CARBOHYDRATE METABOLISM IN THE LEECH *MOOREOBDELLA MICROSTOMA* (ERPOBDELLIDAE)

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The glycolytic pathway has been demonstrated to be the primary energy source in the leech *Mooreobdella microstoma.* While there appeared to be some dependence on a metal cytochrome system, experiments utilizing inhibitors of glycolysis, the Krebs cycle, and the cytochrome system suggest that this leech does not follow the classical mammalian scheme of carbohydrate metabolism.

***Mooreobdella microstoma* is able to survive as long as 24 hours under low oxygen tension, with no indication of an oxygen debt. These data imply that this facultative anaerobe must oxidize NADH via another system as a means of resupplying NAD^+ for the continuation of glycolysis.**

INTRODUCTION

Information concerning metabolic processes of annelids is sparse (1).

Recent research into the physiology of annelids, particularly the Hirudinea, has centered primarily on osmoregulation and electrophysiology. For the most part, metabolic processes of Hirudinea remain unknown (2).

Research on the European leeches has centered primarily on the sanguivorous species (e.g. genus *Hirudo*). Work in this field in North America has dealt with species of the Great Lakes region and has been primarily taxonomic (3,4). Predacious leeches of the family Erpobdellidae have received little attention (3).

No physiological research has been undertaken on the Oklahoma leech fauna.

The purpose of this investigation was to determine which metabolic pathways are used as primary energy sources in *Mooreobdella microstoma*, a representative species of the family Erpobdellidae. This leech was of interest because it occurs in abundant populations in polluted and nonpolluted conditions. Thus, it was thought that its metabolism must be flexible and could be unique.

MATERIALS AND METHODS

Collection and maintenance of *Mooreobdella microstoma* in the laboratory

Mooreobdella microstoma was collected by hand from Bird Creek in Mohawk Park, Tulsa, Oklahoma and dug from substratum, returned to the laboratory, and treated by one of two means: substratum was (a) filtered through a wire screen under running water, or (b) placed in a metal container upon a hot plate; this forced the leeches to the surface (especially effective in winter months when *M. microstoma* was in a lethargic state several inches below the surface). When placed in aquarium water, the leeches resumed normal behavior. Aquarium water was obtained from Lake Yahola, Mohawk Park, Tulsa. The continuously filtered aquarium water had an oxygen tension of 8.0 to 8.5 mg/l, a temperature of 20-25 C, and a pH of 8.0 ± 0.4. Water was changed daily and *Enchytraeus* worms were provided as food twice weekly

In vivo experiments

Respiratory quotients (R.Q.) of fed and starved leeches were determined at three-and four-week intervals via Warburg manometers utilizing the direct total uptake method of Umbreit (5).

Leeches were fed 24 hr prior to each experiment. Their wet weight was determined to the nearest 0.1 mg with a Mettler balance, and each R.Q. was measured in an individual Warburg reaction flask of 7.5 ml volume which contained 1.5 ml of aquarium water (pH 8.0 ± 0.4 at 25 C ± 1). Oxygen uptake and carbon dioxide production were calculated as μl gas/mg wet weight/hr.

Inhibitor experiments

Studies utilizing inhibitors were conducted to test for the presence of glycolysis, the Krebs cycle, and the cytochrome system.

Leeches were again fed 24 hr prior to the experiment, placed in petri dishes containing 15 ml of inhibitor solution (pH 7.0), and observed. Inhibitors utilized were 5×10^{-2} *M* and 5×10^{-3} *M* iodoacetate, sodium fluoride, fluoroacetate, arsenite, malonate, cyanide, and azide and 3.4×10^{-3} *M* seconal. Observations were also made of leeches in a saturated solution of 2,4-dinitrophenol in 1% ethanol and a 1:10 dilution of that solution. All other inhibitors were prepared using aquarium water as the solvent. Controls consisted of leeches in 1% ethanol aquarium water.

