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COMPARATIVE CARBOHYDRATE METABOLISM OF ADULT AND LARVAL *MULTICEPS SERIALIS*

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COMPARATIVE CARBOHYDRATE METABOLISM OF ADULT AND LARVAL *MULTICEPS SERIALIS*

CHAPTER I

Introduction

Investigation of cestode metabolism has been extensive especially with respect to adults. Polysaccharide distribution, carbohydrate content, and carbohydrate dietary requirements have been investigated by Read (1949), Read and Rothman (1957), Fairbairn (1953), and Read, Schiller, and Phifer (1958). As pointed out in von Brand's review (1960), all the enzymes of the Embden-Meyerhof sequence have not been demonstrated in any single species of worm, although it is assumed that the complete cycle is present (Read, 1951, 1952, 1953, 1956; Laurie, 1957, Read and Rothman, 1958, Agosin and Arevena, 1959). Little is known concerning alternate carbohydrate metabolic pathways in cestodes. The work of Agosin and Arevena (1959) indicates that the hexose monophosphate pathway is operative to a significant degree in the scolices of the hydatid cyst of *Echinococcus granulosus*. Investigation of this aerobic pathway in other cestodes has not been made. Enzymes of the citric acid cycle in the form of various dehydrogenases have been reported by Read (1952) and by Goldberg and Nolf (1954). Attempts to demonstrate other enzymes of the TCA cycle have been unsuccessful. Most

of the work cited above, except where noted specifically, has been done on adult Hymenolepis diminuta.

Knowledge of the physiology of adult cestodes may or may not be suggestive of larval metabolic patterns. The physiological consequences involved in the transmission of a larval cestode to the definitive host with subsequent production of the adult would seem to tax the metabolic adaptability of these organisms. However, we know little about this because studies on larval forms, comparable to those noted for adult cestodes have been relatively few.

Heyneman and Voge (1957) demonstrated a polysaccharide (probably glycogen) in cysticercoids of H. diminuta. Hopkins and Hutchinson (1960) showed that glycogen constitutes 43% of the total dry weight of Taenia taeniaeformis larvae. Phifer (1958) demonstrated the presence of aldolase in the larva of Taenia crassiceps. Agosin, et al. (1957), in the first of a series of papers on E. granulosus hydatid cyst scolices, noted that sulfhydryl inhibitors were effective in cutting down oxygen consumption and concluded that certain enzymes of the Embden-Meyerhof sequence were present. They also found that cyanide was an effective inhibitor which suggests the presence of a cytochrome system. Agosin and Arevena (1959) demonstrated four enzymes of a pentose pathway in the scolices of the hydatid cyst of E. granulosus. Recently, Agosin and Repetto (1961) showed that the Embden-Meyerhof sequence accounted for 60% of the metabolism of glucose, the pentose pathway for 20%, and non-triose pathways for 20% in this larva.

With one exception, in all the work undertaken to date, only the larva or the adult has been used in the studies. No comparative work

on the larval and adult forms of the same genus has been carried out except by von Brand, et al. (1961), who performed a series of comparative studies on the strobilocercus and adult of Taenia taeniaeformis. The results of that study indicate the major metabolic between the larva and adult are of a quantitative rather than qualitative nature.

No work has been done on either the adult or the larva of Multiceps serialis. This taenioid cestode lives as an adult in the small intestine of canines and as a larva intermuscularly and subcutaneously in lagomorphs. It would appear that the oxygen tension of the small intestine provides a relatively anaerobic environment for the adult as compared to that provided for the larva by the subcutaneous tissues and voluntary muscle. This apparent environmental difference between the larva and the adult suggests the possibility that the metabolic patterns in the two forms may be different.

This work is based on a partial analysis of the metabolism of the larval and adult forms of Multiceps serialis in an effort to compare and evaluate their carbohydrate metabolism.

CHAPTER II

MATERIALS AND METHODS

Parasitological Methods

Adult *Multiceps serialis* were obtained from female dogs which were infected when approximately six weeks of age. Gravid proglottids which were shed continuously following initial infection were used throughout the experimental determinations as the adult material.

Coenuri were obtained in the field from freshly killed black-tailed jackrabbits (*Lepus californicus*) taken near Ft. Cobb, Caddo County, Oklahoma. They were not completely excised from the host tissue until returned to the laboratory. Upon removal from the rabbit, while still surrounded by the adventitious tissue, they were placed in 0.75% saline. After complete removal, the coenuri were placed in a phosphate buffered (pH 7.2), 0.75% saline. They were then stored in an icebox in covered fingerbowls at 5° to 10° C. until used.

An attempt was made to establish a laboratory-infected colony of domestic rabbits. Oral infection was attempted with both ripe proglottids and free eggs; with hatched oncospheres injected into the abdominal cavity, intramuscularly, subcutaneously, and directly into both the large and small intestine. All of these attempts at artificial infection failed.

Gravid proglottids were collected daily in large numbers from excreta of the infected dogs. Proglottids were used the day of collection whereas the larvae were kept for up to 96 hours before being used. The adults were collected in 0.75% saline, washed several times and then kept in buffered saline at room temperature until utilized.

Tissue Preparation

Coenuri were removed from the host without breaking the cyst wall where possible. At the time of experimentation, the cyst was ruptured and the cyst fluid saved and utilized for enzyme assays described later. The coenurus wall was cut into approximately equal portions having a similar numbers of scolices. The pieces were then utilized for the manometric determinations. The adult cestodes were not cut or otherwise harmed prior to manometric determination. Tissue was blotted, then weighed on a Roller-Smith tissue balance. The quantity of adult tissue varied from 40 to 100 mg tissue per flask while larval tissue varied from 100 to 250 mg tissue per flask.

For some enzyme determinations, the material was homogenized in a Potter-Elvehjem all-glass tissue homogenizer suspended in icewater. The homogenate was centrifuged for ten minutes at approximately 2000 g's. The supernatant was used for enzyme assays. Enzyme activity of tissue is based on mg tissue wet weight per ml whole homogenate prior to centrifugation. The cyst fluid was not altered in any way prior tothe enzymic determinations. The enzyme activity of cyst fluid is based on ml of fluid and not on a weight basis. In some cases, the supernatant and the cyst fluid were stored frozen up to 30 days with no loss of

activity.

