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AN ABSTRACT OF THE THESIS OF D. Mitchell Wolgamott for the Master of Science in Biology presented February 29, 1980.

Title: Storage and Use of Glycogen by Juvenile Carcinonemertes errans.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

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Juvenile C. errans do not feed on particulate matter and yet they can survive, apparently unchanged, for several months before maturing into the adult stage which feeds on the eggs of Cancer magister. The significance of glycogen as an energy source for maintenance of the juvenile form was investigated.

Glycogen was shown to be present and to be confined largely to the columnar cells of the gastrodermis. When animals were

maintained 30 days in filtered sea water at 10 C there was no detectable change in glycogen content on a per worm basis. But when the oxygen tension of the environment was lowered to zero over an eight hour period, the glycogen content of the worms increased 60% in the first four hours. Worms were shown to be capable of surviving at least 21 days of complete anaerobiosis. Iodoacetate at a concentration of 0.5mM greatly reduced tolerance to anoxia.

The findings of this study indicate that glycogen is not essential to the aerobic maintenance of the worm. However, if the oxygen tension is lowered, juvenile C. errans can rapidly increase their glycogen content. They can then survive long periods of anoxia by relying on anaerobic glycolysis as an energy source.

STORAGE AND USE OF GLYCOGEN
BY JUVENILE CARCINONEMERTES ERRANS

by

D. Mitchell Wolgamott

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE
in
BIOLOGY

Portland State University

1980

TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

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ACKNOWLEDGMENTS

I would like to extend thanks to Dr. David Clark, my advisor, and the other members of my Committee for their help and criticism; Dr. Paul Rudy, Director of the Oregon Institute of Marine Biology, for introducing me to Carcinonemertes and for allowing me to work at OIMB; Drs. Robert Terwilliger and Charles Holiday for their enthusiasm and for allowing me to collect worms from crabs they had collected for other purposes at OIMB. Thanks also to Sue E. Orlaske for her criticism and moral support when it was severely needed.

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INTRODUCTION

The Rhynchocoelan genus Carcinonemertes Coe, 1902, consists of five species which live symbiotically¹ with various species of marine crabs where the adult worms feed on the host eggs. Among those crab species infested by the worms are the economically important Cancer magister Dana, 1852, and Callinectes sapidus Rathbun, 1906. Recently, there have been indications that the newly described species, Carcinonemertes errans Wickham, 1978, may play a role in the declining crab fishery along the coast of Northern California (Wickham 1979a).

Host eggs appear to be the only food of adult Carcinonemertes (Kuris 1978, Humes 1942). Juveniles are found on both male and female crabs where they apparently do not feed on particulate matter. For this reason they are considered to be phoretic on the host (Kuris 1978). The energy source for maintenance of the juvenile form, until it is stimulated to mature and begin feeding (when the host undergoes ovaposition), is not known. The stimulus for maturation also is not understood.

This study was undertaken to evaluate the significance of glycogen as an energy source for maintenance of juvenile Carcinonemertes. To do this, the glycogen content of juvenile worms was localized and quantified. The effects of aerobic and anaerobic maintenance on glycogen content of the worms was determined. And the effects, on

¹Symbiosis, as used in this Thesis, refers to any close association between organisms of different species (Pennak 1964).

the intact animal, of the metabolic inhibitors iodoacetate (an inhibitor of glycolysis) and sodium cyanide (an inhibitor of electron transport) were determined.

LITERATURE REVIEW

The genus Carcinonemertes was established by Wesley Coe who described its members as "parasitic nemerteans living on various species of Crustacea" (Coe 1902). This is the only genus included in the family Carcinonemertidae. There are currently five recognized species, one of which is divided into varieties: C. carcinophila var. carcinophila (Kolliker 1845) Coe, 1902, from the South Atlantic Coast of North America, Italy, Belgium, and France; C. Carcinophila var. imminuta, Humes, 1942, from the Atlantic Coast of North America; C. epialti Coe, 1902, from the Pacific Coast of North America; C. mitsukurii Takakura, 1910, from Japan; C. coei Humes, 1942, from the East Coast of Africa; and C. errantia Wickham, 1978, from the Pacific Coast of North America.

Coe (1902) felt that these worms are true parasites feeding on hemolymph from the gills of male and non-ovigerous female hosts and feeding on crab embryos when the host is an ovigerous female. However, Humes (1942) could find no evidence that the worms feed on hemolymph or damage the gills in any other way (except possibly by impeding oxygen exchange when the infestation is severe). He referred to the worms as ectohabitants rather than parasites. He also implied that crab embryos are the only source of nourishment for the worms. Scrocco and Fabianek (1970) also found no evidence that the worms feed on hemolymph or any other tissue of the host. Kuris (1978) has referred to Carcinonemertes as being egg predators.