Leeches were assumed dead when they ceased to respond to touch, began losing color, lay crescent shaped on their sides, and failed to recover when returned to fresh aquarium water. Results are reported as survival time in minutes (ST) and as average survival time (STa).

At least five observations were made for each inhibitor concentration. Experiments were usually terminated after 10 hr, or sooner if the leeches had been ascertained dead.

Manometric determinations of effects of inhibitors on oxygen uptake were carried out as in the R.Q. experiments, except that all solutions were adjusted to pH 7. Initial oxygen uptake was determined in aquarium water, then leeches were incubated individually for 6 hr in 7 ml of one of the following solutions: iodoacetate (5×10^{-3} *M* and 5×10^{-4} *M*), sodium fluoride (5×10^{-3} *M* and 5×10^{-4} *M*), fluoroacetate (5×10^{-2} *M* and 5×10^{-3} *M*), arsenite (5×10^{-2} *M* and 5×10^{-3} *M*), malonate (5×10^{-2} *M*), seconal (5×10^{-5} *M*), cyanide (5×10^{-4} *M*), or azide (5×10^{-5} *M*). Again, at least five determinations were made for each inhibitor solution and the oxygen uptake was measured manometrically after 6 hr. The amounts of oxygen taken up in inhibitor solutions were compared to that taken up in aquarium water.

Low oxygen tension experiments

To determine the presence or absence of the Pasteur effect, initial manometric oxygen consumption measurements were made on leeches that had been fed and were being maintained at normal oxygen tensions (5.8 - 6.1 mg O_2 /l). Oxygen tensions were measured with an IBC oxygen analyzer to an accuracy of ± 2%. Low tension environments were generated by boiling 450 ml aquarium water for 30 min and then adding the hot liquid to flasks which were sealed and allowed to cool. The cooled water was placed in a 500-ml sidearm flask and the pH was adjusted to 6.0. Nitrogen gas was bubbled through the system until oxygen tension and pH were 0.05 to 0.25 mg/l and 7.5 to 8.5, respectively.

Leeches were placed in an anaerobic chamber (450 ml of this deoxygenated water in a 500-ml flask) for three hr. The oxygen tension and pH were periodically measured during this time (the seal was never broken as sensors were placed in the chamber). The oxygen uptake of the leeches was again measured manometrically. Results are reported as μl O_2 taken up/mg wet weight/hr, and compared to the amount of initial uptake.

Mortality experiments

Oxygen concentration effects on survival time of *M. microstoma* were determined in normal to low oxygen tensions produced as described above.

Tensions ranged from 0.25 mg O_2/liter to normal tensions of 5.8 to 6.2 mg O_2/liter. A minimum of three determinations was made for each tension. Leech behavior was observed and date and time of deaths recorded. Final readings of oxygen tension and pH were taken and the results are reported as oxygen concentration (mg/l) vs. survival time.

Metabolic and product analysis

The amounts of lactic acid and pyruvic acid secreted by the leeches under low and normal oxygen tensions were determined using dechlorinated tap water to prevent contamination by extraneous organisms found in normal aquarium water.

Five leeches were weighed and placed in small bottles containing 10 ml of water with an oxygen tension of 0.15 mg/l of oxygen. The bottle was lowered into a 250-ml sidearm flask used as a nitrogen atmospheric chamber. Nitrogen gas was circulated throughout the chamber and the solution containing the test organisms.

An additional group of live leeches was placed in 10 ml of tap water at normal oxygen tension. After a 10-hr incubation peri-

od, the individual solutions were analyzed for traces of lactic and pyruvic acids (6).

A minimum of three determinations was made on the media from low and high oxygen tension incubations.

RESULTS

Mortality experiments

Glycolytic inhibitors. Average survival times in iodoacetate and fluoride were dependent on inhibitor concentration.

Leech behavior was apparently unaffected by 5×10^{-3} *M* concentrations of iodoacetate and fluoride, but they reacted immediately when exposed to 5×10^{-2} *M* fluoride by swimming about rapidly and secreting mucus.

No organism survived the duration of the experiment with 5×10^{-2} *M* iodoacetate and fluoride; STa values were 133 and 35 min respectively.

