EFFECT OF DRUG DERIVATIVES ON THE ELECTRICAL CHARACTERISTICS OF THE ISOLATED GUT EPITHELIUM OF *ASCARIS SUUM*

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CHAPTER I

INTRODUCTION

The presence of the roundworm Ascaris suum in the intestine of pigs and of Ascaris lumbricoides, a physiological variety, in the intestine of humans is reported by the Greeks as early as 1533 B.C. (Chitwood and Chitwood, 1950).

The U.S. Department of Agriculture (1965) estimates that some 70 percent of the pigs and 30 percent of the hogs of breeding age in the United States are infected with Ascaris suum. Stoll (1947) estimated that one out of every four humans is infected with Ascaris lumbricoides. The large adult roundworms often migrate into the bile duct of their host and cause liver damage. Heavy infestations are known to occlude the small intestine. Migrating larval ascarids are known to cause severe pulmonary disease and in this regard it is important to realize that infective eggs of the pig variety hatch in the intestine and migrate to the lungs in man as well as other mammals. Both varieties are a menace to the public health in many parts of the world and the swine variety is responsible for significant economic loss to the farming community.

Ascaris suum adults obtain nutrients from the hog's partially digested intestinal contents. The worm's intestine is responsible for the absorption of nutrients and also plays a role in excretion and osmoregulations. The intestine is a long tube which extends the

length of the worm. It is composed of a single layer of columnar cells which rest on a thick basement membrane and is capped by microvilli. This intestine is easily removed from the worm and is an excellent tissue for the measurement of transepithelial transport.

Studies have been conducted using in vitro preparations of intestine which indicate that carried mediated mechanisms operate to move certain sugars into the intestine against a concentration gradient (Beames, 1971). This transportation system is located at the apical surface of the cells and requires energy derived from the catabolism of carbohydrates in the intestinal cells. The process is sensitive to phlorizen and is dependent upon the presence of sodium in the luminal solution (Schanbacher, 1974). There is a net flux of sodium from the luminal to the pseudocoelomic side of the intestine during the transport of glucose (Harpur and Popkin, 1973). Mebendazole, an effective ascarod anthelmintic, inhibits sugar absorption by the worm, but does not affect the transport of sugar in the vertebrate intestine (Van den Bossche, 1976).

The intestine has been shown to maintain a 15-75mV electrical potential with the pseudocoelomic surface negative to the luminal surface (Merz, 1977). The short circuit current (SCC) required to zero this potential is 15-75 μA and glucose is required on the pseudocoelomic side of the intestine to maintain the potential difference. The addition of glucose to the luminal side of the intestine results in a decrease in the SCC. The decrease in SCC is proportional to the concentration of glucose that is introduced into the solution bathing the isolated intestine (Merz, 1977).

The purpose of this investigation was to observe the electrical properties of the Ascaris intetine and to relate differences in electrical responses to difference in drug mechanism of action. It was suggested from Beames, Merz, and Donahue's (1976) work that transepethelial potentials (TEP) and SCC measurements would provide some insight as to the mode of action of the different benzimidazole derivative. An in vivo mouse screen conducted by Dow Chemical Co. was used in conjunction with the electrical measurements to determine if the in vitro assay could be used as a test for small quantities of proposed anthelmintic drugs.

The results indicate that the electrical measurements of the Ascaris intestine can provide an indication of possible drug activity. The change in SCC response of the intestine to glucose was altered significantly, with some drug treatments indicating a disruption in the normal mechanisms involved in glucose uptake.

Microelectrodes were used to determine normal changes in membrane potential, sodium and potassium activities with time and with drug treatment. Normal decreases in the membrane potential (PD) with time was associated with the decrease in cell potassium. Changes in cell potassium can be used to predict changes in the membrane potential using the Nernst equation. Calculated changes in membrane permeabilitities using the Goldman equation are used to determine changes in sodium and potassium movements across cell membranes in response to drug and glucose treatment. Results of changes in cellular sodium and potassium activities are used to describe the difference in mechanism of action of the closely related benzimidazoles, cambendazole, and mebendazole.

CHAPTER II

LITERATURE REVIEW

Understanding the ability of Ascaris suum to obtain nutrients and regulate its environment is important for the development of rational drug therapy. The following review involves a discussion of the anatomy and biochemistry of the Ascarid. The intestine is of primary interest in this discussion as it is the organ that is largely responsible for the absorptive processes and ion movement that helps maintain the worm's internal environment. Ion movement in this organ may be associated with the generation of electrical activity which can be altered with the addition of certain metabolic inhibitors and anthelmintics (Beames, Merz, and Donahue, 1976). However, it is not known which ions are responsible for this electrical activity. This section reviews these various aspects of the intestine from Ascaris.

Functional Anatomy of Ascaris

Ascaris suum are nematodes which inhabit the intestines of pigs in adult stages. The adult is covered by an outer cuticle and muscle layer. The cuticle is resistant to the normal enzymes in the host's intestine and is relatively impermeable to all but a few ions and some drugs (Read, 1966). Female Ascarids are the larger of the two sexes, with the reproductive organs occupying a major part of the pseudocoelomic cavity. This cavity is filled with a fluid that is rich in a

variety of nutrients. The fluid is always under pressure, forming what is called the "hydrostatic skeleton" of this animal. The skeleton is of great use in locomotion, feeding, and excretion (Lee, 1965).

The intestine of Ascaris extends the length of the body. The muscular pharynx counteracts the positive pressure of the pseudocoelomis fluid allowing food to be pumped into the alimentary canal. The intestine is normally collapsed, due to the pressure of the pseudocoelomic fluid and is only open in the presence of food. The anus is normally closed, like the pharynx, and contraction results in opening and expelling of the fluid feces by the pressure of the hydrostatic skeleton (Harpur and Jackson, 1976).

The intestine is divided structurally and functionally into anterior, middle, and posterior regions. Intestinal cell heights differ in these three regions. The anterior section contains more secretory cell types than the middle or posterior regions (Carpenter, 1952). The intestine is a nonmuscular structure composed of a single layer of columnar cells resting on a thick basement membrane. The intestinal columnar cells have a basally located nucleus and mitochondria concentrations at the apical end of the cell (Sheffield, 1964). Such mitochondrial localizations are generally known to be associated with regions of high metabolic activity.

The apical surface of the columnar cells are covered with microvilli containing an inner core of longitudinal fibers which extend into the cell to become the "terminal web." Secretary granules are often seen in this web region of active cells. These granules are throught to be associated with the development of a glycocalyx coat on

the microvilli (Borgers and DeNullin, 1974). This coat has several hydrolytic and proteolytic enzymes, indicating that the coat may function in the absorption of nutrients (Ito, 1969; Trimble and Thompson, 1975). These secretory granules have been shown to be formed by the golgi apparatus and transported via microtubules to the apical membrane. Worms that have been kept for several hours show a decrease in the number of secretory granules, as well as a drop in glycogen energy reserves (Borgers and DeNullin, 1974).

The apical portions of the intestinal cells are held together by a terminal bar region (Sheffield, 1964). The plasma membranes are highly folded under this region, which probably aids in holding the cells together.

The intercellular spaces fluctuate in volume, depending on the movement of water across the intestine. Harpur and Popkin (1973) demonstrated intercellular dilation after adding glucose to isolated sacs of Ascaris intestine. This increase in intercellular space was observed in conjunction with fluid movement across the intestine, indicating a possible role of the intestine in maintaining the hydrostatic skeleton.

The basilar membrane on which the intestinal cells rest is highly infolded. It has been suggested that cells with such infoldings are engaged in transporting ions and water for waste removal and osmotic equalibrium. The basement membrane is essentially a semipermeable membrane which affords structural stability to the intestinal tissue and may also provide a selective barrier for certain molecules of varying size and electrical charge (Donahue, 1977). The basement membrane is composed of multiple polypeptide components similar to

collagen and amino acids similar to those found in the basement membrane of kidney glomeruli (Peczon, Venable, Beames, and Hudson, 1975).

Environment of Pigs' Intestines

The environment of Ascaris suum consists of the semifluid contents of pig's intestine. The nature of this environment understandably varies with the diet of the host, but generally, certain important factors are maintained relatively constant. These factors include the oxygen and carbon dioxide tension, temperature, and pH.

The partial pressure of oxygen (pO_2) does vary slightly in the host, being higher during fasting than after a meal. The range of pO_2 measured in the intestines of mammals is 0-70mmHg (Barrett, 1981). This partial pressure of O_2 varies within the intestine as well, being greater near the mucosal surface than in the center of the lumen. This low oxygen environment is referred to as being "microaerophilic" (Komuniecki et al., 1980) and necessitates a largely anaerobic metabolism of the parasites.

The partial pressure of CO_2 (pCO_2), on the other hand, is extremely high, ranging from 20 to 60mmHg (Barrett, 1981). It has been suggested that carbon dioxide fixing pathways found in the helminths may be a mechanism for dealing with these high CO_2 levels.

It has also been suggested that these excessive CO_2 levels cause severe acid/base problems for the helminths (Barrett, 1981). Generally, however, the vertebrate intestine has a near neutral pH (5-7.8).

The temperature of the pig intestine is maintained at 37°C. It is generally known that temperature affects the rates of chemical reactions. It is not surprising to find that the activity of the worm

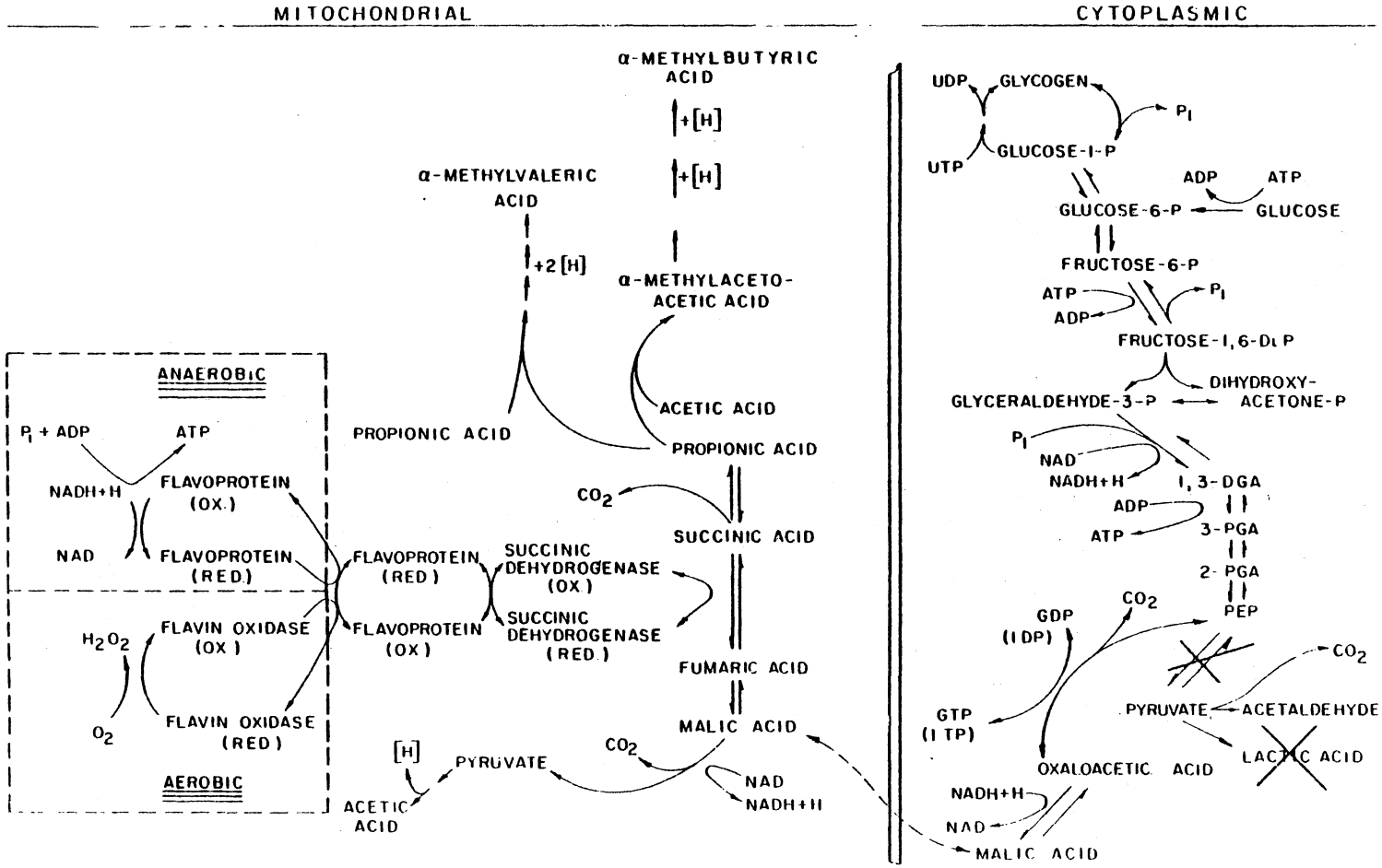
is reduced in temperatures much lower or higher than 37°C. Merz (1977) found that depressing the temperature of *in vitro* intestinal preparations reversibly reduces the electrical activity of this tissue and its response to glucose. How the parasite deals with this environment is further determined in the following discussion of the biochemical pathways found in this worm and the mechanism of nutrient uptake in the Ascarid.

Biochemistry of Ascarids

The pig's intestinal contents contain the nutrients that are utilized by *Ascaris suum* (Rogers and Lazarus, 1949). The following discussion deals with a description of the metabolic pathway present in *Ascaris* and the control of this pathway (Figure 1).

Metabolic Pathway

Ascarids belong to a group of parasitic helminths which excrete succinic acid and volatile fatty acids as the predominant end products of anaerobic metabolism (Srivastrava, Ghatak, and Marti, 1971). Adult Ascarids have a modified glycolytic pathway due to low activities of certain glycolytic enzymes. The modified glycolysis is also coupled with an incomplete and reversed Krebs cycle (Figure 1). This aberrant pathway has proven useful in developing antiparasite agents which do not have a detrimental effect on the host's metabolic processes. Carbohydrate metabolism in way of *Ascaris suum* is not an efficient pathway in that there is incomplete degredation to CO_2 and water. Because of the readily available source of carbohydrates, inefficient



Source: C. G. Beames, Jr., "Movement of hexoses across the midgut of Ascaris," J. Parisitol. (1971).

Figure 1. Carbohydrate Metabolism of Ascaris suum

metabolism is more a curiosity, rather than an impediment to prosperity.

Carbohydrates are converted to energy in Ascarids via glycolysis and the modified Krebs cycle shown in Figure 1. These processes are responsible for the production of energy in the form of adenosine triphosphate (ATP). There is only about one-third the high energy ATP molecules formed, compared to the metabolic pathways encountered in mammalian systems (Borgers and DeNullin, 1974). Glycolysis supplies two-thirds of the ATPs formed in Ascarids; the remaining one-third being of mitochondrial origin (Schanbacher, 1974).

Glucose may be stored as glycogen or may processed through the glycolytic pathway to form phosphoenolpyruvate (PEP). Glycogen can be utilized in times of low food supply. Storage of glycogen is observed in storage granules in fresh intestinal tissue (Borgers and DeNullin, 1974). Tissues taken from starved worms show a decrease in these granules, indicating that they have been utilized in an attempt to maintain the tissue integrity.

Since there is very little pyruvate kinase activity, there is no pyruvate formed from PEP. Instead, PEP carboxykinase fixes CO_2 and forms oxaloacetate (OAA). OAA is reduced by cytoplasmically formed NADH to form malic acid. This reaction is catalyzed by the enzyme malic dehyrogenase. Malic acid enters the mitochondria where it can be used by one of two pathways. In one pathway, NADH+H is obtained by the oxidative decarboxylation of malic acid to pyruvate and CO_2. This reaction is catalyzed by mitochondrial malic dehydrogenase. The pyruvate may serve as a precursor for acetate, which may, in turn, be incorporated into volatile fatty acids. The NADH+H formed in this

reaction is used in the second pathway, which reduces malic acid to succinic acid via fumaric acid and the enzyme fumarate reductase. Under anaerobic conditions succinate formation is accompanied by the production of ATP via an electron transport chain (Figure 1). In the presence of O_2, hydrogen peroxide (H_2O_2) is formed. Enough quantities of peroxide can cause a bleaching of the Ascarid tissue and eventual death.

Succinate formation requires NADH+H from the conversion of malate to pyruvate. Succinate can then be converted to propionate with the aid of succinic dehydrogenase. The reaction then proceeds to the production of various volatile fatty acid byproducts.

Fatty acid byproducts are found in both the feces and pseudocoelomic fluid. The fatty acids that are found in each of these fluids are not identical in concentration (von Brand, 1966). The results of fatty acid analysis along the length of the worm's intestine show that there is a high concentration in the anterior intestine and incorporated into egg reserves. The intestine would then act as a type of circulatory system for fatty acids to get to the reproductive tissues for egg formation. In any event, the concentration of the final excretory products show that there is some selected removal of certain fatty acid byproducts and that the intestine is important in this role.

Control of Metabolism

The control of cellular metabolism understandably depends on a variety of factors. The enzymes required for various steps in the glycolytic pathway have already been mentioned. Enzymatic activity may be altered with changes in environmental pH, CO_2, and O_2, as well

as the availability of substrate. An abnormal amount of any of these factors can alter the rate of the glycolytic pathway.

Landsperger and Harris (1976) found that mitochondrial malic dehydrogenase of Ascaris suum has different affinities for its substrate, malic acid, depending on the pH. They found that a pH increase from 6.5 to 7.5 decreased the affinity for malate.

Uptake of glucose by the Ascarid intestine has been found to correspond with increases of acid production, increases of tissue carbohydrate stores, and CO_2 evolution (Harpur and Jackson, 1975). Lee (1965) stated that most of the digestive enzymes of Ascarid intestines have an activity range between pH 6.0 and 9.4. Carbohydrate and fat digestion can occur at the more alkaline pH's. Metabolism of these substrates makes the intestine more acidic and allows protease digestion to occur at the pH optimum of 6.0.

Schanbacher and Beames (1973) found that altering the luminal pH of isolated Ascarid intestines between pH 5.5 and 8.0 shows the uptake of 3-O-methyl glucose 3-OMG. Movement of 3-OMG was optimized at pH 6.5, and was further stimulated by the addition of glucose. This would indicate that the 3-OMG transport involves a catalytic process that is pH sensitive.

Podesta (1978) found that the cestode Hymenolepis diminuta acidified its environment within the host's intestine by secreting hydrogen ions that were not related to organic acid secretion. Lesser, McCracken, and Lumsden (1975) demonstrated that the rat anterior small intestine with a normal pH of 6.6, becomes acidified to pH 5.7 when parasitized by this cestode. Parasitized rats also had higher glucose concentrations in their posterior intestine. Decreasing the pH of the

rat's intestine limited the uptake of glucose by the rat, although the cestode's ability to absorb glucose was unaffected. Thus, by lowering the pH of the host's intestine, the cestode improves its ability to compete with the host for glucose.

Intestinal glucose uptake in many species occurs with the aid of a sodium-glucose cotransport mechanism. Glucose is moved into the cells with sodium when there is a sodium concentration gradient. Schultz and Curran (1970) suggested that the pH sensitivity of this mechanism may be due to the pK values of sodium binding sites. Lesser, McCracken, and Lumsden (1975) suggested that sodium binding at the transport loci is electrostatis and thus affected by hydrogen ions. Marx (1980) suggested in his review of pH effects on cellular function that pH may act as a regulator in some systems.

The availability of certain gases may also serve to regulate metabolism. When there is an excess of O_2 is present in the environment, the resulting accumulation of hydrogen peroxide can destroy cellular processes in Ascarids and result in the death of the worm (Figure 1). Saz and Lescure (1969) found radioactively labeled CO_2 in several of the byproducts of Ascarid metabolism, including propionic and succinic acids. Available CO_2 is used to carboxylate PEP with the aid of the enzyme PEP carboxykinase to form OAA. Beames (1971) found that the movement of hexoses across the Ascarid intestine *in vitro* were dependent of the availability of CO_2 in the incubating media as well as an adequate supply of glucose. Oxygen completely reduces the movement of hexoses into the cell and would thus limit the activity of the metabolic pathway as well as produce lethal hydrogen peroxide.