Analytical Methods

Standard manometric techniques utilizing Warburg's 'Direct Method' were used (Umbreit et al. 1959). The oxygen consumption was determined in Warburg flasks having a capacity of about 15 ml. The final fluid volume was always 2.7 ml. The reaction medium was phosphate buffered 0.75% saline, pH 7.2. Aerobic determinations were made at 37.5° C with air as the gas phase. Carbon dioxide production was measured at 37.5° C with the anaerobic gas phase being 95%N_2-5%CO_2. The incubation fluid was Krebs-Ringer bicarbonate at pH 7.2. No correction was made for the bound CO_2 for either the larval or adult forms because of the large and variable quantities of CO_2 liberated from calcareous corpuscles. The release of CO_2 from calcareous corpuscles seems to be a common phenomenon associated with taenioid cestodes. Unless otherwise noted, the experiments were terminated after three hours. Additions of carbohydrate substrate from the sidearms were made after a one-hour period of incubation had firmly established an endogenous rate of gas exchange. Other additions are noted where appropriate.

Phosphohexose isomerase activity was measured colorimetrically by the method of Bodansky (1954), where the amount of enzyme activity is related to the amount of fructose-6-phosphate produced by the enzyme under the prescribed conditions of the assay.

Aldolase was measured by the method of Sibley and Lehninger (1949). This test utilized hydrazine to trap the triose phosphates which are then converted by hydrolysis into their characteristic osazones.

The assay of lactic dehydrogenase was carried out colorimetrically following the method of Cabaud and Wroblewski (1958). After the addition of a known quantity of pyruvate followed by a period of incubation with the enzyme, the pyruvate remaining is proportional to the amount of lactic dehydrogenase present in the tissue.

Isocitric dehydrogenase was measured following the colorimetric procedure of Wolfson and Williams-Ashman (1957). It depends on the formation of a characteristically colored osazone of alpha-ketogluarate which can be measured at a wavelength of 400 mμ.

A manometric assay was utilized for the determination of malic dehydrogenase. This procedure was based on the work of Read (1951). The presence of the enzyme is reflected in the oxygen consumption under the specified conditions of the assay.

Unless otherwise noted, all values are expressed as the mean $\pm t^{.975}_{.025}$ x S.E.=95% confidence limits. The number of determinations in any particular series of measurements follows this value in parentheses.

CHAPTER III

RESULTS

Ecological Observations

Certain features of the results of field collections are presented in this section because of their implication in the ecology of larval *M. serialis*.

The coenuri utilized were taken from jackrabbits killed in May through July of 1962 in Caddo County, Oklahoma. Results of the Caddo County collections as well as those made in two other areas are shown in Table 1. One of these was made near the University of Oklahoma Biological Station, Marshall County, Oklahoma, during the months of June and July, 1960. The other was from a large area in southeastern Colorado where collections were made during January through April of 1958.

Lyons *et al.* (1960) reported the coenurus infection rate for jackrabbits in southwestern Kansas to be 19%. This does not appear to be significantly greater than that reported herein for jackrabbits taken in southeastern Colorado (Table 1). It will be noted, however, that both the figures reported for Kansas and for Colorado are considerably lower than those reported for Oklahoma.

Less than half the *L. californicus* collected in the Colorado study

TABLE 1

RESULTS OF FIELD COLLECTIONS

Total no. rabbits	Total no. infected	% rabbits infected	Total no. with sex records	Total no. males	No. males infected	% males infected	Total no. females	No. females infected	% females infected
[a] 99	12	12	15	9	1	11	6	5	83
[b] 81	41	51	70	27	10	37	43	23	54
[c] 180	53	29	85	36	11	31	49	28	57

[a] Colorado collections

[b] Oklahoma collections

[c] Total collections

were sexed and as a result have not been included in data regarding the per cent infection in males and females. The sex records for Oklahoma collections are nearly complete. Table 2 shows the results and significant differences between the rates of infection for the two sexes. A two by two contingency chi square test with Yates correction indicates a significantly higher infection rate in female jackrabbits than in males.

A total of 109 coenuri were taken from 53 infected rabbits in all three areas included in this study. Ninety were obtained from the posterior half of the body (that part of the animal extending posteriorly from the thoracic cavity and the rib cage). This is a definitely favored site of infection. It appears therefore that there may be also a definite route of migration by the larvae of M. serialis in the intermediate host whereby the larvae will usually end up in a particular part of the body. Although a route of infection by the larvae has been suggested by Olsen (1962), no verification can be found in published investigations.

TABLE 2

COMPARISON OF MALE AND FEMALE INFECTION RATES

	Infected	Non-infected	Total
Males	11 (16.02) 1.573	25 (19.98) 1.261	36
Females	28 (22.48) 1.097	21 (26.52) 0.968	49
Total	39	46	85

Chi square, with Yate's correction, 4.899 with 1 degree of freedom. p.> 03.

Manometric Studies

In order to ascertain differences or similarities existing between the gross metabolic behavior of the larva and adult, the oxygen consumption and carbon dioxide production were measured under varying conditions. The gas evolution and consumption were measured with no substrate present and then in the presence of glucose, fructose, galactose, sucrose and xylose.

Oxygen consumption of both larvae and adults was measured as described in the section on materials and methods. After ten minutes of equilibration, followed by a one-hour period used to establish the normal endogenous metabolic rate, substrate was added from the sidearm. Readings were then taken over the next two hours to determine the effect of the particular substrate

TABLE 3

OXYGEN CONSUMPTION

Larvae

Substrate and final molarity	*Q_{O_2} Before addition	Q_{O_2} After addition	t-test Q_{O_2} of mean before addition against mean after addition of substrate
Glucose 0.02M	0.223 ± 0.053 (5)	0.326 ± 0.066 (5)	.01 > p > .005
Fructose 0.02M	0.098 ± 0.020 (6)	0.090 ± 0.007 (6)	.40 < p
Galactose 0.02M	0.160 ± 0.040 (6)	0.166 ± 0.053 (6)	.40 < p
Xylose 0.02M	0.188 ± 0.030 (6)	0.178 ± 0.027 (6)	.30 > p > .20
Sucrose 0.02M	0.134 ± 0.025 (6)	0.117 ± 0.023 (6)	.20 > p > .10

Adults

Substrate and final molarity	Q_{O_2}	t-test Q_{O_2} of mean 'no substrate' controls against mean with substrate
'No substrate' controls	0.477 ± 0.107 (29)	
Glucose 0.02M	0.644 ± 0.100 (12)	.01 > p > .005
Fructose 0.02M	0.769 ± 0.204 (11)	.005>p
Galactose 0.02 M	0.502 ± 0.234 (17)	.40 < p

TABLE 3 CON'D.

