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COMPARISONS OF LARVAE AND ADULT *TRICHINELLA SPIRALIS*: METABOLIC EVENTS AND THEIR POSSIBLE PHYSIOLOGIC SIGNIFICANCE

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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degree of

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BY

JOHN D. FERGUSON

Oklahoma City, Oklahoma

1972

COMPARISONS OF LARVAE AND ADULT *TRICHINELLA SPIRALIS*:

METABOLIC EVENTS AND THEIR POSSIBLE

PHYSIOLOGIC SIGNIFICANCE

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COMPARISONS OF LARVAE AND ADULT *TRICHINELLA SPIRALIS*: METABOLIC EVENTS AND THEIR POSSIBLE PHYSIOLOGIC SIGNIFICANCE

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Trichinosis is a disease affecting numerous species of carnivorous and omniverous animals and is caused by the nematode parasite, *Trichinella spiralis*. In general trichinosis in man and experimental animals may be separated into three phases which are associated with the sequential location of the parasites in the host: (a) the intestinal phase involving adult worms, (b) general extraintestinal phase involving larvae migrating from the gut and invading various tissues, and (c) skeletal muscle phase involving encysted larvae. Descriptive accounts of clinical signs and symptoms attending the various phases of infection are numerous (see Gould, 1970, for a review). Pathologic and symptomatologic changes of infection are part of the host response and are usually described in terms of gross or obvious alterations in host morphology or physiology. However, it is axiomatic from studies in pathology that gross alterations usually reflect underlying biochemical or metabolic defects in injured tissue (Hopps, 1959). The basic cause of various changes induced by *Trichinella* in its host is difficult to assess. It

may be due in part to mechanical or chemical stimuli, or both, from the parasite. Answers to important questions related to the pathogenesis of infection will no doubt depend on a better understanding of the metabolism of the various stages of the parasite.

Experimental evidence (Larsh, 1967a, 1967b; Tanner, 1968; Castro and Fairbairn, 1969a) indicates either directly or indirectly that the elimination of adult Trichinella from the intestine of infected hosts is due to an immune response involving tissue reactions. Larsh (1967a) suggested that an immunologically mediated reaction causes damage to the intestinal mucosa. This damage theoretically stimulates a local inflammatory response. Although inflammation may be responsible for intestinal malfunctions associated with trichinosis (Castro et al., 1967; Castro and Gentner, 1972), it may also create an unsuitable biochemical environment that is responsible for expulsion of intestinal worms (Larsh, 1967a). Larsh's suggestion that worm expulsion is related to alterations in the microenvironment certainly appears rational. However, conditions which constitute a suitable or unsuitable environment have not been established. It can be assumed that an unsuitable environment is one which alters the parasite's normal metabolic or physiologic functions. If so, then it becomes obvious that the biochemistry and physiology of adult Trichinella must be understood in order to answer this important question in relation to immunity.

To date, metabolic studies with Trichinella have been limited and have dealt primarily with excysted larvae, i.e., larvae obtained by digestion of host skeletal muscle and the cyst surrounding the larvae with an acid-pepsin solution. Data are available on various aspects of

biochemistry and physiology of larvae, particularly with regard to energy metabolism and excretory-secretory functions. Agosin and Aravena (1959) produced conclusive evidence for the presence of the glycolytic pathway and the presence of cytochrome C and cytochrome oxidase in excysted larvae. Goldberg (1957) found enzymes of the tricarboxylic acid (TCA) cycle in the same stage. When larvae were maintained aerobically, they consumed oxygen at the rate of 2.24 µl/mg dry weight per hr (Stannard et al., 1938). Various hexoses and pentoses, as well as phosphorylated sugars, increased oxygen consumption (Kozar et al., 1965).

Despite the above findings which indicate the presence of a respiratory metabolism, there are several reports which suggest that larvae utilize oxygen for other than respiratory processes. The suggestion that a large portion of oxygen consumed was involved in the oxidation of endogenous lipids (von Brand et al., 1952) was not supported by results of studies carried out by Castro and Fairbairn (1969a). The latter authors maintained larvae in an equally viable state for 48 hr in the absence of exogenous nutrients under aerobiasis and anaerobiasis. They were unable to demonstrate any decrease in total tissue lipids or qualitative or quantitative changes in various polar lipid or neutral lipid fractions in larvae maintained under either condition.

Maintenance of larvae under both aerobiasis and anaerobiasis was associated with the production and excretion of short chain volatile fatty acids, primarily n-valeric acid, but also lesser quantities of formic-, acetic-, propionic-, n-butyric- and n-hexanoic acids (von Brand et al., 1952; Agosin and Aravena, 1959; Castro and Fairbairn, 1969a). Presumably these acids are products of carbohydrate catabolism. There

is a very large and equivalent decrease in endogenous glycogen and trehalose during aerobic and anaerobic incubation (von Brand et al., 1952; Castro and Fairbairn, 1969b) indicating the absence of the Pasteur effect in this stage. Total endogenous carbohydrates of larvae constitute 19.8% (Kurylo-Borowska and Kozar, 1960) to 21.1% (Castro and Fairbairn, 1969a) of total tissue solids. Glycogen and trehalose make up 15.9% and 4.4% of the dry weight, respectively (Castro and Fairbairn, 1969a). It seems likely that Trichinella larvae metabolize endogenous carbohydrates to phosphoenolpyruvate (PEP) and hence by carboxylation and reduction to succinic acid (Ward et al., 1969), which in turn is a precurser for volatile acids (Fairbairn, 1954; Saz and Weil, 1960, 1962).

Based on available evidence it appears that Trichinella larvae derive energy primarily from carbohydrate fermentation rather than from a respiratory type of metabolism. The fermentation involves the classical Embden-Meyerhof pathway and portions of the Krebs cycle.

In addition to volatile acids, excysted larvae maintained in vitro excrete or secrete both macromolecular and low molecular weight nitrogenous compounds (Weinstein, 1960). Peptide and protein excretion-secretion (ES) products have been identified chemically (Haskins and Weinstein, 1957a). The presence of protein ES products is also suggested by the antigenic nature of larval metabolic antigens (Campbell, 1955; Mills and Kent, 1965), although some of these elaborated antigens are probably polysaccharides (Oliver-Gonzalez, 1954). Trichinella larvae incubated in vitro also excrete amino acids (Haskins and Weinstein, 1957b) and various amines (Haskins and Weinstein, 1957c).

Except for reports dealing with host reactions to larval ES

antigens, little information is available regarding host responses to the excretions and secretions of Trichinella. Reactions to antigenic metabolites are important in explaining specific serological or physiological alterations in the host. In response to ES antigens the host may elicit antibodies which can be detected by various methods and which may or may not be involved in allergic, anaphylactic (Sprent, 1951; Oliver-Gonzalez, 1954; Sharp and Olson, 1962; Briggs et al., 1966) or immune reactions (Soulsby, 1962; Larsh, 1963, 1967a, 1967b). Although there is no strong evidence that ES products are directly toxic, the allergic or anaphylactic reactions which they invoke could lead to physiologic changes and subsequent pathologic alterations in the host.

No significant information of physiologic or biochemical nature exists with regard to intestinal stages of Trichinella. Although the biochemistry and physiology of larvae and adult worms may be similar, a general assumption that such is the case may lead to erroneous conclusions. Several authors have emphasized that the metabolism of larvae and adult stages of the same species can be quite different (Bueding and Most, 1953; Saz and Bueding, 1966; Prichard and Schofield, 1968; Saz, 1971). As a corollary, specific enzymes or enzyme systems may exist in several stages but may be physiologically non-functional in some (Fairbairn, 1970). Thus, accurate conclusions about biochemical and physiological functions of a certain stage of a parasite and their relationship to the host demand a thorough study of that specific stage.

Oliver-Gonzalez and Bueding (1948) and Kozar et al. (1966b) stated that little glycogen is present in adult Trichinella, although quantitative analysis of the carbohydrate composition of adult worms was

not made. Goldberg (1957) demonstrated the presence of both cytochrome C and cytochrome oxidase, but the specific activity was lower than that for larvae. In general little is known about the dependency of adult worms upon oxygen. Adult excreta have been studied only with regard to their contribution to immunity (Chipman, 1957).

The lack of physiological and biochemical studies pertaining to adult Trichinella probably reflects the difficulties encountered in recovering sufficient quantities of worms. With the availability of new and sensitive analytical methods, metabolic studies using only a few worms are feasible. The present investigation was undertaken to compare aspects of energy metabolism and ES products of adult Trichinella to that of excysted larvae, and to relate findings to various physiologic phenomena associated with parasitism.

CHAPTER II

MATERIALS AND METHODS

Stock Infections

The strain of Trichinella used was originally obtained from Carolina Biological Supply Co.,[1] and has been maintained for 6 years in CF-1 mice.[2] Infections of male mice weighing 20 to 30 g were made by oral intubation of viable larvae. Larvae were recovered from mice 40 to 60 days following inoculation as previously described (Castro and Fairbairn, 1969a).

