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A COMPARISON OF TRANS-TEGUMENTAL NUTRIENT UPTAKE AND IONIC REGULATION IN GUT (Ascaris suum) VERSES TISSUE (Dirofilaria immitis) PARASITIC NEMATODES

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Steven Alan Sedrish

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by

STEVEN ALAN SEDRISH

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ABSTRACT

A COMPARISON OF TRANS-TEGUMENTAL NUTRIENT UPTAKE AND IONIC REGULATION IN GUT (*Ascaris suum*) VERSES TISSUE (*Dirofilaria immitis*) PARASITIC NEMATODES

By

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Parasitic nematodes are a very diverse group of organisms that infect nearly every animal known. Despite their diversity very little is known about the mechanisms of nutrient uptake and ionic regulation in these parasites. The tegument has been proposed to play a significant role in these processes however a means of measuring and comparing this quantitatively has not been developed.

We designed a system in which segments of these nematodes were cannulated and perfused in media containing various radioactive compounds. The perfusate was then analyzed to determine the extent to which radioactive compounds could penetrate through the isolated segments.

Using this system we were able to measure mediated transport processes for many nutrients in *Dirofilaria immitis*. These mediated transporters could not be found in *Ascaris suum*. We were also able to measure a significant difference in the movement of ions into these two parasitic nematodes.

To my family for their constant encouragement and for always being there when needed.

I would like to thank my advisor, Dr. James Bennett for all his help. His great ability as an advisor shows in his guidance and patients with graduate students.

I would like to thank Dr Ralph Pax for all his help with the design of the experiments and interpretation of the data. Also I would like to thank the other people in the lab for all their help.

Finally I would like to thank my family for all the encouragement, and a special thanks to my grade school teacher Ms "mickey" lineal for her help and expertise.

Thanks

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List of Abbreviations

A.P.F.....Artificial perienteric fluid

A. suum.....*Ascaris suum*

A. viteae.....*Acanthocheilonema viteae*

B. pahangi.....*Brugia pahangi*

D. immitis.....*Dirofilaria immitis*

I_{RPMI}.....Inorganic $RPMI_{1640}$

$RPMI_{1640}$.....A specific culture media sold by GIBCO

Life Cycles

Dirofilaria immitis, more commonly referred to as heartworm, is an obligate two host tissue dwelling parasitic nematode. The definitive hosts are the domestic dog and cat however foxes, coyotes and other wild canids as well as wild cats serve as a reservoir host for this parasite. The intermediate host is the mosquito and at least fourteen species of mosquitos are proven to be effective vectors for this disease.

Patently infected dogs have circulating microfilariae in the blood. These microfilariae show an interesting phenomenon called periodicity meaning that the microfilaria are present in the blood in higher concentrations (up to fifty times) when the mosquitos are actively feeding. This rhythmic cycle of microfilariae presence in the blood varies depending on the mosquito host as to whether it is a nocturnal or a diurnal rhythm.

The microfilariae are picked up by the mosquito host during a blood meal. While the mosquito is actively feeding the microfilariae are attracted to the bite wound by a positive chemotaxis, thus leading to a higher ingestion of microfilariae than would be expected by the actual concentration of microfilariae in the blood at the time of

the bite. The microfilariae which are actually L_1 larvae then pass with the blood meal into the midgut of the mosquito.

The L_1 larvae then burrows their way (within 24 to 36 hours) through the midgut into the malpighian tubules where they then go through their first ecdysis (or molt) to the L_2 larvae. This larval stage then migrates to the abdominal hemocoel cavity where the second ecdysis occurs to the L_3 larvae. This L_3 or infective stage (for the definitive host) then migrates through the thorax and into the labium. This entire process at optimal conditions takes between ten and fourteen days.

The third stage L_3 larvae is than deposited onto the skin of the definitive host during a blood meal. The L_3 larvae then migrates through the bite wound or burrows through the intact skin to reach the subcutaneous tissue where the larvae undergoes a third ecdysis to the L_4 larvae (approximately three to four days post-infection). This L_4 than begins a subcutaneous migration towards the thorax and undergoes a fourth and final ecdysis to the L_5 larvae or juvenile heartworm (50-70 days post-infection). Then between day 70 and 110 post-infection the juvenile heartworm migrates to the pulmonary arteries, although the exact route is not known, it is thought to be through the systemic circulation. Once in the pulmonary arteries and right ventricle the juveniles then matures into adult heartworms, the entire process taking

between two and four months. The female heartworm does not become fully developed and gravid until about six months post infection when they begin to shed microfilaria.

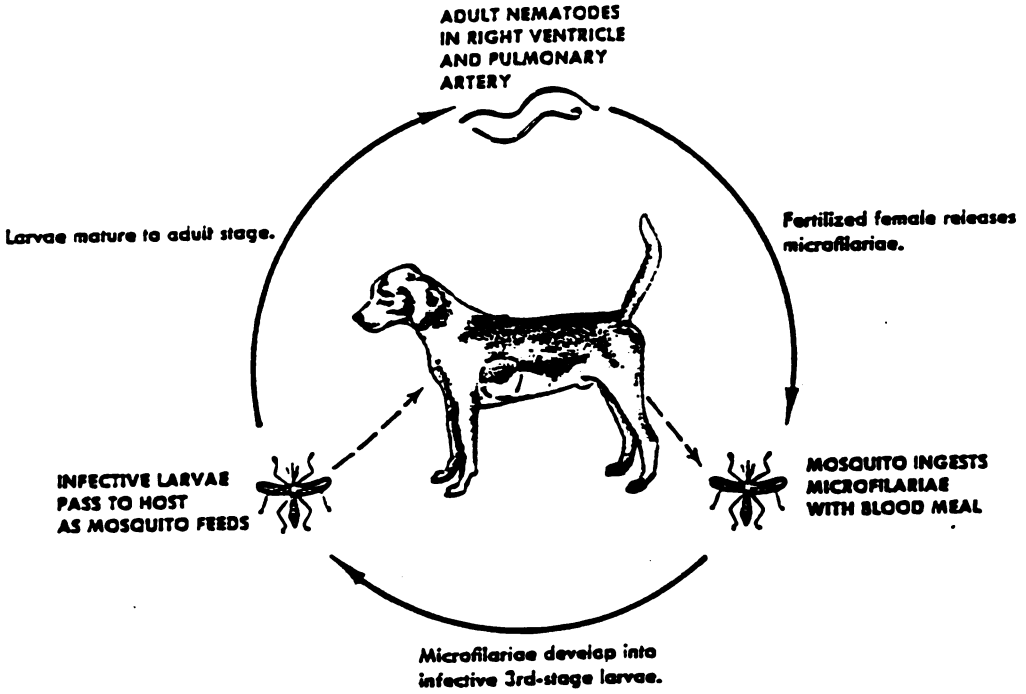


Figure 1: Life cycle of *D. immitis* (Ivene, V.R. 1981)

Ascaris suum is a one host gut dwelling parasitic nematode. The life cycle is direct with the domestic pig as its host. Although there are similarities between the porcine ascarid (*A. suum*) and the human ascarid (*A. lumbricoides*) and each are capable of growing to full maturity in the other's host, they are still considered to be separate parasites.

Adult ascarides live in the small intestine of their host where they are capable of laying huge numbers of eggs per day (up to 200,000). The eggs are passed out with the feces as fertilized but un-embryonated eggs. Once in the soil they embryonate and become infective second stage L_2 larvae. This process takes approximately two weeks to occur.

The L_2 larvae are then swallowed by the host and hatching takes place in the stomach and small intestine, stimulated by temperature, Pco_2, pH, bile salts, and the presence of a non-specific reducing environment. The L_2 larvae then penetrates the lumen of the small intestine and is picked up by the portal circulation and brought to the liver. Once in the liver they migrate throughout the liver causing their characteristic pathology of hepatic scarring.

The larvae then travels through the vena cava to the heart and then through the pulmonary artery to the lungs. Once in the lungs the L_2 larvae molts to become the L_3 larvae and either becomes lodged in the alveolus or travels to the somatic tissues. The larvae that are lodged in the alveolus

eventually breaks loose from the alveolar capillaries into the alveolar space where it actively migrates up the bronchi into the trachea and is swallowed.

The L_3 larvae then goes through two more ecdyses in the small intestine to become the L_5 larvae or immature ascarid. This immature ascarid will then mature to become a mature ascarid, the whole process taking approximately six weeks from ingestion of the infective egg to the passing fertilized eggs in the feces.

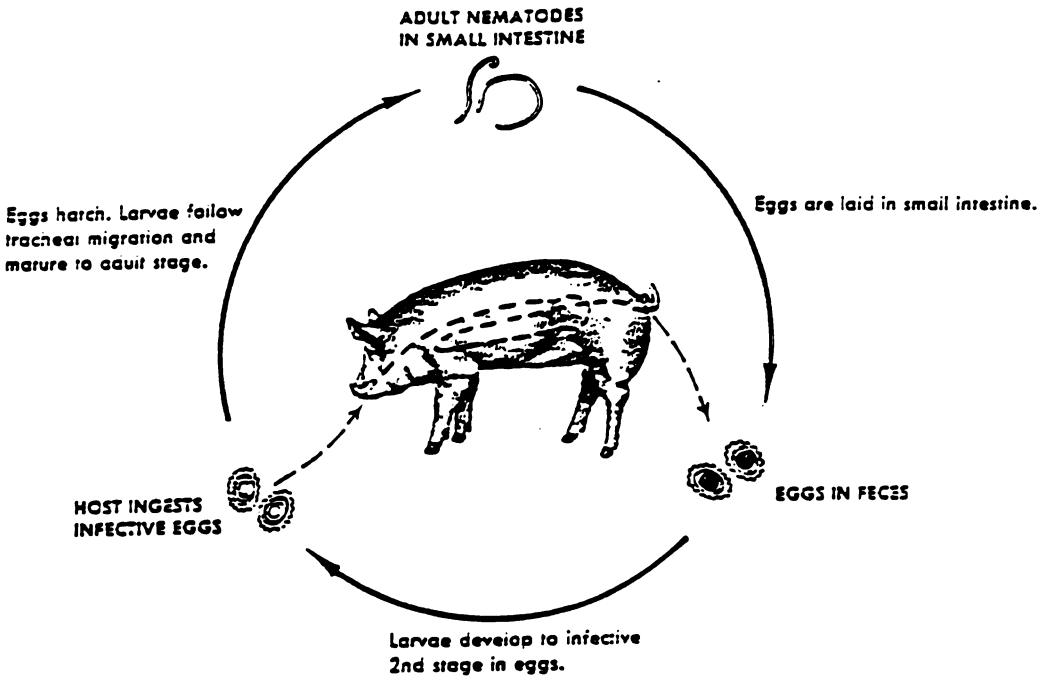


Figure 2: The life cycle of *Ascaris suum*. (Ivene, V.R.1981)

Anatomy of the Nematode's Tegument

The phylum Nematoda is a very diverse group of animals, consisting of many thousands of distinct organisms. As a result of this diversity it is impossible to talk about the anatomy of all nematodes accurately, but rather what follows is a general description of the non-existent "typical" nematode tegument.