Ascarids can only metabolize certain nutrients as may be seen when lactose is the only available food source. Lactose is not utilized by the worm because it has no lactase enzyme. A common old world treatment for ridding a patient of intestinal nematodes is to eat only milk products for several days and thereby starving the intestinal nematodes. When the required nutrients are available, there are a variety of mechanisms available for their uptake, depending on the compound. These absorption processes are effected by similar environmental controls that affect metabolic activity.

Absorptive Processes

The intestine appears to be the principal route for nutrient absorption in Ascaris suum (Fairbairn, 1957; von Brand, 1966). Nutrients are made available to Ascarid metabolism via several mechanisms, depending on the compound. Most of the compounds studied have been carbohydrates and lipids, with some investigation of fluid and ion movements.

Nutrient Uptake

Dissacharides are broken down into smaller units by enzymes located on the brush borders of the intestinal cells of Ascaris suum. These enzymes are deposited on the microvilli by secretory granules formed by the golgi apparatus (Borgers and DeNullin, 1974). The brush border enzymes include acid phosphatases, esterases, leucine aminopeptidases, disaccharidases, and monoglyceride hydrolases. The disaccharideses include sucrase, maltase, trehalase, and platinase (Gentner, Savage, and Castro, 1972). The hydrolyzed byproducts of brush

border enzymes can then be taken into the cell by a variety of mechanisms.

Glucose uptake has long been considered to be dependent on the metabolic activities of the cell. von Brand (1966) observed that lowering the temperature from 37° to 7°C led to a leakage of glucose from the cell. Castro and Fairbairn (1969) demonstrated rapid uptake and metabolism of glucose into glycogen.

Beames (1971) has demonstrated that glucose is probably taken up into the cell against a concentration gradient. He replaced glucose with a similar molecule, 3-O-methyl glucose (3-O-MG). This molecule is dependent on metabolic processes for its transport, but is not metabolized itself. Radioactively labeled 3-O-MG is collected against its concentration gradient and is enhanced when CO_2 and glucose are also present. The stimulatory effect of glucose may be due to the needed energy input to an already exhausted carbohydrate reserve in the cells. Uptake of 3-O-MG and presumaby glucose does not occur equally in all cells. Schanbacher and Beames (1973) found that the anterior intestine took up less 3-O-MG than did the posterior intestine, supporting the concept that there is a functional difference between different sections of the intestine.

Movement of other sugars such as fructose into the cell is similar to glucose, while no significant amount of galactose is taken up by the intestine. Galactose is taken up in mammalian systems, indicating a difference in cell function between the parasite and the host (Sanhueza et al., 1968).

Glucose uptake has been studied at length because of its principal importance in the metabolic scheme. Besides being incorporated

into glycogen reserves, breakdown products of radioactively labelled glucose have been found in various acids, proteins, lipids, nucleic acids, and CO_2 (Entner and Gonzales, 1959). Harpur and Jackson (1975) found that one molecule of CO_2 was given off for every 10 glucose molecules taken up.

Lipids are taken up into the intestinal cells by another mechanism which could best be described as "diffusion." Beames and King (1972) measured the movement of radioactively labeled palmitic acid and found that movement into the cells was a passive process which was increased with bile salts, but that catabolism of carbohydrates was required to move fatty acids into the pseudocoelomic fluid. Fatty acid movement into the pseudocoelom was decreased without glucose or in the presence of oxygen. The addition of IAA also stopped movement of fatty acids outof the cells, but not the movement into the cells of ascarid epithelium.

Uptake of lipid is important to the worm in that only 12% of the lipids passed in the eggs per day is accounted for by de novo synthesis from acetate (Beames, Jacobsen, and Harrington, 1968). The remaining lipid must then be obtained from the worm's diet. Since the female worm produces its own weight in eggs every 10 days, the efficient uptake of lipids would be of prime importance (Fairbairn, 1957).

In conjunction with the movement of glucose, Harpur and Popkin (1973) observed an increased fluid movement and intercellular swelling with glucose uptake. This may be a mechanism for maintaining the hydrostatic skeleton that is necessary for movement in the Ascarid. Harpur and Popkin used sac preparations of the intestines from Ascaris and measured fluid movement from the mucosa to pseudocoelomic surface

under a variety of conditions. In general, fluid will move in response to an osmotic gradient, but under isosmotic conditions, fluid moves from the mucosa to the pseudocoelomic side of the tissue sac. This movement of fluid was enhanced when glucose was added to the sacs of intestines.

Ion Movement

Most cells have mechanisms which enable them to maintain ionic concentrations different from their environment. The production of ion gradients and the maintenance of these gradients often requires metabolic energy. Ions that are moved against a concentration gradient and require energy are actively transported. Other types of ion movement are not directly related to energy requirements and move according to their concentration or electrical gradients, the process being referred to as "passive diffusion." Both the active and passive movement of ions probably occur in Ascarids, but the exact mechanism has yet to be determined.

There is some evidence for the existence of a ouabain sensitive Na^+-K^+ pump in Ascarid intestines (Harpur and Popkin, 1973). However, Schanbacher (1974) found no evidence of a change in uptake of radioactively labeled 3-O-MG with ouabain treated intestines.

A low concentration of sodium in the mammalian intestinal cell has been shown to be necessary for the uptake of glucose (Crane, 1968). This mechanism of glucose uptake involves a carrier mediated cotransport of sodium and glucose. The diffusion gradient of sodium is the force which enables the cells to take up glucose against a

concentration gradient. The passive movement of sodium into the cell is dependent on the active transport of sodium out of the cell.

In mammalian systems, the cotransport of glucose and sodium can be blocked with phlorizen. Phlorizen acts as a competitive inhibitor of glucose absorption by reducing the affinity of the transport system for its substrate (Crane, 1965). Phlorizen in the luminal solutions of Ascaris intestine sac preparations caused a reduction in the uptake of 3-O-MG. The ability of phlorizen to inhibit 3-O-MG uptake suggests that there is a carrier-mediated process in Ascaris that is similar to the mammalian system (Schanbacher, 1974). Merz (1977) also concluded that a carrier system was involved with glucose uptake, as he found that the uptake system for glucose could be saturated.

The uptake of glucose in Ascaris has been shown to require metabolic energy, and it is temperature sensitive (von Brand, 1966). Schanbacher (1974) demonstrated that iodoacetamide and sodium fluoride, inhibitors of glycolysis, depress the uptake of 3-O-MG, thus identifying the metabolic requirements of glucose uptake. Schanbaker also showed that the uptake of 3-O-MG was dependent on the presence of sodium ions in the media. More recently, Beames, Merz, and Donahue (1981) measured an increase in cell sodium with the addition of glucose on the mucosal surface of Ascarid intestinal cells. The normal concentration of sodium ions measure 54±mEq and potassium ions measure 173±35mEq. Thus, the maintenance of a low cell sodium in the Ascarid intestine would provide the gradient for the possible sodium-

dependent uptake of glucose; the glucose uptake being dependent on both the active and passive movement of sodium.

The passive diffusion of ions may change, depending on the integrity of the cell. Cells injured by heat, radiation, electrical overstimulation, and abnormal pH or ion imbalance are generally known to change permeability characteristics. Active transport processes may no longer be able to maintain the normal ionic balances, and death ensues.

Donahue (1977) found that the permeability of the basement membrane of Ascarid intestine to certain ions varied, depending on the pH of the bathing solution. Under acidic or neutral pH the membrane is permeable to Cl^- but not to Na^+ or K^+. At alkaline pH's, the membrane is no longer permeable to Cl^-, but is to Na^+ and K^+. The results indicate that under normal conditions, the basement membrane is more permeable to anions than to cations. Merz (1977) calculated the relative permeability coefficients for radioactive Na^+ to K^+ and Cl^-. He found that the isolated basement membrane was more permeable to Cl^- than the intact intestinal membrane. There were no differences between the two tissues' permeabilities for Na^+ and K^+. He proposed a system where Na^+ is pumped into the lateral spaces between intestinal cells, and Cl^- ions would follow down the electrical gradient. Na^+ would diffuse through the apical junction to the mucosa, while Cl^- would tend to diffuse out of the lateral spaces and basement membrane to the pseudocoelomic side of the tissue. This model of positive movement to the mucosa and negative movement to the pseudocoelomic side is one explanation for the electrically positive nature of the mucosal side of the worm's intestine.

Electrical Activity

The movement of charged molecules across a tissue can be monitored electrically. Ussing and Zerahan (1951) developed a chamber which was used to record electrical changes that develop across a frog skin. In this system, the frog skin acts as a membrane which separates two fluid filled chambers. With this apparatus, small ionic changes can be measured as electrical changes. These are usually measured in millivolts (mV). An unequal distribution of ions results in a potential difference across the tissue. The mV potential differences are monitored by two potassium chloride-agar bridges on either side of the tissue. These bridges are connected to calomel electrodes and a potentiometer. Potential differences across a whole tissue is referred to as the "transepithelial potential," or TEP. Potentials can also be measured across cellular membranes using microelectrodes placed inside the cells. These potentials are called "membrane potentials," or PD.

Current electrodes can be placed on either side of the tissue through which a measured amount of current can be passed. Enough current can be "pushed" through the tissue to drive the potential difference to zero. This is the short circuit current (SCC). The current, measured in microamps (uA), is often considered to be a measure of the quantity of charged ions moving through the cell by active transport. In the case of frog skin, the SCC is due to sodium ion transport. When sodium is replaced in the bathing solution with cesium or choline, the TEP is no longer maintained, indicating that sodium is responsible for the potential and current. Radioactively

labeled sodium fluxes can be measured across the frog skin. Movement against an electrogenic force; i.e., under SCC conditions, indicates that sodium is being actively transported.

The quantity of ions moving in active transport may not always be accurately measured with an SCC. If the ion pump is electrogenic and pumps ions only in one direction, the SCC would provide a more accurate measure of ion movement than when two similar ions are exchanged. Such exchanges exist in some tissues as a one for one exchange of NA^+ for H^+ or a three to two exchange of Na^+ for K^+ (Kidder, 1973). In these cases, radioactively labelled ions can help identify actively transported ions. If radioactively labeled ions are able to move against both a chemical and electrical gradient (under SCC conditions), then there is some reason to suspect active transport of that ion. If this movement of ions, either monitored by radioactive fluxes or electrical measurements, is reduced by certain metabolic inhibitors, then there is an even stronger case for active transport.

Unequal distribution of ions is also possible when a membrane is semipermeable and there can be a difference in the osmotic pressure, hydrostatic pressure, and electrical potential generated by the unequal distribution of ions (Gutknecht, 1970). The potential difference thus generated would be due to the passive diffusion of the permeable ions through the semipermeable membrane. This movement of ions would not proceed if the systems were placed under SCC conditions.

The behavior of ions under SCC conditions has been used by several investigators to identify active or passive movement of ions. Generally, if the molecules or ions do not conform to expected passive behavior, then there may be other forces involved (Gutknecht, 1970).

It has already been mentioned that active transport can often be identified by the movement of ions against an electrochemical gradient. The cotransport of ions with nutrients (i.e., glucose-sodium cotransport) is another example of deviatons from nonpassive behavior. The carrier involved in the cotransport facilitates the diffusive processes.

It is likely that several of these transport processes are occurring simultaneously in the same tissue. In the case of epithelial transport, one is usually dealing with a multimembrane, several compartment system. For example, Na^+-K^+ active transport provides the sodium gradient which powers the facilitated diffusion of glucose while the other ions such as chloride diffuse in response to their electrochemical gradient.

Whether certain ions move across the membranes of the cell or through the junctions between the cells has long been a question. Tissues which have a low transepithelial electrical resistance (6-133ohm-cm^2) are called "leaky" epitheliam and higher resistance tissues (362-2,000 ohm-cm^2) are referred to as "tight" epitheliam (Fromter and Diamond, 1972). The conductance of ions through leaky epitheliam can be high, resulting in low TEPs (0-11mB). Tight junctions typically have 30-100mV TEPs. This increase in ionic conductance is often associated with a high osmotic water permeability. Tissues which are known for their water transporting capabilities are also known to be electrically leaky epitheliam.

Electrical Measurement in *Ascaris* Intestinal Epitheliam

The electrical characteristics of the Ascarid intestinal epitheliam include the development of a 15-30mV TEP with the luminal or mucosal membrane being positive in relation to the pseudocoelomic side (Merz, 1977). The potential is zeroed with a 20-30uA SCC. When glucose is added to the mucosal surface of the isolated intestine there is a corresponding decrease in the negative TEP and SCC. Microelectrodes placed in the intestinal cells record a change in membrane potential and an increase in intercellular sodium when glucose is added to the medium (Beames, Merz, and Donahue, 1981). Beames, Merz, and Donahue suggested that this sodium-glucose uptake is the mechanism involved in establishing an osmotic gradient for the uptake of water that maintains the hydrostatic skeleton. Hypothetically, some chemicals used to kill parasites may function by disrupting the mechanism for maintaining the hydrostatic skeleton, thus reducing parasite movement necessary in feeding and remaining in the hosts' intestine.

Anthelmintics

Almost 100 years ago, Paul Earlich said that the aim of chemotherapy was to use drugs to kill an invading organism without injuring the host (Cohen, 1979). He hypothesized a chemical receptor site on microorganisms whose inhibition would result in specificity of drug action. It is now known that the selectivity of drugs is often the result of the difference between the metabolism of the two organisms, as well as receptor specificity. The ultimate aim of research with

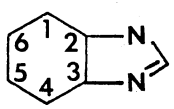
chemotherapeutic agents is to design compounds that act selectively on the parasite (Desowitz, 1971). Since the metabolic mechanisms of helminth parasites are still in the early stage of understanding, only limited access has been obtained with drugs directed to specific receptors.

Structure Activity Relationships

Most currently effective anthelmintic compounds were developed empirically, with the mechanism of action being determined later (Cohen, 1979). The first study which attempted to measure the interdependence of drug activity and structure was done by Overton (1901). He correlated the narcotic action of organic molecules and their oil and water partition coefficient. The more oil soluble compounds were more apt to get through the lipid membranes and affect the cells. A more recent structure analysis was developed by Hansch, Maloney, and Fugita (1962) and is based on the assumption that there is a reduction of variation in biological response when there is a combination of free energy related parameters. This analysis involves cataloging the molar concentration of the compound that gives the desired lethal dose, a lipohydrophilic substitution constant, an electronic substituent constant, and a steric constant. Different types of compounds can be cataloged by this system, also referred to as the "quantitative structure-activity relationship," or QSAR (Tollenare, 1971). Tollenare (1975) compared the inhibition of a series of substituted phenols on succinic dehydrogenase activity from rat liver and Ascaris suum muscle mitochondria. He found that the inhibition of succinic dehydrogenase was enhanced by increasing the lipophilic and electron

withdrawing character of the phenol side groups. The dependence of the activity on the lipophilic character indicated that adsorption from an aqueous media to the side of action is enhanced by the presence of lipophilic groups. Tollenare also found that the phenols with the lowest pKa values were most active. At physiological pHs, then, the most active components are completely ionized.

The group of anthelmintics referred to as the benzimidazoles can also be easily substituted with different side groups. The benzimidazoles, mebendazole, parbendazole, albendazole, and cambendazole, have a carbamate function and all have a group on position 5 of one phenol.



Thiabendazole has a similar phenol nucleus, but it is not substituted in this position and has no carbamate function. Thiabendazole does not effect glycogen utilization in helminths, while the compounds with a carbanate group do effect this cellular function. However, thiabendazole does inhibit fumaric reductase activity. Thus, structural modifications have been shown to either enhance anthelmintic activity or completely change the mechanism of action of the compound.

Mechanism of Action

The mechanism of action of anthelmintics has been divided into groups containing compounds that inhibit muscle contraction and those that interfere with energy processes (Saz, 1970). Some compounds may

affect both functions, but may require very high concentrations for both systems to be effected.

One anthelmintic that has a direct selective toxicity to Ascarid muscle is piperazine. This compound blocks neuromuscular transmission competing with acetylcholine-induced contractions and paralyzes the worm. Alternatively, d-tubocuraire, a potent neuromuscular blocker of mammals has only a very limited effect on Ascaris (Saz, 1970). Another drug, L-tetramesol (levamisol), also acts on the nematodes' nervous system, resulting in the sustained contraction of nematode muscle (Coles, 1977), resulting in the sustained contraction of nematode muscle. The D- isomer is less active (Van den Bossche, 1976). Levamisol and piperazine have reversible effects, the worm regaining activity when the drug is washed from the worm. High concentrations of levamisole are also known to inhibit activiey of isolated fumaric reductase from ascaris mitochondria. However, worms that do not possess fumaric reductase are paralyzed by levamisol, indicating that the primary mode of action of this compound is not related to the disturbance of this enzyme. Verhoeven, Willemsens, and Van den Bossche (1976) found that there was no difference in the uptake of tritiated levamisol when the worm was ligated, suggesting the mechanism of levamisol uptake is mainly through the cuticle and not via the digestive tract.

Benzimidazoles

The benzimidazoles are a class of compounds that interfere with energy processes in round worms (Saz, 1970). As was mentioned earlier, the exact mechanism of action is difficult to determine, as

information on nematode metabolism is still not well defined. Coles (1977) reported that the benzimidazole compounds are known to kill both the adult and egg stages of parasites. Van den Bossche (1976) stated that benzimidazoles are also potent uncouplers of phosphorylation. Mebendazole is one of these compounds which is found to inhibit phosphorylation in Ascarid mitochondria. However, at lower concentrations, this compound inhibits the uptake of glucose into the intestinal cells. Coles reported that mebendazole is found in high concentrations in the intestinal cells, indicating the probable uptake route of the drug and tissue of interference in the intestine. Intracellularly, mebendazole is associated with high molecular weight proteins in the intestinal cells (Van den Bossche, 1976). Ultrastructural changes observed by Borgers, DeNullin, DeBrabander, and Thienport (1975) included the disappearance of cytoplasmic microtubules, blockage of secretory granules resulting in an accumulation in the cytoplasm, followed by cell lysis and degeneration of the intestinal cells. These secretory granules are thought to carry the microvillus coating which may be responsible for the cells' ability to do such activities as take up glucose. The host intestinal cells are not affected either structurally or functionally by mebendazole. The decrease in glucose uptake in the Ascarid intestine is accompanied by a reduction in glycogen stored in the intestinal and muscle cells (Van den Bossche, 1976; Coles, 1977). The decrease in endogenous carbohydrate would be followed by lowering the energy supply and causing death of the organism by starvation. Simpkin and Coles (1975), however, found that Nipastrongelus dubius (N. dubius), a nematode found

in mice, demonstrated no decrease in glycogen before expulsion from the mouse, indicating that mebendazole's action may not be clear cut.

Other benzimidazoles, parbendazole and cambendazole, also reduce glycogen levels in Ascarids and other parasites (Cole, 1977), while thiabendazole does not affect tissue glycogen levels. Thiabendazole resistant Hymenelopis contortus (H. contortus) is also resistant to cambendazole, suggesting some similar enzyme system may be affected. Thiabendazole inhibits fumaric reductase activity, but some parasites do not have fumaric reductase and are still inhibited by this drug (Simpkin and Coles, 1975). Cambendazole also inhibits fumaric reductase activity, but there is no cross resistance to levamesol in H. contortus (Van den Bossche, 1976). Thiabendazole and cambendazole are both more potent inhibitors of fumaric reductase, but levamisol is a more powerful anthelmintic, indicating once again that levamisol acts on other areas.

The variety of effects of the benzimidazoles make it impossible to suggest a single mode of action for all members of this group. Structural modifications around the benzimidazole nucleus may not only change the anthelmintic activity of the drug but may change the mode of action, thiabendazole being 10 times more active as an anthelmintic than mebendazole (Van den Bossche, 1976). All of the benzimidazoles bind to tubulin and prevent microtubular formation. Van den Bossche (1976) suggested that resistance and cross resistance may not be just due to a common mode of action, but may be due to a change in the properties of the target sites, an increased breakdown of benzimidazole, or a failure or drug uptake.

