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THE IN VITRO, MORPHOLOGIC AND METABOLIC EFFECTS
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DISSERTATION

Presented in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy
in the Graduate School of
The Ohio State University

By
Ronald Curtis Chatfield D.V.M., M.Sc.

The Ohio State University
1977

Reading Committee:
Dr. R. A. Yeary
Dr. T. E. Powers
Dr. R. P. Herd

Approved by

Dr. R. A. Yeary
Advisor
Department of Veterinary Physiology and Pharmacology
God has given many blessings for which I am humbly thankful. Included in His gifts are a loving family and many friends. To acknowledge all persons who have been instrumental in helping me reach this point in my life would be an impossible task; however I would like to recognize those persons to whom I feel most indebted.

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VITA

Born . . . . . . . . March 1, 1941 Columbus Ohio

Family . . . . . . . . Wife Peggy
         Children Craig (1960)
         Susan (1965)

Education . . . . . . . D.V.M. The Ohio State University
                   1966
         M.Sc. The Ohio State University
                   1974

Professional Activity. . Small Animal Practice 1966-71
         Instructor, Veterinary Pathobiology
         The Ohio State University
                   1971-74
         Graduate Teaching Associate,
         Veterinary Phys./Pharm.
         The Ohio State University
                   1974-75
         Instructor, Veterinary Phys./Pharm.
         The Ohio State University
                   1975-77

FIELDS OF STUDY

Major Field: Veterinary Physiology and Pharmacology

Studies in Anthelmintic Pharmacology. Dr. R. A. Yeary
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INTRODUCTION

The essence of a successful parasite management program is based upon the implementation of sound control measures in conjunction with the administration of appropriate chemotherapeutic agents. The fact that worldwide helminthiasis is the most common infection of man and animal populations creates a need for continuing research and more cooperative efforts by the scientific community.

Interruption or alteration of a vulnerable period in the life cycle is the basis of parasite control. The most dramatic example of a successful parasite control program is the eradication of the screw worm, Callitroga hominivorax, from the state of Florida in 1955 as described by Baumhover (1) and Lindquist (48). It was based on the principle that these flies mate only once a year and that by releasing sterile male flies fertile breedings were prevented. Although eradication is seldom achieved, a reduction in the parasite burden may be expected where there is a conscientious application of scientific principles that are derived from the environmental requirements of the life cycle. Highly pathogenic parasites may produce disease
however, even when present in small numbers. Therefore, anthelmintics are important aids in the elimination of the infection.

The discovery of anthelmintics is attributed to the Chinese who, in the 6th century, recognized the vermifuge effects derived from chewing the betel nut, the chief alkaloid of which is arecoline (42). Since the early discovery of arecoline, the development of new antiparasitic agents has primarily been the result of empirical information obtained from evaluation the effects of various test compounds on experimentally produced parasitic infections. Although there is merit to this method of drug research, a more rational approach is the selective development of drugs that exert their toxic effects by blocking essential biochemical pathways that are unique to the parasite. That this method of drug development is possible is evident by the identification of a naturally occurring insect juvenile hormone (65) and the retardant effects of various analogs on insect development (80).

An in vitro method to screen and develop chemotherapeutic agents is to first incubate the target organism in a suitable culture media and observe the response to various drug concentrations. The data generated from this type of experimentation is the basis for the LC50, which is the lethal concentration for 50 per cent of the
population within a specified time period, and may be useful in the determination of drug dosage. Appropriate \textit{in vivo} experiments may also be performed to determine the possible mechanisms by which the drug exerts its pharmacologic effect on the organism.

A poor understanding of the nutritional and respiratory requirements of parasitic helminths, coupled with a sketchy knowledge of their metabolic machinery, has hindered not only the development of an \textit{in vitro} parasite model but also the investigations into anthelmintic mechanisms.

The purpose of this investigation was threefold: The first objective was to determine the applicability of the \textit{in vitro} method for \textit{Hymenolepis diminuta} cultivation as described by Schiller (75) and modified by Roberts (62) in the determination of the LC$_{50}$ for bunamidine hydrochloride. The second objective was to determine the effects of bunamidine on four of the enzymes involved in the energy metabolism of \textit{H. diminuta} in an effort to elucidate a possible mechanism of action on the parasite. The third objective was to examine by electron microscopy, morphological alterations that might occur as a result of bunamidine HCL exposure.

\textsuperscript{1}Scolaban, Burroughs Wellcome Co., Research Triangle Park, N.C.
Bunamidine hydrochloride is one of a series of naphthamidines and has been shown to be clinically effective against several cestode species (14,33). The chemical name for this compound is N,N-dibutyl-4-hexyloxynaphthamidine, and has the structural formula shown in Figure 1.

![Structural formula of bunamidine HCL](image)

Figure 1.
Structural formula of bunamidine HCL

Bunamidine hydrochloride is a white, odorless, crystalline solid, soluble in hot water, and melts at 208-211°C. The molecular weight is 419. Bunamidine is irritating to mucous membranes and may produce vomiting after oral administration. It has also been incriminated in sensitizing cardiac muscle to endogenous catecholamines predisposing treated animals to fatal ventricular fibrillation (93).
CHAPTER I

LITERATURE REVIEW

The investigative effort described in the subsequent chapters of this text was made possible by the application of scientific principles as they relate to several medical disciplines. Anthelmintic pharmacology is a young science that bridges parasitology and pharmacology, but its dependence on the related fields of physiology and biochemistry must be recognized. To acknowledge the many pioneering achievements that led to the development of each contributing science would not only represent an enormous undertaking, but would serve no useful purpose. A serious attempt has been made to include in the literature review the major scientific publications that provided the background information for the successful completion of this study.

The introductory comments by Becker (2) in an essay entitled, "How Parasites Tolerate Their Hosts", may well serve the same purpose here, for they describe the complex nature of the normal parasite habitat. "Parasites do maintain themselves in their hosts to a considerable extent through adaptations of structure,
physiology and life cycle. But assuming, firstly, that a particular parasite is admirably equipped with functional mechanical devices for keeping to its wonted place in the host's body; and, secondly, that its fundamental processes are basically salubrious, i.e., it could eat, digest, eliminate, respire, assimilate, grow and reproduce well in its environment if given the chance - there is still a third requirement that it must meet in order to maintain itself inside the host. It is an unwelcomed invader in hostile territory, and as such has constantly to contend with enzymes, antibodies, hormones, foreign proteins, protein-split products, tissue defense reactions and predatory phagocytes. How is the parasite equipped to cope with these factors in its environment?"

The foregoing remarks were directed toward the metazoa, but it is worth noting that there are many parasitic bacteria and protozoa that thrive in the same in vivo environment. It might follow then, that the development of an artificial culture media which supports the growth, maintenance and reproduction of an intestinal microbe, for example, might well serve the same purpose for an intestinal helminth. The adaptability of pathogenic bacteria to appropriate laboratory media is well established; however attempts to cultivate helminths under similar conditions have been less successful.
To further investigate the factors which pose major obstacles to helminth cultivation experiments, it is necessary to examine some of the features that distinguish the helminths from other parasitic forms. Helminths, unlike the protozoa or bacteria, are multicellular organisms that range in size from less than one mm to over 10 meters in length. Most possess definite nervous, digestive, muscular, reproductive and excretory systems but lack distinct circulatory and respiratory systems. Many nematodes have complicated life cycles requiring the stimulus provided by several, but specific hosts to moult from the various larval stages to the adult. The cestodes and trematodes likewise utilize many intermediate hosts prior to their emergence as adults in the final or definitive host. Even helminths that fully develop within one host must undergo ecdyses in prescribed locations of the body.

The complexity of the problem confronting the investigator involved in helminth cultivation is apparent. Creating an *in vitro* environment that not only satisfies all of the nutritional and respiratory needs, but also provides the triggering stimuli for larval development, has been a major challenge and will likely remain as such until more is known regarding their physiological processes. McCoy (52) and Hobson (39) however, have stated that the physiological processes of parasitic helminths have not
been as extensively studied as have other invertebrates because of the inability to maintain them independent of their hosts. It would appear then, that knowledge of helminth physiology and the development of a suitable artificial culture media are so interdependent that each affects the progress of the other.

A. **In Vitro Cultivation of Helminths**

As early as 1883, it was noted that cat ascarids could survive several days outside the host when in an oxygen-free environment (52). In his review, Hobson (39) cited the more notable early efforts at nematode cultivation including the survival periods for each parasitic species. He noted that parasites of cold-blooded hosts survive longer in artificial media than those that parasitize the alimentary canal of warm-blooded animals.

In 1938, Glaser and Stoll (27) incubated first-stage *Haemonchus contortus* larvae aerobically in a media consisting of 0.5 per cent agar, sheep liver extract, ground yeast, sheep blood and sheep kidney in Ringer's solution. Since bacterial sterility appeared to be an absolute requirement for further development, the infective third stage larvae were washed by sedimentation many times in sterile water and in one-to-twenty dilutions of Labarraques solution. Despite later improvements in their
sterilization procedure (28), they were unable to support *H. contortus* beyond a few parasitic fourth stage larvae. Their conclusions were that with slight nutritional modifications of the media, they could complete the life cycle to the adult stage.

Although their optimistic views regarding *H. contortus* did not materialize, Glaser (26) later reported his success in propagating several generations of *Neoaplectana glaseri*, an insect nematode, using sterile rabbit kidney on dextrose agar slants as the culture media. This was the first report of the complete axenic (i.e., free of other living forms) cultivation of a parasitic helminth outside its host environment.

Intrigued with the possibility of raising parasites in stock laboratory cultures, Stoll (81) later developed a liquid media capable of supporting *N. glaseri* through successive life cycles. He suspected the liver extract component of the *H. contortus* media was responsible for his earlier failures and prepared a sterile, acidified, raw liver extract (RLE) that, when added to veal infusion broth, met the nutritional requirements for the complete axenic development of *N. glaseri*. He reported yields of 2,500 to 5,000 *N. glaseri* following the inoculation of 25 third stage larvae, but later reported (82) yields of over 10,000 using the same initial inoculation dose on an enriched media. The larger yields were attributed to the
RLE which had been prepared from the livers of rabbits late in pregnancy or early post-partum.

_Anclostoma caninum, A. duodenale_ and _Nippostrongylus muris_ were cultured from the egg to the filariform stage in the absence of living bacteria by Weinstein (90) on fresh chick embryo or rat liver extracts containing penicillin and streptomycin. He noted that when extracts of chick embryo and rat liver extract were heated to 55°C for 15 minutes, their growth promoting effect was lost. Although Weinstein's culture method proved unsuitable in supporting the complete life cycle of hookworms, it revealed that there were filterable, heat labile components (unidentified) in the RLE necessary for larval development.

