Intermediary metabolism in Ligula intestinalis (Linnaeus, 1758) (Cestoda : Pseudophyllidea)

by

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A thesis submitted
for the degree of Doctor of Philosophy
in the University of London
and for the Diploma of Imperial College

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February, 1980.

ABSTRACT

Studies on the general carbohydrate metabolism of the pseudophyllidean cestode, Ligula intestinalis have been carried out, with emphasis on the larval plerocercoid stage from the body-cavity of roach (Rutilus rutilus) and the adult produced either by in vivo passage through a bird-host, or by in vitro culture. In addition metabolic changes occurring during culture have been examined to determine the nature of the "metabolic switches" which operate during transition from the poikilothermic fish-host to the homeothermic bird-host.

The basic body constituents protein, glycogen, lipid, RNA and DNA, were examined to determine any changes during maturation of the adult. The release of reduced end-products was measured in the presence and absence of glucose and oxygen, at 15°C and 40°C. Additional metabolic studies included measurement of the steady state metabolite levels within the worms, O_2 uptake by Warburg respirometry, analysis of key enzymes by spectrophotometric analysis and characterisation of some enzymes, and in particular of lactate dehydrogenase by isoelectric focusing.

The results indicate that L. intestinalis is essentially a homolactic fermentor, but that CO_2 - fixation coupled with a partial reversal of the TCA cycle may be important in the initial stages of maturation for production and incorporation of fatty acids and other lipids into eggs. Although the metabolism of the parasite is essentially anaerobic, the pathways appear to be very flexible, providing a degree of tolerance to the varying environmental conditions experienced during the host/host transition. Glycogen is the major carbohydrate store in the plerocercoid, and provides the maturing adult with an energy source which is independent of variation in the nutrient supply of the bird gut. It is speculated

that a combination of these factors is of evolutionary advantage to the parasite, allowing a wide host-range to develop, and thus increasing the chances of its survival.

ACKNOWLEDGEMENTS

My thanks are primarily due to my supervisor, Dr D. P. McManus, for his advice, encouragement and discussion throughout this study.

I would also like to thank my advisors, Dr D. J. Wright and Professor J. D. Smyth, who have made valuable comments on the work and manuscript. In addition I am grateful to Dr C. J. Davis for her discussions on histochemistry, and Mr R. G. Davies and Dr P. A. Henderson for their statistical advice.

I am indebted to the Medical Research Council for their financial support, and to the Bailiff of the Royal Parks and the staff of Maxwell's Boathouse for allowing me to collect fish from the Serpentine.

Finally, I would like to thank the technical staff in the Department of Zoology, in particular Mr R. Kraft and Mrs A. Merry for their support, Mrs A. Laird for typing the manuscript, and my parents for their encouragement.

CONTENTS

CHAPTER		PAGE NO.
1.	Introduction	1
2.	Materials and Methods	9
2.1.	Collection of infected fish	9
2.2.	Production of adults by *in vivo* culture	9
2.3.	Production of adults by *in vitro* culture	9
2.4.	Collection of eggs	10
2.5.	Determination of dry/fresh weight ratios and basic body constituents	11
2.5.1.	Dry/fresh weight ratios	11
2.5.2.	Protein	11
2.5.3.	Polysaccharides	11
2.5.4.	Lipids	12
2.5.5.	RNA and DNA	12
2.6.	Determination of metabolic gradients along the length of the plerocercoid worm	12
2.7.	Histochemical investigation of plerocercoids and adults produced *in vivo* and *in vitro*	13
2.7.1.	Neutral mucosubstances, acid mucosubstances, protein, DNA and RNA	13
2.7.2.	Lipids	13
2.7.3.	Vitellaria	13
2.8.	Respiration Studies	14
2.9.	Metabolic end-product release	14
2.10.	Glucose uptake by plerocercoids of *L. intestinalis*	15
2.10.1.	Incubation with C^{14} glucose	15
2.10.2.	Determination of glucose by spectrophotometric analysis	16
2.11.	Glucose uptake during *in vitro* culture	16
2.12.	Steady state metabolite levels	17

		PAGE NO.
2.13.	Enzyme analysis	18
2.13.1.	Pyruvate kinase (E. C. 2.7.1.40.)	18
2.13.2.	Phosphoenolpyruvate carboxykinase (E. C. 4.1.1.3.2.)	18
2.13.3.	Malic enzyme (E. C. 1.1.1.40.)	19
2.13.4.	Phosphofructokinase (E. C. 2.7.1.11.)	19
2.13.5.	Lactate dehydrogenase (E. C. 1.1.1.27.)	19
2.13.6.	Malate dehydrogenase (E. C. 1.1.1.37.)	20
2.13.7.	Succinate dehydrogenase (E. C. 1.3.99.1.)	20
2.13.8.	Protein determinations	20
2.14.	Isoelectric focusing of LDH from plerocercoid and in vitro adult of L. intestinalis	21
2.15.	Isoenzymes and possible "strain" differentiation in L. intestinalis plerocercoids	22
3.	Results	24
3.1.	The dry/fresh weight ratios and basic biochemical composition of plerocercoid, adults and eggs of L. intestinalis	24
3.2.	Glycogen depletion during in vitro culture	33
3.3.	Metabolic gradients along the length of the plerocercoid	35
3.4.	Histochemical investigations of the plerocercoid and adults produced in vitro and in vivo	35
3.5.	Respiration studies	39
3.6.	The release of metabolic end-products by L. intestinalis	41
3.7.	Glucose uptake by plerocercoids of L. intestinalis	49
3.7.1.	Incubation with C^{14} glucose	49
3.7.2.	Determination of glucose by spectrophotmetric analysis	49
3.8.	Glucose uptake during in vitro culture	49
3.9.	Steady state metabolite levels	52
3.10.	Key enzyme analysis	57
3.11.	Enzyme analysis	58

PAGE NO.

3.12.	Isoelectric focusing of LDH from plerocercoid and *in vitro* adult of *L. intestinalis*	62
3.13.	Isoenzymes and possible "strain" differentiation in *L. intestinalis* plerocercoids	63
4.	Discussion	67
4.1.	The dry/fresh weight ratios and basic body constituents	67
4.2.	Respiration studies	72
4.3.	The release of end-products of carbohydrate metabolism by plerocercoids and adults of *L. intestinalis*	77
4.4.	Glucose uptake by plerocercoids of *L. intestinalis*	82
4.5.	Glucose uptake during *in vitro* culture	83
4.6.	Steady-state metabolite levels	84
4.7.	Enzyme activity	88
4.8.	Isoenzymes in *L. intestinalis*	95
4.8.1.	Isoenzyme changes during *in vitro* culture	95
4.8.2.	Isoenzymes and possible "strain" differentiation in plerocercoids of *L. intestinalis*	96
4.9.	Final conclusions	98
	References	105
	Appendices	121

CHAPTER 1.

INTRODUCTION

In order to survive and be successful, a parasite must be metabolically and physically adapted for life within its host. It must also be adapted to the widely differing physiological conditions which ensue during the transition from one host to another. The pseudophyllidean cestode Ligula intestinalis, is an ideal model for the study of such adaptations.

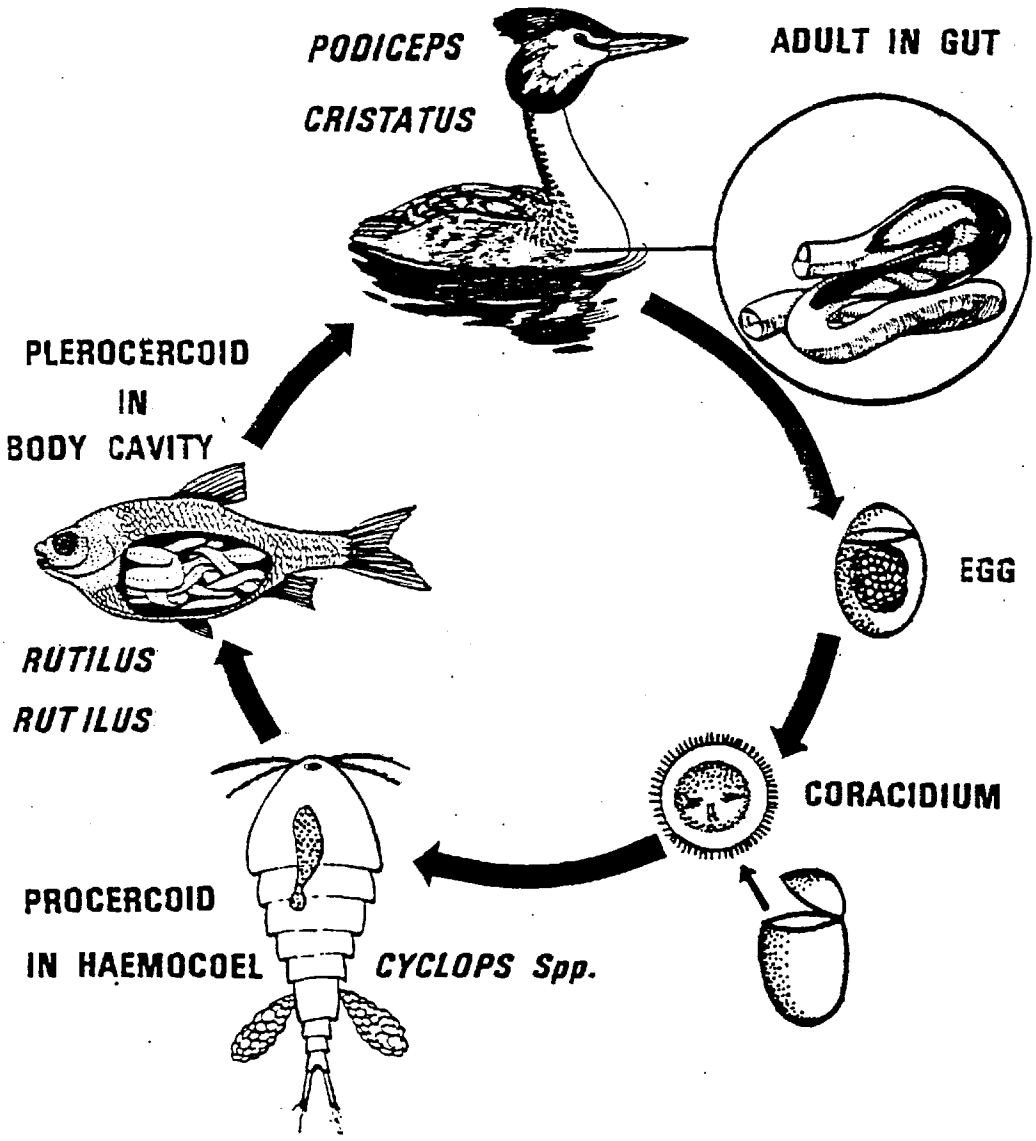
The life-cycle of L. intestinalis is shown in figure 1. The adult worm lives in the small intestine of piscivorous birds such as the Great Crested Grebe (Podiceps cristatus) (Cooper, 1918). The eggs are discharged with the faeces of the definitive host and develop in water (Schaunisland, 1885). The ciliated coracidium hatches, and upon ingestion by a suitable host, develops into the procercoid larva in the haemocoel of a cyclopoid copepod. The 2nd intermediate host is a cyprinid fish; there is a wide host-range with up to 70 species having been recorded (Cooper, 1918; Joyeux & Baer, 1936). When a suitable fish host eats an infected copepod, the larva penetrates the gut wall and develops into the plerocercoid larva in the body cavity. The genital primordia of the plerocercoid reach an advanced stage of development, and upon ingestion of the fish by a bird host, rapid maturation occurs. Egg-production commences 4 - 5 days post-infection.

The plerocercoid and adult of L. intestinalis are suitable for metabolic studies on account of their large size, and because they can be readily cultured in vitro (Smyth, 1947). The transition between the two stages is of particular interest because the physiological environments of the 2nd intermediate host and the definitive host are very different. The most marked difference is one of temperature; the fish host has a temperature varying between

Figure 1

The life-cycle of *Ligula intestinalis* (Cestoda: Pseudophyllidea).

THE LIFE CYCLE OF LIGULA INTESTINALIS



10 - 15°C, compared to 40°C in the bird. In addition, differences are likely to exist between the two hosts, with regards to pH, oxidation-reduction potential (Eh), pO_2, pCO_2 and availability of nutrients. The change between one host environment and the other also takes place rapidly. The parasite must therefore be adapted for rapid changes in its metabolism.

The development of the adult worm from the plerocercoid larva involves no obvious physical metamorphosis and it can be assumed therefore, that the changes are mainly of a metabolic nature. A study of the biochemical composition and metabolism of these two stages of the parasite has thus been carried out to determine how they are adapted to their respective environments. It was anticipated that this would reveal the nature of any 'metabolic switch' taking place. This would be a rapid change from larval to adult metabolism, which could be triggered by one of several of the changing environmental parameters mentioned above.

There are four main approaches available for the determination of metabolic pathways in the tissues of whole organisms. Firstly, the measurement of steady-stage metabolites will give an indication of the major pathways involved in metabolic flux. Secondly, the release of reduced organic end-products of metabolism may be estimated. This type of investigation is of particular value when studying helminth metabolism because it is well known that these worms produce a wide range of end-products as a result of carbohydrate breakdown (Barrett, 1976a). These end-products can vary both quantitatively and qualitatively, depending on the physiological environment experienced by the worms (Bryant, 1978; Reuter, 1967b; Reuter, 1967c). Thirdly, the measurement of optimal activity and characterisation of key enzymes will indicate whether a substrate, if present, will be metabolised and the rate at which the reaction will occur. Lastly the course of radiotracers through the pathways may be monitored, although this type of

approach has not been employed in the present study.

As summarised by Barrett (1967a), the anaerobic pathways of carbohydrate metabolism in helminths can be divided broadly into two categories. Firstly, parasites such as Schistosoma mansoni may be termed homolactic fermenters, where the glycolytic pathway is the main source of energy (Bueding, Peters & Waite, 1947), with lactate the major end-product released by the worm. Secondly, there are parasites such as Ascaris lumbricoides where, at the level of phosphoenolpyrurate (PEP) in the glycolytic pathway, CO_2 fixation occurs under the action of PEP carboxykinase (PEPCK) to oxaloacetate. A partial reversal of the TCA cycle then occurs, with pyruvate and succinate the major end-products. Oxaloacetate is reduced to malate by malate dehydrogenase (MDH), which after entering the mitochondrion is in equilibrium with pyruvate (formed by malic enzyme) and fumarate (formed by fumarase). The fumarate is then reduced to succinate by reductive succinate dehydrogenase (SDH) (i.e. fumarate reductase) and thence to propionate and volatile fatty acids. The pyruvate is converted to acetate and then also to fatty acids. (Saz, 1971). The metabolism of other parasite species need not necessarily fall solely into either category. Rather, it is probable that in vivo, most parasites operate a sequence of mixed pathways. This would be of obvious evolutionary advantage to an organism living in a variable environment as it would facilitate the operation of an efficient overall metabolic flux, whatever the conditions.

Although there is currently a great deal of information regarding the intermediary carbohydrate metabolism of helminths in general (Barrett, 1976a), little is known regarding the biochemistry and physiology of pseudophyllidean cestodes, and in particular L. intestinalis. McManus (1975b) showed that all enzymes of the tricarboxylic acid (TCA) cycle are present in subcellular fractions of the plerocercoid of L. intestinalis.

However, the low activity of aconitase and malate dehydrogenase (MDH) in the forward direction suggested that the TCA cycle was of questionable importance to the parasite *in vivo*. McManus (1975b) also suggested that, as in other helminths and intertidal bivalves (Saz, 1971), succinate production in *L. intestinalis* involves a partial reversal of the TCA cycle. McManus (1975a) studied pyruvate kinase (PK) from plerocercoids of *L. intestinalis*, and found the enzyme active, as was PEPCK. He speculated that co-ordinated changes in intracellular levels of adenosine triphosphate (ATP), malate, fructose-1, 6-diphosphate (FDP) and PEP control PK activity and hence carbohydrate breakdown, *in vivo*. Körting & Barrett (1977) studied the closely related pseudophyllid *Schistocephalus solidus* and found acetate and propionate were major end-products; these authors also suggested that CO_2 fixation and a partial reversal of the TCA cycle were important.

Although it would appear that many helminths including pseudophyllids have anaerobic metabolic pathways, all those so far studied consume oxygen (O_2) *in vitro*, (Barrett, 1976a); this includes the almost total anaerobe *S. mansoni* (Bueding, 1950; Smyth, 1966). It was thus considered appropriate to investigate the O_2 consumption of *L. intestinalis*. Davies & Walkey (1966) investigated the O_2 consumption of the plerocercoid of *S. solidus* and found that the Q_{10} values between 30°C and 40°C were higher than those between 20°C and 30°C. They postulated the existence of two enzyme systems, one operating at the temperature of the fish host, and one operating at the higher temperature of the bird host. This view was supported by the work of Sinha & Hopkins (1967) on *S. solidus*.

Another factor which must be considered, is that parasites are often sizeable organisms; for example, the plerocercoid of *L. intestinalis* frequently weighs 4g, and a worm weighing over

40g has been reported (Baron & Appleton, 1977). Different tissues in such parasites will undoubtedly be exposed to varying levels of pO_2, pCO_2, and metabolic intermediates and end-products. Therefore it would seem advantageous, and indeed necessary for a helminth to operate different metabolic systems in different tissue areas. This presents additional complications for the study of helminth biochemistry, and in particular the estimation of metabolic intermediates and enzyme activities since apparent anomalies may at first be found until the existence of metabolic gradients through the body of the parasite are considered.

The present study has concentrated on end-products previously known to be important in other helminths, i.e. lactate, pyruvate, malate, succinate, propionate and acetate (Saz, 1971). In addition, the intermediates of the pathways that are involved in their formation, including glycolysis, CO_2 fixation and the TCA cycle, have been investigated. The enzymes that are concerned with particular steps in these pathways have also been studied, especially those operating at key metabolic branchpoints.

Fundamental to the understanding of the metabolism of a parasite, is the knowledge of its basic biochemical make-up. The constituents which have thus been examined in the present study are protein, polysaccharides, lipid, RNA and DNA; the absolute levels have been measured, and a histochemical comparison of eggs, plerocercoid and adults, cultured both *in vitro* and *in vivo* made. From this work, it was hoped to confirm that the major energy store for *L. intestinalis* is polysaccharide, and in particular glycogen, as is the case with most helminths (Smyth, 1976).

An additional aim of the study was to compare biochemically adults produced both by *in vivo* and *in vitro* culture. The use of adults produced *in vitro* was considered preferable, since it enabled standardisation of culture procedures and removed possible

errors caused by host differences. It also minimised animal wastage and enabled the variation in culture parameters such as levels in pO_2, pCO_2, Eh and nutrients to be controlled. However, when any organism is removed from its natural environment, normal activities, including metabolism and development may be radically disrupted. It was essential, therefore, to ensure, as far as was possible, that there were no significant metabolic differences between adults produced by either culture method. In vitro culture has been successful for plerocercoids of both L. intestinalis (Smyth, 1947; 1949; 1959) and S. solidus (Smyth, 1954; 1959). It was found that the most important stimulus causing naturation of the genitalia, in both species, was the rise in temperature, assuming that suitable physiological conditions were maintained.

The plerocercoids of L. intestinalis and S. solidus have the function in the life-cycle of building up endogenous energy in the form of glycogen (Orr, 1967; Hopkins, 1950). As already mentioned, the genitalia develop to an advanced state so that maturation to the adult can occur very quickly after ingestion by the definitive bird host. In S. solidus the glycogen store is utilised rapidly by the adult worm as an energy supply for the developing eggs (Hopkins, 1950; 1952), and studies were carried out to determine if a similar situation occurs with L. intestinalis.

As discussed previously, the prime factor involved in triggering naturation of larva to adult, is the rise in temperature; associated with this is a rapid switch from glycogen synthesis to glycogen utilisation (Hopkins, 1950). It has been postulated by Davies & Walkey (1966) that in S. solidus separate enzyme systems are involved in this switch over, but the precise nature of these enzyme differences has not been investigated. Thus a section of the current study is concerned with improving our knowledge of this phenomenon.

The switch on and off of isoenzymes plays a critical rôle

in the development and differentiation of many organisms (Wilkinson, 1970), and it seemed probable that isoenzymes were also important in the transition from larva to adult in L. intestinalis. In Hymenolepis diminuta Carter & Fairbairn (1975) found that the number, and characteristics of PK isoenzymes changed during development. This, these workers argued, enabled the parasites' metabolism to suit the changing physiological environment during its life-cycle. Isoenzymes also vary throughout the individual tissues of many organisms. For example, mammalian LDH has been found to exist in several separate forms (Wieland & Pfleiderer, 1957). Subsequently it has been found that the isoenzymes have a spectrum of properties related to pO_2 and the concentration of pyrurate and lactate. Thus LDH has varying rôles in different parts of the body.

Isoenzymes can present a range of optima not only for pO_2 and concentration of substrate and end-product, but also for temperature and many other parameters (Wilkinson, 1970). In L. intestinalis, such a range of isoenzymes, including those of LDH, would be of particular value, especially during the transition from host to host. It would seem unlikely that a single form of an enzyme would have such a wide temperature range as 15°C (plerocercoid) and 40°C (adult) as two or more isoenzymes with optima at, or near 15°C and 40°C would function more efficiently.

LDH presents an ideal opportunity for the study of isoenzyme changes during the life-cycle of L. intestinalis since it is highly active in both larva and adult. Moreover, LDH systems in other organisms are well documented and present a useful comparison.

CHAPTER 2.

MATERIALS AND METHODS

2. 1. Collection of infected fish

Infected Roach (Rutilus rutilus) were collected from the Serpentine, Hyde Park, London by seine netting during the period October 1976 - April 1979. The fish were transported back to aerated tanks (4' x 4' x 1') and kept in a constant flow of fresh water. They were fed on trout pellets and maintained at 15°C until required for experimentation.

In all experiments, only worms in the range 1 - 4g fresh weight were used. This was considered necessary to eliminate any possible developmental variation between worms of widely differing sizes (Orr, 1967).

2. 2. Production of adults by in vivo culture

Plerocercoids were aseptically removed intact from the body cavity of freshly killed roach (Smyth, 1947), and washed several times in sterile Hanks' medium (see Appendix 1). Single worms were then fed to individual 6 - 8 week old chickens. After 5 days the chickens were killed by cervical dislocation, the intestinal tract removed and opened along its whole length. The adult worms obtained were washed in sterile Hanks' saline at 40°C, and used immediately in experiments.

2. 3. Production of adults by in vitro culture

According to Smyth (1947), all that is necessary to promote maturation of the plerocercoid of Ligula intestinalis is an increase in temperature to 40°C. However, a complex medium was used in these experiments to ensure successful maturation.