Identification of Anthelmintic Activity

In the previous section, reference has been made to various criteria for evaluating anthelmentic activity. Ultrastructural observations indicate that some of the anthelmintics effect the integrity of the cells, while others inhibit enzyme activity, nutrient uptake or muscle mobility. Beames, Merz, and Donahue (1976) have used the uptake of certain ions and changes in electrical activity to evaluate some of these same anthelmintics and a variety of other metabolic inhibitors.

The development of a spontaneous electrical potential across the isolated intestine of Ascaris suum has already been described. Beames, Merz, and Donahue (1976) found that the metabolic inhibitor sodium fluoride and idoacetamide (IAA) both decreased the transepethelial potential difference when compared to the TEP of an untreated section of the same intestine. D-tetramesol had no effect on the TEP, but the L-isomer decreased this TEP as do mebendazole and thiabendazole. Collectively, these data suggest that the TEP is influenced by compounds which are known to inhibit the carbohydrate metabolism of the tissue or the structural integrity.

Measurement of the uptake of radioactive sodium by Beames, Merz, and Donahue (1976) has also given insight into the function of some inhibitors. IAA and levamesol did not change the normal sodium uptake of isolated Ascarid intestines, but sodium uptake by mebendazole treated tissue was different from the controls. This would suggest that mebendazole effects of the intestine by a mechanism different from the metabolic inhibitor, IAA. Transepithelial potentials are

first considered in the following experiments to test the viability of the premise that electrical activity changes in anthelmentic treated intestines do correspond to *in vivo* drug effectiveness. Selected anthelmintics and inhibitory compounds are subsequently used to describe cellular electrical and ion changes in Ascarid intestinal cells. A composite of these data and previous works may then be used to further describe the mechanism of action of these anthelmintics, as their actions effect the ion and electrical balance. These data will be used to further define the normal functions of the intestinal cells of *Ascaris suum*.

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CHAPTER III

METHODS

Tissue Collection

Ascaris suum were collected from pigs at a meat packing plant in Oklahoma City, Perkins, and Arkansas City. Adult female worms were placed in 36°C Harpur's saline for transport (Harpur, 1963). At the meat packing plant the temperature ranged from 35-38°C, and did not drop below 30°C upon return to the laboratory. The worms were maintained in an incubator (36°C) in the laboratory. The saline was changed at least once a day for the life of the worm, which is usually no more than three to four days. The intestine disintegrates when the worms are held under these conditions.

Transepithelial Potential Procedure

To measure the transepithelial potential (TEP) and short-curcuit current (SCC) across adjoining sections of intestine, it was necessary to develop a double chamber which would reduce chamber effects seen in previous studies by Beames (unpublished observation). This chamber is diagrammed in Figures 2 and 3. The chamber allows for equal compression of both sections of tissue and equal temperature control of saline.

The Ascarid's cuticle was slit open from posterior to anterior with surgical scissors. The mid-intestine was removed by cutting it at

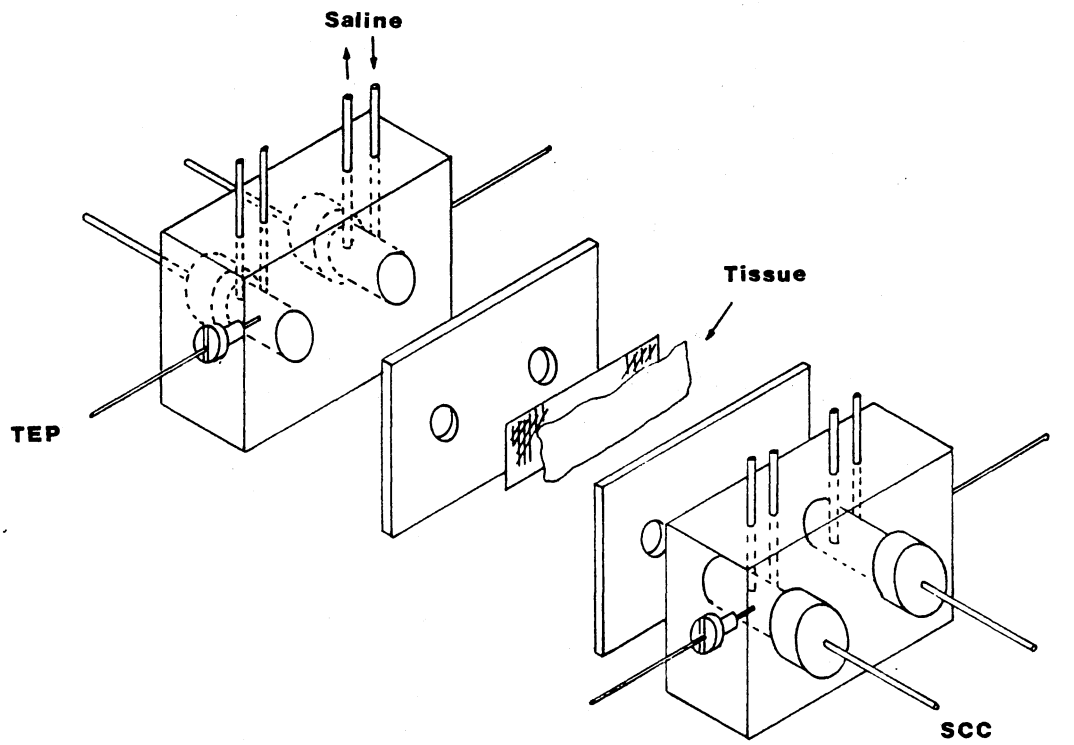

Figure 2. Dissection of Female Ascaris suum Showing the Location of the Mid-Intestine Used in Experiments

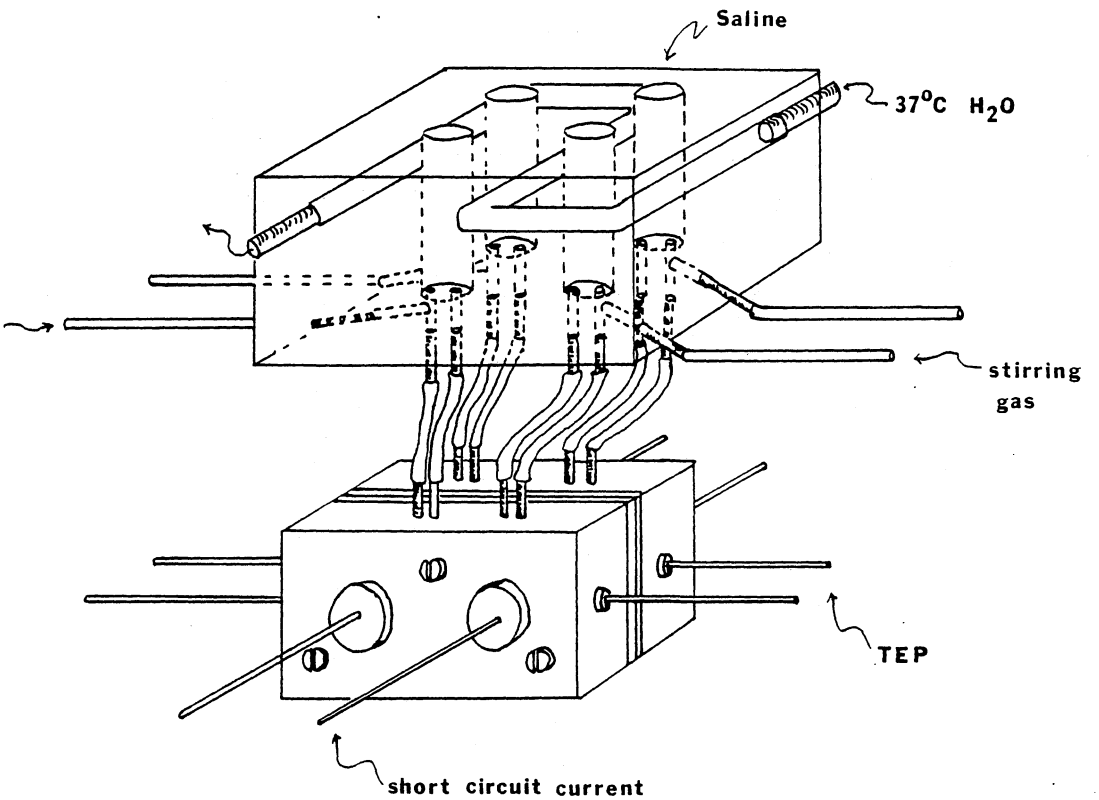

Figure 3. Tissue Preparation of Ascaris suum

the region near the vagina and before the posterior attachment to the body wall (Figure 4). The intestine was gently pulled through the mass of reproductive tissue and placed in a petri dish of room temperature saline. The posterior part of this intestinal preparation was slit open and placed on a piece of silk screen mesh, mucosal surface up (Figure 5). This 1 x 2.5cm ribbon of tissue and screen was placed between the two halves of a double perfussion chamber (Figure 2). Vacuum grease was lightly applied to the surfaces of the chamber in contact with the tissue and silk screen to make a waterproof seal. The three screws which hold the two halves of the chamber together were carefully tightened. The tissue from one worm was thus effectively divided into two adjoining segments. The exposed surface area of each tissue segment was $0.126cm^2$.

Two milliliters of modified Harpur's saline was perfused over the mucosal and pseudocoelomic surfaces of each adjoining section of the intestine (Merz, 1977). Stirring of the solutions was accomplished by a bubble lift pump (Figure 3). A variety of gasses can be used to stir this bathing solution. A gas mixture (5% CO_2, 95% N) was used in drug screening experiments using TEP as a criterion for anthelmintic activity. The gas bubbling rate was adjusted by thumbscrews. The temperature of the saline was maintained at 36°C by a surrounding warm water jacket.

On each side of both paired tissues, 3.0 molar KCl-agar bridges were in contact with the solutions. The TEP was recorded via these bridges as they were in contact with calomel electrodes and voltage followers attached to chart recorders. Two Heathkit chart recorders independently measured the TEP for each adjoining section of intestine.

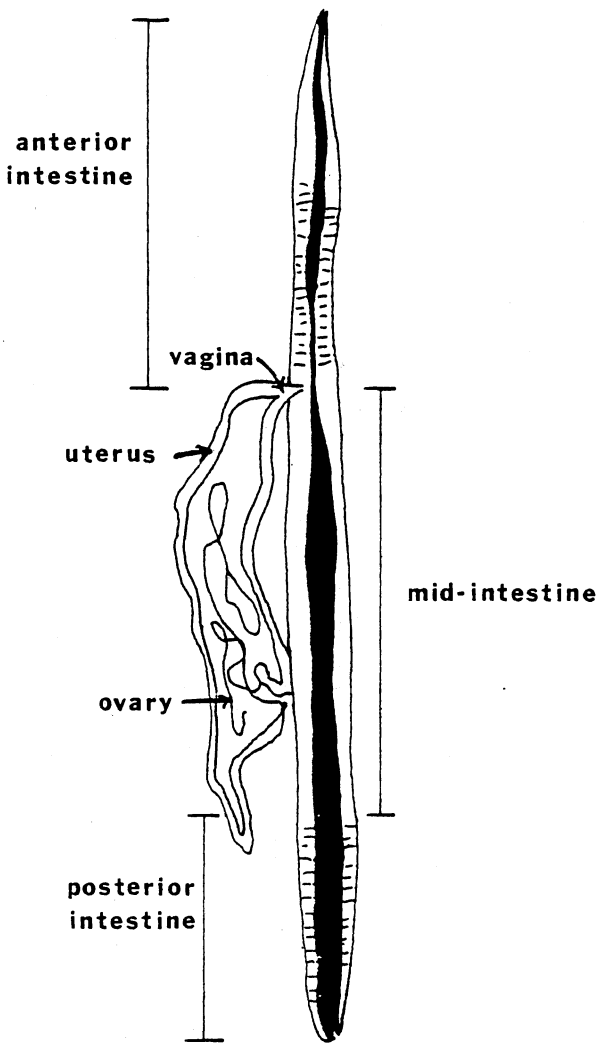

Figure 4. Positioning of Ascaris Intestine in Double Chamber

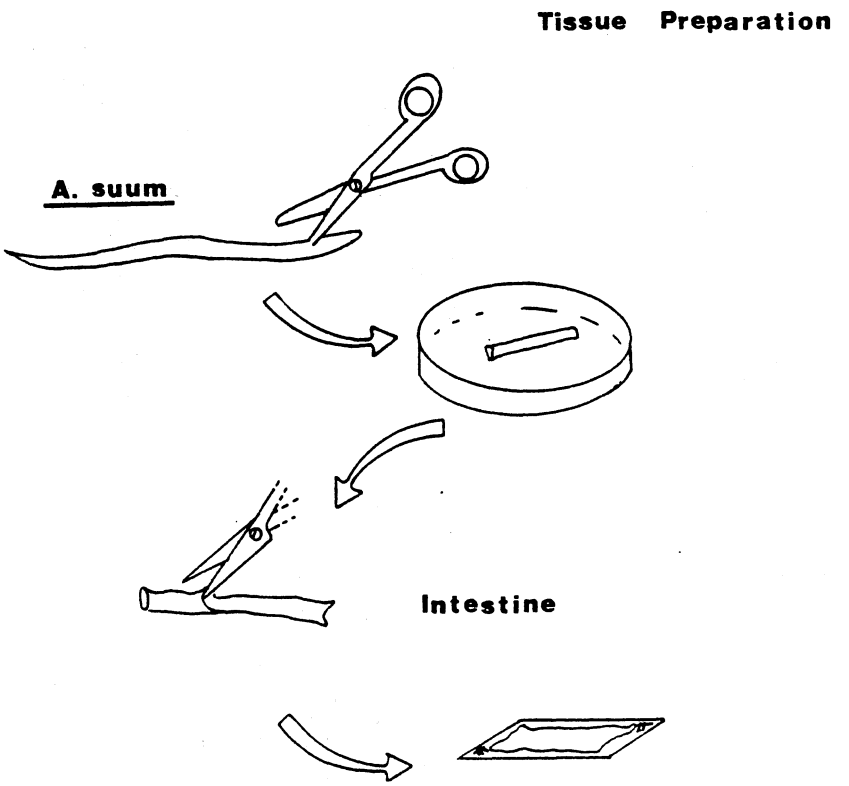

Figure 5. Stirring and Warming Chamber for Perfusing Saline.(Stirring gas was 5%CO_2 95% N_2)

Silver-silver chloride electrodes were also positioned on each side of the tissue. An automatic voltage clamp pushed current via these electrodes through the tissue to zero the TEP. The amount of current needed to zero the potential is called the SCC. This SCC was also displayed on the two chart recorders.

After the initial potential for each tissue section had been recorded and observed to be within 10mV of each other, 10mM glucose was added to the pseudocoelomic solution for each tissue section. The potential generated across an Ascarid intestine has been observed to be more long lived with the addition of glucose to the pseudocoelomic bathing solution (Merz, 1977).

Compounds to be tested in this system were all dissolved in dimethylsulfoxide (DMSO) to avoid differences in drug solubilities (Van den Bosch, 1976). The final concentration of drug, dissolved in 10µl of DMSO, was 0.5mM when added to the two milliliters of saline. The compound was added to the mucosal and pseudocoelomic surface of one section of the intestine. The accompanying section served as the control and was simultaneously treated with 10µl of DMSO on both surfaces.

Drugs or solvents were introduced to the chambers after the potential had reached its peak. The duration of the potential and SCC measurements depended on the initial magnitude and effect of the drug treatment. Large potentials (35-60mV) may last for more than one hour, while smaller initial potentials (10-30mV) are not usually as long lived.

Criterion for discarding a particular preparation were based on the initial similarity between the potential and SCC measurements of

the two adjoining intestinal sections. Large differences may indicate that one of the sections has been damaged in the preparation and would not then be used as a control tissue.

At the end of the TEP recording, 10mM glucose was added to the mucosal surface of both the drug treated and the control issue. SCC measurements were taken before and after the addition of glucose. Changes in the SCC before and after the addition of glucose has been used by Beames, Merz, and Donahue (1976) as a criterion for demonstrating anthelmintic action of certain drugs on the isolated intestine.

In vivo Drug Screen

Many of the compounds tested by the TEP system were also tested in an in vivo mouse screen. The mouse screen is a standard method used by Dow Chemical Co. to test for possible anthelmintic activity in new compounds. Twenty milligrams of the compound being tested were mixed in 40 grams of Purina mouse meal. The mice were infected per os with 20 larvae of Nematospiroides dubius, 200 eggs of Hymenolepis nana, as well as 100 larvae of Nippostrongylus brasiliensis subcutaneously. Mice were then randomly assigned to control and treatment diet groups. Three days later, after one day of fasting, the intestines of the mice were removed and the parasites were counted. Drug efficacy was determined as a percentage reduction in the treated group compared with the average worm burdents in the untreated mice. A similar amount of levamesol used in this mouse screen reduced the parasite population 100% (Hatton, 1980). The percent efficacy from this in vivo screen is later compared to the results of the same compounds used in the TEP in vitro screen.

Microelectrodes

Measurement of the cellular membrane potential (PD), as well as the cellular or activities of sodium and potassium ions, were determined using microelectrodes. A vertical puller was used to produce a glass microelectrode tip from a single barrel borasilicate glass tubing (Omega Dot) that was less than one micron in diameter or which gave eight mega ohms of resistance across the electrode tip. The potential electrode was prepared by backfilling with a 3.0M potassium chloride solution topped with mineral oil to prevent evaporation. The ion sensitive electrodes were prepared by first dipping the tip in a one percent Prosil solution for five seconds and drying in a 110°C oven for several hours. After cooling, the electrodes were backfilled with the ion sensitive resin (Simon's for sodium and Corning 477317 for potassium ion exchange, Beames, 1971). A small dot of resin placed on the end of the barrell quickly moved to the tip. The electrode was then backfilled with 150mM or NaCl solution, depending on the ion sensitive resin used. The ion sensitive electrodes were stored and equilibrated in 150mM solutions of the same ions with which they were backfilled. Silver/silver chloride wire was placed in the barrel of the microelectrode and connected to a high impedance voltage follower (Burr-Brown 3431J Op-Amp). The reference electrode was a 3.0M CKl-agar bridge with a calomel electrode interface. The microelectrode was positioned for cell impalement using a Brinkman motorized micromanipulator (Figure 6). The system was protected from outside electrical interference by placing all the components in a

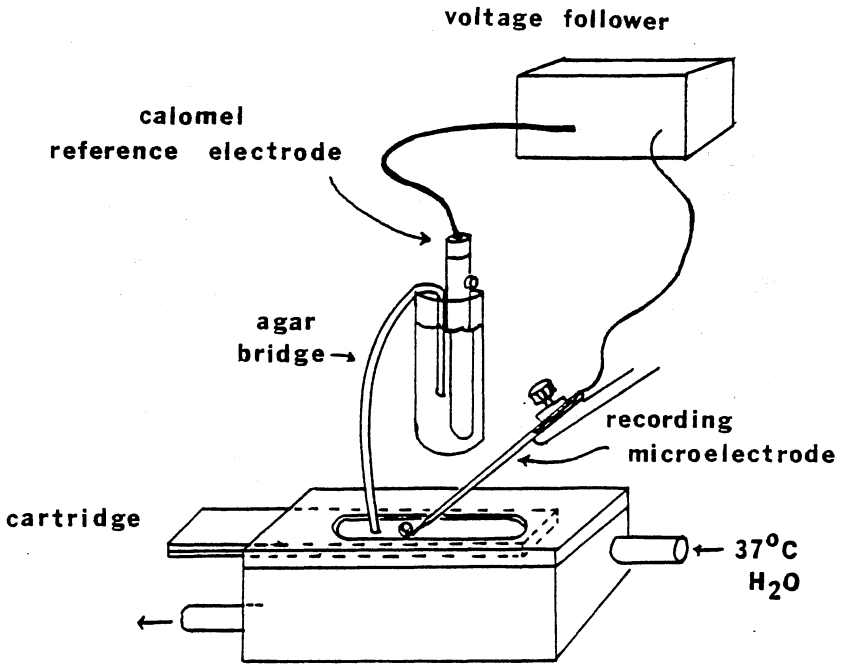

Figure 6. Microelectrode Chamber With Reference Electrode Followed by a High Impedence Burr-Brow Op-Amp and Recorded With a Meter and Chart Recorder

Faraday cage. A digital meter and Heathkit chart recorder are both used to record the output signals.