It was suggested by Dougherty (22) that the axenic cultivation of free-living forms might well provide clues to the problem of growing parasites free from their hosts. He had previously shown (21) that _Rhabditis briggsae_ could be successfully maintained on pieces of chick embryo, but the growth rate was slower in sterile cultures than when a mixed microflora was present. Based on these observations, he concluded that either growth promoting substances were not available, or there were growth inhibitory substances present in sterile chick embryo. Later in the same year, Dougherty (22) reported that fresh chick embryo juice or aqueous liver extract would support _R. briggsae in vitro_, but the "active components" did not
pass through a Visking dialyzing membrane. He termed the necessary growth factor(s) "factor Rb" and discussed some of its physical properties in a subsequent paper (20) but the chemical identification of this component eluded his investigations.

Probably owing to their large size, tapeworms were not used in \textit{in vitro} cultivation experiments until Schiller (75) reported on the development of \textit{Hymenolepis diminuta} cysticercoids to proglottid-shedding adults in a media of defibrinated rabbit blood and Hank's balanced salt solution. Turton (84) and Roberts (62) later showed that defibrinated sheep or horse blood could be substituted for the more expensive rabbit blood with no discernible adverse effects on tapeworm development.

A diphasic medium similar to Schiller's was successfully used by Voge and Coulombe (87) in promoting growth and asexual multiplication of \textit{Mesocestoides corti} tetrathyridia. Other notable achievements in cestode cultivation have been reported (24,34,35,36,79).

The beneficial effects derived from adding a protein supplement such as liver extract, chick embryo extract or blood were evident and became standard components of most parasite culture media (29,30,46,47,58,91,92).

In their review on the \textit{in vitro} cultivation of helminths, Silverman and Hansen (77) show that all the media in which helminths have undergone differentiation,
growth and maturation have contained undefined complex materials such as chick embryo, liver or host's fluids. They also point out the difficulties in reproducibility and stability during storage and incubation when using such undefined components.

Until the growth promoting factors provided by these animal tissues are isolated, purified and identified, investigations involving in vitro helminth cultivations must contend with these limitations and include the crude tissue extracts in the culture media.

B. Helminth Physiology and Biochemistry

Bueding (10) cited the classical experiments of Claude Bernard as being instrumental in developing the concept of the neuromuscular junction and the role of hemoglobin in the transport of oxygen. Recognizing the value of pharmacologically active substances in the study of physiological processes, he noted the effects of curare and carbon monoxide on biological systems and advanced theories to explain his results. Since these early experiments, drugs have become standard investigational tools in physiology and pharmacology and are largely responsible for our current understanding of these respective fields. Evidence for their use in helminth physiological studies was lacking until the middle of this century which
coincides with a period of rapid improvement in \textit{in vitro} cultivation techniques. For an account of the earlier history of helminth biochemistry, the reader is referred to Von Brand's (88) colorful description of eighteenth and nineteenth century investigations.

\textbf{B. 1. Physiological Processes}

Chemotherapeutic agents exert their effect on parasitic organisms by interfering with essential physiological or biochemical processes (72). As has been stated earlier, the goal of anthelmintic research is to develop drugs that interfere with not only essential processes but those that are unique to the invading parasite. Piperazine is an anthelmintic that selectively blocks a physiological process. It was used in the early 1900's as a uricosuric agent in the treatment of gout. Although piperazine is an excellent solvent for uric acid, it proved to be clinically ineffective for this purpose (66). Fayard (66) in 1949, is credited with the discovery of piperazine's anthelmintic properties. It is now widely used in both human and veterinary medicine in the treatment of ascarid and pinworm infections. Piperazine causes a reversible, flaccid paralysis of Ascaris muscle (and presumably Oxyuris muscle as well) and results in the worm's expulsion by the host's intestinal peristaltic movements. The experiments
of Norton and De Beer (56) not only demonstrated the mechanism of action of piperazine, but to some extent characterized the myoneural receptor of Ascaris muscle. While incubating intact worms and split worm segments in a 37°C Ringer's solution to which various drugs and concentrations were added, they made the following observations: (1) Piperazine produced paralysis of Ascaris muscle, but d-tubocurarine had no effect. (2) Acetylcholine (ACH), nicotine alkaloid, succinyl choline, and decamethonium all caused muscle contraction. (3) The onset of piperazine-induced paralysis was more rapid in split worms than in intact worms. Their conclusions were that piperazine is a competitive inhibitor of Ach and that its wide therapeutic safety margin is likely related to subtle differences between mammalian and Ascaris myoneural receptors.

Based on Norton and DeBeer's studies, Fiakpui (25) has studied the effects of piperazine and methyridine on Caenorhabditis briggsae, a free-living nematode. In comparing piperazine, methyridine and Ach, he noted that methyridine and Ach caused a depolarizing paralysis and were additive; whereas, piperazine produced a flaccid paralysis and was a competitive antagonist to methyridine and Ach. Mellanby (53) identified and estimated the Ach content of adult Ascaris lumbricoides and Litmosoides

carinii, and microfilaria of *Dirofilaria repens*, and found that the microfilaria contained the most Ach, followed by *L. carinii* adults. She suggested the physiological significance of the findings may be related to their motile behavior (i.e., microfilaria are active swimmers in the bloodstream, whereas ascarids are much less active in the intestinal lumen) and the amount of nervous tissue in the parasite's body.

That Ach serves as a neurotransmitter in several nematode species is now well established. However, Tomasky, et al. (83) have shown Ach to be a neuroinhibitory substance in the blood fluke *Schistosoma mansoni*. Evidence for this was provided by observing the paralytic effects of direct Ach application, and the stimulatory effects of atropine and mecamylamine. They also noted that 5-hydroxytryptamine was excitatory but catecholamines such as dopamine, epinephrine and nor-epinephrine caused relaxation. Apomorphine, a dopamine agonist, likewise caused relaxation, but the nor-epinephrine partial agonist clonidine, had no effect. Although this study was not conclusive in characterizing trematode receptors, it did suggest the existence of binding sites that may uniquely respond to various cholinergic and adrenergic drugs.

The Ach receptors of *S. mansoni* were visually outlined by Hillman and Gibler (37) using DNS-Chol, a dansylated choline derivative, as a fluorescent indicator.
In a series of elegant experiments, they observed the paralytic effects of Ach and carbachol and the stimulatory effects of DNS-Chol and hycanthone by recording parasite movement on a small-scale monitor connected to a photo-cell. Atropine decreases the amount of DNS-Chol binding and blocks the effects of added Ach (38) which not only verifies the DNS-Chol binding to Ach receptors but suggests that these receptors bear a structural resemblance to the muscarinic receptors of mammalian tissue.

In an earlier report, Hillman and Senft (38) reported that hycanthone is also an inhibitor of Ach esterase (ACHE) from *S. mansoni* but is less effective against mammalian ACHE. In contrast, physostigmine inhibits mammalian enzyme more effectively than it does the helminth enzyme. Based on these observations, it was proposed that mammalian and schistosome ACHE differ and that hycanthone may have a selective affinity for schistosomal cholinergic systems.

The liver fluke Fasciola hepatica was used as the test parasite in a kymographic study of the action of drugs by Chance and Mansour (15). The worms were suspended in Ringer's solution and connected to a kymograph such that their movements in response to added drugs were recorded. The chlorinated hydrocarbons, strychnine, amphetamine and ephedrine all had stimulatory effects on the worm while nicotine and arecholine resulted in a
paralysis that was reversible when amphetamine was introduced into the system. The inhibitory nature of cholinergic drugs on *F. hepatica* is in agreement with similar observations on *S. mansoni*, but the stimulatory effects of alpha and beta adrenergic agonists is in direct conflict.

The occurrence and localization of 5-hydroxytryptamine (5-HT) and catecholamines (CA) have been reported in *S. mansoni* (3,4). Using special fluorescent staining methods, has shown CA to be localized in two bipolar cells in the head region and along the lateral nerve trunks. 5-HT was seen in these same areas but was also found throughout the parenchyma in storage granules. When the worms were incubated in the presence of 5-hydroxytryptophan (5-HTP), the precursor to 5-HT, there was an increase in the amount of 5-HT in storage granules and suggests the presence of 5-HPT decarboxylase as well as a 5-HT uptake mechanism. The uptake mechanism appeared to be specific for 5-HT as determined by the differential fluorescent staining characteristics between 5-HT and CA. The localization of ACHE was shown in a previous report (13) to be closely associated with areas of high 5-HT and CA concentrations.

To give a detailed account of the mechanism by which neurotransmitter and neuroinhibitory substances act in maintaining the functional integrity of helminths is not
possible at this time. Experimental evidence supports the existence of mediators and receptors in helminths that resemble the cholinergic and adrenergic nervous system in vertebrate species. There are apparent functional differences between the "autonomic" nervous system of flatworms (Phylum Platyhelminthes) and roundworms (Nemathelminthes) and may be the reason why most nematode anthelmintics are ineffective against cestodes or trematodes, and vice versa.

B. 2. Biochemical Processes

Parasites are subjected to variable conditions according to their location in the host. For example, the environment of intestinal worm is characterized by low oxygen, high carbon dioxide, variable pH, and a plentiful supply of small particulate foodstuffs; whereas, parasites found in the bloodstream are exposed to high oxygen, low carbon dioxide, a narrow pH range and molecular-sized food particles. In light of these variables it would be fool-hardy to suggest that internal parasites represent an homologous group of organisms with respect to their nutritional and metabolic needs. Unrelated parasites may share common biochemical or metabolic processes but one must exercise caution in making broad generalizations based on one finding.
At the turn of this century, Weinland (89), as cited by Epps (23), reported that *Ascaris lumbricoides* lived longer in an anaerobic environment than under aerobic conditions but exposure to either atmosphere resulted in glycogen depletion. Since carbohydrate was utilized in comparable amounts with or without oxygen, he proposed the revolutionary notion that Ascaris, as well as many other intestinal worms, do not require molecular oxygen. It was suggested by Weinland and later verified by Epps (23) that valeric acid is a major metabolic waste product of Ascaris metabolism and not the result of enteric bacterial fermentation. These observations were made long before the biochemistry of glycolysis was understood for it was not until the mid 1930's that Gustav Embden and Otto Myerhoff first isolated some of the substrates and enzymes of this anaerobic pathway (45).