Plerocercoids were removed aseptically from the body cavity of Roach and cultured as described by Smyth (1947). The parasites were placed individually into sterile glass MD culture vessels containing 50 ml TC199 medium (DIFCO) (see Appendix 2), 20 ml Newborn Calf Serum (Flow laboratories) and 1 ml Penicillin-Streptomycin antibiotic, 5000 l.v./ml and 5000 mcg/ml respectively (Flow laboratories) and cultured at 40°C in a covered water bath shaking 50 times per minute. After every 24 hour period, 50 ml of culture medium was removed from the culture vessels and a further 30 ml of fresh TC199 medium and 20 ml Newborn Calf Serum added under sterile conditions. This was necessary in order to remove the worms' excreted metabolic end-products which if allowed to accumulate would be toxic to the parasites (Smyth, 1947). To study polysaccharide depletion during maturation, wormswere removed from culture after 1, 3 and 5 days and compared with worms cultured in a non-nutrient medium (Hanks' saline without glucose) for the same periods of time. In all other experiments, worms were considered adult after 5 days of culture; they were used only if egg-production had commenced and if the worms were still motile.

For experiments involving eggs, worms were cultured for 10 days, and eggs collected from day 4 onwards.

2.4. Collection of Eggs

Eggs were removed under sterile conditions from the culture vessels containing adult worms, and washed in sterile distilled water. After sedimentation (approximately 30 min.) the eggs were pipetted into fresh sterile distilled water and this washing procedure was repeated. The eggs were then pipetted into pre-weighed glass test tubes and dried at 103°C to constant weight.

2. 5. Determination of dry/fresh weight ratios and basic body constituent

The dry/fresh weight ratios and levels of protein, lipids, polysaccharides, DNA and RNA were determined and compared for whole, fresh plerocercoids, whole adults produced *in vitro* and *in vivo* and for eggs produced *in vitro* as described previously.

Regression lines were plotted for body constituents versus fresh weight of plerocercoids and adult *in vitro* over the whole range of worm sizes encountered, and the composition of body constituents in the dry weight determined for plerocercoids, adults and eggs.

2. 5. 1. Dry/fresh weight ratio

Worms were blotted on hard filter paper, weighed and then dried overnight to constant weight at 103°C. The dry/fresh weight ratio was thus determined.

2. 5. 2. Protein

Worms and eggs were dried to constant weight and 20 - 50 mg of each tissue ground in a pestle and mortar and then dissolved in 5 ml 1M NaOH at 90°C. The solution was then cooled to room temperature and aliquots taken and their protein contents determined by the Hartree (1972) modification of the method of Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumen was used as a standard and the optical densities were measured using a Cecil CE202 spectrophotometer. All subsequent spectrophotometric analyses were carried out using this instrument.

2. 5. 3. Polysaccharides

Worms and eggs were dried and homogenised as described

above. The tissues were dissolved in 30% KOH at 90°C and polysaccharides were precipitated and quantitatively determined by the method of Roe & Dailey (1966).

2.5.4. Lipids

Worms and eggs were blotted dry on hard filter paper, weighed and placed in 20 volumes of Chloroform : Methanol (2 : 1, v/v). The vessels containing the extracts were sealed and placed overnight in a refrigerator at 4°C for lipid extraction. After this period, 100 µl aliquots were pipetted into test tubes and the Chloroform : Methanol removed using a stream of nitrogen. 50 µl H_2SO_4 was then added to each tube and the extracts heated at 100°C for 10 minutes. Total lipids were determined using the colorimetric method of Zöllner & Kirsch (1962).

2.5.5. RNA and DNA

The RNA and DNA contents of the worms and eggs were determined using the methods of Munro & Fleck (1967) and Giles & Myers (1965) respectively.

2.6. Determination of metabolic gradients along the length of the plerocercoid worm

In order to determine possible metabolic gradients along the length of the plerocercoid, the following procedure was carried out. Worms were removed from the body cavity of roach and blotted dry on hard filter paper. They were then placed in sealed containers and frozen to kill and relax the worms. After 10 minutes they were removed, thawed and placed on 1 cm^2 graph paper. 1 cm serial sections were cut along the whole length of each worm, weighed and dried at 103°C to constant weight. The dry/fresh weight ratio and polysaccharide and protein content of

each portion was determined as described previously.

2.7. Histochemical investigations of plerocercoids and adults produced in vivo and in vitro

In order to compare the plerocercoid with the adult stage produced by in vivo and in vitro culture, and to compare the development of the worm under the two types of culture, histochemical examination was performed as described below.

2.7.1. Neutral mucosubstances, acid mucosubstances, protein, DNA and RNA

Worms were fixed in 10% buffered formalin (pH 7.0) and embedded in wax (melting point 56°C) and sectioned at 7 µm. The following staining techniques were used:

Neutral mucosubstances (including glycogen)
either with or without diastase digestion - Periodic acid - Schiff (Bancroft, 1978);

Acid mucosubstances - Alcian blue (Bancroft, 1978);

Protein - aqueous mercury bromophenol blue (Pearse, 1968);

DNA/RNA - Methyl green/pyronin (Bancroft, 1978).

2.7.2. Lipids

Worms were frozen onto a cryostat block and cut at -21°C. The sections were collected on microscope slides and fixed in Formal Saline. Lipids were stained using Oil red O/ Sudan black in isopropanol (Bancroft, 1978).

2.7.3. Vitellaria

Worms were fixed in 70% alcohol and wax-embedded and sectioned as described above. Vitellaria were stained using aqueous Fast Red Salt B (Bell & Smyth, 1958).

2.8. Respiration studies

The O_2 uptake of fresh plerocercoids and adults produced by in vitro culture was determined by Warburg respirometry following the methods described by Umbreit, Burris & Stauffer (1967). The respirometer used was the Braun V166 (B. Braun, Melsungen Appartebau). The O_2 electrode technique was also used in preliminary experiments but was found to damage the parasites. Therefore the Warburg respirometer was used throughout the latter experiments.

Worms were rinsed in Hanks' saline ± glucose, at 15°C, 25°C, 30°C and 40°C according to the experimental conditions employed. The worms were then gently blotted dry and approximately 100 mg portions cut from the central region and weighed accurately. The portions were then placed in the incubation well of the Warburg flasks (approximately 15 ml total volume) in 3 ml Hanks' saline ± glucose.

All flasks and manometers were temperature equilibrated for 15 minutes before the manometer taps were closed. The total respiration period was 1 hr for all experiments and readings were taken every 15 minutes.

2.9. Metabolic end-product release

Fresh plerocercoid worms and 5 day cultured adults produced in vitro were blotted on filter paper, weighed and rinsed several times in Hanks' saline without glucose. The whole worms were then incubated in Hanks' saline for 3 hours under all combinations of the following conditions:-

(a) ± glucose (5.5 mM)

(b) at 15°C or 40°C

(c) under air or 5% CO_2: 95% N_2

In all cases, controls of Hanks' saline ± glucose, but without worms were incubated concurrently under the environ-

mental conditions being investigated. The incubation media before and after the incubation period were analysed enzymatically for the following constituents by the methods indicated:-

Lactate (Gutman & Wahlefield, 1974a);
Malate (Gutman & Wahlefield, 1974b);
Pyruvate (Czok & Lamprecht, 1974);
Succinate (Williamson, 1974);
Acetate and Propionate (Boehringer, Mannheim, personal communication); (see Appendix 3);
Acetate (Methods of enzymatic food analysis, (Boehringer, Mannheim, 1976/7).

The worms were dried to constant weight and their glycogen contents determined as previously described.

2.10. Glucose uptake by plerocercoids of L. intestinalis

2.10.1. Incubation with C^{14} glucose

Plerocercoids were removed from roach and washed in sterile Hanks' saline without glucose. They were then placed in 5 mM glucose in Hanks' saline at 15°C for a pre-incubation period of 5 minutes.

25 µCi of C^{14}glucose (supplied by the Radiochemical Centre, Amersham) were placed in 100 ml 5mM glucose in Hanks' saline. 20 ml of this saline was then placed in experimental vials and worms added after their pre-incubation period. Incubation was at 15°C for 15 minutes, after which time the worms were removed, washed in three separate saline baths, blotted dry and weighed. They were then homogenised with acid-washed sand and extracted overnight in 20 volumes/g of 50% v/v Methanol. 500 µl Methanol was then removed and dried in scintillation vials, after which 5 ml scintillation fluid was added. This consisted of:

1.5g POPOP (1,4 - Di - 2 (5 - phenyloxazolyl) benzene)
8.25 g PPO (2,5 - Diphenyloxazole)
in 500 ml Triton X
+ 1000 ml Toluene

The vials were then counted on a Tracerlab Spectromatic and the counts per minute per whole worm could be calculated. 100 µl of the original incubation medium was also dried in a scintillation vial and the counts per minute measured as described above. Thus, the proportion of initial C^{14} glucose taken-up by the worm could be calculated, and thus the total glucose uptake could be determined.

2.10.2. Determination by spectrophotometric analysis

Fresh plerocercoids were blotted dry on filter-paper, weighed and rinsed several times in Hanks' saline without glucose. Whole worms were then incubated in Hanks' saline containing 5 mM glucose for 3 hours.

After the experimental period, the worms were removed, and the incubation media, together with the original saline (as a standard) were assayed for glucose by the method of Bergmeyer, Bernt, Schmidt & Stork (1974).

2.11. Glucose uptake during in vitro culture

A fresh plerocercoid was weighed aseptically and cultured in vitro to the egg-producing adult, as described previously. After each 24 hour period a 2 ml sample of the old medium was taken and compared with a sample, previously taken at the start of the 24 hour period. Each sample was treated with 10% trichloroacetic acid to precipitate any protein, and neutralised, the volume changes being carefully noted. The glucose content of each sample was determined as described above, and thus the glucose uptake could be determined. The loss in weight of cultured worms was

extrapolated from data on glycogen depletion (figure 7).

2.12. Steady-State metabolite levels

Fresh plerocercoids and 5 day in vitro cultured adult worms were blotted dry on filter paper, weighed and frozen rapidly between aluminium blocks which had been pre-cooled in liquid nitrogen (N_2) (Wollenberger, Ristau & Schoffa, 1960). The parasite tissue was then powdered in a porcelain mortar at -70°C and metabolites extracted by the addition of 4 - 5 volumes of frozen $HClO_4$ (6% w/v). The extraction took place in the mortar, and the continual mixing with the pestle thawed the mixture of $HClO_4$ and frozen parasite powder. The precipitated protein was removed by centrifugation and the extract was neutralised with 3 M $KHCO_3$. The metabolic intermediates were measured enzymatically using the following methods:-

Phosphoenolpyrurate and 2-phosphoglycerate (Czok & Lamprecht, 1974);

Glucose-6-phosphate and Fructose-6-phosphate (Lang & Michal, 1974);

Fructose-1, 6-diphosphate, dihydroxyacetone phosphate and Glyceraldehyde-3-phosphate (Michal & Beutler, 1974);

Oxaloacetate (Wahlefield, 1974);

Adenosine-5'-diphosphate and Adenosine-5'-monophosphate (Jaworek, Gruber & Bergmeyer, 1974);

Adenosine-5'-triphosphate (Lamprecht & Trautschold, 1974);

Glucose-1-phosphate (Bergmeyer & Michal, 1974);

Inorganic phosphate (Fiske & Subbarow, 1925).

Pyruvate, lactate, malate, succinate, acetate, propionate and glucose were estimated as described previously.

2.13. Enzyme analysis

Enzyme analysis was performed on extracts of plerocercoids and 5 day *in vitro* cultured adults.

Worms were washed several times in 0.01M Tris-HCl, pH 7.4 and homogenised in a pre-chilled mortar with acid-washed sand (40 - 100 mesh) and Tris-HCl. The homogenate was subjected to ultrasonic disintegration for 2 minutes using an M.S.E. sonicator in order to release any membrane-bound enzyme activity. The homogenate (10%, w/v) was centrifuged in a M.S.E. High speed 25 centrifuge at 12,000g for 30 min. and then recentrifuged at 109,000g for 30 min. The resulting supernatant was used in the enzyme assays.

Assays were performed at 15°C, 30°C and 40°C for the plerocercoid, but at 30°C and 40°C for the cultured adult. Assays were performed spectrophotometrically using the following methods:-

2.13.1. Pyruvate Kinase (E.C.2.7.1.40.)

The enzyme analysis was based on the method of Bücher & Pfleiderer (1955) but modified as described by McManus (1975a). The reaction mixture in a final volume of 3 ml contained: Tris-HCl, pH 7.0, 100mM; KCl, 75mM; $MnSO_4$ or $MgSO_4$, 4mM; ADP 5 mM; NADH, 0.1mM; PEP, 5mM; LDH, 3.6U. and enzyme supernatant. The reaction was started by the addition of PEP and activity determined from the decrease in extinction at 340 nm.

2.13.2. Phosphoenolpyrurate carboxykinase (PEPCK) (E.C.4.1.1.3.2.)

This assay was based on the method of Behm & Bryant (1975b). The reaction mixture, in a final volume of 3.0 ml contained:

Imidazole, pH 7.2, 100 mM; $MnCl_2$ or $MgCl_2$, 8mM; $NaHCO_3$, 7 mM; KCl, 40 mM; IDP or ADP, 1.7 mM; NADH, 0.07 mM; PEP, 3.3 mM; MDH, 1.8 U and enzyme supernatant.

The reaction was started by the addition of PEP and activity determined from the decrease in extinction at 340 nm.

2.13.3. Malic enzyme (E.C.1.1.1.40.)

The assay was based on the method of Ochoa (1968). The reaction mixture, in a final volume of 3 ml contained: Tris-HCl, pH 7.4, 100 mM; $MgCl_2$ or $MnCl_2$, 7 mM; L-Malate, 15 mM; NADP, 0.5 mM and enzyme supernatant. The reaction was started by the addition of the enzyme supernatant and the activity determined from the increase in extinction at 340 nm.

2.13.4. Phosphofructokinase (E.C.2.7.1.11.)

Based on the method of Racker (1947), the reaction mixture, in a final volume of 2.0 ml, contained: Triethanolamine, pH 8.0, 100 mM; EDTA, 1 mM; NADH, 0.1 mM; $MnCl_2$ or $MgCl_2$, 5 mM; Fructose-6-phosphate, 3 mM; ATP, 0.1 mM; glycerol-3-phosphate dehydrogenase, 0.5 U; Triosephosphate isomerase 5 U; Aldolase, 0.01 U, and enzyme supernatant. The enzyme activity in the presence and absence of ammonium sulphate, 25 mM was compared. The reaction was started with the addition of ATP and the activity determined from the decrease in extinction at 340 nm.

2.13.5. Lactate dehydrogenase (E.C.1.1.1.27.)

This assay was based on the method of Kornberg (1955). The reaction mixture contained phosphate buffer, pH 7.0, 94 mM; Sodium pyruvate, 7.6 mM; NADH, 0.2 mM; Enzyme sample and water to a total volume of 3.0 ml. The reaction was started by

the addition of the enzyme supernatant and the decrease in extinction measured at 340 nm.

2.13.6. Malate dehydrogenase (E. C. 1.1.1.37.)

This assay was based on the method of Shonk & Boxer (1964). The reaction mixture contained phosphate buffer, pH 7.0 94mM; oxaloacetate, 1 mM; NADH, 0.2mM; Enzyme sample and water to a total volume of 3.0 ml. The reaction was started by the addition of the enzyme supernatant and the decrease in extinction measured at 340nm.

2.13.7. Succinate dehydrogenase (E. C. 1.3.99.1.)

Two assay procedures were used to determine the activity of this enzyme. The first, measuring the oxidation of succinate to fumarate, in the presence of ferricyanide, is based on that of Slater & Bonner (1952). This assay mixture contained: phosphate buffer, pH 7.6, 100 mM; $K_3 Fe(CN)_6$, 1 mM; KCN, 1 mM; Succinate, 40 mM; enzyme supernatant and distilled water to 3.0 ml. The reaction was started by the addition of the enzyme supernatant and the activity determined from the decrease in extinction at 340 nm.

The other method, measuring the reduction of fumarate to succinate, is that of Prichard & Schofield (1968). This assay mixture contained: Phosphate buffer, pH 7.6, 42 mM; $CaCl_2$, 0.033 mM; $MgCl_2$, 1.7 mU; fumarate, 33 mM; NADH, 0.057 mM; enzyme supernatant and water to 3.0 ml. The reaction was started by the addition of fumarate and the activity determined from the decrease in extinction at 340 nm.

2.13.8. Protein determinations

The protein content of the enzyme supernatants was deter-

mined by the method of Hartree (1972). Enzyme activities are expressed both in terms of nmole product formed / min / mg protein and nmole product formed / min / mg fresh weight, relating to the worm weight in the original homogenate.

2.14. Isoelectric focusing of LDH from plerocercoid and in vitro adult of L. intestinalis.

Fresh plerocercoids were removed from roach, placed in aluminium foil vessels and frozen in liquid N_2. Additional plerocercoids were removed aseptically from the fish, and cultured in vitro for 5 days as described previously. These worms were also frozen in liquid N_2 until required for further study.

After thawing, the plerocercoid and adult worms were transferred to a chilled pestle and mortar and homogenised with acid-washed sand. The homogenates were then centrifuged at 100,000 gav for 15 min in an M.S.E. High speed 25 centrifuge at 2°C and the clear supernatants used immediately.

The isoelectric focusing experiments were performed using LKB ampholine polyacrylamide gel (PAG) plates, pH range 3.5 to 9.5, on the LKB 2117 multiphor with the LKB 2103 power supply (LKB - Produkter, Bromma, Sweden). 15 µl of the supernatants, containing approximately 250 µg protein (Hartree, 1972), were applied to the gel on supplied filter-paper applications (5 x 10 mm). These applicators were removed 1 hour after commencing the isofocusing run to prevent tailing of absorbed proteins. Haemoglobin controls were applied at intervals along the gel both anodally and cathodally. The cathode wick was soaked in 1M sodium hydroxide and the anode wick was soaked in 1M phosphoric acid. After positioning and trimming the electrode wicks, the PAG plates were transferred to the pre-chilled cooling plate of the multiphor which was kept between 2 and 4°C by circulating ice-cooled water from a Churchill Chiller

thermocirculator. The LKB power pack was set to deliver 1.4 KV, 30 W and maximum current and electrofocusing was complete after two hours.

After the run was complete, the pH gradient was measured across the gel using an Ingold membrane electrode (Pye Unicam), pH readings being taken ever 5 mm. The gel was then removed and placed in an incubation tray and stained for LDH after the method described by Harris & Hopkinson (1976). The reaction mixture contained: 50 ml 0.1M Tris-HCl buffer, pH 7.4; 1500 mg D, L-Lactate (Li); 16 mg NAD; 10 mg nitro blue tetrazolium (NBT); 2 mg phenazine methosulphate (PMS). The gel was stained at 25°C for two hours after which time it was photographed to record the isoenzyme pattern.

2.15. Isoenzymes and possible "strain" differentiation in L. intestinalis plerocercoids.

Fresh plerocercoids were removed from the body cavities of Bleak (Alburnus alburnus), Gudgeon (Gobio gobio) and Roach (Rutilus rutilus). The enzyme extracts and isoelectric focusing runs were performed as described above. Several enzymes were investigated and these included LDH, MDH, phosphoglucose isomerase (PGI) and phosphoglucomutase (PGM). The staining methods were based on the procedures described in Harris & Hopkinson (1976) and the reaction mixtures contained:

LDH - 50 ml 0.1M Tris-HCl buffer, pH 7.4;
1500 mg D, L-Lactate (Li);
16 mg NAD;
10 mg NBT;
2 mg PMS.

MDH - 50 ml 0.1M Tris-HCl buffer, pH 7.4;
900 mg L-Malate Na;
16 mg NAD;
10 mg 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT);
2 mg PMS;
500 mg agar.

PGI - 50 ml 0.1M Tris-HCl buffer, pH 8.0;
26 mg D-fructose-6- phosphate (2 Na);
8 mg NADP;
8 mg MTT;
3 mg PMS;
70 mg $MgCl_2$;
8 U glucose-6-phosphate dehydrogenase (G6PDH)
500 mg agar.

PGM - 50 ml 0.1M Tris-HCl buffer, pH 8.0;
40 mg D-G-1-P (2 Na) with 1% D-glucose-1, 6-diphosphate;
8 mg NADP;
8 mg MTT;
3 mg PMS;
70 mg $Mg\ Cl_2$;
8 U G6PDH;
500 mg agar.

The gel portions were stained for 2 hr (LDH), 1 hr (MDH), and 0.5 hr (PGI and PGM), after which time the plates were photographed.

CHAPTER 3

RESULTS

3.1. The dry/fresh weight ratios and basic biochemical composition of plerocercoids, adults and eggs of Ligula intestinalis

Table 1 shows that the dry/fresh weight ratio of the plerocercoid was significantly greater than that of the adult ($p<0.001$). There was no significant difference between the ratio for the adult produced either *in vivo* or *in vitro*. The water content of the adult was thus significantly greater than in the plerocercoid.

The relationship between the weights of protein, polysaccharide, lipid, RNA and DNA, and the fresh weights of plerocercoid and adult produced *in vitro* are shown in figures 2 - 6. In the case of the plerocercoid, the data for all the above components produced a good fit to a linear regression ($p<0.001$); in the range of all worm sizes examined, the weight of these constituents was a linear function of the weight of worm.

For the adult, produced *in vitro*, the weight of protein, polysaccharide and lipid was also a linear function of the worm weight ($p<0.001$, $0.057>p>0.001$, $p<0.01$, respectively) (figures 2 - 4). However, the data for RNA and DNA in the adult *in vitro* (figures 5 and 6) did not produce a significant fit to a linear regression ($p>0.05$), indicating that over the range of worm sizes sampled, the weights of RNA and DNA were not a linear function of worm weight.

Table 2 shows the biochemical composition of *L. intestinalis*. In the plerocercoid the major component was polysaccharide, but high levels of protein and lipid were also present. However, in the adults both *in vivo* and *in vitro*, the levels of polysaccharide were significantly less than the plerocercoid ($p<0.001$). There was also a significant difference between the polysaccharide content of adults

produced *in vitro* and *in vivo* ($0.05>p>0.01$). The levels of protein and lipid in the adults were significantly greater than in the plerocercoid ($p<0.001$) but there was no significant difference between the protein or lipid levels of adults produced *in vitro* or *in vivo*. In contrast, although the levels of RNA were significantly greater in the adult forms compared to the plerocercoid ($p<0.001$), there was also a significant difference between the adults *in vivo* and *in vitro* ($0.05>p>0.01$). The concentration of DNA was similar in larva and adults ($p>0.05$).

In the eggs, protein was the major component; the level was significantly greater than in the plerocercoid or adults ($p<0.001$). The concentration of polysaccharide was lower than that for protein and was significantly lower than polysaccharide levels in the plerocercoid or adults ($p<0.001$). The concentration of lipid in the eggs was also lower than protein, and was significantly lower than lipid values for plerocercoid ($0.05>p>0.01$) and adults ($p<0.01$). RNA levels were not significantly different from the plerocercoid ($p>0.05$) but were significantly less than both adult forms ($p<0.01$). The level of DNA in the eggs was significantly greater than the plerocercoid and the adults ($0.05>p>0.01$).

In the plerocercoid, the constituents studied formed 91% of the dry weight, whereas with the adult produced *in vitro* the value was 83%. However, with the eggs, the constituents studied formed only 61% of the dry weight.

TABLE 1.

Dry/fresh weight ratios for the plerocercoid and adults of *L. intestinalis*.