Potential electrodes were zeroed in the tissue bathing solution before penetrating the mucosal surface of the tissue. Ion sensitive electrodes were tested for their selectivity by zeroing the potential in a 25mM solution of the ion being tested. When the electrode is placed in a 250mM concentration of the same ion, there should be a 50 to 60mV slope (Nearnstian potential change). An unstable electrode is recognized as the potential will not stabilize with a decade change in ion concentration. Such an electrode is not used. To determine whether sodium ions compete with potassium ions for either the sodium or potassium sensitive electrodes, several dilutions of the competing ion were added to a 100mM solution of the ion for which the electrode is sensitive. Neither the sodium or potassium sensitive electrode were affected by the competing ions. A standard curve was generated when the negative log of the ion activity was plotted against the millivolts measured with the electrode (Figures 7 and 8).

Ion selective microelectrodes record changes in membrane potential as well as changes in ion activity. To compensate for the membrane potentials' addition to the millivolts due to ion activity, the PD was measured separately with a 3.0M KCl potential microelectrode. This PD was subtracted from the potential obtained with the ion sensitive microelectrode. The remaining potential recorded with the ion sensitive microelectrode is then due to the ions within the cell. The actual activity of those ions was found by taking the log of PD due to the ion activity divided by the slope of the microelectrode, multiplied plied by the ion concentration in the saline, as follows (Thomas, 1978):

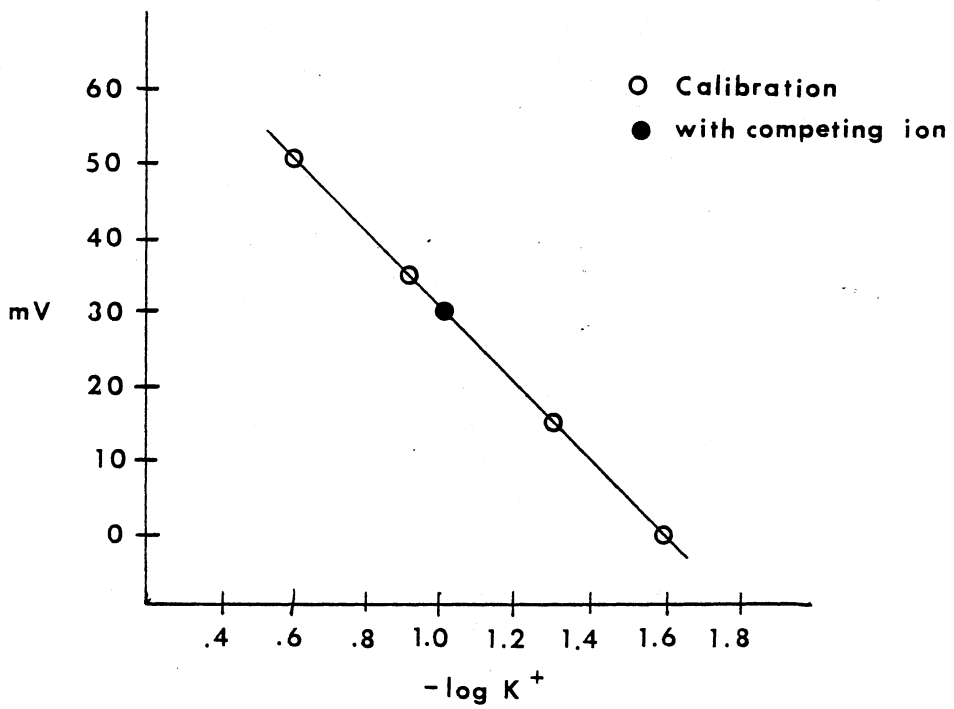

Figure 7. Potassium Ion Selective Calibration Curve From One Electrode

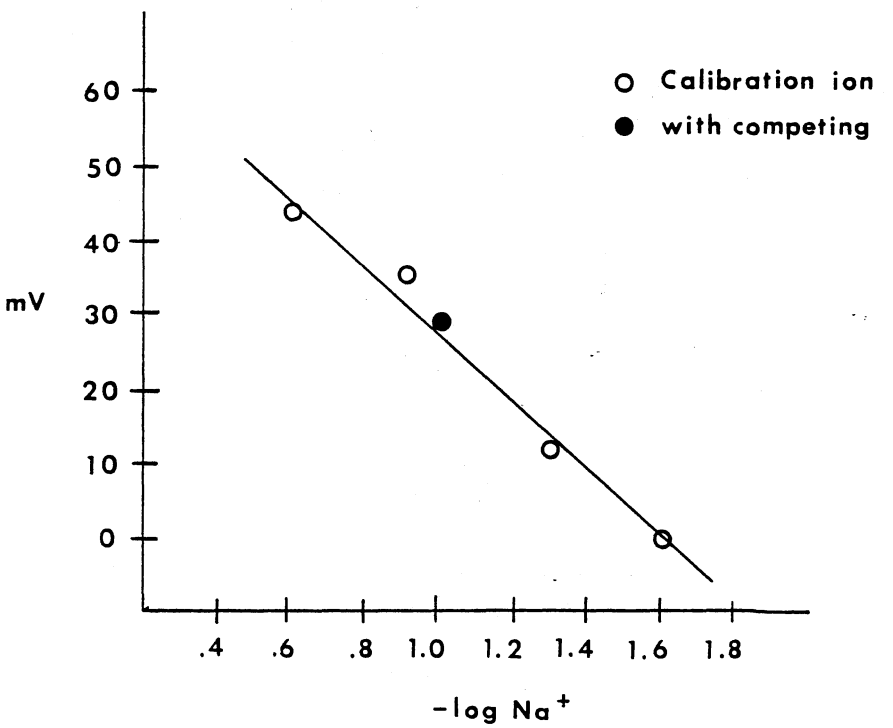

Figure 8. Sodium Ion Selective Calibration Curve From One Electrode

$$e^{mV/slope} \times (ion)\ out$$

Microelectrode Tissue Preparation

Intestines from female Ascaris suum were prepared similarly to those which were used to measure transpithelial potentials. The intestine-silk screen preparation was placed on a cartridge made of two lucite panels with 0.125cm openings. Vacuum grease was used to insure a tight seal. The cartridge was placed in the chamber with the mucosal surface of the intestine on top. When the chamber was prefilled with saline, the cartridge could be pushed into place. The mucosal and pseudocoelomic solutions were thus separated by a 0.125 cm^2 tissue and lucite cartridge. A 100mM $NaPO_4$, 24mM KCl, and 1mM CaCl solution adjusted to pH 5.6 was used to bathe the tissue. The PD, sodium and potassium activity were measured and compared with values from different in vitro ages of worm, tissue incubation time, and different anthelmintics and drugs.

Drug Treatment

Each tissue served as its own control. The initial membrane potential and sodium and potassium activity were measured after the intestine had been allowed to stabilize in the chamber for at least 10 minutes (Figure 9). After the initial determinations were made, the drug was added in 10 microliters of DMSO to give a final 0.5mM concentration on the mucosal surface. While the drug was incubating with the tissue, the slope of the ion sensitive electrode was determined. After 20 minutes, the ion activity and membrane potentials were again

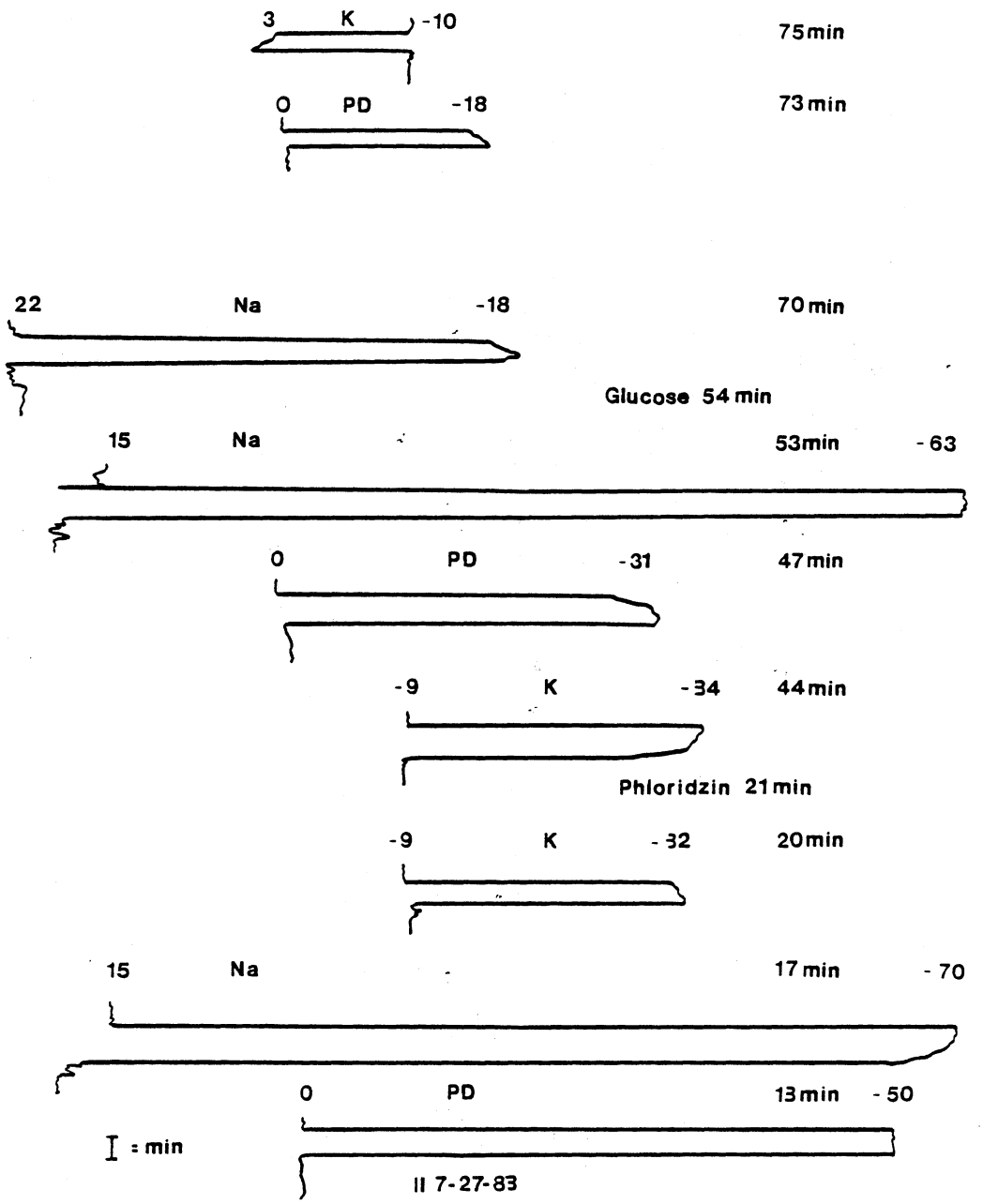

Figure 9. Sample Microelectrode Tracing. (Tissue was preincubated in saline 10 minutes before impalement with PO, Na^+, and K^+ electrodes. Tissue is incubated 20 minutes with 0.05 mm of a drug before similar determinations are again made, and 10 minutes with 20 mm glucose before the last impalements.)

determined. Glucose was then added to the mucosal solution to give a final 20mM glucose concentration. After 10 minutes, the same microelectrode measurements were made. The slope of the microelectrode was was determined before and after each impalement by the individual microelectrodes.

Statistics

Transepithelial Potentials

Six pairs of tissues were used for each compound tested. Chambers serving as the control were alternated to allow for instrumental variation and error in the analysis of the potential curves. Experiments were designed as two-by-two Latin squares repeated three times, for a total of six trials for each drug. Differences between the treatment and control were compared at five significance levels. The first level determined whether the control and treatment TEP were parallel or linear. The second level determined if the two curves were similarly described by a quadratic equation. Other levels involved fitting equations for cubic, quadratic, and higher order curvature.

Short Circuit Current

Selected drugs were measured for the SCC change across the entire tissue before and after the addition of glucose to the mucosal surface. The students' 't' test was used to test the significance of the SCC change at the 0.05 level.

Membrane Potentials and Ion Activities

Results were tested for significance at the 0.05 level using analysis of variance.

CHAPTER IV

RESULTS

Inhibitors and the Transepithelial Potential

Transepithelial potentials were simultaneously measured from the two adjoining sections of intestines in the double chamber. Figure 10 demonstrates the comparison of TEPs. In this figure, (A) is drug and control, while (B) is double control.

The results from the transepithelial measurements using a variety of compounds are listed in Tables I through III. These tables are arranged so that drugs are grouped in classes with certain structural similarities, and are identified by the drug name and structure (the structures may be found in Appendix A). The results from the statistical comparison of the drug treated tissue and their controls are in the adjacent columns. Only the linear and quadratic parameters of curve comparison proved to be significantly different when one section of the intestine was drug treated and the other served as a control. The degree of significance was recorded under the linear and quadratic column headings. There were six different worms used to test each compound, allowing for the statistical analysis of curve differences by a Latin squares design. Those compounds which had no effect on either the linear or quadratic curvature of the TEP curves are recorded as being not significant (ns). Those compounds which did

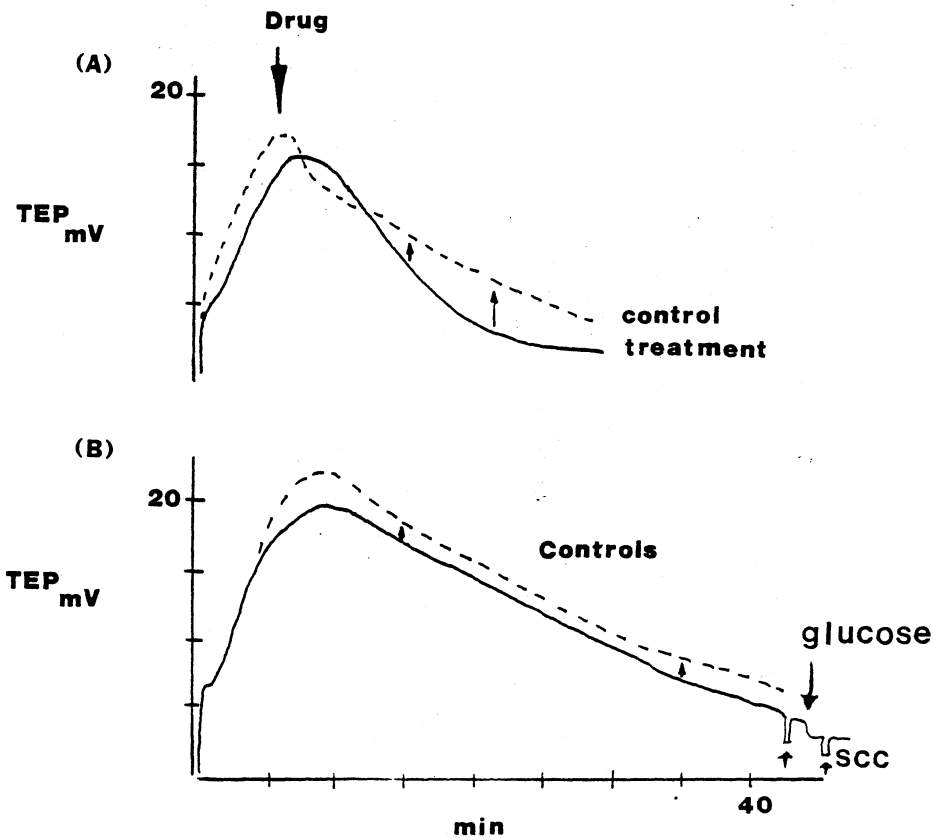

Figure 10. Comparison of Transepethelial Potentials From a Drug Treated and Control Tissue and Two Tissue Sections, Both Treated as Controls. (All experiments are completed by determining SCC before and after glucose is added to the mucosal solutions.)

TABLE I

DEGREE OF SIGNIFICANCE BETWEEN BENZIMIDAZOLE AND CONTROL TRANSEPITHELIAL POTENTIALS

Imidazoles	Degree of Significance	
	Linear	Quadratic
Thiabendazole	0.0001	0.001
Canbendazole	0.0001	ns*
Albendazole	0.07	ns
Parbendazole	ns	ns
Mebendazole	0.02	0.08
Fenbendazole	ns	ns
L-tetramesol	ns	ns
D-tetramesol	ns	ns
Tioxidazole	ns	ns
DMSO	ns	ns
Control (saline)	ns	ns

Note: DMSO was used to dissolve all drugs. A final concentration of 0.5mM was added to both sides of the tissue.

*ns = not significant

TABLE II

DEGREE OF SIGNIFICANCE OF TEP AND PERCENTAGE OF PARASITES KILLED IN IN VIVO DRUG SCREEN OF NEW CHEMICAL DERIVATIVES

	Degree of Significance		in vivo
	Linear	Quadratic	% Effect
Imidazolones			
NSS-I-74	ns*	ns	nt*
NSS-I-75	ns	ns	nt
NSS-I-79	ns	0.03	8% C,** 73% N**
Imidazole-misc.			
NSS-I-75	ns	ns	nt
NSS-I-50	0.10	ns	nt
Pyraoles			
KRR-III-113	ns	ns	59% C, 0% N
KRR-III-46	ns	ns	46% C, 0% N
KRR-III-41	ns	0.01	54% C, 48% N
alpha methylene gama butyrolactones			
NSS-I-52	ns	ns	nt
KRR-III-114	ns	0.09	nt

*ns = not significant; nt = not tested

**C = Cestode; N = Nematode

cause a change in the TEP were recorded according to the degree the TEP curves were significantly different from their controls.

TABLE III

DEGREE OF SIGNIFICANCE OF TEP AND PERCENTAGE OF OF PARASITES KILLED IN *IN VIVO* DRUG SCREEN OF DIHYDROMIDAZO AND THIOZOLE

	Degree of Significance		*in vivo*
	Linear	Quadratic	% Effect
Dihydromidazo (2,1-b) thiozole			
ASR-II-22	ns*	nt*	nt
ASR-I-35	ns	ns	nt
ASR-II-28	ns	ns	nt
ASR-I-50	ns	ns	nt
NSS-I-28	0.001	0.01	0% C,** 39% N**
NSS-I-21	0.0001	ns	nt
ASR-I-47	0.0002	ns	nt
Thiozole			
NSS-I-54	ns	-.11	nt

*ns = not significant; nt = not tested

**C = Cestode; N = Nematode

Table I contains drugs that are presently available for anthelmintic therapy, as well as the DMSO and saline controls used to test the system. DMSO was used to dissolve the drugs before they were introduced into the chamber. Tables II and III contain newly synthesized derivatives that are being tested for their possible anthelmintic activity. The latter two tables have an additional column which records the compounds' activities in the *in vivo* mouse screen. The initial concentration of all the drugs was 0.5mM.

In Table I, the drugs which show both linear and quadratic differences are thiabendazole and mebendazole. Those showing linear changes are cambendazole and albendazole. None of the drugs had only quadratic effects. Parbendazole, fenbendazole, L-tetramesol, D-tetramesol, tioxidazole, and DMSO were not significantly different from their controls. In Tables II and III, the only compound showing linear and quadratic significance was NSS-I-28. Those showing only linear effects were NSS-I-21 and ASR-I-47, and those showing only quadratic significance were NSS-I-79 and KRR-III-41.

in vivo Screen

Only a few of the results were obtained from the *in vivo* mouse screen. The company conducting the mouse screen was looking for compounds that would kill 100 percent of the worm burden at concentrations equivalent to levamisole doses that have this same effect. None of the compounds were this active. A few of the *in vivo* results were given as percentages of worm burdens killed, which could be compared to the TEP *in vitro* screen (Tables II and III).

The percentage kill of the nematodes, Nipastrongelus brasilienses, and N. dubius, as well as the tapeworm Hymenelipis nana were tested in the in vivo screen. Some values were reported as the percentage kill of each of these parasites and some were reported as the percentage of tapeworms or nematodes effected without giving the parasite name.

