5, 25 and 50mM were 2.09, 2.24 and 1.89 respectively, suggesting no dependence of the coupling process on Na^+ concentration in the ambient medium.

Uglem (1976) published evidence for a Na^+ exchange linked to glucose transport in H. diminuta. It was noted that loss or efflux of glucose into the media was enhanced by preloading the worms with glucose and also by removing Na^+ from the incubation medium. The presence of an unstirred layer, however, did not totally favour the reabsorption of leaked glucose, as some was lost to the medium. Glucose leakage was shown to stimulate influx of labelled sodium, but this was balanced by simultaneously increased Na^+ efflux. By using phlorizin, both inward and outward fluxes of labelled sodium were inhibited, and this supported the idea that efflux of sodium occurred by countertransport. The model proposed by Uglem (1976) for glucose transport incorporated all the

salient features as described by in vitro experiments.

In 1975, McCracken and Lumsden investigated the action of phlorizin on glucose uptake in *H. diminuta*. Read (1966) had shown that phlorizin was not itself transported by the worm and it was suggested that its site of action was at the worm surface. A K_i value of 0.015mM was calculated for phlorizin which indicated a far greater affinity for the monosaccharide transport site than glucose (with glucose having a K_t of 0.74mM in this study). They also reiterated the evidence for two mechanisms of glucose uptake; a phlorizin insensitive diffusion element and a phlorizin inhibited mediated portion; it was suggested that the action of phlorizin was to inhibit the Na^+-dependent component. Phloretin, the aglycone of phlorizin, had no effect on glucose uptake, neither did it interfere with phlorizin inhibition. It was proposed that phlorizin was recognised by the monosaccharide uptake site due to its glucose moiety and can bind with this site but is not transported. The formation of bonds between the phlorizin molecule and regions on the membrane near to the carrier has been suggested as immobilizing the carrier and preventing transport (Caspary, Stevenson and Crane,1969). Uglem and Love (1977) also investigated some properties of phlorizin inhibition. They found that phlorizin inhibited glucose uptake over a broad pH range (4.6 - 8.5). A reduction in Na^+ concentration in the medium had the effect of increasing the K_t of phlorizin i.e. lowering its affinity for the uptake site. As a result, it was suggested that phlorizin binding was Na^+-sensitive because the interaction of phlorizin with the uptake site was via its sugar moiety, thus rendering it sensitive to Na^+ concentration.

Podesta and colleagues in 1977 investigated the effect of ouabain on active non-electrolyte uptake in H. diminuta. They worked with both whole and sliced adult worms in vitro. It was noted that ouabain had no effect, as they expected, on the uptake of glucose, galactose or alanine in whole worms. However, concentarions of 1.0, 0.1 and 0.01mM ouabain were sufficient to inhibit uptake of glucose, galactose and alanine in tissue slices. These results suggested a number of possible effects of ouabain on uptake systems. The fact that worms incubated in Na^+-free media took more than 10min to reach control levels when preincubated in media containing Na^+, suggests that the site of Na^+ action is not on the external surface, but at the basal plasma membrane of the syncytium. Na^+ is therefore presumed to be pumped out across the basal plasma membrane of the syncytium. Ouabain is thought to act by inhibiting the Na^+/K^+ ATPase, which is directly involved in generating the energy for the Na^+-pump. However, according to the ion gradient hypothesis, if the action of the Na^+-pump was inhibited by half. then this would reduce the steepness of the gradient and slow down the transport of solutes. This appears not to be the case, as different amounts of ouabain were needed to inhibit the uptake of different subtrates. It was concluded that, although the tegument is polarised with respect to the action of ouabain, hexose uptake must consist of both primary (driven by metabolic energy) and secondary (driven by the electrochemical gradient) active transport (Podesta,1980).

Previously, intrinsic tegumentary phosphohyrolases were shown to be present in H. diminuta by detecting the products of phosphate-ester hydrolysis (Arme and Read, 1970; Dike and

Read,1971). By using fructose phosphates, Arme and Read demonstrated surface hydrolysis yielding free fructose in the medium. In 1971, Dike and Read presented evidence for the hydrolysis of glucose-6-phosphate by a tegumentary phosphohyrolase, to give glucose, which was then transported. The possibility of a kinetic advantage in the uptake of hydrolysis products was also investigated, and it was suggested that the proximity of the monosaccharide transport system and surface phosphohydrolases would confer an advantage in the absorption of the monosaccharide liberated by hydrolysis (Dike and Read, 1971).

The effect of ambient pH changes on glucose absorption by H. diminuta was described by Lesser, McCracken and Lumsden (1975). It was found that a pH range of 5.8 – 8.5 could maintain control levels of glucose uptake, and that this range was broader than that for glucose absorption by the intestine of the rat host (pH range 6.7 - 7.5). It was suggested that, since the presence of worms results in acidification of the intestine (Podesta and Mettrick,1975), the broad pH range for the worm allows it to compete successfully with the host for dietary glucose.

In 1966, Overturf studied glucose transport in vivo and compared it with parallel experiments in vitro. He found that data from in vivo work varied only slightly from invitro studies, but pointed out the limitations of in vivo perfusion experiments. Difficulties arose where both worm and host were removing and adding substances to and from a common pool, so that at a given time, the concentration of substrate available to either animal was unknown. Podesta and Mettrick (1974) extended the work of Overturf to study the components

of glucose uptake *in vivo* in both *H. diminuta* and the intestine of the rat host. Glucose uptake was shown to consist of three components: active transport, diffusion and solvent drag. There appeared to be no saturation of glucose uptake, nor was there any effect of luminal Na^+ concentrations on glucose uptake. From these results they concluded that the role of Na^+ in glucose absorption was intracellular. By increasing the concentration of H^+ (decreasing the pH) in the perfusion fluid, glucose and fluid transport were increased (a direct result of increased solvent drag) and they concluded that this indicated that the worm relied heavily on solvent-coupled glucose transport under near physiological conditions (Podesta and Mettrick,1974).

Galactose uptake by adult *H. diminuta* also occurrs by mediated uptake and both glucose and galactose appear to enter via the same site, as they are reciprocally competitive inhibitors (Read,1961a). Galactose uptake was shown by Read (1967) to be saturable, concentrative and inhibited by phlorizin. The specificity of carbohydrate transport in *H. diminuta* appears rather narrow, with only a small group of closely related monosaccharides transported. Although galactose is readily transported by *H. diminuta* it does not appear to be metabolised (Komuniecki and Roberts,1977b) and therefore does not result, ultimately, in an increase in glycogen levels. When incubating *H. diminuta* under identical conditions with glucose as substrate, there was a 5-fold increase in glycogen level. Komuniecki and Roberts (1977b),however, identified enzymes for galactose utilization in the adult worm and their paper points out the bias by the worm towards using glucose as its energy source, while the role of other metabolisable

sugars remains equivocal (Fairbairn, Wertheim, Harpour and Schiiler,1961; Scheibel and Saz,1966). The enzymes inthe galactose utilization pathway all had low specific activities and g ctose-1-phosphate was itself shown to inhibit galactose phosphorylation. Also, when provided as the sole carbohydrate source, galactose did not support the growth of H. diminuta. These two facts were put forward as reasons for the inadequacy of galactose to serve as an adequate nutritive carbohydrate in H. diminuta development.

The characteristics of glycerol uptake in H. diminuta were determined by Pittman and Fisher (1972). They showed that glycerol uptake was by diffusion at high concentrations and by a mediated system at low concentrations. Inhibitors of glycerol uptake include 1,2,propanediol and glycerophosphate, but not monosaccharides, amino acids or fatty acids. Uptake was thought to occur via one, possibly two distinct carriers with the mediated portion of uptake sensitive to changes in temperature, pH and Na^+ concentrations. Uglem, Pappas and Read (1974) claimed that the mediated component of glycerol uptake consisted of two systems, Na^+-sensitive and Na^+-insensitive. Both showed different kinetic characteristics and different susceptibilities to inhibitors. The inhibition effected by glycerophosphate was relieved when other phosphates were added to the medium e.g. ATP, AMP, G6P and it was deduced that the inhibition was produced by glycerol liberated from the hydrolysis of phosphorylated compounds effected by surface phosphohyrolases.

Uglem, Love and Eubank (1978) illustrated that α-methylglucoside was also transported by the hexose carrier system. It possessed a K_t of 0.73mM, a greater affinity for the uptake site than glucose and this was further supported by

the total competitive inhibition of glucose transport by 0.1mM α-methylglucoside at an inhibitor:substrate ratio of 100:1. α-methylglucoside also exhibited the characteristics shown by glucose transport i.e. Na^+-dependence, sensitivity to phlorizin, transport against an apparent concentration gradient and an increase in worm water during the accumulation of hexose.

Currently, data on carbohydrate transport by the cysticercoid of H. diminuta are limited to a research note (Arme, Middleton and Scott,1973). The authors showed that glucose uptake was non-linear with respect to concentration between 0.2 - 4.0mM and that the K_t was 1.54mM with a V_{max} of 6.6×10^{-4} μmoles glucose/100 cysticercoids/2min. Galactose proved to be an inhibitor of glucose uptake with a K_i of 1.19mM. Other inhibitors included sodium cyanide, sodium phlorizin and Na^+-free media. However, 2,4-dinitrophenol only affected uptake after cysticeroids were preincubated in the inhibitor for 2 h resulting in a 26.3% inhibition.

CHAPTER 4

MATERIALS AND METHODS

A. INTRODUCTION

This chapter describes the methods used in this study and the reasons behind the choice of a particular procedure.

There exists over 30 years' worth of experimentation on membrane transport. The majority of the work is on mammalian small intestine and human erythrocytes, but there is also a substantial volume on tapeworm systems, dating back to the 1950's. Despite modifications in technique, often related to the availability of new technology, the protocol used in most uptake experiments remains essentially the same. To study the ability of a membrane to transport a substance from outside to inside, radiolabelled substrate is usually added to the external medium and the uptake (or otherwise) of that substrate is measured with respect to time, concentration etc. A comparison of uptake of a wide range of substances by endoparasites has been possible due to the use of an established 'standard' technique, originally devised by Read *et al.*(1963). The widespread use of this technique, and variations of it, have enabled comparable experiments to be performed in laboratories all over the world with remarkedly consistent results.

B. CHOICE OF MEDIA

Three main types of media were used in this study and these are described in detail below.

1. DISSECTING MEDIA

The choice of media for the maintenance *in vitro* of any organism including parasites, is critical. A number of criteria are frequently examined to determine the suitability of incubation media. The first is that the medium should resemble that of the 'normal' habitat of the parasite,in the case of *H. diminuta* cysticercoid,the haemolymph of *Tenebrio molitor*. In this connection both ionic composition and osmolarity are especially important. The medium should be capable of maintaining the organism alive for the time period over which experimentation will take place and a number of criteria for judging viability are often employed e.g. ultrastructural integrity, presence of normal physiological processes. The medium should also be stable at temperatures chosen for the experiments and finally, for kinetic experiments, it should not contain substances potentially capable of competing for uptake with substrates under study.

The physico-chemical properties of the habitats of adults and cysticercoids of *H. diminuta* are not fully known. Information concerning the generalised content of the small intestine of rats is comprehensive (Yamacara and Smyth,1972; Smyth,1980), but it is recognised that the composition of

luminal fluid varies according to diet of the rat, its physiological state, the presence of other parasites and disease and stress (Mettrick and Munro,1965; Roberts,1966; Roberts and Platzer,1967; Podesta and Mettrick,1980). In general terms, however, adult H. diminuta reside in a rich mixture of fully and partially digested food, with an abundance of small organic nutritional molecules e.g. monosaccharides, amino acids and fatty acids. The composition of a successful in vitro cultivation medium for cysticercoids is available (Voge and Green,1975; Voge,1978), which is basically a modification of Landreau's medium for the growth of cockroach cells (Landreau,1966,1969). However the composition of 'minimal media' for H. diminuta adults and cysticercoids has not yet been determined. The cultivation medium of Voge and Green (1975) contains 16 amino acids, glucose, trehalose and a variety of salts, and is thus an unsuitable medium for uptake experiments.

The early work of Read et al.(1963) employed the use of Krebs-Ringer saline buffered with 0.25M Tris-maleate. The use of this saline (KRT) has, however, been criticised on a number of counts. Maleate has been implicated in the inhibition of some enzymes and $Tris^+$ is a known competitor of Na^+ for Na^+ receptors. In a study on the uptake of amino acids by H.diminuta cysticercoids, a preliminary investigation into the effect of various salines was performed (Jeffs,1984). Four salines were used and the investigation of five aspects collated;

(a) Infectivity of cysticercoids incubated in saline and then fed to rats

(b) The ability to excyst in vitro following incubation in saline

(c) The effect of incubation on parasite ultrastructure

(d) The rates of uptake in different salines

(e) The amount of protein synthesis of cysticercoids incubated in ^{14}C-L-leucine dissolved in saline. The salines used were KRT, Hanks's, Hoyle's and KRP (Phosphate buffered Krebs-Ringer saline).

The results did not yield an unequivocal answer to the question of which saline was the most suitable for in vitro studies. The best rat infection rate was obtained with cysticercoids incubated in Hoyle's saline whereas protein synthesis was highest in those incubated in Hanks' saline. However, cysticercoids incubated in KRT were unaffected ultrastructurally over short periods of time (< 3 h), the percentage successful excystation was approximately 97% and uptake experiments showed a greater uptake of substrate in KRT than in the other salines. Arme and Coates (1973) also investigated the effect of different salines on cysticercoid uptake using Hedon-Fleig, Tyrode and KRT, and concluded that KRT was the most suitable. In a study on the uptake of glucose by adult H. diminuta in either a balanced electrolyte medium, hymenolepid culture medium or KRT, KRT gave consistently lower results (Podesta, Stallard, Evans, Luisser, Jackson and Mettrick,1977), although these workers used incubation times in excess of 1 h.

It is clear, therefore, that none of the above mentioned salines are ideal in all respects for the purposes of this study. However, KRT was chosen because, on the basis of the available evidence, it was considered the most suitable of the media tested. Since most previous work has been undertaken using KRT, its use here facilitated comparison of data from other workers. The composition of KRT is shown in Appendix A. All dissecting media used in this study were KRT as was the pre-incubation medium, with variations according to the needs of an experiment (e.g. replacement of Na^+ by other cations when needed).

2. INCUBATION MEDIA

The incubation technique is fully described on page 116, but basically it involves the addition of radiolabelled substrate to the incubation medium and measuring the amount absorbed by the parasite during fixed incubation time. Radiochemicals were obtained from Amersham International p.l.c. (Amersham, BERKS. UK). To 50 μCi of labelled substrate was added unlabelled substrate to give a 2mM stock solution of a specific concentration, 2μCi/umole. This was then frozen until used.

The experiments described below usually involved a 30 min pre-incubation in KRT, followed by an incubation in labelled substrate dissolved in KRT, and finally washing, to remove surface adhered radioactive substrate, in three changes of KRT (for further details see page 116). Incubation media were stored frozen and used once only.

When studying the effect of a potential inhibitor on the uptake of a substrate, two types of experiment were usually performed. The first involved maintaining a constant inhibitor concentration while varying that of the substrate and the second varied the inhibitor concentration with a constant concentration of substrate.

By use of suitable standards, uptake was expressed in terms of µmoles absorbed/g ethanol extracted dry wt./time.

Adult H. diminuta were incubated in media with a specific activity of 0.05µCi/umole.

3. EXCYSTATION MEDIA

Excystation of cysticercoids in vitro was performed for two main reasons. The first was to check the ability of cysticercoids to excyst, for example after incubation for long (> 3 h) periods in saline i.e. as an index of viability. The second was to collect the 0-day-old worm for the purposes of uptake experiments or assay of radioactivity that had reached

the inner cysticercoid during incubation. Using the methods of Rothman (1959), parasites were first exposed to pepsin and HCl at 37°C, as a crude simulation of the stomach juices of the rat encompassing its two most important features - acidity and protease activity. The composition of the Pepsin/HCl mixture is given in Appendix A. After approximately 20 min, the cysticercoids were transferred from pepsin/HCl to a 'bile'/trypsin solution at 37°C the composition of which is also given in Appendix A. Although several workers have claimed that H. diminuta can excyst in various other mixtures (for example Schiller (1965) used fresh ox bile, while Berntzen (1961) excysted cysticercoids using only sterile Tyrode's saline at pH 7.8 - 9.0 at 37°C). Rothman's method was used throughout this study.

C. ANIMALS

In this study three groups of animals were used, the definitive host, intermediate host and the parasite.

1. THE PARASITE

The Rice University strain of H. diminuta was used throughout this work. It is noteworthy that 'strain' appears to be of considerable importance, in helminth physiology, although it is often ignored. Although strains of H. diminuta have not been fully characterised in terms of their differences in physiology or biochemistry, such distinctions are known to exist (see Mettrick and Rahman,1984; Bryant, 1983).

2. THE DEFINITIVE HOST

Male albino Wistar rats were used as definitive hosts. They were laboratory bred and maintained 2/3 to a cage on pelletised rodent food and water *ad libitum*. Only males were infected to enable comparison of data between earlier experiments (e.g. Arme, Middleton and Scott,1973). Young rats about 100g in weight-were infected with 30 cysticercoids each, which were themselves obtained from infected *Tenebrio molitor* (the intermediate host). This latter was achieved by making cuts across the thorax of the beetle and the terminal base of two segments of the abdomen. The contents of the insect were then flushed out with KRT saline. The rats to be infected were lightly anaethetised with ether to enable the cysticercoids (contained in a minimum volume of KRT) to be introduced into the throat.

3. THE INTERMEDIATE HOST

Tenebrio molitor was used as the intermediate host, and there are a number of reasons why the grain beetle rather than the flour beetle (*Tribolium confusum*) was used. The first is that *T. molitor* has been used successfully as a host in other studies (e.g. Hurd,1985; Hurd and Arme,1984a,b: Jeffs and Arme, 1984a, 1985a,b). *T. molitor* is also much the larger beetle and can harbour larger infections and, lastly, the variation in size of cysticercoids from *Tenebrio* appears, by visual

inspection, to be less than in those from Tribolium (personal observation).

T. molitor (Rice University strain) were maintained at a constant temperature of 26°C and humidity of c.80%, in bran-filled containers. Infected beetles were kept separately in small containers in an incubator at 26°C, with the humidity maintained by bubbling air into a water-filled trough. Beetles to be infected were collected from the stock colony and starved for three days. H. diminuta eggs extracted from faeces (see below), were filtered to remove excess water and mixed with mashed apple (ratio of c.1:1). The egg/apple mixture was spread evenly on a glass slide and introduced to the beetles. The insects were left to feed on the mixture for 24 h and then removed to a clean dish containing ample bran.

D. EXPERIMENTAL METHODS

1. METHOD FOR OBTAINING *Hymenolepis diminuta* EGGS

A number of methods are currently available for the extraction of H. diminuta eggs. These either involve direct removal from gravid proglottides of adult worms, or extraction from rat faecal pellets (Lethbridge,1970,1971,1972; Roberts, 1966). With the former technique it is impossible to determine the proportion of proglottides that contain infective eggs. Thus, in a suspension obtained in this way, only a certain percentage of eggs will be fully developed. The method also involves sacrificing a rat on each occasion and finally, when

mixed with mashed apple or presented by themselves, these eggs appeared not to be as attractive to beetles as those extracted from faecal pellets (personal observation). Extraction of eggs from faeces was performed by a modification of the salt-flotation method of Lethbridge (1970). Faecal pellets were collected from the cages of infected stock rats and soaked in tapwater for at least 24 h. The resulting suspension was sieved to remove coarse matter (using a sieve with 20 holes in^2, Endecotts Ltd. UK). The liquid remaining was saturated with sodium chloride (SLR grade Fisons Ltd. UK) and then centrifuged at 1000g for 15 min. The froth apparent on the surface of the liquid was removed, together with approximately 20 ml of the fluid immediately below, and the eggs in the froth were left to settle on the bottom of a 2l cylinder of water, which was changed three times. After washing, the eggs were filtered to reduce the volume of water and stored at $4^{o}C$ in distilled water. Eggs prepared in this way remained viable for up to 6 months, although in practice most were used within two weeks. From time to time, eggs were also tested for their ability to 'hatch' under _in vitro_ stimuli.

2. METHOD FOR INCUBATING CYSTICERCOIDS

Although well-tested methods for incubating 10-day-old adult _H. diminuta_ are available, evaluation of methods for cysticercoids is lacking. The only previous work by Arme and Coates (1971,1973) and Arme _et al._(1973), used a method involving the removal of pre-incubation and incubation media using suction filtration methods. This is potentially a simple

and effective method, but one which gave consistently unacceptable background radioactivity when performed in this laboratory. Thus, a system was devised which was capable of measuring amino acid uptake by smaller numbers (25) of cysticercoids (Jeffs,1984). A small length (approximately 3-4cm) of Pyrex glass tubing (diameter, 7mm) was covered at one end by nylon muslin, held in place by a ring of stainless steel wire. Cysticercoids were placed in these tubes and moved from pre-incubation to incubation media with ease. Blotting and rinsing these tubes was easily carried out and resulting background counts proved to be low. Therefore, the preliminary uptake studies were performed using these tubes.

a. Preliminary studies

The first experiments were carried out to establish the working parameters of an uptake experiment e.g. concentration of substrate, level of background radioactivity, number of rinses, incubation time, number of cysticercoids etc. Glucose was chosen as the substrate for these preliminary studies.

The technique employed involved placing the cysticercoids in the aforementioned tubes, pre-incubating in KRT saline (26^{0} for 30min) and incubating for a range of times in radiolabelled glucose. The tubes were then blotted and rinsed by partially dipping them into a 500ml beaker of KRT. However, using a variety of substrate concentrations, numbers of cysticercoids and incubation times, it was not possible, even after many attempts over a 6 month period, to obtain

reproducible results with acceptable standard errors. As a control on techniques employed it was decided to attempt to reproduce experiments performed by Jeffs and Arme (1985a) using labelled cycloleucine as non-metabolisable neutral amino acid. Results virtually identical to those obtained by these workers were obtained, suggesting that the basic techniques and media were not major contributory factors to the lack of reproducibility obtained with glucose, noted above. It was therefore decided to use a metabolically inert monosaccharide, 2-deoxyglucose in order to act as control for the possible effects of glucose metabolism in the previous studies, and other substrates were also tested, including galactose, 3-O-methylglucose and mannose. In all cases a wide variation in results was obtained with a consistently unacceptable degree of reproducibility. In order to determine possible changes in pH of incubation media, cysticercoids were incubated in unlabelled glucose, and pH readings were taken before, during and after the experiment. The maximum variation in pH was 0.2 of a unit (7.3 - 7.5). This variation, on the basis of comparison with data from other workers, was regarded as unlikely to affect uptake, and pH was ruled out as a source of the observed error.

The effect on uptake kinetics of the unstirred layer of adult H. diminuta has been well documented (Podesta, 1977), but reassessed by Lumsden and Murphy (1984). This unstirred layer creates a barrier to the free movemement of substrate to and from the worm surface and can result in inaccurate estimation

of kinetic constants. A range of shaking speeds and duration of agitation was thus employed to investigate possible unstirred layer effects. Data collected over a three month period indicated no difference between agitated and non-agitated experiments. All further experiments were therefore carried out without agitation.

The possible effect of cysticercoid age on the variable results was investigated, with a slight difference in result noted between the 40-day-old and 8-day-old cysticercoids (Chapter 5). However, for all experiments (unless investigating age effects) the age range of cysticercoids was restricted to 12-21 days.

None of the above parameters seemed to be the cause of the variable data with large standard errors. The age and sex of the intermediate host was therefore next investigated as a possible cause. Beetles (measured in days after emergence from the pupa) up to 42 days old were examined, but no significant variation in uptake by cysticercoids from beetles of any age was observed. Individual beetles were sexed and separated at the pupal stage, on the basis of differences in their developing genitalia (by the method of Bhattacharya, Ameel and Waldbauer,1970). Again, there was no difference in the results using cysticercoids obtained from single sex unmated, or mated groups of beetles.

TABLE 4.1 PRELIMINARY RESULTS

Uptake of labelled monosaccharide by *H. diminuta* cysticercoids each point is the average of 30 replicates.

	K_t		V_{max}	
Glucose	0.47mM	± 0.28	0.050	± 0.025
2-Deoxyglucose	0.54mM	± 0.30	0.037	± 0.020
Mannose	0.88mM	± 0.33	0.061	± 0.020

Effect of cysticercoid age on glucose uptake kinetics each point is the average of 10 replicates.

age (days)	K_t		V_{max}	
10	0.55mM	± 0.23	0.033	± 0.017
20	0.29mM	± 0.11	0.061	± 0.015
40	0.47mM	± 0.21	0.047	± 0.021

Effect of intermediate host sex on cysticercoid glucose uptake each point is the average of 10 replicates.

	K_t		V_{max}	
male	0.65mM	± 0.15	0.062	± 0.026
female	0.55mM	± 0.13	0.061	± 0.020

Effect of heat killing cysticercoids on glucose uptake kinetics each point is the average of 10 replicates.

	K_t		V_{max}	
heat killed	0.54mM	± 0.23	0.056	± 0.025
living	0.60mM	± 0.20	0.051	± 0.023

Each figure is given ± standard deviation.
V_{max} is measured in µmoles/50 cysticercoids/30s.

Finally, a set of experiments designed to compare the results of incubating living and heat-killed cysticercoids were performed. Cysticercoids suspended in KRT saline were heat-killed by immersing them (in a test tube) in a waterbath at 60°C for 2 min. Their viability was then checked by attempting to excyst them in vitro. The integrity of the diffusion barrier was shown to be physically intact by electron microscope studies although it is appreciated that this technique can only give a visual inference of the physiological status of the cysticercoid. Wide variations in data were also found in these groups.

A summary of the results obtained from these preliminary experiments is given in Table 4.1. The conclusion drawn from these first experiments was that, with monosaccharides, all the results contained a considerable element of background 'noise'. This 'noise' was extremely variable and persistent, and succeeded in masking any variation in uptake/inhibition that might actually be taking place.

The procedure of washing and rinsing tubes and the nature of the tubes themselves was next investigated and a number of alterations to the experimental procedure were made which enabled consistently reproducible data to be collected.

b. Final standardisation of technique - the method for incubating cysticercoids.

A number of modifications were made to the procedure for incubating cysticercoids; these included increasing the specific concentration of the labelled substrate from 1μCi/umole to 2μCi/umole, increasing the number of washes and rinses after incubation and, perhaps the most important, altering the design of the incubation tubes. Experiments using the glass tubes with stainless steel mesh glued to one end with an epoxy adhesive, rather than the nylon muslin used previously, gave significantly lower background counts (usually *c.* 5 cpm more than background), and this greatly reduced the variability in results obtained.

Thus, a summary of procedure adopted for incubating cysticercoids evolved over many months was as follows:- Cysticercoids from at least 3 beetles were pooled in KRT, and then divided into groups of 50. Each group was then transferred to the wire mesh-bottomed glass tubes which enabled the cysticercoids to be moved from medium to medium with a minimum of carryover. The mesh used was 200 holes/in ^{2} (G.H. Heath and Sons Ltd., Burslem, UK) and this allowed free drainage of media while retaining the cysticercoids. KRT was run through the tubes several times, using the transfer Pasteur pipette, to ensure that no cysticercoids had adhered to the Pasteur pipette or to the sides of each tube. Pre-incubation media consisted of 3 ml KRT, at 26°C in which the tubes were placed for 30 min. When transferring tubes from pre-incubation

to incubation media, they were blotted to remove excess fluid. After an incubation period in labelled substrate, the tubes were blotted and washed in three changes of 400 ml KRT. A wash bottle was also employed to rinse off adhering radioactive media more effectively. Between each wash the tubes were blotted and finally placed in a scintillation vial (Packard Ltd.,UK) containing 1ml of 70% ethanol and extracted for a minimum of 2 h, a time interval shown by previous experiments to be sufficient to extract as much radioactivity as after extraction for 48 h. After the extraction period, 10 ml of a scintillant 'Lumagel' (LKB Ltd. Croydon,Surrey UK) was added directly to the vial (plus the tube) and the samples were counted on a liquid scintillation counter (Packard Tri-carb liquid scintillation spectrometer 2425, Packard Ltd. UK). Any variations of this basic method that were used are described in the text.

3. METHOD FOR INCUBATING 0-DAY-OLD ADULT *Hymenolepis diminuta*

The 0-day-old adult parasite was studied for a variety of reasons. The uptake capabilities of very young worms have never been studied, and little is known of other features of their physiology. Growth of adult *H. diminuta* during the first five days in the rat has been described in detail by Goodchild and Harrison (1961) with data collected from 5 h after infection. They concluded that, although measurements of worm area indicated an exponential growth over the first five days, length measurements only began to increase after the first day.

This, they suggested, was due to the rapid cell proliferation inward to fill the hollow cavity which appears in the posterior region of newly excysted worms. Thus, it appears that the 0-day-old worm is very active physiologically.

The procedure for incubating 0-day-old worms was as follows:-
After approximately 20 min in 'bile'/trypsin solution, the majority of cysticercoids have excysted. All the cysticercoids and excysted adults were then passed through stainless steel mesh (200 holes in ^{2}, G. H. Heath and Sons Ltd., Burslem, UK), into warm (37°C) KRT saline. This filtered out the empty cysticercoid 'cases' and also removed the excysted adult from the 'bile' solution. The KRT now bathing the 0-day-old worms was changed several times, to remove completely any remaining 'bile'/trypsin solution, and the worms were separated into groups of 200. Glass tubes similar to those used for cysticercoids, except with a smaller mesh size (300 holes/in^2), were used to incubate the worms. Pre-incubation was in 3ml of KRT at 37°C, for 30 min. Incubation was followed by washing, as described in the procedure for incubating cysticercoids. Extraction was for a minimum of 2 h in 1ml of 70% ethanol, and samples were then counted on the liquid scintillation spectrometer. All variations and adaptations of this method are described in the text.

4. METHODS OF INCUBATING 5 AND 10-DAY-OLD ADULT WORMS

The uptake characteristics of 10-day-old adult *H. diminuta* have been extensively investigated (see Pappas and Read,1975), largely using the standardised techniques advocated by Read *et al*.(1963). Transport studies on 5-day-old adults have, however, not been carried out, and it was of interest to investigate uptake of monosaccharides in adults of this age, thus providing a range of uptake data for stage 3 and 5 cysticercoids and 0-, 5- and 10-day-old adult *H. diminuta* (this study). The method for incubating adults is described below.

Infected male rats were killed by cervical dislocation. The small intestine was removed from the pyloric sphincter to the ileo-caecal junction. This length of intestine was then flushed through with approximately 40ml of warm ($37^{o}C$) KRT and the contents examined against a dark background. All worms found were transferred to a petri dish of warm KRT, and care was taken to note the number of worms harvested from each rat. Parasites from any host yielding less than 26 worms from an original cysticercoid infection of 30 were discarded. The 10-day-old worms were randomised in groups of 5 and pre-incubated in 10ml of KRT ($37^{o}C$), in 25ml beakers. 5-day-old worms were placed in the glass tubes normally used for cysticercoid incubation, also in groups of five. These tubes had been pre-weighed and pre-incubation was in 4ml of KRT for 30 min. Incubation and washing was performed as for the

cysticercoid, and tubes and worms were extracted in 2ml of 70% ethanol. The 10-day-old adults were transferred between media by the use of a hook and, after incubation, they were washed 3 times and blotted on hard filter paper before being placed in 2ml of 70% ethanol, for a minimum of 2 h. After extraction, 10-day-old worms were removed to pre-weighed aluminium tares and dried in an oven at 90°C to obtain the ethanol-extracted dry weight. A 1ml aliquot of the extraction medium was taken and added to 10ml of lumagel for liquid scintillation counting. 5-day-old worms were also removed from their extraction media and dried at 90°C overnight. They were then weighed in their pre-weighed tubes, and a 1ml aliquot of the extraction medium was added to 10ml of Lumagel for liquid scintillation. For both 10 and 5-day-old adults, media containing labelled substrate at a reduced specific activity was used, because of their predicted greater uptake than the cysticercoid.

E. ANALYTICAL METHODS

1. THE ANALYSIS OF CYSTICERCOID EXTRACT, CYSTICERCOID DIGEST AND INCUBATION MEDIA.

A number of techniques can be employed to analyse components of a complex mixture. A specific assay can be used to measure a particular substance known to be present, and various types of chromatographic methods can separate the components of a mixture. In this study, paper, gas and thin-layer chromatography have all been used and enzymatic assays have been employed to assess the concentration of glucose in

the haemolymph of parasitised and non-parasitised intermediate hosts. The salient features of these techniques, are given in the following pages.

a. Paper chromatography

When placed in incubation media, cysticercoids and adults are known to absorb various substrates (see Pappas and Read, 1975). However, they may also add substances to the media which may subsequently alter its composition, pH etc. Therefore, in order to investigate the composition of media both before and after incubation, paper chromatography was employed, with solvents and locating reagents chosen to detect particular groups of compounds e.g. carbohydrates and amino acids. It was also of importance to identify the radioactive label which was extracted from cysticercoids both to ascertain its purity and to detect whether metabolism has occurred. Finally, any labelled substances not extracted by the ethanol were required to be identified in digests.

In paper chromatography, the cellulose fibres act as a supporting matrix for the liquid phase. The solvent is placed at the bottom of a cylindrcal tank and allowed to equilibrate so that a dense solvent vapour is built up. The paper is then introduced to the tank and stands in the solvent, making sure that the sample spots are just above the surface of the solvent. The solvent then moves up by capillary action and separation of the sample is achieved. The identification of a component is usually made on the basis of the distance moved

during the run relative to that made by the solvent front. The R_f values are therefore defined as:

$$R_f = \frac{\text{the distance moved by solute}}{\text{the distance moved by solvent front}}$$

This value is constant for a particular compound under standard conditions and reflects the distribution coefficient for that compound. For carbohydrates the term R_g is sometimes used and is defined as:

$$R_g = \frac{\text{the distance moved by carbohydrate}}{\text{the distance moved by glucose}}$$

Two types of cysticercoid extract and incubation media were analysed by paper chromatography; undiluted and samples diluted 10 times by water in order to reduce the relative salt content (and thereby reduce interference by salt in the chromatography). All chemicals used were of the purest available - AnalaR and Aristar (supplied by BDH Chemicals Ltd. Poole, Dorset).

The solvent used for investigating carbohydrates was ethyl acetate (55ml), pyridine (25ml) and water (20ml). This was equilibrated in the tank for 1 hour. The tank was cylindrical and lined with Whatman No.1 chromatography paper to aid equilibration (tank supplied by Shandon Ltd., Cheshire UK; Whatman paper by Pierce Chemical Co., Chester, UK). Whatman No 1 paper was also used for the separation of samples, which were

spotted on a line drawn 3.5cm from the bottom. A 10μl capillary tube was used for spotting samples together with a hairdryer for rapid drying. Usually 50μl of sample was applied to spots 7cm apart and < 1mm in diameter. The paper was then curled and clipped in a cylindrical shape and introduced into the solvent tank. The time was noted and the chromatogram left to run until the solvent front was almost at the top of the paper. During this time the locating reagent was made up from the following substances: 0.5g m-phenylenediamene, 1.2g stannous chloride, 20ml acetic acid and 80ml ethanol. This was poured into a dipping tray. The chromatogram was removed from the tank, the solvent front marked and dried in a fume cupboard. When the paper was dry it was dipped into the locating reagent and placed in an oven at 100°C for 5 min. After this time the spots were a yellow/brown colour, and the R_f values were calculated. The same separation procedure was used for radioactive chromatograms, but the paper cut up into 0.5cm strips and each strip was extracted in 1ml 70% ethanol. 'Lumagel' was subsequently added and the samples counted by liquid scintillation. The position of the radioactivity was then compared to the visualised standards.