The nematode tegument can be divided into three main structural components or layers, the cuticle, the hypodermis or epidermis and the muscle. Each of these layers can be divided into separate layers and in fact each of these divisions can be divided structurally and functionally into further divisions. However for this discussion we do not need to and will not go into as much detail as is possible.

The outermost layer of the tegument is the nematode's cuticle. The cuticle is divided into three main layers that are both structurally and presumably functionally distinct. The outer most layer or cortical layer, a medial layer or matrix layer, and an inner layer adjacent to the hypodermis, the basal layer.

The cortical layer needs to be divided further into an outer and an inner cortical layer. The outer cortical layer is considered to be a trilaminate plasma membrane (plasmalemma) of approximately 100 Å in thickness. The outer

layer also contains, as viewed on an electron micrograph a "fuzzy" coating. This coating is thought to be the antigenic component of the tegument. *Ascaris suum* emits the most antigenic protein known to man.

This layer also contains lipids, proteins and carbohydrates. It is formed directly from the cytomembrane of the endoplasmic reticulum.

The internal cortical layer consist mainly of a collagen like protein. This layer is also rich in enzymes, RNA, ascorbic acid, ATP, and acid phosphatases and is presumably metabolically active. This layer is formed from differentiated hypodermal cytoplasm.

The matrix or middle region of the cuticle is a homogenous layer that varies greatly between nematodes in the degree of organization. This layer is also rich in collagen like protein forming rods and canals (at 75° to the long axis of the nematode). To various degree's these rods and cones form the beginning of a lattice network.

The basal layer consists mainly of the same collagen like proteins forming a network of rods and canals. The first layer runs approximately 135° to the rods in the matrix and together with subsequent layers form a strong lattice that gives the nematode shape and allows for the build up of a high internal hydrostatic pressure without the nematode actually exploding

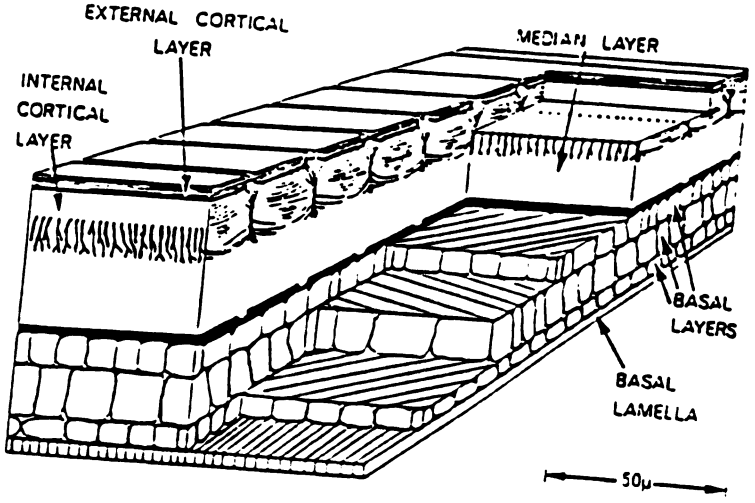


Figure 3: Diagram of a typical Nematode's cuticle (Bird, 1971)

The next main layer, just below the lamella of the cuticle, is the hypodermis (or sometimes called the epidermis). The hypodermis is a cellular or syncytial layer. The main function of the hypodermis is thought to be the secretion of the cuticle.

The most notable characteristic of the hypodermis is that the protoplasm of these layers expands into the pseudocoel cavity. These protrusions form four "cords" containing all the nuclei of the hypodermal layer. Once the adult nematode is formed, after the last ecdysis the number of nuclei remains constant.

The cords are divided into two midlateral, one midventral, and one middorsal cord and extend the entire length of the nematode. The middorsal and midventral cords contains the dorsal and ventral nerve trunks, respectively. The two lateral cords contain the lateral canals of the excretory system

The hypodermal layer is very metabolically active and contain lipids, mitochondria, golgi apparatus, and enzymes. The four cords are especially rich in mitochondria and endoplasmic reticulum.

The third main layer of the nematode's tegument is the muscle layer. The muscle layer consists of longitudinally obliquely striated fibers arranged in bands. The muscle acts

to propel the nematode by contraction against the fluid filled pseudocoel and thus produces a hydrostatic pressure.

The muscle consists of two main types of fibers. Thick fibers (23nm diameter) and thin fibers (5nm diameter). The thick fibers contain myosin while the thin fibers contain actin and contract in a similar fashion as vertebrate striated muscle through actin/myosin interactions.

The base of the muscle fiber, containing the contractile fibers, are against the hypodermal layer. The side of the muscle fiber with the nucleus is towards the pseudocoel. In addition each muscle fiber has a long slender arm like projection. This projection extends to the dorsal or ventral nerve trunk. This is different than the normal situation where the nerve projects to the muscle.

The pseudocoel or sometimes called pseudocoelom although not a part of the tegument is worth mentioning at this point. The pseudocoel is derived from the embryonic blastocoel rather than from an invagination into the endomesoderm. Therefore the pseudocoel does not have a mesoderm lining. The function of the pseudocoel is as a hydrostatic skeleton.

The pseudocoel is filled with a cell free liquid. Any nutrients that are absorbed are presumably transported to this fluid. This liquid contains electrolytes, proteins, albumin, globulins, hemoglobins, enzymes, fats, and

carbohydrates. The most noticeable fact concerning this fluid is that it contains a lower concentration of Cl^- then would be expected from the concentration of positive cations. This electrostatic difference is made up by anionic organic acids. This low Cl^- concentration will be discussed again in this paper in more detail and might be a very important fact.

Another fact about this fluid is that it can maintain a high hydrostatic pressure against the body wall of the parasite. This pressure has been reported to be between 70-120 mm Hg in Ascaris suum.

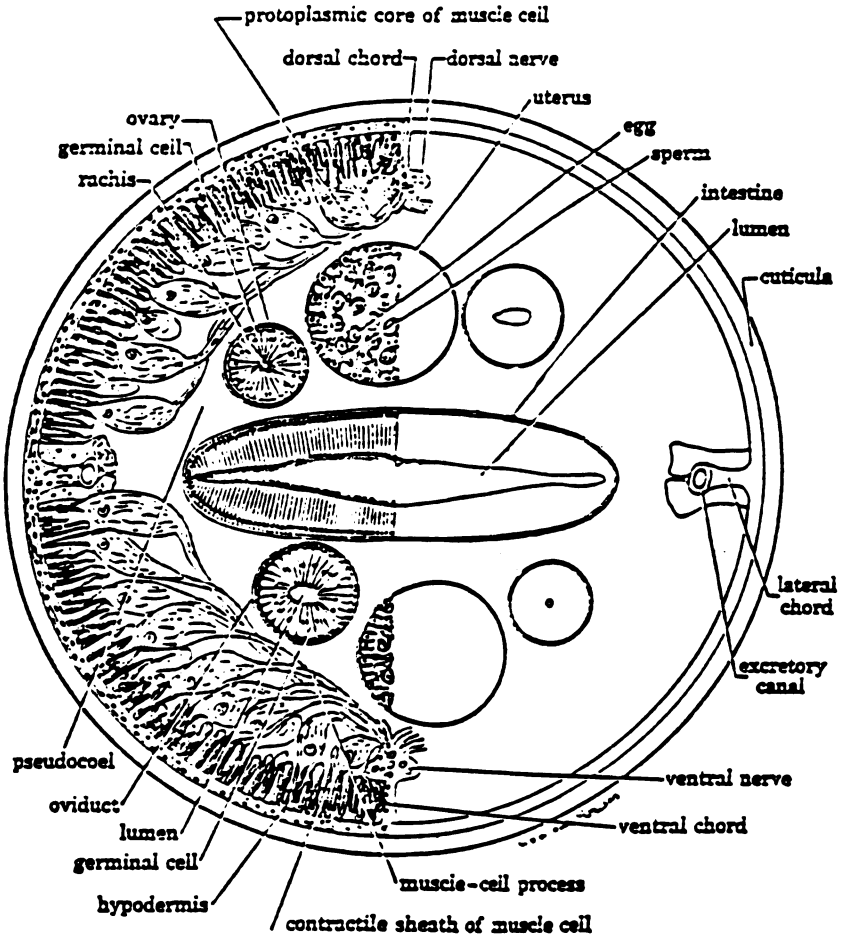
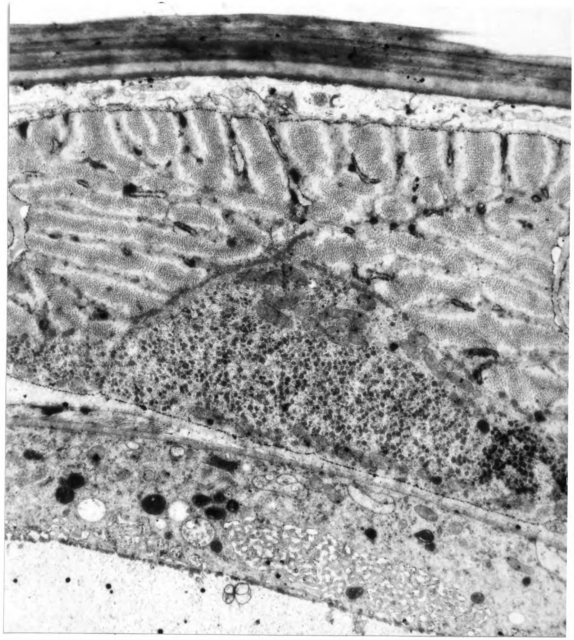


Figure 4: Diagram of a cross section through a nematode in the region of the female gonads. (Noble, E.R. 1974)

Figure 5: Electron micrograph of a cross section through the tegument of an *ascaris*, (Aggarwal, S. Professor of Zoology, Michigan State University).



Introduction

Parasitic nematodes are a very diverse group of organisms. They parasitize almost every vertebrate and invertebrate organism known. The two nematodes, *Dirofilaria immitis* and *Ascaris suum*, used in these experiments are of great economical importance in today's society.

Dirofilaria immitis has been estimated to infect anywhere from 0 to 75% of the canine population in any one place, depending on the climatic conditions. In Ingham County, Michigan 7.5% of the county seizures (dogs picked up by the county animal control agents) were tested positive for circulating microfilaria (approximately 50 dogs were tested). It is also estimated that an additional 30% more would test positive for occult infections (meaning that they are infected but the female is not shedding microfilaria).