The last column in Tables II and III gives the percentage effect of some of the compounds. NSS-I-79 killed 73 percent of the intestinal nematodes (23% N. dubius and 50% N. brasiliensis), and only eight percent of the cestodes (H. nana). This drug also altered the quadratic curvature of the TEP measured from isolated Ascarid intestines. KRR-III-113 and KRR-III-46 had no effect on the mouse nematode population, while both had some effect on cestodes (59% and 46%, respectively). Neither of these compounds had an effect in the TEP screen. KRR-III-41 killed 54% of the tapeworm population and 48% of the nematodes, as well as having a significant effect on the quadratic curvature in the in vivo screen. NSS-I-28 killed 39% of the nematodes (12% N. dubius and 27% N. brasiliensis) and no cestodes. This compound had a significant effect on both the quadratic and linear curvature of the TEP.

Short Circuit Current

The short curcuit current (SCC) was measured before and after 20mM glucose was added to the mucosal surface of the tissue at the end of the TEP determinations (Figure 10). The results from the SCC measurements from the same worms used in the TEP determinations are found in Table IV. The first column contains the compounds which were

TABLE IV

CHANGE IN SCC MEASURED BEFORE AND AFTER GLUCOSE ADDED TO MUCOSA

Compound	$\Delta\mu A/cm^2$ Treatment ($\bar{x} \pm SE$)	$\Delta\mu A/cm^2$ Control ($\bar{x} \pm SE$)	95% sig.	n
Thiabendazole**	5.0±1.2	7.9±2.7	ns*	6
Cambendazole**	4.0±0.4	7.9±0.7	sig*	6
Albendazole**	9.8±3.0	11.4±3.9	ns	5
Parbendazole	9.2±3.2	4.8±1.8	ns	5
Mebendazole**	7.4±1.2	10.3±2.4	ns	6
Fenbendazole	18.5±3.5	19.3±5.0	ns	6
L-tetramesol	10.6±2.2	14.6±2.3	ns	6
D-tetramesol	9.5±1.5	11.4±1.9	ns	6
Tioxidazole	8.5±1.3	6.6±1.9	ns	6
DMSO	6.4±1.1	4.5±0.8	ns	6
NSS-I-74	3.2±2.6	3.5±0.6	ns	6
NSS-I-73	5.4±1.0	6.9±1.0	ns	6
NSS-I-79**	4.2±0.7	6.6±0.9	sig	6
NSS-I-75	3.2±0.6	5.0±0.9	ns	6
NSS-I-50	4.8±0.8	5.0±1.8	ns	6
KRR-III-113	--	--	--	--
KRR-III-46	3.6±1.6	6.8±1.5	ns	5
KRR-III-41**	6.4±2.2	12.0±2.4	ns	5
NSS-I-52	--	--	--	--
KRR-III-144	12.2±1.6	13.1±2.4	ns	7
ASR7-II-22	10.5±2.0	9.5±2.1	ns	6
ASR-I-35	9.0±1.7	6.4±0.9	ns	6
ASR-II-38	--	--	--	--
ASR-I-50	4.8±0.8	5.0±1.8	ns	6
NSS-I-28**	7.4±1.8	11.8±2.5	ns	6
NSS-I-21**	--	--	--	--
ASR-I-47**	3.7±0.7	6.4±1.2	sig	6
NSS-I-54	6.3±1.1	13.3±1.8	sig	8

*ns = not significant; sig = significant

**These compounds are significantly different from controls in TEP measurements.

tested. They are arranged in the same order as they appear in Tables I through III. The mean and standard errors for each treated and control tissue are in the following columns. The values were recorded as the change in microamps per centimeter squared. The values were analyzed statistically with the students' grouped t-test and were tested for their significance at the 95 percent level. The last column contains the number (n) of experiments done to generate these values.

The only known anthelmintic compound that caused a significant change in the SCC was cambendazole. This compound also effected the linear curvature of the TEP. The new derivatives that caused a significant change in the SCC were NSS-I-79, ASR-I-47, and NSS-I-54. All of these compounds caused a significant change in the quadratic, linear, and quadratic curvature of the TEP, respectively.

Microelectrode Determinations

Figure 11 shows the average membrane potential, and the sodium and potassium activities measured in tissues after the initial 10 minute incubation period (PD = -40$\pm$10mV, sodium = 39$\pm$25mEq, and potassium = 166$\pm$64mEq). These same measurements were separated on the basis of *in vitro* worm age. The average values from worms collected and measured on day one are compared with values obtained from worms maintained in the laboratory for two and three days (Figure 12 and Table V). Intestines from worms kept for four or more days tend to fall apart during the tissue preparation. No significant variations were seen in the PD or the potassium or sodium activity in the worms kept for one to three days.

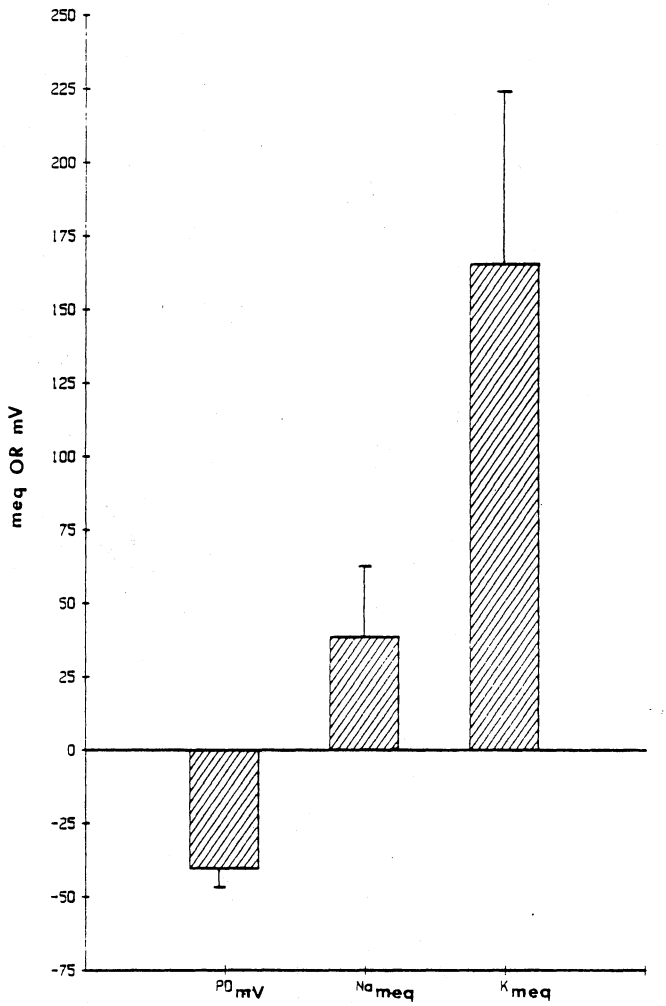

Figure 11. Average Membrane Potential, Sodium and Potassium Activities (n-47)

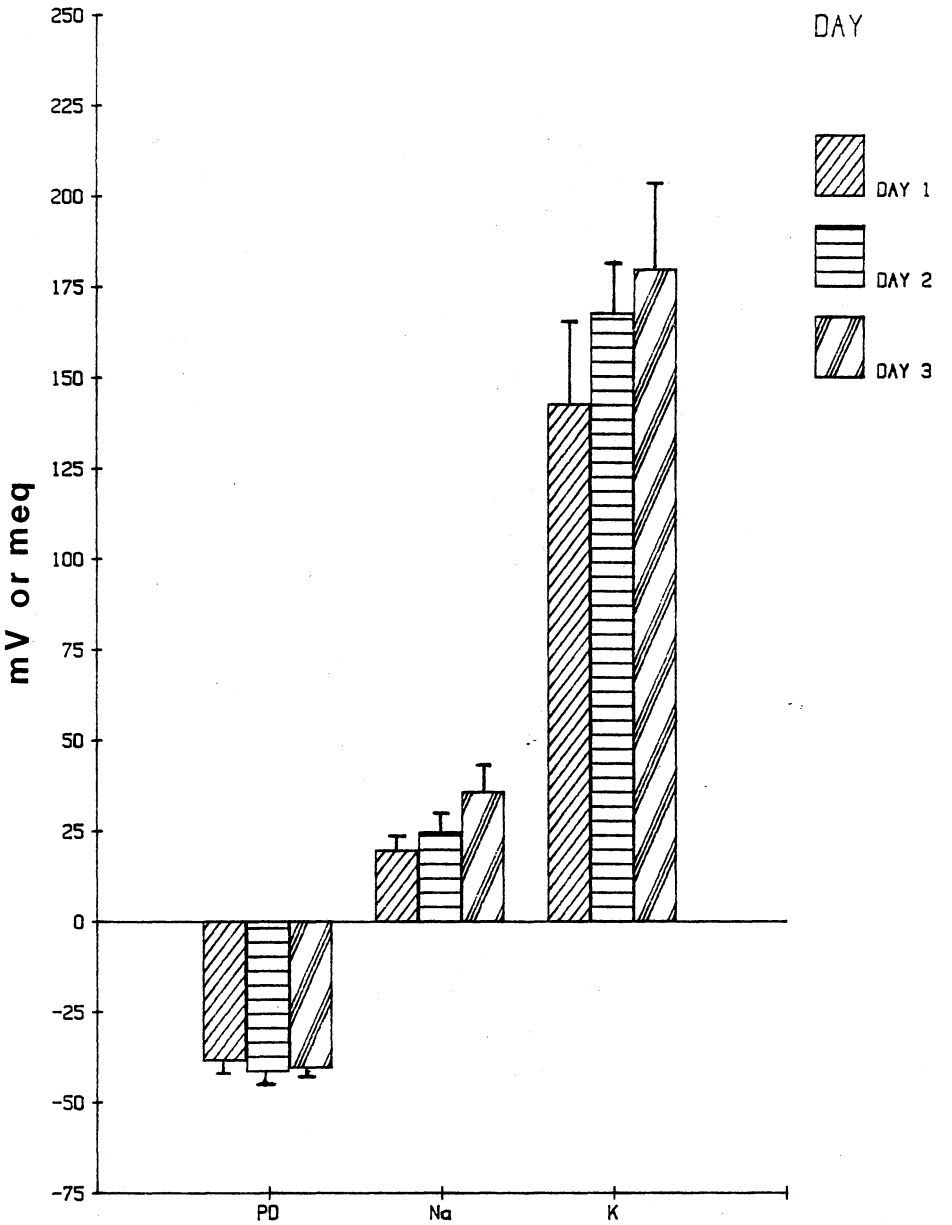

Figure 12. Changes in Membrane Potential, Sodium and Potassium Activities With <u>in vivo</u> Worm Age (n=34)

TABLE V

MICROELECTRODE VALUES COMPARING EFFECTS OF
IN VITRO WORM AGE

	Day 1	Day 2	Day 3
PD_{mV}	-38±(10)	-41±3(20)	-40±2(14)
Na_{mEq}	20±5(7)	25±6(16)	36±7(14)
K_{mEq}	143±21(7)	168±16(15)	180±23(11)

Note: Values are stated ±SE and number of determinations are in parentheses. Grouped t-test indicates indicates no difference in worms kept for one, two, or three days.

Microelectrode values in saline control tissues compared over an average 80 minute incubation periods are in Figures 13 and Table VI. Significant changes are seen in the reduction of cellular potassium after 80 minutes, along with a significant reduction in the membrane potential (becomes less negative). Figure 14 shows the results of multiple impalements (n=6) of one tissue. There is no difference in PD or sodium or potassium activities in cells of one tissue.

Figure 15 and Table VII show the PD, sodium activity, and potassium activity in the DMSO treated tissues. DMSO had no significant effect on the PD or ion activities. DMSO was used to dissolve the drugs in these experiments, as was done in the TEP determinations. The metabolic inhibitor, iodoacetamide (IAA), caused a significant

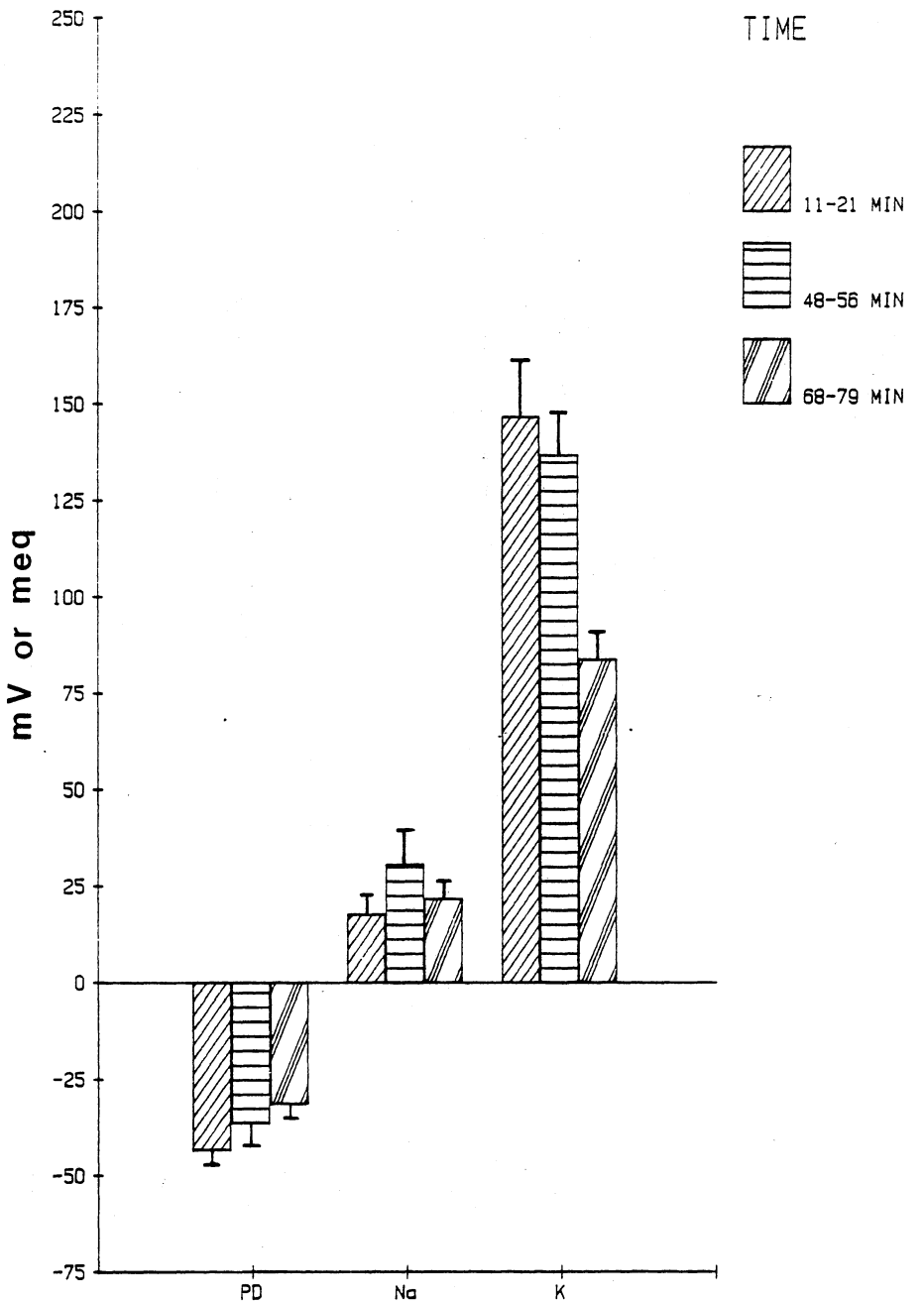

Figure 13. Changes in PD, Sodium, and Potassium Activities of Incubated Intestines With Time in Saline Control (n=6)

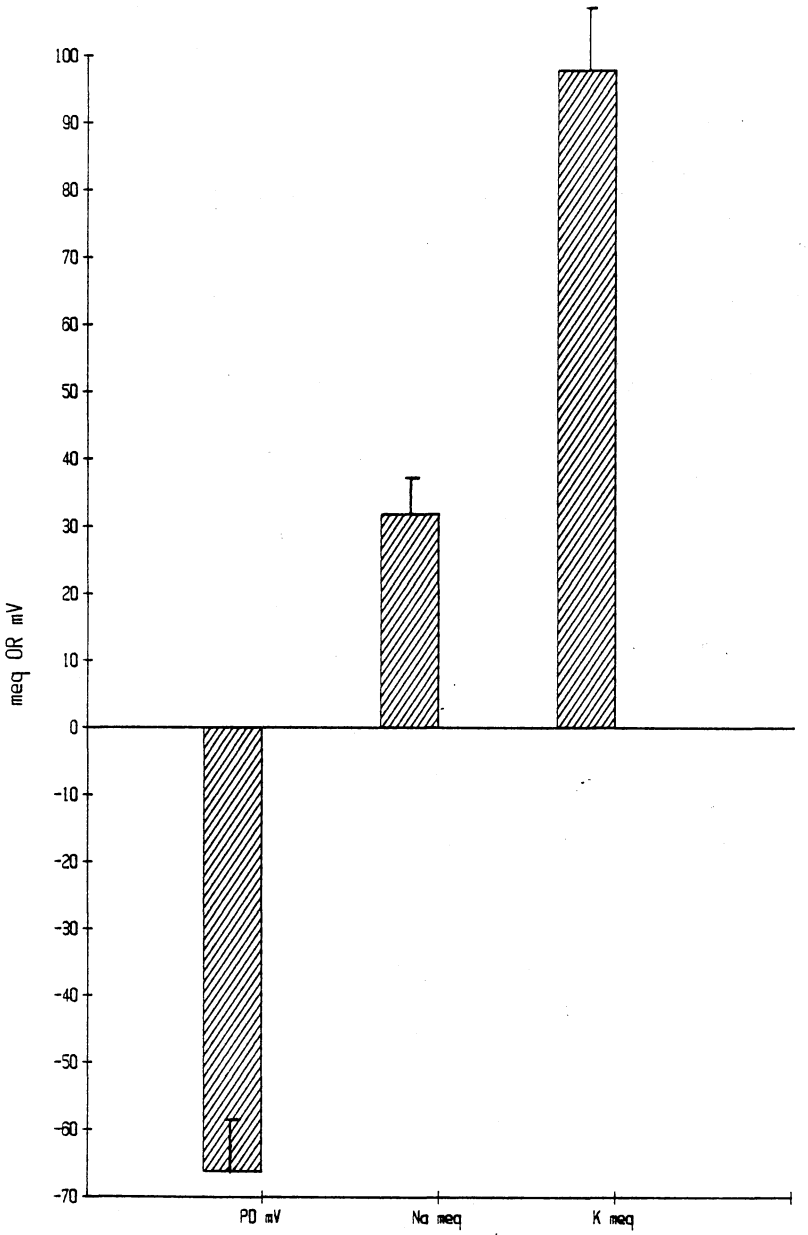

Figure 14. PD, Na+, and K+ in Cells of a Single Tissue (n=6)

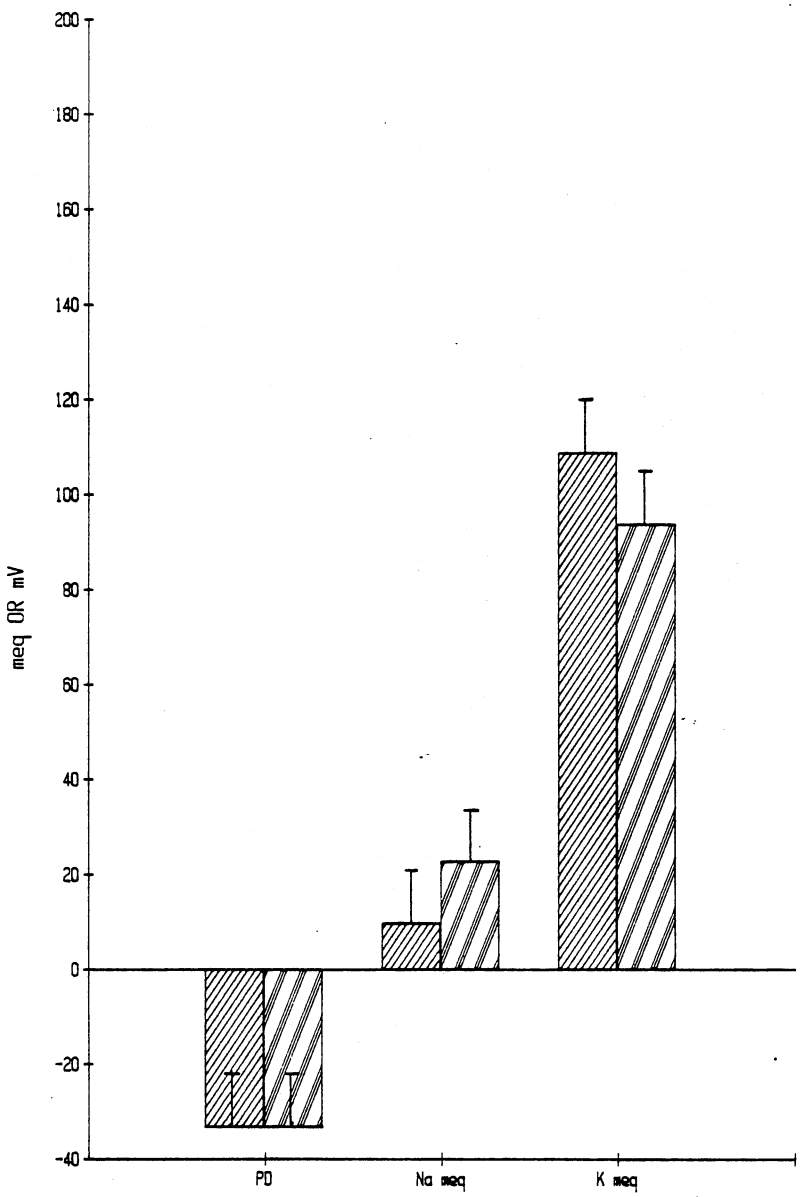

Figure 15. Effect of DMSO on PD, Na+, and K+ in Cells (n=5)

change in the PD (-35±3 to -20±4ml), but did not alter sodium or potassium activities (Figure 16, Table VII).