An account of some experiments involving anyalsis of unlabelled incubation and extraction media is given below following a brief outline of the method used for detecting sugars and amino acids by thin layer chromatography.

b. Thin layer chromatography (TLC)

Incubation and extraction media were analysed for both their carbohydrate and amino acid content. Thin-layer chromatography was used initially for amino acid analysis as a more accurate identification was available using this method rather than using paper chromatography. Three runs using carbohydrates were also performed to check the identification of those substances in paper chromatography. TLC involves the use of glass plates coated with silica gel mixtures which act as the support media for the stationary phase. For amino acid analysis, silica gel G coated plates were used (supplied by Whatman, UK) and the solvent consisted of 60% n-butanol, 15% acetic acid and 25% water (vv). Samples were spotted using capillary tubes and a hairdryer was used to assist drying and keep sample spots to the smallest size possible (a large diameter spot i.e.> 2mm produces tailing and can alter the R_f values). Approximately 50 μl of sample was applied to each spot which was situated on a line above the solvent level. The solvent was allowed to equilibrate in a tank before introducing the plates and, as with paper chromatography, the solvent was allowed to run almost to the top of the plate before being removed. Visualisation of the amino acids was made using a ninhydrin solution which was sprayed onto the plates and developed in an oven at 95°C. Samples and known compounds were run simultaneously, and the results compared. In order to determine the location of the labelled compounds, the plates were marked off in 1cm strips and each section scraped off into

70% ethanol for extraction. Scintillant was then added and all particulate material was allowed to settle before counting.

Cyst digests were obtained by homogenising cysticercoids in 5ml of 5% trichloracetic acid (w/v) in an ice-cold homogeniser for 4 min. The homogenate was then left to stand in ice for 1 h before centrifugation at 2100g for 15 min. The resulting pellet was resuspended in 5% trichloracetic acid and re-centrifuged. This procedure was repeated three times and the pellet was then dissolved in 7ml distilled water and subjected to TLC and radioactive analysis, as described previously.

2. PRELIMINARY EXPERIMENTS INVESTIGATING THE COMPOSITION OF UNLABELLED INCUBATION AND EXTRACTION MEDIA BY PAPER AND THIN LAYER CHROMATOGRAPHY.

A number of preliminary experiments were performed, using unlabelled substrate, in order to evaluate the chromatographic techniques described above before using radiolabelled substrate. Cysticercoids were dissected from 15-day-old beetles and incubated in 1mM glucose for 2, 5, 10, 20 and 60min. Incubation media were analysed after incubation, and extraction media after a minimum 2 h extraction period. The amount of sample applied was 50μl in all cases and identification was made by comparison with known standards. Absolute identification of substances was not possible but comparison between unidentified spots was. The results from these experiments are given in Table 4.2.

TABLE 4.2

ANALYSIS OF INCUBATION AND EXTRACTION MEDIA

(combined results of paper and thin-layer chromotography)

INCUBATION TIME	INCUBATION MEDIA carbohydrates	INCUBATION MEDIA amino acids	EXTRACTION MEDIA carbohydrates	EXTRACTION MEDIA amino acids
2 min	Glucose	-	Glucose	alanine leucine histidine
5 min	Glucose	-	Glucose	"
10 min	Glucose	-	Glucose	"
20 min	Glucose	-	Glucose + unidentified spot	"
1 h	Glucose	proline	Glucose + unidentified spot	" + proline

This was not a quantitative study and in all cases the amount detected in samples appeared small compared to standards of 10mM. Only three amino acids were detectable in extraction media, after short incubation, and in addition proline was present after 1h. Glucose was detected in all extraction media, together with an unidentified spot, which did not correspond with either galactose, 3-0-methylglucose, mannose, trehalose or glucosamine. An unusual result was the detection of proline in the incubation media after 1 h. This experiment was repeated three times with the detection of proline each time. Proline is the most abundant amino acid in T. molitor haemolymph (Hurd and Arme,1984a) and was shown to be lowered in concentration in the haemolymph of parasitised female beetles. Jeffs and Arme (1984) have also shown that proline can be incorporated into protein by cysticercoids. It appears, therefore, that proline may be added to the incubation media from the parasite by efflux mechanisms. This suggestion would need further investigation to substantiate it, but the data serve to illustrate the sensitivity and correct application of the technique.

3. METHODS USED FOR DETECTING CARBOHYDRATES IN Tenebrio molitor HAEMOLYMPH.

a. Glucose assay

Insect haemolymph contains a wide variety of compounds, including proteins, amino acids, fatty acids, salts and sugars. Therefore, when selecting or devising a test for quantifying

free sugars in haemolymph, a number of points must be considered. Tests that are sensitive to the presence of proteins, and compounds other than sugars, are only of use if a good separation of these substances from haemolymph can be effected. Certain types of protein removal procedures, e.g. acid precipitation, are diminished in value if a neutral solution is needed for the next stage (e.g. the Somogyi-Nelson method). Neutralisation could be used, but this results in an increase in total sample volume thus reducing sensitivity. Recently, a number of glucose oxidase assays have been made available. A modification of the Trinder method (1969) was used in this study. In the initial reaction, D-glucose is oxidised by glucose oxidase producing D-gluconolactonic acid and hydrogen peroxide. In the second reaction, hydrogen peroxide is oxidatively coupled with the 4-aminoantipyrin in the presence of p-hydroxybenzoate by peroxidase to yield the quioneimine chromophore with an absorption maximum at 500nm. The change in absorbance at 500nm is directly proportional to the glucose concentration in the sample and is specific for glucose. To assay haemolymph glucose the Statzyme(r) reagent (Worthington Statzyme(r) glucose assay kit 500nm) was reconstituted with water, warmed to 37°C in a waterbath and a small amount was removed for reading initial absorbance on the spectrophotometer (CE272, Cecil Instruments Ltd. Croydon, Surrey UK). Haemolymph was collected from beetles by severing the left prothoracic leg at the base. The exuding haemolymph was collected in a 10µl capillary tube, which was immediately sealed and centrifuged for 5 min in a microhaematocrit centrifuge to remove haemocytes. Protein was then precipitated

with 70% ethanol and the supernatant evaporated and resuspended with 0.9% NaCl. This was then added to the reagent and incubated at 37°C for 10 min. The developed colour was then read on the spectrophotometer at 500nm. A sample blank consisting of 10 μl reagent and 1ml of 0.9% sodium chloride was also read. The spectrophotometer was zeroed with 0.9% sodium chloride and a plot of absorbance of various concentrations of standards was drawn. Calculated glucose values were checked on this standard curve. The calculated glucose concentration from absorbance readings was carried out by using the equation below

$$\text{glucose in mg/100ml} = \frac{\text{(final absorbance of sample - initial absorbance - blank)}}{\text{(final absorbance of standard - initial absorbance)}} \times 100$$

The results of the analysis of Tenebrio molitor haemolymph are given in Chapter 2. This technique has, however, a number of drawbacks. The necessary precipitation of the protein involves a dilution and invariably a loss of sensitivity of the technique as it is detecting variations at the lower end of its range. The colour produced in the reaction is also temporary and therefore reading must be taken quickly and only once. Finally, the reagents involved are unstable and must be kept refrigerated. It was to corroborate the results obtained from these assays and also to analyse cysticercoid digest, that gas chromatography was used.

b. Gas Liquid Chromatography (GLC)

GLC is based on the distribution of compounds between a liquid and gas phase. It is widely used for the qualitative and quantitative analysis of a large number of compounds as it has high sensitivity, reproducibility and speed of resolution, and it is of most use in the separation of compounds with low polarity. A stationary phase of 'liquid' materials is supported on an inert substance such as small particles of silica gel. This material is packed into a glass column and an inert gas of nitrogen is passed through the column. Samples injected onto the column are volatilised instantly by high temperature and carried along the column by the carrier gas. The basis of separation is the difference in the partitiiton coefficient of volatilised compounds between the liquid and gas phases. Detection of components is carried out by a flame ionisation detector which can detect quantities as low as 1ng and has a wide linear-response range. A mixture of hydrogen and air is introduced into the detector to give a flame, the jet of which forms one electrode, whilst the other electrode is a brass wire mounted near the top of the flame. When the sample compounds emerge, they are ionized in the flame causing an increased signal to be passed to the recorder and this results in a peak. Identification of the peak can be made by comparison with standards and quantitation by measuring peak areas, these being directly proportional to the concentration of substance in the sample over a given range. The method used in this study was able to quantify glucose and trehalose in haemolymph.

Pupae of Tenebrio molitor were collected, sexed and placed in an incubator at 26°C. As the pupae hatched, beetles were separated into petri dishes and infected as described above. Beetles were then kept for 12 days to obtain a 12-day-old parasite infection in 15-day-old beetles. Haemolymph was collected from beetles by severing the first prothoracic leg at its base. The resulting haemolymph was collected in a capillary tube, spun down in a microhaematcrit to remove the haemocytes and frozen. When all the samples had been collected they were thawed individually, the haemocyte plug removed and blood added to 0.5ml of 70% ethanol in a centrifuge tube. Samples were spun on an MSE 18 centrifuge (MSE Instruments, Crawley, Sussex) for 20 min. The resulting supernatant was removed, evaporated to dryness in glass reactivials (Supelco Inc. Pennslyvania USA) and resuspended in 10 μl of a silylation reagent Tri-sil'Z' (Pierce Chemical Co. USA). Samples were placed in a waterbath at 70°C for 35 min and, after this silylation process, samples were injected directly onto the column*. The GLC was programmed to run at 200°C for 7 min and then to rise in temperature at a rate of 8°C/min to 290°C where it remained for a further 8 min. This enabled a full development of all peaks. Standards were treated in the same way as samples. Quantitation of peaks was made by measuring the area under peaks of known standard concentrations and comparing sample peaks to these standards.

*column phase was SE30.

The haemolymph of large numbers of male and female parasitised and non-parasitised beetles was examined in order to quantify the sugars found therein. The results of these experiments are discussed in Chapter 2. Chapters 2,5 and 7 describe in detail the results obtained from the implementation of these methods and discuss the conclusions that can be drawn from them.

F. MATHEMATICAL ANALYSIS OF DATA

1. METHOD OF CALCULATING K_t AND V_{max}

This section describes the method used in this study of calculating the K_t and V_{max}. From the data obtained in an uptake experiment, a Lineweaver-Burk double reciprocal plot is drawn, examples of which are shown in Figs. 4.3 and 4.4. A line is then fitted by a method of least squares. Thus, the intercept on the y-axis and the slope of the graph can be calculated. In the estimation of the intercept, any error is quantified by calculating the standard error of estimate (S.E.E.) for each set of data, from the formula given below:

$$S_{x(or\ y)} = \sigma x(or\ y)\sqrt{1-r^2}$$

where ? = the standard deviation of the particular data, and r = the product moment correlation coeffeicient. Thus, a value for C (the intercept on the y-axis) $\pm$ S.E.E. enables a determination of the variation in the K_t and V_{max} values. In 95% of the cases the actual values lie between $\pm$ 2. S.E.E (Moroney,1978). Thus, the ranges for K_t and V_{max} values are always for these 95% confidence limits. It was not necessary to calculate any variation in the gradient within the limits calculated for the y-intercepy, as this will still intercept the y-axis at the same point whatever its value.

2. METHOD OF SEPARATING THE DIFFUSION AND MEDIATED COMPONENTS OF UPTAKE

When more than one uptake process is operative for the uptake of a particular substance, these must be separated to allow the kinetic parameters of the individual processes to be determined.

In the study of mediated uptake in helminths, diffusion is the most important uptake process that is likely to occur at the same time. To determine whether a diffusion component is present parasites are incubated in a fixed amount of substrate but with increasing concentrations of an inhibitor. If the inhibitor is a competitive one, and shares all the transport sites of the substrate, a concentration will be reached where it will totally inhibit the mediated uptake of the substrate. In practice a plot such as Fig 4.5 may be obtained, where further increases in inhibitor concentration do not result in any additional increase in inhibition. The residual uptake therefore represents uptake occurring by diffusion. In certain cases, a plot of substrate absorbed against substrate concentration may contain a linear function, indicating apparent diffusion. However, it is possible that this represents the non-saturated part of a transport system that saturates at concentrations in excess of those used in the experiment.

3. METHOD OF CALCULATING K_i, THE INHIBITOR CONSTANT

Membrane transport is characterised by its susceptibility to inhibition by structurally similar compounds. There are many ways of experimentally testing the inhibition of substrate uptake, but for the calculation of a K_i only one particular experiment is necessary. The concentration of the substrate is varied while that of the inhibitor is kept constant. This produces a varying inhibitor:substrate ratio, and enables both the K_i and the type of inhibition to be determined. If the results of such an experiment are plotted by the method of Lineweaver and Burk (1934), then the following results are possible. Fig.4.3 represents competitive inhibition in which the substrate and inhibitor molecules compete for the same uptake site, and this results in a reduced affinity (i.e. an increase in K_t) but the V_{max} remains constant. Non-competitive inhibition (Fig.4.4), however, results in a reduced uptake rate which is due to the action of the inhibitor at a site other than the uptake site; in this case the V_{max} of the substrate is altered but the K_t remains the same. Quantification of inhibition is achieved by calculating the K_i from the equation below;

$$\frac{K_t}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) = \text{the slope of the inhibited line}$$

Here, the expression $\frac{K_t}{V_{max}}$ = gradient of uninhibited line

FIG. 4.3 COMPETITIVE INHIBITION (Lineweaver - Burk plot)

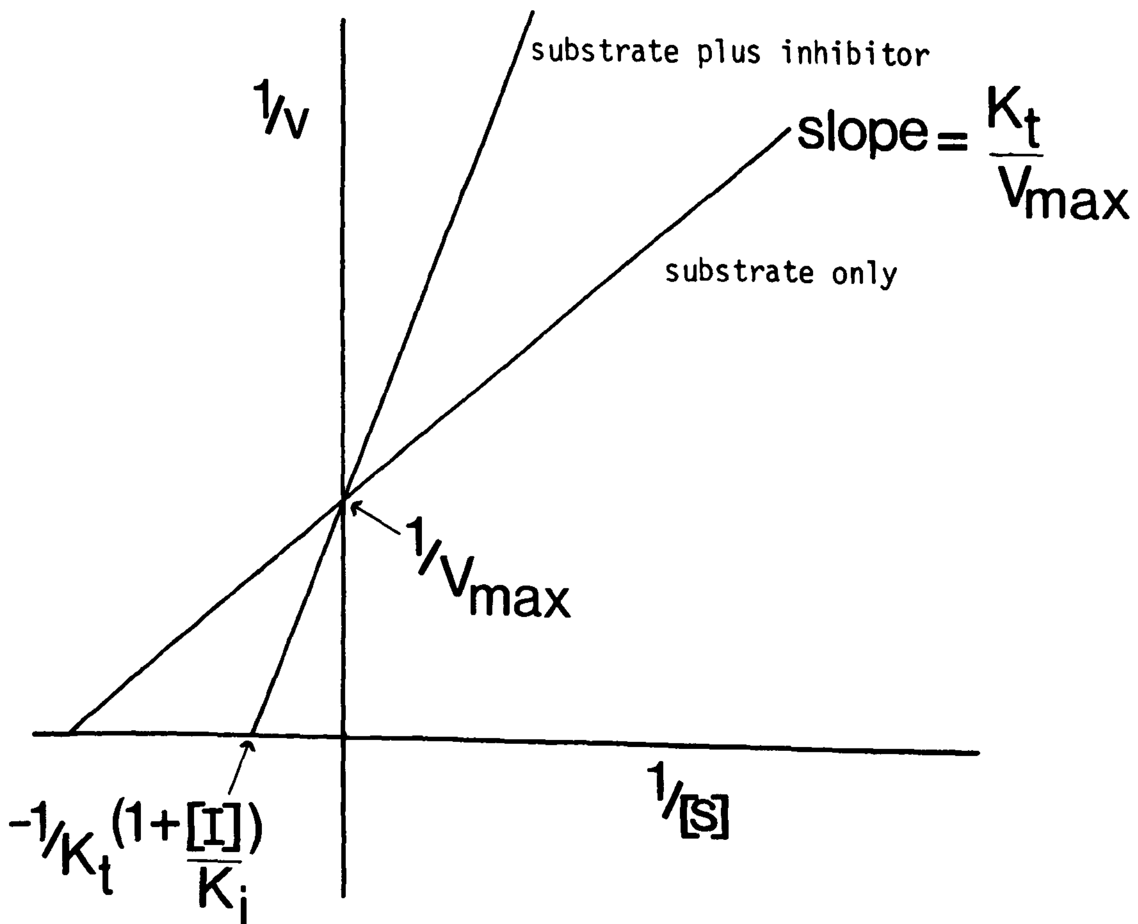


FIG. 4.4 NON-COMPETITIVE INHIBITION (Lineweaver - Burk plot)

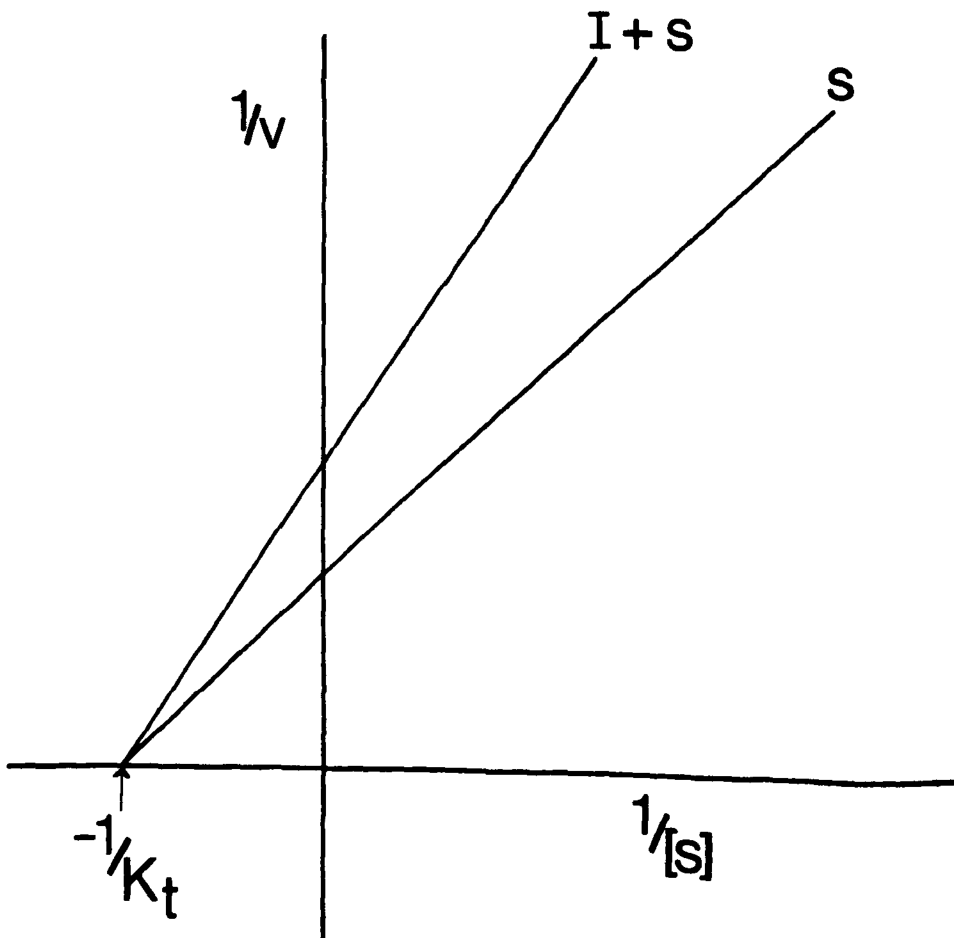
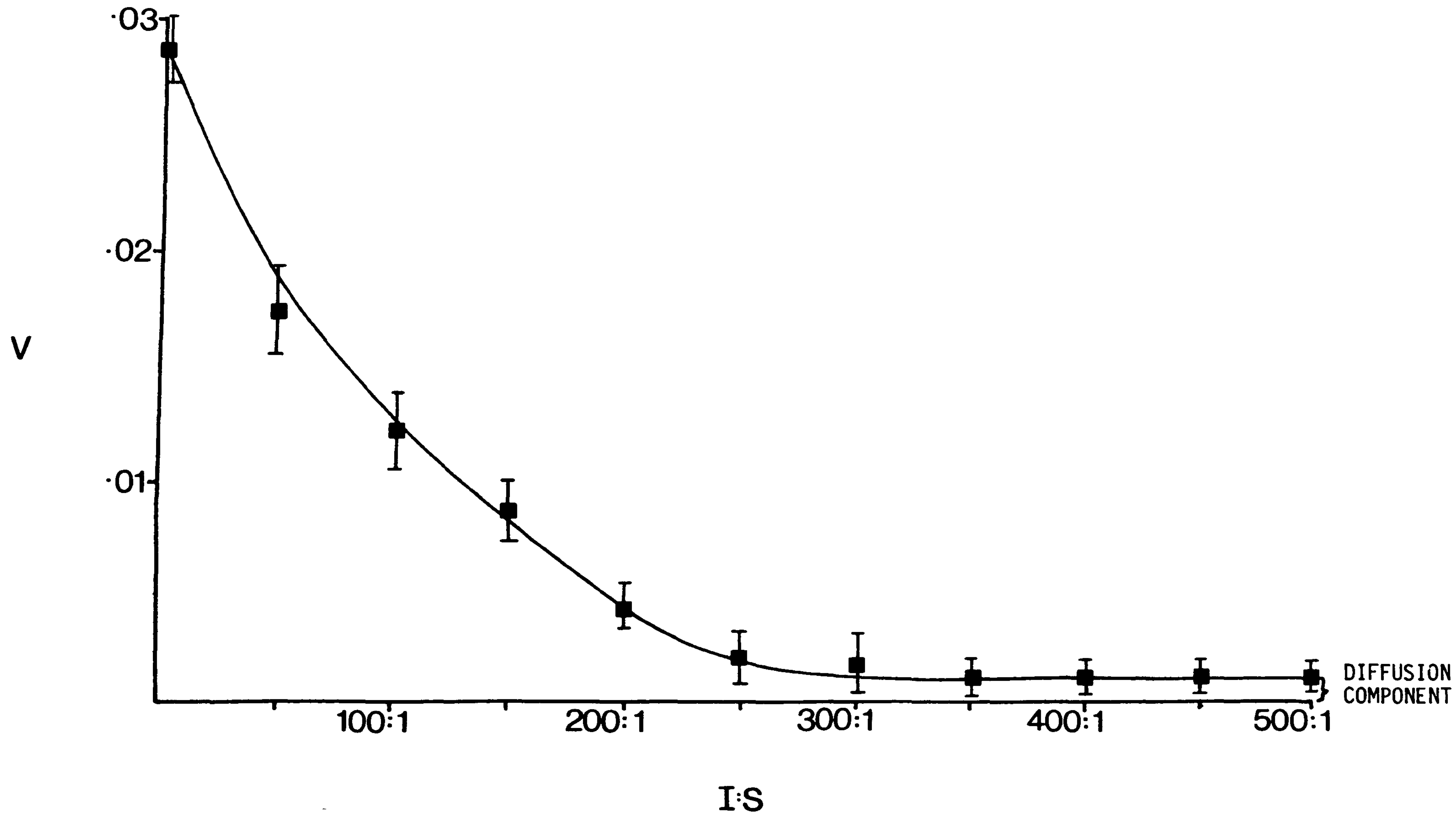


FIGURE 4.5 DIAGRAM SHOWING ALMOST TOTAL INHIBITION OF SUBSTRATE UPTAKE BY AN INHIBITOR (± S.E)



and I = concentration of inhibitor.

4. CALCULATION OF THE CONCENTRATION RATIO

To enable the quantification of substrate accumulation, the concentration ratio must be determined. This is a measure of the concentration of a substrate within the parasite compared to its concentration in the ambient medium. Arme and Coates (1973) determined the mean dry weight : fresh weight ratio of stage 5 cysticercoids of *H. diminuta* as 81% and this value is used throughout this study in the calculation of concentration ratios by the following equation;

$$CR = \frac{\text{concentration of solute in parasite water}}{\text{concentration of solute in medium}}$$

The validity of this ratio is however, subject to many influences including metabolism of absorbed substrate, compartmentalisation of solute in tissue fluids and therefore of absorbed substrate, and efflux. However, it is a parameter that has been used previously in the study of substrate transport by *H. diminuta* cysticercoids (Arme and Coates,1973; Jeffs,1984; Jeffs and Arme,1984a).

CHAPTER 5

UPTAKE STUDIES

A. SPECIFICITY OF THE MONOSACCHARIDE TRANSPORT SYSTEM IN THE CYSTICERCOID OF *Hymenolepis diminuta*.

1. GLUCOSE UPTAKE

According to Phifer (1960a) and Pappas and Read (1975), adult *H. diminuta* possesses a single locus for the transport of glucose and similar monosaccharides. More recent evidence by Starling and Roberts (Roberts,1980) suggests that there are 2 loci in young adults (< 10-days-old); one K^+ -sensitive and one K^+ -insensitive, but these data have yet to be confirmed by other workers. In the cysticercoid, glucose uptake was inhibited by only 32% in the absence of external Na^+, and because of this, it was suggested that the cysticercoids possessed two functional loci - only one of which was Na^+ sensitive (Arme, Middleton and Scott,1973).

To determine uptake in this study, fifty cysticercoids were incubated for 30 sec in a range of labelled monosaccharide concentrations, as described in Chapter 4. Analysis of the results enabled the transport constant (K_t) and the maximum uptake velocity (V_{max}) to be calculated directly from a velocity of uptake *vs.* substrate concentration graph, or by an

equivalent method (see Chapter 4). Table 5.1 illustrates the glucose uptake results. A K_t of 0.26mM and a V_{max} of 0.038 μmoles of glucose absorbed by 50 cysticercoids in 30 sec were obtained, and these data proved to be consistently reproducible. Comparison with data presented by Arme, Middleton and Scott (1973) show that the K_t obtained by the latter authors is approximately six times greater and the V_{max} value two times greater (K_t= 1.54mM; V_{max}= 0.082 μmoles/50 cysticercoids/30 sec, converted from the original figure of 19.8nM of glucose/100 cysticercoids/h). Such a large discrepancy between results is difficult to explain. Arme et al.(1973) used a suction-filter method, whereby radiolabelled substrate was poured onto the cysticercoids, left for the incubation time and suction-filtered off, but both the strain of beetle intermediate host, and H. diminuta used were identical. However, using the method described here, the cysticercoid apparently has a greater affinity for glucose than previously reported.

Absorption of glucose was also studied in recently excysted worms (in this study, recently excysted worms are defined as those which have been excysted in vitro and transferred immediately to the appropriate experimental media; see Chapter 4 for details). Worms were incubated in glucose concentrations of 0.3mM to 1.5mM and Table 5.1 shows the results, a K_t of 0.81mM and a V_{max} of 0.071 μmoles of glucose absorbed/200 worms/2min were calculated. The data from similar experiments on 5 and 10-day-old adult H. diminuta are

shown in Table 5.1 together with summary of the present kinetic data available for glucose absorption by H. diminuta.

From the results (Table 5.1) three points emerge. The first is that the values obtained for the 10-day-old worm in this study, fall within the range of values published by other workers. Secondly, data concerning recently excysted adult glucose absorption are provided for the first time, with the K_t value falling between that of the cysticercoid and the 5- and 10-day-old values. Finally, a gradual increase in the K_t, i.e. a decrease in the affinity of glucose for the uptake site, is observed as the cestode develops from cysticercoid to mature adult worm. The values presented by Starling and Roberts (Roberts,1980), although smaller in absolute terms (i.e. showing a greater affinity), also exhibited a decreased affinity with age. The V_{max} values also decreased, and this may reflect an increase in worm mass without a concomitant increase in the number of uptake loci. Read (1967) and Roberts (1983) have shown that adult H. diminuta has a requirement for glucose as its main energy source and that lack of glucose results in reduced growth, dry weight and tissue glycogen levels. Although no such requirement has been described in the cysticercoid, if it exists, then the affinity of the glucose transport locus would be expected to reflect both the amount required by the cysticercoid and its availability in the environment. The adult worm shows a decrease in affinity for glucose with age (Henderson,1977). Its preferred location also alters so that a position of maximum luminal glucose concentration is occupied during the first 6 days (despite the diurnal migrations) when

TABLE 5.1 A SUMMARY OF KINETIC DATA AVAILABLE FOR GLUCOSE UPTAKE IN *Hymenolepis diminuta*

	K_t	S.D	V_{max}	S.D	REFERENCE
CYSTICERCOIDS	1.54mM	-	0.082μmoles/50 cysticercoids/30s	-	Arme et al.(1973)
	0.26mM	±0.017	0.038μmoles/50 cysticercoids/30s	±0.0017	This study
0-DAY-OLD ADULT	0.81mM	±0.027	0.071μmoles/200 worms/2mins	±0.0019	This study
5-DAY-OLD ADULT	0.95mM	±0.014	576μmoles/g/h	±3.6	This study
6-DAY-OLD ADULT	0.43mM		1494μmole/g/h		Starling and Roberts (in Roberts,1980)
10-DAY-OLD ADULT	0.74mM		603μmoles/g/h		Starling and Roberts (in Roberts,1980)
	1.2mM	±0.143	500μmoles/g/h	±3.5	This study
	1.6mM		-		Read (1961)
	1.4mM		789μmoles/g/h		Read et al. (1974)
	1.0mM		585μmoles/g/h		McCracken and Lumsden (1974)
20-DAY-OLD ADULT	1.84mM		492μmoles/g/h		Starling and Roberts (in Roberts,1980)

the worm is growing exponentially and its glucose requirement is presumably at its highest. The concentration of digested carbohydrate in the form of glucose in the intestine, and along its length, was originally determined in the rat by Clark(1949) who demonstrated a relatively high jejunal concentration, which decreases gradually along its length. Daily fluctuations in this glucose gradient have been suggested as one reason for the circadian migration in the intestine observed by adult worms (Cho and Mettrick,1982; Mettrick and Cho,1981a,b). Table 5.1 shows that the cysticercoid is the stage in the life cycle where the transport loci have the greatest affinity for glucose, and this may in turn reflect glucose availability in its environment. The work described in Chapter 2 suggests concentrations of free glucose in _T. molitor_ haemolymph of less than 0.3mM, so that, if all available glucose were to be utilised, an effective glucose transport locus with high affinity would be required. The adult, recently excysted in the intestine, locates and attaches to a suitable site, and subsequently grows exponentially. To ensure that availability of glucose is never a limiting factor, it locates within an area of high glucose concentration and has also, a transport site with a high affinity for glucose. As the exponential growth phase ends, although the worm may still be located in the same area of the intestine, the affinity of its glucose transport locus decreases and as it grows older there is both reduced affinity of the locus, an apparent reduction in number of uptake loci, and an alteration in the location of the worm,to a site in the intestine of lower glucose concentration.

2. GALACTOSE UPTAKE

Experiments to determine the kinetic parameters of galactose uptake in cysticercoids, recently excysted adults, 5- and 10-day-old adult worms were also carried out. The results are shown in Table 5.2.

The results reveal a number of points of interest. First, they show that the affinity of galactose for the uptake site is less than half that of glucose at every life cycle stage. Secondly, there is a decrease in affinity for galactose with age/life cycle stage from cysticercoid to mature adult worm, as with glucose uptake. The results of Starling (1975) also show a decrease in V_{max}, but the results of this study indicate an increase with age. The similarities in V_{max} values for galactose and glucose uptake in this study strongly suggests that the same number of uptake loci are available to both and that they are therefore possibly transported *via* the same uptake locus. A galactose requirement by *H. diminuta* has not been demonstrated and, although Komuniecki and Roberts (1975) have presented evidence that *H. diminuta* possess the enzymes for galactose utilisation, Read (1965) did not detect these enzymes. The prevalence of galactose in the environment of adult and cysticercoid has not been specifically determined. However, it would be logical to suggest that it does occur in the intestine (dependent upon its presence in food e.g. associated with lactose-milk sugar) and that it is absent in the haemolymph of the intermediate host, since no insect to date has been shown to possess monosaccharides other than

TABLE 5.2 A SUMMARY OF THE KINETIC DATA AVAILABLE FOR GALACTOSE UPTAKE IN *Hymenolepis diminuta*

	K_t	S.D	V_{max}	S.D	REFERENCE
CYSTICERCOID	0.52mM	±0.020	0.038μmoles/50 cysticercoids/30s	±0.018	This study
0-DAY-OLD ADULT	1.2mM	±0.173	512μmoles/200 worms/2min	±4.9	This study
5-DAY-OLD ADULT	1.25mM	±0.144	562μmoles/g/h	±6.3	This study
6-DAY-OLD ADULT	1.89mM		1869μmoles/g/h		Starling (1975)
10-DAY-OLD ADULT	2.7mM		756μmoles/g/h		Starling (1975)
	5.0mM		-		Read (1961)
	3.0mM	±0.210	842μmoles/g/h	±10.0	This study
20-DAY-OLD ADULT	4.27mM		702μmoles /g/h		Starling (1975)

glucose in its haemolymph (Wyatt,1961).

3. UPTAKE OF OTHER CARBOHYDRATES

A range of labelled carbohydrates were used as substrates in absorption studies on cystciercoids and recently excysted adults. Those tested are listed in Table 5.3. Table 5.3 illustrates that transport of all these monosaccharides is through a mediated system (and for those actively transported evidence of accumulation is given on page 164). Glucose has the greatest affinity for the uptake locus with 1-dexoyglucose having the lowest affinity for the locus. Evidence for the presence of a single monosaccharide uptake locus is presented in Chapter 6. Table 5.3 also shows the affinities of some non-metabolisable monosaccharides (i.e. 2-deoxyglucose and 2-deoxygalactose). These monosaccharides appear to have affinities greater than some sugars whose ability to be metabolised, at least in some helminths, has been established (e.g. Starling and Fisher, 1978,1979; Rahman and Mettrick,1982; Read,1956,1959,1961 1967). This suggests that adsorption onto the uptake locus and subsequent transport of substrate is a process which occurs independently of the nature and subsequent fate of the substrate transported. Thus, the data showing transport of non-metabolisable sugars illustrates that they possess the correct configuration for 'attachment' to the carrier locus

not, necessarily, that they are required by the cysticercoid or normally occur in its environment.

Table 5.4 illustrates the equivalent data for adult H. diminuta. Immediately obvious are the differences in specificity illustrated by, for example, the inferred transport of 6-deoxyglucose in adults but not in cysticercoids. The difference in affinities of adult and cysticercoid glucose transport loci have already been noted, and it is evident that differences in specificity also exist. These differences are sufficiently large to suggest that the adult monosaccharide uptake loci are distinct from those in the cysticercoid in both affinity and specificity. A detailed discussion of specificity is included in Chapter 6.

B. FACTORS AFFECTING MONOSACCHARIDE TRANSPORT IN Hymenolepis diminuta CYSTICERCOIDS

1. EFFECT OF TEMPERATURE ON UPTAKE

Temperature is of great importance in active processes. Typically, variations in temperature influence metabolism by altering the rates of enzymatic reactions and by modifying the physical state of cell membranes, in particular their fluidity (Walker and Barrett,1984). The degree of fluidity of the cell membrane influences both membrane transport systems and the properties of membrane-bound enzymes. Thus, temperature variations could significantly alter the absorptive ability of

the cysticercoid. H. diminuta throughout its life cycle experiences three temperature domains, the rat host ($38^{o}C$) the faeces, and the intermediate host (usually between 20 - $26^{o}C$ in the laboratory). In each case, functions which are required to be accomplished at a particular stage are performed by enzymes, the optimum temperatures of which presumably lie within the range encountered. Temperature compensations can be explained in terms of the relationship between the K_m of enzymes for their substrates and temperature, such that as the temperature is reduced, so less substrate is needed to saturate the enzyme. Also, if as the temperature is raised, the K_m also rises, the V_{max} will increase at a proportionally slower rate. Thus, at physiological substrate concentrations, catalytic rates at low temperature will approach those at higher temperatures and therefore a given reaction can be held relatively independent of temperature. The major mechanisms of such acclimations are changes in the concentrations of key enzymes, alterations in isoenzyme patterns and changes in the physical properties of cell membranes. By altering membrane lipid composition in response to temperature change, organisms are able to modulate the response of their membrane-bound enzymes (Walker and Barrett,1984). This latter suggestion that temperature changes can predetermine alterations in the characteristics of cell membranes, may have some significance on the variation in uptake characteristics expressed by the same genome but at different stages in the life cycle.