Ascaris suum is also a very important disease in today's society. It has been estimated that between 20 and 100% of the hogs in the US are infected at any one time. The American pork producers raise 700 tons of Ascaris eggs and 5,000 tons of adult Ascaris yearly (Krull, 1969). These parasites are raised accidentally by consuming feed that would normally be utilized by the porcine host.

Despite the diversity and importance of these parasites little is known concerning the method of nutrient uptake. The

first thing that becomes apparent when one starts to review the literature in this field is that for every nematode there appears to be separate mechanisms involved. This fact has lead to a great deal of confusion in the past since no one generalized rule is available by which all these parasites can be described.

As little as fifteen years ago the tegument of the nematode was thought to be only an outer covering giving shape and integrity to the parasite. In a review by Pappas in 1975, on nutrient uptake in all parasites, the cuticle of nematodes was not even mentioned. In that particular review *Ascaris suum* was used as an example of a typical nematode and all that was talked about was absorption through the gut. As a result of these early reports very little research was performed until recently.

Most of the literature looking at trans-tegumental nutrient absorption in *Ascaris suum* used cuticle preparations that were "stripped" of the muscle (Fetterer, 1986). In these preparations the teguments were normally inverted and then stripped of muscle either physically or with some enzymatic digestion. Although these preparations remained presumably intact by the fact that inulin remained impermeable, it seems unlikely that the tegument would have behaved normally in such a state. These experiments all showed results which

suggested that the tegument of the Ascaris remained impermeable to macromolecules.

Flemming in 1984 conducted a series of experiments using whole intact *Ascaris suum* in which he cannulated at either end and was able to perfuse the pseudocoel cavity. He reported that a significant amount of glucose and 3-O-methyl glucose was able to enter the parasite by a trans-tegumental route. He postulated that possibly the reports of this barrier being impermeable might be incorrect. However when one actually looks at the quantity of glucose entering the parasite and compares it to how much enters through the gut this quantity is insignificant. Also he did not report any data suggesting any process other than simple diffusion occurring.

Many studies have been performed on the gut of *Ascaris suum* (Castro, 1969; Beames, 1971) describing the gut as the main site of nutrient uptake. These reports have demonstrated specific and saturable processes for absorption, not unlike the gut of higher organisms.

Rutherford in two papers, (1974, and 1977) using the nematode *Mermis nigrescens* reported saturable transport mechanisms in the tegument for glucose and certain amino acids. In these experiments it was shown that the glucose transport was saturable and concentration dependent but not energy dependent or coupled to a sodium transporter. This

uptake was shown to be sensitive to 10^{-5}M phloretin and 10^{-4}M dinitrophenol. In the same report he showed the absence of a transporter for trehalose, the major sugar moiety in this parasite's natural host.

His reports stated that this transport mechanism occurred only in the 14 day old larval stage and not in the thicker cuticled 21 day old larvae. This suggested that this mechanism exists on the hypodermal side of the cuticle.

The transport of molecules across the cuticle was in the past described strictly on the basis of size or partition coefficients (Fetterer, 1986).

Many reports of filarial nematodes state that their tegument is permeable. Once again however, no one rule exists for all filariids. Court, in 1983, looked at transport of 3'5'-cyclic adenylate monophosphate (cAMP) and isoproterenol into *Litomosiodes carinii* and *Acanthocheilonema viteae*. This paper stated that both parasites took up cAMP but only A. viteae took up isoproterenol.

Brugia pahangi however was shown to have a correlation of .99 with *A. viteae* when looking at the uptake of several non-electrolytes (Court, 1988).Several experiments were performed on *B. pahangi* and *D. immitis*, by Chen and Howell in 1980, 1981, and 1983. These experiments although very simple in their

design have had a great impact on the thinking on trans-tegumental uptake.

These experiments consisted of incubating parasites in petri dishes, where the parasite's anterior, posterior, and middle regions could be exposed to different environments. Two facts came from these experiments. First, by incubating the anterior and not the posterior region of the parasite with radioactive tracers, they were not able to detect any radioactivity in the posterior region of the parasite. This they demonstrated for both *D. immitis* and A. viteae and postulated that no movement through the gut, in an anterior to posterior direction, occurred.

This was a very interesting observation but the conclusion, although now considered to be true by this author, was inconclusive. This is because *in vitro* they showed no oral absorption of trypan blue dye, which does occur *in vivo*. This signifies that their system was not perfect and needed to be adjusted until concurrent information could be obtained from *in vitro* and *in vivo* data.

The second conclusion from these experiments is that they showed specific trans-tegumental transporters for several macromolecules. Included among them was glucose, adenosine, and several amino acids. Glucose uptake into *D. immitis* was found to be stereospecific in that l-glucose was

not taken up. Also in *D. immitis* the nucleic acid precursor thymidine and the disaccharide sucrose was not shown to be taken up. In *B. pahangi* glycine was shown to be competitive with methionine, valine, and phenylalanine; and to be noncompetitive with arginine.

It appears that *D. immitis*, a filarial parasite, shows the most promise for the measurement of trans-tegumental uptake mechanisms. It also appears that *A. suum*, the classically used nematode, has the most impermeable tegument.

Media

Several media are utilized in my work with regard to both incubations and the perfusion of parasites. To aid in the understanding of this paper the different media will be described. However for the specific media used in each individual experiment refer to the experimental procedure section.

Dirofilaria immitis has in the past been difficult to keep viable in culture. For this reason several variations on the culture media for heartworm (Heartworm media) were tried until a satisfactory media was found. The heartworm media used to keep heartworms alive in culture contained the following $RPMI_{1640}$ with 5% Calf supreme (a fetal calf serum replacement manufactured by Gibco Company) also this media contained 50 mg/L gentamicin sulfate and 2.5 mg/L amphotericin B. This media was adjusted to a pH of 7.4, with the use of 20mM hepes buffer.

The $RPMI_{1640}$ contains 10mg/L phenol red indicator. This indicator at a pH of 7.4 is a bright red color, while in a more acidic solution it turns yellow. This color change allowed us to know when the media needed to be changed due to the acidification of the media by the parasite. The media was changed approximately every three days.

The best culture media for *Ascaris suum* is still a matter of some debate. There are two main media used for this

purpose, Donahue's media and A.P.F. (see table No. 1). The main differences in these media are that both contain Hepes as a buffer but Donahue's media also contain 10 mM $HCO_3^=$ as an additional buffering system. The other main difference is that A.P.F. contains 128 mM acetate, this acetate is added under the belief that *Ascaris suum* are capable of utilizing this acetate for energy. Other differences, although slight are shown in table 1. In all experiments, unless specifically stated differently Donahue's was used as the culture media for *Ascaris*.

One other media used was inorganic RPMI (I_{RPMI}). I_{RPMI} is a solution to simulate the inorganic ions in organic RPMI ($RPMI_{1640}$) without most of the organic added. However I_{RPMI} does contain 11.1mM glucose (except in the experiment looking at dose response curves for glucose uptake) and 20 mM Hepes as a buffering system.

Table 1: Shown here in millimolar concentrations are the inorganic and organic constituents of the three commonly used medias. Also shown is the pH's of the medias

	I_{RPMI}	A.P.F.	Donahue's
Inorganic			
Na^{+}	102.7	131.9	121.0
K^{+}	5.36	24.0	24.0
Ca^{++}	0.44	11.8	1.0
Mg^{++}	0.41	9.8	5.0
Cl^{-}	107.36	71.1	151.0
$NO_3^{=}$	0.88	-	-
PO_4^{-3}	5.64	-	0.5
$SO_4^{=}$	0.41	-	5.0
$HCO_3^{=}$	-	-	10.00
NH_4^{-4}	-	-	0.75
Organic			
Acetate	-	128.0	-
Glucose	11.0	10.0	27.0
Hepes	20.0	5.0	5.0
pH			
pH	7.4	7.0	7.0

Collection and Maintenance of Specimens

Dirofilaria immitis were collected from either random source, naturally occurring microfilaria positive dogs obtained from the local pound or from an NIAID supply contract [1]. In the former case the dogs were tested for the presence of microfilariae by filter occlusion testing of venous return blood obtained from the cephalic vein. If the dogs tested positive for microfilariae the dogs were euthanized by CO_2 asphyxiation in an approximately 90% CO_2 atmosphere (1986 Report of the AVMA Panel on Euthanasia). The heart and lungs were quickly removed and the parasites (heartworms) dissected out. The parasites were then put into a solution of sterile RPMI 1640 with antibiotics (see media section) and transported to the laboratory.

The parasites from the NIAID supply contract were obtained in a similar fashion and shipped in RPMI $_{1640}$ by overnight express to the lab. Once at the lab these parasites were treated in the same manor as the parasites obtained from random source animals.

Prior to placement into incubation flasks, the parasites were rinsed with a sterile saline solution and then placed

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1 Filarial materials used in these experiments were donated by an NIAID supply contact (AI #02642), US-Japan cooperative medical program.

into sterile heartworm media (see media section). The media was adjusted prior to the addition of worms to a pH of 7.4. The media was kept at room temperature until several hours before the experiment when they were put into a 37° incubator. The worms were rinsed with sterile saline and put into fresh media approximately every three days (or as needed) as indicated by the acidification of the media by the parasites.

The heartworms were able to survive in this media for up to one month. The length of the incubation did not affect the experiments in which we were looking at organic transport, however, inorganic permeability did increase significantly after one month of incubation. Therefore all experiments on inorganic transport were done within a week of euthanizing the animal.

Ascaris suum were both obtained from the local abattoir or from lab raised and infected hogs (porcine). In both cases the animals were sacrificed by electrical stunning followed by exsanguination. The parasites (ascarides) were quickly removed from the small intestines and placed into Donahue's media (except in those experiments where a different media, A.P.F., is specifically stated). The parasites were maintained at a constant temperature of 37°C and transported as quickly as possible back to the laboratory. Once at the lab the parasites were rinsed with and placed into fresh

media. The media was maintained at a temperature of 37°C and a pH of 7.0 and changed daily. The parasites were able to be kept in the media for up to one month but were used in all cases within one week.

Materials and Methods

The radiolabeled chemicals were purchased from the following sources. The ^{14}C Leucine (337.1 mCi/mmole), Arginine (270 mC/mmole), Asparagine (129 mCi/mmole), Glutamine (250 mCi/mmole), and glucose (220 mCi/mmole) was purchased from ICN radiochemicals. The ^{14}C tryptophan (119 mCi/mmole) and Acetate (94 mCi/mmole) was purchased from NEN research products. The radioactive ions $^{22}Na^{+}$ (49.65 Ci/mmole, adjusted for decay), $^{36}Cl^{-}$ (1062 Ci/mmole), and $^{45}Ca^{++}$ (3.90 Ci/mmole) as well as the ^{3}H H_2O also came from NEN research products. The ^{3}H (11.1 Ci/mmole) labeled Ivermectin was a gift from the Upjohn company.