TABLE VI

MICROELECTRODE VALUES COMPARING EFFECTS OF INCUBATION TIME

	11-21 Min.	48-56 Min.	68-79 Min.
PD_{mV}	-45±3	-36±4(7)	-30±4*
Na_{mEq}	18±4(7)	31±9(7)	22±4(6)
K_{mEq}	122±18	137±12(6)	84±6(4)*

Note: Microelectrode values comparing effects of incubation time. Values are stated ±SE and number of determinations are in parentheses. Grouped t-test of data indicates a difference (0.05) in PD and K activity in tissues incubated 68-79 minutes (*) when compared to tissues incubated 11-21 minutes (n=6).

Phloridzin, the inhibitor of sodium-glucose uptake in mammalian intestines, was added to prepare Ascarid intestines. Results are shown in Figure 17 and Table VII. All values were not significantly different from the pretreatment controls.

The anthelmintic cambendazole (CBZ) decreased the PD (-43±3 to -24±4). There was a decrease in potassium (171±21 to 54±15 meq) (Figure 18, Table VII), but no effect on sodium activity. Mebendazole (Figure

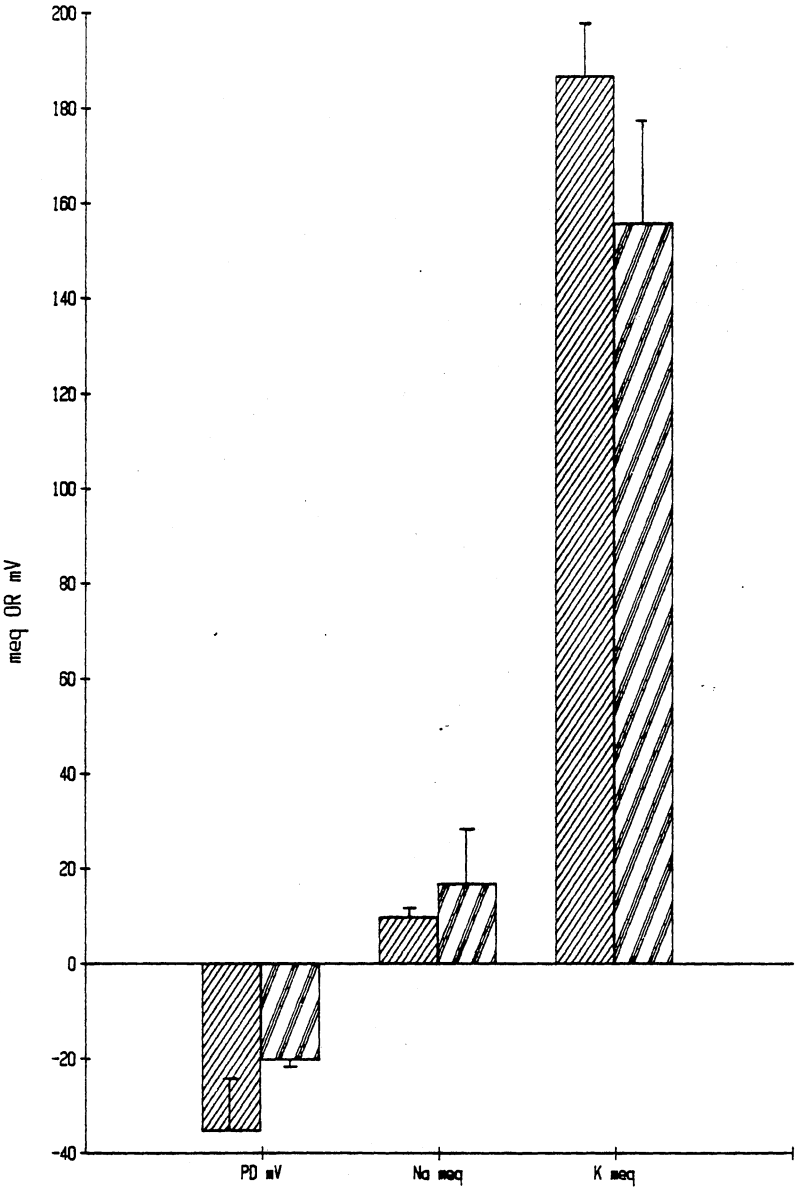

Figure 16. Effect of IAA on PD, Na+, and K+ in Cells (n=6)

TABLE VII

EFFECTS OF DIFFERENT COMPOUNDS ON PD, Na^+, AND K^+ ACTIVITIES

	Initial Potential	Drug Potential	(n)	Initial Sodium	Drug Sodium	(n)	Initial Potassium	Drug Potassium	(n)
Saline	-45±3	-39±4	5	18±6	37±10	5	147±24	125±14	4
DMSO	-31±12	-30±5	5	10±2	23±9	4	116±29	89±26	5
IAA	-35±3	-20±4*	6	10±2	17±10	5	187±13	156±23	6
PHLOR	-47±7	-33±3	6	13±2	18±4	6	142±16	107±27	6
CBZ	-43±3	-24±4	6	28±11	31±14	5	171±21	54±15*	5
MBZ	-38±4	23±2*	6	29±9	26±4	6	226±25	191±26	6

Note: Comparisons were made using AOV, testing at the 0.05 level of significance. Values marked with an asterisk (*) were significant.

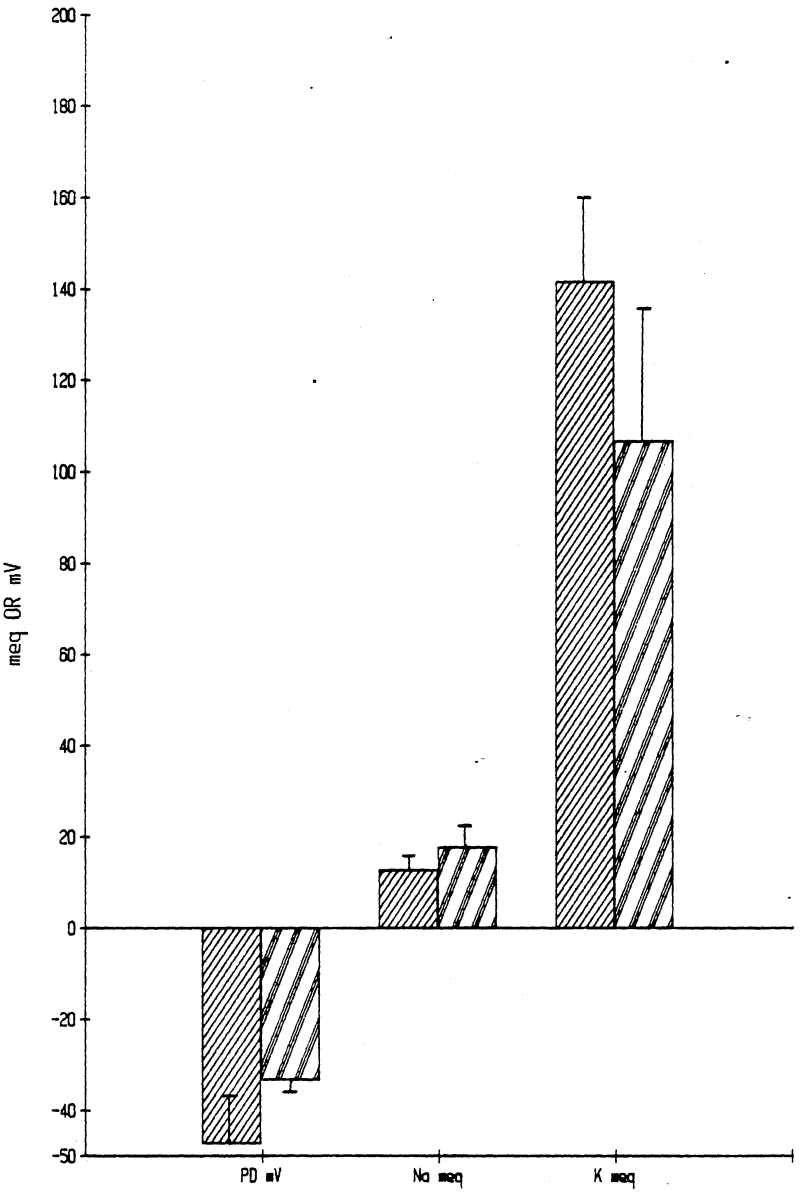

Figure 17. Effect of Phloridzin on PD, Na+, and K+ in Cells (n=6)

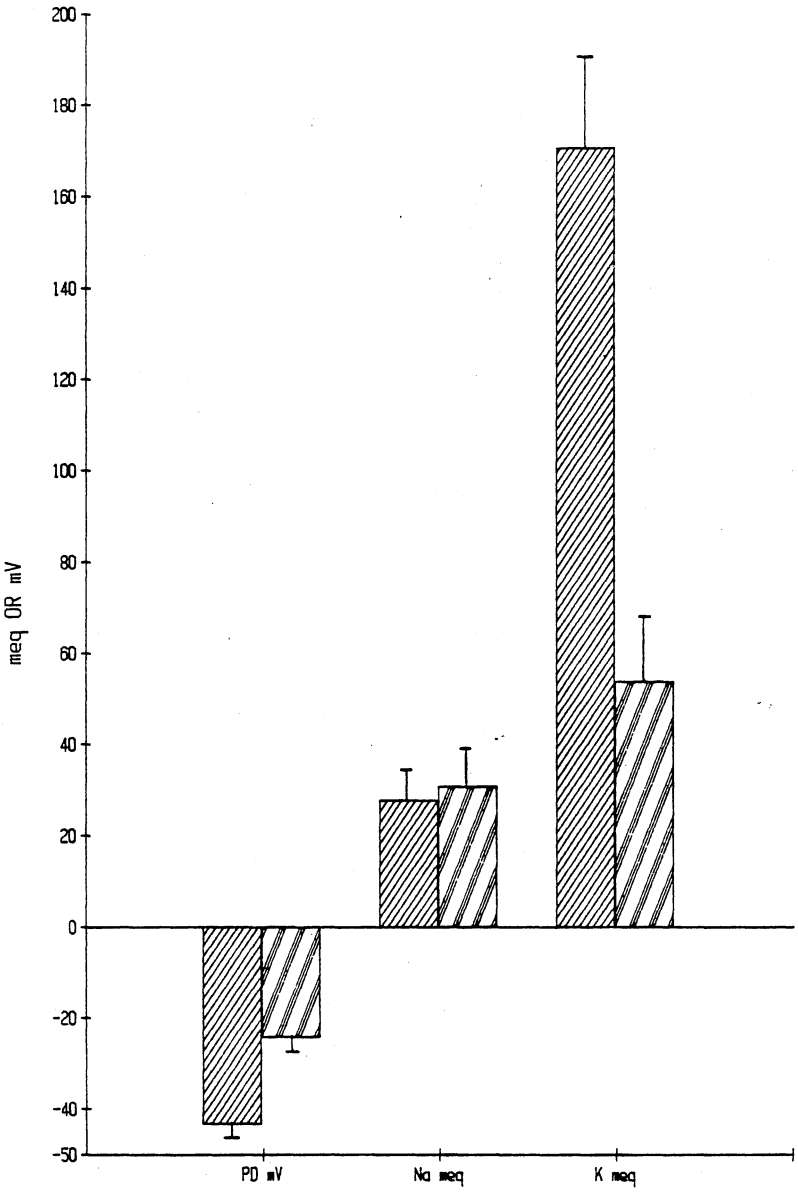

Figure 18. Effect of Cambendazole on PD, Na+, and K+ in Cells (n=6)

19 and Table VII) also had no effect on the ion activities, but caused a decrease (-38±4 to -23±2 mV) in the membrane potential.

After the determinations with the drugs were made, 20mM glucose was added to the mucosal surface of the intestines in a change of warm saline. Figures 20 and 21 are summarized in Table VIII.

In Figure 20, glucose was added to the DMSO treated tissues. There was a decrease in the cellular potential (-30±5 to -9±8mV), but no significant change in sodium or potassium activity.

There were no other differences measured when glucose was added to any of the drug treated tissues. Differences could be observed, however, when cambendazole tissues were compared with values from DMSO controls (Table IX, Figure 21). The ability of the cells to maintain a low sodium activity (39±25mEq, n=47) with external sodium concentrations of 100mM and a high potassium activity (166±64mEq, n=32) with external potassium concentrations of 24mM, suggests the presence of active transport mechanisms. Merz (1977) made this conclusion.

The distribution of these two ions would suggest that they play a role in the production of the membrane potential of -40±10mV (n=47), inside negative. The direct cause of a potential difference is described by Nernst (1888) as the diffusion of ions down a concentration gradient. The indirect cause is the cellular cencentration of negatively charged organic molecules and whatever pump is responsible for the generation of the ion gradient. This ion gradient or concentration difference, when separated by a membrane that is permeable to one ion, produces an electrical potential across that membrane. The Nernst equation can be used in this situation to predict the potential, given the concentrations of the ion on either side of the membrane.

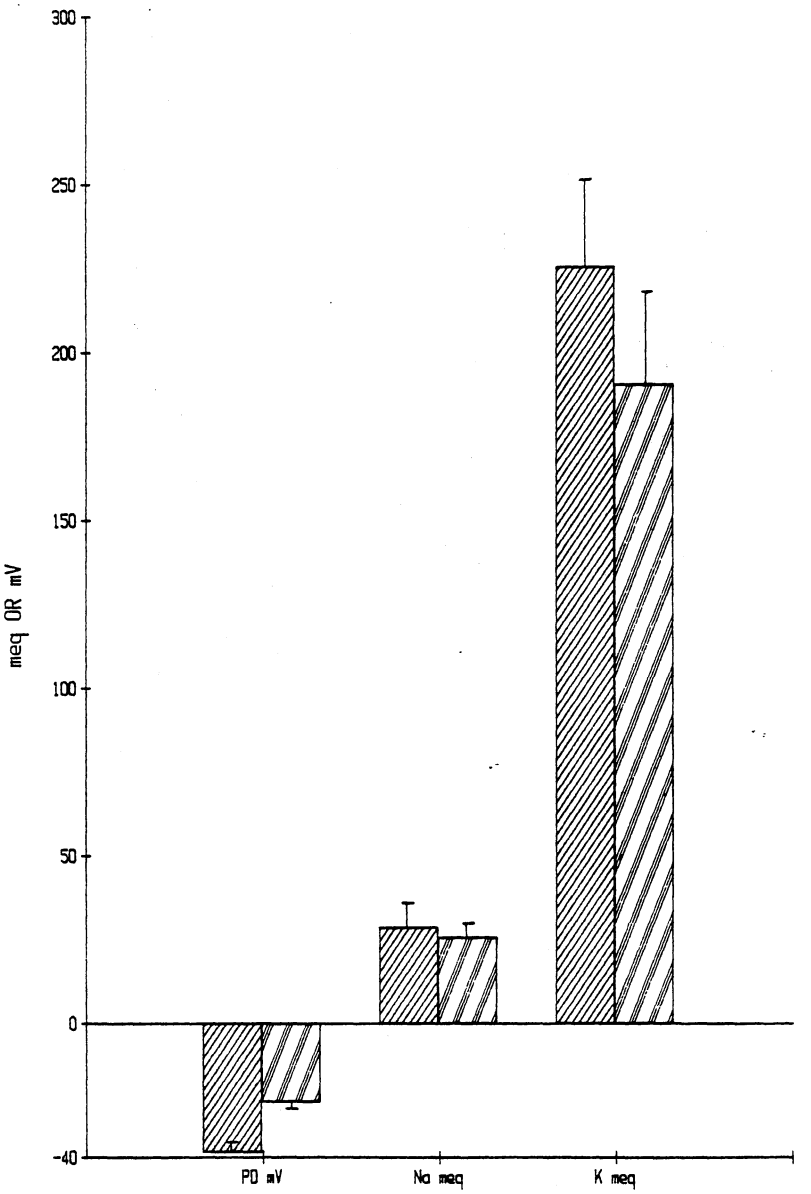

Figure 19. Effect of Mebendazole on PD, Na+, and K+ in Cells (n=6)

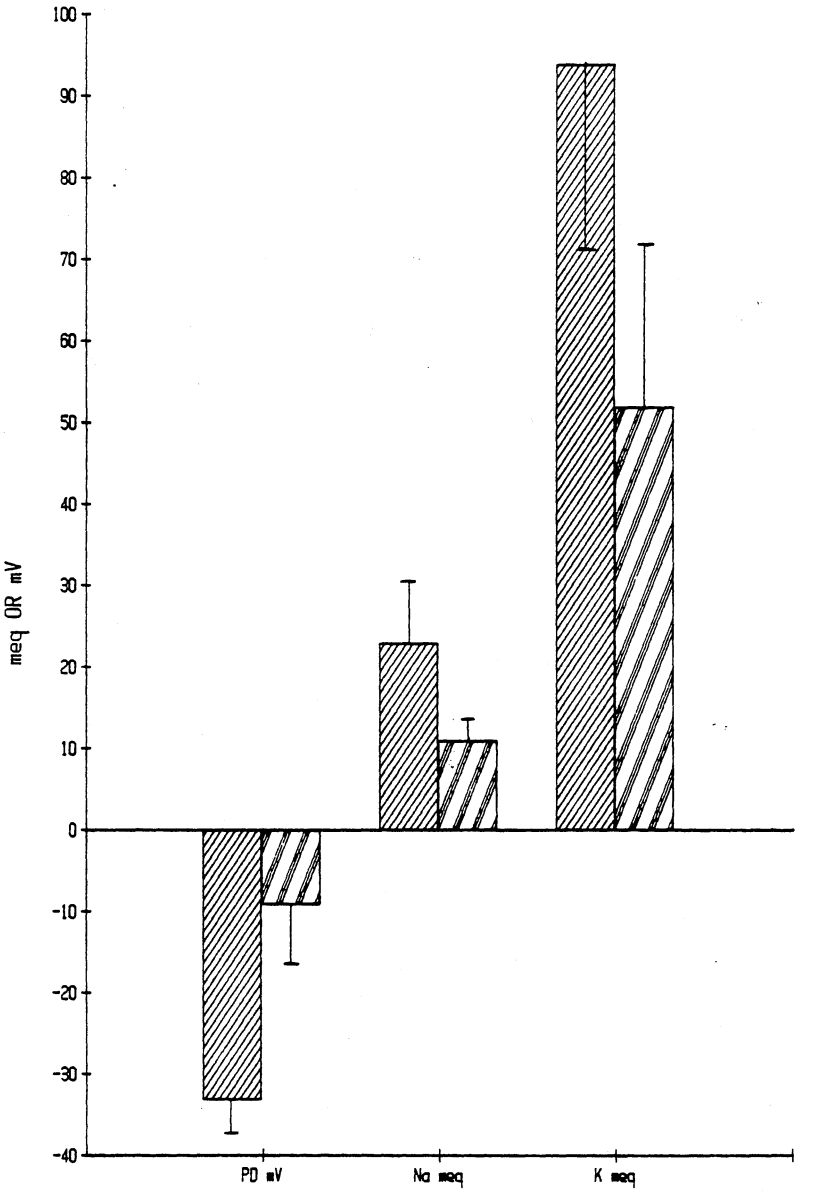

Figure 20. Effect of Glucose on DMSO Treated Cells (n=4)