A series of experiments were carried out on H. diminuta cysticercoids and recently excysted adults at different

temperatures. Incubations were normally carried out at 26°C, but, in order to investigate the effect of temperature on monosaccharide transport, experiments were also carried out at 0°C, 10°C, 20°C and 32°C. At 0°C, the entire experiment was performed on a cooling tray, with glucose and galactose as substrates. In every experiment the age of cysticercoids was kept constant (between 15 - 20 days old - stage 5). The results of all temperature experiments are shown in Table 5.5. The data suggested that there was no appreciable uptake by cysticercoids or recently excysted adults at 0°C. Cysticercoids were, however, viable when held at this temperature for the duration of an experiment as shown by their ability subsequently to excyst in vitro (it was also noted that short periods of freezing had no effect on excystation). The recently excysted adult, however, did not survive the experimental period, so that the results represent transport by recently excysted dead and moribund adults. A further experiment was performed in which cysticercoids were incubated in glucose at 0°C for 1 h, and data were compared with a 2 min incubation at 0°C. Total recovered radioactivity increased only 0.7% from 2 min to 1 h, and this compares with a figure of 17% at 26°C. At 0°C it might be expected that uptake of all kinds would be greatly reduced as there is a concomitant reduction in molecular movement. Diffusion will occur but at a much slower rate. Also, low temperatures will alter the rate of reaction and affect enzyme-substrate, and therefore presumably carrier-substrate binding. In this case 'immediate compensation' (Walker and Barrett,1984) does not appear to

occur, as K_t and V_{max} values are relatively constant at all temperatures between c. 10 and 32°C. A trend in alteration of V_{max} with changing temperature is apparent, however, so that uptake at 0°C probably represents diffusion, which is, at this temperature, less than 0.5% of the 'normal' uptake at 26°C.

At 10°C and 20°C, the cysticercoid showed little alteration in its affinity for both glucose and galactose, but a decrease in V_{max} with decreasing temperature was noted. Similarly, at 32°C, an increase in V_{max} of approximately 15% was noted, but little alteration in K_t. Thus the changing V_{max} probably occurs around 20°C so that decreases in V_{max} are noted below this temperature and increases above. Walker and Barrett (1984) stated that in immediate temperature compensating systems, increasing temperatures generally had the effect of reducing the V_{max} of enzymes and equilibrating the process, so that actual amounts of substrate transported might be expected to be the same over a wide temperature range. An increasing velocity of uptake with increasing temperatures, as shown in the cysticercoids, is accompanied by increase in net uptake. Recently excysted adults also exhibited a constant K_t with changing V_{max}, and the glucose results were similar to those for galactose in both cysticercoids and recently excysted adults. All individuals appeared unaffected by these short incubations at the various temperatures tested, as shown by the cysticercoids ability to excyst, and the visual appearance (under the light microscope) of the recently excysted adults. The 0-day-old adult, which did not survive

TABLE 5.3 CARBOHYDRATES TESTED FOR THEIR ABILITY TO BE TRANSPORTED BY *H. diminuta* CYSTICERCOIDS

	K_t		V_{max}		
Glucose	0.26mM	±0.017	0.038	±0.0017	umoles/50 cysticercoids/30s
Galactose	0.52mM	±0.020	0.058	±0.0018	"
3-O-Methylglucose	0.35mM	±0.020	0.029	±0.0023	"
2-Deoxyglucose	0.30mM	±0.015	0.031	±0.0016	"
α-Methylglucoside	0.41mM	±0.014	0.042	±0.0017	"
β-Methylglucoside	No Transport		-		
6-Deoxyglucose	No Transport		-		
1-Deoxyglucose	0.84mM	±0.016	0.041	±0.0024	"
2-Deoxygalactose	0.76mM	±0.020	0.046	±0.0015	"
Mannose	0.54mM	±0.016	0.038	±0.018	"
Fructose	No Transport		-		
Glycerol	0.64mM	±0.024	0.102	±0.0010	"
Sucrose	No Transport		-		
Maltose	No Transport		-		
Trehalose	No Transport		-		

NB Readings are given ± standard deviation. At least 10 replicates per reading.

TABLE 5.4 A SUMMARY OF THE KINETIC DATA AVAILABLE FOR CARBOHYDRATE TRANSPORT IN ADULT *Hymenolepis diminuta*

	K_t	V_{max}	REFERENCE
Glucose	1.4mM	789μmoles/g/h	Read et al. (1974)
Galactose	5.0mM	-	Read (1961)
Glycerol	0.24mM	84μmoles/g/h	Uglem et al. (1974) (Na^+-dependent)
	0.69mM	91μmoles/g/h	Uglem et al. (1974) (Na^+-independent)
α-Methylglucoside*	-	-	(see Pappas and Read,1975)
1-Deoxyglucose*	-	-	"
3-O-Methylglucose*	-	-	"
6-Deoxyglucose*	-	-	"
Allose*	-	-	"

*= Transport inferred from inhibition experiments

incubation at 0^oC, had no obvious morphological alterations except an appearance of bloatedness and lack of movement.

2. THE EFFECT OF CYSTICERCOID AGE ON UPTAKE

In adult H. diminuta, the K_t for glucose uptake varies with the age of worm (Henderson,1977; Phifer,1960a; Starling, 1975; Roberts,1980), the younger the worm the greater the affinity i.e. the smaller the K_t value (see Table 5.1). Once the worm has matured, and becomes patent, the K_t appears to stabilise, although kinetic data from uptake experiments on older worms (i.e. more than 20-days-old) are unavailable. The V_{max} also alters with age, a gradual decrease being noted in older worms (see Table 5.1). These variations, which are not characteristic of other nutrients e.g. amino acids (Jeffs,1984), are probably related to the apparent absolute dependency of adult H. diminuta for glucose as its main energy source. The adult worm has three main growth periods, the initial period, immediately after excystation and before attachment, when growth is relatively slow, a second period of rapid exponential growth from day 2 - 5, and the final post-patent period when the production of eggs and proglottides becomes the most important function (Goodchild and Harrison, 1961). Current uptake data appear to correspond closely with these periods, with a gradually increasing K_t as the need for glucose is more easily satisfied by adequate glucose concentration in the external media.

The effect of age on cysticercoid glucose and galactose

TABLE 5.5. THE EFFECT OF TEMPERATURE ON GLUCOSE AND GALACTOSE UPTAKE BY CYSTICERCOIDS AND RECENTLY EXCYSTED ADULTS

	CYSTICERCOIDS		R.E.W	
	K_t	V_{max}	K_t	V_{max}
0^oC				
GLUCOSE	0.29mM±0.017	0.007±0.0034	0.80mM±0.027	0.010±0.0040
GALACTOSE	0.56mM±0.020	0.003±0.0020	1.3mM±0.025	0.003±0.004
10^oC				
GLUCOSE	0.28mM±0.015	0.012±0.001	0.83mM±0.025	0.035±0.009
GALACTOSE	0.54mM±0.010	0.035±0.010	1.3mM±0.025	0.009±0.010
20^oC				
GLUCOSE	0.26mM±0.020	0.038±+0.010	0.82mM±0.010	0.073±0.009
GALACTOSE	0.52mM±0.013	0.030±0.010	1.3mM±0.031	0.080±0.008
30^oC				
GLUCOSE	0.26mM±0.018	0.042±0.009	0.81mM±0.018	0.079±0.008
GALACTOSE	0.52mM±0.018	0.038±0.008	1.3mM±0.026	0.085±0.010

R.E.W = recently excysted worm. Figures are given ± standard deviation.

Each figure is the mean of 15 replicates.

V_{max} is measured in µmoles/50 cysticercoids/30s.

uptake was investigated experimentally. An age effect was expected, due to the assumed variation in demand for monosaccharides with the different developmental stages. These developmental stages are described fully in Chapter 1, and are numbered 1 - 5 (according to Heyneman and Voge,1959). Stage 5, being the fully mature infective cysticercoid, is usually attained by 11 days at 26°C. Stage 5 cysticercoids can remain in the intermediate host for periods of 40+ days and, during this time, the principal observed morphological alteration is the continuous build up of a fibrous layer in the outer cysticercoid wall (Richards and Arme,1984a). Some morphometric studies on cysticercoids of different ages are given in Table 5.6 and the increase in width, associated with this fibrous layer, is clearly illustrated (increasing by approximately 8% over each period of 5 days). The significance of the presence of this fibrous layer in the uptake of substrates is unknown, but it may be relevant that absorbed glucose requires at least 10min to reach the presumptive scolex (see later).

The uptake of glucose and galactose was studied in cysticercoids aged 7,10,20 and 30 days. The results are illustrated in Table 5.7. There was no significant alteration in the K_t or V_{max}, although there was a trend towards a decrease in these values. These results show that the affinity for glucose and galactose is only slighty altered as production of the fibrous layer begins and continues, from mature Stage 5 cysticercoids onwards. The V_{max}, gradually decreased as the cysticercoid developed.

TABLE 5.6 CYSTICERCOID MORPHOMETRICS

CYSTICERCOID AGE	a	b	c	d
7 days	0.19mm ± 0.02	0.21mm ± 0.03	0.26mm ± 0.03	0.61mm ± 0.10
10 days	0.22mm ± 0.01	0.27mm ± 0.02	0.28mm ± 0.02	0.72mm ± 0.13
15 days	0.21mm ± 0.02	0.26mm ± 0.02	0.29mm ± 0.03	0.59mm ± 0.08
20 days	0.18mm ± 0.01	0.31mm ± 0.05	0.30mm ± 0.03	0.61mm ± 0.09
25 days	0.21mm ± 0.01	0.31mm ± 0.06	0.30mm ± 0.02	0.65mm ± 0.07
30 days	0.22mm ± 0.01	0.36mm ± 0.03	0.29mm ± 0.02	0.67mm ± 0.10
35 days	0.23mm ± 0.01	0.47mm ± 0.02	0.34mm ± 0.01	0.72mm ± 0.10

Each reading is the average of 300 pooled cysticercoids from each age group.

The readings are given ± standard deviation.

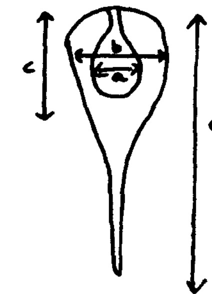


TABLE 5.7 THE EFFECT OF CYSTICERCOID AGE ON GLUCOSE AND GALACTOSE UPTAKE

AGE OF CYSTICERCOID		K_t	V_{max}	
7 days	(glucose)	0.22mM± 0.018	0.039± 0.0018	μmoles/50 cysticercoids/30s.
	(galactose)	0.50mM± 0.014	0.040± 0.0021	"
10 days	(glucose)	0.21mM± 0.019	0.040± 0.0019	"
	(galactose)	0.51mM± 0.015	0.038± 0.0022	"
20 days	(glucose)	0.26mM± 0.020	0.038± 0.0023	"
	(galactose)	0.51mM± 0.016	0.041± 0.0021	"
30 days	(glucose)	0.25mM+ 0.018	0.039± 0.0027	"
	(galactose)	0.52mM± 0.017	0.040+ 0.0021	"

Each reading is the mean of 15 replicates.
V_{max} is measured in μmoles/50 cystciercoids/30s

Each value is given ± standard deviation.

However, although the affinity of the cysticercoid monosaccharide uptake locus is far greater than the adult, the fact that the K_t and V_{max} do not seem related to the expected greater requirements of active growth (i.e. the affinity of the site does not vary with requirement) may imply that exogenous glucose/galactose are not critically important as energy sources during the active growth stages of the cysticercoid and that other substrates e.g. amino acids, may be utilised.

3. EFFECT OF SODIUM IONS ON MONOSACCHARIDE TRANSPORT

Glucose absorption in adult *H. diminuta* is known to be Na^+-dependent and Na^+-coupled (Read, Stewart and Pappas,1974; Uglem *et al.*,1974). This is also true of monosaccharide transport in some other helminths e.g. *Taenia taeniaeformis* (Von Brand and Gibbs,1966), *T. crassiceps* metacestodes (Craig,1983), *Calliobothrium verticillatum* (Pappas and Read,1972a; Read *et al.*,1974), and *H. microstoma* (Pappas and Freeman,1975).

The dependence on the presence of Na^+ in the external medium for sugar transport is a direct result of its role in the actual process of absorption. The active transport process is brought about by the coupled influx of Na^+ and glucose, where Na^+ influx is the result of the maintained asymmetric distribution of Na^+ across the tegument. Chapter 3 describes Na^+-coupled sugar transport in detail and also discusses the results of experiments on adult *H. diminuta* in which the Na^+

concentration in incubation media was altered. Arme *et al.* (1973), in their study on glucose transport by *H. diminuta* cysticercoids, noted that uptake was only inhibited by 32% in the total absence of external sodium. This is in contrast to the almost total inhibition of glucose uptake in adult *H. diminuta* induced by lack of Na^+. They therefore suggested that glucose uptake was only partially Na^+-sensitive, or, because of the strong competitive inhibition of galactose on glucose (97 - 98%) there might exist two uptake loci, one Na^+-sensitive and the other Na^+-insensitive.

An investigation into the effect of sodium ions on glucose transport in the cysticercoid was undertaken. The first experiments established the effect of total replacement of sodium with other cations. Normal KRT saline comprises approximately 154mM Na^+, and forms the basis of all experimental media. Potassium, lithium, choline and Tris were all used as replacement cations and resulted in the following inhibitions of glucose uptake, 36.7, 24.3 31.2 and 35.6% respectively. These results agree well with those of Arme *et al.*(1973) and suggest again, the presence of a partially Na^+-sensitive glucose transport system. Lithium used as replacement cation had the least effect, a result similar to that found in the adult worm (Read, Stewart and Pappas,1974). The replacement of sodium by different proportions of other cations was studied and the results are shown in Table 5.8.

Choline as replacement cation had the effect of increasing the K_t i.e. decreasing the affinity of glucose for the uptake locus, as choline concentrations

increased. The same trends were shown for both lithium and Tris, although with potassium there appeared a decrease in affinity followed by an increase at very high potassium concentrations (i.e. very low Na^+ concentrations). These unusual results were consistently obtained but contradict Crane's hypothesis that inhibitory (or replacement) cations interact with the cation-binding site on the mobile carrier to produce a less efficient carrier than the sodium-loaded one (Crane,1960). Adult H. diminuta showed no alteration in K_t with replacement cations, and only a slight change in V_{max}. Therefore, it appears that there are differences in response of the cysticercoid and adult to reduced and absent Na^+.

A decrease in affinity and capacity of the monosaccharide uptake system with decreasing Na^+ concentrations is thus the trend in the cysticercoid, with an unusual result using potassium. The same experiments were then performed on the recently excysted adult to determine whether they possessed a system similar to either cysticercoid or 10-day-old adult, or one that exhibits intermediate characteristics.

Recently excysted adults were incubated in concentrations of glucose from 0.1 - 1.0mM in which the KRT contained varying concentrations of $Na^+$and K^+, choline$^+$, Tris$^+$ and lithium$^+$ as replacement cations. Table 5.9 shows the results of these experiments. As with the 10-day-old adult, a reduced glucose uptake was noted when Na^+ was replaced by any of the four above-mentioned cations. Unlike the 10-day-old adult, lithium was not significantly better as replacement cation than K^+,

$Tris^+$ or $choline^+$, although it did cause the least percentage reduction in glucose uptake (see Table 5.9.). The percentage reduction in glucose transport brought about by replacment of Na^+, however, was smaller than expected, being half the value calculated for the equivalent data for 10-day-old adult worm. Thus, the 10-day-old adult experiences an almost total reduction in glucose uptake when Na^+ is removed from the incubation media,the recently excysted adult has a reduction of c. 50% and the cysticercoid a reduction of c.30%. This unusual result suggests a gradual increase in sensitivity to Na^+-deletion from cysticercoid to 10-day-old adult, with the recently excysted adult having an intermediate response. There is no evidence from this work, or the work of Read et al. (1974) for the existence of K^+ sensitive/insensitive glucose uptake loci. Starling and Roberts (Roberts,1980) stated that sensitivity to K^+ was found in 6-day adults, was just detectable in 10-day adults and by 20 days post-infection could not be detected. One might expect, therefore, some evidence for K^+-sensitivity in recently excysted adults. Why should the response to Na^+-deletion change with age/life cycle stage? One explanation might be that differences occur in the transport loci so that the cysticercoid has both a Na^+-insensitive and an Na^+-sensitive glucose transport system. If the cysticercoid, however, also had some sensitivity to K^+, as described for the young adult, then the unusual results with K^+ as relacement cation could be explained.

TABLE 5.8 EFFECT OF CATIONS ON GLUCOSE UPTAKE BY H. diminuta CYSTICERCOIDS

REPLACEMENT CATION	$[Na^+]$	K_t	V_{max}	
Control	154mM (KRT)	0.26mM ± 0.018	0.038 ±0.0019	µmoles/50 cysticercoids/30s
Potassium+	10mM	0.12mM ± 0.040	0.047 ±0.0029	"
	25mM	0.27mM ± 0.035	0.080 ±0.0026	"
	50mM	0.42mM ± 0.026	0.065 ±0.0021	"
Lithium+	10mM	0.69mM ± 0.054	0.095 ±0.0039	"
	25mM	0.50mM ± 0.044	0.085 ±0.0032	"
	50mM	0.37mM ± 0.026	0.062 ±0.0021	"
Tris+	10mM	0.39mM ± 0.061	0.063 ±0.0039	"
	25mM	0.44mM ± 0.034	0.088 ±0.0037	"
	50mM	0.34mM ± 0.019	0.101 ±0.0020	"
Choline+	10mM	0.56mM ± 0.049	0.120 ±0.0049	"
	25mM	0.31mM ± 0.032	0.197 ±0.0039	"
	50mM	0.29mM ± 0.025	0.096 ±0.0020	"

Each reading is the mean of 15 replicates and is given ± standard deviation.

TABLE 5.9 EFFECT OF CATIONS ON GLUCOSE UPTAKE IN RECENTLY EXCYSTED ADULT *Hymenolepis diminuta*

REPLACEMENT CATION	$[Na^+]$	K_t	V_{max}		% reduction in glucose uptake (compared with controls)
control	154mM (KRT)	0.81mM ± 0.020	0.071 ±0.0020	µmoles/200 worms/2min	-
Potassium$^+$	10mM	1.34mM ± 0.021	0.066 ±0.0027	"	48.3
	25mM	1.32mM ± 0.022	0.071 ±0.0015	"	37.2
	50mM	0.90mM ± 0.019	0.062 ±0.0014	"	25.1
Lithium$^+$	10mM	1.79mM ± 0.027	0.073 ±0.0011	"	39.2
	25mM	1.43mM ± 0.026	0.059 ±0.0016	"	25.3
	50mM	1.20mM ± 0.025	0.059 ±0.0019	"	19.2
Tris$^+$	10mM	1.62mM ± 0.022	0.059 ±0.0021	"	53.7
	25mM	1.59mM ± 0.022	0.063 ±0.0015	"	42.1
	50mM	1.63mM ± 0.023	0.069 ±0.0019	"	30.9
Choline$^+$	10mM	1.34mM ± 0.029	0.059 ±0.0019	"	47.6
	25mM	1.36mM ± 0.021	0.071 ±0.0016	"	36.6
	50mM	1.28mM ± 0.017	0.072 ±0.0023	"	26.6

Each reading is the mean of 15 replicates and is given ± standard deviation.

C. LOCATION OF MONOSACCHARIDES ABSORBED BY THE CYSTICERCOID

In order to determine how far radio-labelled glucose and galactose penetrated the cysticercoid during an incubation, and to determine that uptake measured is indeed that across the outer cysticercoid membrane, a simple time-course experiment was carried out. This involved incubating cysticercoids for 1,5,10,20 and 30 min in 0.3mM glucose, and then excysting them *in vitro*. The media used for excystation (as described in chapter 5) was supplemented with 0.3mM unlabelled glucose. The excysted worms were removed and extracted in ethanol as were the cysticercoid 'cases', according to the method described in Chapter 5. A control group of cysticercoids, derived from the same original batch, were excysted in the used excysting media and monitored for their radioactivity. The results are shown in Table 5.10, and show that glucose can penetrate to the inner cysticercoid tissue some time between 10 and 20 min when incubated at 26°C. Galactose appears to penetrate more slowly taking between 20 - 30 min to reach the inner cysticercoid. The experiment shows therefore that incubation periods of < 10 min ensure that the uptake figures calculated represent those across the outer cysticercoid membrane and are not influenced by any additional effect of further transport phenomena in the presumptive scolex.

TABLE 5.10 THE LOCATION OF RADIOLABELLED MONOSACCHARIDES IN CYSTICERCOIDS INCUBATED FOR VARYING TIME PERIODS

INCUBATION TIME	1min	5min	10min	20min	30min
Cysticercoid:					
Glucose	74.3 ± 3.9	146.1 ± 4.0	158.3 ± 6.9	160.1 ± 3.4	196.2 ± 4.7
Galactose	60.2 ± 2.0	117.4 ± 2.7	124.7 ± 5.0	120.6 ± 2.6	245.9 ± 3.2
R.E.W:					
Glucose	25.4 ± 1.1	35.4 ± 3.0	42.7 ± 3.6	112.6 + 4.7	134.4 ± 6.9
Galactose	30.3 ± 2.1	35.2 ± 1.1	64.3 ± 4.2	53.4 ± 3.1	124.7 ± 5.7

NB Readings are in counts per minute and are given ±standard deviation.
R.E.W = Recently excysted worm i.e. the amount of radioactivity extracted from the excysted presumptive scolex.

D. ACCUMULATION OF MONOSACCHARIDES BY CYSTICERCOIDS

The Na^+-gradient hypothesis states that the maintenance of an asymmetric distribution of Na^+ across the cell membrane is essential for the accumulation of solutes against their concentration gradient. Therefore, demonstration that accumulation does occur and is inhibited in Na^+-free media, provides strong evidence for the existence of a Na^+-dependent transport system. A series of experiments were carried out to investigate the degree of accumulation of a variety of monosaccharides, and the effect on glucose accumulation of cysticercoid age, temperature and ionic composition of the incubation media. Cysticercoids aged 15-20 days were incubated in a 0.5mM concentration of a number of sugars. The concentration ratios were calculated and are presented in Table 5.11 All the monosaccharides previously shown to be transported by the uptake locus were also shown to be accumulated against a concentration gradient, and those with concentration ratios greater than unity are presumed to be transported actively. Glucose gave the highest ratio and, by calculating the difference between sugar recovered from media, evolved radio-labelled CO_2 and that recovered from worms (including non-ethanol extractable metabolites e.g. glycogen), the amount of sugar metabolised was obtained. Glucose was therefore the only monosaccharide which was apparently metabolised (45%) during the 1 h incubation (for further discussion see E below). It was interesting to note that the non-metabolisable monosaccharides accumulated, were almost totally recovered intact by the subsequent extraction

procedures. The concentration ratios show clearly that the transport of at least 8 monosaccharides by the cysticercoid occurs actively, their being accumulated against the concentration gradient. The ratios attained for all these monosaccharides appear greater than those obtained for many amino acids (in the cysticercoid) especially after 10 min incubations (see Jeffs,1984). The accumulation of carbohydrate substrates by adult H. diminuta has not been extensively studied, but following a 60 min incubation in 5mM glucose, the internal glucose concentration of worms was approximately 25mM (Pappas et al.,1974).

To investigate the effect of cysticercoid age on accumulation, an experiment was performed with glucose as substrate and cysticercoids aged 10,20 and 30 days. One hundred cysticercoids were incubated for 5 min in 0.5mM glucose. The results (Table 5.12) show a decrease in concentration ratios with increasing age, this being consistent with the hypothesis that both the affinity of the uptake locus and the amount of glucose required by cysticercoids decreases with age.

Temperature might be expected to alter accumulation rates and therefore alter the concentration ratios according to the direction of temperature change. In an experiment to investigate this 100 cysticercoids (aged 21 days) were incubated in 0.5mM glucose for 5 min, at various temperatures. The results (Table 5.13) clearly show a relationship between temperature and accumulation. At low temperatures (i.e.10°C)

the concentration ratio is one, indicating no active transport at this temperature. The effect of temperature on membrane fluidity and the properties of membrane-bound proteins has been discussed earlier (see page145).

As stated previously, the ionic composition of incubation media has been shown to be of importance in the transport of monosaccharides across the tegument of *H. diminuta* adults and cysticercoids (Read *et al.*,1974; Uglem *et al.*,1974; Arme *et al.*,1973). Resulting from this, alteration in Na^+ concentration of media used in an accumulation experiment was shown to affect the subsequent accumulation of substrate in cysticercoids. Lithium, potassium, choline and Tris were used as replacment cations in accumulation experiments and the results are shown in Table 5.14. Total deletion of Na^+ did not result in the cessation of glucose absorption and accumulation, and with the exception of choline, the concentration ratios exceeded unity in the absence of Na^+. This data therefore adds to the evidence for the existence of a Na^+-insensitive glucose transport system in cysticercoids. The percentage reduction in accumulation, however, is approximately twice as great as the reduction in uptake in Na^+-depleted media. Potassium was the most effective replacement cation. During these Na^+-free incubations the Na^+-gradient is reversed so that Na^+-dependent transport will occur from the cysticercoid to the outside media, causing some of the glucose accumulated by the proposed Na^+-insensitive system to be effluxed.

TABLE 5.11 CONCENTRATION RATIOS OF SUGARS SHOWN TO BE TRANSPORTED BY H. diminuta CYSTICERCOIDS

Incubation time	1min	5min	10min	60min
Monosaccharides tested				
Glucose	3.52 ± 0.30	6.97 ± 0.63	15.39 ± 0.26	53.49 ± 0.70
Galactose	1.20 ± 0.21	7.95 ± 0.54	10.50 ± 0.51	19.29 ± 0.91
3-0-Methylglucose	3.25 ± 0.26	5.69 ± 0.44	14.36 + 0.46	49.53 ± 0.83
2 Deoxyglucose	1.92 ± 0.21	3.59 ± 0.31	10.22 ± 0.27	38.40 ± 0.95
α-Methylglucoside	2.22 ± 0.19	3.89 ± 0.40	8.12 ± 0.35	25.70 ± 0.69
1-Deoxyglucose	0.95 ± 0.15	2.65 ± 0.39	7.39 ± 0.45	18.32 ± 0.79
2-Deoxygalactose	0.86 ± 0.14	5.20 ± 0.39	5.20 ± 0.41	21.30 ± 0.55
Mannose	1.34 ± 0.18	3.00 ± 0.41	7.62 ± 0.53	37.67 ± 0.31

NB All ratios are given ± standard deviation.

TABLE 5.12 EFFECT OF CYSTICERCOID AGE ON GLUCOSE ACCUMULATION

100 cysticercoids incubated for 5min in 0.5mM D(U^{14}C)Glucose

AGE OF CYSTICERCOIDS	AVERAGE CONCENTRATION RATIO	NUMBER OF REPLICATES	S.D
10 days	8.25	10	0.33
20 days	7.01	10	0.67
30 days	5.10	10	0.77

TABLE 5.13 EFFECT OF TEMPERATURE ON GLUCOSE ACCUMULATION BY CYSTICERCOIDS

100 cysticercoids incubated for 5min in 0.5mM D(U^{14}C)Glucose

TEMPERATURE OF MEDIA	AVERAGE CONCENTRATION RATIO	NUMBER OF REPLICATES	S.D
10°C	1.01	10	0.78
15°C	2.19	10	0.88
20°C	6.32	10	0.64
25°C	6.97	10	0.38
30°C	7.56	10	0.44
32°C	8.79	10	0.41

TABLE 5.14 THE EFFECT OF IONIC COMPOSITION OF INCUBATION MEDIA ON ACCUMULATION OF GLUCOSE BY CYSTICERCOIDS

REPLACEMENT CATION	$[Na^+]$	CONCENTRATION RATIO (± S.D)	% REDUCTION IN GLUCOSE ACCUMULATION
Lithium$^+$	0mM	1.76 ± 0.37	c. 75%
	25mM	2.19 ± 0.46	68
	50mM	2.89 ± 0.91	58
Potassium$^+$	0mM	1.98 ± 0.26	71
	25mM	2.62 ± 0.59	62
	50mM	3.49 ± 0.96	50
Choline$^+$	0mM	0.95 ± 0.47	86
	25mM	2.09 ± 0.67	70
	50mM	2.62 ± 0.99	63
Tris$^+$	0mM	1.69 ± 0.23	76
	25mM	2.23 ± 0.44	68
	50mM	2.97 ± 0.87	57
normal KRT saline	154mM	6.97 ± 0.63	-

E. METABOLISM OF ABSORBED SUBSTRATES BY CYSTICERCOIDS

The fate of absorbed substrates was the subject of experiments in which those substrates, after a period of time, were subsequently extracted and identified. It was of interest also to determine whether glucose was the only monosaccharide capable of being metabolised by the cysticercoid. Adult H. diminuta can only metabolise glucose (Read,1967) although it also possesses enzymes for the utilisation of galactose (see Chapter 3). Therefore the same experiments were also used to study metabolism, where not only the quantity of radioactivity recovered was determined but also the quality. Gas liquid chromatography was used to locate sugar metabolites from the ethanolic extracts of cysticercoids incubated for 15,30,45 min 1 h and 3 h in 0.3mM unlabelled monosaccharide. The ethanolic extracts from each group of 200 cysticercoids were evaporated slowly to dryness, and mixed with Tri-sil Z$^{(R)}$-a silylating reagent (see to Chapter 4). Samples were then injected onto the column and run under the conditions described in Chapter 4. Table 5.15 illustrates the initial results. From the table it appears that only glucose is metabolised as all other sugars gave only one peak after 3 h incubation, which was identified as the original substrate by comparison with known standards. Thus it appears that galactose is not metabolised within 3 h. An attempt was made to identify those substances detected by GLC, by comparison with known substances run under identical conditions. The substances referred to in parentheses (Table 5.15), most closely resemble those unknown peaks present in the ethanolic extracts. Therefore after 10 min at 26°C, it

appears that glucose begins to be metabolised. The rate of metabolism is likely to vary according to the physiological state of the cysticercoid and also the environmental conditions.

The effect of age on metabolism was investigated by performing the same experiment on cysticercoids aged 10,20,30 and 40 days. A sample of 100, 40-day-old cysticercoids was used, because of the difficulty in obtaining 200 cysticercoids of this age. The extracts were taken and analysed as before and the results are given in Table 5.16 Only glucose was used as substrate. The results show that the initial stages of glucose metabolism occur after 10 min in 10-day-old cysticercoids, but only after 1 h in 40-day-old cysticercoids. From the uptake experiments, it is known that glucose penetrates to the inner cysticercoid between 10 - 20 min in 20-day-old cysticercoids. Therefore, if the metabolites detected originated from the presumptive scolex, then we would not expect them to appear until sufficient time had passed to allow glucose to penetrate. However, even in 40-day-old cysticercoids, it would be unlikely to take more than 45 min for glucose to penetrate the tegument which is *c.* 15 nm thick. This possible explanation, that the metabolites detected originate from the scolex and therefore the delay in onset of metabolism reflects penetration time, cannot be discounted.

In order to investigate the origin of the compounds detected, an experiment involving incubation and subsequent excystation of the young adult, followed by extraction and identification of the compounds present, was performed. The results showed the presence of metabolites in both cysticercoid 'cases' and recently excysted adult, indicating that either metabolites diffuse from the scolex into the outer cysticercoid or that both are capable of glucose metabolism. However, it would be of interest to determine whether the compounds found were the products of absorbed glucose or glucose derived from endogenous glycogen reserves.

The effect of temperature on glucose metabolism was next investigated. Fifty cysticercoids were incubated for 30 min and 1 h in glucose at one of four temperatures, 15,20,26 or 32°C. The cysticercoids were then extracted and the compounds present in the extract located. The results showed that only at 26 and 32°C were substances other than glucose detected and at 15 and 20°C there was no appreciable metabolism.

Finally, the effect of altering the ionic composition on metabolism was investigated. From the uptake data, that showed considerable absorption, even in Na^+-free media, it was expected that some metabolism might occur in Na^+-depleted media, proving that the substrate metabolised was that recently absorbed. Fifty cysticercoids were incubated in glucose with choline, lithium, Tris and potassium as replacement cations. Incubation times of 30 min and 1 h were used, with Na^+

concentrations of 0 and 50mM. In all cases, two peaks were detected in the extracts after 1 h incubations, and in all cases except potassium, one peak (corresponding to the substrate) was detected after 30 min. Therefore, metabolism of glucose in cysticercoids does occur in the total absence of external Na^+ in the media. However, it is impossible to state whether the metabolites detected are from endogenous glycogen reserves or from glucose absorbed <u>via</u> a Na^+-insensitive locus.

TABLE 5.15 ANALYSIS OF ETHANOLIC EXTRACTS FROM CYSTICERCOIDS BY GLC

200 cysticercoids incubated in 0.3mM monosaccharide

MONOSACCHARIDE → / INCUBATION TIME ↓	GLUCOSE	GALACTOSE	3OMG	MANNOSE	2DOG
15 min	1 peak (= glucose)	1 peak	1 peak	1 peak	1 peak
30 min	2 peaks (= glucose, glucose-6-phosphate	"	"	"	"
45 min	3 peaks (= glucose, glucose-6-phosphate, lactic acid)	"	"	"	"
1 h	"	"	"	"	"
3h	"	"	"	"	"

3OMG = 3-O-methylglucose
2DOG = 2-deoxyglucose

TABLE 5.16 EFFECT OF AGE ON SUBSTANCES EXTRACTED FROM CYSTICERCOIDS AFTER INCUBATION IN GLUCOSE

200 cysticercoids incubated in 0.3mM glucose

AGE OF CYSTICERCOID (DAYS) →	10	20	30	40*
INCUBATION TIME ↓				
10 min	-	-	-	-
30 min	2 peaks	2 peaks	-	-
45 min	3 peaks	3 peaks	2 peaks	-
1h				2 peaks
3h				

*100 cysticercoids of this age used.

2 peaks = glucose, glucose-6-phosphate

3 peaks = glucose, glucose-6-phosphate and lactic acid

CHAPTER 6

INHIBITOR STUDIES

A. INTRODUCTION

Mediated membrane transport involves the binding of a substrate to a carrier and the subsequent translocation of that substrate across a membrane. The process is therefore open to inhibition at two points; the initial substrate - carrier binding, and the subsequent transport of substrate. There are two types of inhibition frequently described in absorption studies: competitive and non-competitive.

Competitive inhibitors interfere with substrate-carrier binding by competing for the binding site(s). Usually, competitive inhibitors have some structural properties in common with the substrate, and it is this that results in its affinity for the uptake site and hence inhibitory properties. Competitive inhibition is characterised by a raised K_t for the substrate being inhibited, but no alteration in V_{max}. Figure 4.3 llustrates a Lineweaver-Burk (1934) plot for competitive inhibition.

Non-competitive inhibition is that which causes a reduction in V_{max} of the substrate and results from inhibition at a point other than the binding site. Examples of non-competitive inhibitors include metabolic poisons and substances which interfere with metabolic and other processes involved (e.g. the functioning of the Na^+-pump). Figure 4.4 illustrates non-competitive inhibition.

In order to investigate the inhibition of one substance by another, two main types of experiments can be performed. The first is usually employed to test a range of compounds for their inhibitory activity against a substrate, and those which do inhibit are then tested further by the experiment previously described. The I:S(inhibitor:substrate) ratio is usually fixed at a high value e.g. 100:1 - one which is unlikely to be encountered *in vivo*, but that will almost certainly reveal inhibitions. The second involves incubation in varying substrate concentrations with a fixed concentration of inhibitor. Thus, over the range of substrate concentrations the I:S ratio varies. This experiment enables the type of inhibition to be determined and the K_i or inhibitor constant to be calculated.

The K_i is an important parameter in inhibitor studies as it describes the affinity of the inhibitor for the uptake site of the substrate (refer to Chapter 4 for methods of calculating K_i). An inhibitor which has a K_i identical to its own K_t is thought to be transported *via* the system it inhibits. Inhibitor information provided by these methods therefore provides details of the specificity of uptake sites. However, information regarding number of loci can only be obtained by comparing the absorption of a group of structurally similar substrates and also their inhibitory interactions.