All control animals survived to the termination of the experiment.

Krebs cycle inhibitors. Further experiments were performed utilizing 5×10^{-2} *M* concentrations of fluoroacetate, arsenite, and malonate. All leeches survived the 600-min experiments with no unusual behavior, with the exception of that involving arsenite, where they were much more active than usual. Leeches that remained in the inhibitor were alive after 24 hr.

Cytochrome inhibitors. Average survival times established in cyanide (5×10^{-2} *M*), sodium azide (5×10^{-2} *M* and 5×10^{-3} *M*), seconal (3.4×10^{-3} *M*) , and 2,4-dinitrophenol (saturated solution) were significantly less than for the controls.

Leeches reacted immediately in all cytochrome inhibitors (excepting 5×10^{-3} *M* cyanide) by swimming about rapidly and secreting large amounts of mucus.

Results of mortality experiments are summarized in Figure 1.

Effects of low oxygen tensions

Longevity experiments demonstrated a linear relationship between oxygen concentration and survival time (r = 0.99). The ST decreased with a decrease in oxygen concentration. The STa was 234 hr at concentrations of 5.8-6.3 mg O_2/l and 21 hr at

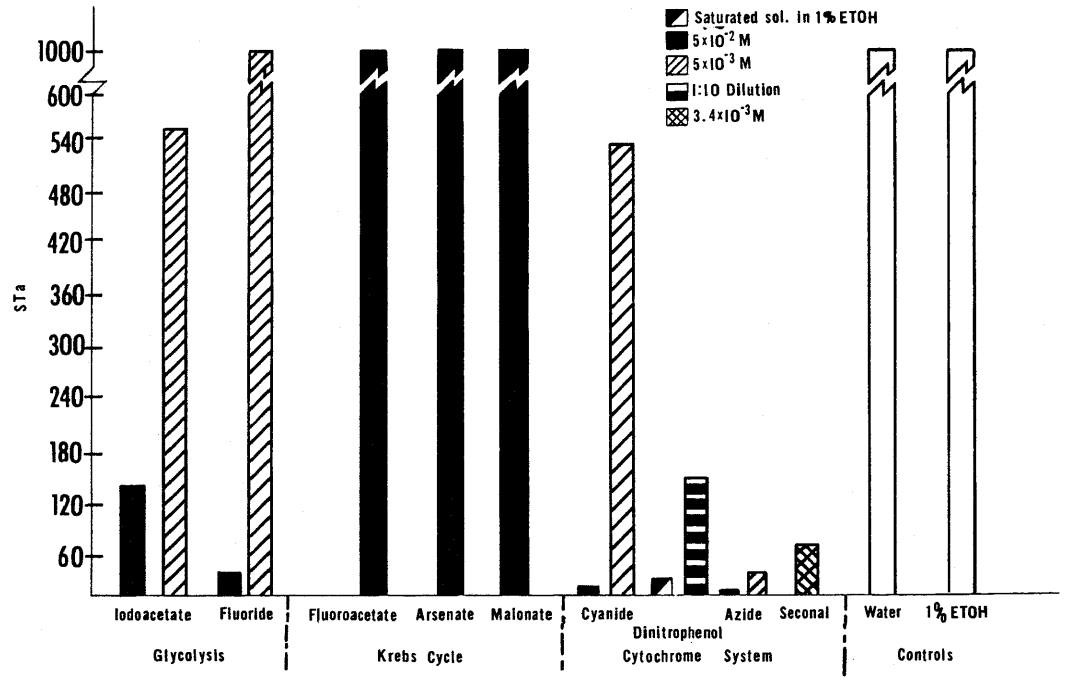

FIGURE 1. Average survival time in minutes (STa) of leeches exposed to inhibitors of glycolysis, the Krebs cycle, and the cytochrome system.

TABLE 1. *Longevity of leeches exposed to various oxygen tensions*[a].