At a 1971 Symposium on Comparative Biochemistry of Parasites, Saz (68) summarized the studies on nematode, cestode and trematode carbohydrate metabolism by stating that all of the helminths studied so far are capable of assimilating oxygen under appropriate conditions, but none are capable of the complete oxidation to carbon dioxide and water. They all accumulate organic metabolic end products which indicate an incomplete terminal pathway. The filarial worm, *Litomosoides carinii*, a parasite found in the pleural cavity of the cotton rat, requires oxygen and
glucose to maintain motility (8). Under aerobic conditions glycogen stores increase while anaerobically they become depleted. The inhibition of glycolysis by the presence of oxygen is termed the Pasteur effect and has also been demonstrated in *Nippostrongylus brasiliensis* (69), a parasite in the rat intestine. Interestingly, in spite of these worm's demands for aerobiosis, lactic acid is the major end-metabolic product in both organisms.

When the rat pinworm, *Syphacia muris*, or the blood fluke, *Schistosoma mansoni*, are incubated either anaerobically or aerobically in the absence of exogenous substrate, their rate of endogenous glycogen utilization is unchanged (9,86). Whereas succinate is the major metabolite of *Syphacia*, lactate is formed by the Embden-Meyerhoff glycolytic sequence and is the sole product formed from glucose in *S. mansoni* (68). *Trichuris vulpis* survives longer in a mixed atmosphere of carbon dioxide and nitrogen than in nitrogen alone, air, or a mixture of carbon dioxide and air. Fifty per cent of the glucose taken up by this worm during aerobic or anaerobic incubation is recovered as lactic, acetic, proprionic or n-valeric acid, carbon dioxide and small quantities of formic and n-butyric acids (11). Dithiazinine, a drug used in the treatment of human and canine whipworm infections, was shown to block glucose absorption in *T. vulpis* (11).
The inability of parasitic organisms to completely metabolize glucose to carbon dioxide and water suggests that the TCA cycle and cytochrome system are either non-functional or are lacking. Experimentally, it has been shown that some parasites have a partial or full complement of TCA cycle enzymes, and cytochrome oxidase has also been reported. Many of the protozoan trypanosomes, while in the insect vector are capable of complete carbohydrate metabolism via the TCA cycle and cytochrome system but in the mammalian host only glycolysis occurs (16). *Dicrocelium dentriticum* likewise possesses all of the enzymes necessary for TCA cycle metabolism but due to low levels of alpha-ketoglutarate dehydrogenase, it plays a minor role in the overall metabolic scheme of the parasite (43). *Ascaris lumbricoides* lacks essential TCA cycle enzymes (76), as does *H. diminuta* (64) and *Litomosoides carinii* (69).

*Ascaris* mitochondria also lack cytochrome activity (76), while in *Syphacia muris*, a phylogenetically related species, it is present in low amounts (86). Scheibel, et al. (74) were unable to detect cytochrome activity in *H. diminuta* but Robinson and Bogitsh (64) later reported the existence of two cytochrome enzymes, one of which was cyanide sensitive and resembled mammalian cytochrome c. The cyanide sensitive enzyme was later considered to be a peroxidase (64). *T. vulpis* has high cytochrome c and
cytochrome oxidase activity and consumes oxygen when incubated in air (11). If either cytochrome enzyme represents a peroxidase, the resultant hydrogen peroxide could account for the shortened aerobic survival time; however *T. vulpis* has catalase activity far in excess of oxygen uptake rate and discounts the possible deleterious effects of hydrogen peroxide in this nematode (11). Mitochondrial peroxidase has been demonstrated in *H. diminuta* (64), and *S. mansoni* (5), but since catalase activity has not been reported, cytotoxic hydrogen peroxide may be formed in sufficient amounts to account for their decreased survival time when oxygen tensions are high.

Carbon dioxide, in contrast to the toxic effects of oxygen, is a requirement for most parasites studied for it is utilized in the anaerobic generation of ATP. Bryant (6) has reviewed the literature on the utilization of carbon dioxide by parasitic helminths and cites the many references that corroborate the dependence of these organisms on carbon dioxide for ATP formation. The incorporation of $^{32}\text{P}$ into ATP by *H. diminuta* was studied anaerobically in the presence and absence of uncouplers of oxidative phosphorylation and various anthelmintic drugs (74). The anticestodal drugs chlorsalicylamide$^3$ and dichlorophen$^4$ and the uncoupler, 2,4-dinitrophenol were shown to inhibit

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3 Yomasan, Haver Lockhart, Shawnee, Kansas
4 Diphenthane 70 Pitman-Moore, Washington Crossing, N.J.
the incorporation of $^{32}$P into ATP under both anaerobic and aerobic conditions. In the presence of carbon dioxide and drug, internal ATP concentrations remained constant in spite of the inhibitory effects of these agents on $^{32}$P incorporation into ATP. When carbon dioxide was removed from the system and in the presence of drug, there was a marked decrease in both $^{32}$P incorporation and ATP levels. Based on these observations, it was concluded that there is an electron transport system present that is associated with anaerobic phosphorylation in H. diminuta and the inhibition of this transport system by anticestodal drugs may account for their mode of action.

It is now widely accepted that of the helminths studied, all ferment carbohydrate via the Embden-Meyerhoff glycolytic pathway to at least the level of phosphoenolpyruvate (PEP) and to this extent resembles mammalian anaerobic glycolysis (70). However, at steps beyond PEP metabolic variations occur which not only metabolically separates the parasite from its mammalian host, but to some extent distinguishes between parasites in their energy producing mechanism. For a given amount of energy production, anaerobic metabolism requires more glucose than aerobic metabolism since the end-products of glycolysis contain considerable chemical energy. Parasites capable of forming succinate have a distinct advantage over those forming only lactic acid by the classical glycolytic
sequence (12). The reason for this advantage is that the reduction of fumarate to succinate by NADH is catalyzed by a mitochondrial electron transport system that generates additional energy in the form of ATP (41, 74, 76). Although the mechanism is poorly understood, it appears that parasites excreting succinate or metabolites derived from succinate share a common energy-yielding electron transport mechanism that utilizes a flavoprotein as the terminal oxidase rather than a cytochrome oxidase (12, 74). The proposed metabolic scheme for carbohydrate metabolism in anaerobic parasites is as follows: In the cytosol fraction, PEP is formed via various intermediates of glucose breakdown. In parasites forming only lactate as a metabolite (S. mansoni, for example), PEP serves as the substrate for pyruvate which in turn forms lactate. ATP is generated from ADP when pyruvate is formed.

In succinate excreting parasites (H. diminuta for example), carbon dioxide is enzymatically incorporated into PEP to form oxaloacetate which in turn is reduced to form malate, thereby regenerating NAD+ from glycolytically formed NADH. Malate then enters the innermembrane space of the mitochondria where it undergoes a dismutation reaction and forms one mole of pyruvate and one mole of fumarate from two moles of malate. In forming pyruvate, NAD+ is reduced and provides the reducing power necessary for the flavin-linked phosphorylation system which
presumably occurs in the mitochondrial matrix. *H. diminuta* differs from *Ascaris* in this reaction in that NADP+ is required for pyruvate formation rather than NAD+. Since the inner mitochondrial membrane is impermeable to pyridine nucleotides, it has been suggested and experimentally supported that an NADH-NAD+ transhydrogenase serves to translocate the hydride ion from the innermembrane space to the matrix side of the membrane where it regenerates the NADH necessary for fumarate reduction to succinate (44, 61, 71). This coupled reaction of NADH oxidation and fumarate reduction generates ATP (44, 76).

The similarities of the metabolic sequence of succinate producing helminths to the glycolytic pathways of mammalian systems has been noted. At steps beyonPEP, there are certain similarities to the TCA cycle. The mammalian TCA cycle proceeds from a succinate to oxaloacetate direction in generating ATP via the electron transport system. This electron transport system is comprised of iron-containing hemoproteins called cytochromes which transfer electrons from flavoproteins to molecular oxygen, and in the process form water and ATP. The helminth counterpart to the TCA cycle operates in an oxaloacetate to succinate direction and anaerobically generates ATP via a flavin-linked fumarate-reductase system operating at the same substrate site as the electron transport system of mammalian cells. Whereas higher forms
of life have more metabolic options to generate the energy necessary for the performance of essential physiological processes, it would appear that most parasitic helminths are limited to a single, unique metabolic pathway that renders them susceptible to attack by chemotherapeutic drugs.

Many anthelmintic drugs have been shown to interfere with the fumarate reductase system of helminth metabolism. The interruption of this system is accompanied by a net decrease in ATP synthesis and may explain the mechanism by which these drugs exert the toxic effect on the parasite (17, 51, 60, 74, 85, 94). Anticestode drugs having no clinical application in treating nematode infections have been shown to inhibit fumarate reductase in A. lumbricoides (67) and implies that the differences in the tegument of cestodes and the cuticle of nematodes may have a role in regulating the drug absorption to the site of action.
Figure 2

ENERGY METABOLISM IN HELMINTHS
CHAPTER II

MATERIALS AND METHODS

There were three phases to this study:

1. The purpose of the experiments in the initial phase was to determine the LC$_{50}$ for bunamidine HCL on *Hymenolepis diminuta* in an *in vitro* culture system. Viability was determined on the basis of worm motility.

2. The purpose of the second phase was to examine worms under electron microscopy to determine the effects of drug exposure on organelle structure.

3. The third phase was designed to evaluate four metabolic enzymes in the presence and absence of bunamidine hydrochloride.