The $RPMI_{1640}$ and the Calf Supreme was purchased from GIBCO Co. All other chemicals were purchased from Sigma chemicals.

In the case of *Dirofilaria immitis* the parasite is allowed to adjust to 37°C for several hours before the start of the experiment. After this equilibration time the parasite is then placed into a petri dish containing I_{RPMI} also at 37°C.

Polyethylene tubing (PE-50) is heated over a soldering iron and pulled out to a fine tip. This allows the tubing to be inserted into the parasite's body cavity.

The heartworm is then cut into a segment of length between .5 and 1.5 cm's. When the parasite is cut the internal organs are expelled due to the internal turgid

pressure. This expulsion can be used to judge the viability of the parasite and if it did not occur the parasite was not used for an experiment.

Once the heartworm is cut into a segment the uteri and gut is removed by gently pulling it through the body cavity with tweezers. Special attention is paid so as to not damage the body wall. Both sides of the segment were then cannulated with the PE-50 tubing prepared with a fine tip. Both ends are then sealed with a cyanoacrylate glue (Figure 6).

In preparing the segment there is a contraction of the body musculature which will again relax when the segment is perfused. To standardize the measurement of the length of segment the length was measured after cannulation and before any perfusion was performed. Once an accurate length has been taken the segment is tested by pushing A.P.F. through it with a syringe. If any leaks are detected the segment is discarded.

For *Ascaris suum* two separate methods of cannulation are used, one for normal segment preparation and a separate method for an inside out segment. In both cases the parasite is placed into a petri dish containing I_{RPMI} or Donohue's media depending on the experiment. The ascarid is then cut using a razor blade and again there is an expulsion of the internal organs that can be used as an estimation of the

viability of the parasite. The intestines and the uterus are then removed similarly to the heartworm.

In the outside out (Fig 6) preparation a length of PE-90 tubing is prepared with a 1 cm length of silastic tubing placed around the tip. The end of the PE-90 tubing with the silastic tubing tip is inserted into the ends of the parasite approximately ½ cm. Both ends are then tied closed with 4-O silk sutures and sealed with cyanoacrylate glue (Figure 6).

In the inside out ascarid preparation one piece of PE-90 tubing is inserted completely through the entire length of the body of the cut and cleaned segment. The far end is tied off with a suture and the body wall of the parasite is feed back over itself. The second piece of tubing is then inserted and tied off. Then both ends of the segment are sealed with cyanoacrylate glue (Figure 6).

Figure 6. This figure shows the various cannulation techniques used. A: For a heartworm. B: For an outside-out ascarid. C: For an inside-out ascarid.

Once the segment is cannulated, the integrity of the seal is tested by perfusing solution through the segment while it is still out of the bath and visually checking for leaks. The integrity of the seal is also tested when the perfusion starts since there is air in the system the appearance of bubbles in the bath signifies a "bad" seal.

One end of the segment is connected to a perfusion pump that is set at a pumping rate of ½ml per minute. The other end of the segment is connected to a fraction collector set to sample at thirty second intervals.

The segment itself is placed into a petri dish containing 15 ml media. The media is either I_{RPMI}, A.P.F., or Donohue's media depending on the individual experiment. The petri dish is placed into a water bath were the temperature is kept constant at 37°C, unless otherwise specifically stated.

Radioactive tracer is then added to the 15ml bath. When working with ^{14}C or ^{3}H 4μCi of radioactive tracer is added. When working with other radioactive isotopes less tracer is used.

The perfusion is started and as stated before the air in the line provides a indicator if the segment is intact. The perfusion is allowed to run for ten to fifteen minutes (unless stated) until the fraction collector is turned on. The

perfusion is then carried out for the time specified in the experiment.

The ½ml fraction is then added to 6-8ml aqueous counting cocktail. The entire amount is then counted for radioactivity with a Beckman scintillation counter.

Once the number of DPM's are known, the actual amount of radioactive solute going across the tegument can be calculated from the specific activity of the solute. Then from the ratio of radioactive to known radioactive solute, the amount of total solute can be calculated.

Results

Our first consideration was, to determine if the process of cannulation damaged the parasite's tegument was damaged. To look at this, a large molecule that is considered to be impermeable, in intact membranes, was used. 4μCi of ^{14}C labeled inulin (mw > 2000) was added to 15ml of I_{RPMI} and both *Ascaris suum* and *Dirofilaria immitis* were perfused with APF at 37°C. As can be seen in figure 7 the membranes of both cannulated preparations were impermeable to inulin. From this result it can be assumed that the tegument of these preparations were not grossly damaged.

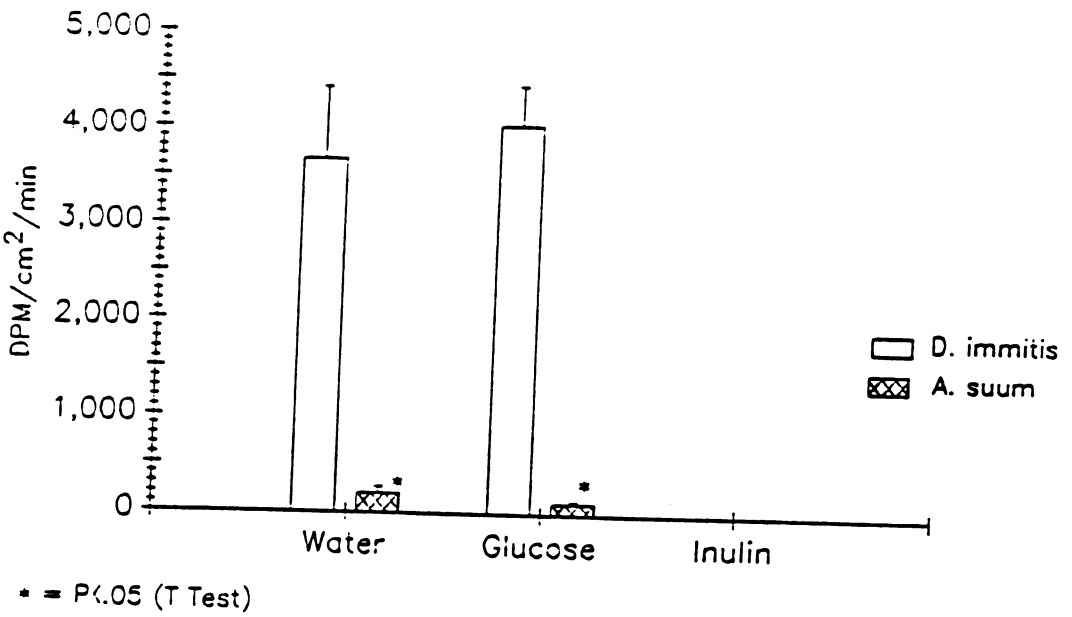


Figure 7: Comparison of the trans-tegumental permeability to water, inulin, and glucose.

The next experiment was to look at the diffusional properties of these segments. 4μCi ^{3}H H_2O was added to 15 ml I_{RPMI} and perfused with A.P.F. at 37°C. In a similar experiment 4μCi ^{14}C Glucose was added instead of the ^{3}H water. The results of both sets of experiments can be seen in figure 7. From this figure it can be observed that the ascaris tegument represents a much greater barrier to both water and glucose than does the heartworm tegument.

The heartworm tegument was approximately 18 times more permeable to water and 34 times more permeable to glucose than was the ascaris tegument (Table 2). The fact that the ratio was not constant indicates the possibility of a transport mechanism for glucose in the heartworm tegument at 37°C. If glucose was being moved across the tegument by simple diffusion you would expect that the glucose would follow along with the water and the ratio would have remained constant.

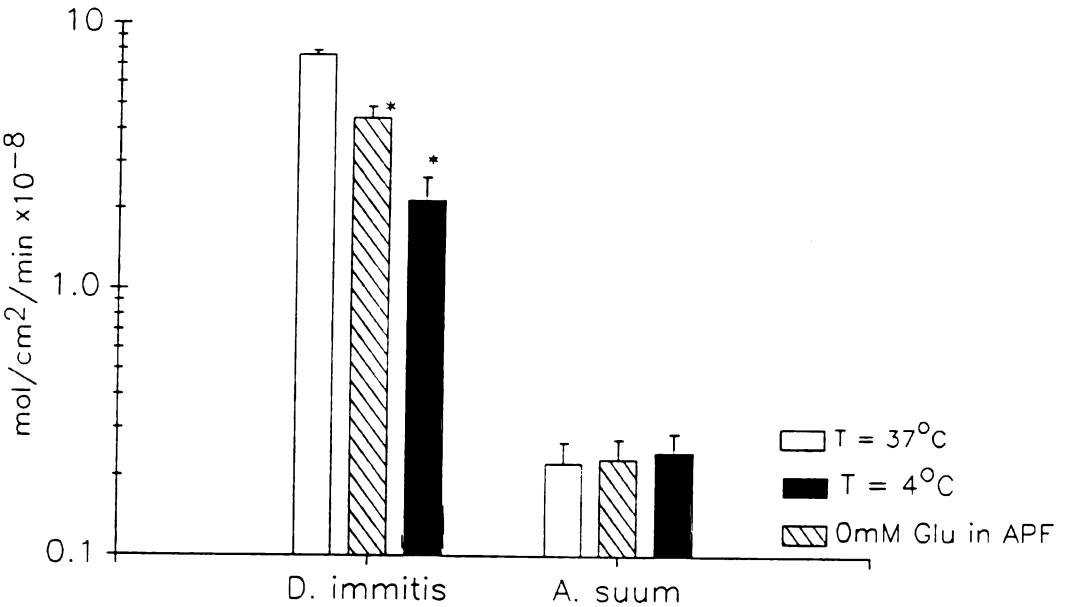
Although in figure 7 the DPM's for water and glucose movement into heartworm and ascaris segments was approximately the same as that for water, the actual movement of solute (both radioactive and non-radioactive) was drastically different when calculated, because the ratio of unlabelled (cold) to labelled (hot) was much greater for

water than glucose. From this it can be assumed that the heartworm is permeable to water but is less, to be shown later, selectively permeable to other molecules.

	Water	Glucose
D. immitis	$3.45x10^{-1} \pm 0.70x10^{-1}$	$7.58x10^{-8} \pm 0.77x10^{-8}$
A. suum	$0.19x10^{-1} \pm 0.06x10^{-1}$	$0.22x10^{-8} \pm 0.04x10^{-8}$
Ratio	18.2	34.0

Table 2: The permeability to water and glucose expressed in mM ± SE concentrations.