This chapter describes the results from experiments performed to investigate the action of inhibitors on monosaccharide absorption by cysticercoids and recently excysted adults of *H. diminuta.*

B. INHIBITION OF GLUCOSE UPTAKE

In order to investigate the specificity of glucose absorption, a range of substances were tested as potential inhibitors. Fifty cysticercoids were incubated in 0.5mM glucose, with the 'inhibitor' compound present at a fixed I:S ratio of 100:1. Substrates other than sugars were tested to investigate whether the specificity of the system was limited to carbohydrates. Table 6.3 illustrates the results in terms of radioactivity recovered (cpm) with and without inhibitor, and the percentage inhibition. It appears from the results that amino acids, fatty acids (short chain) and glycerol have no significant inhibitory activity towards glucose uptake in cysticercoids. Arme *et al.*(1973) studied the absorption of sodium acetate which had no inhibitory effect on glucose uptake and short chain fatty acids were therefore presumed to be transported *via* a kinetically distinct locus. In Chapter 5, glycerol was shown to be transported by the cysticercoid, but the evidence of inhibition experiments suggests that it, too, has an uptake locus distinct from that of glucose and other monosaccharides. Also the disaccharides and pentoses tested had no inhibitory effect on glucose uptake. This provides further evidence against the suggestion that disaccharides are of value in cysticercoid nutrition as they are not absorbed (see Chapter 5) and do not appear to interfere with glucose uptake by supplying monosaccharides, through the action of membrane-bound disaccharidases. The lack of inhibition by pentoses suggests a specificity for hexoses, while the ineffectiveness of L-glucose suggests a strong stereoisomeric

specificity. All the monosaccharides shown previously to be actively absorbed by cysticercoids were capable of inhibiting glucose uptake. Thus,it appears that only very closely related substances are capable of inhibiting glucose absorption in cysticercoids of H. diminuta.

It was of interest to investigate the specificity of the monosaccharide locus of the recently excysted adult, as evidence from Chapter 6 had suggested that it possessed some of the characteristics of the cysticercoid uptake locus (e.g. glucose transport in the absence of Na^{+}). Therefore an experiment was performed to test a wide range of potential inhibitors, and the results shown in Table 6.4, together with some data concerning the 10-day-old adult worm (from Pappas and Read,1975). The similarity between the specificity of cysticercoid and recently excysted adult monosaccharide uptake loci is evident, with only one difference i.e. the inhibition of glucose uptake by 6-deoxyglucose. This sugar was not absorbed by the cysticercoid and unfortunately was not tested in uptake studies on the recently excysted adult. However, it does appear to be the only monosacccharide transported in the adult and not in the cysticercoid (while 3-O-methylglucose appears to be the only sugar transported in the cysticercoid and not in the adult). The inhibition of glucose uptake in recently excysted adults, therefore, also appears restricted to hexoses. Inhibitors such as phlorizin and ouabain etc. are discussed in detail later in this chapter. More detailed studies were next made, to determine some inhibition characteristics of those substances already shown to inhibit glucose uptake.

TABLE 6.3 EFFECTIVENESS OF VARIOUS COMPOUNDS AS INHIBITORS OF GLUCOSE TRANSPORT IN CYSTICERCOIDS

POTENTIAL INHIBITOR	CPM - GLUCOSE ONLY	S.D	CPM - GLUCOSE + INHIBITOR AT I:S of 100:1	S.D	% INHIBITION
Amino Acids					
cycloleucine	53.3 ±	1.2	50.3 ±	3.0	-
lysine	34.0	2.3	38.0	2.9	-
aspartic acid	44.2	3.2	38.7	1.7	-
Fatty acids					
sodium acetate	39.8	6.3	28.4	3.6	-
sodium propionate	44.1	5.2	46.1	4.2	-
Pentoses					
arabinose	39.9	4.5	32.2	1.7	-
xylose	53.0	4.6	37.0	3.9	-
Fructose	49.3	3.9	30.0	4.5	-
Disaccharides					
sucrose	42.1	7.7	40.6	5.5	-
lactose	36.5	6.3	31.2	5.6	-
maltose	37.6	4.1	36.3	5.3	-
trehalose	43.4	4.4	42.2	4.9	-
Glucose analogues					
L-glucose	41.0	3.0	38.5	6.9	-
galactose	46.3	2.6	11.4	1.3	75%
2-deoxyglucose	45.2	1.7	7.1	2.7	84.3%
3-O-methylglucose	36.1	4.5	9.4	2.6	73.9%
1-deoxyglucose	39.3	5.7	11.3	3.1	71.2%
6-deoxyglucose	45.3	3.9	42.3	4.5	-
2-deoxygalactose	41.1	4.8	10.3	2.5	74.9%
α-methylglucoside	48.6	7.6	10.6	2.9	78.1%
β-methylglucoside	47.6	3.5	43.3	3.7	-
mannose	39.9	4.0	12.1	2.7	69.6%
gluconic acid	38.6	3.0	39.0	4.2	-
N-acetylgalactosamine	38.3	2.1	42.0	4.4	-
glucosamine	37.4	4.5	38.6	4.9	-
N-acetylglucosamine	38.0	5.1	36.3	5.3	-
glycerol	45.0	5.2	44.0	2.0	-

TABLE 6.4 EFFECTIVENESS OF VARIOUS COMPOUNDS AS INHIBITORS OF GLUCOSE TRANSPORT IN RECENTLY EXCYSTED ADULTS

POTENTIAL INHIBITOR	CPM - GLUCOSE ONLY		CPM - GLUCOSE + INHIBITOR AT I:S of 100:1		% INHIBITION
		S.D		S.D	
Amino Acids					
cycloleucine	47.6	± 3.0	40.5	± 1.3	-
lysine	44.5	3.2	42.6	1.2	-
aspartic acid	43.2	4.5	44.7	4.3	-
Fatty acids					
sodium acetate	47.5	6.3	40.1	5.9	-
sodium propionate	47.8	1.0	41.8	4.2	-
Pentoses					
arabinose	46.3	5.7	46.3	3.7	-
xylose	45.2	3.2	44.7	3.6	-
Fructose	40.1	3.1	40.0	4.6	-
Disaccharides					
sucrose	43.1	4.4	48.2	2.6	-
lactose	43.3	3.4	41.1	1.3	-
maltose	43.8	5.7	42.7	6.0	-
trehalose	48.1	3.7	46.3	1.9	-
Glucose analogues					
L-glucose	49.7	4.3	48.1	5.2	-
galactose	40.1	5.9	10.0	3.9	75.0%*
2-deoxyglucose	40.6	4.9	13.0	4.1	67.9%
3-0-methylglucose	41.3	3.8	11.6	2.5	71.9%
1-deoxyglucose	47.4	3.7	14.5	2.9	69.4%*
6-deoxyglucose	48.1	4.6	12.4	3.1	74.2%*
2-deoxygalactose	46.1	5.2	13.0	3.4	71.8%
α-methylglucoside	45.3	2.0	11.2	3.5	75.2%*
β-methylglucoside	47.2	4.7	47.1	4.6	-
mannose	49.0	4.9	15.0	4.2	69.3%
glucomic acid	45.0	3.9	44.7	5.0	-
glucosamine	44.4	3.4	43.1	4.5	-
N-acetylglucosamine	43.1	3.3	47.2	5.5	-
glycerol	42.1	2.6	40.3	2.2	-
6-deoxygalactose	47.1	3.0	47.0	4.4	-
allose	48.1	5.7	16.3	5.3	66.1%*
1,5-anhydromannitol	43.0	3.5	42.5	3.6	-

* - *substances which inhibit glucose in 10-day-old adult H. diminuta*

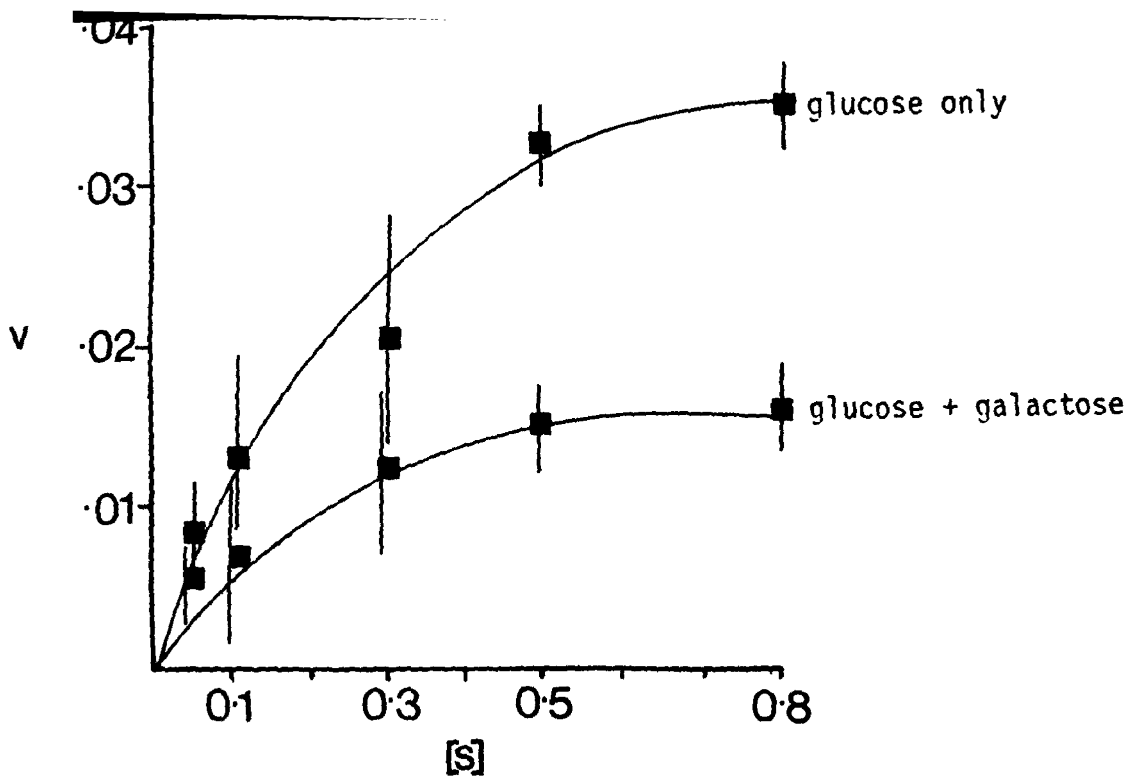
1. INHIBITION OF GLUCOSE UPTAKE BY MONOSACCHARIDES

In order to investigate, in more detail, the inhibition of glucose uptake by structurally similar monosaccharides, experiments involving a varied glucose concentration with a fixed concentration of inhibitor, were carried out. Fifty cysticercoids were incubated in concentrations of glucose of 0.05, 0.1, 0.2, 0.4 and 0.8mM with an inhibitor concentration of 4mM, giving a range of I:S ratios of 80:1, 40:1, 20:1, 10:1 and 5:1. The results of these experiments are shown in the graphs numbered below, together with Table 6.5 showing the calculated K_i values and the type of inhibition; Fig.6.6 = galactose as inhibitor of glucose uptake, Fig.6.7 = 3-O-methylglucose, Fig.6.8 = 2-deoxyglucose, Fig.6.9 = α-methylglucoside, Fig.6.10 = 1-deoxyglucose, Fig.6.11 = 2-deoxygalactose and Fig.6.12 = mannose. The results show that all the monosaccharides tested are competitive inhibitors of glucose uptake in cysticercoids, and the degree of their inhibition reflects their own affinity for the uptake locus. From the graphs depicting I:S ratio against a velocity of uptake it is clear that, in nearly all cases, a relatively large amount of uptake is still occurring at an I:S ratio of 80:1. However, these experiments do not permit the calculation of a diffusion component, as it would be necessary to determine whether a levelling off of the graph at a point above the baseline occurred. Therefore one additional experiment in which the I:S ratio was raised to a maximum of 600:1 was performed. The aim was to determine whether complete

TABLE 6.5 SUMMARY OF GLUCOSE INHIBITION DATA FOR CYSTICERCOIDS

INHIBITORS OF GLUCOSE UPTAKE	K_i	K_t OF COMPOUND	TYPE OF INHIBITION
Galactose	0.51mM ± 0.025	0.52mM ± 0.020	COMPETITIVE
3-O-Methylglucose	0.32mM ± 0.030	0.35mM ± 0.020	"
2-Deoxyglucose	0.29mM ± 0.027	0.30mM ± 0.015	"
α-Methylglucoside	0.42mM ± 0.051	0.41mM ± 0.014	"
1-Deoxyglucose	0.85mM ± 0.033	0.84mM ± 0.016	"
2-Deoxygalactose	0.76mM ± 0.044	0.76mM ± 0.020	"
Mannose	0.55mM ± 0.052	0.54mM ± 0.016	"

Each point is the mean of 20 replicates ± standard deviation.



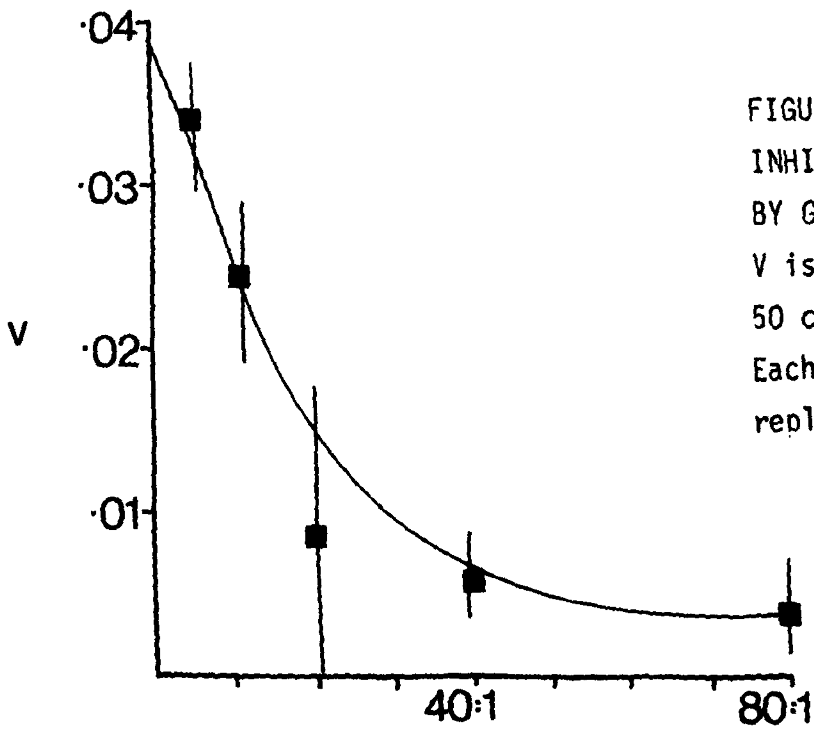
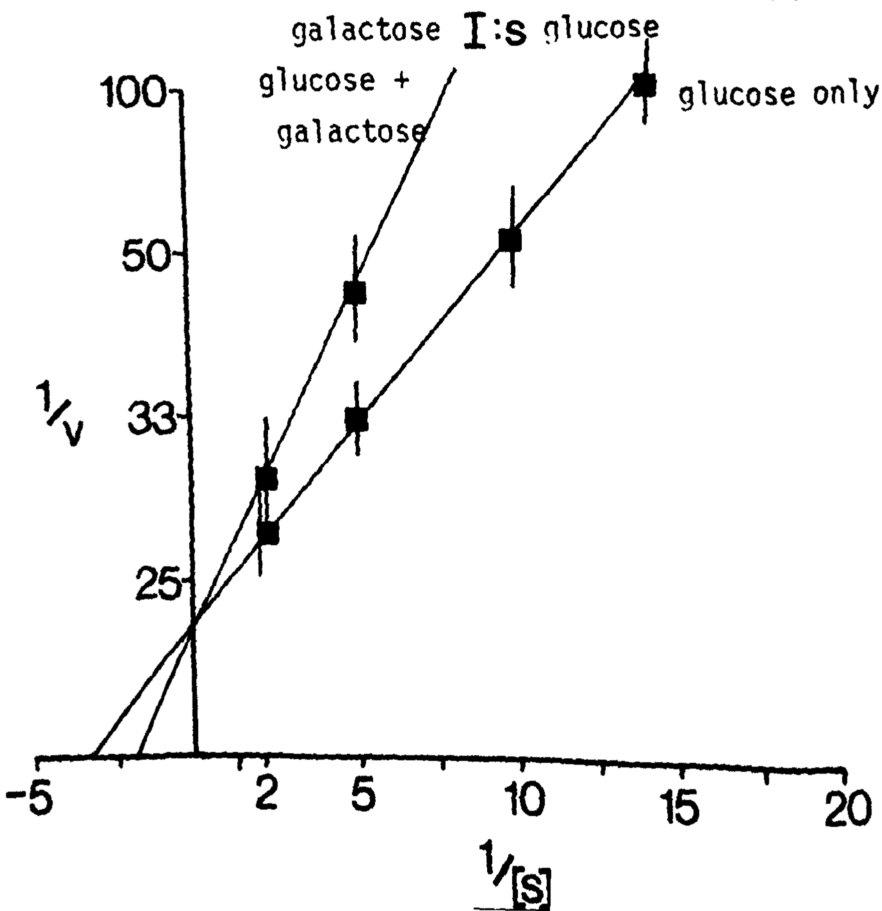
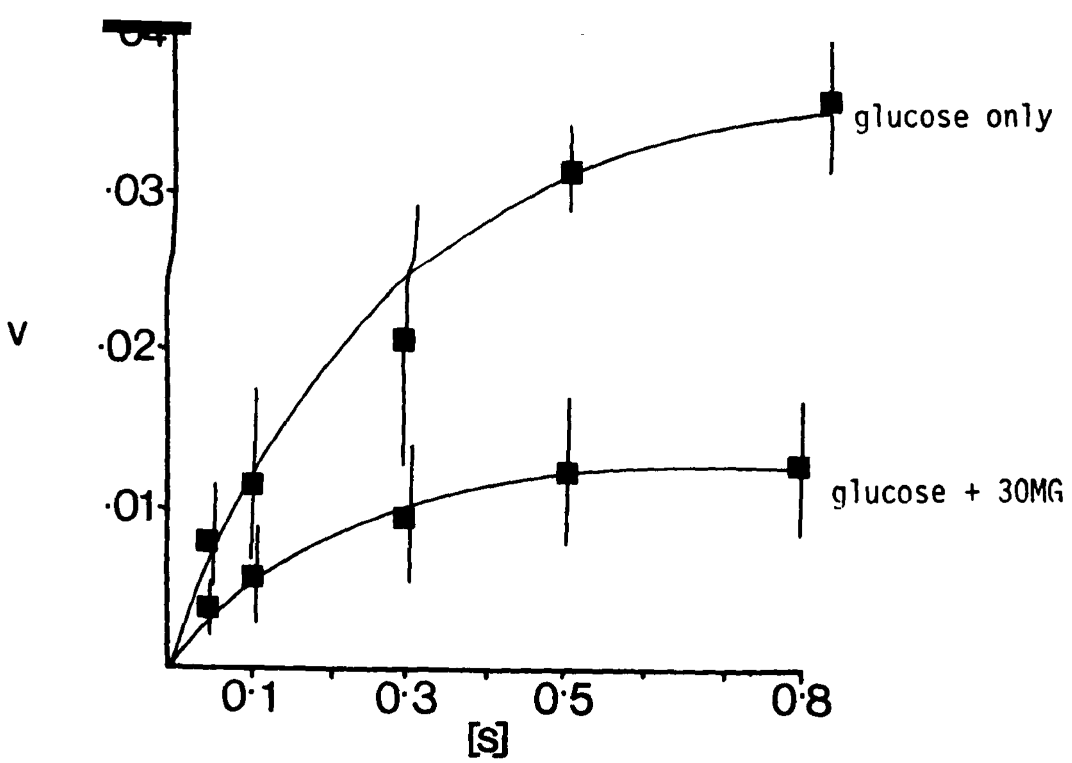
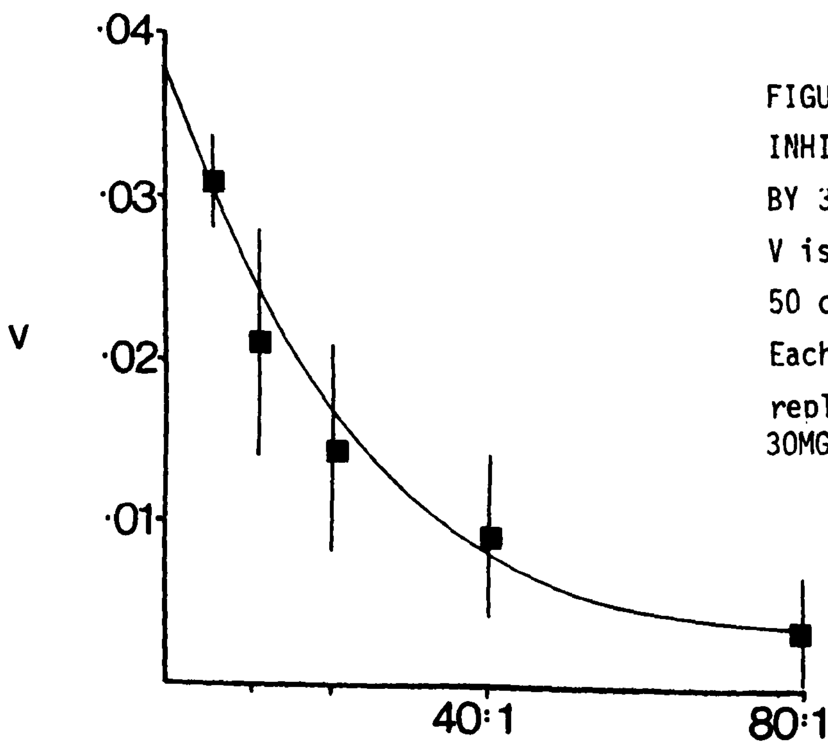


FIGURE 6.6
INHIBITION OF GLUCOSE UPTAKE BY GALACTOSE (4mM).
V is measured in μmoles/ 50 cysticercoids/30s.
Each point is the mean of 8 replicates ± standard error.







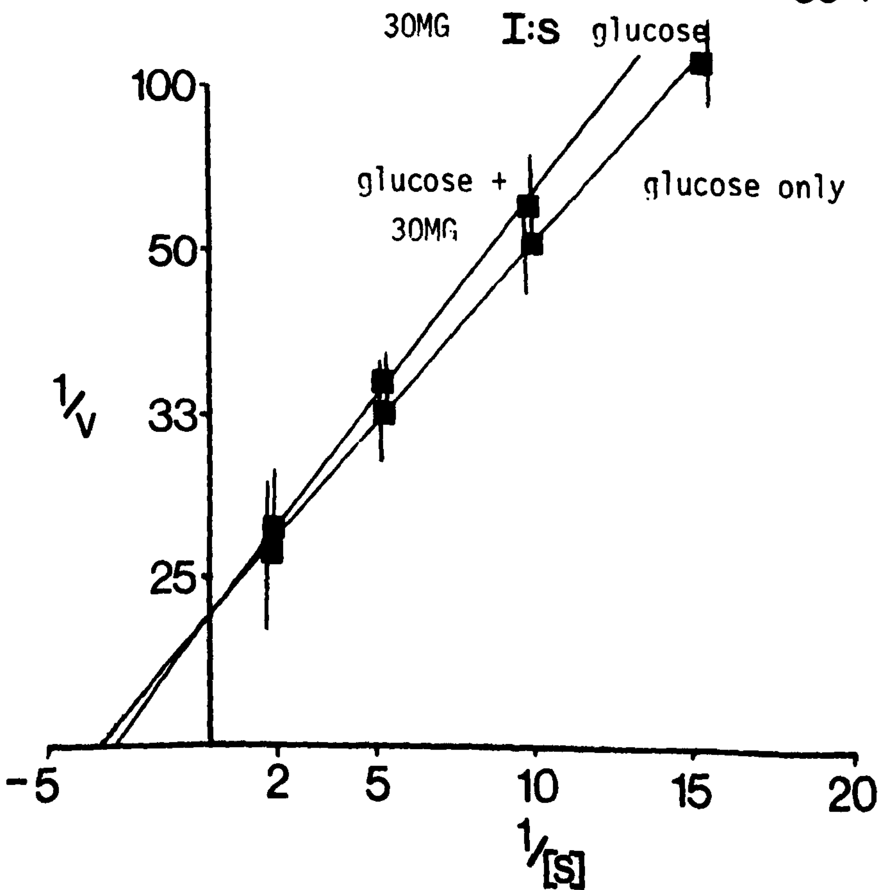
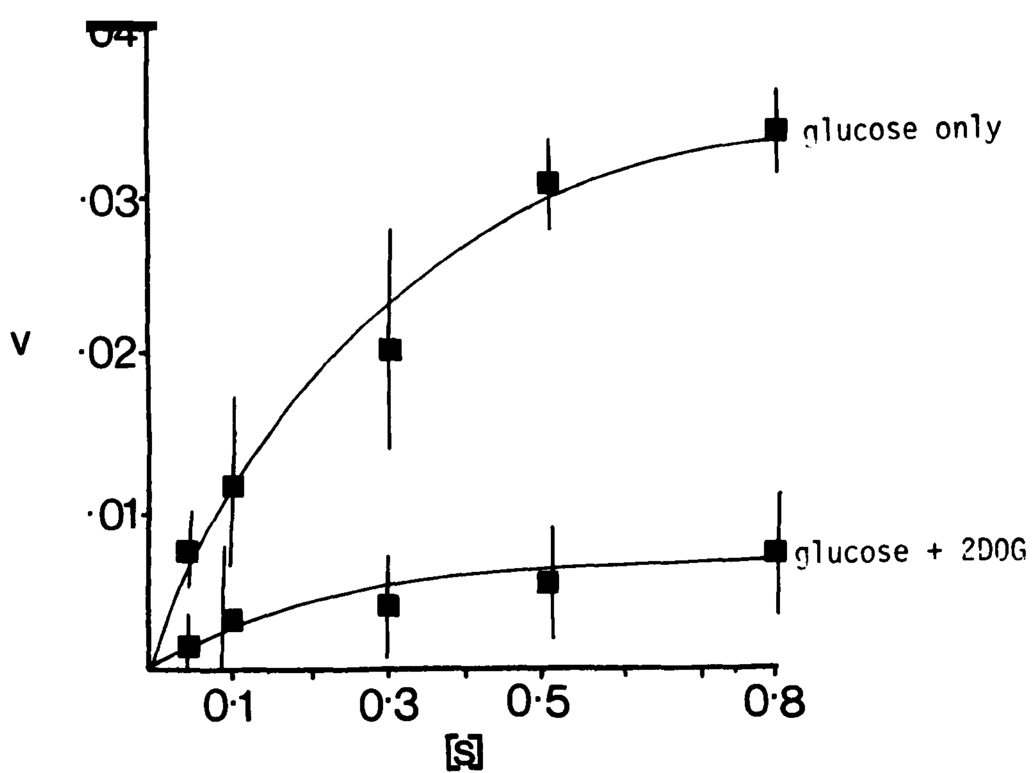


FIGURE 6.7
INHIBITION OF GLUCOSE UPTAKE
BY 3-O-METHYLGLUCOSE (4mM).
V is measured in μmoles/
50 cysticercoids/30s.
Each point is the mean of 8
replicates ± standard error.
30MG = 3-O-Methylglucose.



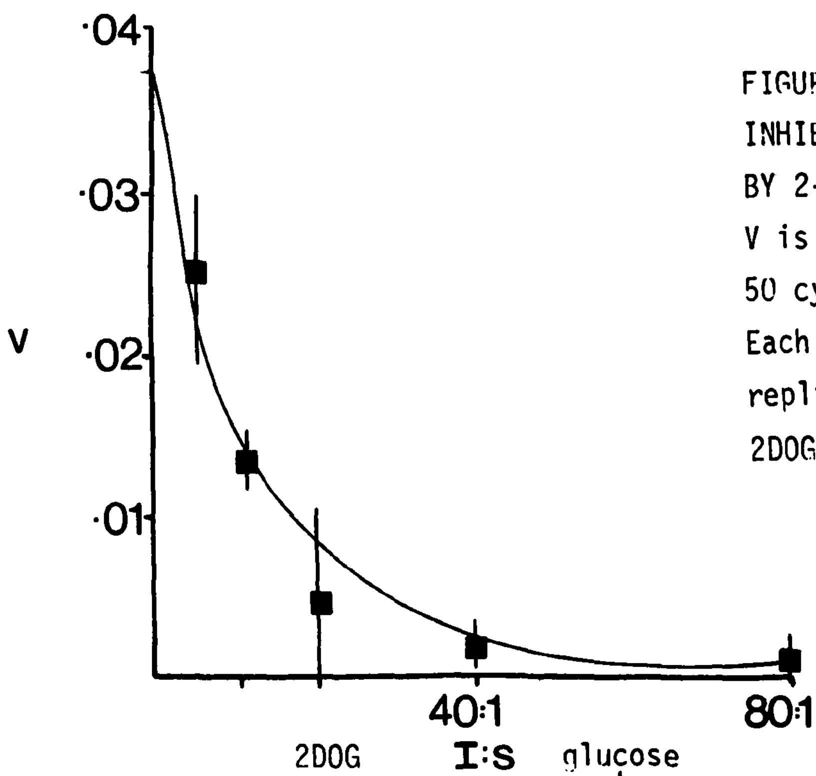
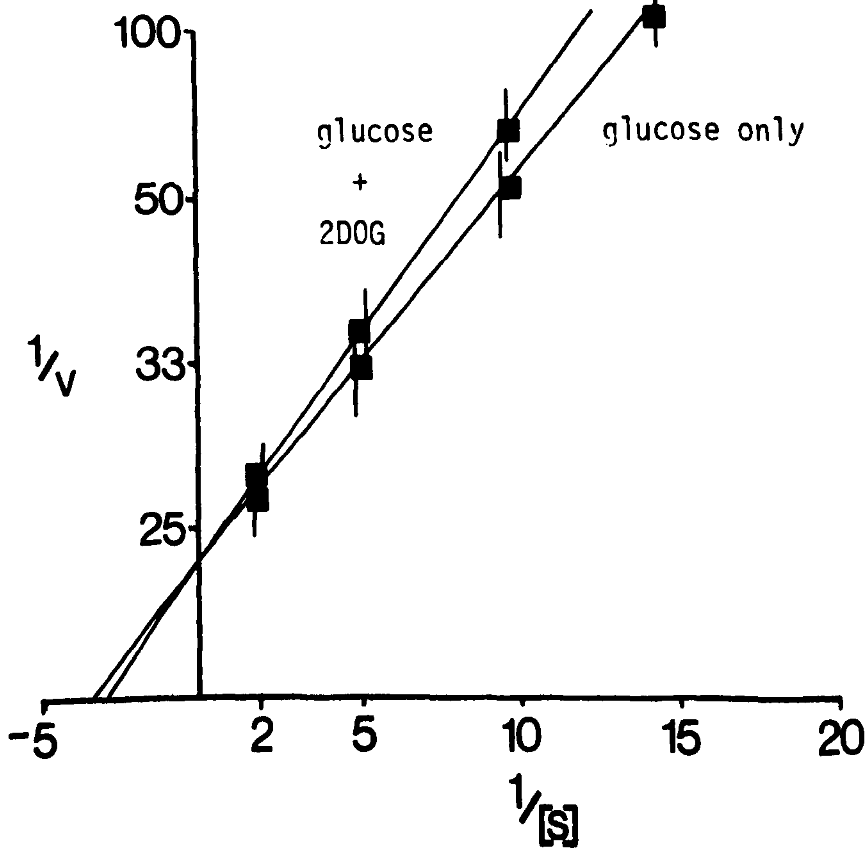
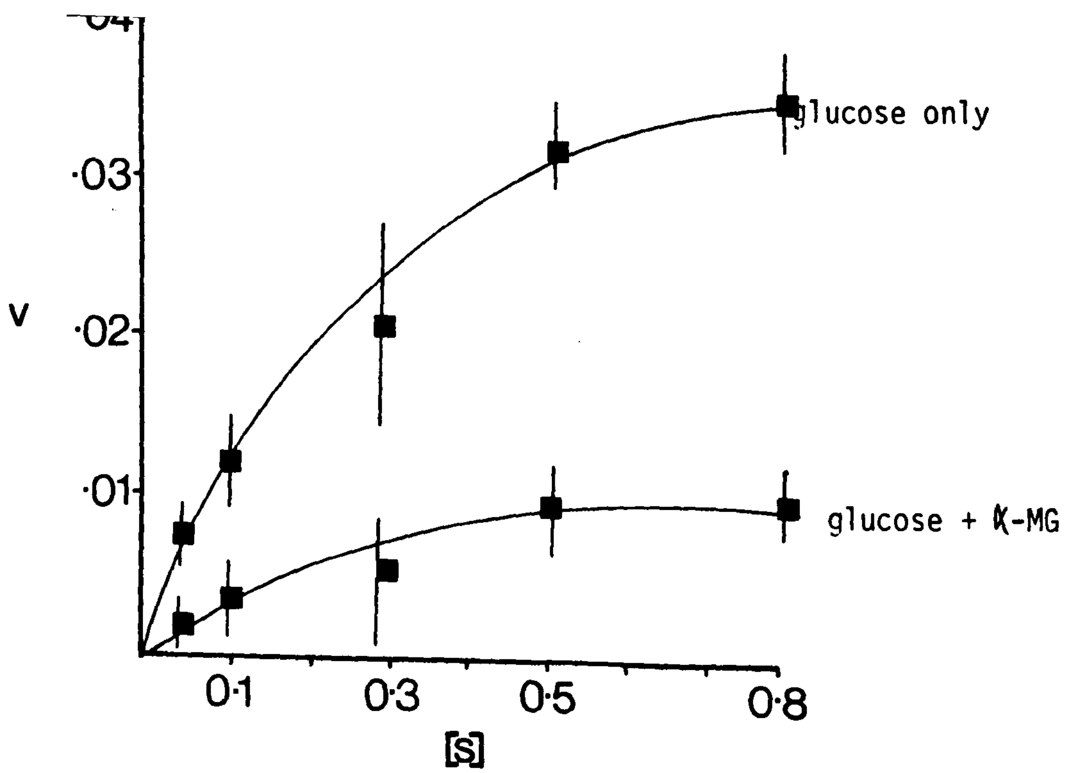
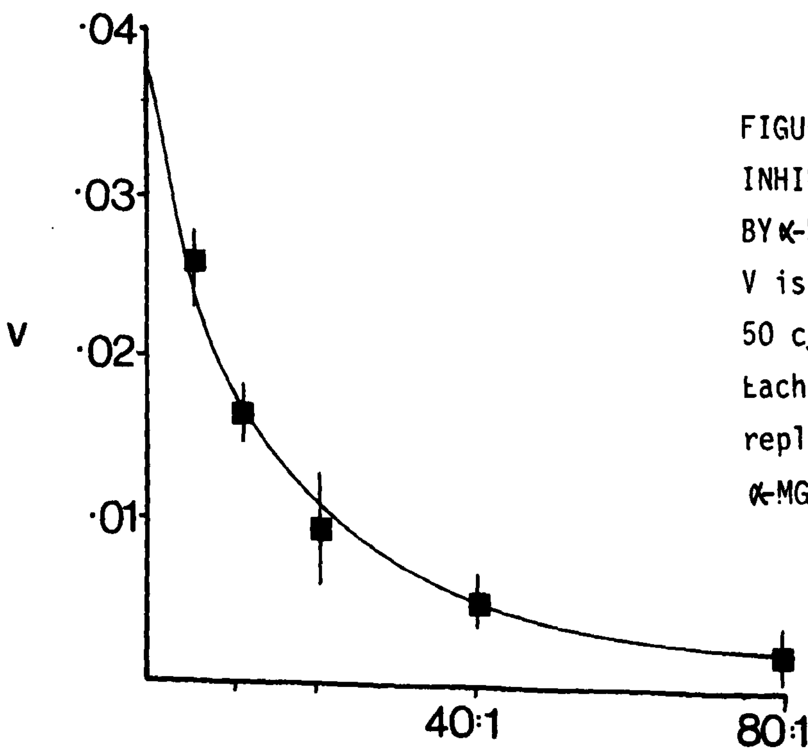