A. Phase I

*Cysticercoid's of* *H. diminuta* were obtained from the laboratory of Dr. Peter Pappas, Dept. of Zoology, The Ohio State University, and orally administered to five unanesthetized female Gunn rats (Wistar strain) using a metal gastric canula. These rats then served as a source

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of tapeworm ova for future experiments. Six weeks following cysticercoid inoculation, a pooled 24-hour fecal collection was mixed with saturated sucrose solution, strained through cheesecloth and centrifuged at 2,000 rpm for 10 minutes in 50 ml centrifuge tubes. The top 10 ml of each of the centrifuged samples was pooled in a large graduated cylinder and diluted to one liter with tap water. Upon overnight standing the top 850 ml of water was removed by suction, leaving the ova which had settled to the bottom. The remaining volume was then centrifuged at 2,000 rpm which concentrated the ova in the form of a pellet. The pellet was then mixed with a small volume of apple (10-15 gms) and fed to 50 starved *Tenebrio molitor* beetles⁵ (common grain beetle). The beetles consumed the ova-laden apple usually within 24 hours and were thereafter maintained at room temperature on a diet of commercial dog biscuit. Gauze surgical sponges were moistened and placed in the open container to increase the humidity and to serve as a source of water for the beetle colony. Nineteen days following the ingestion of ova, mature cysticercoids were harvested from the beetles by removing the head and posterior regions with scissors, then flushing the body contents into a petri dish using a water filled Pasteur pipette fitted with a bulb syringe. With the aid of a

⁵Courtesy of Mr. John McCabe of the Entomology Culture Laboratory, The Ohio State University.
Stereo Zoom "7" microscope (Bausch & Lomb, Rochester, N.Y.),
cysticercoids were counted and divided into doses containing
ten to twenty-five cysticercoids per dose and administered
to rats as described earlier. Rats were sacrificed ten
days post infection by decapitation and the entire small
intestine was removed. The worms were removed by gently
flushing tap water through the intestinal tract and cleared
of rat intestinal contents by swirling the worm through
a 37°C water bath using a small soft bristled brush. The
average worm length was 2½ inches. The surface sterilizat­
ion procedure used in the initial incubations was patterned
after the method of Roberts (62) and involved dipping each
worm for 2-3 seconds in 0.1% hexachlorophene solution
(Phisohex, Winthrop Laboratories, New York, N.Y.) prior to
being placed in the incubation flasks. The surface
sterilization procedure used in the fourth and final
incubation was that described by Dr. R.P. Herd6 (personal
communication). Worms were individually placed in sterile
15 ml vials, each containing 6 ml of a sterilizing solution
comprised of Hank's Balanced Salt Solution (pH 7.4), to
which was added 1,000 units penicillin G, 1 mg streptomycin,
250 units polymixin and 200 units nystatin per ml solution.
The vials containing the worms, as well as the sterilizing

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6Dr. R.P. Herd, Dept. Veterinary Pathobiology,
The Ohio State University.
solution, were maintained at 37°C. At five minute intervals the liquid was removed by suction and replaced with fresh, sterile solution. After six washings, the worms were placed individually in 50 ml screw top culture tubes containing the diphasic culture media described in the next section.

Media Preparation:

1. Blood agar: Trypticose Soy BBL Agar (20 gms), was dissolved in 50 ml of double distilled water by slowly stirring the mixture on a hotplate (Corning Model PC-351) until boiling. Aliquots (9.5ml), were dispensed into 50 ml Erlenmyer flasks or 50 ml culture tubes, using a Cornwall continuous pipette (Becton-Dickinson, Rutherford N.J.). The incubation flasks and tubes were covered with a double thickness of aluminum foil and autoclaved for 20 minutes at 121°C under 15 pounds per square inch pressure. Upon cooling to 45°C, 0.5 ml of bovine blood (collected aseptically) was added by elevating the top aluminum foil cover and inserting an 18 guage needle, fitted to a sterile disposable 3 cc size syringe, through the second cover. Gentle agitation uniformly distributed the blood throughout the liquid agar. The sterile blood agar was kept refrigerated until the day of use.
Liquid Overlay: A liter of liquid culture overlay was prepared by adding 100 ml of a tenfold concentrate of Hank's Balanced Salt Solution (HBSS, prepared by the Reagent Laboratory at The Ohio State University, without NaHCO₃) to 80 ml of 0.1 M NaHCO₃. This solution (pH 7.4), was brought to a final volume by adding double distilled water and sterilized by filtration through a 0.22 micron bacteriological filter (Model 7103, Falcon Division of Becton-Dickenson, Oxnard, Calif.). Penicillin G (1 million units), streptomycin (1 Gm), polymyxin (250,000 units), and nystatin (200,000 units), were added per liter liquid to aid in the control of enteric bacterial overgrowth.

On the day of worm incubations, 9 ml of freshly prepared sterile HBSS was added to the culture vessels containing the blood agar base using a Cornwell continuous pipette. Preliminary incubations consisted of four treatment groups and a control group (one worm per tube). Each group contained 5 worms. The final incubation contained the same number of groups as the preliminary incubations, but each group consisted of 20 worms. The culture vessels were slowly agitated in a Dubnoff metabolic shaking incubator (Precision Scientific Co., Chicago, Ill.) at 37°C. Solutions containing 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ M bunamidine HCl (BUN) were prepared by dissolving the appropriate amount of drug in hot, double-distilled water and sterilized by filtration. One ml of each drug
concentration was then added to the culture flasks while one ml of double distilled water was added to the flasks serving as controls.

In all, four incubation experiments were performed. The results of the preliminary incubation determined a more narrow range of drug concentrations for the second incubation. The results of the second and third study provided the basis for selecting a drug concentration range sufficiently narrow that the LC50 could be determined in the fourth and final incubation.

B. Phase II

Representative worms were selected from control and drug treated groups for electron microscopy studies. All worms selected were alive at the time of fixation in glutaraldehyde to minimize artifactual changes arising from autolysis. Proglottids taken 2 cm distal to the scolex were submitted to the Department of Veterinary Pathobiology for mounting, sectioning and examination under the electron microscope.
B. Phase III

Four of the enzymes involved in carbohydrate metabolism of *H. diminuta* were examined. The enzymes studied were phosphoenolpyruvate carboxykinase (PEPCK) (E.C. 4.1.1.32), pyruvate kinase (PK) (E.C. 2.7.1.40), "malic enzyme" (ME) (E.C. 1.1.1.39) and fumarate reductase (FR) (NADH:fumarate oxidoreductase). PEPCK and PK are located in the cytosol fraction (12) whereas ME and FR are found in the mitochondria (67). Enzyme activity was determined in protein recovered from adult *H. diminuta*. Ten day old worms were not used in the enzyme assay experiments because their small size necessitated the use of prohibitive numbers of worms.

The worms were recovered from the rat intestine as described in Phase I of this chapter, and immediately placed in chilled sucrose solution (0.25M). After blotting, a 10% W/V solution was prepared by placing 2 Gms of worm tissue and 18 ml of 0.25M sucrose-0.005M EDTA solution at 0°C in a glass homogenizing tube. The tissue was homogenized with a Sorvall Omni-Mixer (Model 17150, Ivan Sorvall Inc., Newtown, Conn.) equipped with a teflon tipped pestle. The pestle was inserted to the bottom of the homogenizing tube three times. The milky-white homogenate was then centrifuged under refrigeration (Sorvall Superspeed Centrifuge, Model RC 2-B, Ivan Sorvall Inc., Newtown, Conn.) at
0°C for 10 minutes at 117 g (1,000 rpm). The sediment contained cellular debris and was discarded. Centrifugation of the supernatant at 7,500 rpm (6,780 g) for 30 minutes at 0°C resulted in the formation of a brown pellet that represented the mitochondrial fraction of the worm. This pellet was washed with the sucrose-EDTA solution and was respun at 7,500 g for 30 minutes. The supernatant from the washing was discarded. The supernatant containing the cytosol protein (from the initial 7,500 rpm centrifugation) also had a fluffy white aggregate that was removed by centrifugation at 100,000 g and 4°C in a Beckman Ultra-centrifuge (Model L3-50, Beckman Instruments, Palo Alto, Calif.). Test tubes containing this supernatant, as well as those containing the mitochondrial pellets, were capped with Parafilm (American Can Co., Neenah, Wisc.) and stored at -88°C in a Revco Ultra-Low Temperature freezer (Model ULT-657-3, Revco Inc. Division of Industrial Products, Columbia, S.C.), until the day of use.

On the day of the experiment, mitochondrial or cytosol protein was thawed in an ice bath. Proteins were determined by the method of Lowry, et al. (49). Cytosol proteins averaged about 5mg/ml while mitochondrial proteins averaged 5-6 mg/ml when 3ml sucrose was added to the pellet.
Cytosol Enzymes:

1. PEPCK activity was determined by recording the decrease in optical density associated with NADH oxidation. The analytical procedure used was that described by Bueding and Saz (12). Table (1) lists the concentration of a 3 ml reaction mixture including the concentration of each and the order added. All reactants were kept in a 37°C water bath except the cytosol protein which was maintained at 0°C.

The spectrophotometer cuvettes and cuvette holders were kept a 37°C in a Temp-Blok Module Heater (Model H-2025, Scientific Products, Evanston, Ill.). Eppendorf pipettes (Brinkman Instruments, Inc., Westbury, N.Y.) were used to deliver the reactants to the cuvettes. All reactants through NADH (Table 1) were mixed and allowed to pre-incubate for two minutes at 37°C before PEP was added to initiate the reaction. The cuvette holder and cuvettes were quickly inserted into a Gilford spectrophotometer (Model H-95008, Ft. Washington, Pa.). NADH oxidation was recorded at 340 nm wavelength. A standard curve for NADH optical density in buffer vs. concentration was established to determine the rate of NADH oxidation and was used as the reference standard for all enzyme assays that used this cofactor.
TABLE 1
Components of PEPCK enzyme assay

<table>
<thead>
<tr>
<th>Component (order added)</th>
<th>Concentration (per ml assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Imidazole buffer (pH 7) *</td>
<td>52.0 μmole</td>
</tr>
<tr>
<td>2. Water or bunamidine</td>
<td>70.0 μmole</td>
</tr>
<tr>
<td>3. Cytosol protein</td>
<td>0.18 mg</td>
</tr>
<tr>
<td>4. MnCl2</td>
<td>5.0 μmole</td>
</tr>
<tr>
<td>5. NaHCO₃</td>
<td>17.0 μmole</td>
</tr>
<tr>
<td>6. IDP</td>
<td>1.0 μmole</td>
</tr>
<tr>
<td>7. NADH</td>
<td>0.12 μmole</td>
</tr>
<tr>
<td>8. NaPEP</td>
<td>2.0, 0.66, 0.4, 0.2, 0.1 μmole</td>
</tr>
<tr>
<td>* Malic dehydrogenase in buffer</td>
<td>5.5 units</td>
</tr>
</tbody>
</table>

2. PK activity was measured by following the decrease in optical density associated with NADH oxidation. The analytical procedure was based on the method of Bueding and Saz (12). Table (2) lists the components of the reaction mixture in the order added to a 3 ml cuvette. All reactants were kept at 37°C using a water bath, except the cytosol protein which was kept in an ice bath at 0°C.

The cuvettes and cuvette holder were maintained at 37°C as described for the PEPCK assay method. PEP was added after a two minute incubation at 37°C to initiate the reaction. NADH oxidation was recorded at 340 nm.
TABLE 2

Components of PK enzyme assay

<table>
<thead>
<tr>
<th>Component (order added)</th>
<th>Concentration (per ml assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Imidazole buffer (pH 7.4)*</td>
<td>50.0 μmole</td>
</tr>
<tr>
<td>2. Water or bunamidine</td>
<td>70.0 μmole</td>
</tr>
<tr>
<td>3. Cytosol protein</td>
<td>0.18 mg</td>
</tr>
<tr>
<td>4. ADP</td>
<td>10.0 μmole</td>
</tr>
<tr>
<td>5. KCl</td>
<td>25.0 μmole</td>
</tr>
<tr>
<td>6. MgSO₄</td>
<td>25.0 μmole</td>
</tr>
<tr>
<td>7. NADH</td>
<td>0.12 μmole</td>
</tr>
<tr>
<td>8. NaPEP</td>
<td>6.0, 3.0, 1.5, 0.75, 0.375 μmole</td>
</tr>
<tr>
<td>* Lactic dehydrogenase in buffer</td>
<td>5.5 units</td>
</tr>
</tbody>
</table>

Mitochondrial Enzymes:

1. Malic enzyme activity was determined in the mitochondrial protein according to the procedure described by Saz, et al. (71), but with slight modifications. The constituents of the ME assay as well as the concentration and order each was added to a 3 ml spectrophotometer cuvette, is indicated in Table (3).