Xylose 0.02 M	0.455 ± 0.060 (7)	.40 > p > .30
Sucrose 0.02 M	0.544 ± 0.108 (7)	.20 > p > .10

*Q_{O_2} expressed as mean oxygen consumption in µl/hr/mg tissue wet weight ± $t_{.025}^{.975}$ x S.E. = 95% confidence limits. All substrate additions made after one hour time period and experiments were terminated after one hour incubation with substrate. t-Test based on single tail probabilities.

TABLE 4

CARBON DIOXIDE PRODUCTION

Larvae

Substrate and final molarity	*Q_{CO_2} Before addition	Q_{CO_2} After addition	t-test Q_{CO_2} of mean before addition against mean after addition of substrate
Glucose 0.02M	0.498 ± 0.040 (12)	0.440 ± 0.070 (12)	.10 > p > .05
Fructose 0.02M	0.562 ± 0.159 (6)	0.441 ± 0.152 (6)	.10 > p > .05
Galactose 0.02M	0.551 ± 0.084 (6)	0.536 ± 0.081 (6)	.40 > p > .30
Xylose 0.02M	0.578 ± 0.085 (6)	0.546 ± 0.119 (6)	.40 > p > .30
Sucrose 0.02M	0.612 ± 0.098 (6)		.20 > p > .10

Adults

Substrate and final molarity	Q_{CO_2}	t-test Q_{CO_2} of mean 'no substrate' controls against mean with substrate
'No substrate' controls	1.159 ± 0.113 (24)	
Glucose 0.02M	1.524 ± 0.467 (9)	.05 > p > .025
Fructose 0.02M	1.594 ± 0.217 (10)	.005 > p
Galactose 0.02M	1.489 ± 0.314 (10)	.025 > p > .01
Xylose 0.02M	1.228 ± 0.219 (16)	.30 > p > .20

TABLE 4 CONT'D.

Sucrose 0.02M	1.202 ± 0.164 (21)	.40 > p > .30

*Q_{CO_2} expressed as µl gas production/hr/mg tissue wet weight ± $t^{.975}_{.025}$ times S.E. = 95% confidence limits. All substrate additions were made after one hour incubation and experiments were terminated after one hour incubation with substrate. t-Test based on single tail probabilities.

on the aerobic metabolism. The last one-hour reading after the addition of the substrate was then used in a t-test to evaluate the observed differences between the endogenous oxygen consumption and that recorded in the presence of substrate. The endogenous rate is approximately linear over a three-hour period. The 'no substrate' controls were utilized for comparative evaluation in the t-tests. The adult endogenous O_2 consumption was essentially constant from experiment to experiment, but this was not the case for the larvae. The larvae varied in size, number of scolices, location in intermediate host, etc. Because of these differences, the endogenous oxygen consumption of each larva was first determined before substrate addition. The gas uptake for each individual larva was then used in the t-test along with the uptake readings for the same larva in the presence of substrate.

The results (Table 3) show that the adults utilize both glucose and fructose as reflected by the changes in oxygen consumption in the presence or absence of these two substrates. The larvae on the other hand appear capable of using only glucose. It was also found that the oxygen consumption of the larvae is considerably less than that of the adult.

Carbon dioxide production of the larvae and adults is given in Table 4. Following a ten minute period of gassing by 95%N-5%CO_2 and a ten minute period of equilibration, the endogenous gas evolution under anaerobic conditions was measured. After two hours of endogenous activity, the substrate additions were made, followed by another two hours of measurement. The last hour before substrate addition was then compared with the last hour after substrate addition and the results of the two were then

evaluated by an appropriate t-test.

The results show that glucose, fructose and galactose alter CO_2 production in adult M. serialis. No substrate had any effect on gas production in larvae. These results, obtained under anaerobic conditions, do not parallel those obtained under aerobic conditions when use is made of the same substrates.

Enzyme Studies

Five enzymes were selected for analysis in this investigation. As indicated previously, several enzymes of the Embden-Meyerhof sequence have been found in several different cestodes though not all from any one species. It has also been suggested that the citric acid cycle is probably incomplete in that the presence of several enzymes of the cycle have not been demonstrated, though attempts have been made to establish their presence.

Three enzymes selected are associated with the glycolytic pathway and two are in the TCA cycle. Evidence for the presence of other enzymes in both pathways will be presented in the section on metabolic inhibitors.

The colorimetric assays utilized in this investigation are based primarily on procedures outlined by various Sigma Chemical Company Technical Bulletins.

Phosphohexose Isomerase

This enzyme catalyzes the conversion of G-6-P to F-6-P. The test for this enzyme is based on the fact that F-6-P will give a characteristically colored compound at a wavelength of 490 mμ when treated with resorcinol in

a highly acid medium. With the addition of a known quantity of substrate, F-6-P formed from G-6-P under the influence of phosphohexose isomerase can then be calculated.

Results of these assays shown in Table 5 provide evidence for the presence of phosphohexose isomerase in the gravid proglottids, the coenurus, and the cyst fluid of M. serialis.

Aldolase

Aldolase is responsible for the conversion of fructose diphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The presence of the enzyme is determined by change in the rate of formation of characteristically colored osazones of triose phosphates formed from fructose diphosphate as mediated by the enzyme. The density of color produced by the osazones is read at a wavelength of 540 mμ.

The results (Table 6) show the presence of aldolase in adult gravid proglottids, coenuri, and cyst fluid of M. serialis.

Lactic Dehydrogenase

This enzyme catalyzes the conversion of pyruvate to lactate, DPNH being a necessary coenzyme for the reaction. Pyruvate reacts with 2,4-dinitrophenylhydrazine to give a characteristically colored hydrazone which is measured at a wavelength of 500mμ. A known quantity of substrate (pyruvate) is incubated for a definite period of time and the enzyme activity is terminated by the addition of 2,4-dinotrophenylhydrazine. The quantity of pyruvate remaining after this procedure is a reflection of the activity of lactic dehydrogenase present in the tissue or tissue fluid.