FIGURE 6.8
INHIBITION OF GLUCOSE UPTAKE BY 2-DEOXYGLUCOSE (4mM).
V is measured in μmoles/50 cysticercoids/30s.
Each point is the mean of 8 replicates $\pm$ standard error.
2DOG = 2-Deoxyglucose







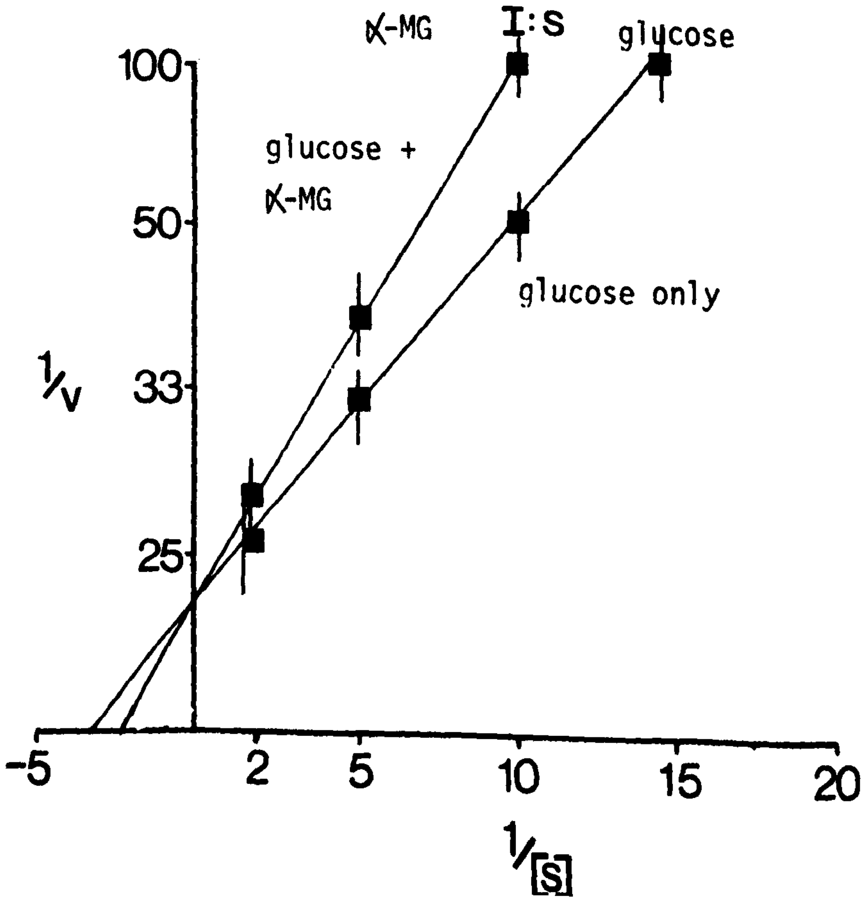
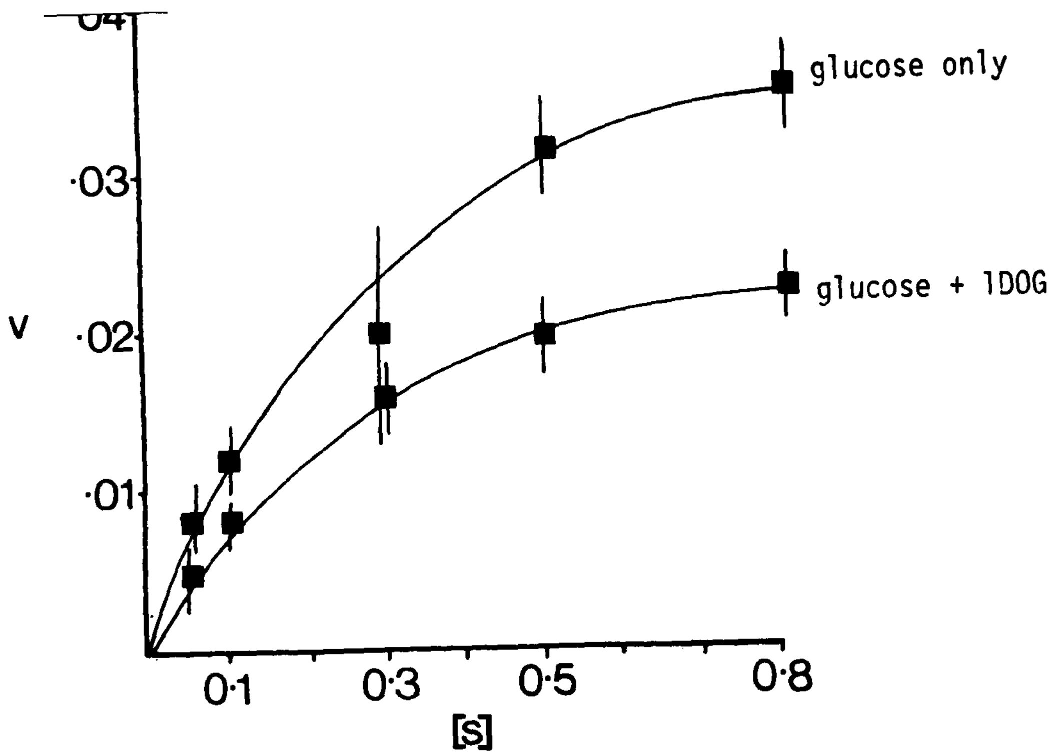


FIGURE 6.9
INHIBITION OF GLUCOSE UPTAKE BY α-METHYLGLUCOSIDE (4mM).
V is measured in μmoles/50 cysticercoids/30s.
Each point is the mean of 8 replicates ± standard error.
α-MG = α-Methylglucoside



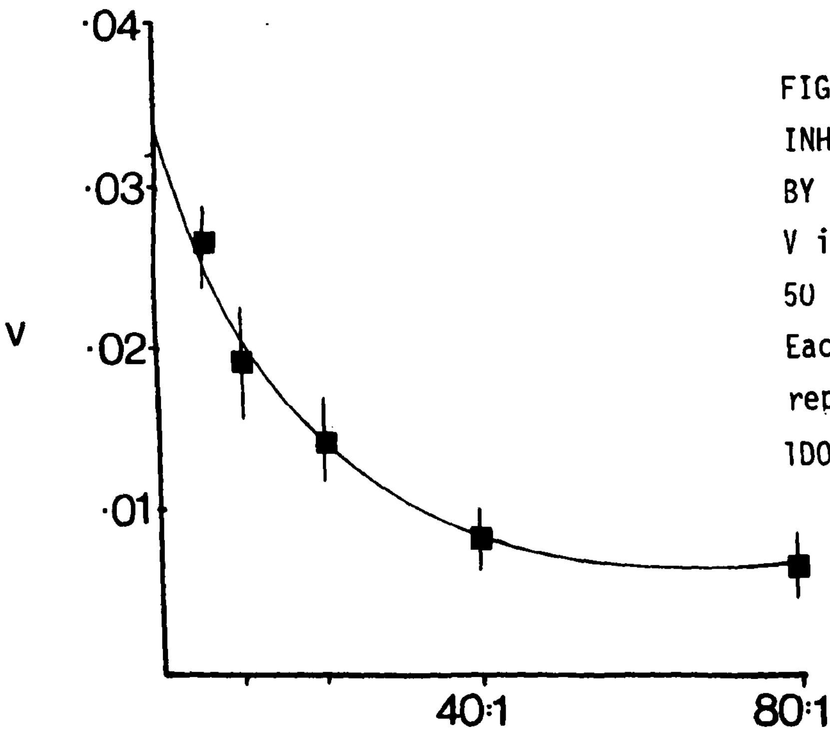
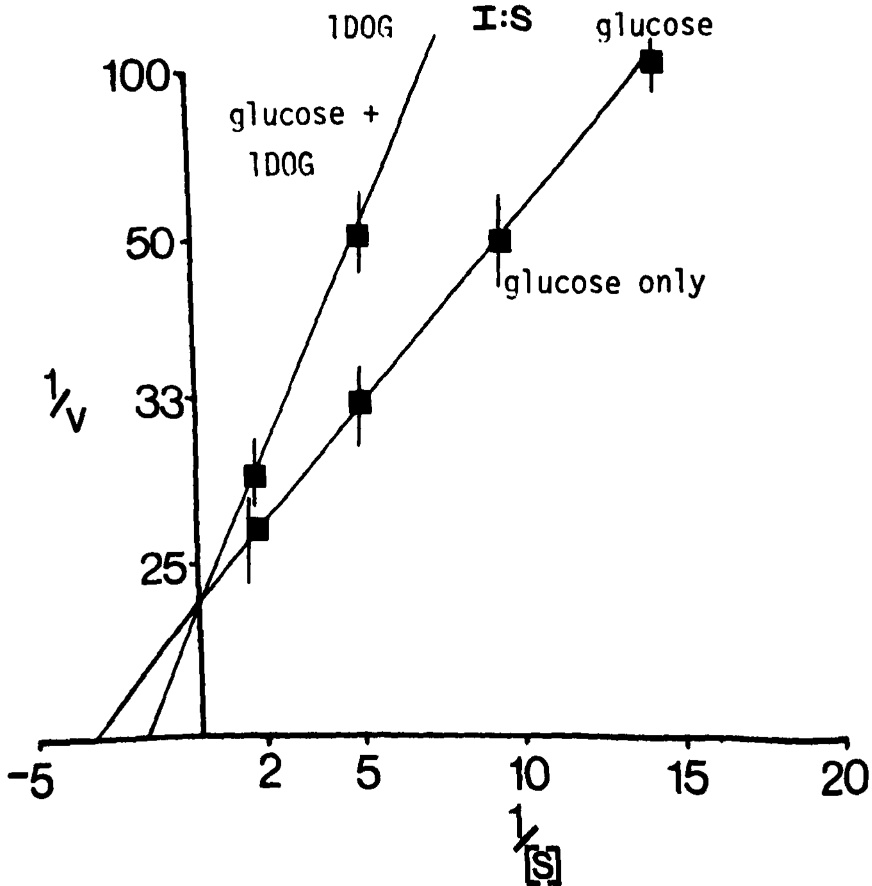
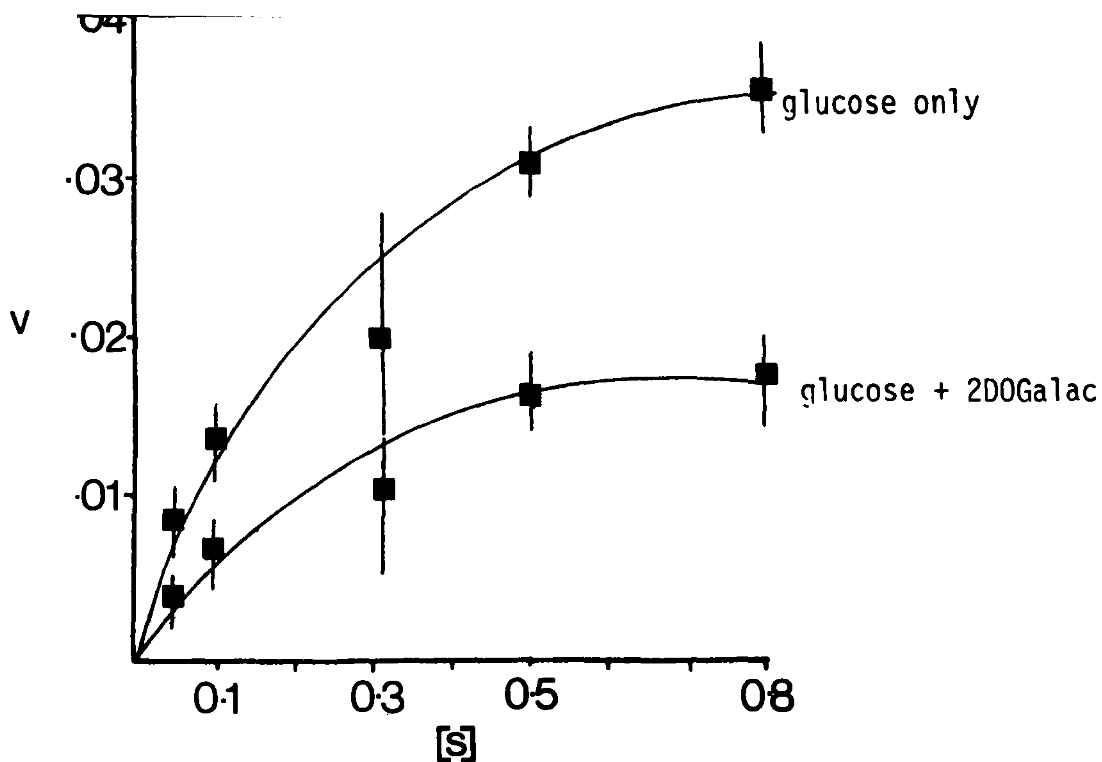


FIGURE 6.10
INHIBITION OF GLUCOSE UPTAKE BY 1-DEOXYGLUCOSE (4mM).
V is measured in μmoles/ 50 cysticercoids/30s.
Each point is the mean of 8 replicates ± standard error.
1DOG = 1-Deoxyglucose





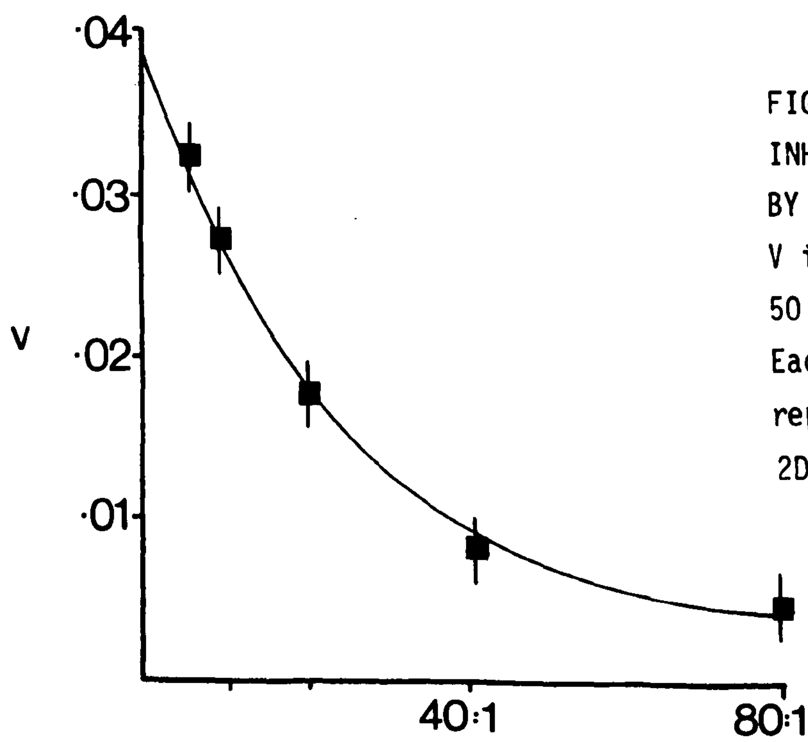
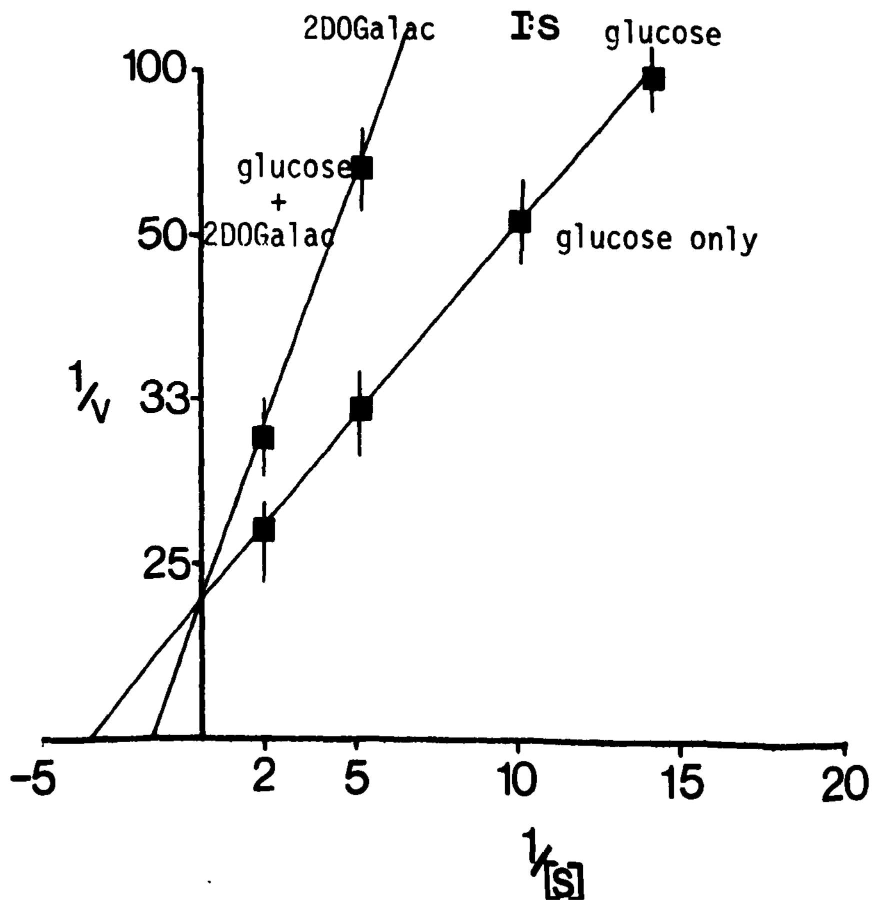
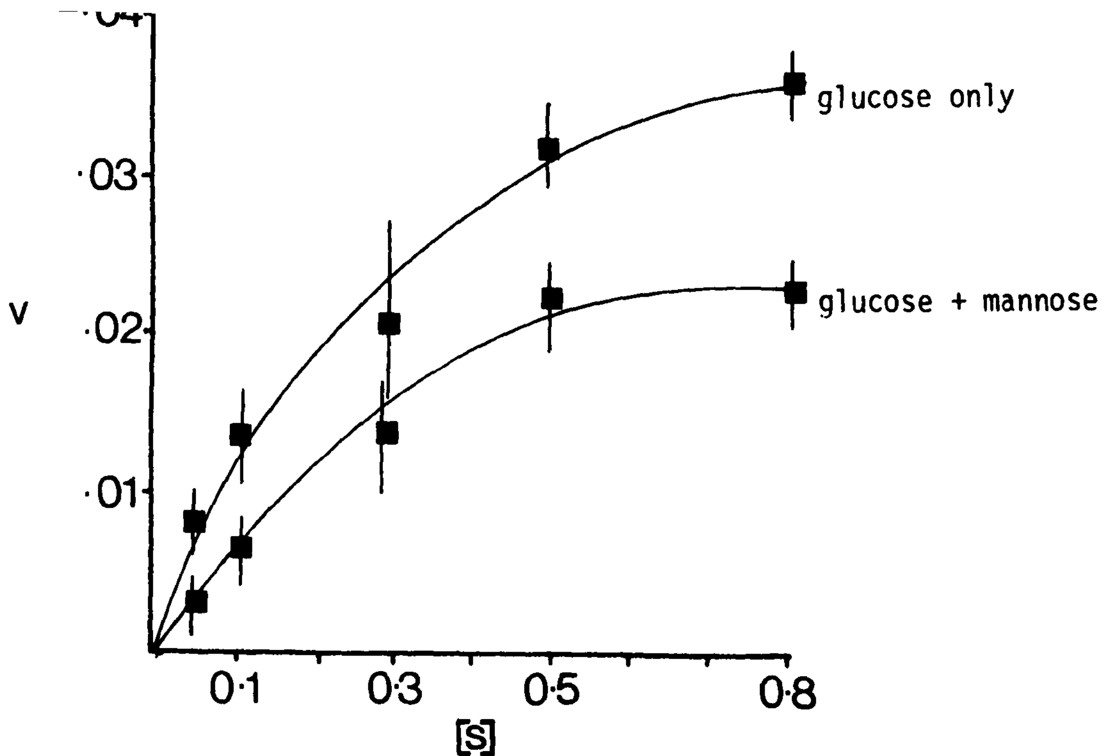


FIGURE 6.11
INHIBITION OF GLUCOSE UPTAKE BY 2-DEOXYGALACTOSE (4mM).
V is measured in μmoles/ 50 cysticercoids/30s.
Each point is the mean of 8 replicates ± standard error.
2DOGalac = 2-Deoxygalactose





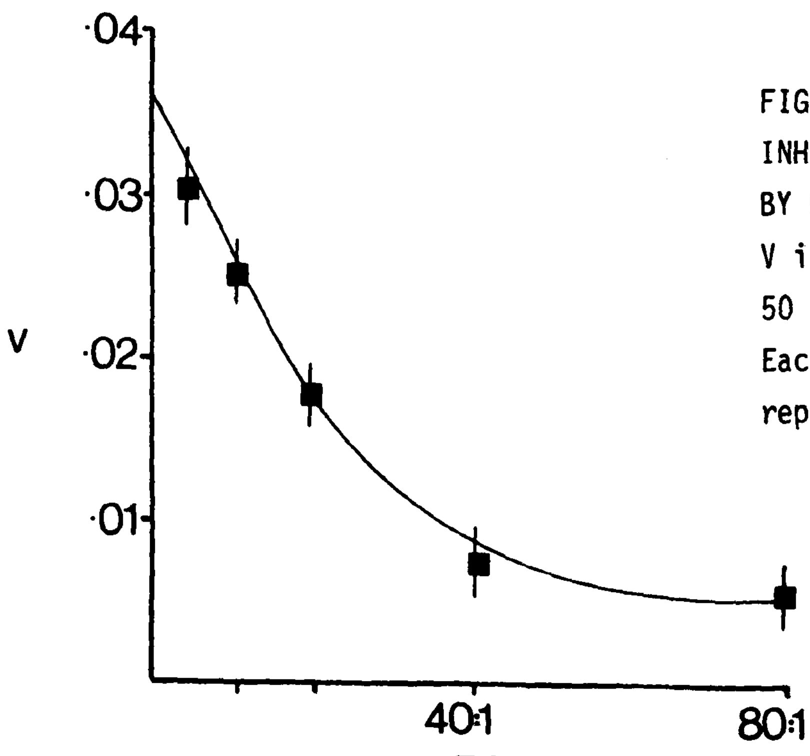
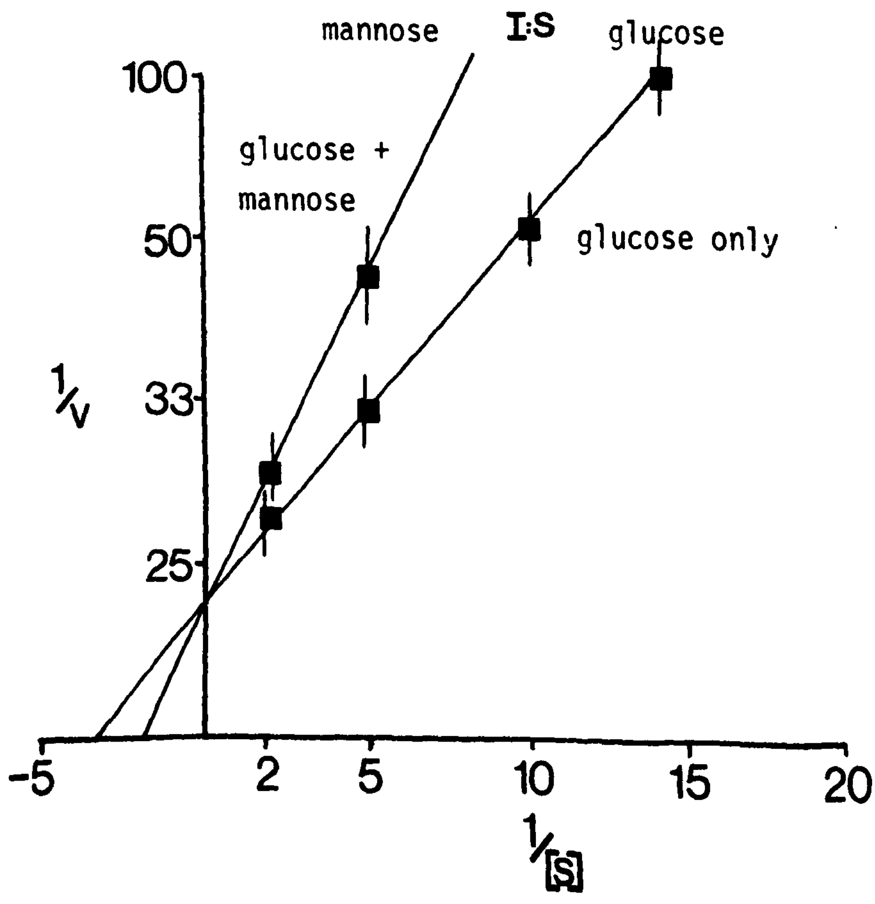


FIGURE 6.12
INHIBITION OF GLUCOSE UPTAKE BY MANNOSE (4mM).
V is measured in μmoles/50 cysticercoids/30s.
Each point is the mean of 8 replicates $\pm$ standard error.



inhibition (i.e. to zero uptake) could be obtained. Galactose was used as the inhibitor and glucose as substrate. Fig.6.13 illustrates the results and shows that even at 600:1 complete inhibition was not achieved. The small amount of uptake present probably represents the diffusion component, but could be the uptake *via* a second Na^+-insensitive locus. From this experiment, however, the K_d was calculated as 0.09 μmoles/mM, less than 2% of control uptake (for method of calculating K_d diffusion coefficient refer to Chapter 4). The inhibition studies carried out here, do not show any evidence for the presence of two monosaccharide transport systems, as suggested by Arme *et al.*(1973). The basis of their suggestion was the continued uptake of glucose in Na^+-free media, and they therefore suggested a Na^+-independent glucose uptake system as well as a Na^+-dependent one. However, if the systems do exist, it appears they have very similar specificities differing only in their operation in conditions of low Na^+ concentration. It is obvious, though, that more work is needed fully to answer the question of two loci (see also Chapter 7).

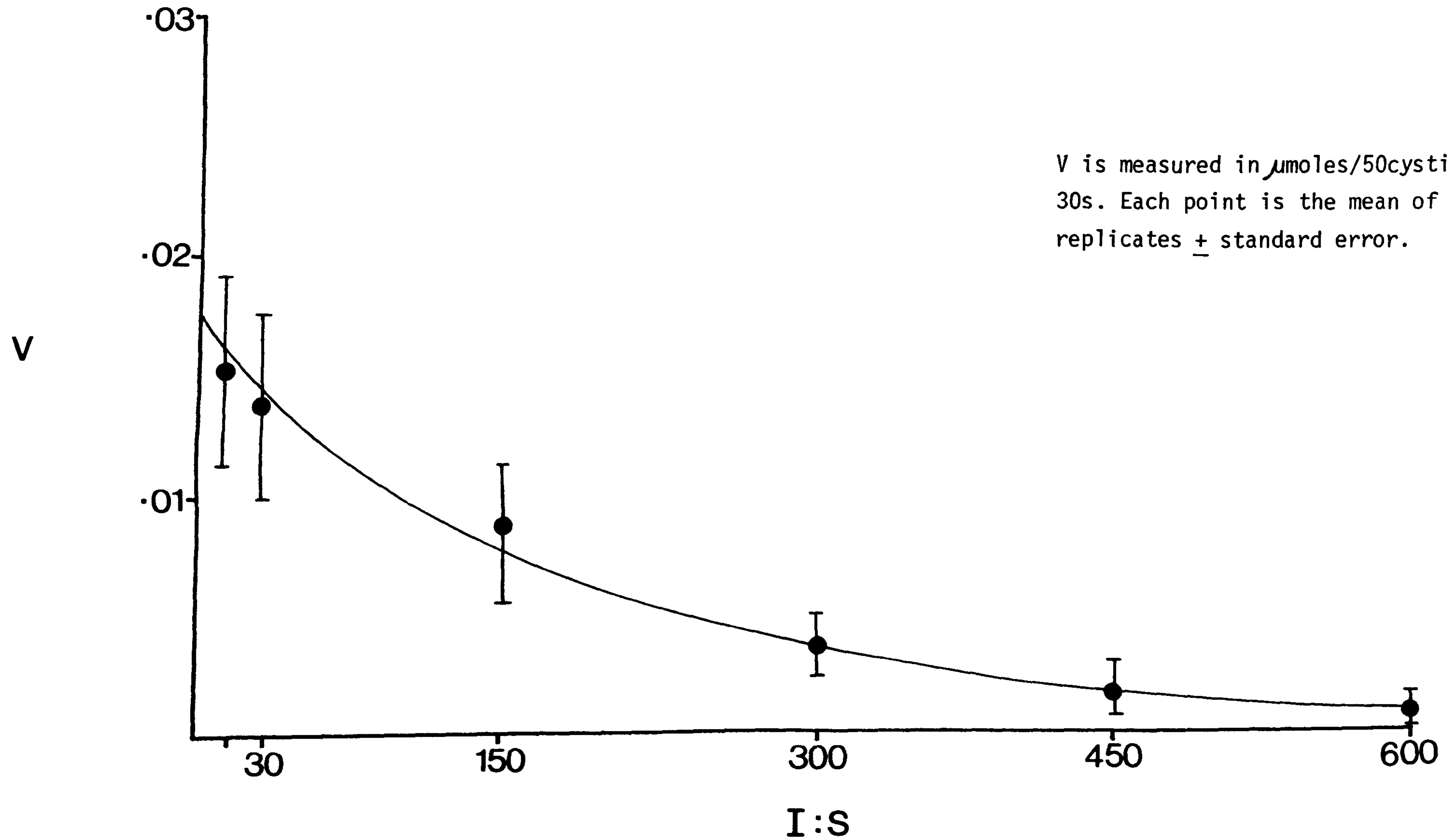
The inhibition of glucose uptake by recently excysted adults was also investigated. Experiments using 200 recently excysted adults, incubated with 4mM inhibitor, were performed, and the list of inhibitors used together with the results are shown in Table 6.14. The results indicate that, as with the cysticercoids, all the monosaccharides which inhibited did so competitively, and their K_i values were close to their values for uptake in recently excysted adults. Also, a decrease in the K_t for glucose was

noted, with no significant alteration V_{max}. The diffusion coefficient of monosaccharide absorption in recently excysted adults was calculated as 0.75 μmole/mM representing approximately 3.5% of 'control' sugar uptake. The effect of galactose on glucose uptake by 5- and 10-day-old adult *H. diminuta* was also investigated. Fig.6.15 and 6.16 illustrate the results and show the competitive inhibition of glucose uptake by galactose in 5- and 10-day-old adults. The K_i values were 1.40mM for 5-day-old and 3.16mM for 10-day-old adults. These figures are comparable with the K_t for galactose uptake which are 1.25mM and 3.0mM respectively. The diffusion coefficients were, as expected, small at 1.06 and 1.10 μmole/mM respectively.

C. RECIPROCAL INHIBITION STUDIES

To corroborate the initial findings on the monosaccharide transport system, a number of further inhibition studies were performed. These involved using monosaccharides shown to be effective inhibitors of glucose uptake and testing them against each other. Selected reciprocal inhibition experiments were therefore carried out and the results of these are listed in Table 6.17. It is interesting to note that the differences between the K_i values and the corresponding K_t values are greater than when these compounds were tested against glucose. However, the inhibition was classified as competitive in all cases and strongly suggests that all monosaccharides are absorbed *via* the same loci.

FIGURE 5.13 INHIBITION OF GLUCOSE UPTAKE BY GALACTOSE AT HIGH I:S RATIOS (CYSTICERCOID)



V is measured in μmoles/50cysticercoids/30s. Each point is the mean of 10 replicates ± standard error.

TABLE 6.14 SUMMARY OF GLUCOSE INHIBITION DATA FOR RECENTLY EXCYSTED ADULTS

INHIBITORS OF GLUCOSE UPTAKE	K_i	K_t OF COMPOUND	TYPE OF INHIBITION
Galactose	1.2mM ± 0.067	1.2mM ± 0.078	COMPETITIVE
Mannose	1.58mM ± 0.101	1.42mM ± 0.088	"
3-0-Methylglucose	1.80mM ± 0.056	1.50mM ± 0.068	"
α-Methylglucoside	1.36mM ± 0.045	1.23mM ± 0.094	"
1-Deoxyglucose	1.20mM ± 0.075	1.20mM ± 0.108	"
2-Deoxyglucose	1.10mM ± 0.098	1.05mM ± 0.110	"
2-Deoxygalactose	1.26mM ± 0.073	1.20mM ± 0.086	"

Each reading is the mean of 15 replicates ± standard deviation.