Parasite stage	Dry/Fresh weight ratio
Plerocercoid	0.318 ± 0.01 (26)
Adult, *in vitro*	0.231 ± 0.015 (7)
Adult, *in vivo*	0.246 ± 0.008 (5)

The results are means ± standard errors. The number of determinations is shown in parentheses.

Figure 2.

A linear regression plot relating the weight of protein to the weight of plerocercoid and *in vitro* adult of *L. intestinalis*.

Plerocercoid	-	$p<0.001$
Adult	-	$p<0.001$

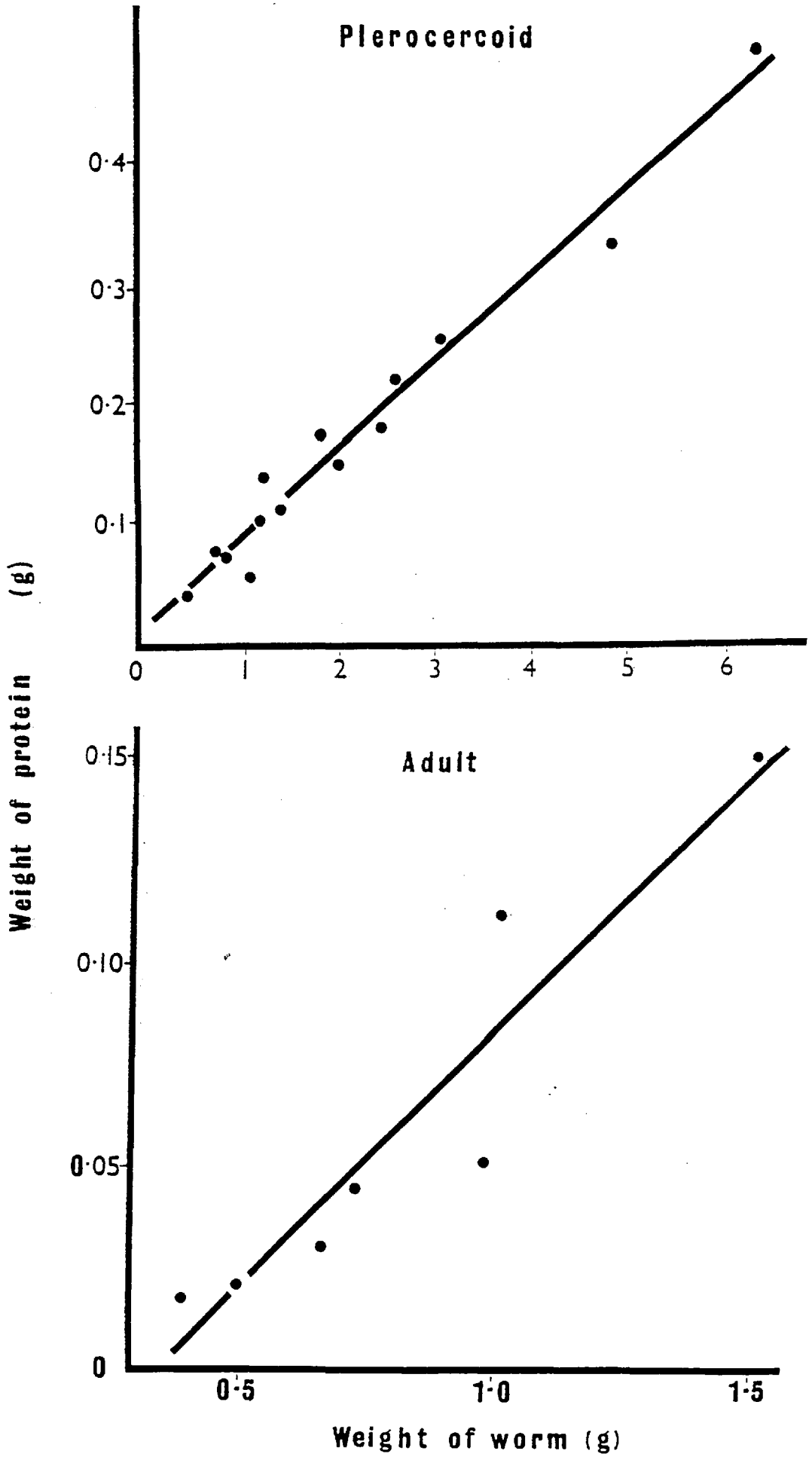

Plerocercoid
0·4
0·3
0·2
0·1
0
1
2
3
4
5
6
Weight of protein (g)
Adult
0·15
0·10
0·05
0
0·5
1·0
1·5
Weight of worm (g)

Figure 3.

A linear regression plot relating the weight of glycogen to the weight of plerocercoid and *in vitro* adult of *L. intestinalis*.

Plerocercoid	-	$p<0.001$
Adult	-	$0.05>p>0.01$

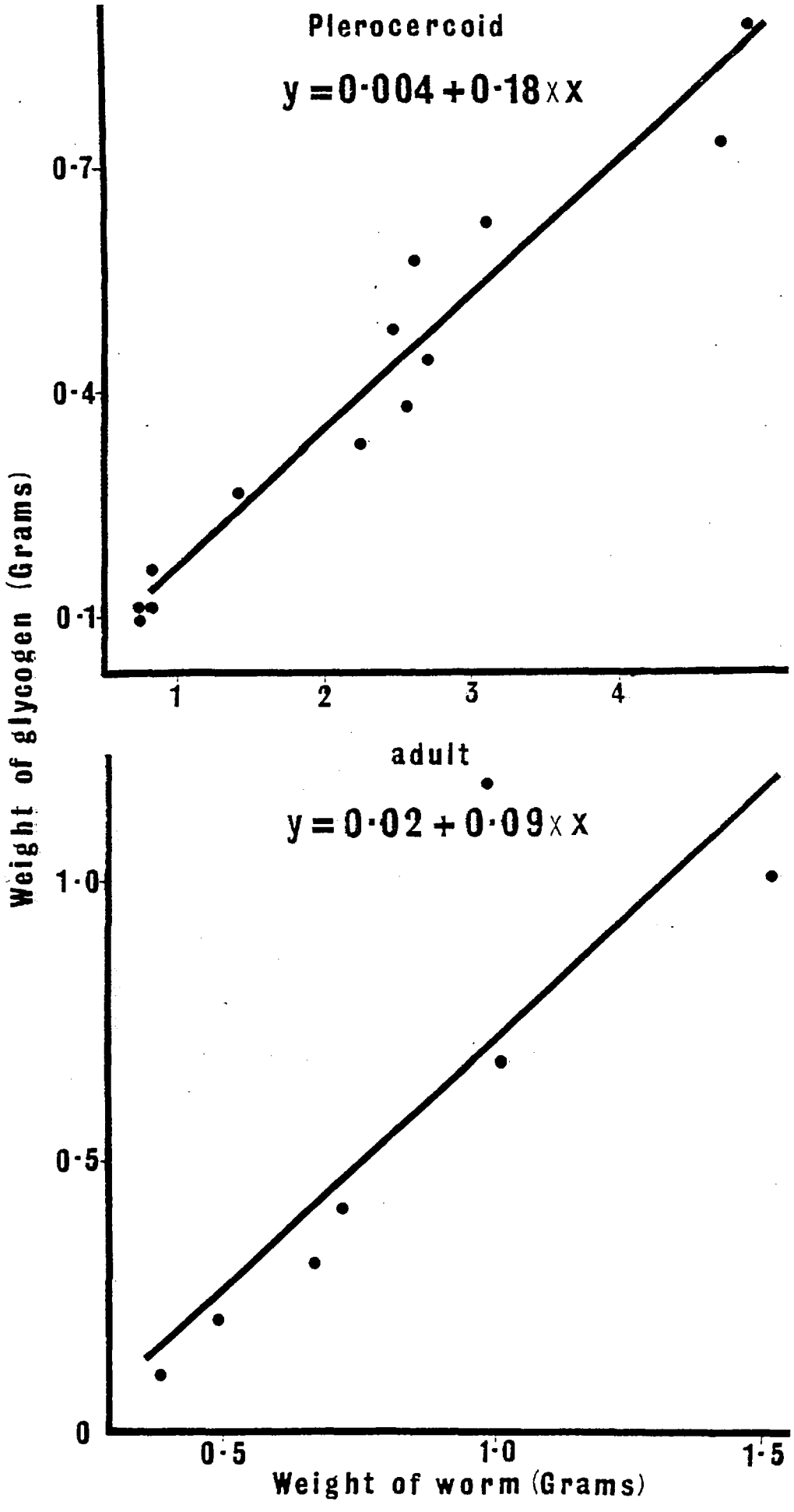

Plerocercoid
y = 0·004 + 0·18 x x
0·7
0·4
0·1
1
2
3
4
adult
y = 0·02 + 0·09 x x
1·0
0·5
0
0·5
1·0
1·5
Weight of glycogen (Grams)
Weight of worm (Grams)

Figure 4.

A linear regression plot relating the weight of lipid to the weight of plerocercoid and *in vitro* adult of *L. intestinalis*.

Plerocercoid	-	$p < 0.001$
Adult	-	$p < 0.001$

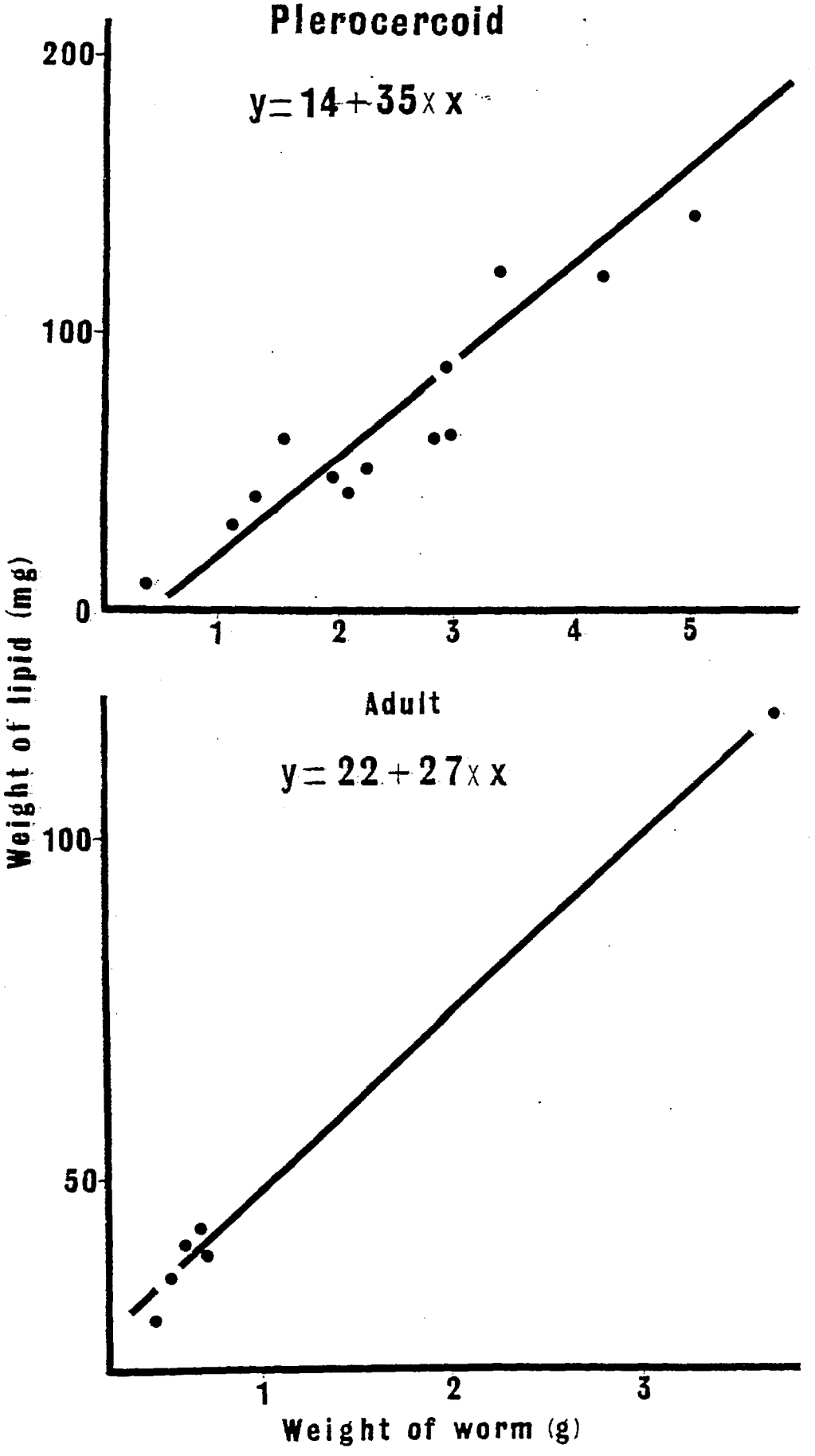

Plerocercoid
y=14+35x x
200
100
0
1
2
3
4
5
Adult
y=22+27x x
100
50
1
2
3
Weight of lipid (mg)
Weight of worm (g)

Figure 5.

A linear regression plot relating the weight of RNA to the weight of plerocercoid and *in vitro* adult of *L. intestinalis*.

Plerocercoid	-	$p<0.001$
Adult	-	$p>0.05$

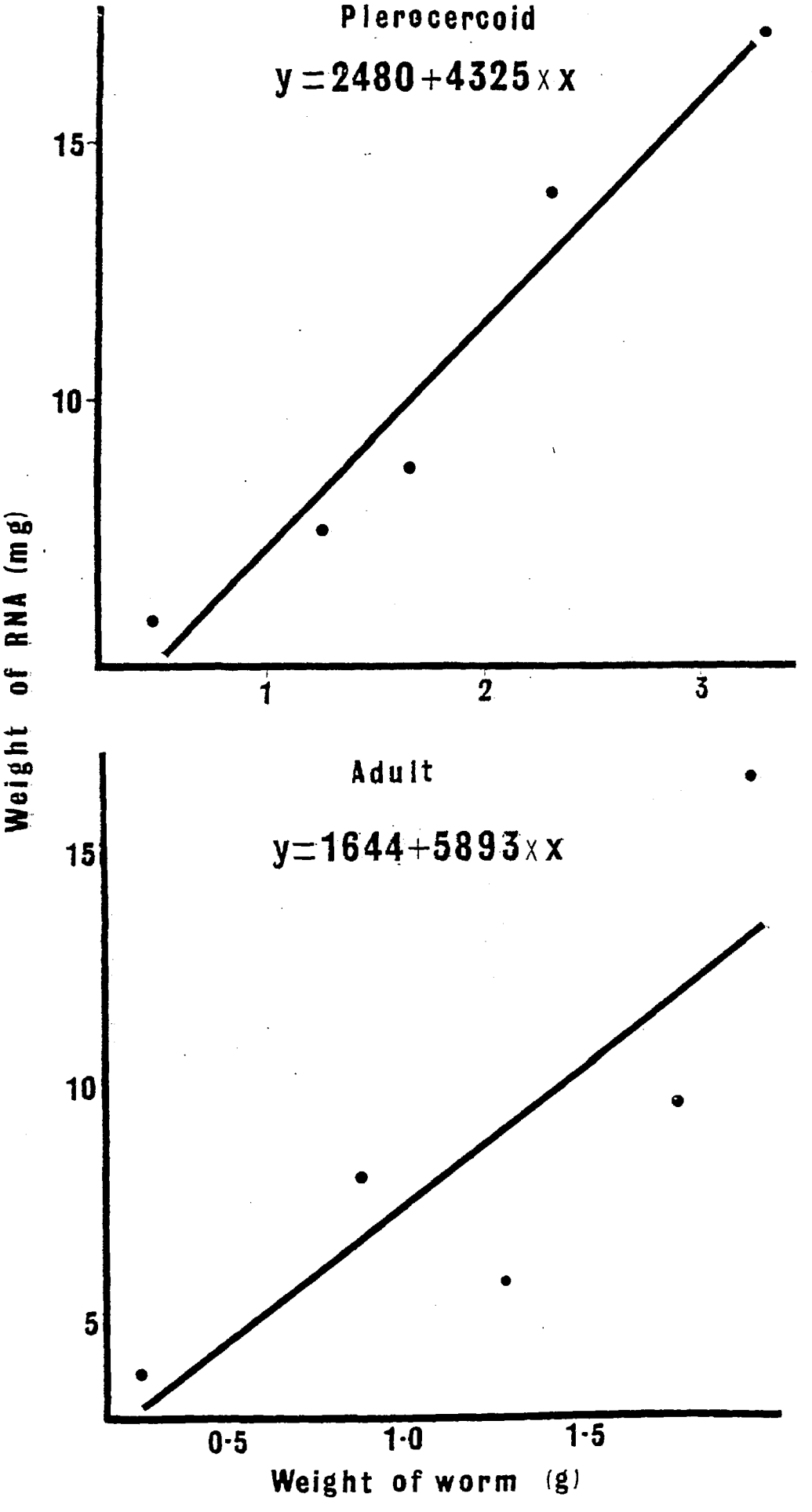

Plerocercoid
y=2480+4325xx
15
10
1
2
3
Adult
y=1644+5893xx
15
10
5
0·5
1·0
1·5
Weight of RNA (mg)
Weight of worm (g)

Figure 6.

A linear regression plot relating the weight of DNA to the weight of plerocercoid and *in vitro* adult of *L. intestinalis*.

Plerocercoid	-	$p<0.001$
Adult	-	$p>0.05$

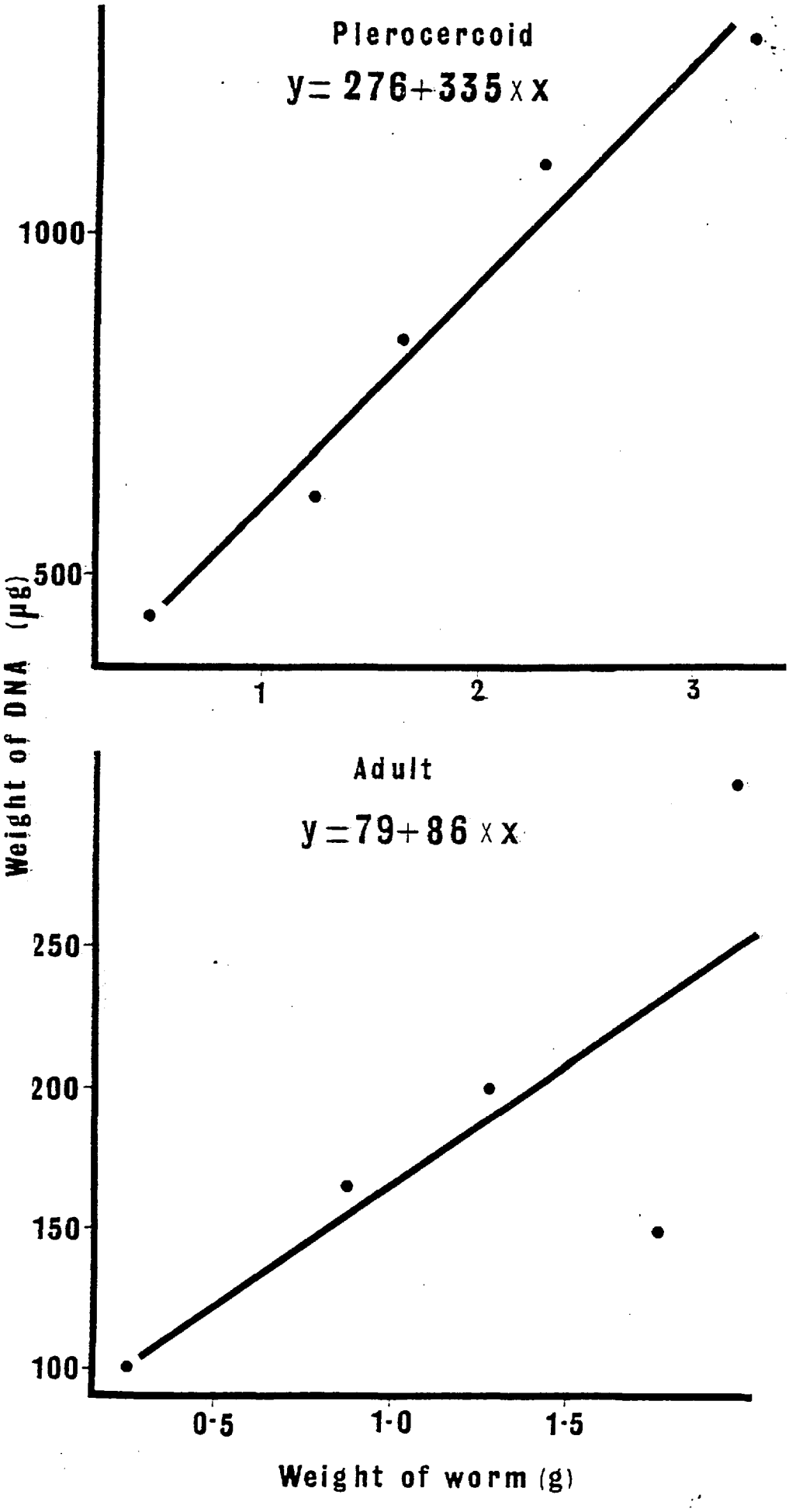

Plerocercoid
y= 276+335 x x
1000
500
Weight of DNA (µg)
1
2
3
Adult
y=79+86 x x
250
200
150
100
0·5
1·0
1·5
Weight of worm (g)

TABLE 2.

The biochemical composition of the plerocercoid, adults and eggs of *L. intestinalis*.

Parasite Stage	Protein	Polysaccharide	Lipid	RNA	DNA
Plerocercoid	256.0 ± 44.0 (14)	552.0 ± 72.0 (14)	93.5 ± 22.6 (14)	8.0 ± 3.0 (5)	0.7 ± 0.3 (5)
Adult, *in vivo*	353.6 ± 9.6 (5)	299.0 ± 15.8 (5)	231.8 ± 28.2 (5)	28.3 ± 2.3 (5)	0.7 ± 0.30 (5)
Adult, *in vitro*	333.4 ± 28.0 (7)	204.8 ± 33.0 (6)	257.0 ± 36.0 (6)	37.6 ± 8.2 (5)	0.8 ± 0.2 (5)
Eggs	513.4 ± 7.0 (5)	27.2 ± 1.8 (5)	62.9 ± 3.0 (5)	7.1 ± 0.1 (5)	1.6 ± 0.1 (5)

The results are expressed in μg/mg dry weight and are means ± standard errors. The number of determinations is shown in parentheses.