TABLE 5

PHOSPHOHEXOSE ISOMERASE ACTIVITY

Enzyme source	*µgm F-6-P/mg tissue	** Units activity/ mg tissue
Adult tissue	23.6±8.8 (5)	24
Larval tissue	7.9± 2.9 (5)	8
Cyst fluid	102.5± 27.5 (6)	149

*µgm F-6-P/ml cyst fluid where cyst fluid used as source of enzyme. ** Units activity expressed as reciprocal of concentration of unknown in cc per cc of reaction mixture that will cause the formation of 25 µgm F-6-P per cc of reaction mixture in 30 minutes. µgm F-6-P in table obtained by subtraction of F-6-P at zero time from that obtained after incubation period. Experimental procedure used in the assay of phosphohexose isomerase is outlined in Sigma Technical Bulletin No. 650 as published by Sigma Chemical Company, St. Louis, Missouri. It is based on a procedure of Bodansky (1954). All reagents are the same as those called for by the technical bulletin except that the tissue homogenate preparations and cyst fluids are substituted for serum when appropriate.

TABLE 6

ALDOLASE ACTIVITY

Enzyme source	* units activity/mg tissue	µgm ALP/hour/mg tissue
Adult tissue	12.3±0.5 (7)	34.4
Larval tissue	2.6±0.6 (6)	7.3
Cyst fluid	18.2±4.3 (6)	50.9

*Units activity expressed as that amount of enzyme which will split one cubic millimeter of fructose-1,6-diphosphate per hour at 37° C under the conditions prescribed under the assay procedure. Units activity expressed in terms of ml cyst fluid when cyst fluid used as a source of enzyme. Units activity expressed in table were obtained after subtraction of units of aldolase at zero time from that obtained after incubation period. Experimental procedure used in assay of aldolase is outlined in Sigma Technical Bulletin No. 750 as published by Sigma Chemical Company, St. Louis, Missouri. It is based on the procedure of Sibley and Lehninger, (1949). All reagents are the same except that the tissue homogenate preparations and cyst fluid were substituted for serum where appropriate.

The results of this series of assays (Table 7) indicate that an active lactic dehydrogenase is present in adult gravid proglottids, the coenurus tissue, and coenurus fluid of *M. serialis*.

Isocitric Dehydrogenase

Isocitric dehydrogenase when present with TPN causes the conversion of isocitrate into alpha-ketoglutarate and CO_2, TPN being reduced to TPNH in the process. When treated with 2,4-dinitrophenylhydrazine in a highly alkaline medium, alpha-ketoglutarate is converted to a hydrazone with a characteristic color which can be measured at a wavelength of 400 mμ.

Results from this assay (Table 8) indicate the presence of the enzyme in the coenurus fluid. Activity of the enzyme in the tissues, as measured by this technique, is questionable. Although some activity was measurable in both larval and adult tissues, it was quite low. The results of inhibitory effects of monofluoracetic acid may aid in a final determination of the enzyme's presence.

Malic Dehydrogenase

Malic dehydrogenase catalyzes the conversion of malate to oxalacetate in the presence of DPN which in the reaction is reduced to DPNH. The enzyme in homogenates of gravid proglottids and coenural tissues was determined by measuring oxygen consumption under controlled conditions after the method of Read (1953). Its presence was ascertained in the homogenates after the addition of methylene blue as an electron acceptor, of cyanide as a binding agent to keep the reaction from estab-

TABLE 7

LACTIC DEHYDROGENASE ACTIVITY

Enzyme source	* units activity/mg tissue
Adult tissue	79.1±2.6 (3)
Larvae tissue	26.8±3.3 (6)
Cyst fluid	365.8±117.9 (6)

*Units activity expressed as that amount of enzyme that would cause a decrease in $O.D._{340}$ of 0.001/minute in a reaction mixture described by Wroblewski (1955). Units activity expressed in terms of ml cyst fluid when cyst fluid used as a source of enzyme. Units activity presented in table were corrected for zero time values in all cases. Experimental procedure used in assay of lactic dehydrogenase is outlined in Sigma Technical Bulletin No. 500 as published by the Sigma Chemical Company, St. Louis, Missouri. Al reagents are the same except that tissue homogenate preparations and cyst fluid are substituted for serum where appropriate. The lactic dehydrogenase activity is based on the procedure of Cabaud and Wroblewski (1958).

TABLE 8

ISOCITRIC DEHYDROGENASE ACTIVITY

Enzyme source	*units activity/ mg tissue
Adult tissue	1.99± 1.05 (7)
Larval tissue	1.86± 0.40 (6)
Cyst fluid	23.25± 10.22 (4)

*Units activity expressed as that amount of enzyme which causes the formation of one millimicromole of alpha-ketoglutarate per hour at 25° C when the test is performed as described in Sigma Technical Bulletin No. 175. Units of activity expressed in terms of ml. cyst fluid when used as a source of enzyme. Experimental tests corrected by subtraction of alphaketoglutarate present at zero time. Experimental procedure used in the assay of isocitric dehydrogenase is outlined in Sigma Technical Bulletin No. 175 as published by Sigma Chemical Company, St. Louis, Missouri. It is based on the procedure of Wolfson and Williams-Ashman (1957). All reagents are the same except that the tissue preparations and cyst fluid are substituted for serum where appropriate.

TABLE 9

MALIC DEHYDROGENASE ACTIVITY

System	Larvae Q_{O_2}	Adult Q_{O_2}
Complete	0.222 (3)	0.574 (6)
Minus malate	0.163 (3)	0.451 (6)
Minus DPN	0.107 (3)	0.302 (6)
Minus methylene blue	0.062 (3)	0.133 (2)

Complete reaction mexture for malic dehydrogenase system as follows: 1 cc 0.1M phosphate buffer (pH 7.5); 0.3 cc 0.5M malic acid; 0.3 cc 0.8M KCN: 1 mg methylene blue; 1 mg DPN in 0.3 cc 0.154M KCl added from sidearm after 10 minutes equilibration; 0.3 cc 20% tissue homogenate prepared in 0.154M KCl. All readings are calculated after equilibration period and represent µl O_2 uptake/mg tissue wet weight/hour. Substitutions made with 0.154M KCl.

lishing equilibrium in the wrong direction, and of DPN as the intermediate electron acceptor.

The results (Table 9) show that this enzyme is present in homogenates of both adult and larval tissues.