FIGURE 6.15 INHIBITION OF GLUCOSE UPTAKE BY GALACTOSE: 5-DAY-OLD ADULT *Hymenolepis diminuta*

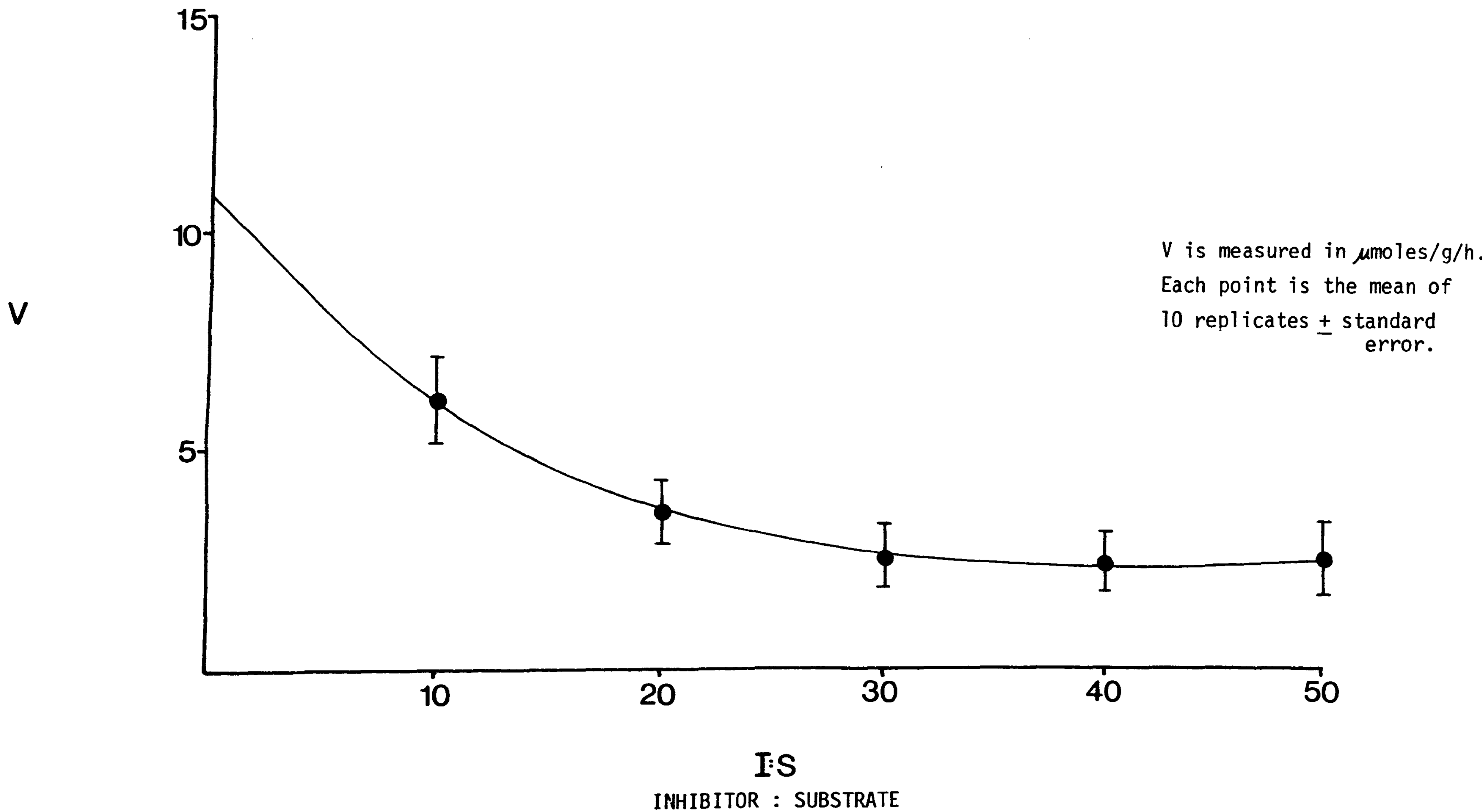


FIGURE 5.16 INHIBITION OF GLUCOSE UPTAKE BY GALACTOSE: 10-DAY-OLD ADULT *Hymenolepis diminuta*

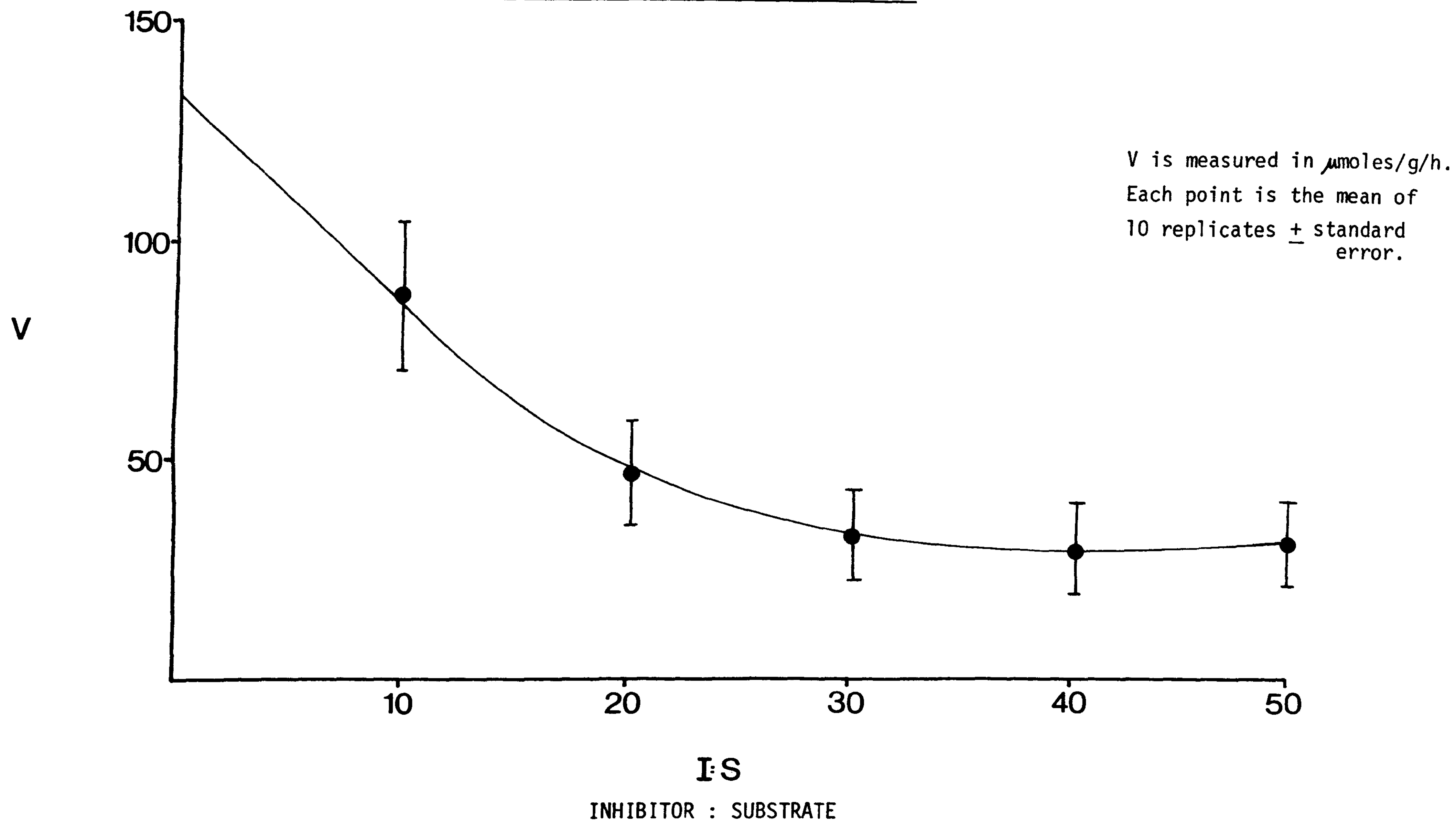


TABLE 6.17 DATA CONCERNING RECIPROCAL INHIBITION STUDIES (CYSTICERCOIDS)

SUBSTRATE	INHIBITOR	K_i	K_t OF INHIBITOR	TYPE OF INHIBITION
Galactose	Mannose	0.65mM $\pm$ 0.022	0.54mM	COMPETITIVE
1-Deoxyglucose	2-Deoxyglucose	0.38mM $\pm$ 0.024	0.30mM	"
2-Deoxygalactose	Galactose	0.63mM $\pm$ 0.032	0.52mM	"
3-O-Methylglucose	α-Methylglucoside	0.45mM $\pm$ 0.033	0.41mM	"
Mannose	1-Deoxyglucose	1.02mM $\pm$ 0.031	0.85mM	"
α-Methylglucoside	3-O-Methylglucose	0.37mM + 0.028	0.35mM	"
2-Deoxyglucose	2-Deoxygalactose	0.84mM $\pm$ 0.022	0.76mM	"

Each reading is the mean of 15 replicates $\pm$ standard deviation.

Pappas and Read (1975) noted that, although experiments using single inhibitors against single substrates were useful in determining the specificity of transport, these studies do not provide information concerning how these systems function in a more complex environment i.e. those containing several potential inhibitors. Read et al.(1963) derived an equation from classical Michaelis-Menten enzyme theory describing the effects of a complex mixture of substrates on the uptake of a single substrate. These authors found that if the K_i was used in the equation (given below) an accurate prediction of the effect of a complex mixture on uptake of a single substrate was possible.

$$V_i = \frac{V_{max}}{\frac{K_t}{S} + 1 + \frac{(K_t)([S_1])}{(K_{t1})(S)} + \frac{(K_t)([S_2])}{(K_{t2})(S)} \ldots\ldots n}$$

where V_i = inhibited uptake velocity

V_{max} = uninhibited maximum velocity of uptake

K_t = transport constant of the substrate for which uptake is being measured.

K_{tn} = transport constants of the inhibitors

[S] = concentration of the substrate being measured

$[S_n]$ = concentration of inhibitors

The K_i values were used instead of K_t to eliminate bias possibly produced by non-productive binding. This term is used when a substrate binds reversibly with the uptake locus without

being transported. It is therefore usually considered as competitive inhibition since competition occurs for the ability to bind to the uptake locus. Examples of non-productive binding in H. diminuta adults are available e.g. purine and pyrimidine bases (Pappas et al.,1973a). Read et al.(1963) suggested that the differences between K_i and K_t may be attributed to non-productive binding as K_t is related directly to solute translocation, while K_i is influenced by both translocation and non-productive binding. It was decided to investigate the possible effects of a complex mixture on glucose uptake, by incubating cysticercoids in 0.1mM glucose plus four other inhibitors; galactose, mannose, 3-O-methylglucose and 2-deoxyglucose, each at a concentration of 0.5mM (I:S ratio of 50:1). From the equation, a predicted value of 0.034 for the velocity of inhibited uptake was calculated, and the experiment using 50 cysticercoids incubated with 0.5mM inhibitor concentrations yielded a value of 0.032µmole /50 cysticercoid/2min. Therefore, in a complex mixture it appears that all the sugars used are competitive inhibitors of glucose uptake.

D. EFFECT OF PHLORIZIN ON GLUCOSE ABSORPTION BY THE CYSTICERCOID AND RECENTLY EXCYSTED ADULT

Phlorizin, a plant glycoside, has been used extensively in the study of active sugar transport. It is a selective inhibitor of carbohydrate uptake in adult H. diminuta (Laurie,1957; Phifer,1960a,b; McCracken and Lumsden,1975; Uglem and Love,1977) and has effects in other parasitic worms e.g.

Calliobothrium verticillatum (Fisher and Read,1971; Pappas and Read,1972); Taenia crassiceps metacestodes (Pappas et al.,1973; Craig,1983), Taenia taeniaeformis (von Brand et al.,1964). It is also a competitive inhibitor of sugar transport in mammalian intestine and kidney (Alvarado and Crane,1962; Silverman,1976). The inhibiting capacity of phlorizin has been shown to be rapidly reversed by washing worms after incubation (Laurie,1957). Read (1966) also demonstrated that negligible amounts of phlorizin enter the parasite so that it is likely that the site of action is at the worm surface. McCracken and Lumsden (1975) suggested that phlorizin is dependent on Na^+ for effect as it competes for the common monosaccharide binding site via its glucose moiety. As a result of this, phlorizin only affects mediated uptake of sugar. It is therefore of interest to ascertain its inhibitory activity in media of low Na^+ concentrations, from which it is known that the cysticercoid is capable of absorbing glucose (see Chapter 5), to determine whether the Na^+- insensitive uptake is mediated.

A number of experiments were therefore designed to examine the effect of phlorizin on glucose transport in the cysticercoid and recently excysted adult. Glucose was used initially as substrate, and also mannose. This latter substrate was also chosen because the uninhibited uptake of monosaccharides other than glucose and galactose, in Na^+-free media, had not been determined, and it was of interest to determine whether the postulated second locus (Na^+-insensitive) transported monosaccharides other than those already tested.

Fifty cysticercoids, or 200 recently excysted adults, were incubated in varying concentrations of glucose or mannose plus 0.05mM phlorizin. The results of these experiments are given in Figs. 6.18, 6.19, 6.20 and 6.21 The graphs clearly show potent inhibition of uptake by phlorizin, and the K_i values obtained reflect this. Phlorizin thus appears to have at least 100 times the affinity for the glucose uptake site than glucose or mannose, and is a competitive inhibitor of uptake. This result is similar to that obtained for the 10-day-old adult (Uglem and Love, 1977; McCracken and Lumsden, 1975). Phlorizin, in the recently excysted adult, appeared to have an even greater affinity (c.200 times that of glucose or mannose) - which is also consistent with the results for 10-day-old adults and the reasons for this greater affinity will be discussed later. Phloretin, the aglycone of phlorizin (see Fig. 6.22 for structures) was also tested on cysticercoids for effect on glucose absorption. The results (Fig. 6.23) show phloretin to have some inhibitory activity on glucose uptake. The percentage inhibition averages approximately 30% and is significant ($P = <0.05$). This result contrasts with work on the adult, but is in keeping with the suggestion that it is the glucose moiety on the phlorizin molecule which is inhibitory. The glucose moiety in phloretin is replaced with 3-O-methylglucose which has been shown by this study to be transported by the cysticercoid. The inhibition of glucose absorption in the cysticercoid by phloretin was competitive in nature. The work of Uglem and Love (1977) stated that phlorizin inhibition was Na^+-dependent. These authors showed

FIGURE 5.18 INHIBITION OF GLUCOSE UPTAKE BY PHLORIZIN: CYSTICERCOIDS

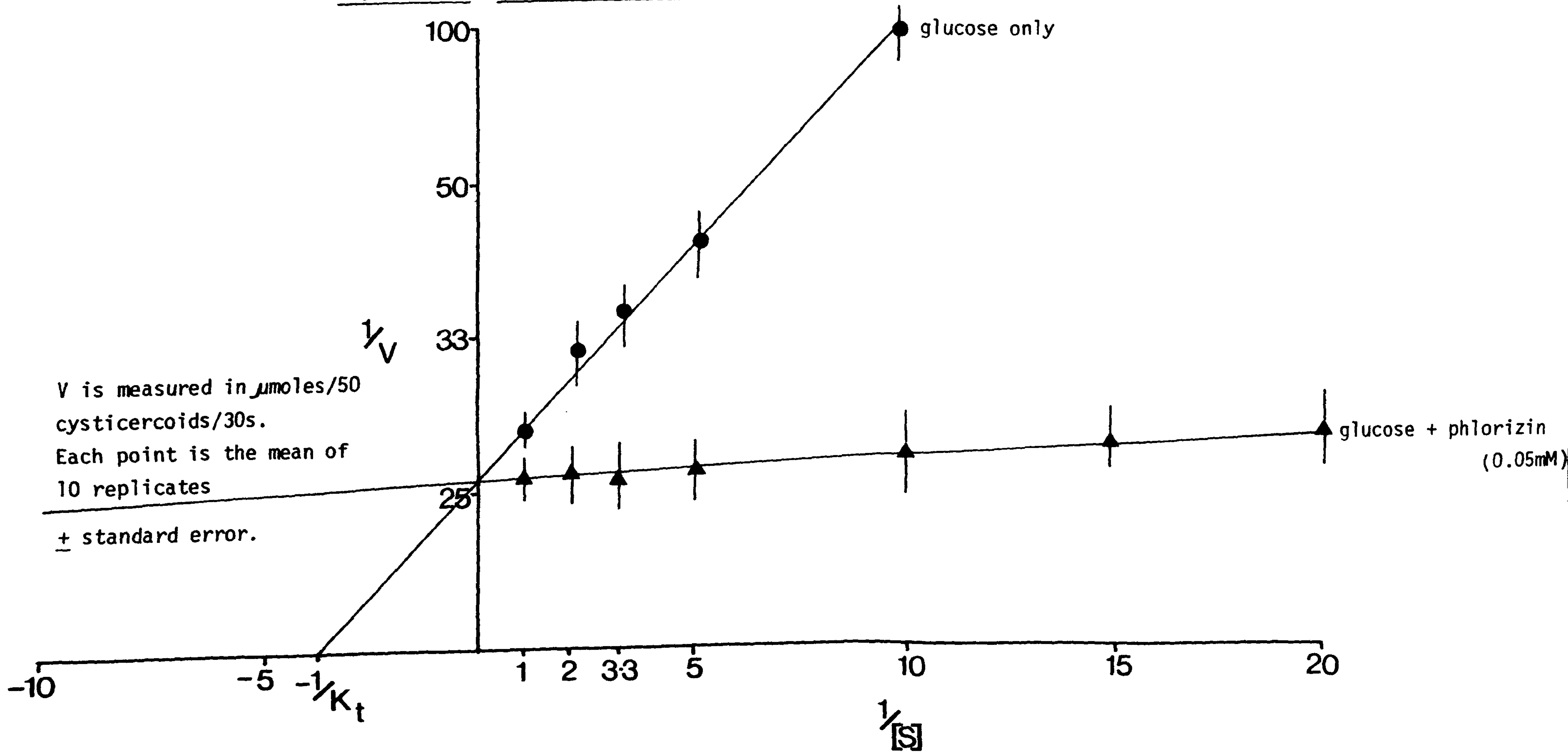


FIGURE 6.19 INHIBITION OF MANNOSE UPTAKE BY PHLORIZIN: CYSTICERCOIDS

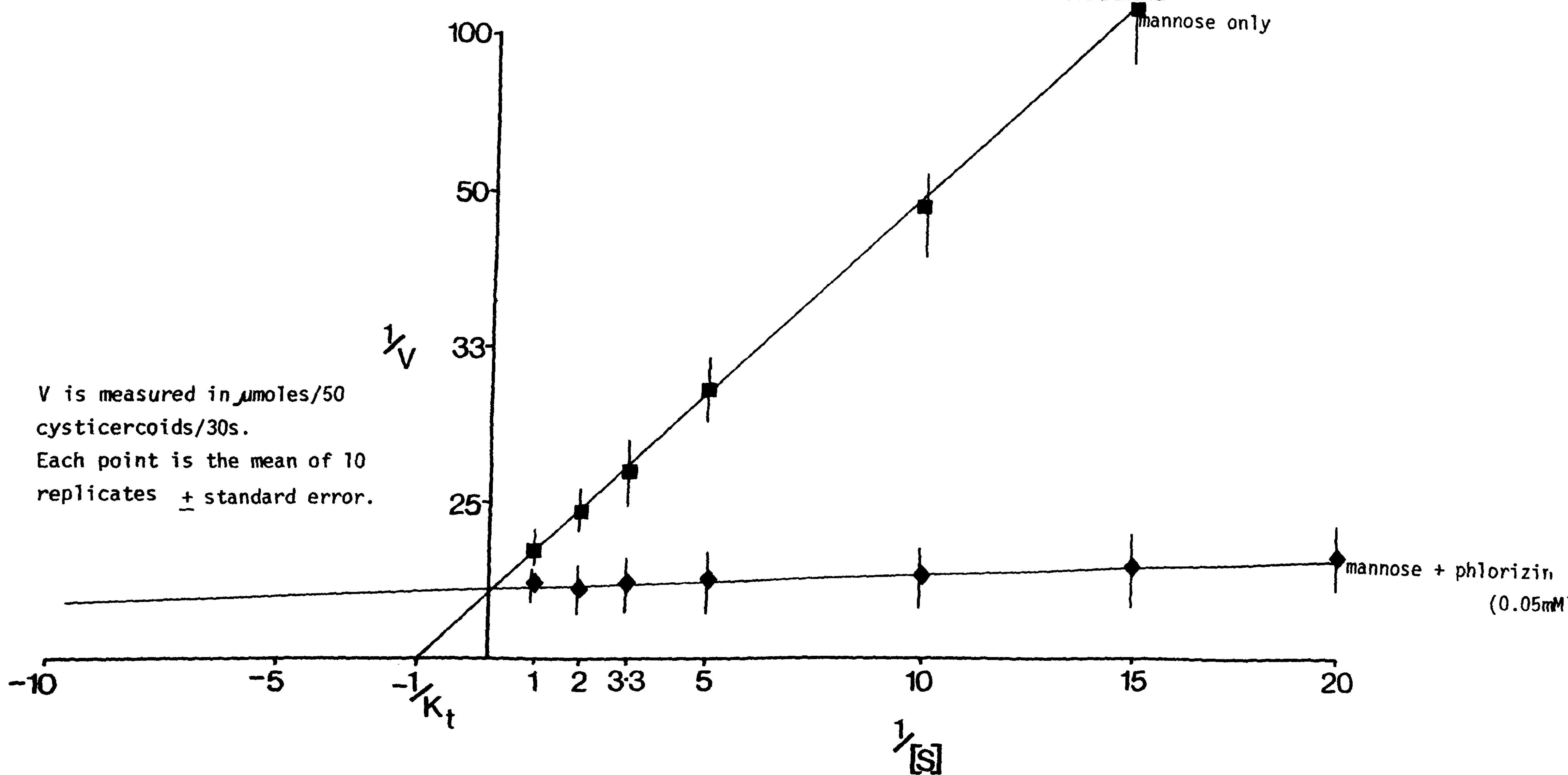
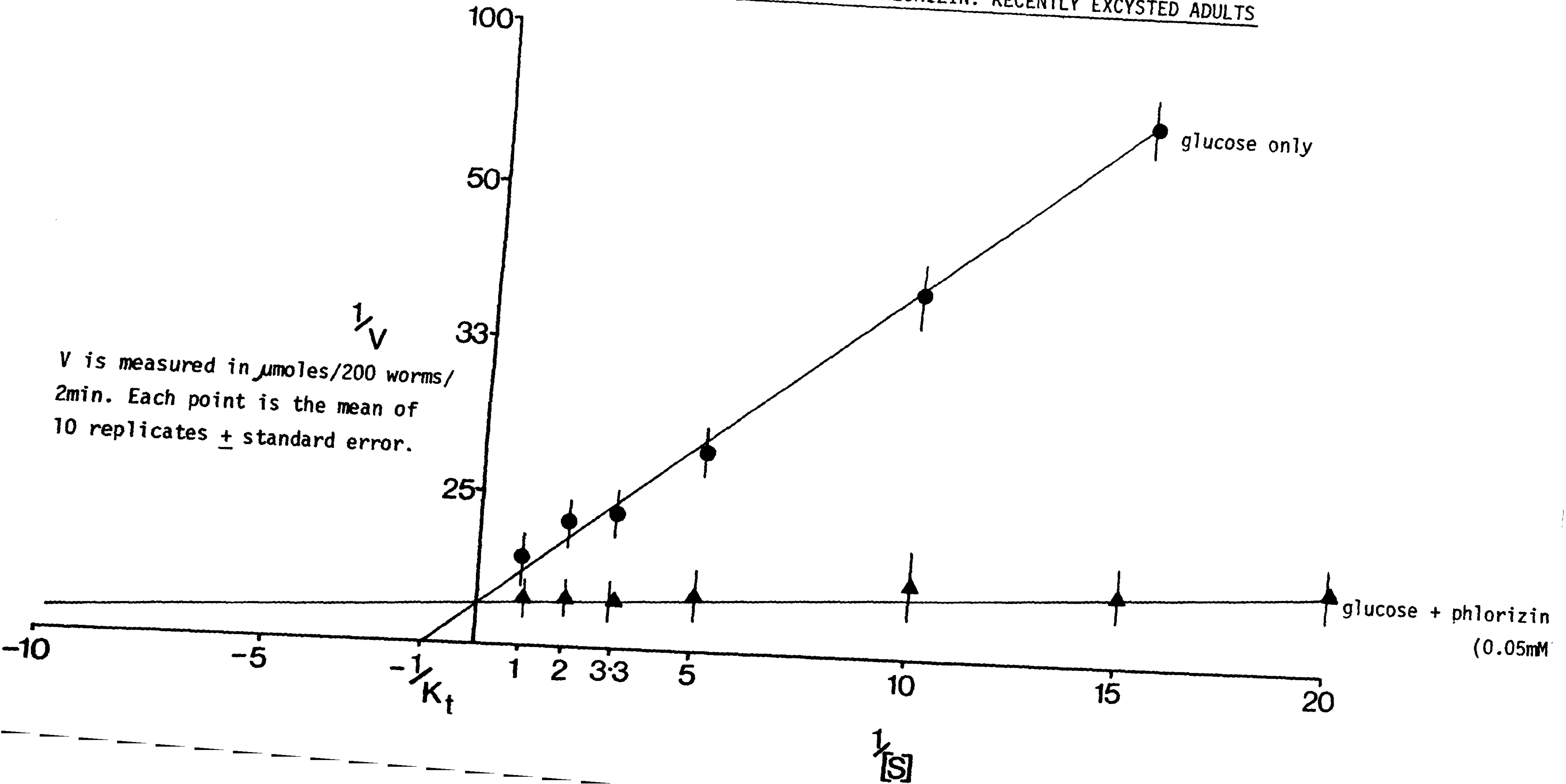


FIGURE 5.20 INHIBITION OF GLUCOSE UPTAKE BY PHLORIZIN: RECENTLY EXCYSTED ADULTS



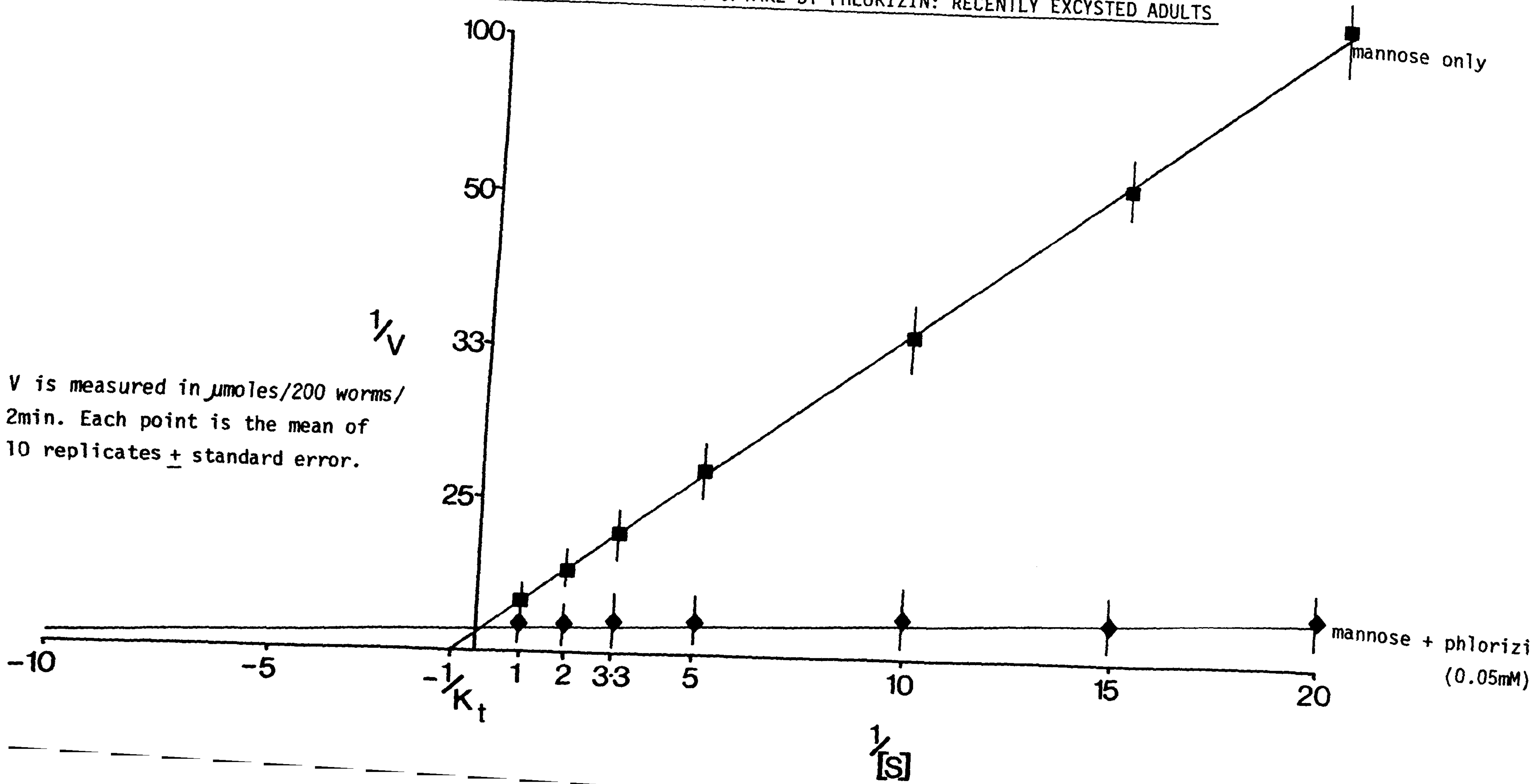
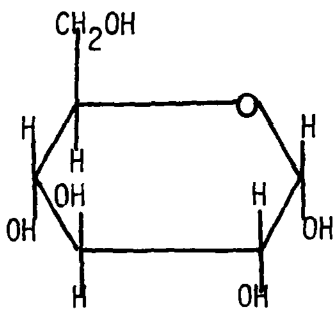
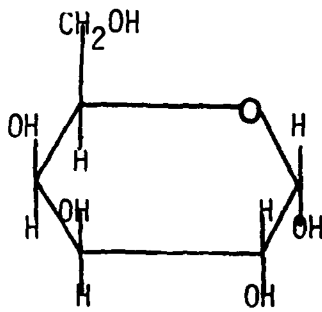


FIGURE 6.21
INHIBITION OF MANNOSE UPTAKE BY PHLORIZIN: RECENTLY EXCYSTED ADULTS
100
50
33
25
1/V
mannose only
V is measured in μmoles/200 worms/
2min. Each point is the mean of
10 replicates ± standard error.
mannose + phlorizi
(0.05mM)
-10
-5
-1
1
2
3·3
5
10
15
20
-1/K_t
1/[S]

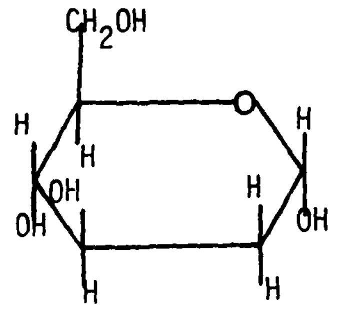
FIGURE 6.22 STRUCTURES OF SUBSTANCES USED IN THIS STUDY



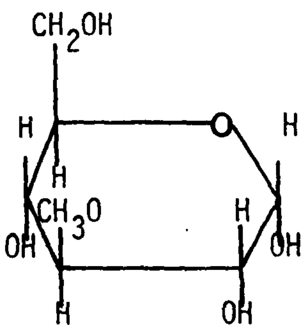
GLUCOSE



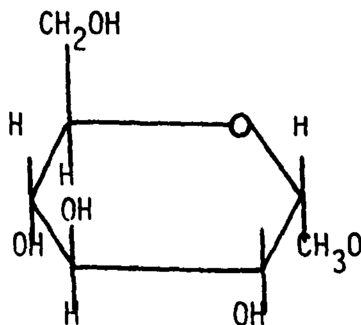
GALACTOSE



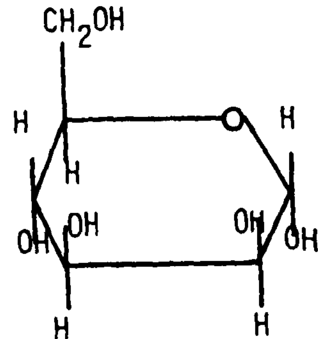
2-DEOXYGLUCOSE



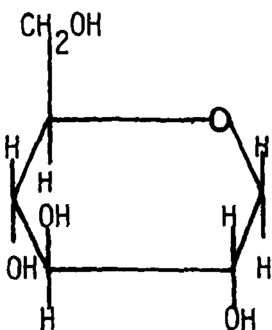
3-O-METHYLGLUCOSE



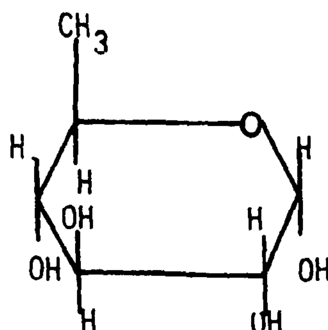
α-METHYLGLUCOSIDE



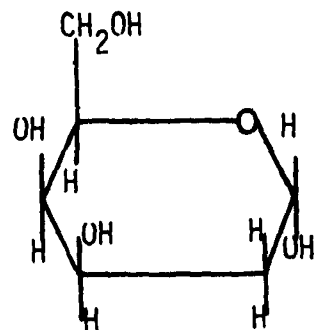
MANNOSE



1-DEOXYGLUCOSE

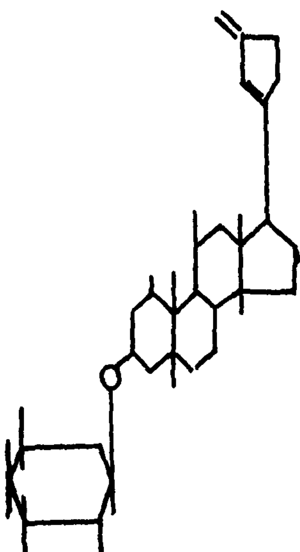


6-DEOXYGLUCOSE

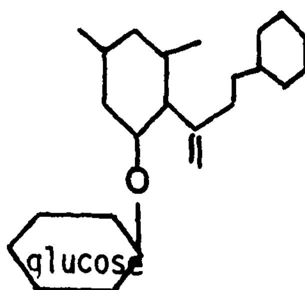


2-DEOXYGALACTOSE

OUABAIN



PHLORIZIN



PHLORETIN

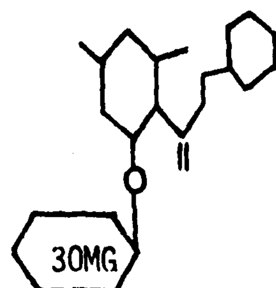
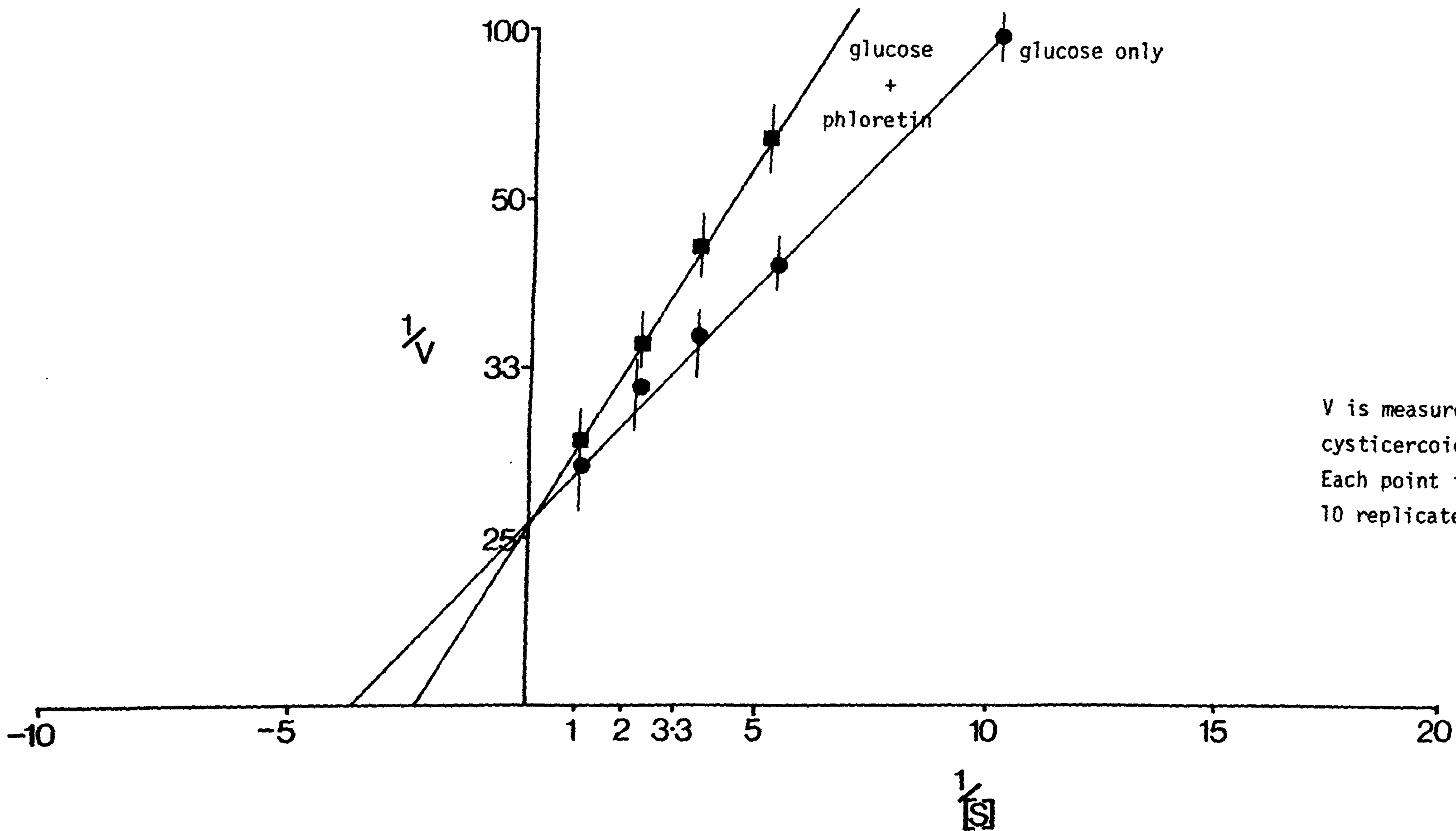


FIGURE 6.23 INHIBITION OF GLUCOSE UPTAKE BY PHLORETIN IN CYSTICERCOIDS



V is measured in μmoles/50 cysticercoids/30s.
Each point is the mean of 10 replicates $\pm$ standard error.

that the affinity of phlorizin decreased (i.e K_t increased) as the Na^+ concentration decreased. They reasoned that if the binding of glucose and phlorizin to the system was Na^+-sensitive to the same degree, then the effect of reducing Na^+ concentration on the K_i of phlorizin and the K_t of glucose should be the same i.e. the ratio of K_i/K_t should remain constant. However, this was not the case, and phlorizin was shown to be less effective at low sodium concentrations. However, because of the magnitude of its potency, it is of value in the study of monosaccharide transport in cysticercoids and its use may help elucidate Na^+-insensitive transport. Fifty cysticercoids, or 200 recently excysted adults, were incubated in glucose plus three concentrations of Na^+ 10,50 and 154mM (normal KRT). Lithium and potassium were used as replacement cations and phlorizin was present at a concentration of 0.05mM. The results are given in Table 6.24, which presents the data for both Lithium and Potassium as replacement cations. The K_i values for phlorizin shown an increase with decreasing Na^+ concentrations indicating that the effectiveness of phlorizin as inhibitor at low Na^+ concentrations is reduced. However, with the cysticercoids, this probably means that the portion of mediated uptake assumed to be Na^+-insensitive is also affected by phlorizin, and the percentages shown in Table 6.24 indicate this to be so. The results with the recently excysted adult also show a decrease in affinity for phlorizin with decreasing Na^+ concentrations; and therefore the data from this study confirm the claim of Na^+-dependency put forward by Uglem and Love (1977). The evidence for a second Na^+-insensitive locus

TABLE 6.24 EFFECT OF REDUCED Na^+ CONCENTRATIONS ON PHLORIZIN INHIBITION OF GLUCOSE ABSORPTION

Phlorizin was present at a concentration of 0.05mM

REPLACEMENT CATION	$[Na^+]$	K_t FOR PHLORIZIN ± S.D	
$Lithium^+$	10mM	0.10mM ± 0.069	(cysticercoid)
		0.052mM ± 0.087	(r.e.w)
	50mM	0.064mM ± 0.091	(cysticercoid)
		0.008mM ± 0.088	(r.e.w)
$Potassium^+$	10mM	0.09mM ± 0.063	(cysticercoid)
		0.061mM ± 0.071	(r.e.w)
	50mM	0.052mM ± 0.099	(cysticercoid)
		0.007mM ± 0.031	(r.e.w)
Control (normal KRT)	154mM	0.025mM ± 0.061	(cysticercoid)
		0.004mM ± 0.077	(r.e.w)

was again present in the controls, but it appears that almost total inhibition by phlorizin is possible and the only evident monosaccharide transport remaining is that occuring by diffusion. Thus if a second locus exists, its specificities and characteristics must be similar to the 'first locus' - with the exception of their functional differences in media with low Na^+ concentrations.