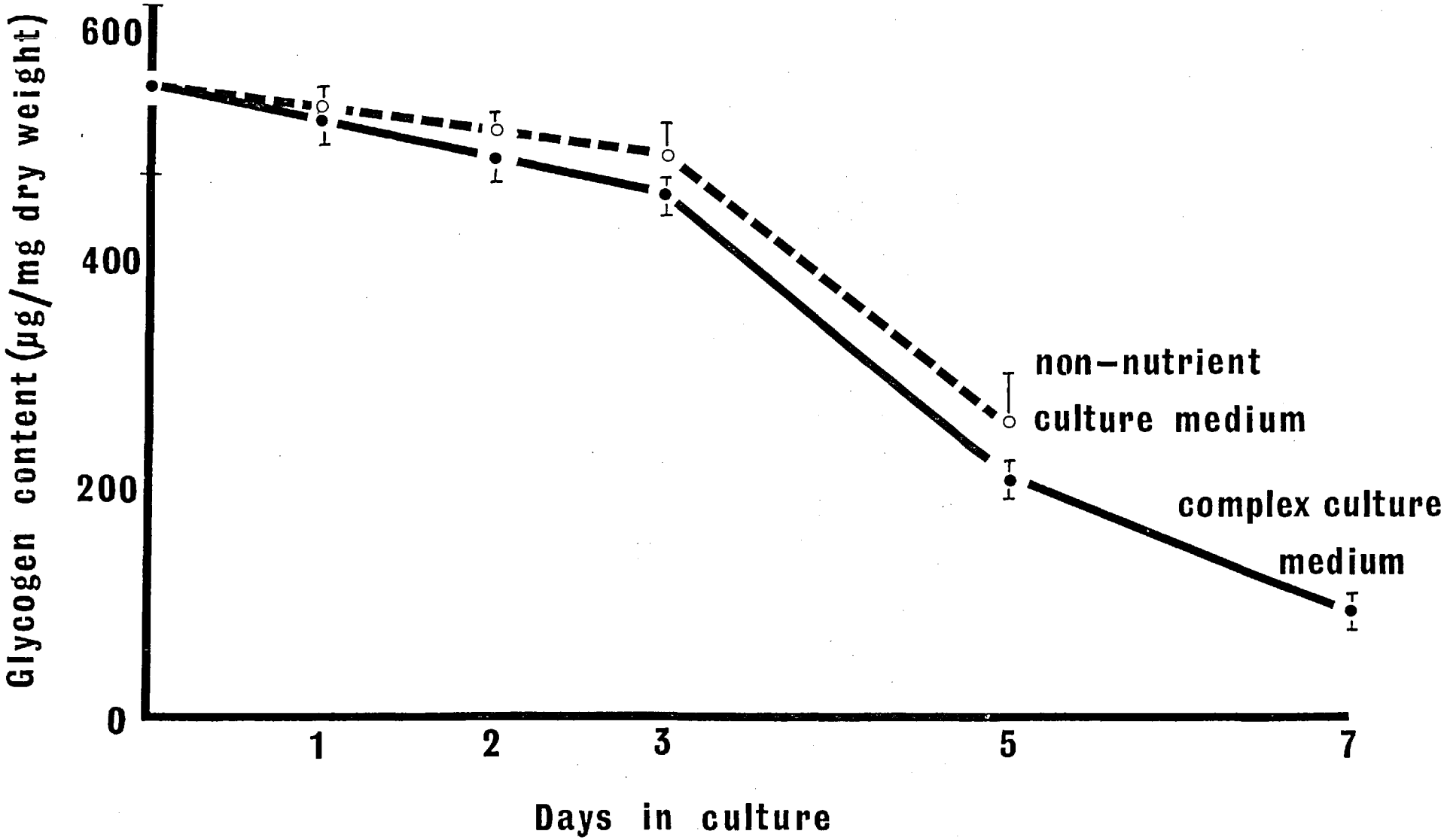
3. 2. Glycogen depletion during in vitro culture

Figure 7 shows the utilisation of glycogen by L. intestinalis during in vitro culture in non-nutrient and complex (nutrient-containing) media. Maximum utilisation commenced at day 3 in both types of culture media which coincided with the initiation of egg-production by the adult worms. Glycogen depletion proceeded at a slightly slower rate in non-nutrient media.

In the complex medium, more than 80% of endogenous glycogen had been consumed by day 7.

Figure 7.

Glycogen depletion in L. intestinalis during in vitro culture from the plerocercoid to the adult stage.


Glycogen content (µg/mg dry weight)
600
400
200
0
1
2
3
5
7
Days in culture
non-nutrient
culture medium
complex culture
medium

3. 3. Metabolic gradients along the length of the plerocercoid

Surprisingly, no significant differences were found in the protein and polysaccharide content along the length of the worm, indicating the absence of metabolic gradients (see Appendix 3).

3. 4. Histochemical investigations of the plerocercoid and adults produced in vitro and in vivo.

Table 3 shows the results of a histochemical study of L. intestinalis; figure 8 indicates the regions and tissues of the worms examined.

The major neutral mucosubstance component present in both the larva and adult was glycogen. In the plerocercoid, glycogen was found in all tissues examined, but there was significantly less glycogen in the adult produced in vitro and in vivo, in all tissues except tegumental cell bodies. This corroborates the biochemical analysis presented earlier. Diastase digestion indicated that a large proportion of the glycogen was lodged in the parenchyma cells, and it was in this tissue that the levels of glycogen dropped most markedly, when the plerocercoid was cultured to the adult.

The intensity of staining in adults produced in vivo and in vitro was identical for all other tissues and stained components, except for acid mucosubstances in the ovary. Acid mucosubstances were present in the plerocercoid only in the anucleate cytoplasm of the tegument. But in the adult they appeared also in the reproductive tissues.

The stain for protein was most intense in the muscles and reproductive tissues of the plerocercoid and adults. Lipids appeared in the muscle, parenchyma cells and reproductive tissues of the plerocercoid, and increased in intensity in the reproductive tissues of the adults.

DNA was found only in the tegumental cell bodies, muscle and reproductive tissues of the plerocercoid and adults, whereas RNA was found throughout the range of tissues examined. Little change occurred in the intensity of RNA staining in adults compared to plerocercoids. Unusually, the vitellaria stain for phenol groups was positive in all the reproductive tissues, but especially in the vitellaria itself, where staining intensity increased markedly in the adults compared to the plerocercoid.

Figure 8.

Transverse sections through the plerocercoid and adult produced in vitro of L. intestinalis to show the parasite tissues studied histochemically.

Key to abbreviations:-

Ta	-	anucleate tegumental cytoplasm
Tc	-	tegumental cell bodies
P	-	parenchyma
M	-	peripheral muscle
Or	-	rudimentary ovary
C	-	cirrus
E	-	mature eggs in uterus
Om	-	mature ovary
V	-	vitellaria
Te	-	testis

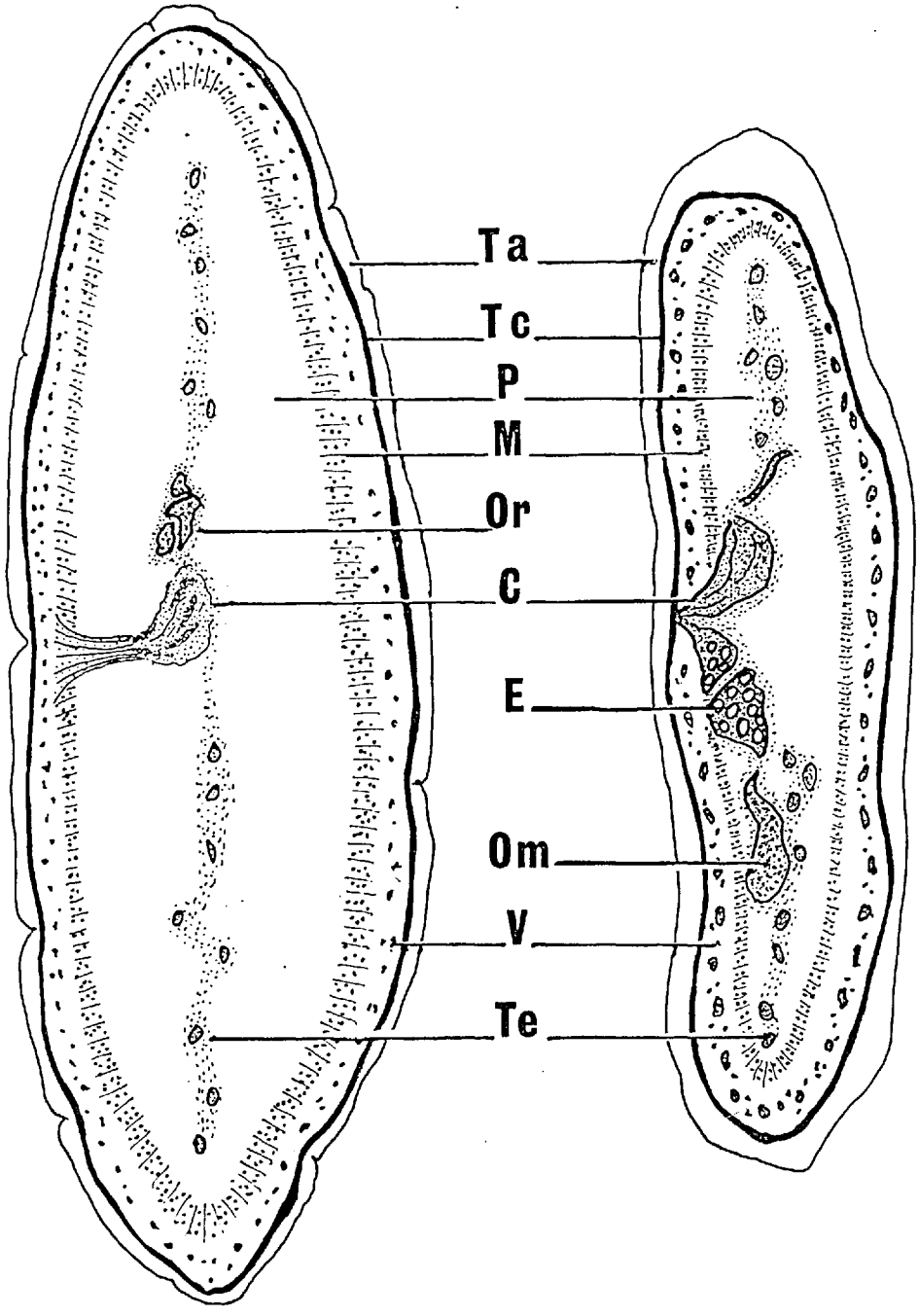

Ta
Tc
P
M
Or
C
E
Om
V
Te
plerocercoid
adult
0
3mm

TABLE 3.

A histochemical investigation of plerocercoid, adult produced *in vitro* and adult produced *in vivo* of *L. intestinalis*.

Key to symbols:

+++	intensely stained
++	moderately stained
+	slightly stained
-	no stain

PARASITE TISSUE

Parasite stage	Parasite Tissue: tegument cell bodies	tegument anucleate cytoplasm	peripheral muscle	parenchyma cells	vitellaria	testis	ovary	reproductive ducts (+ eggs)	Stained component
plerocercoid	+++	+++	+++	+++	+++	++	++	+++	Neutral muco-
adult, in vitro	+++	+++	++	+	++	++	++	++	substances
adult, in vivo	+++	+++	++	+	+	++	++	++	(including glycogen)
plerocercoid	+	+++	+	+	+	+	+	+	Neutral muco-
adult, in vitro	+	++	+	-	+	+	+	+	substances
adult, in vivo	+	++	+	-	+	+	+	+	(+ diastase digestion)
plerocercoid	-	+	-	-	-	-	-	-	
adult, in vitro	-	-	-	-	+	+	+	+	Acid mucosubstances
adult, in vivo	-	-	-	-	+	+	-	+	
plerocercoid	+	+	++	-	++	+	+	+	
adult, in vitro	-	-	+	-	+	++	+	++	Protein
adult, in vivo	-	-	+	-	+	++	+	++	
plerocercoid	-	-	+	-	+	+	+	+	
adult, in vitro	-	-	+	+	++	++	++	++	Lipid
adult, in vivo	-	-	+	+	++	++	++	++	
plerocercoid	+	-	+	-	+	+	+	+	
adult, in vitro	+	-	+	-	++	++	+	+	DNA
adult, in vivo	+	-	+	-	++	++	+	+	
plerocercoid	+++	++	+	++	+++	+++	+++	+++	
adult, in vitro	+	++	+	+	++	+++	+++	+++	RNA
adult, in vivo	+	++	+	+	++	++	+++	+++	
plerocercoid	-	-	-	-	++	+	+	+	
adult, in vitro	-	-	-	-	+++	+	+	+	Vitellaria (phenols)
adult, in vivo	-	-	-	-	+++	+	+	+	

3.5. Respiration Studies

Table 4 shows the result of respiration studies carried out using Warburg respirometry on L. intestinalis. O_2 uptake in the plerocercoid increased with temperature, both in the presence and absence of glucose. There was no significant difference between O_2 uptake at 15°C and 30°C in the presence or absence of glucose in the plerocercoid ($p>0.05$), but it was significantly greater in the presence of glucose at 25 C and 40 C ($p<0.001$).

With the plerocercoid, highest Q_{10} values were obtained at 15 - 25°C in the presence of glucose but at 25 - 30°C in its absence.

The O_2 uptake of the adult produced in vitro in the presence of glucose was significantly lower than that of the plerocercoid at 40°C ($p<0.001$); but there was no significant difference between the adult and plerocercoid in the absence of glucose ($p>0.05$). The adult worm took up a similar amount of O_2 in the presence or absence of glucose.

TABLE 4.

Oxygen uptake by plerocercoids and adults produced *in vitro* of *L. intestinalis*.

PLEROCERCOID

Temperature	+ glucose	Q_{10}	- glucose	Q_{10}
15°C	0.26 + 0.03		0.27 + 0.05	
		3.05		1.98
25°C	0.81 + 0.08		0.54 + 0.02	
		1.31		2.67
30°C	0.93 + 0.06		0.89 + 0.01	
		2.33		1.73
40°C	2.17 + 0.21		1.54 + 0.13	

ADULT (PRODUCED IN VITRO)

Temperature	+ glucose	- glucose
40°C	1.24 + 0.30	1.50 + 0.25

The results are expressed in µl O_2/mg fresh weight/hour as means ± standard errors for 5 determinations with individual worms.

3.6. The release of metabolic end-products by L. intestinalis

The release of end-products of carbohydrate metabolism by plerocercoids and adults produced *in vitro* of *L. intestinalis* are shown respectively in Tables 5 and 6.

At 15°C, the plerocercoid produced mainly lactate, while at 40°C, it produced significantly more succinate, propionate and acetate. The adults produced mainly lactate, but released large concentrations of malate.

A 4-way analysis of variance (ANOVA) was performed on the data in an attempt to determine the levels of significance of the three variables used in this experiment (temperature, $\pm O_2$ and $\pm$ glucose). The method was based on the technique discussed by Hartley (1962), and was run on the Imperial College computer using program Bios. Tables 7 and 8 show the variance ratios and levels of significance from the analysis of variance, with 1st and 2nd order interactions; Tables 9 and 10 summarise the conclusions that can be drawn from these results.

Lactate production was significantly greater under anaerobiosis in the plerocercoid at 15°C and 40°C; it was also greater in the absence of glucose at 15°C. Temperature, O_2 and glucose interacted significantly so that the greatest lactate production occurred in the plerocercoid at 15°C in the absence of glucose and O_2. In contrast, production of pyruvate, malate and succinate were all increased under aerobic conditions, in the presence of glucose, and at 40°C. The production of these three end-products was affected significantly by interactions between aerobic incubation, glucose and incubation temperature. That is, aerobic conditions significantly enhanced the effects of (a) glucose and (b) the temperature of 40°C, upon the release of pyruvate, malate and succinate.

Acetate and propionate were released in greater amounts

at 40°C, compared to 15°C in the plerocercoid, but the presence or absence of glucose or O_2 had no significant effect upon their production. However, propionate production at 40°C was significantly enhanced under aerobic conditions. In the plerocercoid all end-products studied except lactate, showed increased production at 40°C.

The adult worm produced significantly more lactate than the 'activated' plerocercoid, i.e. the larva incubated at 40°C. Similar to the plerocercoid, the adult produced more lactate in the absence of glucose. However, in contrast to the 'activated' plerocercoid, the release of lactate by the adult was unaffected by the presence or absence of glucose. The release of pyruvate, malate and propionate all increased in the presence of O_2, and again there was greater production in the adult compared to the 'activated' plerocercoid. The 2nd order interactions showed that aerobic conditions had a significantly greater effect upon metabolite release in the adult than in the 'activated' plerocercoid. Malate release was increased in the absence of glucose, both in the 'activated' plerocercoid and the adult, as was lactate, but pyruvate and propionate showed greater release in the presence of glucose, in the adult. Release of succinate was increased in both 'activated' plerocercoid and adult under aerobiosis, but the increase was greater in the 'activated' plerocercoid. Acetate, like succinate, was released in greater quantities in the 'activated' plerocercoid, than in the adult, and the release of these two end-products was unaffected by the presence or absence of glucose.

TABLE 5.

The carbohydrate metabolism of plerocercoids of *L. intestinalis*.

	15°C				40°C			
	+ glucose		- glucose		+ glucose		- glucose	
	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic
Lactate	47.7+5.4	17.1+2.3	47.0+1.8	120.1+13.1	32.8+1.0	58.2+2.0	8.2+2.0	70.0+15.0
Succinate	10.5+2.5	13.6+1.7	0	12.0+ 4.0	66.2+5.9	30.2+4.5	42.6+5.5	33.7+ 3.6
Acetate	5.5+0.7	15,0+2.5	0	5.3+ 0.7	74.5+9.8	63.8+8.4	55.8+5.4	68.2+ 8.0
Propionate	1.5+0.5	19.3+5.6	2.0+0.5	11.0+ 2.5	69.7+2.9	15.8+1.1	44.1+7.0	36.5+14.0
Pyruvate	0.7+0.03	0.5+0.03	2.6+0.3	3.6+ 0.4	12.9+1.4	2.5+0.2	7.0+0.2	1.1+ 0.2
Malate	8.3+0.7	3.9+0.2	0	14.6+ 0.9	29.3+0.9	1.1+0.2	1.8+0.2	1.0+ 0.1

The results are expressed as n moles/10 mg dry weight/hour and are means ± standard errors of 5 experiments with individual worms.

TABLE 6.

The carbohydrate metabolism of *in vitro* adults of *L. intestinalis* at 40°C.

	+ glucose		- glucose	
	aerobic	anaerobic	aerobic	anaerobic
Lactate	214.2 ± 11.1	228.4 ± 41.0	405.2 ± 40.0	321.8 ± 42.5
Succinate	10.4 ± 1.8	11.5 ± 1.0	8.6 ± 1.3	12.9 ± 6.1
Acetate	25.9 ± 4.0	60.2 ± 18.0	60.0 ± 4.7	33.1 ± 10.0
Propionate	105.5 ± 11.0	26.8 ± 5.2	82.0 ± 2.0	27.4 ± 8.1
Pyruvate	3.1 ± 0.3	3.6 ± 1.0	11.4 ± 1.3	11.1 ± 1.6
Malate	73.1 ± 5.6	24.6 ± 3.9	129.8 ± 23.0	78.6 ± 17.3

Results are expressed as means ± standard errors in nmoles/10 mg dry weight/hr. and are for 5 experiments with individual worms.

TABLE 7.

The variance ratios and probability values for the ANOVA on pleroceroid end-product release at 15°C and 40°C.

	Lactate	Succinate	Acetate	Propionate	Pyruvate	Malate
1st Order Interactions:						
O_2	15.7 ***	7.75 **	0.9 NS	3.2 NS	36.1 ***	137.2 ***
Glucose	48.3 ***	8.8 **	3.2 NS	0.4 NS	26.9 ***	269.7 ***
Temperature	7.3 *	158.0 ***	204.5 ***	42.1 ***	36.7 ***	16.7 ***
2nd Order Interactions:						
O_2 x Glucose	33.4 ***	10.8 **	1.35 NS	3.5 NS	50.6 ***	870.4 ***
O_2 x Temperature	0.3 NS	29.5 ***	0.6 NS	17.7 ***	49.0 ***	631.0 ***
Glucose x Temperature	10.6 **	0.52 NS	0.008 NS	0.03 NS	128.4 ***	373.0 ***

Levels of Significance:

***	$p<0.001$
**	$0.01>p>0.001$
*	$0.05>p>0.01$
NS	$p>0.05$ Not significant

TABLE 8.

The variance ratios and probability values for the ANOVA of plerocercoid and *in vitro* adult end-product release at 40°C.

	Lactate	Succinate	Acetate	Propionate	Pyruvate	Malate
1st Order Interactions:						
Oxygen	0.26 NS	10.5 **	0.09 NS	61.5 ***	7.3 *	27.7 ***
Glucose	20.9 ***	2.8 NS	0.06 NS	1.13 NS	0.04 NS	11.5 **
Stage	199.3 ***	112.5 ***	8.1 **	9.6 **	10.4 **	125.0 ***
2nd Order Interactions:						
O_2 x Glucose	1.5 NS	6.1 *	1.74 NS	8.0 **	12.2 **	1.0 NS
O_2 x Stage	2.1 NS	17.0 ***	0.03 NS	8.4 **	15.1 ***	8.5 **
Glucose x Stage	12.5 **	2.6 NS	0.56 NS	0.44 NS	72.9 ***	32.2 ***

Levels of Significance:

***	$p<0.001$
**	$0.01>p>70.001$
*	$0.05>p>0.01$
NS	$p>0.05$ - not significant

TABLE 9.

Overall conclusions drawn from the ANOVA of end-product release by plerocercoid at 15°C and 40°C.

	1st Order Interactions	2nd Order Interactions
Lactate	Greater production under anaerobiosis Greater production in absence of glucose Greater production at 15°C	Greater production under anaerobiosis without glucose Greater production with glucose present at 15°C
Pyruvate	Greater production under aerobiosis Greater production in presence of glucose Greater production at 40°C	Greater production under aerobiosis in the presence of glucose Greater production under aerobiosis at 40°C Greater production + glucose at 40°C
Malate	Greater production under aerobiosis Greater production in presence of glucose Greater production at 40°C	Greater production under aerobiosis in the presence of glucose Greater production under aerobiosis at 40°C Greater production + glucose at 40°C
Succinate	Greater production under aerobiosis Greater production in presence of glucose Greater production at 40°C	Greater production under aerobiosis in the presence of glucose Greater production under aerobiosis at 40°C
Acetate	Greater production at 40°C	-
Propionate	Greater production at 40°C	Greater production under aerobiosis at 40°C

TABLE 10.

Overall conclusions drawn from ANOVA of end-product release by plerocercoid and adult, *in vitro* at 40°C.

	1st Order Interactions	2nd Order Interactions
Lactate	Greater production in absence of glucose Greater production in adult	Greater production in adult in absence of glucose
Pyruvate	Greater production under aerobiosis Greater production in adult	Greater production under aerobiosis in the presence of glucose Greater production under aerobiosis in adult Greater production in absence of glucose in adult
Malate	Greater production under aerobiosis Greater production in absence of glucose Greater production in adult	Greater production under aerobiosis in adult Greater production in absence of glucose in adult
Succinate	Greater production under aerobiosis Greater production in plerocercoid	Greater production under aerobiosis in plerocercoid
Acetate	Greater production in plerocercoid	-
Propionate	Greater production under aerobiosis Greater production in adult	Greater production under aerobiosis in adult Greater production under aerobiosis in presence of glucose

3.7. Glucose uptake by plerocercoids of L. intestinalis

3.7.1. Incubation with C^{14} glucose

Results are shown in Table 11. Plerocercoids of L. intestinalis can be clearly seen to have taken up glucose under aerobic conditions at 15°C.