Metabolic Inhibition

Metabolic inhibitors provide evidence relative to the presence of particular enzymes and enzyme systems associated with both the Embden-Meyerhof sequence and the Krebs cycle.

All values for determining the percentage inhibition for any particular inhibitor are based on the change in oxygen consumption or in carbon dioxide production. The change in activity is expressed as a change in gas uptake or output during the last 30 minute period of endogenous activity versus the gas uptake or output during the last 30 minutes following the addition of the inhibitor. The last 30 minute period of endogenous activity was selected for comparison since it represents the one of minimum activity for the tissue. The last 30 minute period of activity following addition of the inhibitor was used for comparison with the endogenous rate because it represents the highest level of effectiveness of the inhibitor. The treatment of control flasks was identical with that of the experimentals except that buffered pH 7.2 physiological saline or Krebs-Ringer bicarbonate pH 7.2 was added instead of the inhibitor.

The data in Table 10 represent the results of the effects of metabolic inhibitors on the oxygen consumption of the gravid proglottids and coenuri.

TABLE 10

AEROBIC INHIBITION

Larvae

Inhibitor and final molarity	*O_2 consumption during last 30 minutes before addition of inhibitor	*O_2 consumption during last 30 minutes after addition of inhibitor	% change
Malonate 0.01M	0.080±0.022(6)	0.079±0.019	-0.9%
Malonate 0.05M	0.097±0.033(9)	0.038±0.017	-60.8%
Monofluoroacetate 0.005M	0.068±0.023(12)	0.050±0.013	-27.5%
dl-Glyceraldehyde 0.01M	0.065±0.013(6)	0.069±0.022	+ 5.8%
Sodium Fluoride 0.02M	0.084±0.028(6)	0.055±0.027	±34.5%
Sodium Fluoride 0.1M	0.070±0.020(12)	0.044±0.017	-37.1%
Iodoacetate 0.001M	0.080±0.017(6)	0.014±0.007	-82.5%
para-HMBRA 0.01M	0.052±0.018(6)	0.015±0.004	-71.2%
Iodoacetamide 0.01M	0.060±0.015(6)	0.029±0.008	-51.7%
Control (no inhibitor)	0.071±0.019(87)	0.078±0.005(18)	+9.1%

*O_2 consumption expressed as µl gas uptake/30 minutes/mg tissue wet weight± $^{.975}_{.025}$ x S.E.=95% confidence limits. Larval per cent change based on mean of last 30 minute reading before addition of inhibitor versus mean of last 30 minute reading prior to termination of experiment after addition of inhibitor. This is based on fact that individual coenuri varied from one another in regard to size, number of scolices, location in intermediate host, etc. Except where specifically noted in text, all inhibitors employed in the experiments were significant at the 5% level of significance when evaluated by a t-test.

TABLE 10 (CONT'D.)

AEROBIC INHIBITION

Adults

Inhibitor and final molarity	*O_2 consumption during last 30 minutes before addition of inhibitor	*O_2 consumption during last 30 minutes after addition of inhibitor	% change
Malonate 0.01M	0.291±0.042(11)	0.284±0.049	+5.6%
Malonate 0.05M	0.229±0.044(6)	0.0884±0.035	-67.2%
Monofluoroacetate 0.005M	0.245±0.024(14)	0.174±0.044	-35.1%
dl-Glyceraldehyde 0.01M	0.249±0.028(18)	0.310±0.039	+13.5%
Sodium Fluoride 0.02M	0.235±0.032(12)	0.158±0.047	-41.0%
Sodium Fluoride 0.1M	0.333±0.060(6)	0.136±0.047	-49.3%
Iodoacetate 0.001M	0.335±0.027(7)	0.109±0.065	-59.3%
para-HMBA 0.01M	0.309±0.045(7)	0.055±0.037	-79.5%
Iodoacetamide 0.01M	0.250±0.21(4)	0.050±0.042	-81.3%
Control (No inhibitor)	0.268± 0.025(18)	0.284±0.027	+5.6%

*O_2 consumption expressed as µl gas uptake/30 minutes/mg tissue wet weight ±t.975 x S.E.=95% confidence limits. Adult per cent change based on mean .025 of last 30 minute reading before addition of inhibitor versus mean of last 30 minute reading prior to termination of experiment after addition of inhibitor. This is based on comparison of readings with inhibitor and readings of control experiments. Except where specifically noted in text, all inhibitors employed were tested with the control readings prior to addition from sidearm and were found significant at the 5% level when evaluated by a t-test.

Since no attempts were made to evaluate any enzyme in terms of exact kinetic values, it is not possible to draw conclusions regarding the comparative effectiveness of the inhibitors on larval or adult gas exchanges. Inhibitors were utilized, therefore, only to provide evidence for the presence or absence of particular enzymes through the determination of change in respiratory activity following the addition of the inhibitor.

Following the addition of the sidearm contents, readings were made at 30-minute intervals for a period of two hours. In some cases, a reproducible fluctuation in gas exchange was observed during the 30-minute time periods, all of which appeared to be complemental with respect to one another. Control readings did not coincide and in fact appeared to be quite steady over the two-hour period following sidearm addition. No explanation can be given for such fluctuations. Differential permeability of the tissue to the inhibitor or enzyme mechanics in response to specific inhibitors may possibly influence these fluctuations. No attempt was made to determine the cause of the fluctuations since this would require a complete analysis of the kinetics of each enzyme and this is not an objective of this thesis.

From the results in Table 10, certain evaluations may be made. It appears that sulfhydryl group inhibitors such as iodoacetate, iodacetamide, and parahydroxymercuribenzoate are especially effective under aerobic conditions, in both larval and adult tissues. Sodium fluoride which is inhibitory of the enzyme enolase also has a definite effect both on adult gravid proglottids and on coenuri.

dl-Glyceraldehyde, which is effective against glucokinase, does not appear to change respiratory activity in larval tissues. However, in several experiments utilizing gravid proglottids, dl-glyceraldehyde evoked a significant increase in oxygen consumption. It is possible that the inhibitor in this case was actually oxidized by the tissues and thus was used as a substrate for certain metabolic activity.

Malonate did not inhibit succinic dehydrogenase in either adult or larval tissues when used in low concentrations. However, when the concentration was increased fivefold, inhibition was definite.