The protein fraction was kept in an ice bath while the remaining components were maintained at 37°C in a water bath. Malate was added after a two minute pre-incubation at 37°C to initiate the reaction which was read at 340 nm
and followed the increased optical density associated with nucleotide reduction. Plotting various NADP concentrations in buffer as a function of optical density established a standard curve which was used in determining the reaction velocity of mitochondrial ME.

TABLE 3
Components of ME enzyme assay

<table>
<thead>
<tr>
<th>Component (order added)</th>
<th>Concentration (per ml assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Imidazole buffer (pH 8)</td>
<td>40.0 μmole</td>
</tr>
<tr>
<td>2. Mitochondrial protein</td>
<td>0.02 mg</td>
</tr>
<tr>
<td>3. NADP</td>
<td>1.0 μmole</td>
</tr>
<tr>
<td>4. MnCl₂</td>
<td>1.0 μmole</td>
</tr>
<tr>
<td>5. Water or bunamidine</td>
<td>70.0 μmole</td>
</tr>
<tr>
<td>6. Malate</td>
<td>0.8, 0.53, 0.4, 0.2, 0.1 μmole</td>
</tr>
</tbody>
</table>

2. Fumarate reductase activity was measured according to the method described by Pritchard (60). The decreased optical density associated with NADH oxidation was recorded at 340 nm in the reaction mixture outlined in Table (4). All reactants were maintained at 37°C except the protein fraction which was kept at 0°C.

The components, through NADH, were mixed and incubated for two minutes at 37°C before fumarate was
added to initiate the reaction.

PEP, malate, fumarate, NADH, NADP, IDP, ADP, imidazole, lactic dehydrogenase and malic dehydrogenase were obtained from Sigma Chemical Company, St. Louis Missouri. Bunamidine was donated courtesy of Burroughs Wellcome Co., Research Triangle Park, N.C.

**TABLE 4**

Components of FR enzyme assay

<table>
<thead>
<tr>
<th>Component (order added)</th>
<th>Concentration (per ml assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. KPO₄ buffer (pH 7)</td>
<td>400 µmole</td>
</tr>
<tr>
<td>2. Mitochondrial protein</td>
<td>0.07 mg</td>
</tr>
<tr>
<td>3. MgCl₂</td>
<td>3.3 µmole</td>
</tr>
<tr>
<td>4. NADH</td>
<td>0.1 µmole</td>
</tr>
<tr>
<td>5. Water or bunamidine</td>
<td>2.7 µmole</td>
</tr>
<tr>
<td>6. Na fumarate</td>
<td>3.3, 1.7, 0.8, 0.4 µmole</td>
</tr>
</tbody>
</table>
CHAPTER III

RESULTS

The results of each experimental phase are presented separately.

1. In Vitro Cultivation Results

The diphasic medium consisting of a blood agar base overlaid with an equal volume of HBSS proved suitable in supporting H. diminuta for the 24-hour experimental period. The white appearance of the worms against the red background of the media presented a striking color contrast that allowed quick and easy determinations of motility.

The results obtained in the three preliminary incubations indicated that many of the culture flasks were contaminated with bacteria as evidenced by the discoloration of the media to a dark-reddish brown and the presence of particulate matter that imparted a cloudy appearance to the HBSS. Flasks containing high concentrations of bunamidine ($10^{-3}$M) showed no evidence of bacterial contamination, but when the drug was added at these levels it immediately precipitated from solution and settled on the agar phase within two minutes. The bacterial
contamination was attributed to incomplete surface sterilization or to an inadequate antibiotic mixture in the HBSS. Since penicillin and streptomycin were the only antibiotics added to the preliminary cultures, it was decided to increase their concentration tenfold (to one million units penicillin and one gram streptomycin/L) and to include polymyxin and nystatin in the HBSS overlay. This solution was also used for the surface sterilization procedure in the final incubation (as described in Chapter II, page 30) in place of hexachlorophene. The results of the fourth incubation indicated that these modifications in the incubation procedure were adequate to maintain sterility in the culture tubes for at least 24 hrs. The sterility was verified by inoculating a small aliquot of the HBSS from randomly selected culture tubes following 24 hrs. incubation, into beef-heart infusion broth.

The control worms in all four incubation studies remained motile throughout the 24 hr. observation period. The results of the initial in vitro cultivation experiment are presented in Table (5). Worms that became immobile within the first hour of exposure to $10^{-3}$ and $10^{-4}M$ bunamidine were rigid and coiled while the immobilized worms in flasks containing $10^{-5}M$ drug remained flaccid and uncoiled. By eight hr. of incubation, the two higher drug concentrations appeared to produce an amber discoloration of the worms which persisted throughout the 24-hour
TABLE 5

First preliminary incubation of *H. diminuta* in *vitro* in the presence of bunamidine HCL showing the per cent mortality of worms at various drug concentrations.

<table>
<thead>
<tr>
<th>Hours Incubation</th>
<th>Bunamidine HCL Conc. (M/L)</th>
<th>No. Alive</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10^{-3}</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^{-4}</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^{-5}</td>
<td>1/5</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>10^{-6}</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10^{-3}</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^{-4}</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^{-5}</td>
<td>1/5</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>10^{-6}</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>10^{-3}</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^{-4}</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^{-5}</td>
<td>1/5</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>10^{-6}</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td></td>
<td>10^{-3}</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^{-4}</td>
<td>0/5</td>
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<td>10^{-5}</td>
<td>1/5</td>
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<td>10^{-6}</td>
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<td>Control</td>
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<tr>
<td></td>
<td>10^{-3}</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^{-4}</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^{-5}</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10^{-6}</td>
<td>5/5</td>
<td>0</td>
</tr>
</tbody>
</table>
observation period. All of the worms incubated in the presence of $10^{-5}$M bunamidine were immobile by eight hours, but did not coil or become discolored by the termination of the experiment. Following the 24-hour incubation, representative worms from the $10^{-3}$, $10^{-4}$ and $10^{-5}$M drug treatment group were placed in fresh, sterile drug-free media to determine if the immobilization was reversible. No movement was detected after 24 hrs. of additional incubation.

From the preliminary incubation experiment the LC$_{50}$ value for bunamidine appeared to be between $10^{-5}$ and $10^{-6}$M. These two molar concentrations were expressed as µg/ml of HBSS and plotted on log-probability graph paper as representing 97.5 and 2.5 per cent mortality respectively (Figure 3) according to the method of Miller and Tainter (54). The apparent LC$_{50}$, based on a tenfold difference in drug concentrations, was 1.3 µg/ml ($3.1 \times 10^{-6}$M). Four equally spaced drug concentrations that fell between the limits of 4.5 ($10^{-5}$M) and 0.45 µg/ml ($10^{-6}$M) were chosen for the second incubation experiment, the results of which are presented in Table (6).

By 12 hrs. of incubation, there was evidence of bacterial contamination in some of the flasks but there were many that remained clear and normal in color. The few worms that became immobile appeared normal with respect to color and configuration. Figure (4) is a log-probability
Figure 3

Log-probit plot of toxicity data from incubating *H. diminuta* in the presence of bunamidine, 4.2 µg/ml, (10^{-5}M) and 0.42 µg/ml, (10^{-6}M) for 24 hours.
BUNAMIDINE CONC. (µg/ml media)

APPARENT LC₅₀ = 1.3 µg/ml

% MORTALITY

Figure 3

BUNAMIDINE CONC. (µg/ml media)
TABLE 6
Second preliminary incubation of *H. diminuta* in vitro in the presence of bunamidine HCL showing the per cent mortality of worms at various drug concentrations.

<table>
<thead>
<tr>
<th>Hours Incubation</th>
<th>Bunamidine HCL Conc. (µg/ml)</th>
<th>No. Alive</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.0 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.0 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4.0 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Control 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.0 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.0 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4.0 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Control 5/5</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.0 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.0 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4.0 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Control 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.0 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.0 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4.0 4/5</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>24</td>
<td>Control 5/5</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>0.5 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1.0 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.0 5/5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4.0 2/5</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>
graph depicting the LC$_{50}$ value for bunamidine when 2 and 4 µg/ml concentrations are plotted respectively as 2.5 and 60 per cent mortality. The LC$_{50}$ value determined from the second incubation study was 3.65 µg/ml ($8.7 \times 10^{-6}$M).

Since the highest drug concentration (4 µg/ml) used in the second incubation resulted in only 60 per cent mortality at 24 hrs., a third experiment was designed to include more doses above the expected LC$_{50}$ value. The results of the third incubation are presented in Table (7).

An LC$_{50}$ for bunamidine was determined by pooling the data from the second and third incubation and by a weighted method of least squares, plotting the results on log-probability graph paper (Figure 5). The LC$_{50}$ value determined on the basis of the second and third incubation was 2.7 µg/ml ($6.4 \times 10^{-6}$M). However since many of the culture tubes used in determining this LC$_{50}$ appeared contaminated, it was decided to perform a fourth incubation using the modifications in the culture procedure described on page 42 of this chapter, to determine what effect the presence of bacteria had on the LC$_{50}$ value for bunamidine. Sterility of the culture tubes in the final incubation was confirmed by laboratory culture methods. The results of the fourth incubation are presented in Table (8). A weighted method of least squares was used to plot the data on a log-probit graph (Figure 6) which then provided the
Figure 4

Log-probit plot of toxicity data from incubating *H. diminuta* in the presence of bunamidine, 2 μg/ml (4.8 x 10⁻⁶M) and 4 μg/ml (9.5 x 10⁻⁶M) for 24 hours.
APPARENT LC₅₀ = 3.65 µg/ml
TABLE 7

Third incubation of *H. diminuta* in *vitro* in the presence of bunamidine HCL showing the per cent mortality of worms at various drug concentrations.