3.7.2. Determination by spectrophotometric analysis

This method failed to produce satisfactory results. The data indicated wide-ranging values for glucose uptake under identical conditions, and on some occasions, even glucose release appeared to be occurring (see Appendix 5). This could perhaps have been due to water absorption by the worm, thus increasing the concentration in the medium.

3.8. Glucose uptake during in vitro culture

The results are presented in Table 12. During in vitro culture, the uptake of glucose decreased to a minimum during 48 - 72 hours after the start of culture. The uptake then increased to a maximum of 62 nmoles glucose/10 mg dry weight/hr during the period 96 - 120 hours after the start of culture.

TABLE 11.

Uptake of glucose by plerocercoids of *L. intestinalis* at 15°C, determined by incubation with C^{14} glucose.

Fresh weight of worm (g)	Glucose uptake (n moles/10 mg dry weight/hr)
1.27	97.0
3.70	51.5
1.02	79.2
3.25	85.5
	$\bar{x}$ = 78.3 ± 8.7 (S.E.M.)

TABLE 12.

The uptake of glucose by *L. intestinalis* during *in vitro* culture from the plerocercoid to the adult

Period after the start of *in vitro* culture (hr)	Glucose uptake n moles/10 mg dry weight/hr
0 - 24	38.5
24 - 48	23.1
48 - 72	11.5
72 - 96	53.9
96 - 120	52.0

3.9. Steady-state metabolite levels

The steady-state metabolite levels of L. intestinalis are shown in Table 13.

Lactate was by far the major tissue metabolite in the plerocercoid, apart from glucose, and its level significantly increased ($p < 0.001$) in the adult. All other metabolites were found in the tissues at moderately high levels, compared with lactate, except for fructose-6-phosphate (F6P) in the plerocercoid and glucose-1-phosphate (G1P) in the adult. This suggests a rapid flux of these intermediates in the glycolytic pathway. It is perhaps understandable that G1P should have occurred at low levels in the adult, since by the time the worm has reached 5 days culture in vitro, a significant proportion of its endogenous glycogen store has been utilised (see Figure 7). Thus, the rate of conversion of G1P to glucose-6-phosphate (G6P) may exceed the rate of formation of G1P from glycogen, because of depletion of the latter.

It is interesting to compare the levels of intermediates in the plerocercoid with those in the adult. All the glycolytic intermediates decreased in the adult, except G6P, F6P and lactate, while the TCA cycle intermediates, with the exception of malate, decreased only slightly or increased.

The content of adenosine nucleotides and inorganic phosphate is shown in Table 14. The adenylate charge is a measure of the energy available to the parasite in terms of nucleotide phosphate (Atkinson, 1971) and is also shown in Table 14. The high value of 0.86 in the plerocercoid indicated that as much ATP is being regenerated as utilised. In the 'activated' plerocercoid and adult, the values are 0.775 and 0.72 respectively; this indicated that more ATP was being utilised than regenerated. This net energy consumption was probably a result of synthetic processes, including egg-production, occurring during maturation.

Also the maturing adult will require energy for maintaining its position in the intestine of the definitive host.

TABLE 13.

Steady state metabolite levels

in *L. intestinalis*

Steady state metabolite	Parasite stage	
	Plerocercoid	Adult
Citrate	127 ± 3	167 ± 17
Succinate	716 ± 139	675 ± 93
Malate	187 ± 26	80 ± 16
Oxaloacetate (OAA)	50 ± 5	84 ± 10
Glucose-1-phosphate (GIP)	50 ± 6	18 ± 2
Glucose-6-phosphate (G6P)	583 ± 78	586 ± 127
Fructose-6-phosphate (F6P)	17 ± 4	51 ± 5
Fructose-1,6-diphosphate (FDP)	800 ± 96	150 ± 16
Di-hydroxyacetone phosphate (DHAP)	150 ± 8	56 ± 4
Glyceraldehyde phosphate (GAP)	593 ± 19	99 ± 4
2-phosphoglycerate (2PG)	314 ± 58	94 ± 13
Phosphoenolpyruvate (PEP)	514 ± 23	100 ± 11
Pyruvate	116 ± 13	76 ± 8
Lactate	1181 ± 155	1536 ± 251
Acetate	80 ± 4	395 ± 75
Propionate	680 ± 43	1496 ± 113
Glucose	7270 ± 1013	18515 ± 989

The values for steady state metabolite levels are given in nmoles/g fresh weight and are means ± standard errors for 3 determinations.

TABLE 14.

The content of Adenosine nucleotides and inorganic phosphate in L. intestinalis.

Content *(nmoles/g fresh weight)

	ATP	ADP	AMP	Pi	Adenylate Charge	Phosphorylation Potential	$\frac{(ATP)}{(ADP)^2}\frac{(AMP)}{}$
Fresh Plerocercoids	1672 ± 90	261 ± 12	152 ± 9	10,685 ± 707	0.86	599	3.73
Activated Plerocercoids	1536 ± 130	629 ± 28	215 ± 7	8,452 ± 338	0.775	288	0.83
Adult, produced in vitro	1157 ± 100	493 ± 43	300 ± 9	6,644 ± 817	0.72	353	1.43

* Mean ± SEM for 3 determinations

TABLE 15.

The mass-action ratios and apparent equilibrium constants for glycolytic enzymes of *L. intestinalis*.

Enzyme	** Apparent equilibrium constant(K′)	Mass action ratio		Mass action ratio K′	
		Plerocercoid	Adult	Plerocercoid	Adult
Hexokinase	5500	0.001	0.013	18×10^{-8} *	236×10^{-8} *
Phosphoglucose isomerase	0.47	0.029	0.087	0.06	0.185
Phosphofructokinase	1200	7.69	1.24	0.006 *	0.001 *
Aldolase	68 - 90	111.2	37.5	1.6	0.54
Triosephosphate isomerase	0.036	3.95	1.78	109	49.4
Enolase	1.4	1.64	1.07	1.17	0.76
Pyruvate kinase	15,000	1.35	1.78	9×10^{-5} *	11.8×10^{-5} *
Phosphoglucomutase	0.055	11.66	31.7	212	575.8

* possible rate limiting enzymes

** from Barrett & Beis (1973b) and Newsholme & Start (1973)

3.10. Key enzyme analysis

The mass-action ratios (M.A.R.) have been calculated from metabolite levels presented in Table 15 and metabolite concentrations (Appendix 7), are shown in Table 15. In addition, Table 15 shows apparent equilibrium constants (K′) and $\frac{M.A.R.}{K'}$ for several key glycolytic and TCA cycle enzymes of L. intestinalis. The ratio, $\frac{M.A.R.}{K'}$ indicates which enzymes are out of equilibrium, and which may be rate-limiting (Behm & Bryant, 1975a). In both plerocercoid and adult, hexokinase (HK), phosphofructokinase (PFK) and PK were non-equilibrium, and thus may play critical rôles in the energy metabolism of L. intestinalis.

3.11. Enzyme analysis

Optimal enzyme activities, obtained by using saturating substrate concentrations, are presented in Tables 16 and 17.

In the plerocercoid, the activity of succinate dehydrogenase (SDM) proceeded at a faster rate in the reverse direction (reduction of fumarate) than in the forward direction (oxidation of succinate) ($0.05>p>0.01$). However, at 30°C and 40°C, the forward direction was preferred ($p<0.001$). The activity of SDH in the adult at 30°C and 40°C was significantly less, in both the forward and reverse directions, than in the plerocercoid ($p<0.001$). The SDH activities were similar in the forward and reverse directions in the adult form.

The activities of LDH and MDH were significantly greater in the adult, than in the plerocercoid ($p<0.001$), the activity of the latter enzyme, greatly exceeding that of the former.

The activity of PK significantly increased between 15°C and 30°C in the plerocercoid ($0.05>p>0.01$) but there was no difference between the activity of the enzyme at 30°C and 40°C ($p>0.05$). In the adult, the activity was less than half that of the plerocercoid at both 30°C and 40°C ($p<0.001$).

In the plerocercoid, the activity of PEPCK was activated to a greater extent by Mn^+ than Mg^+ ($p<0.001$ at 30°C) and by IDP rather than ADP. The enzyme was most active at 30°C in the plerocercoid, but at 40°C in the adult. The activity of the adult enzyme at 40°C was significantly higher than the plerocercoid enzyme under identical conditions, ($p<0.001$).

PFK was activated by ammonium sulphate, and to a greater extent by Mn^+ than Mg^+ ($0.01>p>0.001$). Again, in the plerocercoid, activity was highest at 30°C, whereas in the adult the activity was greater at 40°C. The activity of the adult enzyme was also significantly lower than the plerocercoid under the same

conditions ($p<0.001$).

Malic enzyme (ME) activity was very low in both plerocercoid and adult, and was activated to a greater extent by Mn^{+} than Mg^{+}. The activity increased with temperature; maximum activity was at 40°C in plerocercoid and adult. The activity of the adult enzyme was lower than that of the plerocercoid under identical conditions.

TABLE 16.

The activities of some dehydrogenase enzymes of *L. intestinalis*.

Enzyme	Plerocercoid		Adult	
	n moles/mg protein/min	n moles/mg wet weight/min	n moles/mg protein/min	n moles/mg wet weight/min
Succinate dehydrogenase (Suc. → Fum.)				
15°C	2.66 ± 0.56	0.07 ± 0.014	-	-
30°C	* 130.9	-	5.66 ± 1.03	0.18 ± 0.03
40°C	130.0 ± 8.2	5.29 ± 0.34	5.95 ± 0.47	0.20 ± 0.02
Succinate dehydrogenase (Fum. → Suc.)				
15°C	3.08 ± 0.16	0.08 ± 0.004	-	-
30°C	* 27.1	-	3.38 ± 0.25	0.11 ± 0.07
40°C	26.1 ± 1.4	1.07 ± 0.05	6.5 ± 0.57	0.21 ± 0.07
Lactate dehydrogenase				
30°C	113.2 ± 6.6	-	443.8 ± 13.0	-
Malate dehydrogenase				
30°C	2845.4 ± 102.3	-	4386.7 ± 159.0	-

The results are expressed as n moles product formed/mg protein/min and as n moles product formed/mg wet weight/min, and are means ± standard errors for determinations on 4 individual worms.

* Results from McManus, (1975b)

TABLE 17.

The effect of certain modulators on the activity of some key enzymes of L. intestinalis.

Enzyme	Plerocercoid		Adult	
	nmoles/mg protein/min	nmoles/mg wet weight/min	nmoles/mg protein/min	nmoles/mg wet weight/min
Pyruvate kinase				
15°C	54.0 ± 4.7	1.38 ± 0.12	-	-
30°C	65.25 ± 6.55	2.67 ± 0.26	23.2 ± 1.64	0.767 ± 0.06
40°C	65.4 ± 7.2	2.66 ± 0.3	26.6 ± 3.7	0.88 ± 0.12
Phosphoenol pyruvate carboxykinase				
15°C, Mn^{2+}, IDP	19.7 ± 2.7	0.5 ± 0.07	-	-
30°C, Mn^{2+}, ADP	16.7 ± 0.35	0.86 ± 0.02	-	-
30°C, Mg^{2+}, ADP	5.34 ± 0.54	0.27 ± 0.03	-	-
30°C, Mn^{2+}, IDP	70.3 ± 4.5	3.66 ± 0.23	43.1 ± 6.6	1.45 ± 0.25
30°C, Mn^{2+}, -	7.86 ± 0.45	0.39 ± 0.02	-	-
40°C, Mn^{2+}, IDP	52.4 ± 5.6	2.59 ± 0.28	151.3 ± 12.2	5.05 ± 0.50
Phospho fructokinase				
15°C, Mn^{2+}, A.S.	42.1 ± 3.2	1.06 ± 0.08	-	-
30°C, Mn^{2+}, -	56.7 ± 4.8	1.84 ± 0.15	-	-
30°C, Mg^{2+}, -	34.5 ± 3.6	1.11 ± 0.11	-	-
30°C, Mn^{2+}, A.S.	157.0 ± 11.8	5.1 ± 0.38	27.8 ± 4.0	0.94 ± 0.16
40°C, Mn^{2+}, A.S.	119.0 ± 6.6	3.85 ± 0.21	67.5 ± 16.6	2.25 ± 0.56
Malic enzyme				
15°C, Mg^{2+}	1.63 ± 0.20	0.041 ± 0.005		
15°C, Mn^{2+}	3.88 ± 0.41	0.099 ± 0.009		
30°C, Mn^{2+}	3.66 ± 0.42	0.15 ± 0.02	1.75 ± 0.22	0.057 ± 0.005
40°C, Mn^{2+}	4.43 ± 0.68	0.17 ± 0.03	2.83 ± 0.14	0.097 ± 0.007

The results are expressed as nmoles product formed/mg protein/min and as nmoles product formed/mg wet weight/min, and are means ± standard errors for 4 determinations on individual worms.

3.12. Isoelectric focusing of LDH from plerocercoid and in vitro adult of L. intestinalis

The results are shown in Plate 1. The zymogram clearly shows that in the in vitro adult, additional bands were present. Although these bands appeared as a single block in the photograph, 2 separate bands were visible in the gel itself.

3.13. Isoenzymes and possible "strain" differentiation in L. intestinalis plerocercoids

The results are shown in Plates 2 and 3. PGM was the only enzyme to exhibit any isoenzyme profile differences between different host-species (Plate 3). The PGM patterns from gudgeon and roach origin were identical and 11 major isoenzymes were detectable. In contrast, the PGM of L. intestinalis from bleak indicated a qualitatively different pattern and in two individuals an extra isoenzyme was apparent.

Plate 1.

Photograph of electrophoretic pattern obtained with larval and adult, *in vitro* *L. intestinalis* for lactate dehydrogenase.

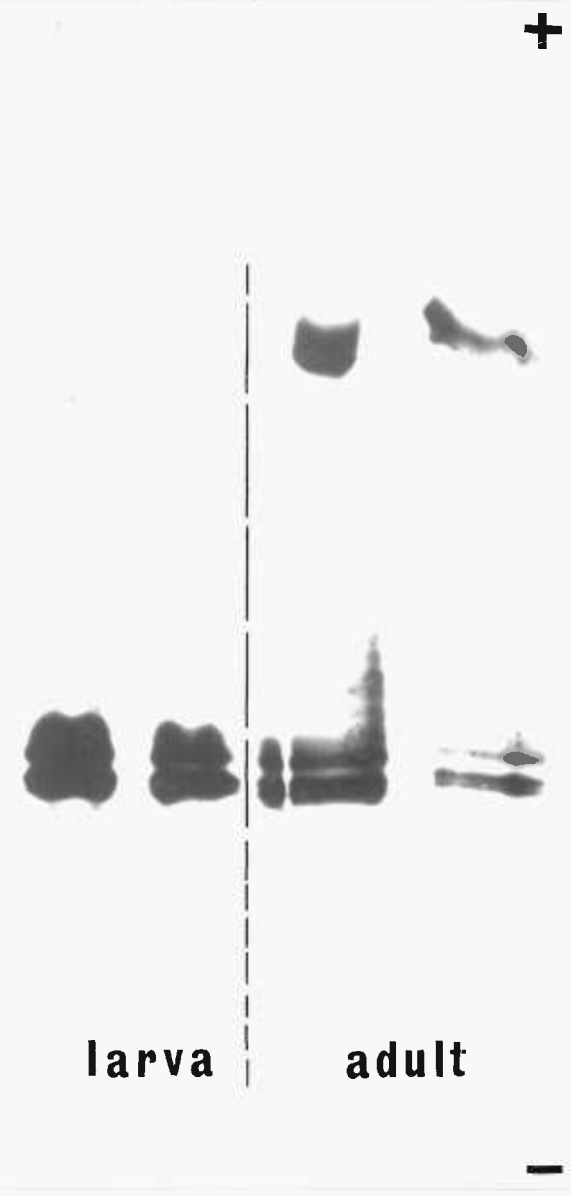
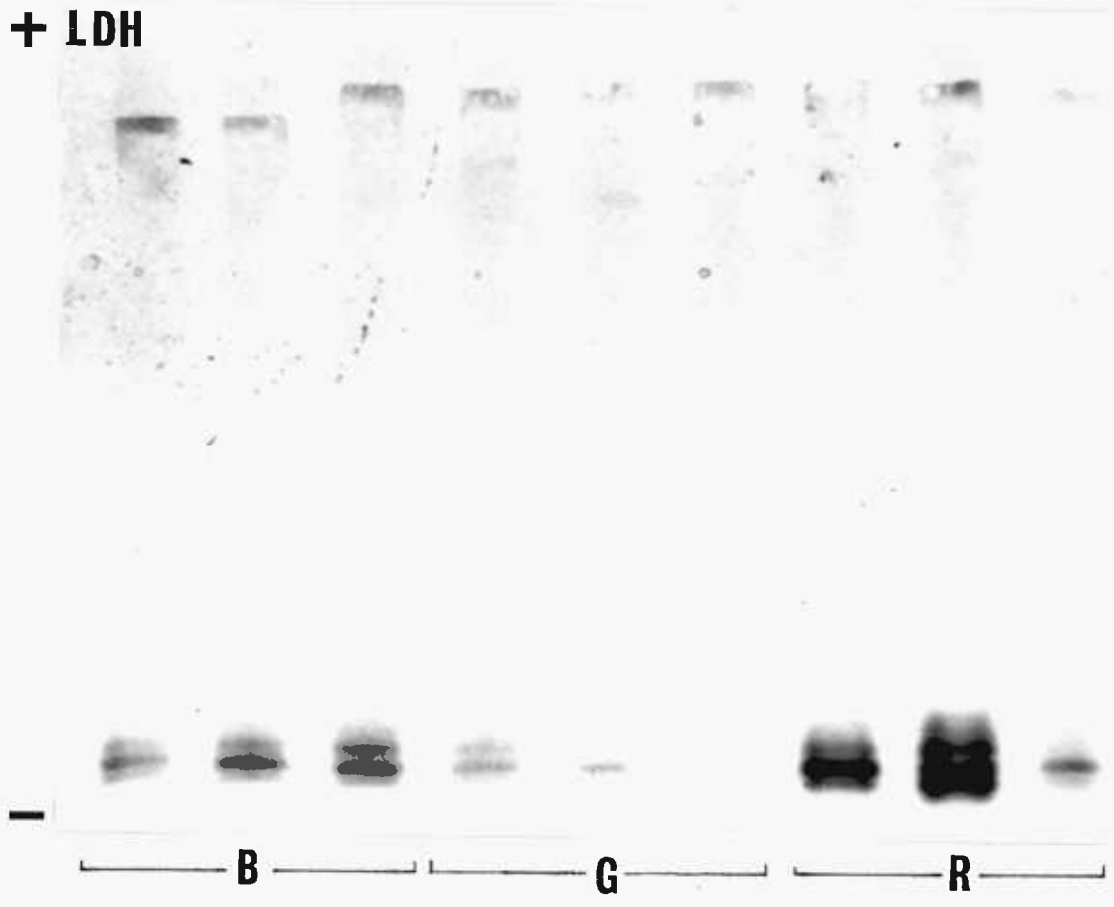

+
larva
adult
-

Plate 2.

Photographs of electrophoretic patterns obtained with extracts of plerocercoids of L. intestinalis for lactate dehydrogenase (LDH) and malate dehydrogenase (MDH).

Plerocercoids were removed from the following fish-hosts:

R -	Roach (Rutilus rutilus)	- from Serpentine
G -	Gudgeon (Gobio gobio)	- from Wierwood reservoir, Sussex
B -	Bleak (Alburnus alburnus)	- from Langley Gravel Pit, Slough.


+ LDH
−
B
G
R

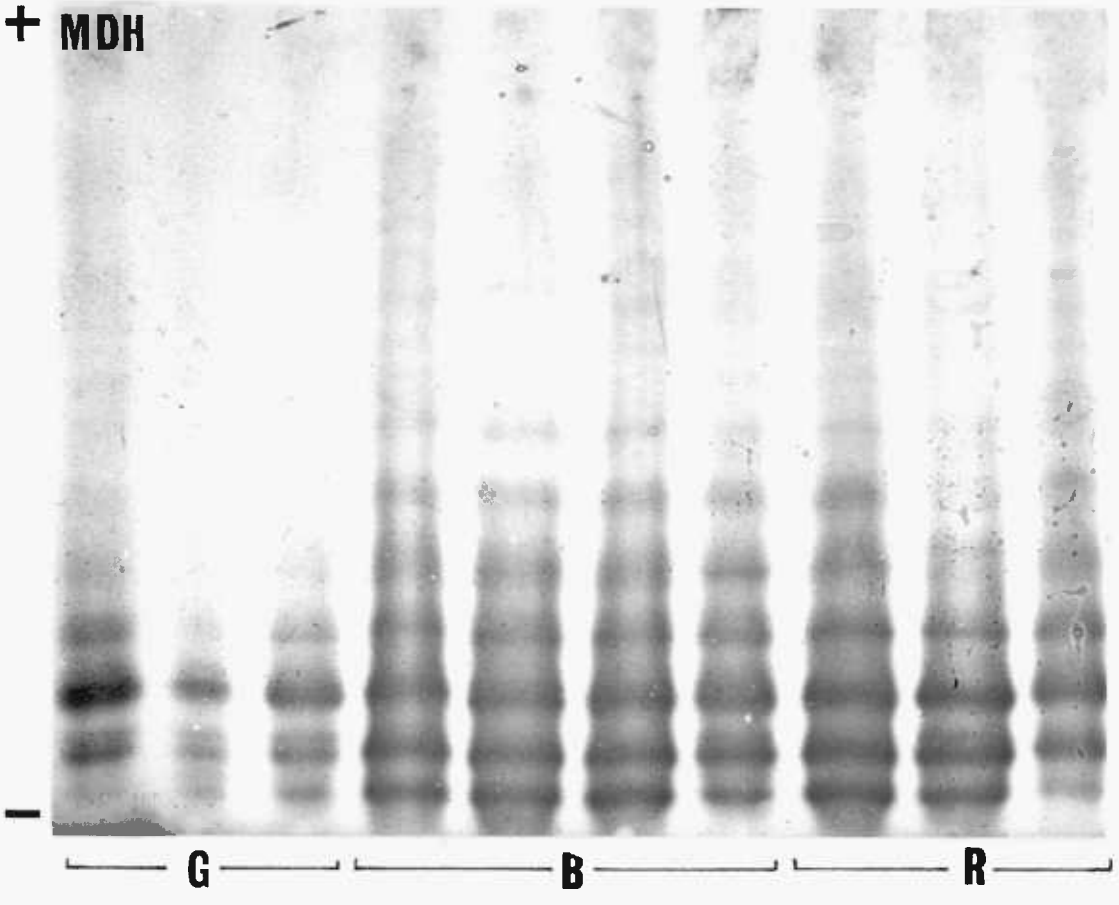

+ MDH
−
G
B
R

Plate 3.