To decide if the uptake of glucose was a transport dependent process the same experiment was conducted with glucose both at 37°C and 4°C. Also in the same experiment the effect of removing the glucose from the APF, which is normally in 10mM concentration, was performed. Removing the glucose from APF made the inside of the segment devoid of glucose. The experiment was run with and without 10mM glucose in APF at 37°C and with 10mM glucose in the APF at 4°C. If the process was controlled by a transport mechanism there should be a significant decrease in uptake at 4°C.



* = P<.05 (Paired t Test)

Figure 8: Uptake of glucose under various conditions.

Shown in figure 8 are the results from the experiment. There was a significant decrease in glucose when the glucose was removed from the inside of the preparation in the heartworm, while in ascaris there was no effect. Likewise there was even a further decrease in glucose uptake in heartworm, both significant from control and from the 0 mM glucose when the temperature was decreased to 4°C. This decrease again did not occur in ascaris.

Several known organic and inorganic poisons were examined for their ability to inhibit the transport of glucose into the heartworm segment. Among these were 10^{-4}M Ouabain, 10^{-5}M Thio-D-glucose, 10^{-3} KCN, 10^{-3} Sodium azide, 10^{-4} antimony potassium tartrate, 10^{-5} Phlorizin, 10^{-3} Phloretin, and low sodium in the external media.

The only compound that had a significant and repeatable effect on glucose uptake was Caparsolate^{R} (Thiacetarsamide). Thiacetarsamide is an adultacide for heartworm and is presently the drug of choice for the disease. Shown in Figure 9 is the results from one experiment looking at 1.5mM thiacetarsamide.

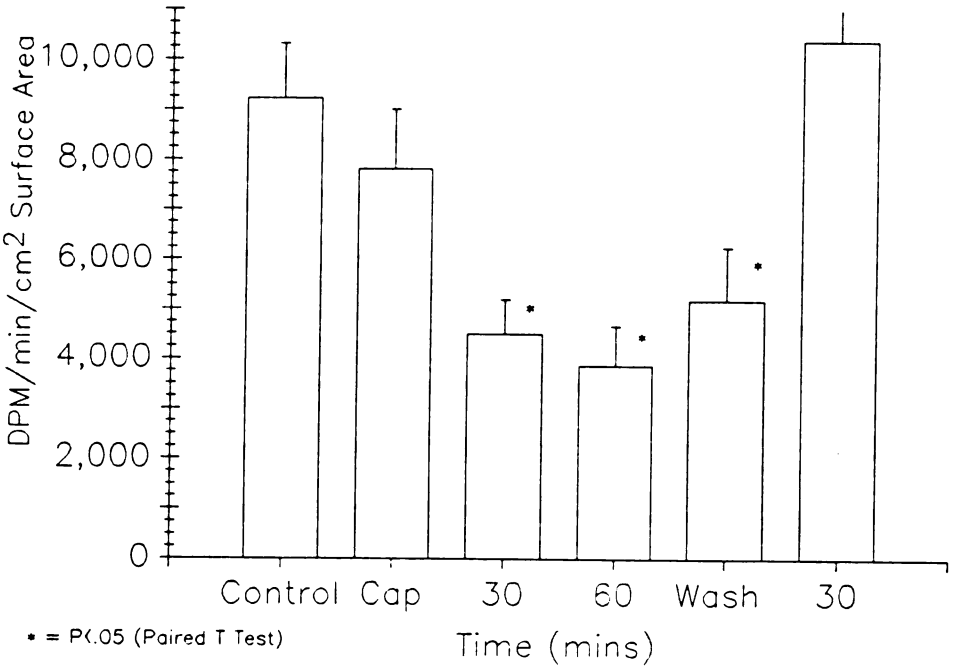


Figure 9. The affect of 1.5mM thiacetarsamide on glucose uptake in *Dirofilaria immitis*.

As can be seen in figure 8, thiacetarsamide rapidly decreased the uptake of glucose into a heartworm segment, which was significant within 30 minutes. The effect of the arsenical was reversible however at 60 minutes.

Since pH is an important variable in the transport of nutrients across biological membranes we analyzed its affect on glucose uptake. I_{RPMI}, is normally on the outside of the worm in this system, and has a pH of 7.4. APF, which is normally on the inside of the heartworm in this system, has a pH of 7.0. Experiments involving the changing of pH's of both medias were performed and the results can be observed in figure 10. Changing the pH of the external environment, in either direction, caused a significant decrease in glucose uptake. Changing the pH of the internal environment (APF) from 7.0 to either 6.0 or 8.0 had little if any effect on glucose uptake.

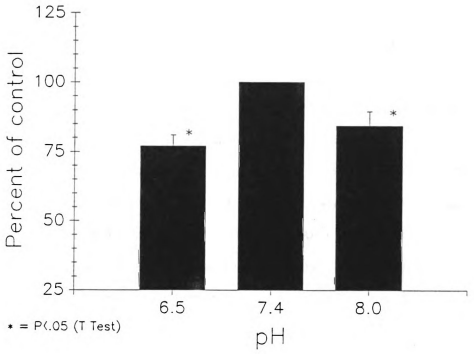


Figure 10: Affects of external pH on glucose uptake in heartworm.

The obvious effect of temperature on glucose uptake when the temperature was decreased from 37°C to 4°C led us to try to quantify the affect. 4μCi ^{14}C glucose was added to 15ml I_{RPMI} and several heartworm segments were perfused with APF. The temperature of the water bath was set at 4°C to start with and raised approximately 5 to 10°C every ten minutes. In similar experiments inulin was used instead of glucose to determine to what point the temperature could be raised without damaging the tegument membranes.

As is shown in figure 11 there was a temperature dependent increase in glucose uptake. However a T_{max} could not be determined because the transport of glucose was still increasing at a temperature where the tegument began to break down i.e., between 50 and 60°C inulin began to pass through the membrane into the APF perfusate.

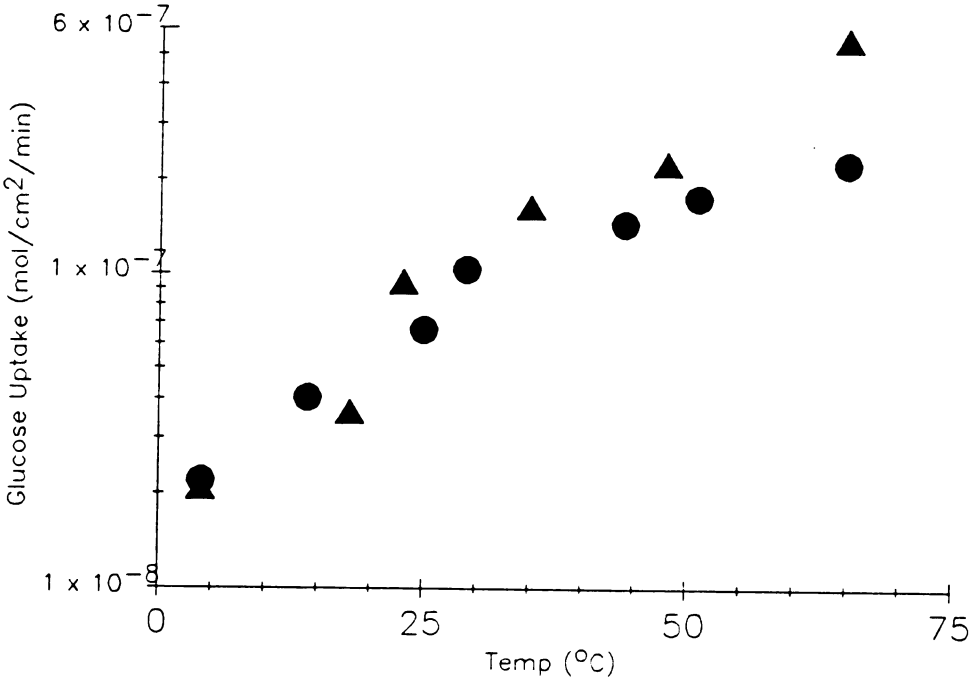


Figure 11: Glucose uptake into heartworm segments as a function of temperature.

Lately there has been much interest with acetate in nematodes. Several investigators have recently shown that ascaris are capable of utilizing acetate as an energy source. It was previously thought that acetate was strictly an excretory product that was eliminated by the parasite. With this in mind and with the information previously gathered on the absence of a glucose transporter in ascaris tegument the experiments were performed to determine the presence or absence of an acetate transporter in these parasites.

As was shown in the media section I_{RPMI} normally does not contain acetate while APF contains 128mM acetate. 4μCi of ^{14}C labeled acetate as well as 1mM cold acetate was added to the incubation bath of 15ml I_{RPMI} and the segments were perfused with APF at 37°C and 4°C. The results of these experiments are presented in Figure 12.

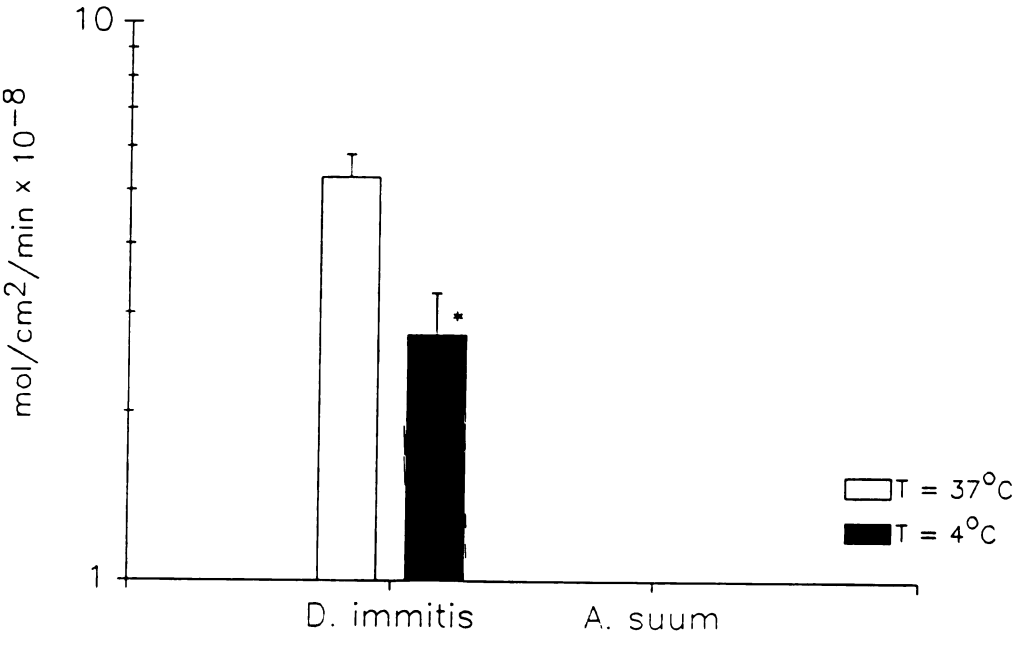


Figure 12: Acetate uptake into *Dirofilaria immitis* and *Ascaris suum* segment.