E. EFFECT OF OUABAIN ON MONOSACCHARIDE TRANSPORT IN CYSTICERCOIDS AND RECENTLY EXCYSTED ADULTS

The action of ouabain (strophanthin G) upon monosaccharide absorption is quite different from that of phlorizin. Ouabain is a cardiac glycoside - effectively an inhibitor of Na^+/K^+ stimulated Mg^+-dependent ATPase. This enzyme is located in the cellular membrane and is intimately involved in the energy-dependent transfer of sodium across the membrane (Skou,1965). Ouabain does have, however, a differential effect on sodium-dependent hexose transport in intestines, and this has led to the concept of a functionally polaraized epithelial cell (Schultz and Zalusky,1964; Newey, Sandford and Smyth,1968; Crane,1968). Podesta, Evans and Stallard (1974) showed that ouabain, when added to the medium, had no effect on glucose or galactose uptake in *H. diminuta* or *H. microstoma* adults. However, when using sliced worms to allow the ouabain to act on the basal plasma membrane, inhibition was effected at ouabain concentrations of 0.1mM (for glucose inhibition) and 1.0mM (for galactose inhibition) in *H. diminuta.* The results of these latter authors confirmed suggestions of a functional as well as

a morphological polarity in adult H. diminuta tegument - at least in terms of ouabain sensitivity. Schultz and Curran (1970) described the extensive inhibition by ouabain of sugar transport in a variety of mammalian tissues, and it has been shown to be an inhibitor also in Calliobothrium verticillatum (Fisher and Read,1971). A number of experiments were performed to investigate the action of ouabain on cysticercoids and recently excysted adult worm hexose absorption. An inhibition experiment with varying substrate concentration and a fixed concentration of ouabain (0.5mM) was performed both with and without preincubation in KRT saline plus 0.5mM ouabain. The results are presented in Table 6.25 and show that uptake of monosaccharides in intact cysticercoids and young adults, is unaffected by the presence of ouabain in the media.

F. EFFECT OF METABOLIC INHIBITORS ON HEXOSE ABSORPTION IN CYSTICERCOIDS AND RECENTLY EXCYSTED ADULTS

A number of known metabolic inhibitors were tested for their ability to inhibit monosaccharide transport in cysticercoids and recently excysted adults. They included iodoacetate, 2,4,-dinitrophenol, p-chloromercuribenzoate and cycloheximide. The results are illustrated in Table 6.26

The inhibitory activity of 2,4-dinitrophenol and iodoacetate, initially described by Arme et al.1973 was confirmed. The activity of p-chloromercuribenzoate was also demonstrated and this has been shown to inhibit glucose absorption in adult H. diminuta (Pappas and Read,1975).

TABLE 6.25 EFFECT OF OUABAIN ON GLUCOSE AND GALACTOSE UPTALE IN CYSTICERCOIDS AND RECENTLY EXCYSTED ADULTS

		K_t	V_{max}	C.R
GLUCOSE:				
control	C	0.25mM ± 0.017	0.038 ± 0.0018	7.02 ± 0.62
	R	0.81mM ± 0.026	0.071 ± 0.0045	10.09 ±0.93
glucose + ouabain (no preincubation)	C	0.27mM ± 0.020	0.040 ± 0.0026	6.95 ± 0.89
	R	0.85mM ± 0.037	0.077 ± 0.0031	10.62 ± 1.01
glucose + ouabain (+ preincubation)	C	0.26mM ± 0.022	0.038 ± 0.0025	7.10 ± 1.20
	R	0.82mM ± 0.040	0.082 ± 0.0044	10.52 ±0.66
GALACTOSE:				
control	C	0.53mM ± 0.018	0.039 ± 0.0020	7.35 ± 0.71
	R	1.20mM ± 0.179	0.081 ± 0.0081	10.10 ± 1.31
galactose + ouabain (no preincubation)	C	0.52mM ± 0.022	0.041 ± 0.0027	7.24 ± 1.24
	R	1.25mM ± 0.186	0.070 ± 0.0097	10.24 ± 1.70
galactose + ouabain (+ preincubation)	C	0.52mM ± 0.029	0.039 ± 0.0031	7.15 ± 0.96
	R	1.26mM ± 0.200	0.072 ± 0.0101	10.34 ± 1.59

C= Cysticercoid R= Recently excysted adult
Absorption of 1mM monosaccharide in the presence of 0.5mM ouabain (5min incubations)
Each reading is the mean of 8 replicates and is given ± standard deviation.

TABLE 6.26 THE EFFECT OF METABOLIC INHIBITORS ON GLUCOSE UPTAKE IN CYSTICERCOIDS

Cysticercoids were incubated in 0.5mM glucose with 0.1mM inhibitor for 30s.

COMPOUND	PERCENTAGE REDUCTION IN UPTAKE (compared to controls) S.D
Iodoacetate	15% ± 2.9
2,4-Dinitrophenol	21.4% ± 3.0
p-Chloromercuribenzoate	17.6% ± 3.4
Cyclohexamide	20.1% ± 2.5

Cycloheximide was shown to inhibit amino acid incorporation into protein in cysticercoids (Jeffs,1984), and also appears strongly to inhibit glucose absorption by cysticercoids.

G. DISCUSSION

The main aim of this thesis was to provide some information concerning the characteristics and specificity of monosaccharide absorption by cysticercoids in *H. diminuta*. Initially the uptake characteristics were studied in order to obtain information regarding the structural requirements of the hexose transport system, and these data can now be collated with the evidence presented in this chapter on the inhibition of monosaccaride transport.

The evidence provided by kinetic experiments shows that the 8 monosaccharides tested are absorbed by carrier-mediated processes in *H. diminuta* cysticercoids. Table 5.3 lists the kinetic data appertaining to these monosaccharides, whilst Table 5.4 gives some similar information on the adult. From these data some inferences can be made. The similarity between the maximal influx rates (V_{max}) of all 8 monosaccharides implies that the same number of uptake sites are available to all hexoses and this, together with the results of inhibitor studies, indicates that they are transferred *via* a common carrier. The K_t values vary between the monosaccharides, with the list below providing the order of affinity for the hexose locus beginning with the one with greatest affinity; glucose > 2-deoxyglucose > 3-O-methylglucose> α-methylglucoside

galactose > mannose > 2-deoxygalactose > 1-deoxyglucose.

It is of interest some that non-metabolisable monosaccharides have strong affinities for the carrier, indicating that there is no relationship between the affinity of a substrate and its post-absorptive fate. i.e. that molecular structure alone is the basis for its binding and subsequent translocation. The K_t for glucose was calculated as 0.26mM (V_{max} 0.038) and that for 1-deoxyglucose 0.84mM (V_{max} 0.047). The affinities calculated and presented here are considerably greater than those for adult H. diminuta monosaccharide absorption locus (refer to Table 5.1, Chapter 5). Thus, the differences between adult and cysticercoid hexose transport systems appear to be pronounced. First, there is the c. 10 fold difference in K_t values for the uptake loci. Secondly, the range of monosaccharides shown to be actively transported in cysticercoids is greater than that shown to date in adults, although further study may modify this view. Thirdly, not only is the range larger but there are differences in specificities. The active transport of 3-O-methylglucose by cysticercoids may differ from adults, although Read (1961) presented evidence for the inhibition of glucose uptake by this compound. Starling and Roberts (Roberts,1980), however, stated that 3-O-methylglucose is not transported in adult H. diminuta. Another analogue, 6-deoxyglucose, was not transported by cysticercoids, but showed some inhibitory activity towards glucose absorption in adults. Similarly, mannose has only previously been reported as being absorbed by simple diffusion in adult H. diminuta (Phifer,1960c; Read and Simmons,1963), yet in this study mannose has proved to be actively absorbed in the

cysticercoid. Although these latter conclusions could be tentatively drawn from the initial data, it was considered wise to perform inhibition studies to clarify the uptake system further. Therefore, glucose uptake was tested for its susceptibility to inhibition by other hexoses, and also these hexoses were tested against one another. The results (e.g. Figs.6.6 - 6.12, Table 6.17) show that inhibition of all monosaccharide uptake was inhibited competitively by the presence of the other hexoses. K_i values, a measure of the affinity for the uptake site of the substrate, are very similar to K_t values. The failure to inhibit completely glucose uptake with an I:S ratio of 600:1, using galactose as inhibitor, suggests the presence of a small diffusion component which was calculated as 0.09 μmole/mM, for glucose transport in cysticercoids. The diffusion coefficient was shown to increase, with increasing age of adult, from 0.75 (recently excysted adult), 1.06 (5-day-old adult) to 1.10 (10-day-old adult). These figures compare with values of 2.16 and 2.50 μmole. mM^{-1} presented by Podesta (1977a) for 10-day-old adult _H. diminuta_ and 1.44 - 1.12 μmole.mM^{-1} given for _Taenia crassiceps_ larvae (Craig,1983).

Phlorizin has been shown to be an effective inhibitor of mediated hexose transport in a variety of vertebrate and invertebrate tissues (e.g. Alvarado,1967; Bamford and Gingles,1974; Silverman,1976; Gomme,1981). It is suggested that phlorizin functions by competing with D-glucose for binding to the hexose uptake locus. The molecule possesses a

D-glucopyranoside ring and it is this glucose moiety which is thought to be of most importance (McCracken and Lumsden,1975; Uglem and Love,1977). Phloretin, the aglycone of phlorizin, has this glucose moiety replaced by 3-O-methylglucose, and it has been suggested that this is why it has no effect on hexose absorption in adult H. diminuta, as this helminth does not absorb 3-O-methylglucose (according to Uglem and Love,1977, although this is contrary to Read (1961), Phifer (1960a) and this study). The evidence presented in this chapter supports this suggestion, because, when phloretin was tested against hexose absorption in cysticercoids, it had some inhibitiory effect, and 3-O-methylglucose is actively absorbed by the cysticercoid. Phlorizin was shown to reduce glucose uptake in cysticercoids by c. 90%, has a K_i of 0.025mM and this value for K_i increased as the Na^+ concentration decreased. Phloretin also inhibited glucose uptake, but to a lesser extent - c. 30% reduction, and it was not tested in media of low Na^+ concentrations. Ouabain had no effect on intact cysticercoids or recently excysted adults, whether with a pre-incubation or not - a result which parallels that of the adult (Podesta et al.,1977). The metabolic inhibitors tested all inhibited glucose uptake by c. 82% suggesting the dependence of hexose absorption on metabolic energy.

Absorption of glucose and galactose was shown to be Na^+-sensitive, but other monosaccharides were not tested in media of reduced Na^+ concentrations. Uptake of glucose and galactose was not, however, totally Na^+-dependent, being reduced by only c. 30% in the total absence of Na^+. Apart

from this latter result and those on accumulation and metabolism in reduced Na^+ concentrations, the experiments (both uptake and inhibition experiments) have not shown any evidence for the existence of two uptake loci as shown by, for example, absorption of a monosaccharide by a kinetically distinct locus with a corresponding lack of effect by the presence of others etc. If two sites do exist, the evidence suggests that their specificities are identical and they differ only in their function in conditions of low sodium concentration in the incubation media.

The data presented in this study allow certain limited conclusions to be drawn concerning the structural requirements of the monosaccharide transport system in H. diminuta cysticercoids. The structural configuration of each monosaccharide tested is given in Fig.6.22 together with that of phlorizin, phloretin, ouabain and other sugars relevant to this discussion. It was the original work of Crane (1960), on the structural requirements of sugar transport in mammalian intestine, which led to the idea of a minimum structural requirement for transported sugars, and therefore "Crane specificity". The requirements he suggested for transport were a 6 carbon (pyranose) ring, a free hydroxyl group in the D-glucose configuration at C-2, and an additional carbon, C-6, attached to a C-5 (a methyl or methyl substituted group).

A number of uptake studies were subsequently carried out using a variety of natural and substituted sugars which proved the importance of this basic structural unit. However, they also revealed that

the requirement for attachment to the hexose carrier may be less precise (Csaky and Lassen,1964; Alvarado,1966; Neale and Wiseman,1968). As a result, a category of "minimal entry specificity" was suggested as an absolute requirement for transport *via* mammalian hexose carrier systems. In the cysticercoid of *H. diminuta* however, the 'picture' of specificity when examined closely looks less clear cut. Strong evidence for the binding and transport of 8 monosaccharides *via* a common hexose transport locus is available. From the structural configurations presented in Fig.6.22 it appears that not all the monosaccharides possess the entry requirements of Crane (1960) and, unlike *Taenia crassiceps* cysticerci (Craig,1983), cysticercoids of *H. diminuta* do not express the same specificity for hexose transport as mammalian intestine. Glucose has the greatest affinity for the uptake locus, of the monosaccharides tested, and also achieves the greatest concentration ratio over a fixed period of time.
2-deoxyglucose differs from glucose by the absence of oxygen from the hydroxyl group at the carbon 2 position. This results in it possessing the second highest affinity for the hexose carrier. It is interesting to note that the other deoxyhexoses i.e. 1-deoxyglucose and 2-deoxygalactose, do not have strong affinities, even though their difference from glucose is equally small, with the presence of two hydrogen atoms at the C-1 position (1-deoxyglucose) and two at the C-2 position of a galactose molecule (2-deoxygalactose). The relatively low affinity of 2-deoxygalactose could, however, be due to the galactose moiety, since galactose has only the fifth greatest affinity for the uptake locus. Galactose is the diastereoisomer

of glucose, where the position of the hydroxyl and hydrogen groups on C-4 are reversed relative to their orientation in D-glucose. Although sharing a common locus with glucose, its poorer affinity for the carrier, and lower rate of accumulation, probably results from this single alteration in structure. 3-O-methylglucose and α-methylglucoside have the third and fourth greatest affinity for the hexose locus. The former differs from glucose by the presence of a methylated hydroxyl group at C-3 position. This has been shown in some tapeworms e.g. *Taenia crassiceps* cysticerci, to reduce its specificity considerably (glucose having a K_t of 0.26mM and 3-O-methylglucose a K_t of 2mM (Craig,1983)). The importance of the hydroxyl group associated with C_3 of the pyranose ring in sugar transport has been previously noted (Phifer,1960c; Fisher and Read,1971; Pappas *et al.*,1973; Pappas and Freeman,1975). However, it appears that in the cysticercoid of *H. diminuta*, 3-O-methyglucose is readily absorbed and accumulated. The compound α -methylglucoside possesses a methyl group at the C-1 position, and this too has no deleterious effect on its absorption when compared to glucose. Finally, D-mannose, a diastereoisomer of glucose with an inversion at C-2, is also apparently absorbed *via* the hexose carrier. This sugar has not previously been reported as transported by other cestodes, and in *T. crassiceps* cysticerci it was shown to have no uptake properties (Craig,1983). It was suggested by Barnett, Jarvis and Munday (1968) that the hydroxyl group on C-2 is obligatory for transport by an Na^+-dependent intestinal sugar carrier, but this is not the case in *H. diminuta* cysticercoids. Work on kidney tubules,

however, led to the suggestion that mannose is transported via a carrier distinct from that of glucose, which is also shared by fructose (Silverman,1976).

It is not possible from the evidence of these studies to postulate a minimal structural requirement, as was done by Crane(1960), because alterations at each carbon position of the glucose molecule do not render it incapable of being transported by cysticercoids. However, an alteration to the C-5 position as in gluconic acid - did result in the failure of this compound to act as an inhibitor. Similarly, larger substitutions, e.g. in glucosamine and N-acetylglucosamine, also failed to produce inhibition of glucose absorption. Therefore the specificity of the monosaccharide transport system in H. diminuta cysticercoid is restricted to hexoses with a 6-carbon ring, and substances without large and strongly ionisable substitutions on that ring.

CHAPTER 7

GENERAL DISCUSSION

The aim of this discussion is to compare data from this study with that already known about uptake systems in other helminths and in H. diminuta in particular. Of particular interest are the characteristics and specificities of the uptake of low molecular weight carbohydrates by cysticercoids, and the similarity or otherwise with those of adult H. diminuta and other species of parasitic helminths. Pertinent to the comparison of data collected from different systems will be the differences between those systems e.g. in membrane morphology and physiology, worm habitat and general biology. The factors affecting monosaccharide absorption are also discussed leading to a resume of carbohydrate transport specificity in cysticercoids. Finally, the conclusion summarizes the findings and discusses the adaptation of parasites to their life style.

The work of Read (1959) led him to conclude that adult H. diminuta had a "requirement for carbohydrate". This 'requirement' was based on data available to Read (for example; Chandler,1943; Read and Rothman,1957a,c) and has since been confirmed by other studies. The reduction or removal of carbohydrate from the diet of the rat host, results in a stunting of worm growth and a reduction in numbers establishing

(Mettrick and Munro,1965; Roberts,1966). Diets containing less than sufficient concentrations of starch are suboptimal with respect to carbohydrate, and replacement of starch with glucose, sucrose,fructose and dextrins-maltose were all deleterious to worm growth (Roberts,1966; Roberts and Platzer,1967; Read and Rothman,1957a,c). These suboptimal diets produced stunted worms, inhibited proglottis formation and reduced the amount of stored carbohydrate, even though the diets were adequate to support the host (Roberts,1966; Roberts and Platzer,1967). The suggested reason for this is that the rat host intestine can absorb mono- and disaccharides so quickly that the worm is an unsuccessful competitor (Roberts,1984). However, if rats are given protein-free diets i.e. ones which are nutritionally inadequate for the host, and glucose is provided as carbohydrate, larger worms are produced than with a starch containing (protein-free) diet (Dunkley and Mettrick,1969). Evidence that other hymenolepids have a carbohydrate requirement is available for _H. citelli_ and _H. nana_ (Read,1959; Read and Rothman,1957b) and Roberts, working on _H. microstoma_ (unpublished in Roberts,1984), demonstrated that a carbohydrate-free diet almost completely suppressed growth. However, a diet containing sucrose instead of starch did not affect _H. microstoma_ as seriously as it would have affected _H. diminuta_ under the same conditions, and the reasons for this are unknown. Carbohydrate requirement in other worms has been demonstrated, but the evidence is less convincing. The spiral value parasite of dogfish sharks, _Lacistorhynchus tenuis_ were shown to reduce in number and size

if starved and the administration of both starch and glucose reduced this effect considerably (Read and Rothman,1957a). *Raillietina cesticillus* and *Davainea proglottina* were shown to destrobilate when carbohydrate was restricted in their diet (Levine,1938; Reid,1942). Roberts (1984) pointed out that most of the helminths shown to have a carbohydrate requirement were parasitic in herbivores whose diets would, of course, be expected to contain considerable amounts of starch. He stated that it is "not obvious how tapeworms found in carnivores could obtain sufficient carbohydrate if their requirement is comparable to that of *H. diminuta.*" One suggestion was that amino acids were more glycogenic in some cestodes than in others, and also metacestodes, located in host tissue, could probably obtain adequate carbohydrate from host body fluids. It appears, therefore, that all adult cestodes examined to date, under normal circumstances, store large amounts of glycogen which is usually rapidly used up under conditions of reduced carbohydrate in the host diet.

Before continuing to consider the factors affecting monosaccharide uptake and how these factors differentially affect the system described, a consideration of other differences between adult and cysticercoid will be made.

The similarities and differences between adult and cysticercoid *H. diminuta* have been described in Chapter 1. The importance of the tegument when considering the characteristics of monosaccharide absorption must not be overlooked. Within the tegument are the protein carriers, which contain the actual loci which are responsible for the transport of substrate from

outside to inside the cell. Also, there are membrane-bound enzymes (e.g. Arme and Read,1970) which play a part in digestion. The tegument not only serves as a barrier to the outside media and maintains the integrity of the organism, but it also mediates the absorption and efflux of all substrates. It is therefore of crucial importance to the biology of the worm.

In adult H. diminuta the brush border membrane (referred to as such because of its resemblance to the mucosal epithelium of mammalian intestine) is overlain by the glycocalyx which, because of the presence of sialic acid residues, has been shown to possess a net negative charge (Lumsden,1977). Cations are therefore attracted to it and Lumsden (1977) suggested that it acts as a "cation-exchange resin". The glycocalyx may be the location of some membrane-bound enzymes and also adsorbed enzymes of host origin, some of which have been shown to exhibit enhanced activity in the presence of divalent cations. The glycocalyx may also prevent subsequent diffusion away from the membrane (and the transport loci) of hydrolysis products and efflux substances. The glycocalyx and its associated proteins may also act as a rate-limiting step to the transport of solutes from the intestinal lumen to the transport loci. No evidence for this has been presented but Jeffs (1984) suggested that this rate-limiting step could represent part of the the 'unstirred water layer effect' observed by Podesta (1977a). There is however no evidence for the presence of a glycocalyx in cysticercoids or even of adsorbed host proteins. Also, the effects of the unstirred water layer were

undetectable in both this study and that of Jeffs (1984) on amino acid absorption in H. diminuta cysticercoids. Moczon (1973,1977) described the presence of phosphatase activity in H. diminuta cysticercoids, but no alkaline phosphatase was present in the tegument of the cysticercoid. Alkaline phosphatase was present, however, in the presumptive scolex region and this suggested to Moczon that the presumptive scolex is the only region in the cysticercoid that is active in absorption. However, results of this study have shown that both the outer cysticercoid and the inner presumptive scolex (when excysted in vitro) are absorptive and have mediated uptake systems for monosaccharides. The presence of membrane-bound enzymes in the adult has been shown to aid in the presentation of nutrients to the transport loci. Adult H. diminuta is impermeable to phosphorylated monosaccharides nucleotides and glycerophosphates, but the products of their hydrolysis are readily absorbed (Dike and Read,1971; Pappas and Read,1974; Kuo,1979; Uglem, Pappas and Read,1974). Read (1971) also suggested that the proximity of some enzymes to transport loci confered a kinetic advantage to the products of hydrolysis in their subsequent absorption. The environments of cysticercoid and adult H. diminuta are both similar (i.e. rich media with large amounts of small organic molecules) and different (i.e. one homoiothermic the other poikilothermic). However, considerably more is known about the environment of the adult worm than that of the cysticercoid. Indeed, there is a wealth of literature on mammalian intestinal absorption of nutrients so that the kinetic properties of the loci with which H. diminuta competes are also available. The affinity of all

transport systems in adult H. diminuta are known to exceed those of the rat host i.e. the K_t values are smaller for amino acid (MacInnis, Graffe, Kilejian and Read,1976), and glucose absorption (Podesta and Mettrick,1976). Work by Wilson and Dietschy (1974) has led to the conclusion that the rat gut would have twelve times more absorptive capacity per unit weight than H. diminuta if it were not for the proportional effects of the unstirred water layer. The presence of this layer, on both H. diminuta and host, is to reduce the absorptive capacity so that H. diminuta maintains a 3 - 4 times greater absorptive surface than the rat (Befus and Podesta,1976). The location of adult worms is age-dependent and affected by its nutrient requirement (Ulmer,1977; Holmes,1973; Crompton,1973). The preferred site varies according to species of worm, size of infection, other species of worm present but it provides optimal conditions that support maximum growth and fecundity (Mettrick and Dunkley,1969). Mettrick and Podesta (1974) suggested that adult cestodes locate in accordance with carbohydrate gradients and vary their positions in relation to gradient variation. Initially, the adult is located in the second quarter of the intestine (Goodchild and Harrison,1961) but, by 16 - 18 days post infection, they are found at least half way down the small intestine (Bråten and Hopkins,1969; Cannon and Mettrick,1970). Adult H. diminuta do, however, undergo a diurnal migration forwards and backwards along the small intestine, which can be correlated with the feeding patterns of the host (Read and Kilejian,1969; Arai,1980). The worm population is located in the first third of the small intestine after host feeding to

take advantage of glucose which is in high concentrations in this region. Mettrick and Cho (1981a,b) have shown that the migratory activity of H. diminuta is correlated with the concentrations of 5-hydroxytryptamine, which is secreted from the rat gut cells upon feeding and is known to stimulate muscular movement in H. diminuta. Bråten and Hopkins (1969) suggested that the worm acts on stimuli received through various points all over the worm, which provide clues to its position. In a later study by Hopkins and Allen (1979) they confirmed this and suggested that the position of the worms is determined by balancing the input of adverse stimuli from its 'tail' and 'head' ends. Thus by surgically shortening worms they were induced to move posteriorly to the preferred site of younger (shorter) worms. In Chapter 1 the factors which affect the location of adult H. diminuta were discussed. The most important include intraspecific and interspecific competition. The former involves high densities of adult H. diminuta with the same host and describes an inverse relationship between population density and parasite size and fecundity. The cause of the 'crowding effect' is still unclear, but it is probably the result of a combination of factors. However, competition for available carbohydrate is most probably the main cause of reduced fitness (Roberts,1966; Roberts and Mong,1968). Interspecific competition describes that which occurs when H. diminuta is present in conjunction with other helminths e.g. Moniliformis dubius, other hymenolepids etc. The effects of interspecific competition will depend largely on the species of helminth in concurrent infection with H.diminuta. However, there are usually two effects: displacement of H. diminuta from

its preferred site and, as a result, a reduction in growth/size of worms. Reduced fecundity is also sometimes found. Therefore, it is clear that the location of adult H. diminuta is primarily governed by the quest to optimise its nutrient uptake, although it is also affected by the factors mentioned above.

The location of H. diminuta cysticercoids within the intermediate host is known, but the factors which determine and affect their location are not. Generally, cysticercoids are found in the thorax and abdominal regions of the intermediate host, with the majority located at or near their point of entry i.e. the point at which they burrowed through the gut wall. Observations of dissected insects show that they are found associated with the serosal surface of the gut and also with pieces of fat body, with the 'head' region apparently attached to these surfaces and the tail 'floating' in the haemolymph (personal observation). Thus, initial observations suggest that the site selected is simply the point of entry. However, there is evidence for the existence of sensory structures in H. diminuta cysticercoids (Ubelaker,1980), and muscles are present for at least the first two weeks of development (Richards and Arme,1984a). The presence of these structures might suggest that the cysticercoid is capable of both detecting nutritionally favourable sites and moving away from unfavourable sites. MacDondald and Wilson (1964) showed that in low densities, H. diminuta, cysticercoids located in the mesothorax of Tribolium confusum, whereas at higher densities

they located in the abdomen, and therefore by inference suggested the presence of site selection. Also, 'privileged sites' have been shown in insects e.g. the legs of *Periplaneta americana* where *H. diminuta* cysticercoids can grow and develop unaffected by the normal severe host response, which is presumed to be prohibited by the lack of sufficient space for the haemocytes to circulate in the legs. However, *H. diminuta* cysticercoids experience no obvious host response to their presence in *T. molitor* and their location is, as far as is known, unaffected by an immune reaction. The haemolymph is a circulating fluid into which nutrients are absorbed directly from the intestinal lumen. In terms of carbohydrate there exists a concentration difference from the lumen into the haemolymph which is maintained by the rapid conversion of absorbed glucose into trehalose. The resulting trehalose is transported around the body and can then be converted back into glucose (as shown by the universal presence of trehalase throughout the insect). Therefore, it was not surprising that there are low concentrations of free glucose in *Tenebrio molitor* haemolymph, although it is possible that higher concentrations might be found in proximity to the serosal surface. Therefore, if the cysticercoid selected a site, on the basis of requirement for glucose (as it cannot absorb the principal carbohydrate present in *Tenebrio molitor* haemolymph-trehalose), then it might be expected to be in association with the serosal surface. However, if glucose requirement is not paramount then any site, which offers • adequate space and in which the haemolymph can circulate

freely, will suffice. The results do not give a clear indication of active site selection and, supporting the evidence against, mature H. diminuta cysticercoids have only occasionally been known to move in vitro (usually a slight movement of the "tail"), with no indication of movement active enough to enable a change from one site to another in vivo. It is probable that recently penetrated oncospheres can be moved by the circulation of the haemolymph, so that those found in the privileged sites of inhospitable hosts could have been taken there by the circulating haemolymph.

The effect of inter- and intraspecific competition on the location and other factors in the development of cysticercoids has not been fully investigated. Jeffs (1984) investigated the effect of density of infection on the subsequent in vitro uptake characteristics of H. diminuta cysticercoids. This author noted that high densities of infection in T. molitor had a growth-retarding effect, which was particularly evident in the cercomer length. The effect of crowding on kinetic parameters was to reduce the V_{max} as the density of infection increased, but there was no effect on the K_t value. Bolla and Roberts (1971) found that adult H. diminuta from high density infections have lower rates of protein synthesis. If this were also true for cysticercoids, then a reduction in V_{max} might be expected. The uptake loci are probably located on the tegument of cysticercoids, which, if similar to the adult, would be totally replaced at least every 8 h (the glycocalyx is replaced every 6-8h, Oaks and Lumsden,1971). Therefore,

assuming the uptake loci are proteinaceous, this involves the synthesis of 'new' loci, which is dependent on the rate of RNA synthesis, which is in turn dependent upon the supply of nucleotides. Competition for available nucleotides could reduce the rate of protein synthesis and ultimately reduce growth by creating a shortage of transport proteins. Heyneman and Voge (1971) studied *Tribolium confusum* infected with *H. diminuta*, *H. microstoma* and *H. citelli* simultaneously. They found that all parasites developed normally, in spite of the presence of others, and without any significant host reaction. However, it would be of interest to determine whether the kinetic properties and specificity of *H. diminuta* cysticercoids hexose absorption loci, grown in the presence of other hymenolepid species, are the same as those presented in this study, and if, with large numbers of cysticercoids, one species benefits over the others.

The role of carbohydrate in the biology of adult *H. diminuta* is therefore paramount for normal growth and development. This study aimed to investigate the role of carbohydrate in the cysticercoid biology which began with some initial observations on the types of sugars transported and the mechanism involved.

The cysticercoid of *Hymenolepis diminuta* has been shown to absorb and accumulate the following carbohydrates: glucose, galactose, 2-deoxyglucose, 2-deoxygalactose, 3-0-methylglucose, α-methylglucoside, 1-deoxyglucose and mannose. The kinetic data for the transport of these carbohydrates is presented in Table 5.3 and the equivalent data for adult *H. diminuta* is given in Table 5.4.

Unlike the results for cycloleucine absorption in cysticercoids (Jeffs,1984: Jeffs and Arme,1985b) there appears little similarity between the kinetics and specificities of monosaccharide uptake in adults and cysticercoids of *H. diminuta*. Glucose and galactose are the only monosaccharides shown to be absorbed by mediated uptake, by adult *H. diminuta*, although there is some dispute over the absorption of 3-0-methylglucose (30MG). Read (1961) showed 30MG to be a competitive inhibitor of glucose uptake but Starling and Roberts (Roberts,1980) stated that 30MG was not absorbed by adults and this explains the ineffectiveness of phloretin as an inhibitor. Other monosaccharides are thought to be transported by adults, but the evidence is only indirect and derived from their effect as inhibitors of glucose uptake. The inhibitory activity of 6-deoxyglucose on adult glucose uptake highlights another difference between adult and cysticercoid, as this compound has no effect in the latter (Read,1961; Uglem, Love and Eubank,1978). A small diffusion component was detected and measured for the uptake of glucose in adults and cysticercoids. For mature stage 5 cysticercoids the K_d was calculated as $0.09 \mu mole.mM^{-1}$, recently excysted adult $K_d = 0.75 \mu mole.mM^{-1}$, 5-day-old adult

$K_d = 1.01\mu mole.mM^{-1}$ and 10-day-old adults $K_d = 1.10\mu mole.mM^{-1}$. There is, therefore an increase in the size of the diffusion component with age, with an extremely small diffusion component for glucose uptake in cysticercoids. Generally, however, diffusion does not play a major part in the uptake of glucose (or other monosaccharides) by H. diminuta. This is in agreement with McCracken and Lumsden (1975) who concluded that, under normal physiological conditions, diffusion played no significant part in glucose absorption by adult H. diminuta. However, the size of a diffusion component is likely to vary according to the magnitude of the glucose gradient between the parasite and the media so that a small diffusion component will occur where a high initial concentration inside the worm prevails. Hymenolepis microstoma (Pappas and Freeman,1975), H. diminuta (McCracken and Lumsden, 1975), C. verticillatum (Fisher and Read,1971) and T. crassiceps metacestodes (Pappas et al.,1973; Craig,1983), all have negligible diffusion components, and therefore the 'active' absorption of monosaccharides is probably an indication of their importance in the biology of these helminths.

This study also investigated some uptake characteristics of the recently excysted adult. It is known that the adult tegument is formed while the presumptive scolex is in the cysticercoid (Richards and Arme,1984a), but the presence of a similar structure does not automatically imply the presence of the uptake loci characteristic of adults. Indeed, the results presented show that the recently excysted adult has hexose

absorption loci whose kinetic characteristics fall between those of the cysticercoid and the adult. However, with regard to specificity, the recently excysted adult hexose absorption loci were most similar to those in the cysticercoid, with the active absorption of 3OMG and the lack of inhibition of glucose uptake by 6-deoxyglucose (see Table 6.3) This is a surprising result, as it suggests that although the morphology of the tegument is adult, physiologically it behaves like the cysticercoid tegument. If, however, the cysticercoid and recently excysted adult possessed precisely the same uptake loci, then we would also expect the same kinetic properties - which have not been demonstrated. Walker and Barrett (1984) discussed the alterations in membranes and membrane-bound enzymes that might result from temperature variations in the environment. Thus, with some speculative extrapolation, might the differences in kinetics between hexose absorption by cysticercoids and recently excysted adults be induced by the different temperatures experienced _in vitro_ and _in vivo_?. However, cysticercoids incubated at 32°C shown no alteration, in the K_t value (with a slight increase in V_{max}, this study) suggesting that this is not the case. The recently excysted adult tegument, however, is morphologically adult and therefore its lipid/protein ratio may be considerably different from that of the cysticercoid. An increase in temperature therefore could affect the uptake locus by altering the fluidity of the membrane surrounding it and perhaps the spatial relationship of the site, so that the affinities could be altered.

The recently excysted adult has cysticercoid monosaccharide absorption chracteristics, but we know that 10-days post-infection the characteristics of the loci are different. Are the initial uptake loci gradually replaced by adult loci over the first ten days, or are we observing characteristics peculiar to the first functional adult tegument or just that covering the scolex region?. Further studies are needed to answer these questions to ascertain at what point the uptake characteristics of young adult H. diminuta take on the properties clearly demonstrable at 10-days post-infection.