Recovery of Trichinella Adults

Living Trichinella adult worms were recovered from 200- to 300 g Holtzman hybrid rats[3] infected 1 to 4 days previously with 3×10^4 to 5×10^4 larvae. The small intestine was removed by cutting its junction with the stomach and caecum, and was perfused with 25 ml of Krebs-Ringer bicarbonate (KRB) buffer, pH 7.4 (Umbreit et al., 1964). The intestine was stripped from the mesentery and washed in a porcelain evaporating dish containing 20 ml of KRB buffer. The gut was then everted (Crane

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[1]Carolina Biological Supply Co., Burlington, N. C.

[2]Carworth Farms, Rock City, New York.

[3]Stanley and Gumbreck, Oklahoma City, Oklahoma.

and Wilson, 1958) and placed in a 250 ml beaker containing 100 ml of buffer. Intestines from two animals were placed in each beaker maintained at 37 C in a Dubnoff Metabolic Shaking Incubator for 1 hr. After 1 hr, the gut segment was removed, and its mucosa was scraped and added to the beaker. This material was poured through a Buchner funnel with a 9.0 cm Whatman #1 filter pad at the bottom. The funnel was 10.0 cm in diameter. The filter pad was sucked dry as lightly as possible. The pad was removed, placed face-down in a 10.0 cm petri dish containing 30 to 40 ml of buffer and incubated at 37 C in a water-jacketed incubator (a modification of a technique by Ross, 1948, for collecting live Trichinella adults).

After 1 hr incubation, the pads were carefully removed from the petri dishes so that debris and mucous were not shaken loose. The adult worms which fell into the buffer were filtered through an 80-mesh stainless steel wire screen into 50 ml conical graduated centrifuge tubes. Worms were washed three times by suspending and sedimenting to remove fine debris. Recovery rates of 15 to 20 per cent were obtained by this method.

Incubation of Adult Trichinella

Batches of worms were collected and incubated in KRB buffer containing 100 μg of streptomycin and 300 units of penicillin per ml. Worms were incubated at 37 C and gassed continuously with a humidified gas mixture of 90% N_2:5% CO_2:5% O_2 or 95% N_2:5% CO_2. The N_2:CO_2 mixture was passed through hot copper filings to remove any contaminating traces of oxygen (Ginger and Fairbairn, 1966). Gas flow rates varied from 15 to 25 ml per min. A trace of silicone defoamer (Dow Corning Antifoam AF

emulsion)[4] was added to each tube.

Carbohydrate Analysis of Adult Trichinella

Adult worms collected 24-, 48- and 96-hours post-infection were washed three times with distilled water. They were homogenized in a hand driven Potter-Elvehjem homogenizer. Final homogenate volume was 5 ml. Total carbohydrate, alkali stable carbohydrate, and glycogen were isolated according to published methods (Fairbairn, 1958; Roberts and Fairbairn, 1965); the amount of each was determined colorimetrically (Mokrasch, 1954). For dry weight determinations, 1.0 ml samples of worm homogenate were dried to constant weight in a vacuum dessicator under a residual pressure of 18 mm Hg.

Determination of Volatile Amines Excreted by Adult Trichinella

Incubation of adult worms was carried out as previously mentioned under aerobic and anaerobic gas phases for 48 hr. The exhaust gas was bubbled through 0.1 N HCl to trap volatile amines blown off by the gassing procedure (Figure 1). Following incubation the medium was passed through a coarse glass sintered funnel to remove worms. The filtrate was made alkaline with 0.1 N NaOH. Amines were extracted from 5.0 ml aliquots of the alkaline solution with ether using a continuous liquid-liquid extractor. One ml of 12 N HCl was added to 150 ml of ether in the receiving flask, and the extraction was carried out for 18 hr. At the end of the extraction period the ether was evaporated to dryness in a rotary vacuum (18 mm residual pressure) apparatus. Amine hydrochlorides

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[4]Dow Chemical Corp., Midland, Michigan.

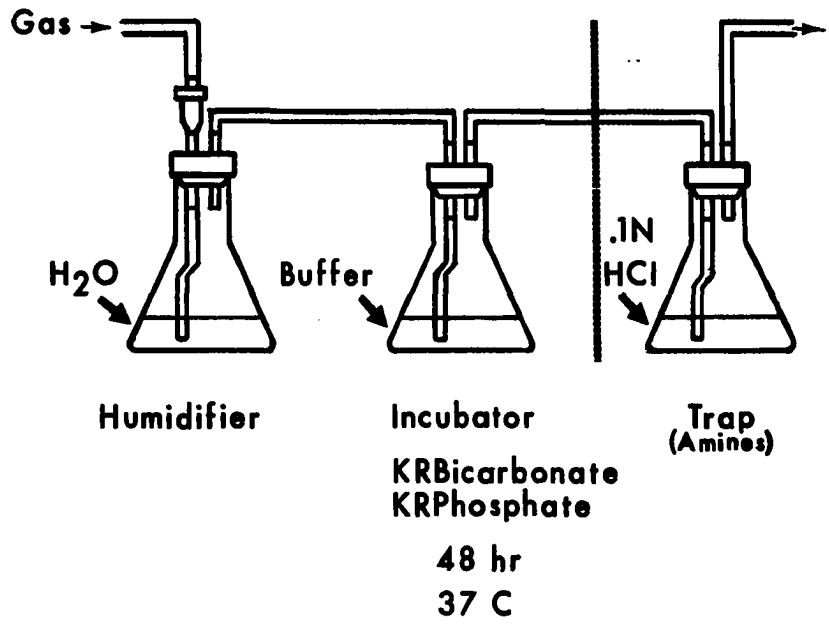

Figure 1. General incubation procedure used. Trap was used in experiments involving collection of amines.

were dissolved in 2 ml of water and identified using thin-layer chromatographic (TLC) techniques. Chromatography was on 20 cm x 20 cm glass plates coated to a thickness of 0.5 mm with microcrystalline cellulose (Sigmacell type 19) obtained from Sigma Chemical Co.[5] Plates were developed in the upper phase of a mixture of n-butanol, acetic acid and water (4:1:5, v/v), and the amines were visualized by spraying with 0.1% ninhydrin in n-butanol followed by heating for 10 min at 90 C. Unknown amines were identified by comparing R_F values to those of authentic reference compounds obtained from Eastman Organic Chemicals and J. T. Baker Chemical Co.[6] Controls included extraction and TLC analysis of medium gassed for 48 hr but containing no worms. Quantitative analysis of amine production was performed using the colorimetric ninhydrin reaction (Matthews et al., 1964). Dry weights of worms were determined as previously described (Castro and Fairbairn, 1969a).

Determination of Fermentation Acids Excreted by Adult Trichinella

Worms were removed from the incubation medium by filtration through a glass sintered Buchner funnel. The medium was acidified to pH 3 using 0.1 N HCl. The fluid was then extracted with ether for 18 hr. The extract was neutralized with 2 N NaOH, dried en vacuo, dissolved in 2.0 ml of water, acidified (pH 3) with phosphoric acid and analyzed by gas-liquid chromatography (GLC). A 6-ft column (1/8 in inside diameter) in a model 1700 Varian Aerograph with a flame detector was packed with

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[5]Sigma Chemical Co., St. Louis, Mo.

[6]Eastman Organic Chemicals, Rochester, N. Y.; J. T. Baker Chemical Co., Phillipsburg, N. J.

Chromosorb 102 (60-80 mesh)[7] and operated at 200 C with a carrier gas (N_2) flow rate of 120 ml/min. Volatile acids were identified by comparing their retention times to those of authentic reference acids (J. T. Baker Chemical Co.).[6] Quantitation was by multiplication of retention time x peak height (Carroll, 1961). Conversion of peak areas to mass was made using propionic acid as an external standard.

Measurement of Oxygen Uptake

Oxygen uptake of a free-living nematode (Rhabditella sp.) and both Trichinella adults (24-, 48- and 96-hours post-infection) and larvae was measured using a Gilson differential respirometer (Model G-14). The basic incubation system consisted of Krebs-Ringer phosphate (KRP) buffer (pH 7.4), 5.5 mM glucose, 300 units of penicillin/ml, and 100 μg of streptomycin/ml, all in a final volume of 1.8 ml. Systems were incubated at 37 C for 30 min, and measurements were taken at 5 min intervals. After 30 min elapsed time, the vessels were promptly removed from the respirometer, and 0.2 ml of a 1.0 M sodium cyanide solution was added yielding a final concentration of 0.1 M. After allowing 10 min for the systems to equilibrate, oxygen uptake was recorded for another 30 min in the presence of the cyanide inhibitor. Oxygen uptake was measured as μl O_2/mg protein/hr. Protein content was measured according to Lowry et al. (1951).

Microscopic Differentiation Between Live and Dead Worms

Decrease in viability of adult worms was used as a criterion to

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[7]Applied Science Laboratories, Inc., State College, Pa.

determine the effect of various experimental treatments on the parasite *in vitro*. In this sense it was assumed that viability was reflected by the presence or lack of motility or movement, i.e., motile worms were considered alive, non-motile worms were considered dead. To document observed differences between living and dead worms as seen microscopically, timed photographs were taken of 96-hour *Trichinella* adults (Experiment I) and also of a free-living nematode, *Rhabditella sp.* (Experiment II). Using an E. Leitz Ortholux Research Microscope, photographs were taken of both live nematodes and nematodes that were killed by treatment with 0.1 M sodium cyanide for 30 min. A 15 sec time interval was used to take the pictures. Living and dead worms could be differentiated by observing the presence or absence of movement and coiling.