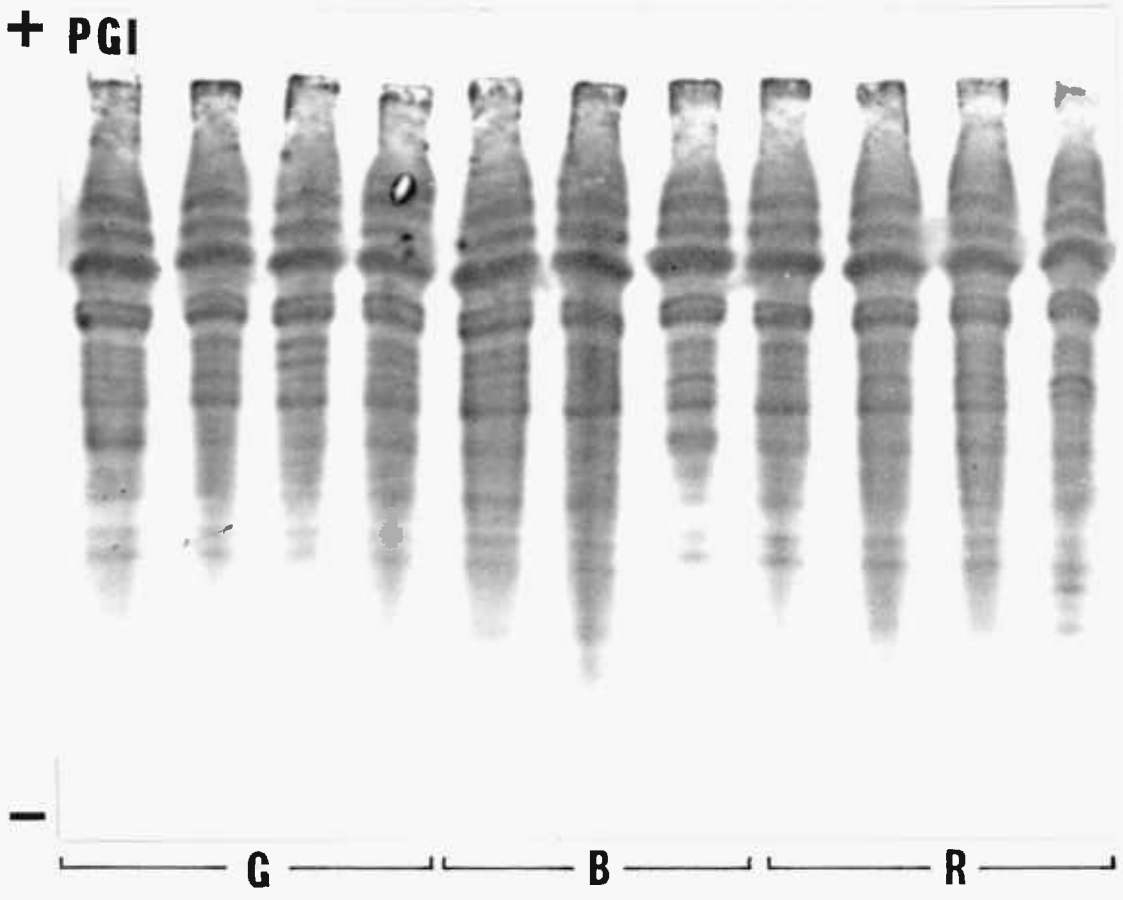
Photographs of electrophoretic patterns obtained with extracts of plerocercoids of L. intestinalis for phosphoglucose-isomerase (PGI) and phosphoglucomutase (PGM).

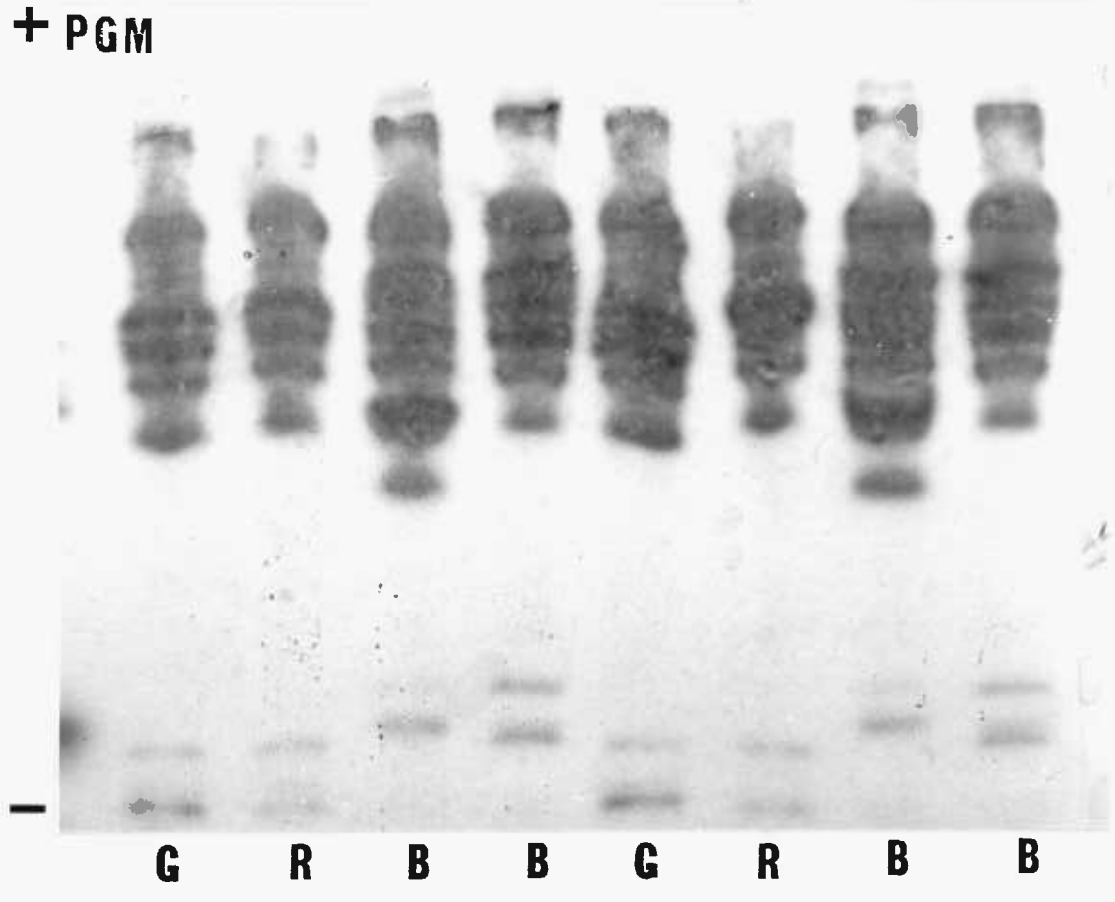
Plerocercoids were removed from the following fish-hosts:

R - Roach (R. rutilus) - from the Serpentine, London

G - Gudgeon (G. gobio) - from Wierwood reservoir, Sussex

B - Bleak (A. alburnus) - from Langley Gravel Pit, Slough.


+ PGI
−
G
B
R


+ PGM
−
G
R
B
B
G
R
B
B

CHAPTER 4.

DISCUSSION

4.1. The dry/fresh weight ratios and basic body constituents

The dry/fresh weight ratios for the plerocercoid of L. intestinalis was higher than the value of 0.29 obtained by Markov (1939), but was the same as the value obtained for the plerocercoid of Schistocephalus solidus (Hopkins, 1950).

The dry/fresh weight ratio decreased in both in vivo and in vitro produced adults (Table 1) indicating that the water content of the adult worm was greater than the plerocercoid; this may be a reflection of the decrease in glycogen content in the adult stage, associated with vast egg-production, but this will be discussed later. In S. solidus, Hopkins (1950) found that the dry/fresh weight ratio increased in the adult in vivo and it is curious that two such closely related species should differ so markedly in this respect. Again, in the pseudophyllid Diphyllobothrium dendriticum, the plerocercoid had a dry/fresh weight ratio of 0.27 (Reuter, 1967a), but this fell to a value of 0.09 in the adult (von Brand, 1952). Thus, it seems that the decrease in dry/fresh weight ratio from plerocercoid to adult may not be peculiar to L. intestinalis among the pseudophyllid cestodes.

The levels of protein, glycogen, lipid, RNA and DNA were linear functions of the weight of worm in the plerocercoid (Figures 2 - 6); this indicates that any plerocercoids within the sample range investigated had identical concentrations of the above constituents. It was of prime importance to determine this fact, because during the course of other experiments, worms of differing sizes had to be used, although a range of 1 - 4g was employed wherever possible. Thus, any variation produced by experimentation was not necessarily a result of the variation in size of

experimental worms. This size variation might have represented developmental differences of the plerocercoid, as Orr (1967) recognised 4 stages in the development of L. intestinalis from the Rudd (Scardinius erythropthalamus). The size range examined in this investigation is roughly equivalent to the third stage described by Orr (1967) (0.246 - 10g) where the plerocercoid is fully developed and infective to the definitive host.

The levels of protein, glycogen and lipid in the adult produced by in vitro culture were also linear functions of worm weight. Again, this indicates that any variation found in subsequent experiments (particularly those where enzyme activity was related to weight of protein) may not have been attributed necessarily to a variation in weight of experimental worms. In contrast, the amounts of RNA and DNA in the adult, produced in vitro, were not linear functions of worm weight. This may reflect differences in the rates of maturation and egg-production of worms from different fish-host individuals.

Glycogen was the major biochemical constituent of the plerocercoid of L. intestinalis (Table 2), and the value obtained (552. µg/mg dry weight) was higher than the range of 380 - 520 µg/mg dry weight found by Markov (1939) for this species. Glycogen is also a major energy store in many other cestodes, including S. solidus (Hopkins, 1950, 1952, von Brand, 1979). The glycogen levels of this latter species, as well as L. intestinalis drop markedly during maturation to the adult. This rapid utilisation is necessary for providing energy for the maturation of the adult, egg-production and also for maintenance of the adult's position within the intestine of the definitive host. Maximum glycogen depletion occurred between days 3 - 5 of culture, and corresponds to the commencement of egg-production (Figure 7); most of the glycogen was consumed by day 7. It is important to remember that the adult worm is short-lived and is geared to rapid and efficient

egg-production. There is no obvious requirement for large quantities of exogenous nutrients and as soon as egg-production has ceased, the rôle of the adult is essentially complete.

The protein levels in the plerocercoid (Table 2) were lower than those reported by Markov (1939). This difference may not have been entirely due to differing experimental techniques because as will be discussed later, preliminary work has indicated the possibility of "strain" differences within L. intestinalis. It is therefore possible that different "strains" may have been used in the two investigations.

The level of protein increased in the adult (Table 2), and this presumably was associated with increased protein synthesis during egg-production. It is interesting to note that the eggs of L. intestinalis had even greater levels of protein, and reduced levels of glycogen compared to the adult. This suggests that the coracidium larva which emerges has only a limited energy supply and thus will remain infective for only a short period. Guttawa & Moczoń (1974) provided histochemical evidence that the metabolism of the coracidia of D. latum, L. intestinalis and Triaenophorus nodulosus were anaerobic with glycolytic pathways providing energy via degradation of polysaccharides. Glycogen is thought to be the major energy reserve in coracidia of pseudophyllid cestodes (Grabiec, Guttawa & Michajlow, 1963; Ginetsinskaya & Purtseladze, 1971). However, as will be discussed below, lipid may also be utilised as an extra energy substrate.

The level of lipid present in the plerocercoid of L. intestinalis (93 µg/mg dry weight; Table 2) was similar to the value obtained for the plerocercoid of S. solidus (Vysotskaya & Sidarov, 1973); but less than the level found by these authors in plerocercoids of L. intestinalis from Roach. It is known that the lipid content of helminths may vary in different hosts; for example Warren & Daugherty (1957) found that in H. diminuta from hamsters, the lipid

content was 9.5%, whereas from rats it was 16.5%. Moreover the lipid content may also vary with the nutrition of the host. Although both the lipid content of L. intestinalis presented in Table 2, and the value reported by Vysotskaya & Sidorov (1973) were obtained from Roach, the two fish populations may have been nutritionally different, thus causing the difference in the values obtained. These workers also found seasonal variation in T. nodulosus which varied in different lakes. The data presented in this investigation was taken throughout the three year period, but it is unknown when data for L. intestinalis was collected by the other workers; thus seasonal variations in L. intestinalis may have contributed to the different values obtained.

The lipid level of L. intestinalis increased in the adults (Table 2) and this may be associated with fatty-acid mobilisation for energy required in egg-production and also for incorporation within the eggs themselves. Grabiec, Guttawa & Michajlow (1962) presented histochemical evidence that lipid may be involved as an energy reserve in coracidia of T. nodulosus. However, the lipid levels in the eggs of L. intestinalis were relatively low (Table 2). Also, the percentage dry weight accounted for in the eggs by the studied constituents was low. It is perhaps possible that some lipids were lost in extraction and so the true level of lipid in the eggs may be somewhat higher.

DNA levels were similar in the plerocercoid and adult of L. intestinalis, but were greater in the eggs (Table 2). The higher levels in the eggs are perhaps to be expected since a greater nuclei : cytoplasm ratio will undoubtedly be present. RNA levels were always greater than DNA in L. intestinalis (Table 2), but were greatly increased in the adult, which is presumably associated with the increase in biosynthetic processes occurring during vitellogenesis and egg-production.

For all the constituents studied, except DNA, the adults are significantly different from the plerocercoid, which would be expected in two stages of a life-cycle which differ so markedly in their rôles. The plerocercoid is essentially a growth stage, where energy stores, in the form of glycogen are laid down, and sexual development is advanced. In contrast, the adult is a short-lived, egg-producing stage concerned solely with the purpose of producing many eggs in a very short period of time. There were differences, however, between the in vivo and in vitro adults, where the glycogen levels differed significantly. This may be due to differing rates of development, or could be due to disturbed metabolism caused by the in vitro culture techniques. The glycogen level in the adult, produced in vitro was lower than the adult, produced in vivo. During in vitro culture the worm is removed from the fish host and immediately starts development at 40°C, whereas when in vivo infection takes place, a delay will inevitably occur while the parasite is transported to the correct position within the intestine; it may be that during this period of transportation, conditions are not ideal for maturation to occur at the maximum rate, and development of the adult is somewhat retarded. The histochemical investigation showed no apparent difference between the two adults for the characters examined, and it can therefore be concluded that differences between in vivo and in vitro adults are probably due to differing rates of maturation, and are not necessarily of a morphological nature.

The histochemical investigation confirmed the biochemical analysis and indicated that, as in other helminths, glycogen was by far the major polysaccharide stored and utilised in both the plerocercoid and adult. Protein and lipids, which increased in the adult (Table 3) occurred mainly in the reproductive tissues, confirming their rôles in egg-synthesis and production.

4. 2. Respiration studies

Both plerocercoids and adults of L. intestinalis utilised O_2 under in vitro conditions (Table 4). This is to be expected since all parasitic helminths so far studied have been shown to consume O_2 in vitro (Barrett, 1976a).

O_2 uptake increased with temperature in the plerocercoid, both in the presence and absence of glucose. However, the increase in consumption was not a linear function of temperature increase (Table 4). This is shown by the Q_{10} values where the Q_{10} between 30 - 40 C was lower than the Q_{10} for 15 - 25°C, both in the presence and absence of glucose. These results are in direct contrast to those of Davies & Walkey (1966), working with the closely related pseudophyllid S. solidus. In this parasite the Q_{10} value between 30 - 40°C was higher than that between 20 - 30°C. These authors postulated the existence of 2 enzyme systems, each operating at the different temperature range. However, this need not necessarily be the case, since a single enzyme may be operating with a temperature optimum closer to 40°C than 20°C. The contrasting Q_{10} values for L. intestinalis and S. solidus could be a result of differences in the maturation time of the adults. Incubation at 40 C for O_2 uptake will lead to the initiation of maturation of the plerocercoid to the adult. S. solidus matures in 36 - 48 hours (Smyth, 1976) whereas the present investigation shows that L. intestinalis was not mature until 72 hours after the commencement of culture. Therefore fundamental differences may exist in the metabolism of the two worms as a result of their differing developmental states.

Plerocercoids of S. solidus utilised 2 µl O_2/mg dry weight/hr at 40°C in the absence of glucose (Walkey & Davies, 1968). Under similar conditions plerocercoids of L. intestinalis utilised 1. 54 µl O_2/mg fresh weight/hr which corresponded to 4. 18 µl O_2/

mg dry weight/hr. In another pseudophyllid, D. dendriticum, Reuter, (1967c) reported an uptake of 3 - 4 µl O_2/mg dry weight/hr for the plerocercoid at 39°C, The O_2 uptake of plerocercoids of L. intestinalis thus appears to be greater than that of other pseudophyllids at 40°C. Friedheim & Baer (1933) showed an uptake of 0.67 µl O_2/mg dry weight/hr in the plerocercoids of D. latum at 22°C in the presence of glucose. Under similar conditions at 25°C, plerocercoids of L. intestinalis took up 0.81 µl O_2/mg fresh weight/hr, which corresponded to 1.7 µl O_2/ mg dry weight/hr. Thus it appears that L. intestinalis may have a greater O_2 requirement than other pseudophyllids, in vitro, at temperatures close to that of the 2nd intermediate host. However, direct comparisons between these species may not be fully justified because differing experimental procedures used by the various authors may have caused, in part, some of the apparent differences in results.

Friedheim & Baer (1933) also studied O_2 uptake in adults of D. latum. These workers showed that at 37°C in the absence of glucose 2.7 µl O_2/mg dry weight/hr were used, whereas in the presence of glucose 15 µl O_2/mg dry weight/hr were used. In contrast, the values for O_2 uptake in adults of L. intestinalis were lower, and unaffected by the presence of glucose (Table 4). This suggests that oxidative processes in adults of L. intestinalis are not being stimulated by glucose.

The adults of D. latum and L. intestinalis are intestinal parasites of birds. A comparison of their O_2 uptake with that of the cyclophyllidean cestode Hymenolepis diminuta, an intestinal parasite of rodents, thus seemed appropriate. Read (1956) reported an uptake of 3.0 and 1.2 µl/O_2/mg dry weight/hr in the presence and absence of glucose respectively in H. diminuta at 37°C. Again, as in D. latum, glucose stimulates the uptake of O_2, in contrast to L. intestinalis. That both H. diminuta and

D. latum show glucose stimulation of O_2 uptake, may be a reflection of their life-cycle strategies. Both these species infect the definitive host whilst in a state of development that is far less advanced than L. intestinalis. Additionally, in contrast to this species, both the former parasites undergo rapid and dramatic periods of growth in the definitive host before maturation can occur. Therefore, the glucose stimulation of O_2 uptake in these species may indicate its use in the synthetic processes necessary for growth and development, as well as in energy yielding pathways.

McManus (1975b) studied the TCA cycle enzymes of L. intestinalis and found that although all were present, the activities of some key enzymes were low, and this worker questioned the role of a normally functioning pathway in the parasite. However, Table 4 shows that L. intestinalis does take up O_2 in vitro; but are the quantities consumed sufficient for a fully functional oxidative pathway? Davies & Walkey (1966) suggested that S. solidus consumes less O_2 than a free-living animal of similar weight. However, Whitney (1942) reported that the turbellarian Crenobia alpina consumes 0.12 $\mu l\ O_2$/mg dry weight/hr at 14.5°C. Petitpren & Knight (1970) quoted the O_2 uptake of the larva of the dragonfly Anax junius as 0.37 and 1.89 $\mu l\ O_2$/mg dry weight/hr at 13°C and 34°C respectively. Clearly then, the O_2 consumption of L. intestinalis in vitro was of the same order as some free-living animals of similar size and weight.

Before drawing conclusions from in vitro experiments, one must decide whether the in vitro conditions encountered by a parasite bear any relation to those in vivo. Little is known regarding the gaseous environment within the coelomic cavity of fish. According to Körting (1976), fish, and the fish intestinal system will have a higher oxygen content than that of warm blooded vertebrates, but the author offered no data, and as far as I am aware, no such data has been published.

The fish body-cavity will have no direct oxygen supply other than diffusion through the body wall from the surrounding water environment, the walls of the internal organs and blood vessels. The O_2 tension will probably be greater in the fish body-cavity than in the small intestine of the bird host. Crompton, Shrimpton & Silver (1965) measured the O_2 tension in the small intestine of the domestic duck, by means of an O_2 electrode. They found that close to the villi, the tension was 25 mm Hg, but this dropped to 0.5 mm Hg in the centre of the lumen. More recently, Podesta & Mettrick (1974) found an O_2 tension of 40 - 50 mm Hg in the aqueous phase of the lumenal contents of the rat intestine. They also found that the presence of H. diminuta in the intestine affected the O_2 tensions in this, and other parts of the gut to some degree.

It seems, therefore, that the study of O_2 tensions in parasite environments may not be as simple as first appears. It is difficult to evaluate how the presence of the adult of L. intestinalis affects the O_2 tensions in the bird gut, since it lies coiled, almost blocking the small intestine (Flockart, 1979). This blockage will inevitably cause a build-up of food material passing down the gut; it is possible O_2 tensions may build up on this anterior site of the blockage. However, conditions of anoxia may occur in the posterior side of the blockage. Since the adult parasite is large, its outer tissues may thus be in contact with the intestinal mucosa and these outer tissues may obtain O_2 via diffusion from the mucosal blood supply. In fact, it is likely that O_2, if available, may only be of importance to the outer tegument of the worm, since it is here that most of the mitochondria lie (Smyth, 1976). Additional evidence that it is the outer layers only that are aerobic was provided by Davies & McManus (personal communication) who demonstrated histochemically that the TCA cycle enzymes SDH & MDH were found only in the tegumental mitochondria.

It would seem likely, therefore, that L. intestinalis has a limited use for molecular O_2 in vivo, except in its outer regions, although it is consumed under in vitro conditions. This conclusion can be drawn partly from the fact that some key TCA cycle enzymes exhibit low activities indicating that an operational TCA cycle is of limited importance. Also, the mitochondria, the sites of oxidative metabolism are located mainly in the peripheral regions of the worm. Even if the parasite were able to operate oxidative pathways throughout its body, there would be the additional problem of O_2 supply to all tissue regions. Cestodes possess no respiratory or circulatory systems, and in a parasite as large as L. intestinalis diffusion of O_2 would be slow and limited. In all probability, therefore, L. intestinalis, which possesses flexible metabolic pathways (as will be discussed below), will probably utilise some O_2 should it become available, in combination with less efficient anaerobic pathways. It is likely that O_2 may be of more importance to the parasite in synthetic processes such as egg-production.

4. 3. The release of end-products of carbohydrate metabolism by plerocercoids and adults of L. intestinalis

The study of end-products of carbohydrate metabolism can often give useful information as to the pathways operating within a tissue or organism. Some helminths produce a limited range of end-products; S. mansoni for example produces mainly lactate (Bueding, Peters & Waite, 1947), indicating that glycolysis is the major pathway of carbohydrate breakdown. The production of lactate in this species is interesting since the parasite lives in the mesenteric veins of mammals, a comparatively O_2-rich environment. Therefore it might be expected that the parasite would have sufficient O_2 to operate more efficient pathways of oxidative metabolism than by glycolysis. One must be wary, therefore, of classifying the metabolism of a parasite simply by the environment in which it is found. Other parasites may produce a wide range of organic end-products, and these may vary according to the conditions in which the parasite exists (Bryant, 1978).