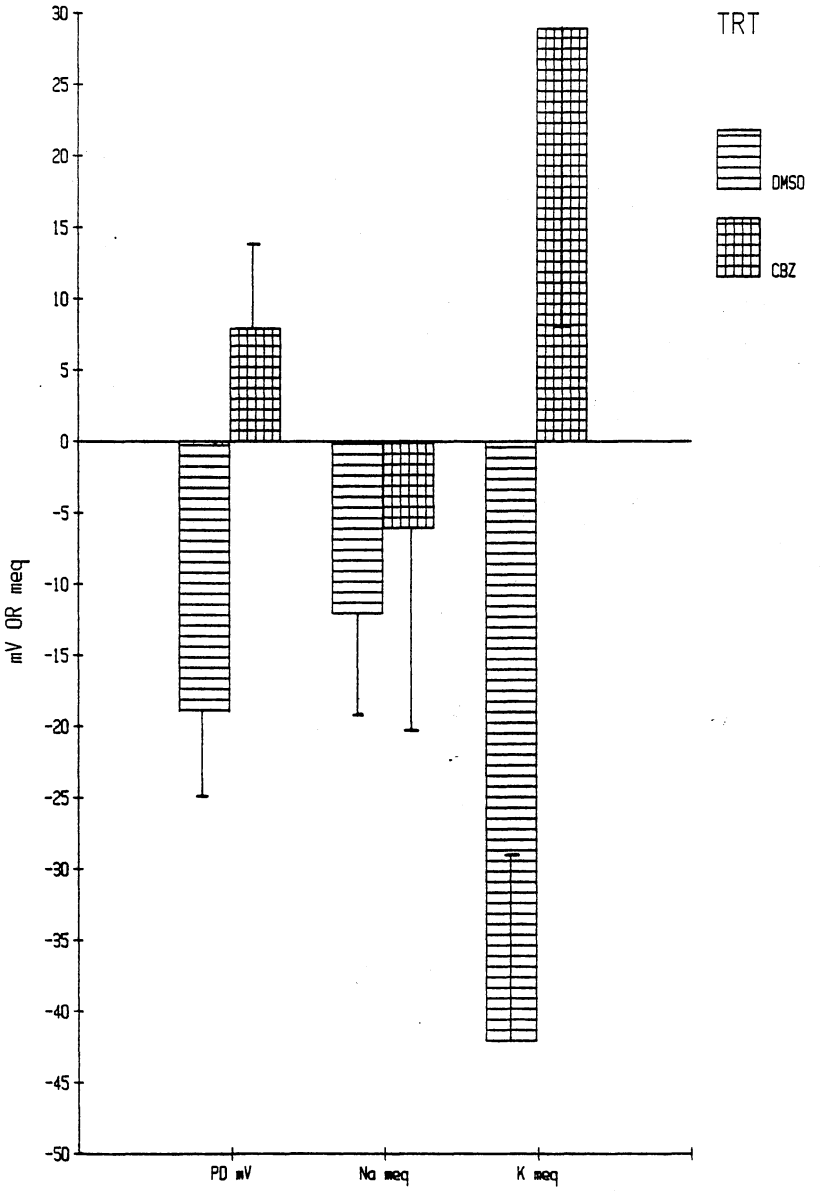

Figure 21. Comparison of Glucose Changes in DMSO and Cambendazole Treated Cells (n=4)

TABLE VIII

EFFECT OF ADDING SALINE WITH 20mM GLUCOSE TO TISSUES PREVIOUSLY TREATED WITH DRUGS

	Drug Potential	Glucose Potential	(n)	Drug Sodium	Glucose Sodium	(n)	Drug Potassium	Glucose Potassium	(n)
Saline	-39±4	-30±4	5	37±11	23±5	5	125±14	84+6(*)	4
DMSO	-30±5	-9±8*	5	23±9	11±3	4	89±26	52±23	5
IAA	-20±4	-14±4	6	17±10	20±7	5	156±23	123±22	6
PHLOR	-33+3	-21±6	6	18±4	24±5	6	107±27	93±11	5
CBZ	-24±4	-32±4	6	31±14	25±6	5	54±15	83±25	5
MBZ	-23±2	-20±6	6	26±4	24±2	5	191±26	146±26	6

Note: Comparisons were made using AOV, testing at the 0.05 level of significance. Values marked with an asterisk (*) were significant.

TABLE IX

COMPARISON OF GLUCOSE INDUCED CHANGES IN DMSO TREATED TISSUES AND TISSUES TREATED WITH DRUGS

	PD Change		Na+ Change		K+ Change	
	DMSO		DMSO		DMSO	
	-19±6	(4)	-12±7	(4)	-42±13	(4)
IAA	-6±4	(6)	+4±14	(5)	-33±9	(6)
PHLOR	-12±4	(6)	+5±6	(6)	-13±28	(5)
CBZ	+9±6*	(6)	+5±14	(5)	+29±21*	(5)
MBZ	-3±6	(6)	-1±7	(5)	-45±45	(6)

Note: Changes were determined by subtracting the PD, Na+, and K+ values resulting from the 200 mM glucose treatment from the drug treatment values. Minus signs indicate a decrease in ion activity or a decrease in the negative PD. AOV was used to compare changes in DMSO treated tissues with changes occurring in drug treated tissues. Asterisk (*) values were significant at the 0.05 level.

$$V = RT/F \log \frac{C_{out}}{C_{in}} \qquad \text{Equation 1}$$

where RT/F is 62 in systems where the temperature is 37°C, C_{in} is the ion concentration in the cell, and C_{out} is the extracellular ion concentration. Substituting the cellular sodium activity of 39mEq from the average in Figure 11, and 100mEq extracellular sodium, the equation would be:

$$V = 62 \log \frac{100}{39}$$

The calculated V would be +25mV. However, if potassium is assumed to be the permeable ion, the calculated V would be -52mV.

$$-52mV = 62 \log \frac{24}{166}$$

The Nernst potential calculated for potassium is closer to the measured -40mV observed in the cells. This suggests that potassium is the major permeating ion in Ascarid intestinal cells.

The Nernst potential calculations may also be used to determine which ion is responsible for the decay in PD with time. The measured PD in saline controlled tissues is $-45 \pm 3_{mV}$ at 10 minutes. This potential decreased to -30 ± 4mV at 70 minutes (a change of 15 ± 3mV). This calculated Nernst potential at 10 minutes is -48 ± 5mV and at 70 minutes is -33 ± 2mV (a change of 15 ± 7mV). There is no difference in the change observed with the potential recording microelectrode and the Nernst potential for potassium, indicating that the decay in potential is due to the decrease in cellular potassium activity.

It is unlikely, however, that such a living system is only permeable to one ion. Permeability is generally measured as the number

of molecules crossing a unit area of a membrane in unit time when a concentration difference is applied. The ability of ions to permeate a membrane is related to properties of the membrane and of the particle (Baker and Hall, 1977).

Goldman (1943) used the ratios of the two main permeating ions to produce a permeability coefficient (b). This coefficient is added to an equation called the "Goldman-Katz-Hodgkin-Huxley equation," which considers how the various permeabilities of these ions can interact to form a membrane potential.

$$V = RT/F \ln \frac{K_o + b\ Na_o}{K_i + b\ Na_i} \qquad \text{Equation 2}$$

where b is the permeability of sodium divided by the permeability of potassium. Other ions may be added to this equation if sodium and potassium ions do not account for the PD. When b is small (less than 0.1), the PD will be similar to the Nernst potential for potassium. A larger b (greater than 10) would approach the Nernst potential for sodium. Thus, if one obtained a permeability coefficient of 10, the Goldman equation, solved by substituting the ion concentrations used in the Nernst equation, would be equal to the values obtained in the Nernst equation when sodium was assumed to be the permeating ion. Variations in ion permeabilities would be reflected in a change in b. Experimentally, an increase in b from a control measurement would relate to an increase in sodium permeability in relation to potassium. Similarly, a decrease in b would indicate an increased potassium permeability.

One can estimate the permeability coefficient of the Ascarid intestinal cell membrane using the ion activities and PD measured with

microelectrodes. For this estimation, the Goldman equation is solved for b (see Appendix D for derivation).

$$b = \frac{K_o - e^{mV/62} K_i}{e^{mV/62} Na_i - Na_o}$$

Using the same values as were used in the Nernst equation, the solution becomes:

$$b = \frac{24 - e^{-40/62}\ 166}{e^{-40/62}\ 39 - 100} = \frac{-13.6}{-91.2}$$

The calculated permeability coefficient is 0.1491--a value which suggests, once again, that potassium is the major permeating ion in this system.

The microelectrode results for each drug treatment can be evaluated using the calculated permeability coefficients along with the observed changes in ion activity and membrane potential. Table X shows the calculated permeability coefficients before treatment, after treatment, and after glucose. Variation in before treatment permeabilities is due to tissue variations or technique. It was for this variation among tissues that all drug measurements, whether TEP, PD, or ion activities, were based on each intestine being its own control.

Permeability coefficients continue to approach values which show greater membrane permeability to potassium (0.01 to 0.71). Changes in b within this range, however, indicated variations in membrane

permeability with different tretments, where an increase in (b) indicates an increase in membrane permeability to sodium or a decreased permability to potassium.

TABLE X

PERMEABILITY COEFFICIENTS CALCULATED FOR MICROELECTRODE DATA

Trt.	Init b_1	Drug b_2	Glucose b_3
Saline	0.01±0.02	0.04±0.04	(0.04±0.06)*
DMSO	0.16±0.11	0.09±0.12	0.23±0.16
IAA	0.45±0.12	0.7±0.21	0.70±21
PHLOR	0.11±0.05	0.03±0.12	0.32±0.11
CBZ	0.12±0.07	-0.03±0.05	0.13±0.10
MBZ	0.38±0.12	0.75±0.26	0.58±0.14

Note: Values not significant $p < 0.05$.

*Saline was not treated with glucose.

CHAPTER V

DISCUSSION

The major finding from this study was that the changes in electrical activity in the intestine of Ascaris suum may be used to determine differences in drug action and predict drug activity in vivo. The differences in action and the normal in vitro changes can be used to determine the cellular mechanisms involved in the production of electrical activity. The major ion, namely K^+, producing the cellular electrical potential is also the ion responsible for the potential decay characteristically observed in this system. Alterations in the permeability of the intestine to this ion occurs with certain drug treatments and remains unchanged by other compounds in the same drug class.

The transepethelial potential is altered by some benzimidazole compounds, but some compound derivatives either have no effect on the TEP response and/or alter the normal SCC change after glucose is added to the mucosal solution. Compounds tested that are efficacious anthelmentics and cause an altered TEP response include thiabendazole, cambendazole, albendazole, and mebendazole. Parbendazole and fenbendazole did not change the TEP, nor did the levamisole compound. Both thiabendazole and mebendazole caused a decrease in the TEP in a study conducted by Beames, Merz, and Donahue (1976); however, they also observed a TEP effect with levamisole. Levamisole has been found to

inhibit mitochondrial fumeric reductase in large concentrations and inhibits muscle contractions in nematodes at much lower concentration. Thus, the lack of effect on the intestinal transport by Levamisole is not unlikely. This conclusion is further supported by Schanbacher's (1974) observation that inhibition of mitochondrial oxidative phosphorylation by 2,4-dinitrophenol did not alter the ability of Ascarid intestines to meet energy requirements necessary for the uptake of glucose. However, he did find that the inhibition of glycolysis by iodacetamide decreased glucose uptake in intestinal sac preparation. Glycolysis is known to provide two-thirds of the total ATP produced by anaerobic carbohydrate metabolism. It is thus likely that the inhibition of cellular functions utilized in the transport of ions will result in greater TEP changes, particularly if those ions are involved in the production of the TEP. The benzimidazole compounds that decreased the TEP are thus able to alter the movement of ions responsible for electrical activity. The other benzimidazoles tested have a mode of action that does not involve disruption of the intestine's ability to maintain a separation of ion charges necessary for an electrical potential.

The change in SCC after the addition of glucose to the mucosal saline is decreased in intestines treated with cambendazole. This result suggests that the compound has a mechanism of action that is not shared by the other benzimidazoles tested. The uptake of glucose by the Ascaris suum intestine requires extracellular sodium (Schanbacher, 1974) and is associated with the cellular uptake of sodium (Beames, Merz, and Donahue, 1981) in a manner similar to sodium-glucose uptake mechanisms in mammalian intestines. The change in SCC

upon the addition of glucose, then, reflects the uptake of sodium (Merz, 1977). The cambendazole alteration of the normal SCC change after glucose may be due either to a change in the electrochemical gradient for sodium or disruption of the cellular membrane or glucose-sodium carrier. Data collected with microelectrodes provide evidence to support the former conclusion.

These data do not support the suggestion by Beames (1971) that thiabendazole and levamisole also alter the SCC response to glucose. It is possible that variation among responses by worms in this study were greater than the change in the worms used in the 1976 observation. It is more likely, however, that Ascarids are losing their sensitivity to these compounds as the increase in anthelmintic resistance is well known. Mebendazole's lack of change in SCC response supports Beames, Merz, and Donahue's (1976) findings that this compound alters the TEP while not affecting the normal SCC response to glucose. These data indicate the benzimidazoles have a different effect on the electrical activity generated by isolated intestines and thus have a different action on the Ascarid intestine.

Newly derived benzimidazole compounds are also seen to have various effects on the TEP and SCC. Comparisons with *in vivo* studies by Dow Chemical Company using mouse nematodes indicate that compounds showing significant electrical changes in *Ascaris suum* intestines also kill nematodes *in vivo*. All compounds found to kill nematodes in mice also affected the electrical characteristics of *Ascaris* in the present study. Information concerning some drugs tested *in vivo* may not have been reported to this investigator. There may be some compounds that do not alter the electrical characteristics of the intestine but are

still effective *in vivo*. The derivative study indicates that the mode of action that is responsible for altering the electrical characteristics may also be responsible for killing the parasite.

Thus, the study with Benzimidazole derivatives indicates that the ability of a compound to alter the ability of the *Ascaris suum* intestine to maintain electrical activity can be used to predict *in vivo* activity against parasites of the same phylum.

Normal changes in membrane potentials and intercellular ion activities measured with microelectrodes demonstrate that potassium is the major ion responsible for producing the membrane potential. Results also show that the decay in this PD with time is due to the decrease in cellular potassium activity. The role sodium ions play in producing the cellular potential can be estimated by calculating the permeability ratio (b) from the Goldman-Katz-Hodgkin-Huxley equation. The results calculated from pretreatment ion measurements indicate that b = 0.15 or 15 meq sodium ions per every 100 meq potassium ions are involved in producing the membrane potential. This ratio should decrease when there is an increase in sodium or a decrease in potassium permeability, but potassium ions remain the major permeating ion normally producing the membrane potential, and are responsible for the decay of the potential. The lack of change in permeability ratios measured with saline treatment over time (see Table IX) gives furthur evidence of the role of potassium ions in the normal potential measurements.

DMSO treatment did not alter PD, sodium, or potassium activity measurements, but glucose added to the mucosal solution produced a more positive PD with no significant changes in ion activity. This

lack of significant ion change may be due to the 10 minute time delay after glucose was added, and before the measurements were taken. There even seems to be a reduction in sodium in these measurements. This observation is supported by a recent observation by Hudson and Schultz (1984) in the Necturus small intestine that sodium sensitive microelectrodes measure an immediate increase in cell sodium with galactose treatment followed by a significant decrease in cell sodium within two minutes. Previous findings do indicate that sodium is necessary for glucose uptake in Ascaris suum (Schanbacher, 1974), and that there is an expected increase in cellular sodium immediately after glucose is added to the bathing media (Beams, Merz, and Donahue, 1981).

These results indicate that DMSO used as a drug solvent does not alter the normal cell ion activities and potential. Treatment with glucose causes a more positive PD and an enlargement of the permeability ratio, which supports previous findings that sodium activity does begin to play a part in the production of the membrane potential.

The uptake of glucose (radioactive 3-O-methyl glucose) was depressed in the presence of the competitive inhibitor, phlorizin (Schanbacher, 1974). The present study did not substantiate these findings by showing significant changes in cell ion activities with administration of this drug. There was, however, a reduction of the permeability ratio after phlorizing treatment, suggesting that the normal sodium influx may be associated with a phlorizin sensitive carrier. The lack of significant ion activity changes associated with phlorizin may be due to the low concentration used. Only 0.5mM phlorizin was added to the mucosal solution, and 20mM glucose was later

added. This large concentration of glucose would thus increase the chances of glucose attaching to the carrier in the place of phlorizin. An elevated (b) indicates possible sodium uptake after glucose treatment. Phlorizin was removed from the tissue before the addition of glucose. It is known that phlorizin's effect on tissues is reversible. Rinsing the tissue post-treatment would overwhelm any remaining competitive effect phlorizin may have had on the uptake of glucose. Therefore, it is probably that any drug effect that mimics phlorizin might also be reversible and not affect the Ascaris suum intestine at the concentration administered.

Administration of iodoacetamide, a glycolytic inhibitor, decreases the membrane potential. This event is not associated with an elevated permeability ratio (only 0.5 to 0.7) or significant changes in ion activity. These data confirm observations that IAA depresses the transepithelial potential while not altering the flux of radioactive sodium (Beames, Merz, and Donahue, 1976).

There was no change in ion activities or PD after the addition of glucose, an observation which is confirmed by the lack of change in the permeability ratio (0.70 to 0.71). The microelectrode findings support the observation made by Beames, Merz, and Donahue (1976) that IAA depressed the SCC change normally observed with the addition of glucose. It was once thought that this reduced response to glucose might be due to an increase in cell sodium activity, an event that might also be associated with a drug induced decrease in the energy required to pump sodium out of the cell. An alternative explanation might be that it is not the alteration in a sodium gradient that is responsible for the lack of glucose response, but rather a reduction

in the electrical gradient for sodium uptake. The negative membrane potential from previous microelectrode data is now known to be mostly due to potassium ion movements out of the cells. However, other ions may compete with potassium ions for the production of the membrane potential. Such would be the case if there was an increase in some negative ion in the cell (Cl^- or HCO_3^-) or a decrease in another positive ion (H^+). Another explanation would be a loss of significance due to high variability with the sodium electrode' IAA-treated Na+ activity was 17±10meq, and the pretreatment value was 10±2meq.

The results of the microelectrode determinations with iodoacetamide-treated tissues indicated that this metabolic inhibitor decreased membrane potentials without significantly altering sodium or potassium activities. The tissues are not responsive to glucose treatment. A combination of these observations and reports from other researchers (Beames, Merz, and Donahue, 1976; Schanbacher, 1974) indicated that IAA reduces the electrical gradient for sodium to diffuse into the cell, thereby decreasing PD, TEP, and SCC response to glucose, and uptake of radioactive 3-O-methyl glucose.