N[b]	Initial pH	Final pH	Initial oxygen tension (mg/l)	Final oxygen tension (mg/l)	Longevity, average (hr)
3	7.8	8.6	0.15*	0.15	21
3	8.2	7.65	0.15	0.60	31
3	8.1	7.4	0.20	0.15	42
3	7.7	7.6	0.35	0.30	35
3	8.0	7.6	1.10	0.65	75
3	8.1	7.6	2.25	0.25	124
3	8.2	7.55	3.10	0.75	141.5
2 (controls)	8.3	7.24	5.8-6.3	0.75	234

[a] Nitrogen gas bubbled through system.
[b] N = number of experiments.

0.15 mg O_2/l. The pH and oxygen concentrations of the incubation medium also decreased with time (Table 1).

Leeches exposed to oxygen concentrations below 1.0 mg/l had increased ventilation rate (undulating with anal sucker attached).

Metabolic end product analysis

The average pyruvate concentration of the incubation medium decreased 10.9% when the oxygen tension was decreased from the normal value to 0.15 mg/l from 0.0083 ± .0033 μmole/ml to 0.0074 ±.0033 μmole/ml).

The lactic acid concentration after 10 hr under normal tension was 36 times that of pyruvic acid, and 19% greater than under low tension. The difference in concentrations of lactic acid was significant (0.291 ± .004 μmole/ml and 0.363 ± .042 μmole/ml respectively; $P < .05$).

Manometric experiments

The mean R.Q. of leeches fed 24 hr prior to uptake experiments was 1.22 ± .331 (mean Q_{O_2} 0.471 ± .104 and Q_{CO_2} 0.585 ± .213). Leeches starved for 3 and 4 weeks had mean R.Q. of 1.45 ± .223 (mean Q_{O_2} 0.687 ± .150 and Q_{CO_2} 0.989 ± .345) and 1.21 ± .482 (mean Q_{O_2} 0.383 ± .096 and Q_{CO_2} 0.456 ± .166) respectively.

Difference between the mean Q_{O_2} uptake with (final Q_{O_2}) and without (initial Q_{O_2}) iodoacetate (5×10^{-4} *M*) was not significant (0.2172 ± .0537 and 0.2732 ± .1382: P = .13), (the correlated t test was performed on all comparative data at the .01 level of significance), nor was it significant at 5×10^{-3} *M* (0.3932 ± .1541 and 0.3496 ± .1313; P = .02).

In sodium fluoride concentrations of 5×10^{-3} *M* and 5×10^{-4} *M*, respiration inhibition was approximately 50%. The mean amount of oxygen taken up decreased significantly and the difference in the means (control vs. inhibitor) was significant (0.3839 ± .1191 vs. 0.1981 ± .0667 @ 5×10^{-3} *M* and 0.2717 ± .1079 vs. 0.1246 ± .0356 @ 5×10^{-4} *M*; P = .007).

There was no significant difference between oxygen uptake means of leeches exposed to fluoroacetate and those in the absence of the inhibitor (0.2175 ± .1129 vs. 0.2465 ± .0934 @ 5×10^{-2} *M*, and 0.1520 ± .0578 vs. 0.1420 ± .0817 @ 5×10^{-3} *M*; P = .313 and .776 respectively).

Difference between the means in the presence and absence of arsenite was not significant (0.4567 ± .0863 vs. 0.4750 ± .0663 @ 5×10^{-2} *M*, and 0.2493 ± .1060 vs. 0.3321 ± .1511 @ 5×10^{-3} *M*; P = .76 and .029 respectively), nor between the mean uptake by leeches exposed to 5×10^{-2} *M* concentrations of malonate (0.4622 ± .2735 vs. 0.5158 ± .2762; P = .06) compared to their initial uptake in aquarium water.

Difference in the mean oxygen uptake in the presence and absence of cyanide (5×10^{-4} *M*) was significant (0.2122 ± .0803 vs. 0.3957 ± .1021; *P* = .0004).