<table>
<thead>
<tr>
<th>Hours Incubation</th>
<th>Bunamidine HCL Conc. (µg/ml)</th>
<th>No. Alive</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>3/5</td>
<td>40</td>
</tr>
<tr>
<td>18</td>
<td>Control</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>4/5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>4/5</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td>24</td>
<td>Control</td>
<td>5/5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>2/5</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>3/5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>0/5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>0/5</td>
<td>100</td>
</tr>
</tbody>
</table>
basis for determining the LC$_{50}$ for bunamidine HCL on *H. diminuta*. The weighted least squares method, complete with calculations, is presented in the Appendix.

**TABLE 8**

<table>
<thead>
<tr>
<th>Hours Incubation</th>
<th>Bunamidine HCL Conc. (µg/ml)</th>
<th>No. Alive</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1.0</td>
<td>18/19</td>
<td>5</td>
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<tr>
<td></td>
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<td></td>
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<td>70</td>
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<tr>
<td></td>
<td>8.0</td>
<td>0/20</td>
<td>100</td>
</tr>
</tbody>
</table>

2. Electron Microscopy Results

The morphologic changes associated with the incubation of *H. diminuta* in the presence of bunamidine HCL were determined by electron microscopy. When worms were exposed to 2 µg/ml (4.8 x 10$^{-6}$M) drug, there were resulting structural alterations indicative of cellular degeneration. Higher drug concentrations (4µg/ml or 9.5 x 10$^{-6}$M) produced more dramatic lesions which suggested that the cytotoxic mechanism of bunamidine is a dose-related process.
Figure 5

Log-probit plot of toxicity data from the second and third in vitro culture of *H. diminuta* in the presence of 1, 2, 4 and 6 μg/ml bunamidine.
Figure 5

BUNAMIDINE CONC. (µg/ml media)

% MORTALITY

97.5

30 50 66

LC50 = 27 µg/ml
Figure 6

Log-probit plot of the toxicity data from the fourth in vitro culture of *H. diminuta* under aseptic conditions, in the presence of 1, 2, 4 and 8 μg/ml bunamidine.
LC₅₀: 2.7 µg/ml

BUNAMIDINE CONC. (µg/ml media)

% MORTALITY

5 25 50 75 95
Outer Body Wall:

Figures (7) and (8) are electron photomicrographs of the tegument, sub-tegument and outer parenchymal region of a proglottid from a 10 day old worm incubated for 24 hrs. in a drug-free diphasic culture media. The trilaminar tegument consists of an outer microvilli layer (microtriches), a middle zone of vacuolated ground substance and an inner mitochondrial-rich area. There is a well defined basement membrane that underlies the tegument layer. The sub-tegument area contains circular and longitudinal muscle fibers. The deeper parenchymal region contains two types of cells: (1) Large electron-dense cells and (2) Smaller electron-light cells. There are connecting tubules that communicate between the outer tegument and the parenchymal cells and appear to serve as a transport system. The vacuolated outer tegument suggests that pinocytosis may be involved in the translocation of nutrients from the host's intestinal lumen into the worm's body. Figure (9) shows the drug effects of 2 µg/ml bunamidine on the outer proglottid wall of a 10 day old worm. The tegment, basement membrane and muscle layers appear normal when compared to the control worms, but the deeper parenchymal cells show evidence of degeneration and cell death. Although the plasma membrane appears intact, the cytosol and nuclear materials have been destroyed in many cells. This effect is more pronounced in the smaller electron-light
Figure 7

Tegument and sub-tegument of *H. diminuta* incubated for 24 hours in the absence of bunamidine. Magnification, 8,500x.

M - microtriches
T - tegument
B - basement membrane
C - connecting channel
Mu- muscle bundles
Figure 7
Figure 8

Tegument and sub-tegument of *H. diminuta* incubated for 24 hours in the absence of bunamidine. Magnification, 15,000x.

M - microtriches
T - tegument
B - basement membrane
C - connecting channel
Mu- muscle bundles
Figure 9

Tegument and sub-tegument of *H. diminuta* incubated with bunamidine, 2µg/ml (4.8 x 10^{-6} M), for 24 hours. Magnification, 20,235x.

M - microtriches
T - tegument
B - basement membrane
C - connecting channel
Mu - muscle bundles
cells. Other than a reduction in electron density, the large parenchymal cells appear normal.

The pronounced cellular destruction resulting from exposure to 4 μg/ml drug are depicted in Figure (10). The outer tegument surface including the connecting tubules, is completely gone. The basement membrane, although present, is disrupted in many areas. The muscle fibers are well-defined and appear normal, but the parenchymal cells for the most part are destroyed. Those cells that are still present are undergoing degenerative changes.

Nephridial Canals:

The morphology of the nephridial system (osmoregulatory or excretory canals) and deeper parenchymal regions of a non-drug treated H. diminuta cestode under electron microscopy is shown in Figure (11). The surface epithelial cells that line this tubular network are developed into microvilli which have a bead-like appearance on cross-section. The nuclear portion of these cells extends through the well-developed basement membrane and lie adjacent to it. The portion of the cytoplasm lying beneath the basement membrane contains numerous mitochondria, Golgi complexes and endoplasmic reticula. The smaller collecting ductules have the same general appearance as the larger ducts, but the basement membrane is thinner.

The structural alterations in the epithelium and basement membrane of the nephridial system of a worm exposed
Figure 10

Outer body surface of *H. diminuta* incubated with bunamidine, 4 μg/ml (9.5 x 10^-6 M), for 24 hours. The outer tegumental surface has been eroded away. Magnification, 20,235x.

B - basement membrane
Mu- muscle bundles
Figure 11

Nephridial canal of *H. diminuta* incubated for 24 hours in the absence of bunamidine. Magnification, 15,000x.

NC - lumen of nephridial canal
Mv - microvilli
B - basement membrane
OR - "organelle-rich" epithelial cells
Figure 11
to 2 and 4 μg/ml bunamidine are shown in Figures (12) and (13) respectively. The basement membrane has been greatly diminished while the epithelial cells have been destroyed. The microvillus tips to the epithelial cells are still evident at 2 μg/ml, but are only present in sparse numbers at 4 μg/ml drug. The parenchymal cells likewise show decreased electron density at the 2 μg/ml concentration and are destroyed at 4 μg/ml levels. Muscle fibers are readily delineated in the drug treatment worms and appear unaltered.

3. Results of Metabolic Enzyme Studies

The effect of bunamidine HCL on the rate of NADH oxidation in PEPCK, PK, and FR catalyzed reactions and in the rate of NADP reduction in ME reactions are summarized in Table (9). Maximal soluble drug concentrations of \(7 \times 10^{-5}\) M, (30 μg/ml), did not alter the velocity of the PEPCK, PK, or ME reactions, but there was a complete inhibition of the FR reaction. When the concentration of bunamidine was reduced to \(6.4 \times 10^{-6}\) M, NADH oxidation occurred but at a slower rate than the non-treated controls. The two cytosol enzyme reactions were usually completed within two minutes, while the mitochondrial reactions proceeded for several minutes. The initial portion of nucleotide decay recordings appeared linear and was used to determine the velocity of each reaction. Table (10)
Figure 12

Nephridial canal of *H. diminuta* incubated with bunamidine, 2 μg/ml, for 24 hours. Magnification, 15,000x.

NC - lumen of nephridial canal
Mv - microvilli
Figure 13

Epithelial erosion from the nephridial canal in *H. diminuta* incubated with bunamidine, 4 µg/ml, for 24 hrs. Magnification, 15,000x.

NC - lumen of nephridial canal
lists the apparent $K_m$ and the $V_{max}$ values for these enzymes as determined from the respective Lineweaver-Burk graphs (Figures 14 through 17).

**TABLE 9**

Nucleotide recordings for PEPCK, PK, ME and FR using different substrate concentrations in presence and absence of bunamidine.

<table>
<thead>
<tr>
<th>Enzyme Assay</th>
<th>Substrate Conc. (1 x $10^{-6}$M)</th>
<th>Nucleotide Measured</th>
<th>umole/min/mg protein Control Drug Treat.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEPCK</td>
<td>PEP 2.0</td>
<td>NADH</td>
<td>818</td>
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<tr>
<td></td>
<td>0.66</td>
<td>Oxidation</td>
<td>695</td>
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<tr>
<td></td>
<td>0.4</td>
<td>572</td>
<td>532</td>
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<td></td>
<td>0.2</td>
<td>409</td>
<td>429</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>286</td>
<td>286</td>
</tr>
<tr>
<td>PK</td>
<td>PEP 3.0</td>
<td>NADH</td>
<td>1554</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>Oxidation</td>
<td>818</td>
</tr>
<tr>
<td></td>
<td>0.375</td>
<td>327</td>
<td>245</td>
</tr>
<tr>
<td>ME</td>
<td>MALATE 0.8</td>
<td>NADP</td>
<td>9880</td>
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<td>0.53</td>
<td>Reduction</td>
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</tr>
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<td>0.1</td>
<td>2736</td>
<td>3344</td>
</tr>
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<td>FR*</td>
<td>FUMARATE 3.33</td>
<td>NADH</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>3.33</td>
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<td>1.7</td>
<td>48</td>
<td>28</td>
</tr>
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<td>0.8</td>
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<td>35</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>29</td>
<td>14</td>
</tr>
</tbody>
</table>

*20.8 umoles of NADH/min/mg protein were oxidized in the absence of added fumarate and was subtracted from the observed oxidation values.
TABLE 10

Km and Vmax values as determined for PEPCK, PK, ME and FR from Lineweaver-Burk plots.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Apparent Km for Substrate (M)</th>
<th>Vmax (M/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEPCK</td>
<td>$2.4 \times 10^{-4}$</td>
<td>$9.7 \times 10^{-4}$</td>
</tr>
<tr>
<td>PK</td>
<td>$3.8 \times 10^{-3}$</td>
<td>$1.9 \times 10^{-3}$</td>
</tr>
<tr>
<td>ME</td>
<td>$3.1 \times 10^{-4}$</td>
<td>$10.6 \times 10^{-3}$</td>
</tr>
<tr>
<td>FR (Control)</td>
<td>$4.5 \times 10^{-4}$</td>
<td>$6.3 \times 10^{-5}$</td>
</tr>
<tr>
<td>FR (6.4 x 10^{-6}M Bunamidine)</td>
<td>$9.1 \times 10^{-4}$</td>
<td>$4.9 \times 10^{-5}$</td>
</tr>
</tbody>
</table>
Figure 14

Comparison of control and bunamidine treated phosphoenolpyruvate carboxykinase reactions on Lineeweaver-Burk plot. Line was fitted by least squares method.
**Figure 14**

$V = \text{umoles NADH ox. min}^{-1} \cdot \text{mg protein}^{-1}$

$\frac{1}{V_{\text{max}}} = \frac{b}{K_{\text{m}}} + c$

- $\square$ = CONTROL
- $\circ$ = BUNAMIDINE TREATED ($7 \times 10^{-5} \text{M}$)