Monofluoroacetate is an effective inhibitor of citrate metabolism. Since citrate metabolism is inextricably coupled with the enzyme isocitric dehydrogenase, it was thought that the use of monofluoracetate would provide further information regarding the presence or absence of the enzyme in the tissues of this cestode. Data in Table 10 show that oxygen consumption in the adult tissues is decreased in the presence of monofluoracetate. The gas uptake in the larvae before and after addition of this inhibitor is below the five per cent level of significance, thus indicating a lack of inhibition. It must be noted, however, that there is a significant decrease in the oxygen consumption when the level of inhibition is compared to the overall mean of 87 control vessels. This decrease is significant when subjected to a t-test, at more than the 5% level. There is therefore, positive evidence for citrate metabolism in adult worms but no positive evidence for it in larval tissues in light of evidence presented here.

Inhibition of carbon dioxide production was studied in the same man-

ner as described for the experiments using carbohydrate substrates, the time duration being the same as that employed in the study of the effects of metabolic inhibitors on oxygen consumption.

Data in Table 11 present definite indication of inhibition of CO_2 production by iodoacetamide in both larvae and adults. dl-Glyceraldehyde does not appear to inhibit CO_2 production in either adult or coenural tissue. Sodium fluoride had no noticeable effect on gas production in the adults but did evoke a definite inhibition in larval tissues.

TABLE 11

ANAEROBIC INHIBITION

Larvae

Inhibitor and final molarity	*CO_2 production last 30 minutes before addition	*CO_2 production last 30 minutes after addition	% change
Sodium Fluoride 0.02M	0.251 ± 0.033 (12)	0.212 ± 0.028	-15.6%
dl-Glyceralydehyde 0.01M	0.171 ± 0.029 (10)	0.158 ± 0.027	-7.6%
Iodoacetamide 0.01M	0.198 ± 0.077 (6)	0.032 ± 0.040	-84.0%
	Adults		
Sodium fluoride 0.02M	0.572 ± 0.167 (5)	0.557 ± 0.089	-2.8%
dl-Glyceraldehyde 0.01M	0.503 ± 0.057 (15)	0.504 ± 0.057	+0.2%
Iodoacetamide 0.01M	0.463 ± 0.093 (6)	0.031 ± 0.031	-94.6%

All values expressed as µl CO_2 production/30 minutes/mg tissue wet weight ± t.975 times S.E. = 95% confidence limits. Per cent change based on mean of .025 last 30 minute reading before addition of inhibitor versus mean of last 30 minutes after addition of inhibitor prior to termination of experiment.

CHAPTER 4

DISCUSSION

Results of field investigations in this study provide a basis for several implications regarding the host-parasite relationship existing between Lepus californicus and the larvae of M. serialis.

Initially it was intended that experimental work on coenuri would be carried out on larvae grown in domestic rabbits. After a series of attempts failed in experimental infection (described previously), larvae were collected from wild hosts.

While Lyons et al. (1960) found that 19% of a black-tailed jackrabbit population in southwestern Kansas was infected by coenuri, I found that only 12% were infected in Colorado. During 1962, the infection rate in Oklahoma was more than twice that of either Kansas or Colorado. Such a difference in infection rates may be a reflection of normal year-to-year fluctuations. However, differences as large as those reported in the three collecting areas of Oklahoma, Kansas, and Colorado appear to require further field investigation before a suitable explanation can be given.

In performing autopsies on each animal, it was found that 85% of all the larvae in the rabbits were in the posterior portion of the body. This suggests the possibility of a definite route of migration by the onchosphere or that there is some kind of physiological attractant for the onchosphere

in the posterior half of the body.

With the posterior half of the body appearing to be the preferential site of infection for coenuri one wonders if there is a survival value to the larvae finding their way to this portion of the animal. The initial result of such survival value would be the successful completion of the life cycle and, as a final consequence, the propagation of this particular type of coenurus.

Since there is a definite site of infection in the intermediate host by coenuri a definite route of infection seems undoubtedly to occur. Contrary to what Olsen (1962) indicates, however, no published work can be found to substantiate a route of infection. Therefore, a definite migration route remains to be determined. The pathology of coenuriasis, the nature of a physiological attractant (if one exists), the process of development after reaching the final site of infection remain unexplained.

The respiratory characteristics were studied in an effort to determine what differences, if any, occur between the larvae and adults of M. serialis. Numerous studies on both larvae and adults of other cestodes have been reported in the literature. However, only one other study relating larval and adult cestodes of the same species has been reported to date. In this investigation by von Brand and Bowman (1961), they determined the respiratory characteristics and the chemical constituents of the strobilcercus and adult of Taenia taeniaeformis. They found that the two forms varied only slightly qualitatively while the quantitative variations were quite significantly different. These authors found that the rates of aerobic and anaerobic gas exhanges occured at a much lower level in larvae

as compared to adults. Both utilized glucose. It was also determined that both forms excrete some type of carbohydrate into the incubation medium at the same time that glucose is having its effect on the oxygen consumption.

Read (1959) has summarized the data available on carbohydrate metabolism of adult cestodes. He states that of nine species representing three orders of cestodes, none was reported as being capable of utilizing fructose as a substrate. It is further stated that glucose and galactose are the sugars of favor in carbohydrate metabolism of cestodes. Agosin, et al. (1957), report that oxygen consumption by E. granulosus hydatid cyst scolices does not appear to be altered by glucose.

The results from my investigation indicate that both the adults and larvae of M. serialis are capable of utilizing glucose under aerobic conditions. Oxygen consumption by the adult is also altered by fructose. That the adult is capable of utilizing fructose does not seem surprising when the diet and exposure to substrate of the adult and larval forms are analyzed. Fructose is present in a large part of the diet of coyotes during the summer months (Karschgen, 1957). The coenuri located intermuscularly are exposed only to that quantity of fructose circulating in the blood stream which would, under normal circumstances, be negligible.

Herein is the possible answer to the question of which substrate any particular cestode is capable of utilizing for energy purposes. It would appear that those substrates to which the cestode is continously exposed would be those utilized in maintaining metabolic requirements.

In the case of T. taeniaeformis, the major differences in aerobic

metabolism between the larva and adult are of a quantitative rather than qualitative nature. This seems also to be the case for M. serialis. The mean oxygen consumption of the adult far exceeds that of the larva. The same relative comparison can be made for carbon dioxide production of the adult and larva under anaerobic conditions.