The intestine of Ascaris suum responds very strongly to treatment with the benzimidazole, cambendazole. This compound causes a decrease in PD and a decrease in cell potassium. The permeability ratio becomes negative. Negative (b) values may be due to low potassium activity. One of the actions of CBZ is thus to greatly increase the permeability of the membrane to potassium resulting in low potassium activity (Figure 22). There was no change in the cellular activity of sodium in CBZ-treated tissues. This would not be unexpected, even if CBZ increased membrane permeability to all cations. As in the case of

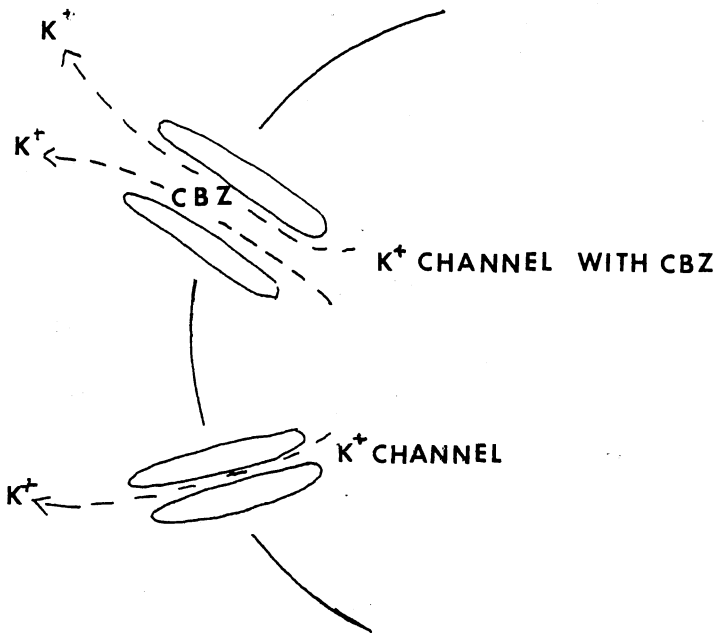

Figure 22. Model Demonstrating Cambendazole's Ability to Increase the Permeability of the Cell Membrane to Potassium Ions. (This event is diagrammed as if CBZ enlarges a potassium channel.)

IAA, there is an electrical gradient for sodium influx as well as a high variation in sodium activity measurements. The former explanation seems more probable because there is a definite increase in potassium activity and not just a theoretical cation decrease as hypothesized with IAA treatment.

The addition of glucose produces a dramatic increase in cell potassium and an associated increase in the negative cellular potential. This result is observed when changes in CBZ-treated tissue are compared with changes in DMSO-treated tissue. The stimulation of potassium uptake by glucose indicates that CBZ action does not alter the mechanism involved in glucose uptake and that the glycolytic pathway is still able to function. Potassium uptake is thus probably related to the ability of the cell to produce ATP needed to run a potassium pump.

This observation is supported by Schanbacher's (1974) that incubated Ascaris suum intestine loses carbohydrate reserves after 10 minutes of incubation. However, these microelectrode results with glucose do not support Van den Bossche and DeNullin's (1973) observation that CBZ (and MBZ) causes a fall in muscle glycogen of whole worms incubated with cambendazole due to reduction in the uptake of glucose. These microelectrode data suggest that glucose uptake may still occur, but glucose is rapidly utilized by glycolytic mechanisms in an attempt to produce energy needed for potassium pumping. Thus, little or no glucose would be available for glycogen storage in Ascarid muscle.

Alternatively, it may be that glucose uptake results in cell swelling. Hudson and Schultz (1984) reported that treatment of

Necturus intestine with either hypotonic saline or galactose results in cell swelling and stimulation of potassium pumping. Intestinal cells are then able to prevent large changes in cell volume associated with glucose uptake. It would seem particularly important for Ascarid intestines to regulate fluid uptake due to the importance of the worms' hydrostatic skeletons. It would be interesting to see if treatment of Ascaris intestine with hypotonic saline would cause an increase in cell potassium.

Large variations in sodium values defy attempts to show the reduction expected if glucose also stimulates the removal of cell sodium. An alternative explanation would be the existence of separate sodium and potassium pumps. Schanbacher (1974) was unable to inhibit Ascaris intestine with ouabain, an inhibitor classically known to inhibit sodium and potassium exchange pumps in other systems. It is still possible, however, that sodium-potassium exchange may occur via pumps that are ouabain-insensitive.

One of cambendazole's major actions is thus related to its ability to increase membrane permeability to potassium. There is a subsequent alteration in the PD and TEP, and a variation in the normal response to glucose. This compound does not alter the ability of the cell to sequester potassium given glucose. This glucose, however, does not accumulate for use by other Ascaris tissues.

Mebendazole causes a decrease in the membrane potential without significantly changing potassium or sodium activities in the cells. This is similar to the results from the IAA experiments where changes in ion activities were not significant and could not be used to account for potential changes. Mebendazole, however, is the only

compound used in this study that may cause a reduction in sodium activity. This can only be stated as a trend because of lack of significance at $p < 0.05$. This trend, however, supports Beames, Merz, and Donahue's (1976) observation that mebendazole treatment of *Ascaris* intestines reduces radioactive sodium flux. This conclusion may also be supported by interpreting the increased permeability ratio (0.4 to 0.8) with MBZ-treatment as a reduction in potassium rather than an increase in sodium permeability. The action of Mebendazole may be related to a decrease in ion permeabilities rather than the increase in permeability to potassium as was observed with CBZ.

The fact that the SCC response to glucose is not altered in MBZ-treated tissues indicates that the cells are able to respond to glucose as long as there is an electrochemical gradient for sodium-glucose uptake and a membrane that will support that gradient. The disruption of the intestinal membrane is observed to occur in 24 hours with worms treated *in vivo* (Borgers, DeNullin, DeBrabander, and Thienport, 1975). It is possible that the mebendazole action involves disruption of the membrane integrity, which affects membrane permeability characteristics and thereby decreases membrane potential measurements. Eventually, the membrane would reach a state where it would no longer support glucose uptake. This conclusion supports the observation of Van den Bossche (1976) that tissue glycogen is significantly depleted within 24 hours in worms treated with mebendazole. From his observations, Van den Bossche hypothesized that the action of Mebendazole was to reduce glucose uptake by the worm, leading to starvation. There is a lack of change (0.8-0.6) in the calculated permeability ratio after glucose treatment from the present data.

This indicates that while enough sodium may enter with glucose to produce a SCC change, there is not enough change in sodium permeability to alter the permeability ratio.

The results from the microelectrode determinations thus support previous evidence that one of the actions of mebendazole is to reduce membrane permeability (Figure 23). Certainly, the most significant finding is that mebendazole action is greatly different than the related compound, cambendazole.

However, it is known that both of these benzimidazoles are cross resistant *in vivo* (Coles, 1977). Their common action may be related to their attachment to structural components found in the membrane or organells directly involved with the maintenance of the membrane. These structural components may be proteins involved in ion channels. Mebendazole would decrease, while cambendazole would increase the channel's permeability to ions. Genetic alterations in the parasite proteins may then alter the ability of both of these compounds to affect membrane permeability. This difference in protein structure may also be the reason that these same compounds do not affect mammalian intestinal cells.

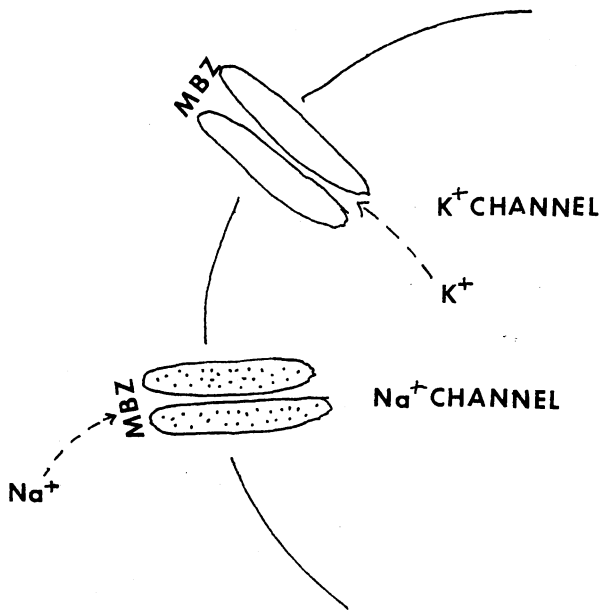

Figure 23. Model Demonstrating Attachment of Mebendazole to Ion Channels in Such a Way as to Reduce Ion Permeability

CHAPTER VI

SUMMARY

The results of this study indicate that potassium is the major permeating ion that produces the membrane potential in cells of isolated intestines of Ascaris suum. The decay of potential with time in vitro correlates with a decrease in intracellular potassium activity while sodium activity remains unchanged. Transepithelial potentials, measured simultaneously in adjoining sections of intestine, are not significantly different. Similarly, several impalements with potential and ion sensitive microelectrodes indicate that the intestine is made up of cells that have similar electrical and ion activities. DMSO does not alter the normal TEP, PD, or ion activities. Glucose addition to the saline bathing the mucosal surface of the intestine causes the production of a more positive PD with no significant changes in ion activities when measurements are made 10 minutes after glucose is added. There is some indication that there is actually a decrease in cell sodium after 10 minutes.

Cambendazole is the only benzimidazole anthelmentic that produces a decrease in the SCC response to the mucosal addition of glucose. This compound also causes a significant decrease in the TEP, PD, and potassium ion activity. Glucose added to the mucosal saline of CBZ treated cells is associated with a dramatic increase in cell potassium activity. Thus, it is concluded that cambendazole acts to increase

membrane permeability to post potassium while not affecting the ability of the cells to sequester potassium with the addition of glucose.

Mebendazole also causes a decrease in TEP and PD, but it does not alter the normal SCC response to glucose or cell ion activities. Calculation of the permeability ratio of sodium and potassium indicate that this compound acts by altering the membrane integrity and decreasing its permeability to ions. Neither MBZ nor CBZ treated tissues respond in a manner that would indicate that their action might be similar to either iodoacetamide or phlorizin. IAA causes a decrease in PD without significantly altering sodium or potassium activities. IAA treated tissues also did not respond to glucose and are known to cause a reduction in SCC response to glucose (Beames, Merz, and Donahue, 1976). Phlorizin does not alter the PD or ion activities of the intestinal cells, nor is there a significant alteration in the glucose response.

Other benzimidazoles that cause a change in TEP are thiabendazole and albendazole. Parbendazole and fenbendazole did not change the TEP, nor did levamisole. This difference in TEP response among compounds that are closely related in chemical structure indicates that they may also have a difference in their mode of action. Newly synthesized benzimidazoles used in the EP measurements and correlated with *in vivo* tests indicate that the measurements of electrical activity in *Ascaris suum* intestine may give an indication of possible anthelmintic action *in vivo*.

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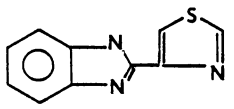
APPENDIXES

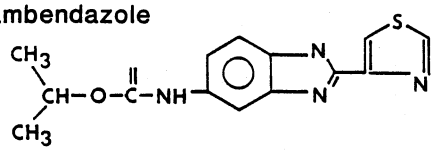
APPENDIX A

CHEMICAL STRUCTURES OF COMPOUNDS USED IN TEP STUDY

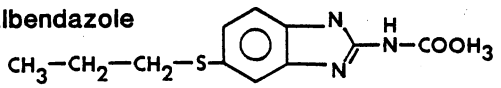
Thiabendazole



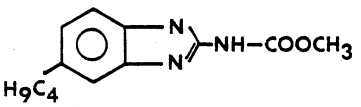
Cambendazole



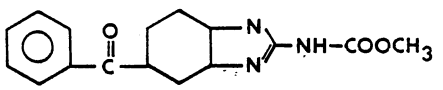
Albendazole



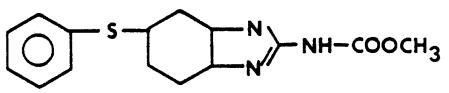
Parbendazole



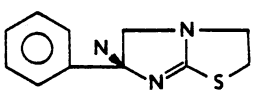
Mebendazole



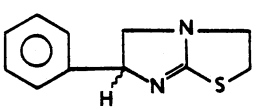
Fenbendazole



L-tetramesole



D-tetramesole



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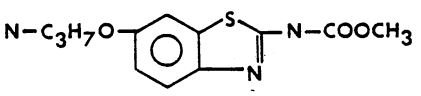

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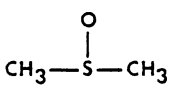

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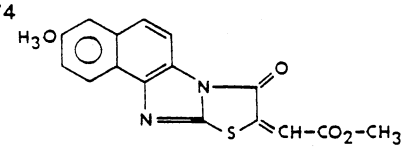

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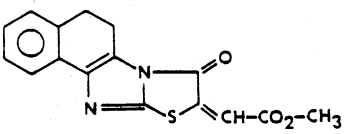

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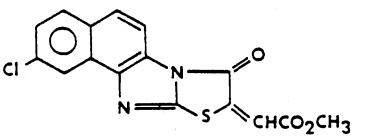

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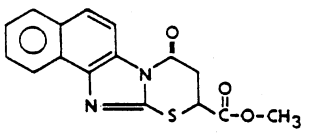

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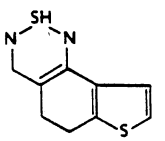

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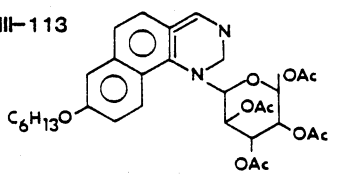

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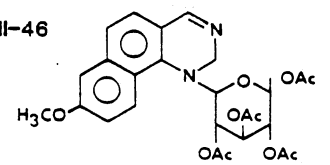

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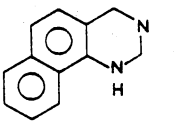

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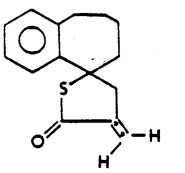

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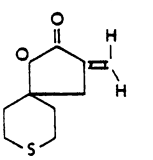

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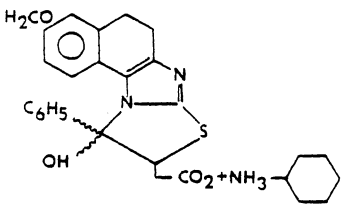

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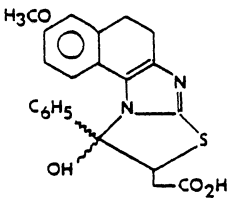

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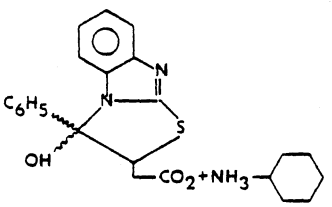

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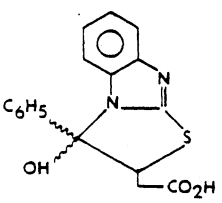

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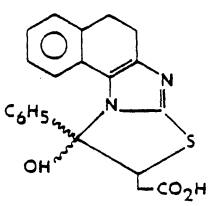

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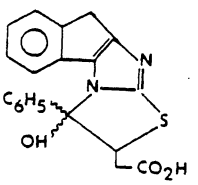

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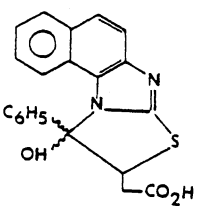

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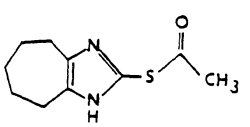

APPENDIX B

CALCULATION OF PERMEABILITY RATIO (b)

Goldman-Hodgkin-Katz Equation $V=RT/F \ln \frac{K_o + b(Na_o)}{K_i + b(Na_i)}$

Where RT/F = 62 at 37°C

if $K_o = 24$, $Na_o = 100$, $K_i = 147$, $Na_i = 31$, and $V = -43mV$

$$-43 = 62 \ln \frac{24 + b(100)}{147 + b(31)}$$

$$e^{-43/62} = \frac{24 + b(100)}{147 + b(31)}$$

$$e^{-43/62}(147 + b(31) = 24 + b(100)$$

$$e^{-43/62}(147) + e^{-43/62}(31)b = 24 + b(100)$$

$$e^{-43/62}(b31) - (b100) = 24 = e^{-43/62}(147)$$

$$b(e^{-43/62}31-100) = 24 - e^{-43/62}(147)$$

$$b = \frac{24 - e^{-43/62}(147)}{e^{-43/62}(31) - 100} = \frac{-5.76}{-93.7} = 0.0614$$

$$b = \frac{K_o - e^{mV/62}(K_i)}{e^{mV/62}Na_i - Na_o}$$

APPENDIX C

RAW DATA FOR MICROELECTRODE EXPERIMENTS

Saline

DATE	WORM	PD_1	PD_2	(PD_3)	Na_1	Na_2	(Na_3)	K_1	K_2	(K_3)
II6-27-83	6-27	-42	-28	-24	11	18	18	-	-	-
I 6-24-83	6-23	-50	-48	-43	10	15	11	191	126	83
II6-28-83	6-27	-50	-42	-25	42	35	42	182	159	66
I 6-28-83	6-27	-47	-46	-34	11	76	18	122	93	93
III6-28-83	6-27	-36	-31	-22	17	41	25	93	121	94

DMSO

DATE	WORM	PD_1	PD_2	PD_3	Na_1	Na_2	Na_3	K_1	K_2	K_3
II7-13-83	7-11	-25	-25	-9	16	11	14	151	182	109
III7-12-83	7-11	-35	-47	11	8	30	11	53	71	19
II7-12-83	7-11	-32	-35	-30	6	45	17	190	94	61
I 6-23-83	6-23	-38	-25	-6	8	5	2	43	27	17
II6-23-83	6-23	-27	-18	-	-	-	-	141	69	-

IAA

DATE	WORM	PD_1	PD_2	PD_3	Na_1	Na_2	Na_3	K_1	K_2	K_3
IV9-10-83	9-8	-35	-21	-3	17	56	10	207	135	74
III9-10-83	9-8	-42	-31	-32	4	4	22	72	52	45
II9-10-83	9-8	-26	-7	-13	8	10	12	293	197	164
I 9-10-83	9-8	-45	-25	-12	8	6	11	111	172	137
III9-9-83	9-8	-29	-12	-11	12	7	46	229	180	129
II9-9-83	9-8	-35	-23	-11	-	-	-	208	197	188

PHLOR

DATE	WORM	PD_1	PD_2	PD_3	Na_1	Na_2	Na_3	K_1	K_2	K_3
II7-27-83	7-27	-50	-31	-18	8	4	33	95	35	110
I 7-27-83	7-27	-57	-32	-31	11	13	15	187	78	50
I 7-26-83	7-25	-29	-24	-11	21	21	13	164	135	101
III7-23-83	7-22	-30	-26	-9	11	22	18	124	192	99
II7-23-83	7-22	-45	-35	-10	13	19	17	140	93	106
I 7-23-83	7-22	-72	-47	-44	16	31	46	-	-	-

CBZ

DATE	WORM	PD_1	PD_2	PD_3	Na_1	Na_2	Na_3	K_1	K_2	K_3
I 7-18-83	7-18	-42	-25	-29	9	9	13	150	63	86
II7-18-83	7-18	-32	-22	-38	4	14	45	200	14	83
II7-16-83	7-14	-41	-23	-18	67	83	26	101	27	53
II7-15-83	7-14	-50	-33	-24	25	35	27	218	64	21
I 7-15-83	7-14	-41	-8	-37	35	13	16	-	-	-
I 7-16-83	7-14	-50	-30	-46	-	-	-	188	101	172

MBZ

DATE	WORM	PD_1	PD_2	PD_3	Na_1	Na_2	Na_3	K_1	K_2	K_3
IV7-20-83	7-18	-45	-29	-18	18	36	-	157	181	94
III7-20-83	7-18	-32	-23	-44	37	18	26	144	157	156
V 7-20-83	7-18	-50	-27	-10	37	28	17	289	196	88
I 7-20-83	7-18	-45	-26	-28	65	17	26	263	104	263
II7-19-83	7-18	-24	-14	-8	4	39	21	263	290	150
I 7-19-83	7-18	-29	-18	-10	15	15	31	240	219	124

VITA

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