$\frac{1}{S} (10^{-3} \text{M PEP})$
Figure 15

Comparison of control and bunamidine treated pyruvic kinase reactions on Lineweaver-Burk plot. Line was fitted by least squares method.
Figure 15

V = μmoles NADH oxi. min⁻¹ mg protein⁻¹

1/V

1/Km

1/S (10⁻³ M PEP)

□ = CONTROL
○ = BUNAMIDINE TREATED (7 x 10⁻⁵ M)

79
Figure 16

Comparison of control and bunamidine treated malic enzyme reactions on Lineweaver-Burk plot. Line was fitted by least squares method.
Figure 16

The graph shows the relationship between the reciprocal of the substrate concentration (1/S) and the reciprocal of the reaction rate constant (V/Vmax). The data points represent the activity of the enzyme in control and bunamidine-treated conditions. The symbols used are:

- □ = CONTROL
- ○ = BUNAMIDINE TREATED (7 × 10^-5 M)

The x-axis represents 1/S (10^-3 M MALATE), and the y-axis represents V/Vmax. The lines are fitted to the data points to illustrate the enzyme kinetics.
Figure 17

Comparison of control and bunamidine treated fumarate reductase reactions on Lineweaver-Burk plot. Lines were fitted by least squares method.
Figure 17

Graph showing the relationship between $1/V_{\text{max}}$ and $1/S$ (in M). The x-axis represents $1/S$ (in $10^{-3}$ M fumarate), and the y-axis represents $1/V_{\text{max}}$. The graph includes data points for control and bunamidine-treated conditions (6.4 x $10^{-6}$ M).

- • = CONTROL
- ○ = BUNAMIDINE TREATED ($6.4 \times 10^{-6}$ M)
CHAPTER IV

DISCUSSION

Anthelmintics, like other drugs, must reach the site of action in the parasite in a pharmacologically active form before they can exert their effect. To be clinically useful in the oral treatment of helminthiasis, drugs must also be tolerated by the host and withstand gastric degradation or hepatic biotransformation to ineffective metabolites. Bunamidine hydrochloride is a drug that is effective in the treatment of tapeworm infections of domestic animals (14, 33). The results of this investigation show that the drug is likewise effective against *in vitro* cultivated *H. diminuta* and indicate that unless the drug is metabolized to a more active form by the worm, the parent compound is responsible for the biological response. Its anthelmintic property may be related to two mechanisms: (1) The inhibition of fumarate reductase with a concurrent decrease in ATP formation and (2) A non-specific cytotoxicity that results in the erosion of the tegumental and nephridial epithelium as well
as the destruction of parenchymal cells. It is not possible to determine which drug effect is predominant, but it is likely that both exert adverse effects in the cestode.

A common feature among tapeworms is the absence of an alimentary canal. The tegument resembles an inverted intestinal epithelium complete with microvilli (microtriches) and provides the surface through which nutrients (and drugs) are absorbed. In addition to nutrient absorption, the body wall performs other essential functions for the worm such as protection against digestion by the host, attachment to the intestinal wall and protein synthesis (57).

The adverse effects of bunamidine HCL on the outer body wall of _H. diminuta_ are evident from the electron photomicrographs (Figures 9 & 10, Chapter III) and agrees with similar effects on _H. nana_ as reported by Hart et al. (31). The implication of tegument destruction is that it can no longer perform the vital functions necessary for the maintenance of the parasite's life. The significance of the slightly increased vacuolization in the outer ground substance portion associated with 2 μg/ml drug concentration is speculative, but Hart noted that increased vacuolization was followed by sloughing of the tegumental layer in _H. nana_.

The decreased electron density of the large parenchymal cells from worms incubated in the presence of 2 μg/ml bunamidine appears to be more dramatic than the vacuolization of the tegument (Figure 9). If these structural changes are truly indicative of early destruction then it is possible that the parenchymal cell death precedes tegumental sloughing. Support for this hypothesis comes from the fact that the tegumental surface is actually comprised of cytoplasmic extensions of the deeper electron-dense parenchymal cells (78), and that destruction of the cell body likewise results in destruction of its cytoplasmic processes. Although further investigations into the time course of outer body wall destruction were not performed, presumably it could be determined by the sequential examination of worms exposed to higher drug concentrations for shorter time intervals.

The nephridial system (osmoregulatory or excretory canals) is a tubular network that extends the length of the cestode. The function of this system has not been thoroughly investigated, but it has been postulated that it serves as an excretory system and/or a system for maintaining osmotic pressures. The terminal ductules are connected proximally with primary collecting ducts and distally are capped by flame cells (40). The duct walls are formed from epithelial cells which are associated with a well developed basement membrane. Cytoplasmic extensions
project through the basement membrane and form microvilli.

The effects of bunamidine HCL on the deeper parenchymal cells and nephridial system are demonstrated in Figures (12) and (13). Whereas low concentrations of drug (2 ug/ml) had only minor visible effects on the tegument, similar concentrations appeared to dramatically affect the nephridial system and the surrounding cellular components. These findings support the hypothesis that bunamidine is absorbed through the tegument and moves toward the nephridial tubules causing more extensive damage as it courses toward the interior of the worm. The implication of a gradient in cellular damage is that, if the nephridial ducts serve an excretory function for metabolic wastes or foreign materials, there would likely be an accumulation of drug in these areas allowing more damage to occur. Another possible explanation for the apparent difference in the cell damage is an increased sensitivity of the deep parenchymal cells and nephridial epithelium to the adverse effects of the drug.

The mechanism by which bunamidine promotes cell lysis is at best speculative. Hart et al. (31) indicated that at pH 7 bunamidine, with a pKa of 10.6, is highly protonated and has a hydrophilic cationic end. Lumsden and Berger (50) have demonstrated anionic charges on the tegumental microvilli which could then serve as binding sites for the drug. This binding may, as proposed in
detergent effects in bacterial cell walls, alter the parasite's cell membrane causing a loss of its selective permeability which then results in lysis. It is recognized that this hypothesis on the mode of action based on cationic detergent effects on the tegumental microvilli suggests that the gradient for cell destruction should proceed opposite to that proposed by the electron photomicrograph interpretation (i.e. nephridial to tegumental direction). However, it is possible that there are anionic or other binding sites in the deeper layers of the worm that result in a detergent-like lysis when occupied by bunamidine. The fact that none of the preliminary incubation flasks containing high concentrations of bunamidine (10^{-3}M) showed evidence of bacterial contamination suggests that the drug may also have a bactericidal or bacteriostatic property related to a cationic detergent effect.

The applicability of the diphasic culture media consisting of blood agar overlaid with HBSS in the determination of the 24 hr. effects of anthelmintic on H. diminuta appears valid, since all of the control parasites remained motile at the termination of the experiment. The incubation under aerobiosis had no obvious deleterious effects and agrees with the observations made by Turton (84) and Roberts and Mong (63) who reported that H. diminuta increased in both wet weight and numbers of
proglottids when incubated under an atmosphere of air for 12 days in a diphasic media.

The presence of contaminants in the three preliminary culture flasks introduced an unwanted variable in the incubation study. Although no attempt was made to identify these organisms, it is likely that they were penicillin and streptomycin-resistant coliforms from the rat intestine that were not removed during the preliminary washing phase with hexachlorophene. There were fewer contaminated flasks in the second and third incubation than in the first, which may be a reflection of the modification made in the hexachlorophene washing procedure. In an effort to minimize the time required for transferring worms from the intestine to the culture flasks of the first incubation experiment, several worms were washed together which reduced the surface exposure of many worms to the hexachlorophene solution. In the second and third incubations, worms were individually washed which exposed the entire worm surface to the disinfectant. The culture tubes of the final incubation were not contaminated after 24 hrs. The substitution of repeated worm washings in HBSS containing antibiotics for the brief rinse in hexachlorophene solution and the modification in antibiotic content of the culture liquid overlay proved superior in maintaining culture sterility for 24 hr. incubations. The interdependence of changing the pre-incubation washing
and the antibiotic content of the culture liquid overlay to 24 hr. culture sterility was not determined since both modifications were made in the final incubation.

Worms that were immobile at the end of the incubation period were recorded as dead. A disadvantage in using motility as the sole criteria for determining viability is that it does not distinguish between paralyzed and dead worms. Some drugs competitively inhibit neurotransmitter substances which allows the parasite to recover muscle movement when the drug is removed from the environment. The *H. diminuta* that were immobilized by bunamide in this investigation remained immobile following 24 hrs. of additional incubation in a drug-free media, which suggests that the worms were either dead or irreversibly paralyzed. The evidence provided by the electron microscopy examination implies that the worms were dead as determined by the drug-induced cellular damage.

Worms incubated in the presence of high drug concentrations (10^-3 M) in the first incubation were rigid within an hour while those immobilized by lower concentrations remained flaccid. It appears unlikely that a drug would produce a spastic paralysis at high concentrations and a flaccid paralysis at lower concentrations. The reason for the difference in the type of immobility seen as a function of drug concentration may be related to the ability of cationic detergents to denature proteins as
well as produce cell lysis (32). Although experimental evidence is lacking, it is possible that there is a dose-response relationship between bunamidine concentration and the type of injury produced in living tissues whereby high concentrations produce denaturation of protein contractile elements in the tapeworm. A detergent effect may also explain the irritant properties of bunamidine on mammalian mucous membranes.

The enzymes assayed in this investigation were chosen on the basis that their inhibition would quantitatively affect ATP formation in *H. diminuta*. The inhibition of an essential but unique metabolic pathway is an example of the selective approach to chemotherapy. The effects of many anthelmintics on the ATP generating fumarate reductase (FR) system is well established (Chapter I); however information regarding the effects of anthelmintics on enzymes preceding this terminal respiratory reaction is lacking.

Phosphoenolpyruvate (PEP) serves as the common substrate for pyruvic kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK) in the formation of pyruvate and oxaloacetate respectively in many helminths, including *H. diminuta*. Since NADH is oxidized at the next substrate level in both reactions (i.e. pyruvate to lactate and oxaloacetate to malate), it was necessary to verify that the reaction under consideration was proceeding in the
intended direction. Omitting IDP, an essential co-factor in the PEPCK catalyzed reaction, prevented NADH oxidation whereas its addition to the reaction mixture resulted in a rapid decline in the nucleotide recording and agrees with the results of Bueding (12). That PEP was consumed in forming pyruvate was verified by observing the optical density decrease associated with NADH oxidation only after adding ADP to the reaction mixture. The presence of bunamidine in concentrations exceeding 25 times the LC50 had no discernible effects on either of these reactions (Figures 14 & 15 and Table 9 Chapter III).