These findings do not confirm the prediction made in the introduction that there should be a greater rate of oxygen consumption in larvae in response to the relatively higher oxygen tension in voluntary muscle. It must be pointed out that some investigators (Read, 1950) consider that the paramucosa of the small intestine offers an environment similar in oxygen tension to that of intercellular spaces. If this be true, then it is quite possible that the larvae and adults of M. serialis exist in a very similar environment.

Studies on substrate utilization by the larvae and adults under anaerobic conditions produced qualitatively different results to those obtained aerobically. Adults appear capable of utilizing galactose as well as glucose and fructose anerobically. This apparent deviation from the established aerobic pattern is not readily explained.

No substrate studied in this investigation altered the CO_2 production by the larvae of M. serialis. This finding may be related to the fact that carbon dioxide and other gases are continually released from the calcareous corpuscles of this and several other taenioid cestodes (von Brand and Bowman, 1961; Agosin, et al. 1957). It has been postulated that the calcareous corpuscles function as a buffering mechanism for the benefit of the tapeworm while completing its life cycle. Through a cursory examination of the

larvae and adults it has been determined that calcareous corpuscles are most abundant in the larval form which may account for the discrepancy between the substrate effects observed aerobically and anaerobically.

Enzymes of the Embden-Meyerhof pathway have been demonstrated in several different cestodes, though the entire pathway is not known to occur in any single species. Read (1951) found phosphorylase, phosphohexose isomerase, aldolase, triose phosphate dehydrogenase, and lactic dehydrogenase in adults of H. diminuta. Agosin and Arevena (1959) in a somewhat similar study, found the same enzymes in hydatid cyst scolices of E. granulosus. Thus, two different investigations have demonstrated the presence of glycolytic enzymes in an adult and a larval cestode of different species. Except for these two investigations, little other additional information on the presence or absence of the classical Embden-Meyerhof enzymes is available from work with cestodes.

My data show that the coenurus tissue and the gravid proglottids of M. serialis definitely contain phosphohexose isomerase, aldolase, and lactic dehydrogenase.

In the assays performed for these three enzymes, all conditions were the same except for the tissue homogenates. As a result, certain conclusions regarding the relative activity of the enzymes in both tissues can be made. Since only the source and presumably therefore only the quantity of enzyme present was changed, the relative activity of the enzyme in adults and larvae can be deduced and compared on the same level. If these assumptions are true, then the activity of phosphohexose isomerase, aldolase and lactic dehydrogenase is greater in adult tissues than in larval

tissues.

This finding further substantiates the result obtained in the study of endogenous respiratory levels of activity. In both oxygen consumption and carbon dioxide production, the level of activity in the adult tissues is substantially higher than that for larval tissues. Herein lies additional evidence that there is a greater metabolic activity in adult tissues than in larval ones. This further negates the premise made at the beginning that the larvae might be expected to operate at a higher arabic metabolic rate than the adults because of their aerobic environment.

The contents of hydatid cyst fluid has long been under scrutiny with regard to its origin, function, and chemical content (Lemaire and Ribere, 1935). Recent studies have dealt with the analysis of protein constituents of the cyst fluid in comparison with those of the host serum (Goodchild and Kagan, 1961). These studies indicate that the fluid of hydatid cysts is a transudate of the host serum. Since there are many host serum proteins in the cyst fluid it seems reasonable to assume that the fluid also should contain those glycolytic enzymes commonly found circulating free in the blood. My work has shown this to be the case. The relative activity of these three enzymes cannot be compared to that of the tissue homogenates since there is no common ground for comparison. That the enzymes are present in the fluid is not surprising if in fact the fluid within the coenurus is a transudate of host serum. It is reasonable to assume that if proteins of relatively high molecular weight such as albumin and serum globulin can make their way into hydatid cyst fluid from the host blood, then proteins of the size of circulating glycolytic enzymes might also be found in the coenuri. The

only other possible source of the cyst fluid enzymes would be from the tissues of the parasite itself. This is believed not to be the case since only those coenuri which were whole prior to controlled rupture were utilized as a source of fluid.

Two enzymes associated with the TCA cycle were also investigated. Read (1952, 1953) demonstrated the presence of malic dehydrogenase, succinic dehydrogenase, and cytochrome oxidase in H. diminuta. He further indicated that isocitric and citric dehydrogenases are probably not present in this worm. Goldberg and Nolf (1954) found an active succinic dehydrogenase in H. nana. The use of specific metabolic inhibitors and specific TCA cycle intermediates in the study of metabolic pathways in cestodes has also been extensive.

Evidence presented in Table 9 indicates the presence of malic dehydrogenase in M. serialis. This finding is based on manometric assay techniques following the methods of Read (1953), who found the same enzyme in fortified homogenates of H. diminuta.

Read (loc. cit.), also investigated the presence of several other dehydrogenases in H. diminuta. He was unable to demonstrate isocitric dehydrogenase while my investigations (Table 8) suggest its presence in the cyst fluid of coenuri. It is interesting that the level of activity in both adult and larval forms was found to be identical and quite low. The same degree of activity of isocitric dehydrogenase in larvae and adults is quite different from the pattern established for other enzymes of the glycolytic and aerobic pathways in adults and larvae of M. serialis. Because of the differences found in this instance, the reliability of the colorimetric isocitric dehydrogenase assay employed was tested on mammalian

liver homogenates. Excellent reproducibility was obtained thereby indicating reliability of the procedure. Other tests were also performed on the tissue homogenates of M. serialis in which the concentration of substrate, the coenzyme, and the tissue homogenate itself were altered. In each case, the presence of the enzyme was indicated as evidenced by variations of activity reflecting these alterations.

I also investigated the possibility that alpha-ketoglutarate was being produced through conversion of glutamic acid by action of glutamic dehydrogenase. However, since there was a change in the quantity of alpha-ketoglutarate produced even when isocitrate concentration was altered, it must be assumed that glutamic dehydrogenase is not responsible for most of the alpha-ketoglutarate produced. All of these tests convince one of the reliability of the assay procedure and it must be assumed that the enzyme isocitric dehydrogenase is present in both larval and adult tissues.