Differentiation of Live and Dead Worms by Passive Transfer

To further show that viability of worms was correlated with the presence or absence of movement and coiling, passive transfers of worms possessing these characteristics were carried out. Both treated and untreated *Rhabditella* were transferred to 5% blood agar slants to determine whether lack of movement affected the worm's ability to propagate. Adult *Trichinella* were collected and separated into two equal groups of approximately 10^4 worms. The control group was incubated for 30 min at 37 C in KRP buffer, antibiotics and 5 mM glucose. The treated group was incubated in the same type system with the addition of 0.1 M sodium cyanide. After 30 min incubation both groups were washed 3 times with KRP buffer and examined microscopically to determine presence or absence of movement. Adult worms from each group were passively transferred to the small

intestine of 3 male mice. The transfer involved anesthetizing the mice with ether, making an abdominal incision in a para-sagittal plane 1 cm from the midline, and injecting approximately 3,000 adult worms into the exposed small intestine with the aid of a disposable syringe equipped with a 22 gauge needle. Wounds were closed with stainless-steel clamps. Mice were killed 48 hr later, and adult worms were collected as previously described and counted.

Effect of Metabolic Inhibitors on Survival of Trichinella Adults and Larvae

Larvae and adult worms were collected as described and incubated for 48 hr at 37 C in KRB buffer with different metabolic inhibitors. These included 0.001 M iodoacetamide, 0.001 M 2,4-dinitrophenol, 0.1 M sodium cyanide, and 0.1 M sodium azide. The total molarity was higher than normal KRB buffer. Thus a 0.1 M NaCl control was used to determine the effects of osmolarity. Adult worms were examined microscopically and counted during the incubation at 24, 36, and 48 hr. Viability of larvae following treatment was determined by infecting rats and mice, and recovering and counting the number of adults and larvae developing in the rat and mouse host, respectively.

Effect of pH on Survival of Trichinella Adults

Adult worms were collected and separated into six equal lots and incubated for 48 hr at 37 C in KRB buffer. The pH of the buffer was varied from 5.5 to 8.0 at 0.5 intervals by changing the sodium bicarbonate concentration (Umbreit *et al.*, 1964). Total molarity of normal KRB buffer was maintained by appropriately altering the NaCl concentration. Other components in the buffer were not changed.

The effect of pH was studied in intestinal worms collected 24-, 48- and 96-hr post-infection. For each pH interval, 100 worms were counted, and the percentage of live worms was calculated.

Metabolism of (U-^{14}C) Palmitic Acid by Trichinella Larvae and Adults

Fatty acid oxidation was determined by measuring the $^{14}CO_2$ produced when living Trichinella larvae and adults, and homogenates of these worms were incubated with (U-^{14}C) palmitic acid.[8] Larvae and adults were divided into two equal groups. One group of each was homogenized. Homogenates were prepared by grinding worms in a Potter-Elvehjem homogenizer by hand. Worm preparations were incubated at 37 C in 5 ml of Krebs-Ringer phosphate (KRP) buffer with 2.13 μC_i of (U-^{14}C) palmitic acid with a specific activity of 542 μC_i/mole.

$^{14}CO_2$ liberated was trapped in KOH placed in a well suspended from the stopper of the flask. Radioactivity in the KOH following incubation was determined according to the method of Ward and Fairbairn (1970a).

Incorporation of absorbed, labelled palmitic acid into various lipid fractions was determined after extracting the total lipids (Folch et al., 1957). Total lipids were fractionated on TLC plates coated with silica gel G according to Freeman and West (1966). Lipid bands were sprayed with 0.005% Rhodamine 6-G and visualized under ultraviolet light. The bands were scraped from the plate into a glass-sintered funnel, eluted with 20 ml of ether into a scintillation vial and dried under a stream of nitrogen.

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[8]New England Nuclear, Boston, Massachusetts.

Measurement of radioactivity was carried out in Aquasol[8] using a Nuclear Chicago Liquid Scintillation Spectrometer, equipped with a barium-133 external standard. Quenching was determined by comparing an external standard channels ratio of the radioactivity in the experimental vial with that of standard ^{14}C quenched samples (Nuclear Chicago Corp.).[9]

Purification of (U-^{14}C) Palmitic Acid

Prior to use in metabolic studies, (U-^{14}C) palmitic acid was purified by TLC using hexane:ether:acetic acid (70:30:1) as a solvent. The band corresponding to the standard palmitic acid (Applied Science Laboratories)[10] was located by radioautography using Royal Blue X-ray film.[11] Palmitic acid was isolated as the free acid band on 20 x 20 cm glass plates coated with 0.25 mm of silica gel G.[12] The band was scraped from the plate and eluted with ether. The ether was dried *en vacuo*, and the palmitic acid was collected in 1 ml of hexane. Only the radioactive compound corresponding to a known standard was retained. Purified (U-^{14}C) palmitic acid was diluted with previously purified unlabeled palmitic acid. The diluted mixture was re-purified immediately before use.

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[9]Nuclear Chicago Corp., Des Plaines, Illinois.

[10]Applied Sciences Laboratories, State College, Pa.

[11]Eastman Kodak Co., Rochester, N. Y.

[12]Brinkman Instruments, Westbury, N. Y.

CHAPTER III

RESULTS

Problems were encountered initially in keeping adult stages of Trichinella alive for more than 12 hr in KRB buffer. The possibility that hydrogen ion concentration affected viability of adult worms recovered 24-, 48- and 96-hr post-infection was examined. Worms were maintained for 48 hr in a gradient from pH 5.5 to pH 8.0 (Figure 2). Worms were adversely affected by a pH below 6.5. However, survival rate was maximal between 7.0 and 8.0. Consequently, it was concluded that the very slight deviation (usually less than 0.2 units) from the initial pH of the medium which might occur during a 48 hr period was not responsible for the decreased survival rate. It was eventually determined that the survival rate of all post-larval stages could be maintained at essentially 100% for a minimum of 48 hr by the addition of glucose (5.5 mM) to the buffer.

The effect of gas phases on adult worm survival in KRB buffer was also investigated. Worms were maintained under an atmosphere of 90% N_2:5% CO_2:5% O_2 or 95% N_2:5% CO_2. Worms in both gas phases were viable at the end of 48 hr. However, those in the presence of oxygen were clearly more active. Those in the absence of O_2 were coiled but showed little or no movement.

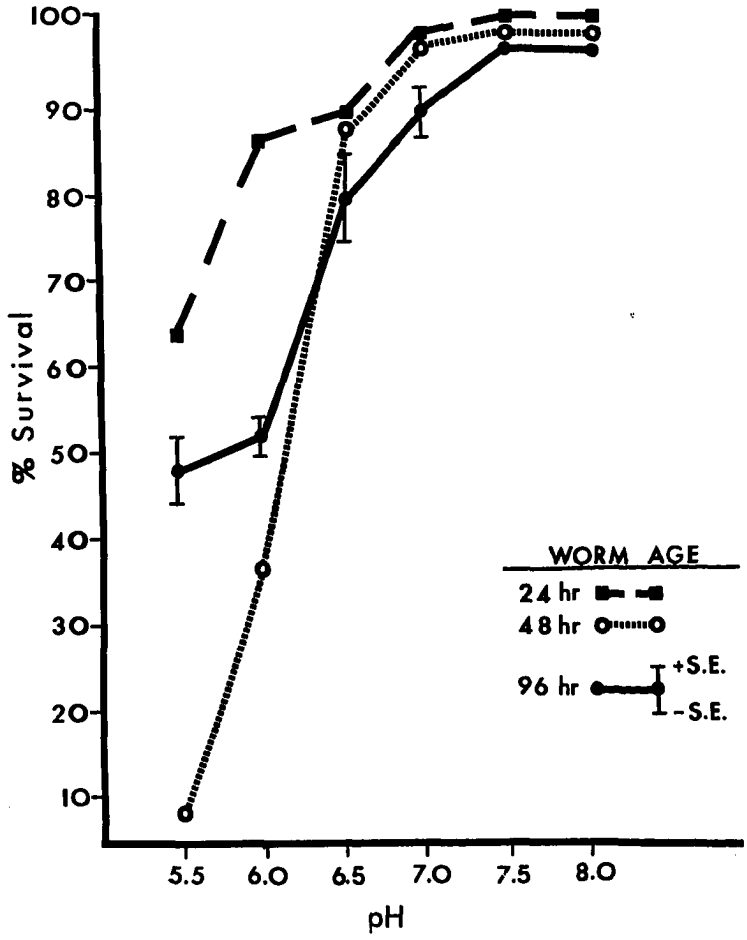

Figure 2. Effect of pH on survival of adult Trichinella. Adults (24-, 48- and 96-hr) were incubated for 48 hr at 37 C in KRB buffer. The pH of the buffer was varied from 5.5 to 8.0 at 0.5 intervals by changing the sodium bicarbonate concentration. Total molarity of normal KRB buffer was maintained by altering the NaCl concentration. Percent survival was calculated from a count of 100 worms.