The apparent Km value for PEPCK (2.4 x 10^{-4}M) is similar to that reported for Ascaris muscle (2.6 x 10^{-4}M) by Saz (73). The ratio of PK to PEPCK was 1.9 when the PK reaction contained 6 umoles/ml PEP and the PEPCK reaction contained 2 umoles/ml PEP. Bueding (12) reported PK and PEPCK activities of 105 and 583 umoles/min/mg protein respectively in H. diminuta when the PK reaction contained 12 umoles/ml and the PEPCK reaction contained 2 umoles/ml, or a PK/PEPCK ratio of 0.18. At identical PEP concentrations the PEPCK activity reported by Bueding is comparable to that found in this study (818 umoles/min/mg protein). However, there are wide differences in PK activity (105 compared to 1554 umoles/min/mg protein) between these studies especially in consideration that the concentration of PEP was only on half that used by Bueding.
Moon et al. (55) has shown that CO₂ activates PEPCK but inhibits PK, which was expected since PEPCK is favored in the PEP carboxylation direction. They also reported that a peak in PEPCK activity occurred at pH 5.5 but was decreased by one half at pH 7, while the PK activity was essentially unaltered over a similar pH range. On the basis of these findings it is likely that the PEPCK assay in this study was performed under less than optimal conditions (i.e. CO₂ was not provided either in the form of a bicarbonate buffer or as a gas, and the assay pH was 7); whereas the PK method performed under more optimal conditions, led to a more accurate activity value.

Malate, like PEP, serves as a common substrate for two enzymatic reactions; however unlike the two PEP reactions, malate undergoes a dismutation to yield equimolar quantities of fumarate and pyruvate in the presence of fumarase and malic enzyme (ME). Fumarate is converted to succinate by FR in the presence of Mg⁺⁺ and NADH while the malate to pyruvate reaction requires NADP and Mn⁺⁺ (59). On the basis of the differences in co-factor and divalent cation requirements for each of these enzyme reactions their direction was not verified by omitting assay constituents. The dependence of ME on NADP as a co-factor in this coupled reaction was evident from the decrease in reaction velocity when NAD was substituted for NADP. The addition of bunamidine HCL in concentrations far
in excess of the LC50 had no effect on the ME catalyzed reaction (Figure 16 and Table 9) and led to the conclusion that the drug's mechanism of action was unrelated to this metabolic enzyme.

The reduction of fumarate in the presence of NADH to form succinate is associated with the generation of ATP by a poorly understood mechanism that involves a flavin-linked FR system (44). The results of the FR assay experiment are shown in Table 8, Chapter III. There appears to be an NADH oxidase operable in H. diminuta mitochondria that oxidizes NADH in the absence of added fumarate. The velocity of this fumarate-independent reaction was subtracted from each of the control as well as drug treated reactions to obtain the nucleotide reductase velocity associated with the FR catalyzed reaction. Pritchard (60) has also shown an NADH oxidase mechanism operable in Haemonchus contortus that reduces nucleotide in the absence of added fumarate.

The effect of bunamidine HCL on the FR reaction was analyzed by a Lineweaver-Burk (L-B) double reciprocal plot of the velocity and substrate concentration. A non-competitive inhibited reaction that obeys Michaelis-Menten kinetics is characterized by identical Km but different Vmax values when comparisons are made between control and drug-inhibited reactions on a L-B plot. An enzyme inhibitor may also alter both the Km and Vmax values
by a combination of competitive and non-competitive mechanisms and is termed a mixed inhibition.

An examination of the plotted data from the FR assay shown in Figure (17) is more consistent with a mixed type of inhibition than a purely non-competitive inhibition. The linearity of the $\frac{1}{V}$ and $\frac{1}{S}$ values in the control enzyme assay is more apparent than in the presence of drug. In an effort to obtain better linearity, the FR assay was repeated many times with bunamidine but each subsequent assay produced a similar scatter in the data points. The straight lines representing the control and drug inhibited reactions were determined by a non-weighted least squares method of analysis which was based on the four highest substrate concentrations. On the basis of the similarities of the apparent rate constant values and the non-weighted method used in their determination, it is difficult to characterize the true nature of the drug-inhibition by a L-B treatment of the data.

Dixon (19) described a graphic method for determining a $K_i$ (which is an inhibitory rate constant analogous to the $K_m$ of a non-inhibited reaction) directly from plotting the reciprocal of the reaction velocity using a single substrate concentration as a function of variable inhibitor concentrations. Comparing the $K_i$ as determined by this method with the $K_m$ determined by a L-B plot should further illucidate the inhibitory effects of bunamidine on
the FR reaction. In a true non-competitive inhibition the 
Ki and Km values are expected to be the same. However when 
the FR assays were performed using a single substrate 
concentration in the presence of several bunamidine 
concentrations, the resulting reaction velocities were so 
variable that a meaningful Ki could not be determined. 
Further efforts were not made to analyze the inhibitory 
mechanism but it is evident from the data presented that 
in vitro the drug exerts an inhibitory effect on FR that is 
partially, if not entirely, non-competitive in nature. The 
consequences of this inhibition on intact H. diminuta is 
uncertain since drug transport was not determined.

In addition to the FR system, ATP is generated 
from the PEP to lactate (via pyruvate + ATP) reactions. 
Parasites such as Ascaris appear to rely on the FR system 
for most, if not all, of their energy demands, since they 
possess only trace quantities of PK (12) and excrete large 
quantities of succinate. In contrast, S. mansoni excretes 
only lactate which implies the reliance of this parasite 
on PK for meeting energy requirements (68). H. diminuta 
is an example of a parasite capable of forming either 
lactate or succinate and as such has two mechanisms 
available for energy production. Moon et al. (55) have 
suggested that the HCO₃⁻:CO₂ ratio determines the pathway 
by which ATP is formed in H. diminuta, i.e. elevated pCO₂ 
favors succinate and low pCO₂ favors lactate excretion.
Coles and Simpkin (18) have reported a metabolic
gradient in *H. diminuta* whereby the first 2 cm of the worm
consumes more oxygen and produces mainly lactate, while in
the more distal proglottids, succinate is the major
metabolic end product. The implication of this finding
is that drugs that act by FR inhibition alone may be less
toxic to the scolex end of the worm and may account for
anthelmintic failures.

In light of the metabolic option available to
*H. diminuta* in the synthesis of ATP, it appears that the
inhibition of FR is more significant in those parasites
capable of excreting only succinate. However, since the
relative molar quantity of ATP produced by the FR pathway
in *H. diminuta* has not been reported, it is difficult to
evaluate the toxicological significance of FR inhibition
by bunamidine.
CHAPTER V

SUMMARY AND CONCLUSIONS

The in vitro, morphologic and metabolic effects of bunamidine HCL on Hymenolepis diminuta were examined. The results of these investigations provide the basis for the following summary and conclusion.

Summary:

1. When incubated for 24 hrs. at 37 C in a diphasic culture media consisting of blood agar and Hank's Balanced Salt Solution, the LC50 for bunamidine on H. diminuta is 2.7 µg/ml or 6.4 x 10^-6M.

2. Bunamidine exerts a cytotoxic effect on the parasite as evidenced by the erosion of the tegumental and nephridial epithelium as well as lysis of parenchymal cells. The mechanism by which the drug destroys these cellular elements may be related to a cationic detergent effect on the cell membrane which alters the permeability such that lysis occurs.

3. Of the four metabolic enzymes examined (phosphoenolpyruvate carboxykinase, pyruvic kinase, malic enzyme and fumarate reductase), only fumarate reductase...
was inhibited.

Conclusion:

The mechanism of action of bunamidine HCL is primarily related to a direct cytotoxic effect on cestodes. Although the drug also inhibits fumarate reductase, an enzyme necessary for the generation of ATP via an electron transport system, this parasite has an alternate pathway available for energy production. The exposure of worms to high concentrations of drug ($10^{-3}$M), resulted in parasite death within an hour which is difficult to explain on the basis of fumarate reductase inhibition alone. It is likely that the cellular destruction that is seen under electron microscopy is due mainly to non-specific cytotoxicity, but subcellular changes resulting from decreased ATP levels cannot be discounted.
APPENDIX

TABLE II

Weighted Least Squares Method

<table>
<thead>
<tr>
<th>Dose (X)</th>
<th>Log Dose X</th>
<th>% Mortality</th>
<th>Probit Y*</th>
<th>X-X</th>
<th>Y(X-X)</th>
<th>(X-X)^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ug/ml</td>
<td>0</td>
<td>5</td>
<td>3.36</td>
<td>-.450</td>
<td>-1.51</td>
<td>.203</td>
</tr>
<tr>
<td>2 ug/ml</td>
<td>.301</td>
<td>32</td>
<td>4.53</td>
<td>-.149</td>
<td>.67</td>
<td>.022</td>
</tr>
<tr>
<td>4 ug/ml</td>
<td>.602</td>
<td>70</td>
<td>5.52</td>
<td>.152</td>
<td>.84</td>
<td>.023</td>
</tr>
<tr>
<td>8 ug/ml</td>
<td>.903</td>
<td>97.5</td>
<td>6.97</td>
<td>.453</td>
<td>3.16</td>
<td>.205</td>
</tr>
<tr>
<td>Sum =</td>
<td>1.81</td>
<td></td>
<td></td>
<td></td>
<td>Sum =</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.38</td>
<td></td>
<td></td>
<td></td>
<td>Sum =</td>
<td>.453</td>
</tr>
</tbody>
</table>

* From Table of Transformation of Percentages to Probits

\[ \bar{X} = 0.45 ; \quad \bar{Y} = 5.1 \]

Equation for Straight Line: \[ Y = mx + b ; \]

In this case, form is: \[ Y = a + b(X-X) , \text{ where;} \]

\[ a = \bar{Y} \text{ or } 5.1 \] and

\[ b = \frac{\text{Sum } Y(X-X)}{\text{Sum } (X-X)^2} \text{ or } 4.02 \]

<table>
<thead>
<tr>
<th>Dose (ug/ml)</th>
<th>Weighted Mortality*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>[ Y = 5.1 + 4.02(-.450) = 3.3 ] 4%</td>
</tr>
<tr>
<td>2.0</td>
<td>[ Y = 5.1 + 4.02(-.149) = 4.5 ] 31%</td>
</tr>
<tr>
<td>4.0</td>
<td>[ Y = 5.1 + 4.02(.152) = 5.71 ] 76%</td>
</tr>
<tr>
<td>8.0</td>
<td>[ Y = 5.1 + 4.02(.453) = 6.92 ] 97%</td>
</tr>
</tbody>
</table>

* From Table of Transformation of Percentages to Probits
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