Information about end-products released by fish-parasites, and in particular psuedophyllids, is limited. Smyth (1950) reported an accumulation of acidic substances during the in vitro culture of L. intestinalis and S. solidus plerocercoids. More recently, the work of McManus (1975b) indicated that succinate is a major end-product of anaerobic glucose metabolism in L. intestinalis. Körting & Barrett (1977), studied the organic end-products of S. solidus in more detail. They found that aerobically 22% of the glycogen utilised was catabolised to organic acids, whereas anaerobically 70% of the carbohydrate metabolised was produced as organic acids. The major end-products in this species were acetate and propionate, with acetate predominating under aerobic conditions, and propionate under anaerobic conditions. The production of acetate from glycogen results in the formation of 2 molecules of NADH. The authors stated that this pathway would result in an

acetate : propionate ratio of 1 : 2 under anaerobic conditions. Since in S. solidus they found a ratio of 1 : 3, they argued that the additional NADH must come from another source. Under aerobic conditions Körting & Barrett (1977) found an acetate : propionate ratio of 4 : 1 which they believed was understandable since the NADH could be oxidised by molecular O_2. As is often the case with other parasites (Barrett, 1976a), glucose changed the pattern of the end-products; lactate became important, and these authors suggested that this may have been an 'overflow' as a result of the low oxidative capacity of the parasite. Since aerobically only 22% of the glycogen was released as reduced end-products, the authors suggested that a functional TCA cycle may be important under aerobic conditions. More recently, Beis & Barrett (1979) have presented data on TCA cycle intermediates in S. solidus which corroborates the previous work. The rôle of oxidative processes in this parasite and in L. intestinalis will be discussed later.

In the present investigation, six possible end-products of metabolism in L. intestinalis were studied; these had been shown to be released by other parasites in varying quantities (von Brand, 1973). Pyruvate and lactate would originate via the glycolytic pathway being the penultimate metabolite and end-product respectively. The other four compounds were likely to originate via the pathways of CO_2 fixation and the partial reversal of the TCA cycle; these were malate, succinate, acetate and propionate.

In order to gain an understanding of the range of pathways, and the flexibility within the system, 3 environmental criteria were varied. The temperature used was either 15°C or 40°C; the former approximates to the temperature of the fish intermediate-host of the plerocercoid, and this represents the 'resting' stage of the larva. 40°C is the temperature of the definitive bird host and represents the 'activation' temperature of the plerocercoid as

well as the adult-environmental temperature. Little is known about the gaseous environment of the fish body-cavity, and thus two extremes were employed; worms were incubated either under anaerobic conditions (95% O_2 : 5% CO_2) or atomospheric air. In order to assess the effects of nutrients to the parasite, glucose was either present or absent in the experimental saline.

Reuter (1967c) examined the effects of media of different osmotic conditions upon the release of lactate and succinate by plerocercoids of D. dendriticum. The author found that hypertonic Hanks' saline (2.5 times normal) caused a decrease in succinate production, but an increase in lactate production. However, this parameter was not examined here, and normal Hanks' saline was employed throughout.

In the plerocercoid of L. intestinalis, lactate was a major end-product (Table 5) indicating that glycolysis was an important pathway of carbohydrate breakdown. Since pyruvate was not a major end-product under any conditions, this would indicate that glycolysis continued to completion. In the plerocercoid at 15°C, anaerobiosis enhanced lactate production (Table 5), as a result of increased glycolytic flux. This is typical of this pathway because glycolysis requires no O_2, and the NADH produced is oxidised to NAD during lactate production. Alternative oxidative pathways involving molecular O_2 would be excluded under anaerobiosis leaving increased pyruvate levels for conversion to lactate. Similarly, Bryant & Behm (1976) reported a 50% increase in lactate production under anaerobic conditions in Moniezia expansa. They also reported that succinate production was unaffected by anaerobiosis, but that there was a 16% increase in overall end-product release. In contrast to these results, Reuter (1967b) found no difference in lactate and succinate production by plerocercoids of D. dendriticum under aerobic and anaerobic conditions, providing the gaseous environment

contained CO_2; he also noted that succinate production was far greater than lactate production.

As already mentioned, the presence of glucose in the incubation medium can have a dramatic effect upon end-product release, both quantitatively and qualitatively. In the plerocercoid of L. intestinalis, the presence of glucose decreased lactate production (Table 5). But it is interesting to note, in contrast, that malate and succinate excretion are enhanced by the presence of this sugar. If the pathways leading to their formation, i.e. CO_2 fixation and a partial reversal of the TCA cycle are enhanced by glucose, then possibly its absence would favour glycolysis indirectly at the PEP branchpoint. Pyruvate production in the plerocercoid at 15°C is also enhanced in the presence of glucose, in contrast to lactate production (Tables 5 and 9). This is unusual, since pyruvate might be expected to be derived via glycolysis and thus to be affected in the same way as lactate production. Possibly, in the presence of glucose, the lowered glycolytic flux would allow a slow build-up of pyruvate. Alternatively, as discussed by Barrett (1976a), the pyruvate may originate via the same route as malate, being decarboxylated from malate by malic enzyme.

Reuter (1967d) showed that the production of lactate and succinate by plerocercoids of D. dendriticum increased with temperature. He attributed the increase in lactate production to damage of the worm. Table 5 shows that in the 'activated' plerocercoid of L. intestinalis, i.e. incubated at 40°C, lactate production decreased; this is unlikely to be as a result of worm damage. In contrast, the release of those end-products derived from a partial reverse of the TCA cycle increases. This would indicate that these pathways play a more important rôle in the maturing plerocercoid. Since these end-products were also more sensitive to the effects of O_2 and glucose in the 'activated' plerocercoid (Table 7), it may be that the rôle of these pathways is

to allow an efficient overall metabolic flux during the varying conditions that will be experienced during transition from one host to another. It is vital that, at this period in the life-cycle, development occurs rapidly and is in no way affected by reduced metabolic flux. It is interesting to note that the factors enhancing lactate production in the plerocercoid are opposite to the factors enhancing the production of the other end-products (Table 7).

In the mature adult worm, lactate was again the major end-product (Table 6), and was produced in significantly greater quantities than in the 'activated' plerocercoid (Tables 8 and 10). This would indicate that as in the 'resting' plerocercoid, glycolysis was the major energy-yielding metabolic pathway. Again there was greater production in the absence of glucose, but in contrast to the plerocercoid, lactate production was independent of the presence or absence of oxygen. This may reflect a degree of independence of the parasite's metabolism from the host environment. The release of pyruvate, malate and propionate increased in the adult, compared to the 'activated' plerocercoid, whereas the reverse was true for acetate and succinate (Table 10). With the latter two end-products it may indicate that some proportion of their levels may be directed towards fatty acid production and other non energy-yielding pathways, rather than being concerned simply with energy production.

In summary, end-product release in the adult appeared to be less sensitive to the effects of glucose and O_2, possibly reflecting a greater independence of the adult metabolic pathways. This may be advantageous to the parasite since it would allow maturation to continue in a wide range of environmental conditions.

4.4. Glucose uptake by plerocercoids of L. intestinalis

The mean value of glucose uptake by plerocercoids of L. intestinalis (Table 11) must be treated with some caution. Since the incubation period was 15 min, some radiolabelled glucose may have been incorporated into glycogen, lipid or protein during this period. Extraction in 50% methanol would remove free-pool compounds, but may have failed to release all label incorporated into the compounds mentioned above. In addition, some labelled carbon may have been metabolised via aerobic and anaerobic processes to CO_2. Thus it is likely that the value presented may be an under-estimate.

Glucose uptake by helminths is generally by active processes (von Brand, 1979) and in many species these processes are responsible for considerable and rapid uptake of this sugar. For example, under aerobic conditions, adult H. diminuta took up 4,300 nmoles glucose/10 mg dry tissue/hr (Fairbairn, Wertheim, Harpur & Schiller, 1961). This contrasts markedly with the value of 78.3 nmoles/10 mg dry weight/hr obtained for the plerocercoid of L. intestinalis.

It is interesting to note that the low glucose uptake by L. intestinalis at 15°C (Table 11) approximately balanced the release of organic end-products (Table 5). This would indicate that, at this stage in the life-cycle, the metabolism was operating at a relatively low level. The plerocercoids examined in these experiments would be infective to the definitive host, and would have fully developed glycogen stores. Therefore, the metabolic pathways of this stage in the life-cycle would probably be required to supply the immediate energy requirements of the parasite, with relatively little being needed for biosynthetic processes.

4. 5. Glucose uptake during *in vitro* culture

When the glucose uptake of the maturing adult, *in vitro* (Table 12) is compared to the consumption of endogenous glycogen (Figure 7) expressed as molar equivalents (Appendix 6), glycogen is by far the major source of energy. It is interesting that minimum glucose uptake occurred at a time when egg-production commenced (48 - 72 hr after the start of culture). This also corresponded to the period of maximum glycogen consumption (Figure 7). Glucose uptake then increased thereafter for the duration of *in vitro* culture. Thus glucose probably acts merely as an energy supplement to the glycogen stores in the developing adult, but may have a rôle in the synthesis of glycogen within the eggs, as an energy supply.

4.6. Steady-State metabolite levels

As in adult M. expansa (Behm & Brant, 1975a), all glycolytic intermediates were present in the larva and in vitro adult of L. intestinalis; also endogenous glucose levels were very high in both species. Moreover, all the TCA cycle intermediates investigated in L. intestinalis were shown to be present, indicating that this pathway is operational.

A comparison of metabolite levels in plerocercoid and adult revealed that all glycolytic intermediates except F6P and lactate either decreased or were unchanged. However, of the TCA cycle intermediates measured, citrate and OAA increased while succinate and malate decreased. This contrasted with the recent work of Beis & Barrett (1979) who found that upon 'activation' of the larva of S. solidus all TCA cycle intermediates increased in concentration 2 or 3 fold. These authors also found that of the glycolytic intermediates F6P, FDP, 3-phosphoglycerate, PEP and lactate decreased upon 'activation' while the rest remained unchanged. The comparison of results for the 'activated' larva of S. solidus (Beis & Barrett, 1979) may not be directly comparable with the results for the adult of L. intestinalis (Table 13). Plerocercoids of S. solidus were 'activated' for 12 hours, and so would not have attained the same state of maturation as L. intestinalis adults.

Of the TCA cycle intermediates, citrate and malate levels (Table 13) are of the same order as those found by Beis & Barrett (1979) in S. solidus. However, succinate and OAA levels (Table 13) were much higher than reported in S. solidus. Beis & Barrett (1979) stated that the levels of TCA cycle intermediates in S. solidus were of the same order as those reported by Williamson & Brosnan (1974) for tissues with an active TCA cycle. Corroborative evidence that the TCA cycle was important in S. solidus was shown by Körting & Barrett (1977) where 75% of

glycogen catabolised aerobically may have been broken down via the TCA cycle. In L. intestinalis, the present study did not measure levels of isocitrate, 2-oxyglutarate or glutamate (an intermediate associated with the TCA cycle) and so a full comparison with the TCA cycle of other tissues was not possible. However, carbon balances for L. intestinalis were calculated (Appendix 8) and it appeared that no glycogen was available for breakdown via the TCA cycle. Indeed, it appeared that the release of end-products greatly exceeded the utilisation of glycogen and glucose in the 'activated' larva; this obviously was an anomalous result. Glucose and glycogen consumption over the first 24 hours of culture were calculated from Appendix 6, whereas data for end-product release were calculated from Table 5. In this latter case the experimental data was derived from the first 3 hours of 'activation' and multiplied to give a value for a 24 hour period. Thus, if the release of end-products dropped after the initial 3 hour period then the data in Appendix 8 would appear unusually high. However, despite difficulties in interpreting the data, the results suggested that the TCA cycle may not have the same importance in aerobic metabolism as in S. solidus.

Incubation of M. expansa with 10 mM glucose elevated the internal glucose concentration significantly. Similarly, after the in vitro cultivation of L. intestinalis, the glucose concentration in the adult was more than twice that of the plerocercoid (Table 13) indicating that the parasite takes up glucose during prolonged in vitro culture. It is curious that incubation of the plerocercoid for 3 hour periods produced little detectable glucose uptake (see results section). Possibly, the high glucose levels in the plerocercoid were the result of a slow build-up during this stage in the life-cycle. Little utilisation of glucose would occur since HK levels are low (McManus, personal communication) and it is a potential rate-limiter (Table 15). The elevated glucose levels in

the adult may have been built up by glucose uptake occurring after maturation of the adult has commenced.

The adenosine nucleotide levels in L. intestinalis, shown in Table 14, are also expressed as the adenylate charge. A value of 0.8 can be expected in normal, healthy tissue (Atkinson, 1971). Barrett & Beis (1973b) described the adenylate charge of H. diminuta as 0.71, and the authors reported that this value is high enough to indicate a healthy tissue. Similarly, Behm & Bryant (1975a) quoted a value of between 0.70 and 0.76 for M. expansa under aerobic or anaerobic conditions, suggesting healthy tissue. The very high value of 0.86 in the plerocercoid of L. intestinalis (Table 14) again indicated healthy tissue. This might be expected for the plerocercoid, since it is during this stage in the life-cycle of pseudophyllids that energy stores are normally built up, and once the plerocercoid is fully developed it effectively becomes a 'resting' stage until infection of the definitive host occurs. The 'activated' plerocercoid showed a lower adenylate charge value of 0.775 while the adult value was even lower at 0.72. This would be connected with the rapid utilisation of energy during the maturation of the adult and subsequently egg-production. Beis & Barrett (1979) also found that, the adenylate charge of S. solidus dropped during 'activation' of the plerocercoid. Similarly, the phosphorylation potential and $\frac{(ATP)(AMP)}{(ADP)^2}$ values were high in the plerocercoid and decreased in the 'activated' plerocercoid (Table 14), again indicating that a large amount of energy was being expended. The values for the adult were higher than for the 'activated' plerocercoid (Table 14), which may reflect a stabilisation of the energy yielding systems in the mature adult worm.

The calculation of mass-action ratios and comparison with apparent equilibrium constants indicated that HK, PFK and PK

are potential rate-limiting enzymes. However, HK may be of limited importance in L. intestinalis since it is probable that a large proportion of the G-6-P pool, present in the tissues of the worm, comes not from glucose (the reaction which HK catalyses), but from glycogen; thus HK may not limit the glycolytic flux in L. intestinalis. Barrett (1976b) reported that these three enzymes may also be rate-limiting in Ascaris eggs, and that phosphorylase may also be important. Moreover, in the scolices of M. expansa, HK, PFK and PK (Behm & Brant, 1975a) and possibly PEPCK (Bryant, 1978) may be important rate-limiting enzymes. In a recent paper by Beis & Barrett (1979) PFK and PK were found to be key regulatory enzymes in S. solidus. It was also suggested that PEPCK may be important, but as discussed by these authors, its rôle is difficult to assess.

4.7. Enzyme activity

The enzyme pyruvate kinase (PK) occupies an important position in the glycolytic pathway, catalysing the conversion of PEP to pyruvate. As discussed previously, PK is a non-equilibrium enzyme in L. intestinalis and is therefore a potential regulator of carbohydrate metabolism. PK has been investigated in some detail in a number of parasites. Behm & Bryant (1975c) studied PK from M. expansa and found that ATP inhibits the enzyme and that ATP competes with ADP when it is inactivated by FDP. More recently, Bryant & Behm (1976) proposed a regulatory system for M. expansa where, under aerobic conditions, increased concentrations of malate and ATP depress PK and consequently the lactate-producing branchpoint in glycolysis. Conversely, under aerobiosis the lactate-producing pathway is favoured.

Podesta, Mustafa, Moon, Hulbert & Mettrick (1976) investigated PK and PEPCK in H. diminuta. They reported that PEPCK had a low pH optimum and was activated by HCO_3^-. In contrast, PK was inhibited by HCO_3^- and had a low affinity for PEP at acid pHs. Thus, they suggested high CO_2 levels would favour activation of PEPCK and inhibition of PK, and this could control the PEP branchpoint; glycolysis would be favoured at low levels of CO_2, and CO_2 fixation would occur when higher CO_2 concentrations were available.

Other authors have also suggested that the PEP branchpoint plays an important role in metabolic regulation in parasites. Körting & Barrett (1977) reported high PK activity in S. solidus and suggested that the PEP branchpoint could be important. Earlier, Körting (1976) had reported PK from S. solidus and determined that it was 3 times more active than PEPCK in the plerocercoid under the in vitro conditions employed. The same author also studied Bothriocephalus gowkongensis, Khawia sinensis, and adults and plerocercoids of Triaenophorus crassus and found

PK and PEPCK present in these worms. PEPCK was slightly more active than PK, which required FDP.

In Mesocestoides corti, Köhler & Hanselmann (1974) reported low activity of PK which was regulated by FDP. In Dicrocoelium dendriticum Köhler (1974) again reported that PK was activated by FDP. Mg^{2+} or Mn^{2+} also regulated the enzyme, but the author suggested that Mg^{+} was probably more important in vivo. ATP was an inhibitor of the enzyme, and malate inhibited HK in the absence of FDP.

McManus (1975a) studied PK from the plerocercoid of L. intestinalis. He reported a pH optimum of 7.0 which is similar to the enzymes from other helminths and rat liver. The enzyme had a high affinity for Mn^{2+} as did the enzyme from M. expansa (Bryant, 1972) and ATP and malate inhibited the enzyme co-operatively. The author also reported an active PEPCK from L. intestinalis. In the present investigation, analysis of PK activity from L. intestinalis was performed under the optimum conditions reported by McManus (1975a). However, as the author investigated the enzyme only at 30°C, it was felt that a study of activity at the temperature of the plerocercoid in the fish-host (15°C) and at the temperature of the adult in the bird-host (40°C) was appropriate. The increased PK activity in the plerocercoid between 15°C and 30°C with no significant increase between 30°C and 40°C (Table 17), may indicate that the glycolytic pathway is particularly important during the initial stages of maturation of the plerocercoid. The activity of the adult PK was less than half that of the 'activated' plerocercoid. This indicated that the glycolytic pathway may be subject to greater control by PK in the adult than in the 'activated' plerocercoid. However, as discussed previously, lactate production was significantly greater in the adult compared to the 'activated' plerocercoid (Table 10). These two results may at first appear anomalous, but it may be that, in

the adult worm, alternative pathways to glycolysis are less active than in the plerocercoid, allowing an increased glycolytic flux, despite decreased PK activity. It is interesting to note that the activity of PK was greater than PEPCK at 15°C and 40°C in the plerocercoid but lower at 30°C (Table 17). It is obvious that the PEP branchpoint is important in the developing plerocercoid with a fine balance between glycolysis and CO_2 fixation. In the adult, however, PEPCK was far more active than PK, especially at 40°C, which may reflect the greater importance of CO_2 fixation to the adult. If this is the case, then CO_2 fixation may not be concerned solely with energy production since lactate was the major end-product in the adult; biosynthetic processes may require substrates derived from CO_2 fixation and the partial reversal of the TCA cycle, and hence these substrates would not appear as released end-products.

PEPCK has also been investigated extensively in helminths. Behm & Bryant (1975a) reported PEPCK from both cytosolic and mitochondrial fractions of M. expansa and in a later paper (Behm & Bryant, 1975b) showed that the two fractions contained different PEPCKs; both enzymes were activated by Mn^{2+} but cytosolic activity was also activated by Mg^{2+}. The pH optimum lay between 6.4 - 7.0. GDP gave slightly higher activity than IDP, whereas little activity was found with ADP. The authors noted that PK of M. expansa used IDP and GDP but that ADP gave highest activity. This has obvious implications for the control of the PEP branchpoint. PEPCK had similar rates in the forward and reverse directions, suggesting that the enzyme may be readily reversible in vivo.

Körting (1976) reported PEPCK activity for B. gowkongensis, K. sinensis and adults and plerocercoids of T. crassus. In addition, the importance of PEPCK and CO_2 fixation in the metabolic flux of S. solidus were suggsted by Körting & Barrett (1977);

they found PEPCK and fumarate reductase activity but relatively low activities of aconitase and isocitrate dehydrogenase (IDH), suggesting limited TCA cycle activity in the forward direction.

In L. intestinalis, PEPCK activity with Mn^{2+} is greater than Mg^{2+} and IDP promotes greater activity than ADP (Table 14), thus agreeing with the results of Behm & Bryant (1975b) discussed previously. Highest activity in the plerocercoid was at 30°C suggesting that CO_2 fixation, as well as glycolysis may be important in the initial stages of maturation. In the adult, activity at 30°C was less than in the plerocercoid, whereas at 40°C the activity was much higher. This would indicate the importance of CO_2 fixation to the mature adult. Also the temperature responses of the enzyme from the adult and plerocercoid are entirely different which suggests that two different enzyme systems may be involved in the two stages.

Phosphofructokinase (PFK) catalyses the reaction between F6P and FDP, and as discussed earlier may be rate-limiting in L. intestinalis (Table 15). Similarly, Behm & Bryant (1975a, c) found PFK was non-equilibrium in M. expansa. The authors found a pH optimum between pH 7.4 - 8.0 and activation by Mg^{2+} and Mn^{2+}. Unlike mammalian systems they found sigmoid kinetics with F6P; ATP was shown to decrease the affinity of the enzyme for F6P. This inhibition was relieved by F6P, AMP and ammonium ions. Bryant & Behm (1976) proposed a model of metabolic control during the transition from aerobic to anaerobic metabolism in M. expansa. The model system is finely controlled by adenosine nucelotides; during the transition, the aerobic production of ATP decreases, removing the inhibition of PFK. This allows increased carbon flow through glycolysis and other anaerobic pathways which in turn permits increased anaerobic production.

PFK was highly active in L. intestinalis (Table 17).

Ammonium ions stimulate PFK and greater activity was achieved with Mn^{2+} than Mg^{2+}. In these respects the enzyme differs little from M. expansa and it is probably subject to the same modulation as in this and other helminths (Bryant, 1978). Maximum activity was found at 30°C in the plerocercoid, although the activity was still very high at 40°C; at both 30°C and 40°C the activity of the plerocercoid was much higher than in the adult. It may be that during the initial stages of maturation, a high flux through PFK is necessary to provide an immediate energy source. In the mature adult, the PFK may have a more important regulatory function in the breakdown of glycogen.

Malic enzyme (ME) catalyses the decarboxylation of malate to pyruvate, with the consequent production of NADH. In Tubifex spp. the decarboyxlation reaction occurs primarily in in vivo (Hoffmann, Mustafa & Jorgensen, 1979), and Barrett (1976b) reported that this also occurs in the mitchondria of Ascaris. The malate, produced via MDH, will also be in equilibrium with fumarate, via fumarase, the NADH formed by the malic enzyme being reoxidised by the reduction of fumarate to succinate. In some parasites, however, ME is of limited importance. Bryant (1978) reported low ME activity in M. expansa in the cytosol and mitochondrial fractions; the reaction is primarily in the carboxylation direction. Körting (1976) reported low ME activity from B. gowkongensis, K. sinensis, T. crassus and S. solidus; the enzyme is NADP linked, and there was little detectable activity with NAD. Similarly ME from L. intestinalis also showed low activity (Table 12). Mn^{2+} ions stimulated greater activity than Mg^{2+} and there was an increase in activity with temperature in the plerocercoids, unlike some other enzymes from L. intestinalis (Table 12). It may be that, in L. intestinalis, the pathways via ME are not the most active, but nevertheless it is

likely that they will be important in providing substrates for biosynthetic processes and also in balancing redox couples.