The absorption of monosaccharides by other helminths was discussed in Chapter 4, and it is apparent that most data are available for adult H. diminuta. Hymenolepis microstoma absorbs D-glucose by a carrier-mediated system and this absorption was inhibited by galactose, α- and β-methylglucoside (Pappas and Freeman,1975). Taenia crassiceps metacestodes absorb glucose and galactose actively against a concentration gradient. D-fructose is absorbed by simple diffusion, whilst only α- and β-methylglucoside had any inhibitory action on glucose uptake. It is noteworthy that mannose and 3OMG had no effect on glucose uptake (Craig,1983). Taenia taeniaeformis has been shown capable of absorbing glucose (Von Brand et al.,1964) but no other sugars have been tested. Calliobothrium verticillatum absorbs glucose and galactose, and glucose uptake was inhibited by galactose and α-methylglucoside. 3OMG was absorbed by simple diffusion, and fructose and mannose not at all (Fisher and Read,1977). Therefore, it is evident

that the monosaccharide uptake loci in the above tapeworms are very similar in some respects (e.g. sensitivity to Na^+ - see later) but are somewhat different in their specificities. Table 7.1 attempts to draw together all the available information on monosaccharide transport in parasitic helminths. From the species of helminth shown in Table 7.1, it is clear that a very narrow range of monosaccharides are absorbed. H. diminuta cysticercoids absorb the greatest variety of monosaccharides, probably reflecting the fact that detailed studies have been undertaken on this species. It is, however, interesting to note the narrow range of specificity and even uptake kinetic data over such a diverse group of parasitic worms - cestodes, trematodes and acanthocephalans often inhabiting different environments.

In this study, 3 main factors which affect monosaccharide absorption were investigated, age of parasite, temperature of incubation media and the ionic composition of incubation media.

Age of cysticercoid has relatively little effect upon its absorptive capacity. A slight reduction in affinity of glucose for the uptake locus was noted, and a small decrease in the V_{max} value. Together, these effects will slightly reduce the capacity of the monosaccharide uptake locus. Would we expect an age effect on uptake? The maximum life of a cysticercoid is governed by the life of its host, but in the laboratory, T. molitor can be kept for a maximum of c. 50 days at 26°C. Therefore laboratory reared cysticercoids older than c. 47 days are unusual. During these 47 days at 26°C the first 12 days are spent actively growing and differentiating so that after 12 days a mature stage 5 cysticercoid is observed. From days 12

TABLE 7.1

Group	Species	Stage	glucose	galactose	mannose	1-deoxyglucose	2-deoxyglucose	2-deoxygalactose	3-O-methylglucose	α-methylglucoseide	β-methylglucoside	6-deoxyglucose	fructose	allose	fucose	glucosamine	ribose	N-acetylglucosamine
CESTODA	*Hymenolepis diminuta*	cysticercoid	*	*	*	*	*	*	*	*								
		0-day-old adult	*	*	*	*	*	*	*	*								
		5-day-old adult	*	*														
		10-day-old adult	*	*		*				*		*		*				
	Hymenolepis microstoma	adult	*	*						*	*							
	Taenia crassiceps	cysticerci	*	*					*	*	*	*			*			
	Calliobothrium verticillatum	adult	*	*						*								
TREMATODA	*Fasciola hepatica*	adult	*		*											*	*	
	Schistosoma mansoni	adult	*	*	*		*		*							*	*	
ACANTHOCEPHALA	*Polymorphus minutus*	adult	*															
	Moniliformis dubius	adult	*	*	*		*		*				*					*

*= Compound absorbed by parasite

to 47, therefore the only observable alteration in size is due to the build up of a fibrous layer in the outer cysticercoid wall. This layer should not alter the absorptive properties of the monosaccharide uptake locus directly but it could serve as a barrier to the free movement of absorbed solute. Studies on the adult worm have revealed considerable difference in both uptake specificity and characteristics with age (Table 5.1, Chapter 5). The results presented in this thesis indicate that the recently excysted adult monosaccharide uptake locus has the same specificities as that of the cysticercoid, although the kinetic data more closely resemble that of adult worms. Changes in the affinity of an uptake locus can be explained in terms of additional steric influences from surrounding molecules in a membrane or slight alterations in membrane composition. Likewise, a change in the specificity of an uptake locus appears also to reflect a membrane alteration. Although the presumptive scolex (and the recently excysted adult) have already developed microtriches (Richards and Arme,1984a) it appears that the uptake loci present at this time are different from those of the older 10-day-old adult. The microtriches present in the scolex region are, however, morphologically different from those found on the strobila of worms, and this morphological difference may infer a variation in membrane composition which in turn could result in altered specificities of uptake loci. Therefore, it is possible that only after formation of the strobila that the characteristics and specificities typical of the adult worm hexose absorption locus are evident. It would be of interest to investigate the specificity of 1-6-day-old adult hexose uptake loci to determine

whether the presence of the strobila does in fact mask the specificity of the uptake loci on the scolex, or indeed to study uptake in the scolex region only. The results of glucose and galactose uptake by 5- and 10-day-old adult worms presented in this study, indicate that as the worm ages, so the K_t value increases i.e. the affinity falls, while the V_{max} also decreases. This was also shown by Starling and Roberts (Roberts,1980) although they suggested that this was due to changing proportions of kinetically distinct loci, the distinct loci representing the 'standard' glucose uptake loci plus a K^+-insensitive loci. This K^+-insensitive component was apparent in 6-day-old worms, less apparent in 10-day-old worms and could not be detected in 20-day-old worms. The changes in proportion suggested were found to occur along the entire length of the strobila of 6,10 and 20-day-old worms, representing whole worm development,not that confined to new proglottides produced by older worms. Therefore, although no evidence for the presence of a K^+-insensitive transport locus in adult H. diminuta was detected in this study, Starling and Roberts (Roberts,1980) have provided evidence for the variation in specificity of uptake loci along the length of the worm, which makes the previous suggestion of differences between scolex and strobila located uptake loci more acceptable. Honegger and Semenza (1971), working on hamster small intestine, also noted and increase in K_t with age and proved it to be due to the presence of two populations of uptake loci, one with high, the other with low affinity for glucose. A study on the effect of age on amino acid uptake by H. diminuta cysticercoids has been made by Jeffs (1984). He found that

the pattern of cycloleucine and methionine uptake changed with age, with the K_t remaining the same and a reduction in V_{max}, and suggested that this may "reflect the need for a constant amount of amino acids throughout the life of the parasite". Other studies on the effect of age on monosaccharide absorption are few, although some evidence can be collated from different studies using different aged individuals (e.g. Pappas *et al.*,1973; Craig,1983).

Temperature as a factor affecting monosaccharide uptake was also investigated in this study. From the results, it was obvious that temperature only affected the V_{max}, which decreased with decreasing temperature. One interesting point was that cysticercoids were able to withstand short periods of freezing, and yet were capable of subsequent excystation *in vitro*. On several occasions, cysticercoids suspended in KRT saline plus glucose/galactose were accidentally frozen for periods of up to 10min. However, when treated with excystation media at $37^{o}C$, all were excysted. This highlights the extraordinary resilience of these cysticercoids to adverse conditions, and the extent to which physical and biochemical properties of the cysticercoid ensure the continuation of the life cycle against unfavourable conditions. It would be of interest to determine how long a period of freezing the cysticercoid could survive, the temperature of freezing, the viability *in vivo*, the subsequent uptake capabilities and the

effect of age with regards to the fibrous layer which may have a protective role.

The effect of temperature on hexose absorption in adult cestodes has not been investigated. However, a short study on metacestodes of T. crassiceps has been carried out (Craig,1983). This author found that uptake of D-glucose at $10^{o}C$ represented only 2% of the control uptake value. However, the lack of data on this subject precludes further discussion.

In 1977, Podesta demonstrated that the presence of an unstirred layer in proximity to the surface of H. diminuta leads to an underestimation of the passive diffusion component K_d and to an overestimation of the Michaelis constant K_t for carrier-mediated transport of organic substrates. The majority of membrane transport studies on parasites have not taken into account the possible effects of the unstirred water layers on absorption parameters, and Podesta claimed that more accurate estimates of kinetic parameters are obtained if the unstirred water layer is reduced by stirring the media. There is considerable evidence to support this, particularly in work on mammalian intestine. Fluid adjacent to, and lying between, the microvilli present significant resistance to the passage of nutrient molecules (Wilson and Dietschy,1972a,1974; Westergaard and Dietschy,1974; Dugas, Ramaswamy and Crane,1975; Thomson and Dietschy,1980a). The most important consequence of this layer is that the fluid adjacent to the epithelium has a lower

concentration of substrate than the bulk medium. The tegument of adult and cysticercoids of H. diminuta bears microtriches or microvilli which resemble the brush border membrane of mammalian intestine. Therefore the possibility of an unstirred water layer effect should not be ruled out. However, in a wide range of substrate concentrations, shaking rates, and incubation times, no difference in the absorption kinetics of glucose or galactose in H. diminuta cysticercoid could be detected. In a similar study on T. crassiceps metacestodes (Craig,1983) it was concluded that there was no effect of unstirred water layer on mediated glucose uptake. In fact, the latter author observed differences dependent on the method of stirring employed (oscillation or spinning). Podesta (1977) concluded that the true value for glucose uptake by adult H.diminuta was 0.21mM, a value almost identical to that of the cysticercoid. The spin/oscillation rates employed by Podesta (1977) and Dugas et al.(1974 working on mammalian intestine) range from approximately 0-1200 rev.min^{-1} with a decrease in K_t noted for increasing spin speed (Podesta,1977). However, it is unlikely that adult H. diminuta experiences movement of fluid in relation to its surface, of such speed in vivo, and therefore its relationship to actual in vivo kinetic values is questionable. Indeed, Murphy and Lumsden (1984) have questioned the validity of Podesta's original work on the basis of his use of absorbable extracellular space markers, and insufficient rinsing and blotting procedures. These latter authors did not dispute the presence of this unstirred layer, merely its magnitude. They also suggested that unstirred layers may be reduced by movement of the microtriches, as has

been suggested for intestinal microvilli (Mooseker,1976). However, this requires further investigation. Also, as a result of the lack of unstirred layer effects in this study, and the desire to continue the uniformity of studies on H.diminuta cysticercoids, no shaking of the media was carried out during this study except for an initial 3 month test period.

The effect of Na^+ on sugar transport has been widely described in many systems (e.g.Read et al.,1974; Crane,1962; Kimmich,1973; Silverman,1976), and there are available several comprehensive review articles (e.g.Schultz and Curran,1970; Mettrick and Podesta,1974). It is apparent that, although sugar transport varies considerably in its specificities and characteristics from species to species, one common effect is that induced by varying the amount of Na^+ in the external media.

The reason for the common effect of Na^+ on sugar transport is based upon the Na^+-gradient hypothesis (see Chapter 3), which assumes that a carrier catalyses a reversible reaction whose asymmetry is maintained by the asymmetric distribution of ions (Crane,1962). The idea that ion gradients could provide the energy for accumulation of non-electrolytes was originally devised by Crane (1962) and expanded by Shultz and Curran (1970). The hypothesis suggests that the influx of Na^+ and, for example, sugar is energetically coupled, possibly through the formation of a ternary complex between membrane carrier, sugar and ion at a fixed stoichiometry. There are many data which support the

hypothesis but there are also ill-fitting data which has led some authors to discount the theory as it is presented to date (e.g. Podesta,1982). Observations which support the hypothesis include the following: the absorption and efflux of substrate is dependent upon concentrations of Na^+ in the media and within the tissues. The inhibition of Na^+ influx also inhibits substrate absorption. The influx and efflux of Na^+ is accelerated by the presence of substrate in the external medium (former) and intracellularly (latter). There is a fixed relationship between substrate-dependent Na^+ influx and Na^+-dependent substrate influx. Absorption of Na^+-dependent substrate is accompanied by membrane depolarization and,finally,the reversal of the normal direction of the Na^+-gradient causes efflux of substrate. In this study, the effect of Na^+ concentration on glucose and galactose absorption by cysticercoids and recently excysted adults was investigated. The results for cysticercoids indicate a decrease in the affinity of the uptake site for substrate as the Na^+ concentration falls, with a concomitant fall in the V_{max}. However, when K^+ was used as replacement cation an increase in affinity was noted at very low Na^+ concentrations (and high K^+ concentrations). This, however, had no effect on accumulation rate, or metabolism of substrate under these conditions. Starling and Roberts (Roberts,1980) suggested the presence of a K^+-sensitive system in adult *H. diminuta*, which diminished in importance, with age of worm. These data therefore suggest the operation of K^+-sensitive uptake loci at high K^+ concentrations (i.e. low Na^+ concentrations) but there are insufficient data to develop this

hypothesis further.

It appears that cysticercoid and adult H. diminuta monosaccharide uptake systems differ in their reaction to conditions of low Na^+ and to the replacement of Na^+ by other cations. Adult H. diminuta hexose absorption is Na^+-dependent and Na^+-sensitive, and lithium is considerably better as replacement cation than choline, Tris or potassium. The cysticercoid absorbs 70% of control glucose uptake in the total absence of Na^+, and can also accumulate and metabolise solute under these conditions. However, inhibitor studies and reciprocal inhibition studies between sugars have not indicated the presence of two different sugar uptake sites, therefore the evidence points to a monosaccharide absorption site with special capabilities under conditions of low Na^+ concentrations. These data do not support the Na^+-gradient hypothesis as the inhibition of Na^+ influx does not totally inhibit substrate absorption and suggests the presence and activation of another mechanism in conditions of low extracellular Na^+. The results presented for the recently excysted adult are even more intriguing. It appears that newly excysted adults also have the ability to absorb at least 30% of control glucose uptake in the absence of Na^+ in the external media. Although a greater amount of hexose absorption is inhibited in the recently excysted adult (70%) compared to cysticercoid (30%), the ability to accumulate and metabolise substrate under these conditions is likewise present. Apart from these data, there is no evidence from studies on specificity, for the presence of two uptake sites as suggested (Arme et al., 1973; Pappas and Read, 1975). In all

experiments a pre-incubation in Na^+-free media of 30min was carried out. Pre-incubation of cysticercoids and young adults was primarily to allow the tissues to equilibrate in vitro, and to attempt to bring all cysticercoids (from different hosts) to a standard physiological condition. Therefore, there was presumably ample time for the affect of Na^+-free media to reach the tissues. However, the hexose absorption systems in H. diminuta cysticercoids and recently excysted adults have the ability to operate in Na^+-free media, while by 5-days post-infection in the definitive host, the absorption sites are totally Na^+-dependent and therefore Na^+-sensitive. This finding brings to mind two main questions; the first asks why a Na^+-insensitive uptake locus is needed by cysticercoids and young adults and the second question refers to the Na^+-gradient hypothesis and its role in monosaccharide transport. What is the relevance of a Na^+-insensitive monosaccharide absorption system to cysticercoids and young adults in vivo? The evidence suggests none. Both T. molitor haemolymph and the intestinal lumen of rats contains ample Na^+ and although fluctuations will occur, Na^+ absence is unlikely to occur in these two environments. It is therefore unlikely that, in their natural environments, despite differences in location within hosts, or hosts themselves, cysticercoids would experience conditions of low Na^+concentration (i.e. $< 10mM\ Na^+$). However, one explanation could be that in both habitats, transport of a variety of substrates across membranes is occurring all around the parasite and some of this will be Na^+-dependent. Therefore at the molecular level, there could exist local

shortages of free Na^+ as a result of the capture of Na^+ by other Na^+-dependent systems. Thus Na^+ effluxed from a system finds itself with a kinetic advantage for influx. It is located in the correct place and , because of the restraints of the unstirred layer on free diffusion of molecules away from the surface, it is effectively cycled between influx and efflux. This could result in local shortages, at the molecular level, of Na^+. The second question concerns the validity of the Na^+-gradient hypothesis. Podesta (1980,1982) has presented some clear evidence against the validity of this hypothesis, and the data of this study also question its function as presently suggested. The inability adequately to demonstrate Na^+-dependent efflux of substrate against its chemical potential gradient (when the Na^+-gradient is reversed) is interesting, and Johnstone (1975) showed that when it did occur, it was smaller than expected and often uptake still occurred, suggesting that factors other than the Na^+-gradient had contributed to the vectorial operation of the uptake system (Johnstone,1975). From the thermodynamic or energetic viewpoint, the Na^+-gradient hypothesis stands firm (Armstrong,1975; Curran,1975; Heinz, Geck and Pietrzyk,1975). There is enough energy derived from the electrical gradient to support transport and also to energise accumulation of substrate, possibly explaining the continued uptake of substrate even when the Na^+-gradient is reversed (Heinz *et al.*,1975). The uptake of glucose (and alanine) across depolarised vesicles of isolated intestinal brush border has been demonstrated (Murer, Sigrist-nelson and Hopfer,1975)

strongly suggesting that factors other than a Na^+ electrochemical potential gradient are involved. However, Silverman (1976) demonstrated Na^+-coupled glucose transport across vesicles made of isolated intestinal and kidney brush border - strong evidence for the Na^+-gradient hypothesis. A diffusive-convective model of ion dependent sugar transport has been postulated by Podesta (1982 - see Chapter 3) which is consistent with much of the data derived from adult *H. diminuta*. Podesta (1982) concluded that the results from sugar uptake studies in cestodes have been either consistent or inconsistent with the predictions of the Na^+-gradient hypothesis, so that "it cannot yet be considered as the unique explanation of Na^+-dependent uptake in cestodes". The results of this study also bring into question the Na^+-gradient hypothesis as an adequate explanation of hexose absorptive mechanisms in *H. diminuta* cysticercoids and young adults.

An extensive range of inhibition experiments using structurally similar compounds, specific inhibitors e.g. phlorizin, ouabain, and metabolic inhibitors will provide ample data for the specificity of an uptake system to be determined. Such a study was carried out and shows that at least 8 monosaccharides are actually transported *via* a single uptake locus or group of uptake loci which are fully interactive in *H. diminuta* cysticercoids. All 8 sugars (listed in Table 6.5) are competitve inhibitors of each other. Glucose has the greatest affinity for the uptake locus and 1-deoxyglucose the least. The uptake of each hexose was shown to contain a small diffusion component ranging from a K_d of $0.09 \mu mole.mM^{-1}$

(glucose) to a K_d of 0.18 μmole.mM^{-1} (1-deoxyglucose) - with the K_d increasing with decreasing affinity of substrate for the absorption site. Read (1961) carried out some inhibition studies on glucose uptake in adult H. diminuta. The results (see Pappas and Read,1975) highlight the differences in specificity between adult and cysticercoid, with inhibitory action shown by 6-deoxyglucose but not phloretin. The situation is reversed in cysticercoids with active inhibition of glucose uptake by phloretin, but not 6-deoxyglucose. Fructose had no inhibitory activity, neither was it shown to be absorbed by cysticercoids - a result similar to the adult worm (Arme and Read,1971). A summary of the inhibition data for H. diminuta is given in Table 6.5 . The action of phlorizin and phloretin on hexose absorption in adults and cysticercoids also points out some dissimilarities. Phlorizin was a potent inhibitor of monosaccharide absorption in both cysticercoids and adults, and the inhibition was classified as competitive (McCracken and Lumsden,1974; this study). This is in contrast with the results presented for H. microstoma, which show that while glucose uptake was inhibited it was characterised as partially competitive (Pappas and Freeman,1975). Phlorizin affinity for the hexose absorption site of the cysticercoid (H. diminuta) was also shown to decrease with decreasing Na^+ in the media. Phloretin, the aglycone of phlorizin, differs from phlorizin by the replacement of the glucose moiety by 3-O-methylglucose. Presumably resulting from this, it has no inhibitory action on glucose absorption by adult H. diminuta, suggesting that the worm is impermeable to 3-O-methylglucose. Phloretin did however inhibit hexose uptake in cysticercoids

and *H. microstoma* adults. In the latter case the inhibition was mixed in nature - suggesting that phloretin not only affects the binding of glucose to the uptake locus but also affects the mechanism of the transport system. If the suggestion that the uptake loci specificities change with age in the adult is correct, and bearing in mind the noncompetitive portion of inhibition detected in *H. microstoma*, the inhibitory activity of phloretin on recently excysted adults can be explained.

The effect of ouabain on hexose uptake was the same for both adult and cysticercoid. Ouabain had no effect on uptake in intact parasites. It has been shown that tissue slices of adult *H. diminuta* allow ouabain to penetrate to the basal plasma membrane where it can take effect (Podesta *et al.*,1980). This gives a strong indication that the ouabain sensitive mechanism of transport i.e. the Na^+-pump dependent upon Na^+/K^+-dependent ATPase is located on the basal plasma membrane. These findings add a new dimension to the Na^+-gradient hypothesis as the Na^+-pump appears to be located at the base of the tegumental syncytium - suggesting the presence of an Na^+-gradient throughout the syncytium and across the tegument. It would be of interest to investigate the action of ouabain on cysticercoid 'cases' i.e. that remaining when the presumptive scolex has excysted, to ascertain whether ouabain can reach the basal plasma membrane and take effect. It would however be difficult to slice the cysticercoid in the same way as the adult in order to investigate the action of ouabain.

In Chapter 5 and 6 an attempt was made to draw together the data on monosaccharide uptake in cysticercoids and to make some tentative conclusions concerning the specificity of the uptake locus. It is clear that the cysticercoid does not exhibit all the aspects of 'Crane specificity' or 'minimal entry specificity' as defined for sugar transport in mammalian small intestine (Crane,1960,1962; Csaky and Lassen,1964). It is possible to say, however, that only 6-membered rings are absorbed. Furthermore only those hexoses without alteration at the C5 position, and without very large strongly ionisable substitutions elsewhere on the ring, are transported. The specificity of hexose absorption in H. diminuta cysticercoids does not therefore hinge upon maintaining a specific structural configuartion around one carbon position in the hexose ring. Alterations at all carbon positions have not rendered substrates unable to be absorbed.

The data concerning recently excysted adult shows that the specificity of its hexose locus is identical to that of the cysticercoid. It has been suggested that the differences between young, and 10-day-old adults reflects a difference in specificity of uptake loci located in the scolex region and on the strobila - which at 0-days of age is non-existent. However, there is no evidence for this and it therefore requires further investigation. Table 6.6 summarizes the differences between cysticercoid, recently excysted adult and 10-day adult hexose uptake loci. The differences in specificity of cysticercoid and adult hexose absorption sites

are not great and are unlikely to be of importance *in vivo*. This is particularly true when taking into account the types of hexoses likely to be encountered by cysticercoids and adults. Pappas and Read (1975) discussed the advantage in having a small number of loci types with wide specificities over a large number of loci with narrow specificities. They stated that less genetic capacity is required for the continued synthesis of a small number of loci types and studies on bacteria have shown that bacteria employing this strategy have an energetic advantage over their competitors. It appears that the cysticercoid possesses a broad hexose specific system, allowing it to absorb most hexoses. In view of the small quantity of free monosaccharide in *Tenebrio molitor* haemolymph, a broad hexose system would enable the cysticercoid to take full advantage of any hexose or hexose containing compound present. Jeffs (1984) stated that broadly specific loci can also prevent flooding of a parasite with a compound which is in high concentrations in the environment, and this, together with a lowering of the affinity of an uptake locus as concentration of compound increases, effectively regulates influx. An example is proline uptake in *H. diminuta* cysticercoids. Proline is the most abundant amino acid in *T. molitor* haemolymph (Hurd and Arme,1984a). Cysticercoids raised in these beetles show a raised K_t value over that of the adult worm (bearing in mind the similarity of kinetic parameters governing amino acid absorption in cysticercoids and adults - see Jeffs,1984). Therefore, a combination of affinity and specificity can 'regulate' the composition of internal pools of metabolites. This suggests that environment may influence the

characteristics of an uptake site. Read et al.(1963) found such an influence when they reared H. diminuta in golden hamsters. The worms obtained from these animals, after 10-days of growth, had different uptake characteristics, compared with those reared in rats. They interpreted these findings as a host induced change in the number of loci responsible for the uptake of various compounds.

Although this study confirmed the presence of glucose uptake by cysticercoids in Na^+-free media, there was no other evidence for the presence of 2 sites. If two sites are present, they cannot be separated by the experiments performed in this study and differ only in their operation under conditions of low external Na^+.

There is, as yet, no evidence for a requirement for exogenous carbohydrate by cysticercoids. However, several points lead to the suggestion that free monosaccharide does not play a vital role. The first notes the paucity of free monosaccharides in the environment of the cysticercoid. The second notes the low K_d values obtained for the uptake of sugars by cysticercoids. This shows a small diffusion component but also implies the presence of a large concentration of carbohydrate already inside the cysticercoid. This carbohydrate (if it exists) could be endogenous (unlikely because of its use in active growth) or derived from absorbed substrates e.g. monosaccharides (not present in high concentration) or glycogenic amino acids - which are present in T. molitor haemolymph (Hurd and Arme,1984a). It is clear that

the determination of a 'requirement' for carbohydrate by cysticercoids is needed.

In a review of biochemical adaptations of helminths to their life style, Fairbairn (1970) defined adaptation as goal-directedness of an organism, resulting from natural selection. The adaptations concerned with growth and development of a helminth throughout its life cycle are referred to as epigenetic adaptations, which are particularly evident in helminths. This latter type of adaptation allows biochemical processes to be switched 'on' and 'off' in response to different demands made on the parasite by its life cycle. Helminths also show exploitive adaptation, a genetically determined capacity of organisms to acclimatise to new situations. This is shown by e.g. different enzyme kinetics of the lactate dehydrogenase possessed by the cysticercoid and adult worm (Walkey and Fairbairn,1973), and the presence of 2 isoenzymes of pyruvate kinase in the cysticercoid compared to 5 in the adult (Carter and Fairbairn,1975). A genetically determined capacity to deal with 'new situations' is of great importance to parasites which may have a wide range of intermediate hosts e.g. H. diminuta cysticercoids. It also implies the tailoring of uptake systems to meet the need of the cysticercoid in relation to the presence of substances in the environment. Arme and Coates (1973) suggested that the similarity of amino acid uptake in cysticercoids and adults of H. diminuta was induced by a similarity in the environments of the two life cycle stages. The results of this study suggest the opposite for low molecular weight carbohydrate uptake, the

differences seen reflect differences in the environment and possibly also the requirement for carbohydrate. The cysticercoid of *H. diminuta* has the ability to actively absorb monosaccharide *in vitro*, but also has the genetic flexibility to grow and develop in situations of low available carbohydrate *in vivo*.

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APPENDIX A

MEDIA COMPOSITION

Composition of Krebs-Ringer-Tris saline (KRT)

1 volume of Krebs-Ringer saline (x10)

1 volume of Tris-maleate buffer (x10)

8 volumes of distilled water

Krebs-Ringer saline (x10)

The following compounds dissolved in 800ml of distilled water;

70.20g NaCl

3.59g KCl

3.19g $MgSO_4.7H_2O$

To this is added 5.65g $CaCl_2.6H_2O$

The volume is adjusted to 1000ml with distilled water and stored in the fridge.

1M Tris-maleic acid solution

1 mole of Tris(hydroxymethyl)aminomethane base = 121.1g

1 mole of maleic acid = 116.0g

Add the above together with distilled water to 1000ml.

This can be stored at room temperature.

0.25M Tris-maleate buffer

Add 500ml of 1M Tris-maleic acid solution to 800ml of distilled water. The pH is then adjusted to 7.5 with NaOH solution. Adjust the volume to 2000ml, the pH should then be 7.4. Store in the fridge.

COMPOSITION OF MEDIA USED FOR EXCYSTING CYSTICERCOIDS

Pepsin/HCl

0.1g pepsin in 100ml saline (in this study the saline used was KRT)

Add 0.5ml glacial HCl and store at 37°C. Use within 1 day.

Trypsin/bile

0.25g sodium tauroglycocholate in 20ml of saline

0.1g trypsin in 20ml of saline

mix the above solutions together and bring up to 50ml.

Store at 37°C and use within 1 day.

APPENDIX B

SOME FACTS SURROUNDING THE APPEARANCE OF SURFACE BLEBS IN THE CYSTICERCOID TEGUMENT

Introduction

During the last 2 months of this project, it was noticed that cysticercoids developed surfac blebs on contact with *in vitro* media i.e. upon removal from their hosts, *Tenebrio molitor*. Initially, these blebs were only visible under the binocular microscope (x16), but they were later visible with the naked eye. Due to limitations of time, it was not possible to determine the precise nature of the blebs, their exact cause or their effects on the physiology of the cysticercoid. However, the conditions under which they developed and other factors associated with these blebs were determined and are discussed here.

It is important to note that these surface blebs first appeared after the completion of the experimental work presented in this thesis, but they did prevent replication of some experiments. The surface blebs continued to appear in cysticercoid stocks for several months (c.5 months), but current stocks do not exhibit blebbing or any other unusual feature (C.Arme, pers. comm.).

Appearance of the blebs

Two types of bleb were apparent; large bladder-like blebs visible to the naked eye and smaller 'cytoplasmic' blebs visible under the electron microscope. Both types have recently

been described in detail by Richards and Arme (1985).
The blebs are referred to by these latter authors as occurring on 'surface-stressed' cysticercoids. The smaller blebs typically contained numerous microtubules and although bearing microvilli, the terminal web normally present, was ill-defined. The larger blebs were also bound by the microvillus border and consisted of small amounts of cytoplasm and large vacuoles. Photomicrographs of both blebs are presented in Richards and Arme (1985).

Occurence of the blebs

Initial observations of blebbing were made when cysticercoids were dissected into KRT. Subsequently, a variety of media were tested for their ability to induce blebbing. Table B1 lists the media tested, and shows that all media tested induced blebs despite the fact that most are, at best, capable of maintaining viable cysticercoids for several hours. The composition of the media was subsequently scrutinised and samples of each medium analysed on an osmometer (fisons,ltd,U.K). The results proved that all media were at their expected osmolarity and suggested that the blebs were not the result of 'osmotic shock'. The chemicals used to constitute these media were all AnalaR or Aristar grade and as far as could be determined they were not contaminated. Freshly distilled water was used in all cases.

The variation of these blebs with age of cysticercoid was next investigated, with the result that older cysticercoids (i.e. 23 days) produced more and larger blebs than younger cysticercoids (i.e. 23 days). However, during the 5 month

period in which blebs were observed, cysticercoids of stage 4 (day 7 at 26°C) onwards produced some blebbing.

It was noted that by varying the temperature of the media into which cysticercoids were dissected, the time taken for blebs to appear could be altered. Thus, media at temperatures of 30 and 32°C induced blebs after 5-10 min. Temperatures of 26°C and below produced virtual instant blebbing. Also, cysticercoids transferred from media at 26°C to 32°C did not produce any further blebs and those blebs already present did not increase in size.

The density of infection of *Tenebrio molitor* did not have any effect on the type or degree of blebbing produced by the cysticercoids, although larger cysticercoids generally produced more blebs.

The osmolarity of *T. molitor* haemolymph had already been determined (Hurd and Arme,1984a) as 520mOsm. It was decided to investigate the osmolarity of the haemolymph of current beetle stocks, in order to determine whether a significant alteration in osmolarity of the haemolymph was associated with the appearance of the surface blebs. The osmolarity of 20 non-infected beetle haemolymph was tested on the osmometer and gave an average reading of 518mOsm, with a standard error of 1.6%.

All aspects of the maintenance of beetle colonies were investigated for alterations which may have affected the beetle stocks. Checks were made on the humidity of the 'beetle room' and incubators, and also the quality of the bran fed *ad libitum*, but no unusual features were found.

Changes in the viability of cysticercoids associated with the appearance of surface blebs.

The most obvious physiological alteration of the cysticercoid which also occurred with the appearance of the blebs, was their inability to excyst *in vitro*. Excystation media were made up using a selection of the salines tested previously and experiments were performed both with and without the pepsin/HCl prime (see Chapter 5 for method of excysting cysticercoids). In all cases no more than 5% excysted successfully. A common observation was that of half-excysted adults which managed to extrude their anterior end, but then rapidly decreased in activity and became moribund soon after. The percentage successful excystment was slightly increased when the pepsin/HCl stage was omitted. Also, in most cases, the presumptive adult was clearly active within the cysticercoid (as observed under the light microscope), but did not manage to even break out of the inner capsule.

Discussion

Strong evidence for the exact cause of these surface blebs is lacking, however, the accumulation of observations and other factors associated with these blebs enables us to draw some conclusions concerning their origin and cause.

The evidence suggests that variations in osmolarity of experimental media are not the prime cause. It is, however, true that KRT does vary considerably in osmolarity from *T. molitor* haemolymph. This is not normally a problem,

although it does reflect the inadequacies of current salines for uptake experiments. A more suitable saline for such experiments has been sought for some time and with the recent information concerning *T. molitor* haemolymph composition (Hurd, 1985), the basis for a more appropriate saline is now available. Jeffs (1984) however, in a study on the effects of different salines on *H. diminuta* cysticercoids, concluded that KRT caused the least visible disruption to the cysticercoid tegument over a period of time. Thus it appears that the cysticercoids which produce blebs are indeed 'surface-stressed' and this becomes obvious when they are dissected into saline which for 'normal' cysticercoids produces no such effect. As an addition to these data, the osmolarity of *T. molitor* haemolymph was also shown to be 'normal'.

It was shown that age of cysticercoid affected the appearance of blebs, so that after c.23 days considerably more blebs were produced. In normal cysticercoids the integrity of the tegument is maintained for at least 40 days as illustrated by uptake studies on 40-day-old cysticercoids. However, 'surface-stressed' individuals appear to lose their tegumental integrity (and therefore increase their susceptibilty to blebbing) much earlier.

The effect of temperature on blebbing can be explained in terms of permeability of the plasma membrane. Therefore, if the larger blebs are a result of influx of fluid, this is dependent upon the permeability of the membrane which can be altered by the temperature of the media.

The suggested cause of blebbing put forward by Richards and Arme (1985) has, however, the most credance. They suggested that the localised imperfections in the tegument resulted in the blebs evident in *in vitro* media, and that this surface imperfection could have resulted from defective tegumentary cytons. Thus, one or more adjacent defective tegumentary cytons could produce a patch of different tegument which provides the 'first tier' recognition resulting in the initiation of a host cellular response. This explains the proximity of haemocytes noted by these authors to 'defective' tegument. These authors also noted phagocytosis of microvilli but siggested it to be the microvilli shed in tegument turnover, not the phagocytosis of living microvilli. Membrane turnover in adult *H. diminuta* has been demonstrated (e.g. Oaks and Lumsden,1971), but little work has been done on the cysticercoid. However, if membrane turnover occurs in the cysticerocid then microvilli will be shed into the surrounding media.

Perhaps the most striking effect of this breakdown of tegumental integrity is its effect on the excystment of cysticercoids *in vitro*. The most likely explanation is that the pepsin/HCl and bile/trypsin solutions reach the presumptive scolex quicker and therefore begin to digest the presumptive scolex before it has excysted. Personal observations have shown that recently excysted worms do not have the ability to prevent themselves from being digested by these enzymes. If they are transferred to saline they remain active for at least 6 h. The ability of adult *H. diminuta* to inactivate some digestive enzymes has been demonstrated (Schroeder,

Pappas and Means,1981; Uglem and Just,1983). However, the artificially high concentrations of these enzymes in *in vitro* media combined with surface-impaired cysticercoids could explain their inability to excyst. It would have been of interest to investigate the viability of these cysticercoids *in vivo*.

Therefore it is suggested that the cause of the blebs and the cysticercoids inability to excyst are directly and indirectly the result of a defective surface, which in turn is produced by defective tegumentary cytons. The cause of this defection could be genetic mutation, possibly restricted to one adult worm. Since the appearance of these blebs, new larger groups of infected stock rats have been established which may have eliminated this problem.

TABLE B1

MEDIA IN WHICH *Hymenolepis diminuta* CYSTICERCOIDS PRODUCED BLEBS

- KRT
- KRP (phosphate-buffered Krebs-Ringer)
- Phosphate buffered saline
- Locke's solution
- Hank's
- Hoyle's
- Insect Ringer
- 0.9% NaCl
- Distilled water