It can be seen in figure 12 that no acetate was transported into the ascaris segment even in the presence of a large concentration gradient. The heartworm segment was however able to transport acetate into the segment. This transport was significantly decreased at 4°C. It should also be noted that the radioactive acetate could be displaced from entering the preparation if nonradioactive acetate was added. As noted before this transport took place against a concentration gradient of over one hundred fold and still occurred at a very high rate, comparable to glucose.

Four amino acids were picked to represent the four different "R" groups which are, nonpolar and uncharged, polar but uncharged, polar and negatively charged, and polar and positively charged. Leucine, asparagine, glutamic acid and arginine were used respectively. 4μCi of ^{14}C labeled amino acids were added to 15 ml of $RPMI_{1640}$ which contains unlabeled amino acids. The segments were than perfused with APF and the perfusate counted for radioactivity. This was done in both *Dirofilaria immitis* and *Ascaris suum*.

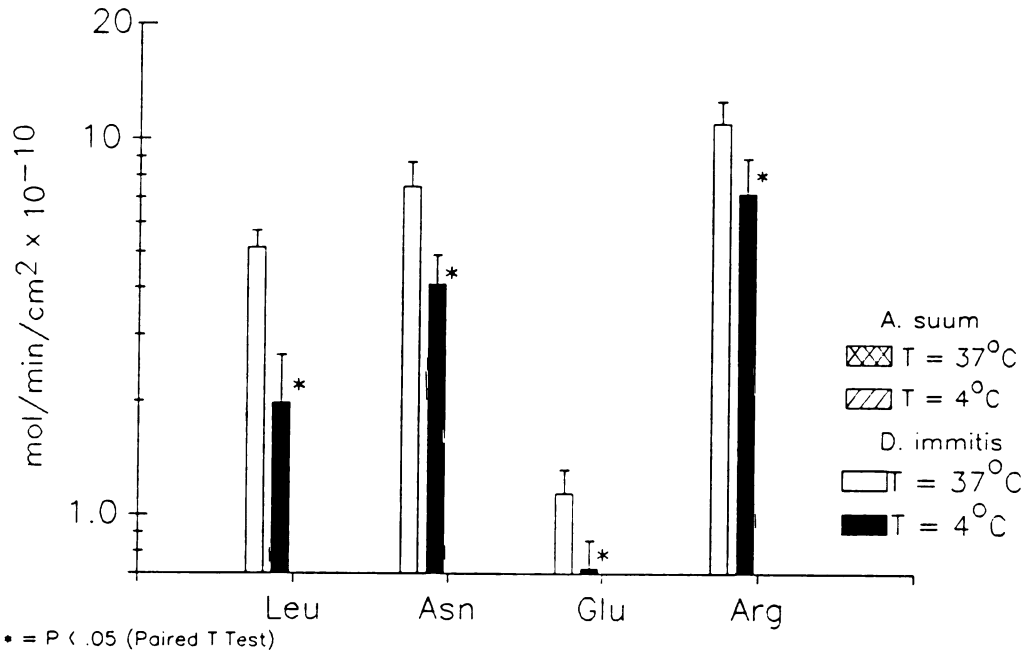


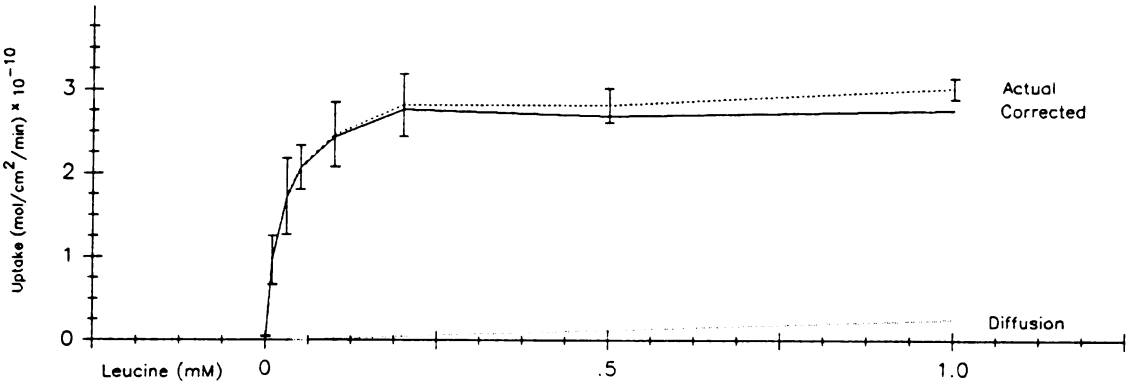
Figure 13: Amino acid uptake into *Dirofilaria immitis* and *Ascaris suum* segments.

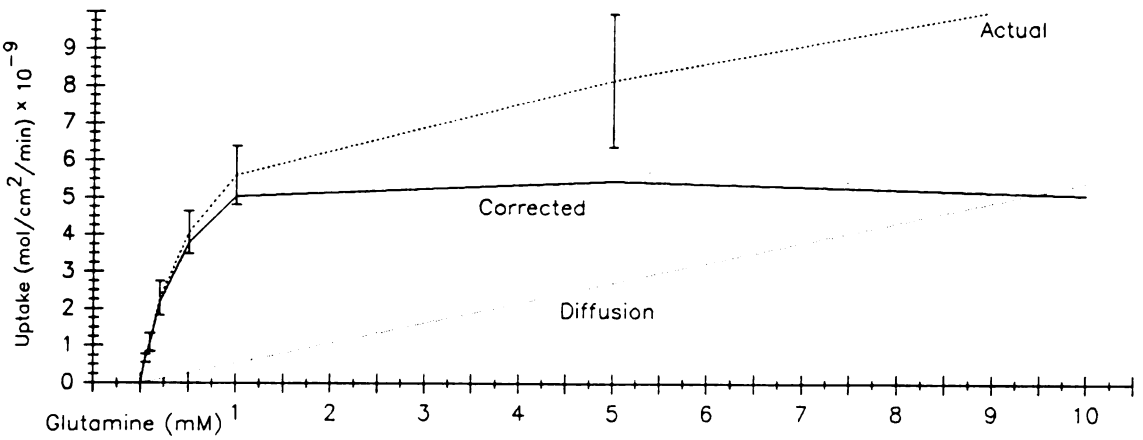
Figure 13 shows the results from these experiments. As can be seen on the graph the bars for ascaris are missing this is because under these conditions radiation above control was not detected, signifying that the amino acids were unable to enter the ascaris preparation.

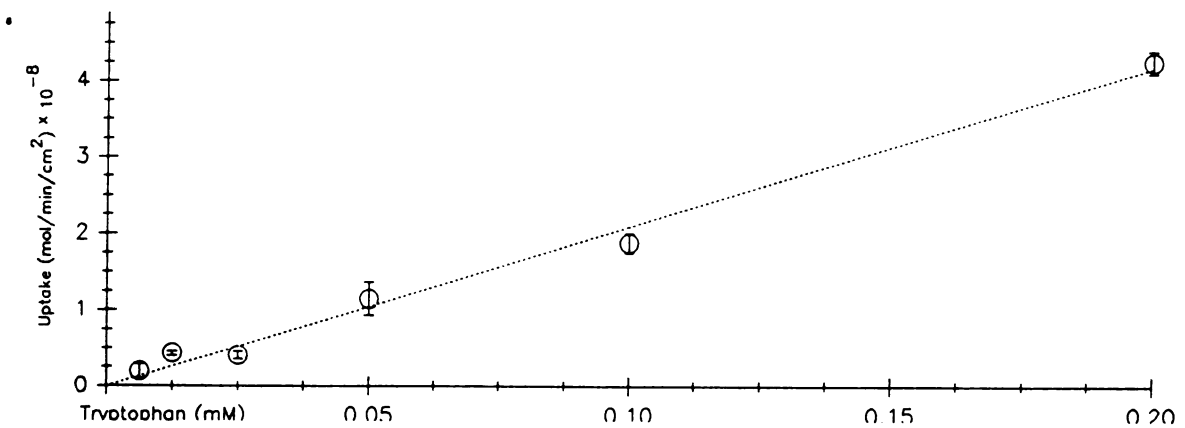
This figure also shows that a significant amount of each amino acid was transported into heartworm segments under these conditions. Transport significantly decreased when the temperature was decreased to 4°C. This decrease was reversible when the temperature was raised back to 37°C.

The next set of experiments were designed to determine to what extent the transport of nutrients into heartworm segments was concentration dependent. Three amino acids as well as glucose were used. Amino acids used were leucine (an uncharged and non-polar amino acid), glutamine (an uncharged and polar amino acid), and tryptophan (also a non-polar and uncharged but somewhat larger amino acid). All three amino acids used were ^{14}C labeled as was glucose.

Figure 14;A,B,C: concentration curves for the uptake into heartworm segments of leucine, Glutamine and Tryptophan. The ------ line represent the actual experimental values obtained. The ..... line represents the calculated diffusional component of transport. The ——— line represents the corrected transport.


Uptake (mol/cm^2/min) x 10^{-10}
3
2
1
0
Actual
Corrected
Diffusion
Leucine (mM)
0
.5
1.0


Uptake (mol/cm^2/min) x 10^{-9}
9
8
7
6
5
4
3
2
1
0
Actual
Corrected
Diffusion
Glutamine (mM)
1
2
3
4
5
6
7
8
9
10


Uptake (mol/min/cm^2) x 10^{-8}
4
3
2
1
0
Tryptophan (mM)
0.05
0.10
0.15
0.20

These three graphs, Figures 14:A,B,C show the results from the amino acid experiments. Figure 14:A shows the results for leucine. The ------ line shows the actual experimental values obtained. The ..... shows the values that were calculated for how much of the actual transport can be attributed to simple diffusion. The ———— line shows what is left of the actual experimental data when the diffusional part is subtracted. From this a value for the K_m (or half maximal concentration) was calculated to be .68mM and a value for the V_{max} (or maximum rate of transport) was calculated to be 2.72×10^{-8} (shown in table 3).

Figure 14B shows similar graphs for glutamine. The values for K_m and V_{max} are shown in table 3. As is shown in table 3 there is a significant variation in K_m and V_{max} values between the various amino acids. This would be expected to vary depending on the individual need for each nutrient.

The third graph, figure 14C, shows the experimental values for tryptophan. The amount transported into the heartworm segments was linearly related to concentration. Addition of large amount of unlabeled tryptophan was not able to displace any of the labeled tryptophan from entering the tegument. Also when at the end of the experiment the temperature was decreased to 4°C no decrease in uptake occurred. From this data and as can be seen on the graph

there was only a diffusional portion of the transport and no active process that can be measured.