With strong evidence for the presence of isocitric dehydrogenase, there arise certain questions regarding its relative activity in both larval and adult metabolic pathways. The level of activity associated with larval and adult tissues is quite low but is similar in grade. Because the difference in activity of this enzyme between larvae and adults is negligible, there is a distinct possibility that it may not play a major role in the metabolism of M. serialis. The demonstration of this enzyme, regardless of its presumed low level of activity, makes a re-evaluation of the entire TCA cycle and its role in the metabolism of cestodes in general a definite necessity.

Although Read (1953) reported that he was unable to demonstrate the presence of isocitric and other dehydrogenases, he later states (1961) that the failure to demonstrate the presence of these enzymes can not be taken as clear evidence for their absence. It appears from my experiments that isocitric dehydrogenase definitely is present in M. serialis and this is further substantiated by enzyme inhibition studies.

While the results of enzyme inhibitor studies are not quantitative, it was found that sulfhydryl binding inhibitors such as para-hydroxymercuribenzoate, iodoacetate, and iodoacetamide are effective under both aerobic and anaerobic conditions. Sodium fluoride inhibition indicates the presence of enolase in both adult and larval forms. Why fluoride was without effect anaerobically on adult tissues while the larvae under the same conditions appeared to be inhibited is not readily explainable. These results along with those obtained through specific enzyme assays provide strong evidence for an active glycolytic pathway in M. serialis.

Aerobic respiration was not altered by the presence of dl-glyceradehyde a glucokinase inhibitor. Gravid proglottids reacted to it by increasing oxygen consumption. Anaerobically, dl-glyceraldehyde had no effect on either larvae or adults. The lack of inhibition by dl-glyceraldehyde is expected since the inhibitor normally would have its effect on glucokinase, which is responsible for the initial phosphorylation of glucose upon its removal from the incubation medium by the cestode. Since however, there is no glucose present in the incubation medium and since it is assumed that glycogen is utilized as an energy source, dl-glyceraldehyde would have no opportunity to operate on glucokinase in these experiments.

Even if it were to actively block the enzyme, no measurable effect of such inhibition would be detectable unless an exogenous source of glucose were available in the incubation medium.

Agosin, et al. (1957), reported that malonate is ineffective against oxygen consumption by hydatid cyst scolices. It was suggested that this lack of inhibition might be due to the absence of succinic dehydrogenase. In my work, malonate did not effect the oxygen consumption at the same concentration as that used by Agosin, et al. (loc. cit.). However, a fivefold increase in the concentration of malonate had an appreciable effect. Both larvae and adults were motionless following their exposure to the inhibitor, an effect comparable to that observed under sulfhydryl inhibition.

Since a definite inhibition by malonate was observed, it is concluded that an active succinic dehydrogenase is present in both adult and larval tissues. With the demonstration of the presence of malic dehydrogenase in this cestode, I feel justified in assuming the presence of at least a portion of the terminal sequence of the TCA cycle.

While Agosin, et al. (1957), were unable to show an inhibitory effect for hydatid cyst scolices by malonate, they did demonstrate a definite inhibition of citrate metabolism by monofluoracetate. Using this inhibitor at the same concentration, I was able to demonstrate inhibition of oxygen consumption in adults. Larval tissues also appear to be inhibited by monofluoreacetate, but the decrease in oxygen consumption must be evaluated carefully in light of the results obtained through statistical analysis. Monofluoroacetate has an inhibitory effect on

citrate metabolism which is coupled with the enzyme isocitric dehydrogenase. Because of this association between citrate metabolism and isocitric dehydrogenase and since monofluoroacetate appears to have an inhibitory effect on both larval and adult tissues, further evidence for the presence of isocitric dehydorgenase is indicated.

Several conclusions regarding intermediary carbohydrate metabolism of M. serialis seem justifiable. There is significant evidence that the Embden-Meyerhof sequence is operative in both larvae and adults. Whether the pathway is complete in the classical sense has not been determined even though its effectiveness as an integral part of the intermediary metabolism of this cestode has been clearly established. There is also strong evidence that the terminal portion of the TCA cycle is present and that other portions of the cycle are probably present. If the cycle as revealed here is essentially correct for M. serialis, it would be basically comparable to that operating in most vertebrates. This alters somewhat the prior concept that only a partial cycle is operative in most cestodes.

CHAPTER 5

SUMMARY

1. Field collections of Multiceps serialis in the black-tailed jackrabbit (Lepus californicus), revealed that the infection rate in Oklahoma was substantially higher than in L. californicus collected in either Colorado or Kansas. It was further determined that the infection rate in females was significantly higher than that in males. Approximately 90% of all the larvae recovered were taken from the posterior half of the body suggesting a definite route of infection to that area for migrating larvae.

2. Manometric studies on the respiration M. serialis show a substartially higher rate of gas exchange occurring in the adult as compared with the larval forms.

3. The adults appear capable of utilizing glucose and fructose aerobically and galactose as well as glucose and fructose anaerobically.

4. Coenuri were shown to utilize only glucose aerobically and no substrate utilization at all could be demonstrated anaerobically.

5. Phosphohexose isomerase, aldolase, and lactic dehydrogenase were demonstrated in homogenates of adult and larval tissues with activity being higher in adult tissues.

6. The same enzymes were found to occur in the fluid of coenuri. Thus there is a suggestion that the cyst fluid is a transudate of the host serum.

7. Malic dehydrogenase was found to be present in both adult and larval tissues. Isocitric dehydrogenase was demonstrated in the cyst fluid and a low level of activity of the enzyme was found in adult and larval tissue homogenates.

8. Metabolic inhibitors were used in an effort to further evaluate the presence of certain enzymes. Sulfhydryl inhibitors were especially effective against respiratory activities of both adults and larvae. This further substantiates the presence of a glycolytic pathway in M. serialis. Sodium fluoride inhibited respiratory activity suggesting the presence of enolase.

9. Malonate was effective against both larval and adult oxygen consumption. Thus, succinic dehydrogenase is probably present in M. serialis. Monofluoraacetate inhibition offers further support for the presence of isocitric dehydrogenase.

10. Evidence is strong for a functional Embden-Meyerhof pathway in part and for the terminal portion of the TCA cycle. Evidence for citrate metabolism was found through studies of monofluoroacetate inhibition.

11. The respiratory differences between adult and larva are considered largely as quantitative in nature. It appears that the role of the environment does not play as great a part in the determination of respiratory activity in M. serialis as was thought prior to the initiation of this investigation.

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