For the above study it was necessary to establish criteria to designate whether worms were alive or dead. Worms were considered alive if they were moving or coiling upon microscopic examination. Worms were considered dead if they showed no signs of motion. Figures 3 and 4 depict these characteristics. Figure 3 shows photomicrographs of adult Trichinella collected 96-hr post-infection. Figure 4 shows mixed stages of a free-living nematode, Rhabditella sp.[13] The photographs represent both untreated worms (photomicrographs 1a and 1b in both Figure 3 and 4) and worms treated with 0.1 M sodium cyanide for 30 min (photomicrographs 2a and 2b in both Figure 3 and 4). Photomicrographs 1b and 2b in each figure were taken 15 sec after 1a and 2a, respectively. In each Figure (3 and 4), note the coiling and movement of untreated worms in contrast to the lack of such in the cyanide-treated group.

As a check on the validity of this criterion, treated and untreated Rhabditella were transferred to 5% blood agar slants, and their viability estimated by their ability to propagate on this media. Results of this experiment are shown in Table 1. Data indicate that those worms treated with cyanide were immotile and unable to propagate, whereas untreated, motile worms did develop into teeming colonies.

A second check on the validity of the above criterion for differentiating between living and dead worms was carried out by implanting equal numbers of cyanide-treated or untreated adult Trichinella into the small intestine of two groups of three mice. Mice from both groups were killed 48 hr later for recovery of implanted worms. Data in Table 2

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[13]Obtained from Dr. Leroy J. Olson, Department of Microbiology, University of Texas Medical Branch, Galveston, Texas.

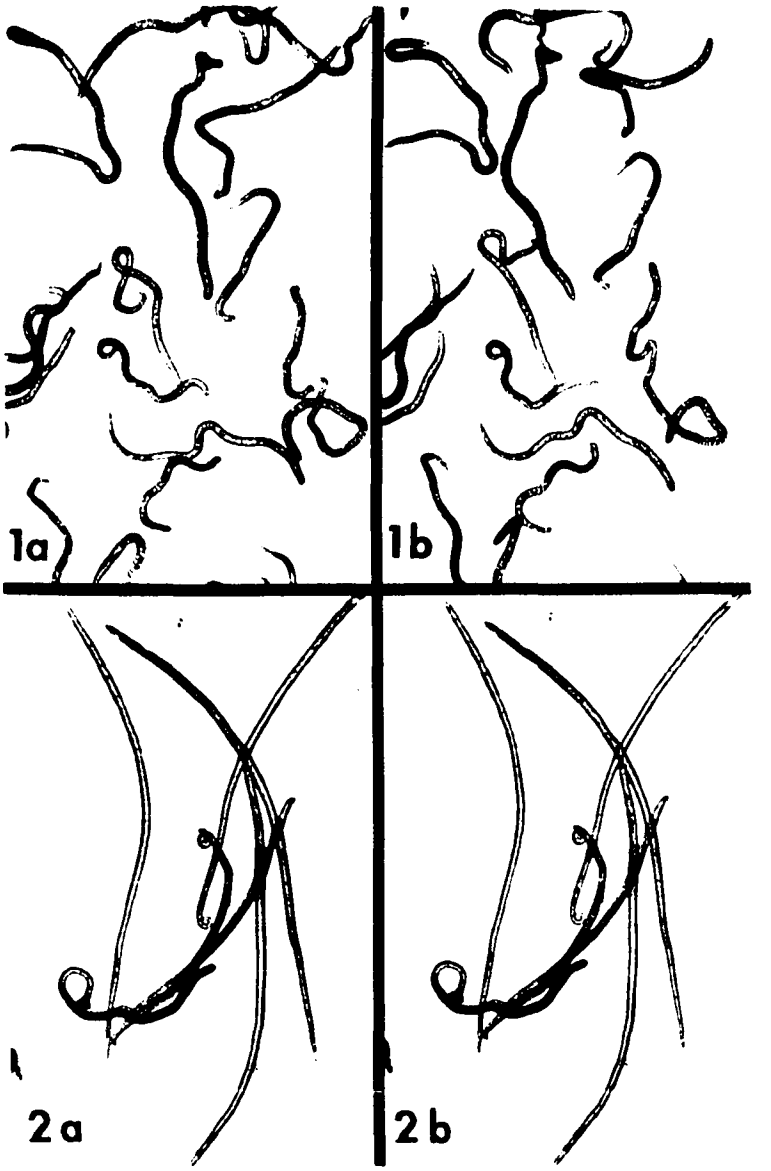

Figure 3. Photomicrographs showing differences between living and dead worms (Experiment I). The first two photographs (1a, 1b) were taken of 96-hr Trichinella adults freshly isolated and placed in KRP buffer. The second set of photographs (2a, 2b) were taken of adult worms which were placed in KRP buffer containing 0.1 M sodium cyanide for 30 min. A 15 sec time interval elapsed between photographs a and b in each group. Worms in 1a and 1b were considered living; those in 2a and 2b were considered dead.

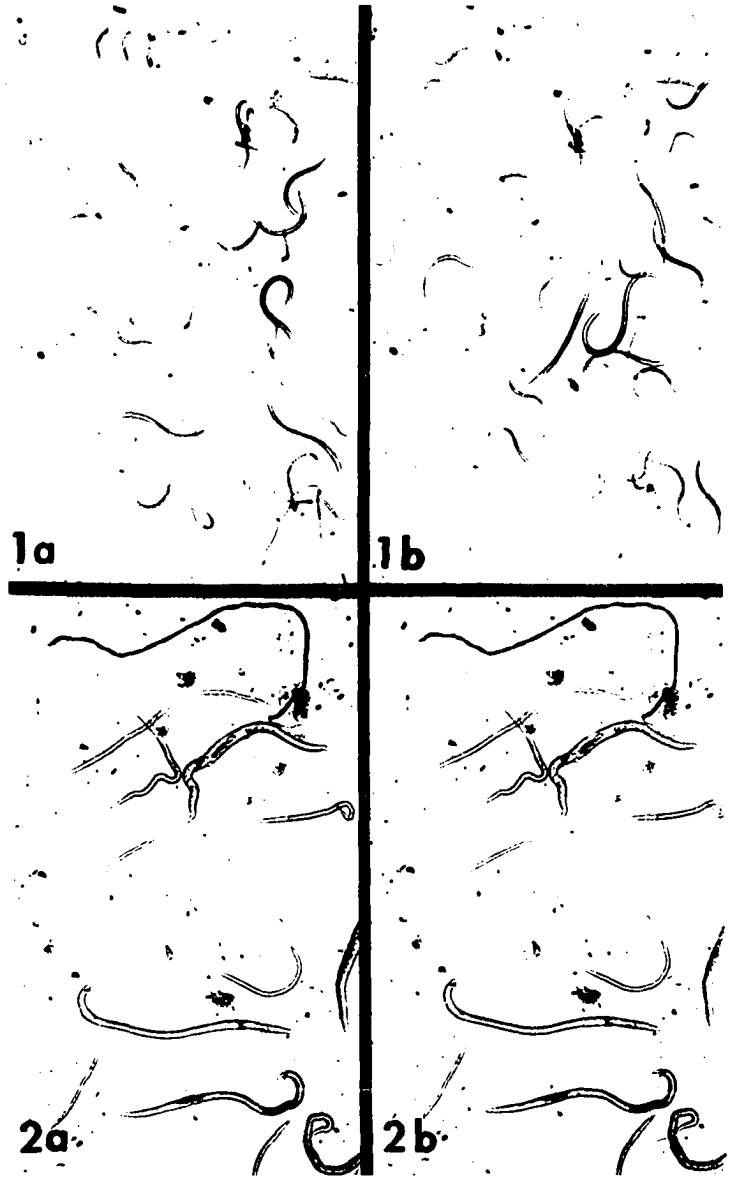

Figure 4. Photomicrographs showing differences between living and dead worms (Experiment II). The first two photographs (1a, 1b) were taken of a free-living nematode, Rhabditella, placed in KRP buffer. The second set of photographs (2a, 2b) were taken of Rhabditella that had been placed in KRP buffer containing 0.1 M sodium cyanide for 30 min. A 15 sec time interval elapsed between photographs a and b in each group. Worms in 1a and 1b were considered living; those in 2a and 2b were considered dead.

TABLE 1

PROPAGATION OF RHABDITELLA FOLLOWING INHIBITION OF MOTILITY WITH SODIUM CYANIDE

Incubation medium	Percent motile ("living")	Percent immotile ("dead")	Ability to propagate[a]
KRP buffer[b]	100	0	+
0.1 M Sodium cyanide in KRP buffer	0	100	-

[a]Rhabditella were transferred to 5% blood agar slants upon which they are known to propagate.

[b]KRP buffer contained antibiotics and 5 mM glucose. Incubation was for 30 min at 37 C.