This next graph, Figure 15, shows a similar graph for the concentration dependency of glucose on uptake. This graph shows clearly, the diffusional and active transport parts for the experimental values. Values for K_m and for V_{max} were obtained and can be seen in table 3.

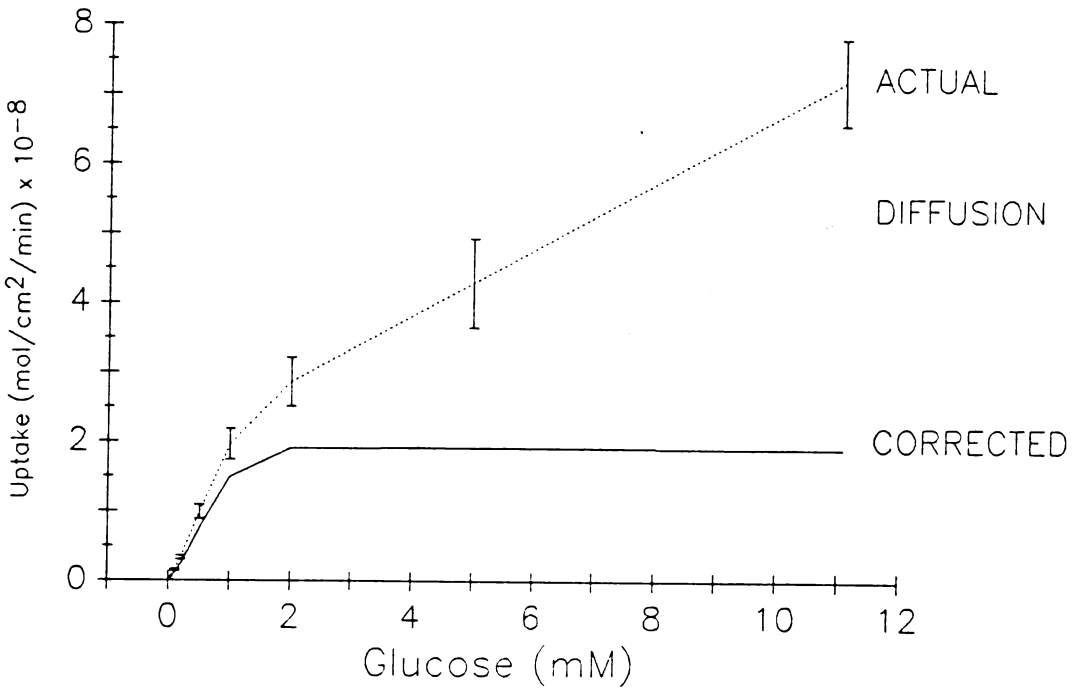


Figure 15: The concentration curve for uptake of glucose into the heartworm segments. The ------ line represent the actual experimental values obtained. The ..... line represents the calculated diffusional component of transport. The ——— line represents the corrected transport.

Nutrient	K_m (mM)	V_{max} ($mM/min/cm^2$)
Glucose	0.68	$2.72x10^{-8}$
Glutamine	0.14	$2.87x10^{-9}$
Leucine	0.014	$2.73x10^{-10}$

Table 3: K_m and V_{max} values for leucine, glutamine and glucose uptake into heartworm segments.

The last macromolecule that was looked at for permeability was the drug ivermectin. Ivermectin is a potent anthelmintic that has been used for heartworm prevention at low doses. At these doses the drug does not have an effect on the adult heartworm. Ivermectin has also been used to control roundworm in several species. It was therefore postulated that the difference might be in its ability to enter the parasite. Both *Ascaris suum* and *D. immitis* segments were perfused with APF while incubating in I_{RPMI} with radioactive ivermectin. The perfusion was carried out over a one hour period per segment. Following the perfusion the perfusion pump was turned off and the segment left in place for 20 minutes to allow for the possible accumulation of ivermectin in the segments. The results of these experiments are shown in Figure 16.

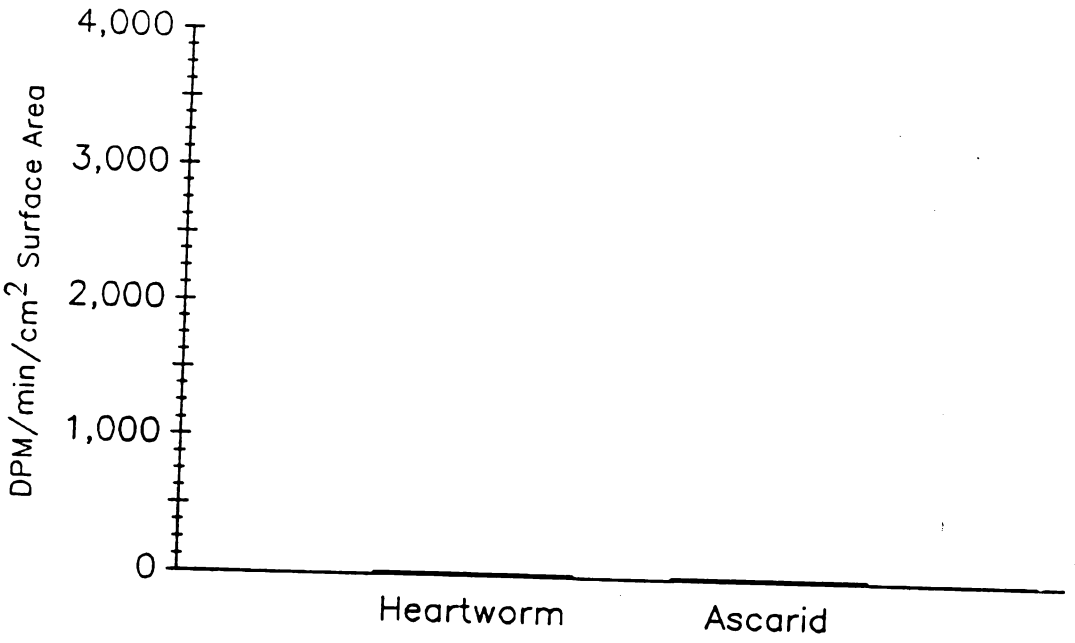


Figure 16: The permeability of both *Ascaris suum* and *Dirofilaria immitis* to the drug ivermectin.

Figure 16 shows the results of the ivermectin experiments. As can been seen the bars for both *Ascaris suum* and *Dirofilaria immitis* are missing this is because in neither case did any ivermectin enter the segments. When the segments were allowed to "soak" in the incubation bath for twenty minutes still no ivermectin entered either segment.

The next series of experiments involved the permeability of both the heartworm and ascaris to different ions. The ions looked at were $^{22}Na^{+}$, $^{36}Cl^{-}$, and $^{45}Ca^{++}$. 4μCi of radioactive ion was added to I_{RPMI} and the segments were perfused with APF. The results for these experiments are shown in figure 17 and table 4. As can be seen there is quite a large difference to the permeability to the ions between the two parasite. The ascaris seems to remain quite impermeable to ions as compared to heartworm.

	D. immitis	*A. suum*
Cl^-	2085 ± 196	28 ± 8
Na^+	795 ± 100	9 ± 2
Ca^{++}	544 ± 191	42 ± 17

Table 4: The permeability of both *Dirofilaria immitis* and *Ascaris suum* to ions expressed in DPM's.

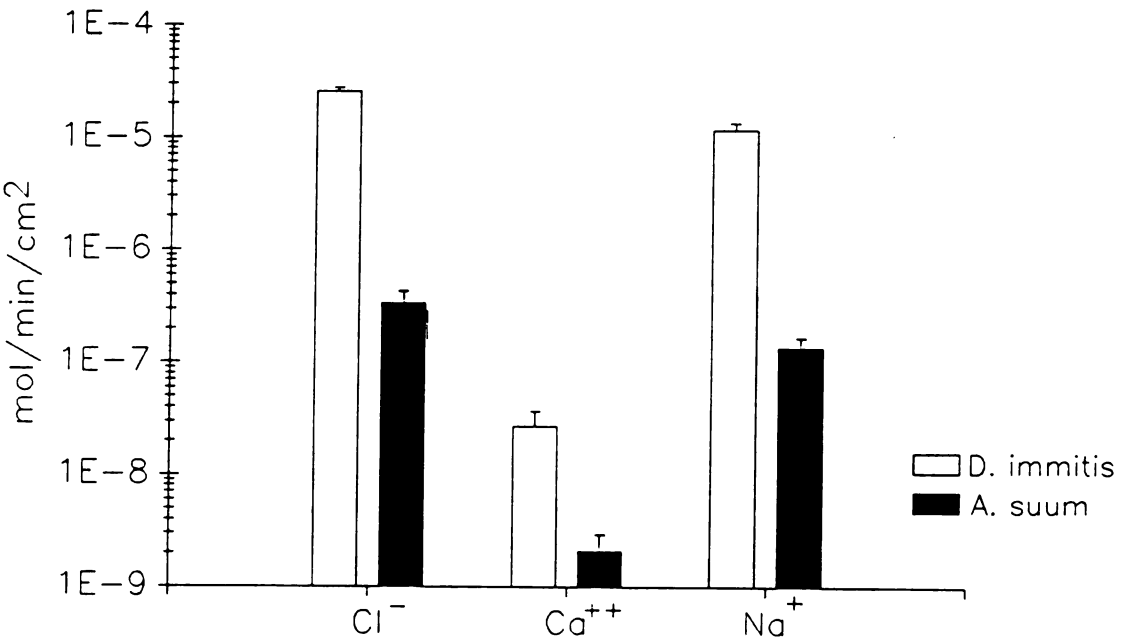


Figure 17: The permeability of both *Dirofilaria immitis* and *Ascaris suum* to ions.

The ascaris tegument was inverted and perfused, as described in the methods section. In this preparation APF was on the outside of the tegument which represents the inside of the parasite. I_{RPMI} was on the inside of the preparation being perfused through the system but this represents the outside of the parasite. The radioactivity was placed on the outside of the system in the APF and the perfusate was measured for the presence of the radioactive ions. Figure 18 and Table 5 shows the results from these experiments.

	Into	Out-of
NA^+	9 ± 2	18 ± 4
Cl^-	28 ± 8	53 ± 6

Table 5: Net movement of ions into and out-of *Ascaris suum* expressed as DPM's.