Oxygen uptake decreased from 0.4786 ± .1559 to 0.2918 ± .1468 μl/mg wet weight/ hr after exposure to azide (5×10^{-5} *M*), a significant decrease of 39% (*P* = .015 in light of the mortality experiments.

Experiments with seconal (5×10^5 *M*) did not produce results different from the controls at the μ .01 level of significance (0.2586 ± .0785 vs. 0.2008 ± .0801; P = .24).

It should be noted that while some of these inhibitors did not give significant results at extremely low concentrations, they produced dramatic effects at higher ones, as demonstrated in the mortality experiments.

Oxygen uptake of control organisms was measured by using aquarium water in place of inhibitor solutions. After 6 hr exposure to a limited supply of aquarium water, uptake decreased from 0.1958 ± .0699 to 0.1837 ± .0650 l/mg wet weight/hr. Difference between the means was not statistically significant (P = .06).

These experiments showed a wide range of oxygen uptake readings within the groups under study. Comparison of oxygen consumption rates related to body weight showed that smaller leeches had a higher rate of consumption.

Pasteur effect. Eleven leeches incubated for three hours at oxygen tensions of 0.10 to 0.25 mg O_2/l and then returned to normal tensions showed no evidence of a Pasteur effect (Q_{O_2} 0.2541 ± .1235 vs. 0.2266 ± .1067; (P = .15). Three of the leeches showed a slight increase in uptake after exposure to low tensions, while the others showed a decrease in means.

Results of an incubation period of 5 hr again indicated no Pasteur effect with only a slight increase in uptake after exposure to low oxygen tension (Q_{O_2} 0.1802 ± .0621 vs. 0.1906 ± .0552). Results were not significant (P = .80).

DISCUSSION

Metabolism of *Mooreobdella microstoma* does not appear to follow classical patterns of carbohydrate metabolism. Experimental evidence indicates that *M. microstoma* depends primarily on glycolysis and the cytochrome system as energy production sources. However, the importance of the Krebs cycle and its associated enzymes is still in question.

Mooreobdella microstoma is found in polluted substrata where oxygen tension is low, and it would seem a necessity for it to be able to rely upon the pathway of glycolysis for energy production, a theory supported by evidence from experiments with R.Q. of *M. microstoma* and other organisms. Prosser (7) states that high R.Q. values occur with reliance on glycolysis and oxidation of intermediate acids. The R.Q. of *M. microstoma* is very high (> 1.0), which may well indicate the animal relies upon glycolytic degradation of carbohydrates as a energy source.

Experiments demonstrated that *M. microstoma* is able to survive in low oxygen tensions (< 1.0 mg/l) for 24 hours or more. Few animals can obtain sufficient energy from purely glycolytic metabolism and will utilize some oxygen even in low tensions (7), as *M. microstoma* did in tensions as low as 0.20 mg/l.

There is still good evidence that the metabolism of *M. microstoma* is primarily glycolytic, as exemplified in the Pasteur effect experiments. *M. microstoma* excretes the end products of glycolysis (pyruvate and lactate), as evidenced by pH changes and quantitative analysis, rather than storing them for future oxidation. Some annelids which utilize the Krebs cycle (e.g. *Lumbricus terrestris)* repay an oxygen debt after exposure to an anaerobic environment (8). Others, such as the gnathobdellid leech *Hirudo medicinalis,* do not exhibit this behavior (9). Daily oxygen consumption rhythms have been observed in *L. terrestris* (10). As with other metabolic functions, the Pasteur effect varies among annelid species.

M. microstoma seems to fit into the category which can rely on glycolysis temporarily and excrete or later oxidize intermediate acids. These organisms are generally able to survive longer in low oxygen tensions (2, 7). In *M. microstoma,* lactic acid excretion increases under low oxygen tensions while pyruvic acid excretion decreases, suggesting its reduction to lactic acid by the enzyme lactic acid dehydrogenase (LDH) with the oxidation of NADH. This conversion is not an energy conservation reaction, but a means of maintaining the NAD^+ needed for the continuation of glycolysis (11).