TABLE 2

DIFFERENTIATION BETWEEN LIVE AND DEAD TRICHINELLA ADULTS BY IMPLANTATION INTO THE SMALL INTESTINES OF MICE

Incubation medium	No. of mice per group	Adult Worm Implantation		Adult Worms Recovered[b]	
		No. implanted per mouse	Total No. implanted	Total No. recovered from 3 mice	Percent recovered
KRP[a] buffer	3	3,000	9,000	428	4.8
0.1 M Sodium cyanide in KRP buffer	3	3,000	9,000	0	0

[a]KRP contained antibiotics and 5 mM glucose. Incubation was for 30 min at 37 C.

[b]Mice were killed 48 hr post-implantation.

show that some of the untreated motile worms were able to reestablish in a second host upon passive transfer, whereas the non-motile worms which had been treated with cyanide were unable to reestablish. Thus, the criterion of basing survival on the absence or presence of motility was deemed valid.

Total carbohydrates, alkali-stable carbohydrates and glycogen content of intestinal worms were determined directly by chemical methods (Table 3). Although trehalose was not measured directly, it was presumed to be present and was calculated as the difference between alkali-stable carbohydrate and glycogen (Castro and Fairbairn, 1969a). It was immediately obvious upon analysis that the tissues of the post-larval stages examined possess only small quantities of metabolizable carbohydrates. Alkali stable carbohydrates comprised 3.0%, 1.4% and 1.0% of the dry weight of worms recovered 96-, 48- and 24-hr post-infection, respectively.

In view of the fact that most parasitic stages of helminths examined produce some fermentation products, the incubation medium in several experiments was examined for the presence of fermentation acids Gas-liquid chromatograms of volatile fatty acids excreted by Trichinella larvae and adults incubated for 48 hr are presented in Figure 5. All stages produced formic-, acetic-, propionic-, n-butyric-, n-valeric- and n-hexanoic acid. However, the per cent composition varied considerably among the different stages. Larvae excreted about three times as much acid as equal numbers of adult worms (Table 4). Data illustrating the quantitative differences in acid output among the different stages examined are shown in Table 5.

TABLE 3

CARBOHYDRATES OF INTESTINAL STAGES OF *TRICHINELLA*

Carbohydrate	Carbohydrate as Percent Dry Weight		
	4-day Adults[a]	2-day Adults[b]	1-day Adults[b]
Total[c]	3.3 ± 0.1	1.9	1.3
Alkali stable	3.0 ± 0.2	1.4	1.0
Glycogen	1.3 ± 0.1	1.1	0.7
Trehalose	1.7 ± 0.1	0.3	0.3

[a]Means ± Standard Error (SE) for 3 samples of adult *T. spiralis*.

[b]Values represent a single determination.

[c]Total carbohydrate, alkali-stable carbohydrate and glycogen were determined by the anthrone reaction (Mokrasch, 1954). Trehalose was estimated as the difference between alkali-stable carbohydrate and glycogen.

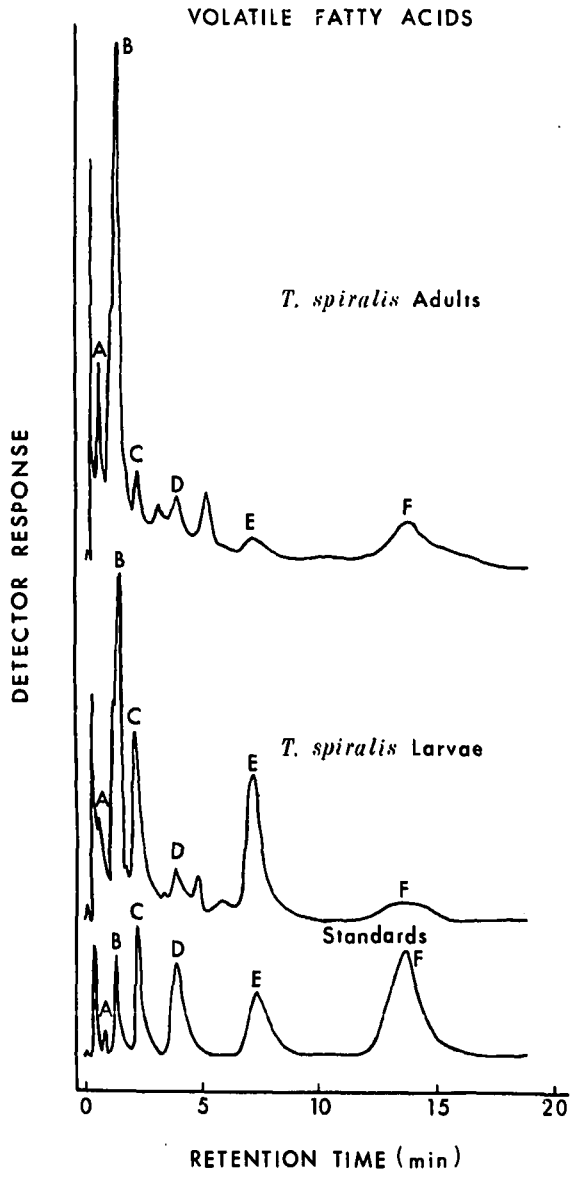

Figure 5. Gas-liquid chromatograms of volatile fatty acids excreted by Trichinella adults and larvae, and of authentic reference acids. The column was packed with 60- to 80-mesh Chromosorb 102 and operated at 210 C with a carrier gas (N_2) flow rate of 120 ml/min.

TABLE 4

FERMENTATION ACID OUTPUT BY TRICHINELLA LARVAE AND ADULTS

Stage	Total acid output[a]
Larvae	28.8 μg
Adults	
24-hr	9.1 μg
48-hr	10.2 μg
96-hr	8.6 μg

[a]A single determination for 3×10^4 larval or adult worms incubated in KRB buffer and gassed with 90% N_2:5% CO_2 for 48 hr at 37 C. Values represent the amount of formic-, acetic-, propionic-, n-butyric-, n-valeric- and n-hexanoic acid excreted as determined by quantitative GLC.

TABLE 5

QUANTITATIVE ANALYSIS OF FERMENTATION ACIDS PRODUCED BY *TRICHINELLA* ADULTS AND LARVAE

Stage[a]	Weight Percent of Free Acids Placed on Column					
	Formic	Acetic	Propionic	Butyric	Valeric	Hexanoic
Larvae	1.5	19.7	22.2	7.4	41.6	7.6
Adults						
24-hr	2.7	38.8	12.1	9.8	9.8	26.8
48-hr	4.2	32.0	10.5	13.0	9.7	30.6
96-hr	15.8	11.8	36.9	8.5	16.7	10.4

[a]A single determination for equal numbers (3×10^4) larval and adult worms maintained for 48 hr at 37 C.

The aliphatic amines excreted by 6 x 10^4 to 9 x 10^4 96-hr intestinal worms were recovered from the medium as hydrochloride salts and were chromatographed on thin-layer plates coated with microcrystalline cellulose. Plate development was complete within 5 hr. Dibasic amines were effectively separated from monobasic compounds, and the individual components in the latter group were clearly discernable (Figure 6). R_F values for the amines in Figure 6 were as follows: ethylenediamine, .15; 1,5-pentanediamine, .15; methyl, .25; ethyl, .32; propyl, .42; n-butyl, .57; n-pentyl, .70; n-hexyl, .77; n-heptyl, .82. Since diamines migrated as a group to a position only slightly above the origin on thin-layer chromatograms, the presence of more than one dibasic amine in worm excretions could not be determined using this approach. Worms produced 256 $\pm$ 41 and 599.7 $\pm$ 115 µg amine nitrogen/mg dry wt per 48 hr under a 90% N_2:5% CO_2:5% O_2 and a 95% N_2:5% CO_2 gas phase, respectively.

Oxygen consumption was measured for Trichinella larvae and intestinal worms. Rhabditella was used for comparative purposes. Measurements were taken at 5 min intervals for 30 min in both the absence and presence of 0.1 M sodium cyanide. Measurements were recorded as µl O_2 uptake/mg worm protein per hr after adjusting to standard temperature and pressure. Data show that Rhabditella consumed O_2 at a rate about 3 times that of intestinal stages of Trichinella (Table 6). In none of three replicates was O_2 consumption measurable for Trichinella larvae. Oxygen uptake in all cases was inhibited almost completely by the addition of cyanide to the incubation flask.

The purpose of the next experiment was to investigate the effect of various metabolic inhibitors on survival of intestinal stages of



Figure 6. Thin-layer chromatograph of standard amines and amines excreted by Trichinella adults (96 hr). The center sample is the standard mixture. The sample on the left represents amines from adults maintained aerobically, and the one on the right represents amines produced under anaerobic conditions. TLC plates were coated with microcrystalline cellulose (0.25 mm thickness). The solvent phase consisted of butanol:acetic acid:water (4:1:5). Plates were visualized by spraying with 0.1% ninhydrin in n-butanol and heating to 90 C for 10 min. (1) ethylenediamine and 1,5-pentanediamine; (2) methyl-; (3) ethyl-; (4) propyl-; (5) butyl-; (6) pentyl-; (7) hexyl-; and (8) heptylamine.