Succinate dehydrogenase (SDH) can operate in parasite tissues in both the forward and reverse directions. In the forward direction, succinate is oxidised to fumarate whereas in the reverse direction fumarate is reduced to succinate (fumarate reductase). McManus (1975b) studied SDH in plerocercoids of L. intestinalis and found a succinate oxidation : fumarate reduction ratio of 4.5:1; this resembled other facultative anaerobes where succinate is formed as a means of reoxidising NADH formed during glycolysis (Barrett, 1976a; Saz, 1971). SDH activity in L. intestinalis increased with temperature in the plerocercoid between 15°C and 30°C, in both directions, while the activities were similar at 30°C and 40°C (Table 16). This emphasises the importance of the TCA cycle in both directions to the maturing parasite, and also indicates the flexibility present within the overall metabolic system. SDH activities were significantly lower in the adult compared to the plerocercoid, which may indicate a greater regulatory function of SDH in the adult. Associated with SDH in the partial reversal of the TCA cycle is the enzyme MDH, operating in the reverse direction from oxaloacetate to malate. NADH is converted to NAD during this reaction and a redox couple can operate between the MDH step and the conversion of 2-glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate during glycolysis. McManus (1975b) examined MDH from the plerocercoids of L. intestinalis in the forward and reverse directions. He found much higher activity in the reverse directions compared to the forward. The values of MDH in the reverse direction (Table 16) were higher than those found by McManus (1975b) in the plerocercoid, and extremely high in the adult. This indicated that the reaction is important in both plerocercoid and adult, and the high activity in the adult is

reflected in increased malate production as an end-product (Table 6).

Lactate dehydrogenase catalyses the final reaction in the glycolytic pathway i.e. the conversion of pyruvate to lactate. Since lactate was a major end-product in L. intestinalis (Tables 5 - 6) it is not surprising, therefore, that LDH was active in this parasite. LDH has been demonstrated in a wide range of parasitic helminths (von Brand, 1973) and is particularly active in parasites whose environment and metabolism are anaerobic.

4.8. Isoenzymes in L. intestinalis

4.8.1. Isoenzyme changes during in vitro culture

LDH is an ideal enzyme for the investigation of isoenzyme chan es associated with tissue or whole organism development. As previously discussed, this enzyme occurs as a tetramer in mammalian systems, and the genetic control of intra specific isoenzyme differences has frequently been assessed (Wilkinson, 1970).

In the house fly (Musca domestica), Agatsuma, Kawamoto & Takeuchi (1977) found that LDH activity changed during the life-cycle; activity was high in the larva and low in the pupa and adult. The authors suggested that this was a beneficial adaptation, allowing the larvae to survive under essentially anaerobic conditions. These authors also found that the changes in LDH activity was connected with changes in the intensity of an isoenzyme controlled by one gene. This introduces the concept of separate genes controlling different stages in the life-cycle.

Parasites often have complex life-cycles. In many cases, such as L. intestinalis, the physiochemical environments experienced will vary dramatically at each stage of the life-cycle and it is likely that isoenzyme changes will be a necessary adaptation to these differing conditions. Thus, parasites are in many ways ideal as models for the study of isoenzyme changesd during their development.

H. diminuta has been studied for isoenzyme changes during its life cycle. Walkey & Fairbairn (1973) performed kinetic studies on LDH and suggested the presence of 2 isoenzymes, one in eggs and cysticercoids and one in the anterior proglottids and pre-patent worms. Their work was supported by the investigations of Logan, Ubelaker & Vrijenhoek (1977), who found two isoenzymes of LDH, A and B. A and B occurred together in the eggs and cysticercoids while A alone was active in the adult and pre-patent worms. The authors suggested that the changes were genetically controlled by 2 gene loci. As discussed previously,

Carter & Fairbairn (1975) found varying numbers of PK isoenzymes throughout the life-cycle of H. diminuta. Thus, in H. diminuta, the isoenzymes of at least two enzymes change throughout its life-cycle and the metabolism of the cestode is thus modified to suit the environment of each stage of the parasite.

In L. intestinalis the appearance of two new isoenzyme bands in the in vitro adult (Plate 1) may represent isoenzymes which are more suited to the physiological conditions found in the bird-host. As mentioned above, many isoenzyme changes are genetically controlled, and this may occur in L. intestinalis. The 'swtich' from larval to adult metabolism appears to occur rapidly. If isoenzyme changes are necessary for an enzyme, such as LDH, to function efficiently in the adult, then it would seem advantageous for this alteration to occur equally quickly. This would be possible if the isoenzyme change were genetically pre-programmed.

These results are preliminary but it is hoped, in future work to isolate the isoenzymes of LDH from plerocercoid and adult of L. intestinalis. The characteristics of each isoenzyme could then be determined, and thus their roles in relation to the life-cycle stage could be assessed.

4.8.2. Isoenzymes and possible "strain" differentiation in plerocercoids of L. intestinalis

Many parasites can infect a wide-range of host species, which is beneficial to the survival of the parasite. However, if isolated populations of the same parasite species are exposed to different host species, then this could give rise to "strain" formation. Each "strain" would be best adapted to its particular host species.

The existence of intraspecific "strains" is perhaps best documented in Echinococcus granulosus (Smyth, 1976). Both horse and sheep "strains" occur in the British Isles and develop-

mental and physiological differences have been demonstrated (Smyth & Davies, 1974; McManus & Smyth, 1978). More recently, McManus & Smyth (1979) have shown isoenzyme differences between the "strains" for several enzymes.

Although only PGM exhibited intraspecific variation in L. intestinalis (Plate 3), it is possible that "strain" variations may be indicated. Since two bleak samples were identical to plerocercoids from gudgeon and roach, these may all originate from the same parasite population. The two bleak samples which differed from the others may have arisen from the introduction of a separate parasite population from an external source. This introduction could easily be caused by the definitive host travelling from a bleak-only environment to Langley gravel pit (which contained all 3 fish species).

All three parasite locations are in South-East England, and the existence of large areas of water in this region is a relatively recent occurrence. Thus, populations of fish-hosts may not have been isolated for sufficient time to allow complex "strain" differences to develop. Hence differences in PGM alone may be explained. Clearly more work is required in this area to evaluate the extent and nature of these "strains" as each "strain" may exhibit fundamental differences in metabolism. It is important to remember that the parasites used in the present study of metabolism in L. intestinalis all originated from the same roach population at the Serpentine. As there was no indication of any isoenzyme variation with these worms, it can be assumed that they represent a single "strain". However, the fact that strains of L. intestinalis may exist in different host species and/or differing geographical locales should be borne in mind for any future studies on this parasite.

4.9. Final conclusions

The present studies on the metabolism of L. intestinalis have revealed that the parasite uses active anaerobic pathways for carbohydrate breakdown. Glycolysis, although essential, is not the only means of obtaining energy as the TCA cycle and pathways of CO_2 fixation and a partial reversal of the TCA cycle also operate. However, the relative importance of these different pathways changes during the maturation of the plerocercoid to the adult, and it is these metabolic modifications which could account for the parasite's success.

As discussed, L. intestinalis probably has a limited use for O_2 in vivo, although it was consumed in vitro by both the plerocercoid and adult. In a parasite such as L. intestinalis which is large and has no circulatory system, only the peripheral tissues, which possess any significant concentration of mitochondria, could utilise O_2; it is perhaps more important in biosynthetic processes such as the tanning of eggs, than to energy-producing pathways.

As is often the case with mainly anaerobic organisms, glycogen was the major energy reserve in L. intestinalis, and although glucose was present at relatively high levels in the plerocercoid, there was no evidence to indicate significant uptake of the sugar by this stage of the life-cycle. Presumably, however, a slow uptake of glucose occurs into the plerocercoid while it is developing in the fish. In any case, a large capacity for glucose uptake by the parasite would imply a large utilisation. This was not evident in L. intestinalis since HK activity was low and was potentially rate-limiting. The role of the plerocercoid is to build up glycogen stores. As HK is involved in the conversion of glucose to G6P, which then may be used in glycogen synthesis, it would seem probable that synthesis of the large glycogen reserves occurs gradually over a long period, while other metabolic processes are operating at a low level. In the adult, very high levels of glucose

were found (Table 13) implying that glucose uptake occurs during maturation. However, HK was again a potential rate-limiting enzyme, and so glucose probably acts merely as a supplement to the energy derived from endogenous glycogen stores. The free hexose might be involved in the synthesis of the limited glycogen reserves within the eggs (Table 2).

In the 'resting' plerocercoid , i. e. a fully developed larva within the fish, glycolysis was the major pathway for carbohydrate breakdown. It would seem likely that, in this stage of the life-cycle, exogenous hexoses, including glucose, rather than glycogen, are the major dietary sources of energy. If hexoses are a substrate for the pathway mentioned above, then a lack of glucose during incubation of the parasite would be expected to affect the end-products released. Indeed, at 15°C an absence of glucose enhanced lactate production. A possible, though unsubstantiated explanation for this observation is that glucose might inhibit the activity of glycogen phosphorylase or phosphoglucomutase (PGM), which would in turn inhibit glycogen breakdown in the presence of the hexose, and thus favour synthesis. Under conditions where glucose is absent glycogen breakdown, and thus glycolysis could operate unhindered, possibly at a faster rate than if glucose were present. An alternative explanation is that, because the synthesis of G-6-P from glucose requires ATP, an absence of glucose would free more ATP which would then be available for the conversion of fructose-6-phosphate (F6P) to FDP; the glycolytic flux would, therefore, increase in the absence of glucose. Corroborative evidence that glycolysis occurs in the plerocercoid is provided by the presence of glycolytic intermediates (Table 13) and the fact that all enzymes of the pathway have been detected and are highly active (McManus, personal communication).

In addition to the presence of glycolytic intermediates, all the TCA cycle intermediates investigated, were found in both larva and adult. As discussed previously, molecular O_2 may be of limited

importance to the parasite and a fully functional TCA cycle is unlikely to be very active. Indeed the work of McManus (1975b) reported low activities of some critical TCA cycle enzymes. Additionally, carbon balances (Appendix 8) suggested that little, if any glycogen would be available to the 'activated' larva for operation of the TCA cycle. This is in contrast to the results of Beis & Barrett (1979) on S. solidus. It is probable, therefore, that the intermediates found are primarily concerned with CO_2 fixation and a partially-reversed TCA cycle in L. intestinalis. Malate levels exceeded those of OAA in the plerocercoid which reflects the high activity of MDH operating in the reverse direction. Propionate and succinate occurred at very high concentrations in the plerocercoid (Table 13) which corroborates the evidence that the pathway via fumarase and SDH is an active one. However, as discussed previously, a redox balance is required, and levels of pyruvate and acetate were low. ME activity was also low. It is probable, therefore, that the step via ME limits the flux of pathways from the level of malate in the plerocercoid.

As glycolysis, CO_2 fixation and a partial reversal of the TCA cycle operate in the plerocercoid, the PEP branchpoint is obviously of critical importance to the parasite. As previously described, PK catalyses the conversion of PEP to pyruvate. Since the characteristics of this enzyme were studied in detail by McManus (1975a) a comparison of its properties with the metabolite concentrations within the tissues was possible, and seemed appropriate. Millimolar metabolite concentrations (Appendix 7) were determined from the metabolite levels (Table 13) and dry/fresh weight ratios (Table 1). The activity of the adult enzyme was less than that of the plerocercoid, and its properties may be different. Thus the direct comparison of adult metabolite concentrations with plerocercoid PK must be

treated with some caution. However, the steady state concentration of PEP in the plerocercoid was close to the saturation concentration at which McManus (1975a) found maximum activity. In the adult, however, the concentration of PEP was significantly lower and lies within the ascending portion of the sigmoid curve as shown by McManus (1975a). Therefore, in the adult it is likely that small changes in the concentration of PEP would cause far more drastic changes in enzyme activity than in the plerocercoid. The concentration of malate in the tissues of the larva and adult was low (Table 13), and the results of McManus (1975a) would suggest that malate has little modulatory effect on PK activity in vivo. However, it is interesting to note that in the adult in particular, malate was released as an end-product in large amounts (Table 6). It is possible, and indeed probable that it was excreted in order that its concentration did not build up and inhibit PK. In this way the activity of glycolysis is ensured, and energy production via this pathway guaranteed. During the transition from larva to adult in L. intestinalis the activity of PK decreased (Table 17) but the isoenzymes may change as well. In H. diminuta, Carter & Fairbairn (1975) reported isoenzyme changes during the life-cycle; which they proposed controlled the specific concentration of the excreted end-products. If similar changes in isoenzymes occur in L. intestinalis then the interpretation of the above relationships will be further complicated.

In the 'activated' larva, the rise in temperature triggers maturation to the adult stage. It appears from the results of end-product release (Table 5) that maturation occurs rapidly after incubation at 40°C, since the relative levels of the end-products changed immediately. Succinate, acetate and propionate were released in increased concentrations, which suggests an increase in activity of the partial reversal of the TCA cycle and associated processes. This is corroborated by increased PEPCK and SDH

activities in the 'activated' plerocercoid (Tables 16 and 17). PFK activity also increased indicating an overall increase in the metabolic flux through the tissues of the parasite. The release of end-products was more sensitive quantitatively and qualitatively to the effects of glucose and O_2. This suggests a more flexible system of metabolism in the developing adult. This would be advantageous to the parasite, since a variety of environments will be experienced during host-transition in vivo. Firstly, after the fish is eaten by the definitive bird-host, the parasite will reside within the dead fish until digestion allows its release. This could take several hours, during which time the parasite will experience an increase in temperature, and possibly a decrease in pO_2. On emergence from the fish, the parasite will be exposed to a low pH until migration results in its reaching the small intestine. In its final position the parasite will experience not only a return to a neutral pH (Crompton, 1969) but also changes in pO_2, pCO_2, Eh and availability of nutrients. Thus flexibility in the metabolism would allow maturation to commence as soon as the temperature rose, and to continue unhindered during the transition. Obviously, by some means the glycogen store of the plerocercoid must immediately become available to the developing adult. Until host-transition occurs, glycogen synthesis rather than breakdown predominates, and it is possible that glycogen synthetase in L. intestinalis operates with a temperature optimum close to 15°C (the temperature of the fish-host), whereas glycogen phosphorylase may have a higher temperature optimum.

In the mature, egg-producing adult, glycolysis is still the major pathway of energy metabolism. It is probable that the large glycogen stores of L. intestinalis are sufficient to supply all the energy requirements of the adult via glycolysis alone. This is a relatively inefficient pathway with regards to energy

yield, but has the advantage that neither O_2 nor CO_2 are required for its functioning. PEPCK was much more active in the adult than in the plerocercoid which indicates that O_2 fixation is more important in the adult. This pathway may be more important qualitatively than quantitatively since it could give rise, via a partial reversal of the TCA cycle, to substrates for fatty-acid production; these in turn could be used as energy stores by the eggs of the parasite.

As already discussed, MDH was extremely active in the adult and this was reflected in the high concentrations of malate excreted. The release of malate allows a redox couple to be maintained, provides some malate necessary for the operation of the partial reversal of the TCA cycle and may reduce the modulatory effect upon PK. The pathways leading to the formation of acetate from malate are linked by a redox couple to those which result in propionate and succinate formation (Barrett, 1976a). Since, in the adult, there was a relative increase in the release of propionate compared to the 'activated' larva, it is possible that acetate is an important substrate for fatty-acid synthesis and therefore was not released; thus propionate would be produced and released to balance the redox potential, being less important as a substrate.

Enzymes are inevitably in control of the metabolic pathways operating in an organism. It is remarkable that similar pathways are able to operate successfully in a single organism under the physiological conditions encountered by both larva and adult of L. intestinalis. What is even more remarkable is that the parasite can cope with the 'switch' between the two stages, which takes place so rapidly. Obviously, the activity of the parasite's enzymes is likely to change during this 'switch'. With L. intestinalis, the activity of the enzymes in the plerocercoid generally increased with temperature (Tables 16 and 17). However, under

identical conditions, none of the enzymes studied showed similar activities in larva and adult. This indicates that during the maturation period of the adult worm, the enzymes altered.

The present study indicated that LDH possessed more isoenzymes in the adult (Plate 1) and this explains the increase in activity found in the adult: the characteristics of the adult isoenzymes presumably are more suited to the physiological conditions of the definitive host. However, with MDH, recent work, (McManus, personal communication) has indicated identical isoenzyme patters in larva and adult L. intestinalis. But the activity of the adult enzyme was far greater than in the larva (Table 16). This suggests that the characteristics of the enzyme have been modified without altering the isoenzyme structure extensively.

In L. intestinalis can be seen a metabolic system well adapted to the environments of the two stages studied. The metabolism also has to allow for the rapid transition from larva to adult, and its apparent metabolic 'flexibility' obviously helps. By relying upon glycolysis to a major extent for its carbohydrate breakdown the parasite may also gain a degree of independence from the physiological environment; this would certainly aid the rapid maturation of the adult. These factors in combination probably enable the rapid metabolic 'switch' to take place, and account for the parasite's success.

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APPENDIX 1

The composition of Hanks' saline.

Component	g/l
NaCl	8.000
KCl	0.400
$Na_2HPO_4 \cdot 7H_2O$	0.090
KH_2PO_4	0.060
$MgSO_4 \cdot 7H_2O$	0.100
$CaCl_2$	0.140
Glucose	1.000
$MgCl_2 \cdot 6H_2O$	0.100
$NaHCO_3$	0.350
Phenol Red	0.010

APPENDIX 2

The composition of tissue culture medium 199.

Component		mg/l
Hanks' saline salts		(see Appendix 1)
$Fe(NO_3)_3$		0.1
Adenine sulphate		10.000
Adenosinetriphosphate (Disodium salt)		1.000
Adenylic acid		0.200
alpha tocopherol phosphate (sodium salt)		0.010
Cholesterol		0.200
Deoxyribose		0.500
Glutathione		0.050
Guanine HCl		0.300
Hypoxanthine	(.354 Na salt)a	0.300
Phenol red		20.0
Ribose		0.500
Sodium acetate		50.000
Thymine		0.300
Tween 80c		20.000
Uracil		0.300
Xanthine	(.344 Na salt)a	0.300
DL-Alpha-Alanine		50.000
L-Arginne HCl		70.000
DL-Aspartic acid		60.000
L-Cysteine HCl		0.100
L-Cystine	26.00 (2 HCl)a	20.000
DL-Glutamic acid	H_2O	150.000
L-Glutamine		100.000
Glycine		50.000
L-Histidine HCl		20.000
L-Hydroxyproline		10.000
DL-Isoleucine		40.000
DL-Leucine		120.000
L-Lysine HCl		70.000
DL-Methionine		30.000
DL-Phenylalanine		50.000
L-Proline		40.000
DL-Serine		50.000
DL-Threonine		60.000
DL-Tryptophane		20.000
L-Tyrosine	57.88 (2 Na)a	40.000
DL-Valine		50.000
Ascorbic acid		0.050
d-Biotin		0.010
Calciferol		0.100
Ca pantothenate		0.010
Choline chloride		0.500
Folic acid		0.010
i-Inositol		0.050
Menadione		0.010
Niacin		0.025
Niacinamide		0.025
Para-aminobenzoic acid		0.050
Pyridoxal HCl		0.025
Pyridoxine HCl		0.025
Riboflavin		0.010
Thiamine HCl		0.010
Vitamin A (acetate)d		0.14

APPENDIX 3

Data on metabolic gradients along the length of plerocercoid worms.

	Percentage length along worm			
	0 - 25	25 - 50	50 - 75	75 - 100
Protein	25.9 ± 8.0	22.0 ± 6.0	19.5 ± 3.8	20.5 ± 6.6
Glycogen	45.6 ± 9.3	55.7 ± 10.4	56.1 ± 8.6	58.2 ± 6.6
Dry/fresh weight ratio	0.32 ± 0.04	0.34 ± 0.03	0.35 ± 0.03	0.35 ± 0.03

The results are means ± standard errors for 5 determinations. Data for protein and glycogen levels are expressed as % dry weight of worm tissue.

APPENDIX 4

Method for the determination of acetate and propionate by spectrophometric analysis (Boehringer, Mannheim; personal communication).

The reaction mixture contained:

Triethanolamine buffer	125mM, pH 7.0;
$MgSO_4$	5mM;
Coenzyme A	6mM;
NADH	15mM;
ATP	30mM;
PEP	30mM;
LDH	50 U
MK	18 U
PK	10 U

Incubation medium + H_2O to a total volume of 3.0 ml

The reaction was started by the addition of 0.375 U acetyl CoA synthetase, and the decrease in absorbance measured at 340 nm.

APPENDIX 5

Glucose uptake determined by spectrophotometric assay.

Weight of worm	Glucose uptake (n moles/10 mg dry weight/hr)
0.57	14.4
1.28	6.9
1.33	14.3
1.60	28.0
0.96	2.4
1.55	0.9
2.56	+ 8.2

+ - this denotes an apparent release of glucose into the incubation medium.

APPENDIX 6

Glucose and glycogen consumption by L. intestinalis during in vitro culture.

Period of in vitro culture	Glucose uptake (n moles/ 10 mg dry wt.)	Glycogen (mg/g dry wt.)	Consumption (* n moles/ dry wt.)
0 - 3 days	73.6	115.6	578.3
3 - 5 days	115.9	247.4	1238.2
5 - 7 days	-	198.6	994.2

The glucose uptake was determined from data in Table 12.

The glycogen consumption was determined by calculating the glycogen content of worms after 0, 3, 5 and 7 days of in vitro culture and thus extrapolating the consumption.

* expressed as glucose equivalents - determined by:

$$\text{glycogen (glucose equivalents)} = \text{wt of glycogen} \times \frac{1.11}{180}$$

APPENDIX 7

Concentration of metabolites in the tissues of plerocercoids and adults of L. intestinalis.

Metabolite	Molarity in tissue (mM) Plerocercoid	Adult
Citrate	0.18	0.22
Succinate	1.05	0.88
Malate	0.27	0.1
Oxalo	0.07	0.1
G6P	0.85	0.76
F6P	0.025	0.066
F16DP	1.2	0.2
DHAP	0.22	0.07
GAP	0.87	0.13
2PG	0.46	0.12
PEP	0.75	0.13
Pyruvate	0.17	0.1
Lactate	1.7	2.0
Acetate	0.12	0.5
Propionate	1.0	1.9
ATP	2.45	1.5
ADP	0.4	0.64
AMP	0.22	0.4
Glucose	10.6	24
GIP	0.07	0.024
Pi	16	6.6

APPENDIX 8

Glucose and glycogen utilisation and acid end-product release by 'activated' plerocercoids of L. intestinalis.

		Change (n moles/mg dry weight/24h)
*	Acidic end-products	+ 684.9
**	Glycogen	- 192.7
**	Glucose	- 2.45

* Calculated from data in Table 5: Total end-product release by plerocercoid, in the presence of glucose, under aerobic conditions.

** Calculated from Appendix 6.