In the classical scheme of energy metabolism, pyruvic acid can be oxidized in the mitochondria via the Krebs cycle, or reduced to lactic acid and excreted, depending

on the organism in question and the enzyme systems present (15).

M. microstoma must be capable of NADH oxidation via one or more of the following systems: LDH, the mitochondria, or the succinate-propionate scheme (13). Such is indicated by low oxygen tension experiments, R.Q. values, lack of an oxygen debt, and increase of lactic acid excretion under anaerobic conditions. *M. microstoma* utilizes available oxygen, yet may not oxydize intermediate products via the Krebs cycle. It is suggested by the data that *M. microstoma* utilizes the cytochrome system whenever possible to oxidize NADH, increasing the efficiency of energy derivation via glycolysis.

This hypothesis is suggested by the results obtained via experiments utilizing metabolic inhibitors of glycolysis, the Krebs cycle, and the cytochrome system. Results of survival experiments using iodoacetate and fluoride (especially at 5×10^{-2} *M*) suggest the presence of the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase and enolase, respectively (14).

There was no significant effect upon oxygen uptake using dilute concentrations of iodoacetate; concentrations of 5×10^{-2} *M* caused dramatic reduction in survival times, thus demonstrating the necessity of maintaining an intact glycolytic pathway in *M. microstoma*.

Results of mortality and oxygen uptake experiments utilizing fluoride indicated the latter stages of glycolysis to be functional and necessary. This stage is particularly important since it precedes an energy-deriving point where conversion of phosphoenolpyruvic acid to pyruvic acid, with the production of ATP, is catalyzed by the enzyme pyruvic kinase (15). This evidence also indicates a reliance upon glycolysis in *M. microstoma*.

Experiments indicating lack of an oxygen debt, and those utilizing Krebs cycle inhibitors, imply *M. microstoma* does not rely on the Krebs cycle for energy production to any measurable extent. Fluoroacetate, arsenite, and malonate had no significant effect upon either survival time or oxygen uptake. Webb (16) states that some inhibitors (e.g. malonate) penetrate the integument less readily than others and this should be considered when examining the effectiveness of a particular inhibitor upon whole organisms. Cumulative results of our investigation suggest *M. microstoma* does not rely in the classical sense on the Krebs cycle for energy production. However, until problems utilizing homogenates can be resolved and other experimental approaches (e.g. histochemistry and tracer studies) applied, caution should be used in interpreting results.

Experimental results indicate *M. microstoma* has some dependency upon a heavy metal cytochrome system. Cyanide and azide were highly effective inhibitors, indicating that the enzyme cytochrome oxidase must be functional to maintain normal metabolism for extended periods of time.

Mattisson (17) showed by spectrophotometric analysis and cyanide inhibition the presence of cytochrome oxidase in *Lumbricus* and the polychaete *Nereis* sp. In regenerating *Tubifex* worms there is an increase in oxygen consumption associated with differentiation. However, this system is insensitive to cyanide at 10^{-4} *M* and 10^{-5} *M* concentrations (18). Low cyanide concentrations (2×10^{-4} *M* to 2×10^{-6} *M*) did not affect respiration at any stage of *Tubifex* regeneration (19) while concentrations of 2×10^{-2} *M* and 2×10^{-3} *M* did prove toxic in these animals. Like these other annelids, in the presence of oxygen *M. microstoma* apparently oxidizes NADH via a heavy metal cytochrome system, but since complete inhibition of oxygen uptake could not be achieved, there must be non-cytochrome oxygen utilization as well.

Results of the mortality experiments with 2,4-dinitrophenol suggest *M. microstoma* is dependent to some degree upon ATP production by the cytochrome system and can use this pathway to oxidize NADH.

Roles of lipid and protein metabolism were not investigated. The present investigation does not eliminate the utilization of those molecules by *M. microstoma*, but there are strong indications of primary dependence on carbohydrates as the main energy source. Future metabolic research on *M. microstoma* should include studies of lipid and protein metabolism, and further studies of carbohydrate metabolism perhaps utilizing radioactive tracers.

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