TABLE 6

OXYGEN CONSUMPTION BY *TRICHINELLA* LARVAE AND ADULTS

Organism Stage	Presence or absence of 0.1 M sodium cyanide	µl O_2 uptake/mg protein per hr[a]
Trichinella		
Larvae	-	0[b]
	+	0
Adults		
96-hr	-	13.4 ± 0.8[c]
	+	0.4 ± 0.4
48-hr	-	13.7[d]
	+	0
24-hr	-	13.6[d]
	+	0
Rhabditella sp.	-	44.4 ± 1.3[e]
(mixed stages)	+	0.5 ± 0.4

[a]Oxygen uptake measurements were adjusted to standard temperature and pressure. Worms were maintained in KRP buffer containing antibiotics and glucose.

[b]Represents mean value from 3 determinations.

[c]Means ± SE for 3 replicates.

[d]Values represent a single determination.

[e]Means ± SE for 4 replicates.

Trichinella. The nematodes were incubated in KRB buffer, 0.001 M iodoacetamide, 0.001 M 2,4-dinitrophenol, 0.1 M sodium cyanide, and 0.1 M sodium azide and gassed continuously with 90% N_2:5% CO_2:5% O_2. All solutions contained antibiotics and 5 mM glucose. Viability of worms was determined microscopically at 24, 36 and 48 hr, and the results were recorded (Table 7). It was obvious that all inhibitors except 2,4-dinitrophenol killed 100% of the worms within 24 hr.

The results of the previous experiment (Table 7) showed that various metabolic inhibitors had detrimental effects on adult Trichinella. The next two experiments were designed to examine the effects of these same inhibitors on viability of larvae as measured by alterations in their infectivity rate and reproductive capacity (Tables 8 and 9). Iodoacetamide killed 100% of the larvae within 48 hr. The other inhibitors appeared to have no adverse effect upon the larvae after 48-hr incubation as determined by microscopic observation. However, the development of these larvae following infection was significantly impaired. Both the number of adult worms and second generation larvae recovered from mice following infective doses was lower when the mice were infected with larvae previously incubated in the presence of 2,4-dinitrophenol, cyanide and azide as compared to untreated control larvae (Tables 8 and 9).

The final experiment was designed to investigate the ability of Trichinella larvae and adults to utilize (U-^{14}C) palmitic acid as a substrate. Oxidation of lipids can be expected to be tightly coupled to the TCA cycle (Ward and Fairbairn, 1970). Thus, oxidation of palmitate to CO_2 would indirectly support the conclusion that a functional TCA cycle and respiratory type of energy metabolism exists in Trichinella.

TABLE 7

EFFECT OF METABOLIC INHIBITORS ON SURVIVAL OF TRICHINELLA ADULTS

Inhibitor		Percent Worms Living After:		
Compound	Molar concentration[b]	24 hr	36 hr	48 hr
KRB buffer[a]	0	100	100	100
Iodoacetamide	10^{-3}	0	0	0
2,4-dinitrophenol	10^{-3}	42	35	17
Sodium cyanide	10^{-1}	0	0	0
Sodium azide	10^{-1}	0	0	0

[a]KRB contained antibiotics and 5 mM glucose and was gassed continuously with 90% N_2:5% CO_2:5% O_2.

[b]All inhibitors were solubilized in KRB buffer.

TABLE 8

EFFECT OF METABOLIC INHIBITORS ON INFECTIVITY AND REPRODUCTIVE CAPACITY OF *TRICHINELLA* LARVAE

Treatment[a]	Total no. of larvae administered to 3 rats ($\times 10^3$)	Total no. of adults recovered ($\times 10^3$)	Percent recovery
KRB buffer[c]	127.5 ± 1.1	17.3 ± 0.1	13.6
Iodoacetamide	107.5	0	0
2,4-dinitrophenol	108.5	0.1	0.1
Sodium cyanide	73.5	0.3	0.4
Sodium azide	104.5	0.5	0.5
Sodium chloride[d]	120.0	22.0	18.3

[a]All samples were incubated in KRB buffer gassed continuously with 90% N_2:5% CO_2:5% O_2 for 48 hr at 37 C. Metabolic inhibitors were solubilized in KRB buffer at a concentration of 0.1 M.

[b]Adult worms were recovered 4 days post-infection.

[c]Means ± SE for 2 samples of *Trichinella* larvae.

[d]The 0.1 M sodium chloride solution represents an osmolarity control and is not to be considered a metabolic inhibitor.

TABLE 9

EFFECT OF METABOLIC INHIBITORS ON *TRICHINELLA* LARVAE AS MEASURED BY THE RECOVERY OF SECOND GENERATION LARVAE

Treatment[a]	No. of larvae administered per mouse	No. of muscle larvae recovered per mouse ($\times 10^3$)[b]
KRB buffer	500	52.0 ± 6.5
Iodoacetamide (0.1 M)	500	0
2,4-dinitrophenol (0.1 M)	500	0.2 ± 0.0
Sodium cyanide (0.1 M)	500	1.7 ± 0.7
Sodium azide (0.1 M)	500	0.1 ± 0.0

[a]Inhibitors were solubilized in KRB buffer with antibiotics and 5 mM glucose. The buffer was gassed with 90% N_2:5% CO_2:5% O_2 for 48 hr at 37 C.

[b]Values represent means ± SE for 10 mice. Larvae were recovered 45 days post-infection.

Both intact worms and homogenates of Trichinella larvae and adults were incubated at 37 C in 5 ml of KRP buffer containing 2.13 μC_i of palmitic acid with a specific activity of 542 μC_i/mole. Intact adult Trichinella and adult worm homogenate metabolized absorbed (U-^{14}C) palmitic acid to $^{14}CO_2$. In contrast only trace amounts of $^{14}CO_2$ were produced when larvae were incubated with labelled palmitic acid (Table 10).

To determine if palmitate was actually being absorbed by the nematodes, total lipids were extracted and fractionated on TLC plates coated with Silica Gel G. Figure 7 shows the lipid spots. Lipids were visualized under ultraviolet light following spraying with Rhodamine 6-G. Individual bands were scraped into a glass-sintered funnel, eluted with ether into a scintillation vial and counted. As seen in Table 11, the majority of the label was found in the free fatty acid fraction for both larvae and adult worms. However, in the case of adult worms there was some distribution in glycerides and in the cholesterol and sterol ester fraction. In the larvae, label was found only in the phospholipid fraction in addition to the free fatty acid band.

TABLE 10

$^{14}CO_2$ EVOLVED DURING INCUBATION OF TRICHINELLA ADULTS AND LARVAE WITH (U-^{14}C) PALMITIC ACID[a]

Stage	$^{14}CO_2$ Evolved	
	dpm[b]	dpm/gm wet wt. tissue[c]
Adult (96-hr)		
Intact	1654 ± 16	23,907 ± 1785
Homogenate	295 ± 74	4,176 ± 1203
Larvae		
Intact	0	0
Homogenate	16 ± 6	28 ± 6

[a]Larvae and adults, and homogenates of each, were incubated for 3 hr at 37 C in 5 ml of KRP buffer containing 2.13 μC_i/mg of (U-^{14}C) palmitic acid with a specific activity of 514 μC_i/mole.

[b]Means ± SE for 2 replicates.

[c]Means ± SE for 2 replicates.



Figure 7. Thin-layer chromatograph of neutral lipids of Trichinella larvae and adults. The center sample is the standard. The sample on the left represents neutral lipids obtained from larvae, and the one on the right represents neutral lipids of adult worms. Fractions were analyzed on silica gel G plates (0.25 mm thickness). Spots were visualized by charring at 180 C after spraying with 50% sulfuric acid saturated with potassium dichromate. (1) Phospholipids; (2) Monoglycerides; (3) Free fatty acids; (4) Cholesterol; (5) Diglycerides; (6) Triglycerides; and (7) Cholesterol esters.

TABLE 11

INCORPORATION OF ABSORBED (U-^{14}C) PALMITIC ACID INTO THE LIPIDS OF TRICHINELLA ADULTS AND LARVAE

Stage	Total dpm recovered	Radioactivity (dpm)[a]						
		Phospho-lipids	Mono-glycerides	Free fatty acids	Di-glycerides	Tri-glycerides	Cholesterol	Cholesterol esters
96-hr adult worms	2,456	0	3.7 ± 3.2	92.3 ± 4.4	0.6 ± 0.4	1.2 ± 0.3	1.9 ± 1.4	0.4 ± 0.3
Larvae	1,794	5.7 ± 5.7	0	94.3 ± 5.7	0	0	0	0

[a]Analysis of two samples of total lipids. Neutral lipids were fractionated by TLC. Worms were incubated as mentioned in the footnotes for Table 10.

CHAPTER IV

DISCUSSION

The limited metabolic studies done in the past with Trichinella dealt primarily with excysted larvae. No significant information exists with regard to the biochemistry and physiology of other stages of this parasite. In view of this, comparisons were made throughout this study to determine similarities and dissimilarities in metabolism between intestinal stages and larvae of Trichinella.