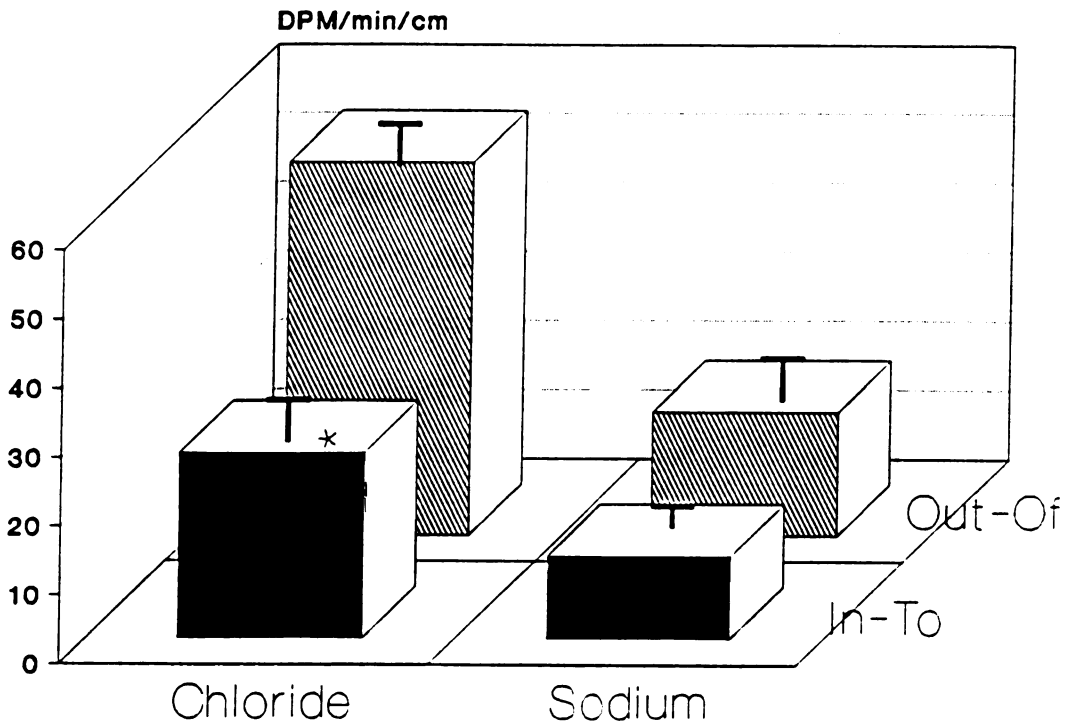


Figure 18: Net movement of ions into and out-of *Ascaris suum* expressed in Molar concentrations.

The movement of Na^+ into the tegument of the ascarid was not significantly different from the movement of sodium out-of the preparation. In either case the measurement of radioactive sodium was barely larger than control radiation. Therefore the actual numbers might contain a higher degree of error than if they were much larger than control.

Chloride movement out-of the tegument was significantly larger than the chloride movement into the tegument. This movement also occurred against a concentration gradient, however the electrochemical gradient was not known.

Conclusions and Discussions

The tegument of both parasites remained intact and undamaged throughout these experiments. This was demonstrated by the fact that inulin was not able to enter the tegument of either parasite.

Water was able, however, to enter both parasites. This movement of water into the heartworm segment was significantly greater than that into the ascaris segment. Looking back at table 2, heartworm was over 18 times more permeable to water than the *ascaris*. This difference could be explained by the difference in the thicknesses of these two parasites. However, this difference in thickness could not explain the other differences seen throughout these experiments

The first nutrient that was looked at was glucose. As was seen in table 2, glucose was much more (approximately 30 times) permeable in the heartworm than the ascaris segments. The glucose uptake in the heartworm segments was sensitive to temperature. When the temperature was decreased from 37°C to 4°C there was a significant decrease in glucose uptake which was reversible when the temperature was returned to 37°C. Figure 11 showed a temperature response curve for glucose uptake in heartworm segments. However the T_{max} could not be calculated since the uptake of glucose was still

increasing at a temperature at which tegument began to breakdown. This temperature dependency did not occur in *Ascaris suum* where no decrease in glucose uptake occurred as a result of lowering temperature.

APF contains glucose at a concentration of 10mM. When the glucose was removed from the APF in the same experiments there was a significant decrease in glucose uptake in the heartworm segments. Removing the glucose from the APF, made the inside of the parasite devoid of glucose and actually increased the glucose gradient. This fact signifies that the uptake of glucose is sensitive to internal glucose and may, in fact, depend upon it. This again didn't occur in ascaris segments.

Glucose uptake into *Dirofilaria immitis* was also concentration dependent. Glucose at concentrations between 0 and 11.1 mM, in I_{RPMI}, was examined for its effects on glucose uptake. Since the amount of "hot" or radioactive glucose remained constant in the incubation bath and the cold glucose could displace it from being taken up. This signifies a saturable mechanism. Figure 14 showed the curve that was calculated for this series of experiments. The actual experimental uptake could be broken down into a diffusional part, that was linear with respect to concentration; and a presumably mediated process, that was saturable. From this mediated part of

uptake apparent enzymo-kinetic parameters could be calculated, and was shown in table 3.

Thiacetarsamide, which is an arsenical used for the treatment of heartworm infection in dogs, had a significant but reversible affect on glucose uptake. The fact that this inhibition was reversible might explain the necessity of treating infected dogs for 3 consecutive days with this compound to ensure complete efficacy in killing the adult heartworms. The mechanism of action of this arsenical is believed to be in its ability to inhibit sulfhydryl containing enzymes (Booth, Veterinary Pharmacology and Therapeutics).

Trans-tegumental glucose uptake into heartworm segments is now believed (by this author) to be a process mediated by a transport protein because the trans-tegumental uptake of glucose into heartworm segments:

1) is temperature sensitive.
2) is concentration sensitive
3) is stereospecific for d-glucose
4) is sensitive to internal glucose concentration.
5) is sensitive to external but not internal pH.
6) is sensitive to the sulfhydryl inhibitor thiacetarsamide.
7) is not sensitive to classical glucose transport inhibitors applied to the outside of the tegument.
8) is not coupled to an external sodium transport.

Flemming (1984) reported that ascarides might be more permeable to macromolecules than previously reported because they were able to measure glucose entering their preparation. However the amount entering was small and corresponds nicely with the values that I obtained in these experiments. Unlike heartworm segments this amount was small and was not sensitive to temperature or concentration, and therefore is probably due to a simple diffusional process.

Acetate has received a lot of interest lately because it is now believed that ascaris is capable of not only excreting acetate but also, under glucose deprivation conditions, they are able to utilize it as an energy source. Ascaris was not able to transport any acetate into their body but heartworm showed a very active mechanism for trans-tegumental uptake. This uptake:

1) was temperature sensitive

2) was concentration sensitive, in that excess cold acetate could displace the hot acetate.

3) also occurred against a large concentration gradient of over 125 fold.

With regard to amino acid uptake by both these parasites, we examined the transport of several amino acids, representing the various "R" groups. The amino acids used were leucine (which is non-polar and uncharged), tryptophan (which is also non-polar and uncharged but somewhat larger), asparagine (which is polar but uncharged), glutamine (which

is polar and uncharged, glutamic acid (which is polar and negatively charged), and arginine (which is polar and positively charged).

In the ascaris segments none of these amino acids were able to enter through the tegument. In the heartworm segments all of amino acids were able to enter through the tegument. They all showed both concentration and temperature sensitivity except for tryptophan. For leucine and glutamine actual kinetic values (V_{max} and K_m) could be calculated for their uptake. Tryptophan on the other hand didn't show any concentration sensitivity in the range tested. The graph of uptake verses concentration was linear, signifying this was a diffusional mechanism.

In heartworm segments the uptake of most amino acids tested appeared to be a controlled process because:

1) It is temperature sensitive

2) It concentration sensitive, in as much as excess cold nutrient could displace the uptake of the labelled nutrient

Although the results are not shown several experiments were performed measuring the accumulation of glucose, acetate, and various amino acids by ascaris segments in order to try to maximize the experimental conditions for uptake. There are several recent reports of different media's being used for ascaris incubation so it was decided to try several of these in our system. We examined 1) APF on both sides of

the segment, 2) I_{RPMI} on the inside and APF on the outside and 3) Donohue's on the outside with APF on the inside. All of these configurations provided the same results with regard to trans-tegumental uptake.

Also, time has been reported to be a possible factor in that a delay might exist between the start of the experiment and the start of trans-tegumental uptake. Therefore, the segments were also perfused for up to one hour. Increased perfusion time didn't change the amount or rate of labelled material passing through the parasite's tegument.

One experiment that needs to be done is to try a new APF media reported to show interesting affects on ascaris muscle preparations. This media was reported by Holden-Dye (1988) with the main difference between in and Donohue's media, being a high concentration of Mg^{++} and a slightly higher pH (pH=7.6). Although I suspect that this new media will not affect the results significantly, the experiment still needs to be done.

Ivermectin is a rather interesting drug. It is used in low concentrations in dogs for heartworm prevention. At these concentrations the drug has no affect on the adult heartworm but is a powerful microfilariacide. However at higher doses ivermectin is known to have a detrimental affect on spermatogenesis and oogenesis. Ivermectin is also used for

the control (or treatment) of roundworm (ascaris) infections in many species.

The reason for this difference in its efficacy for the treatment of heartworm and ascaris infection is not known. One theory is that there is a difference in the parasites ability to take up this drug and thus allow it access to its site of action, which at this point in time is believed to be its pharynx. Ivermectin's ability to penetrate these parasites was tested and I observed no trans-tegumental transport. A theory for this difference is that if ivermectin's site of action is the pharynx, access to it in the case of ascaris might be by oral ingestion. While in heartworm if the parasite takes up all its nutrients trans-tegumentally and oral uptake does not normally occur then the access to the pharynx is therefore limited

The last group of experiments that was done was to look at the permeability of ions in our preparations. Sodium ($^{22}NA^{++}$), chloride ($^{36}Cl^{-}$), and calcium ($^{45}Ca^{++}$) were used. In all three cases the heartworm segments were much more permeable to ions than was the ascaris segments. Heartworm was approximately 100 times more permeable to Cl^{-}, 800 times more permeable to Na^{+}, and 10 times more permeable to Ca^{++} than ascaris, which appeared to remain impermeable to calcium and sodium.

In ascaris the amount of radioactive sodium and calcium was barely above background radiation. Chloride permeability was higher than sodium and calcium. In the last series of experiments ascaris segments were inverted and perfused as described in the results section and the permeability to sodium and chloride was determined. Sodium movement in either direction (both into and out-of) the segment was statistically the same. While chloride movement was significantly higher in an outward direction as compared to an inward direction. In either direction this movement was barely above control. This net movement of chloride outward occurred against a concentration gradient. Although the electrochemical gradient was and is still not known. It has been reported (Wright 1987) that chloride concentration in the pseudocoel cavity is lower than expected simply from the concentration of positive ions. It has also been reported that chloride might play an important role in the maintenance of this internal turgid pressure and from this data that possibility seems quite feasible

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