The intestinal stage of Trichinella is usually referred to as the "adult" stage. However, this nematode, like all others, must undergo developmental changes or molts before it matures and is capable of reproducing. Considerable controversy exists concerning the development of Trichinella. Berntzen (1965) reported only one molt, Shanta and Meerovitch (1967a) observed two molts and Ali Kahn (1966) observed four molts during the intestinal phase. While there was disagreement about the number and timing of molts, all authors agreed that mature adults develop within 72 hr. In the present investigation intestinal worms recovered 24-, 48- and 96-hr post-infection were studied. The younger intestinal stages (24- and 48-hr) were examined to detect immediate changes that took place from the time muscle larvae were liberated from their cysts until they became fully mature (96-hr).

During initial incubation experiments striking differences were observed with regard to the motility and physical appearance of adult Trichinella maintained in the presence or absence of glucose or metabolic poisons. To evaluate the suitability of incubation conditions on worm survival and to estimate the effect of various treatments on worms in vitro, it was necessary to establish criteria for classifying adult parasites as either living or dead.

The designation of motile worms as alive and non-motile worms as dead was justified on the following basis. Motile Rhabditella were able to propagate on blood agar, and motile adult Trichinella were able to reestablish in mouse recipients upon passive transfer (Figures 3 and 4; Tables 1 and 2). Non-motile worms neither propagated on blood agar nor reestablished, respectively.

In the passive transfer experiments, four percent of the untreated Trichinella adults reestablished in the new host. When mice are given an infection by oral intubation of larvae, adult worm recovery rate by the method of collection used is 15 to 20%. However, a decrease in recovery of adult worms passively transferred is not unusual since Katz (1960) had difficulty in getting more than 7% of 72-hr intestinal worms to reestablish following oral transplantation.

Problems were encountered initially in keeping adult stages of Trichinella alive for more than 12 hr in KRB buffer. Little has been published with regard to nutritional requirements of the adult stage. Oliver-Gonzalez and Bueding (1948) reported that adult worms did not utilize glucose present in a medium. In contrast, intestinal worms in the present study remained viable for 48 hr or longer in a simple buffer

system (KRB or KRP, pH 7.4) containing antibiotics and 5 mM glucose. The increased survival of adults in the presence of glucose indicates thay they can metabolize this carbohydrate.

The *in vitro* glucose requirement of larvae was markedly different than that observed for adult worms. Larvae incubated for 48 hr required no exogenous energy source, utilizing their endogenous carbohydrate stores of glycogen and trehalose (von Brand *et al.*, 1952; Castro and Fairbairn, 1969a) for energy. Alkali-stable carbohydrates make up 20.7% of the dry weight of larvae, indicating a considerable reserve source of energy. Castro and Fairbairn (1969a) reported that larvae maintained *in vitro* consume 75% of their carbohydrate within 48 hr. In contrast, the tissues of post-larval stages possess only small quantities of metabolizable carbohydrates. Alkali-stable carbohydrates represented 3.0%, 1.4% and 1.0% of the dry weight of worms recovered 96-, 48- and 24-hours post-infection, respectively (Table 3). These findings demonstrate that *Trichinella* utilize endogenous carbohydrates rapidly, and offer a suggestion as to why intestinal stages require an exogenous energy source.

The effect of pH on worm survival was examined. Generally, nematodes tolerate wide pH ranges. This finding has been explained in terms of an impermeable cuticle rather than metabolic systems themselves being insensitive (Barrett, 1969b). Adult worms were adversely affected by a pH below 6.5, and survival was maximal between 7.0 and 8.0 (Figure 2). For subsequent studies, the KRB and KRP buffer used to incubate the worms was adjusted to pH 7.4. During incubation, the pH never changed more than 0.2 units.

The effect of a low pH on specific physiological and biochemical functions of this parasite is unknown. Larsh (1967a) suggested that accumulation of lactic acid in tissue during inflammation may be harmful to the parasite. There is documented evidence that tissue pH can drop to 6.0 during subacute and chronic inflammation (Menkin, 1956). Since hydrogen ion concentration adversely affected worm survival in this study, it is proposed that local acidosis in intestinal tissue could alter the biochemical environment of adult Trichinella, resulting in premature termination of the intestinal phase of infection.

Considering that acidosis is deleterious to the intestinal stage of Trichinella, it is interesting to speculate that amine excretory products of this parasite might compensate for or offset a low pH in the environment. The reason for higher amine output under anaerobic conditions is obscure. The hypothesis that amine production by adults may serve as a mechanism to offset an acidic environment is supported by work with bacterial systems. Hanke and Koessler (1919) suggested that amine formation in bacteria might be a physiological mechanism to counteract acidic environments. Gale et al. (1946) demonstrated clearly that amine formation by bacteria resulted from the action of specific inducible amino acid decarboxylases. Maximal production of these enzymes occurred when growth was carried out in an acidic medium. Amino acid decarboxylases have been demonstrated in the tissues of some nematodes, and amines have been identified as excretory products of some nematode larvae. The observation that adult Trichinella excretes amines represents the first report of amine production by an adult nematode parasite of animals.

All nematodes, as well as cestodes and trematodes examined thus

far, are capable of taking up oxygen *in vitro*. However, the significance of this uptake remains in doubt in the case of those worms which normally dwell in essentially anaerobic environments such as the intestinal lumen. No helminths studied to date, regardless of their oxygen requirements, completely oxidize substrates to carbon dioxide and water. Incomplete oxidation is suggested by the fact that all excrete or secrete organic end-products. It would appear that terminal respiration is either absent or rate-limiting in this entire group of organisms (Saz, 1971).

The significance of aerobiasis versus anaerobiasis in some species remains unknown. Von Brand (1946) stated that some worms living in the gut may lead aerobic lives while others in the same environment lead anaerobic lives. Perhaps a number of apparently puzzling observations can be interpreted in terms of development. For example, *Trichinella* larvae are fermenters (von Brand *et al.*, 1952; Castro and Fairbairn, 1969a). Nevertheless, they exhibit more Krebs cycle and cytochrome oxidase activity (Agosin, 1956; Goldberg, 1957) than do most helminths. Fairbairn (1970) gave a possible explanation for this phenomena. In the muscle of the host, these larvae are in a resting stage which may continue for years. At any time the muscle is ingested by a predator, this stage may be interrupted. They then develop within 72 hrs into reproductively mature intestinal worms. Although little is known about adult metabolism, *in vitro* culture studies showed that growth and development from larvae to adults required oxygen (Berntzen, 1965). The presence of aerobic systems in the larvae may therefore indicate preparation for the next stage of development.

Larvae have been reported to consume oxygen at a rate of 2.24 µl

oxygen/mg dry substance per hr (Stannard et al., 1938; von Brand et al., 1952). Kozar et al. (1965) on the other hand reported a value nearly four times as high. These investigators used sterile tyrode's solution and saline in the absence of antibiotics to measure oxygen uptake. In the present study oxygen consumption by larvae was not measurable, whereas adult worms consumed oxygen readily (Table 6). In comparison to mixed stages of Rhabditella which are free-living and are known to require oxygen, all intestinal stages of Trichinella consumed oxygen at one-third the rate of the free-living nematode. The use of antibiotics in these findings may account for the differences between this study and earlier studies. It is possible that previous studies by other authors could have measured oxygen consumption by bacteria rather than larvae. Another possible explanation for this conflicting report is that oxygen uptake by larval stages may be inhibited by the antibiotics. A report referring to this type of inhibition was made by Barrett (1969b) while studying Strongyloides ratti.

The fact that adults consume oxygen does not necessarily indicate that they obtain all their energy by means of respiration. Fairbairn (1970) stated: "If some species (nematode) should ultimately prove to have an oxygen requirement for their energy metabolism, it remains undeniable that most of their energy requirements can be satisfied by fermentations."

Fairbairn's view is supported by the fact that adult Trichinella excreted fermentation acids. Volatile fatty acids excreted by equal numbers of Trichinella larvae and adults were identified and quantitated by GLC as shown in Figure 5. All stages produced formic-, acetic-,

propionic-, n-butyric-, n-valeric- and n-hexanoic acid. However the percent composition varied considerably. Larvae excreted three times as much acid as adult worms (Table 4). The quantity of each acid produced by different stages differed markedly (Table 5). Valeric acid is the predominating acid for larvae, which agrees with the findings of Castro and Fairbairn (1969a). On the other hand, 24- and 48-hr adults excrete primarily acetic and hexanoic acid, and 96-hr intestinal worms excrete primarily propionic acid along with equal amounts of the other acids. Obviously, fermentation acids of larvae are qualitatively similar but quantitatively different from those produced by adult worms. The finding of fermentation acids in adults does not support an earlier report by Oliver-Gonzalez and Bueding (1948) that adults do not produce acids in a salt medium containing glucose. Perhaps fermentations in adult worms explain how they survive *in vitro* for 48 hr in KRB buffer gassed continuously with 95% N_2:5% CO_2 (anaerobic), but do not move as actively as those incubated in oxygen. Adults may carry out fermentations while they are free in the gut lumen, yet have the potential to produce energy aerobically while in contact with the mucosa where the oxygen tensior is relatively high.

The greater motility of worms under aerobic conditions suggested that the amount of energy obtained anaerobically was too low to support muscle movement. This contention was proposed for larvae by von Brand *et al.* (1952), but was not supported by the findings of Castro and Fairbairn (1969a). To determine if this is the case for adult worms, acid production should be examined to determine if anaerobiasis increases the output of fermentation acids.

In a previous experiment, both Trichinella adults and mixed stages of Rhabditella took up oxygen readily. This oxygen uptake was also cyanide sensitive (Table 6). Stannard et al. (1938) reported that larvae oxygen uptake was strongly inhibited by cyanide. However, since oxygen uptake was insignificant for the larval stage in the present study, the effects of cyanide could not be measured.

The preliminary experiment with cyanide, an irreversible metabolic inhibitor of cytochrome oxidase, aroused interest in other metabolic inhibitors which might inhibit glycolysis (iodoacetamide), the cytochrome system (azide), and oxidative phosphorylation (2,4-dinitrophenol). Trichinella intestinal stages were incubated in KRB buffer containing 0.001 M iodoacetamide, 0.001 2,4-dinitrophenol, 0.1 M sodium cyanide or 0.1 M sodium azide and gassed continuously with 90% N_2:5% CO_2:5% O_2. With the exception of 2,4-dinitrophenol, all inhibitors killed 100% of the worms within 24 hr (Table 7). Since 2,4-dinitrophenol is known to uncouple oxidative phosphorylation and electron transport and since the worm is able to survive in the presence of this inhibitor, it is evident that glycolysis can supply enough energy for a short period of time.

Since metabolic inhibitors had detrimental effects on adult Trichinella, the next two experiments were designed to examine the effects of these same inhibitors on viability of larvae as measured by alterations in their infectivity rate and reproductive capacity (Tables 8 and 9). Iodoacetamide killed 100% of the larvae within 48 hr, which may indicate that glycolysis was inhibited. Although other inhibitors appeared to have little or no effect upon larvae after 48 hr incubation as

determined microscopically, development of larvae following infection was significantly impaired. Many parasites are anaerobic in the restricted sense that they are able to satisfy their needs for energy by means of fermentation. Whether they are strictly anaerobic requires demonstration that they grow and develop in an atmosphere known to be oxygen-free; or alternatively that enzymes catalysing syntheses in which molecular oxygen is a reactant are absent or non-functional (Fairbairn, 1970). Trichinella requires oxygen for development from the larval to adult stages (Berntzen, 1965), and it is apparent from the previous experiments that irreversible cytochrome inhibitors like cyanide and azide, although they do not kill larvae, do prevent development to maturity.

Mills and Kent (1965) suggested that adverse effects on Trichinella larvae in an immune host relate to circumstances which impair oxygen transport or utilization. The fact that larvae depend on fermentative metabolism for energy does not mean that larvae are strictly anaerobic organisms. It is clear, however, that tissue hypoxia per se would have little adverse effect on energy production by the larvae. On the other hand, adults are known to take up oxygen readily, and this same oxygen uptake is cyanide sensitive. Apparently, post-larval stages switch from a fermentative to a respiratory type of energy metabolism. The partial pressure of oxygen that may develop during infection could be detrimental to the intestinal stage. However, oxygen partial pressures have not been measured during inflammatory responses in the gut, and information on oxygen uptake by adult worms at different pressures is unavailable. Thus, it is not clear how lower oxygen tensions that develop during infection might affect the parasite.

Since adult respiration was completely inhibited by 0.1 M cyanide, electron transport via the cytochrome system was considered to be of physiological importance to this stage. Goldberg (1957) reported that both larvae and adult Trichinella contain components of the classical cytochrome system, namely cytochrome C and cytochrome oxidase. The presence of these components in both stages is difficult to interpret unless they are related to some overall change or process which can be measured. Development in helminth parasites implies a capacity for orderly release of information to be used in the next stage. As each stage may require an extremely different environment, preparation for it may lead to puzzling observations, such as the existence of aerobic electron transport systems in a stage whose energy metabolism is fermentative. The attribution of useful function on the basis of the determination of enzyme activities alone is undesirable, and in developing organisms, can be misleading. Thus, the concept of epigenetic adaptations is useful for interpreting these observations. Epigenetic adaptation entails repression or derepression of genes so that orderly growth and development occurs throughout a life cycle (Fairbairn, 1970). Changes in the external environment may, if the organism is capable of responding, lead to changes in control mechanisms, metabolic pathways, enzyme activity and mobilization of energy reserves (Rao, 1967).

As previously stated, the presence of aerobic systems in the larvae may indicate preparation for the next stage of development. This possibility was examined by comparing functional activity of the aerobic systems in larvae and adult Trichinella. The possible presence of aerobic metabolism can be measured functionally by means of absorption and

oxidation of (U-^{14}C) palmitate. If palmitic acid is absorbed as shown by incorporation into lipids and gives rise to $^{14}CO_2$, one must assume that a functional TCA cycle is operating. When palmitate is absorbed but not oxidized, one of the TCA cycle or β-oxidation enzymes is absent (Ward and Fairbairn, 1970a, 1970b). Both intact worms and homogenates of Trichinella larvae and adults were incubated in KRP buffer containing 2.13 μC_i of palmitic acid with a specific activity of 542 μC_i/mole. Intact adult Trichinella and adult worm homogenates metabolized absorbed (U-^{14}C) palmitic acid to $^{14}CO_2$. In contrast only trace amounts of $^{14}CO_2$ were produced by larvae incubated with labelled palmitic acid (Table 10). Palmitic acid had been purified from other carbon sources by TLC as described in Chapter II.

To determine if palmitate was actually being absorbed by the nematodes, total lipids were extracted and fractionated on TLC plates coated with Silica Gel G. Figure 7 shows the lipid spots. Each spot was removed from the plate, and lipids were eluted into a scintillation vial with ether. Table 11 indicates that the majority of label was found in the free fatty acid fraction for both larvae and adult worms. In the case of adult worms, there was some label found in glycerides, cholesterol and sterol ester fractions. In the larvae some label was found in the phospholipid fraction in addition to the free fatty acid band.

The above findings indicate that both larvae and adults differ with respect to energy metabolism. It seems reasonable to conclude that even though the larval stage has been reported to be anaerobic in terms of energy production, the adult stage is aerobic.

CHAPTER V

SUMMARY

Metabolism in adult Trichinella spiralis was investigated. Results were compared to previous reports dealing with the biochemical and physiological processes in the larval stage. Metabolic events were discussed in regard to their role in the development of the parasite within its host.

Hydrogen ion concentration, a factor which is altered in tissue during infection, was studied as to its influence on survival of adult worms. Intestinal worms recovered 24-, 48- and 96-hr post-infection lost 50 to 90% of their viability at pH 6.0 after 48 hr, whereas worms maintained at pH 7.0 to 8.0 retained their viability. It is proposed that local acidosis in gut tissue may contribute to termination of the intestinal phase of infection. The excretion of aliphatic amines by intestinal worms is the first report of amine excretion by an adult animal parasite and may serve as an adaptive mechanism aimed at offsetting a drop in tissue pH.

Excysted Trichinella larvae can survive anaerobiasis for several days by fermenting endogenous carbohydrates. Adult worms cannot live beyond 12 hr without exogenous nutrients. The shorter survival period for adults may be related to the observed lower level of utilizable tissue

carbohydrates.

Experiments with (U-^{14}C) palmitate indicated that adult worms, but not larvae, could utilize stored lipid to some degree for energy production. (U-^{14}C) palmitate was absorbed and oxidized to $^{14}CO_2$ only by the adults. This presumably indicates that β-oxidation of lipids occurs in adult worms. In other nematodes, this oxidation system has been found to be closely associated with a functional TCA cycle.

Intestinal worms collected 96-hr post-infection consumed 13.4 µl oxygen/mg protein per hr. Other intestinal stages (24- and 48-hr) took up oxygen at approximately the same rate. Oxygen uptake of larvae was not measurable under similar conditions, whereas mixed stages of a free-living nematode, Rhabditella sp., consumed 44.4 µl/mg protein/hr. Oxygen uptake by both Trichinella adults and Rhabditella was inhibited by cyanide.

Incubation for 48 hr in 0.1 M cyanide and azide killed adult worms. Larvae were not killed by these cytochrome inhibitors, but their infectivity was decreased significantly. Iodoacetamide, an inhibitor of glycolysis, killed both larvae and adults exposed to a concentration of 0.001 M.

The complement of volatile acids excreted by adults was qualitatively similar but quantitatively different from larvae. In contrast to larvae, the adult stage of Trichinella appears to be dependent primarily upon a respiratory type of metabolism for energy production.

Results of this investigation indicate that larval and adult stages of Trichinella are basically dissimilar in regard to specific aspects of metabolism. It supports the proposal that accurate conclusions

about biochemical and physiological functions or processes in a certain parasite or stage of a parasite demand the study of that specific parasite or stage.

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