Regardless of whether the species of Carcinonemertes are considered to be commensals or true parasites, they do display several structural and behavioral modifications related to their symbiotic mode of life. These include: reduction in number of eyes as compared to many free-living nemertean species, increase in number of gonads, reduced proboscis, absence of cephalic grooves or other cerebral sense organs, excessive development of cephalic glands, positive photaxis of the larvae (Humes 1942, Gibson 1972).

The juvenile or non-sexual phase of Carcinonemertes is found encysted in mucoid capsules in the branchial chamber, on the gills, or on the exoskeleton of the host crab (Humes 1942, Kuris 1978) with the possible exception of C. errans which, according to Wickham (1978a), does not build mucous sheaths and is never found on the gills.

The worms begin their feeding, growing, sexual phase upon receiving some signal at the time of extrusion of the egg mass by a female crab. At this time the worms leave their capsules and migrate to the abdomen of the host where they take up residence in or near the egg mass. Here they begin a period of rapid growth and maturation. When mucous tubes are constructed, individuals usually inhabit separate tubes, but occasionally a male and female are found together in the same tubes. After mating, the female deposits her eggs in the closed end of her mucous tube. The eggs hatch as free-swimming larvae which eventually escape from the mucous tube. Roe (1979) has shown for C. epialti that not all larvae from a particular tube hatch at once. Instead, they hatch a few at a time over a period of several days. The larvae may represent a dispersal stage which must settle on the

same or on a new host to survive. Once a suitable substrate is located, the larvae presumably lose their swimming cilia and become juvenile worms. Host-to-host transmission of juvenile worms has been shown to occur under experimental conditions (Humes 1942, Kuris 1978). Crabs of both sexes become infested with juveniles, but the life cycle can only be completed on an ovigerous female. Hopkins (1947) has reported that the spawning history of female Callinectes sapidus can be determined by the presence or absence of mature Carcinonemertes in the gills. He observed that mature worms are not found in the gills unless the crab has spawned at least once.

Juvenile Carcinonemertes are phoretic on the host crab (Kuris 1978). They do not appear to feed on particulate matter until after the host has undergone ovaposition, at which time they migrate to the egg mass, start the maturation process, and begin feeding on crab embryos. This being the case, the juvenile worms must be capable of surviving long periods without feeding when the host is nonovigerous. Several genera of nemerteans can withstand long periods without ingesting particulate food; some can live more than a year without feeding (Gibson 1972). In many of these cases various structures and tissues are gradually absorbed. After the starvation period the animal may have a volume of as little as one percent of the original. Juvenile Carcinonemertes, however, can survive starvation for over two months with no apparent change in internal structure or size (Humes 1942, Wickham 1979b). Although these worms are often encysted during the period of time the crab is nonovigerous, they still must expend energy to maintain life. Therefore, they must have some method of

gaining or storing energy. This method is apparently distinct from absorption of the individuals' own tissues.

Fisher and Cramer (1968) showed the nemertean, Lineus ruber, to be capable of active uptake of organic molecules at concentrations found in sea water. This absorption apparently occurs at microvilli on the epidermis of the worm. Juvenile C. errans are capable of uptake of amino acids (Roe, et al 1980) and microvilli have been observed on the epidermis of Carcinonemertes (personal communication, David Metge, California State College, Stanislaus).

The ability to survive long periods of starvation by relying on food reserves is common among invertebrates (Reid 1950). Many parasitic worms store glycogen and have a metabolism based on this carbohydrate (Von Brand 1966). This is especially true for endoparasites which live in oxygen-poor environments (i.e., Fasciola hepatica in the bile duct), but even those endoparasites living in relatively oxygen-rich environments (i.e., species of Schistosomes in blood vessels) often contain large amounts of glycogen (Halton 1967). A metabolism based on incomplete oxidation of sugars is essential for those species with limited access to oxygen (Von Brand 1966). For species with ample access to oxygen, incomplete oxidation of sugars may seem wasteful; however, it must be realized that most adult parasites have easy access to a surplus of food. Failure to extract all available energy is no disadvantage.

Two ectoparasitic trematodes living on the gills of fish have been shown to store glycogen (Halton 1967). However, they store smaller amounts than the endoparasitic trematodes. Jennings (1971) has shown that endocommensal turbellarians store much more glycogen

than do their free-living relatives even though their habitat is not anaerobic. These data may support the view that the amount of glycogen stored is related to life style and/or availability of oxygen.

Food reserves in nemerteans have received little attention, but available information indicates that glycogen is only a secondary reserve in most nemerteans (Gibson 1972). Lipids appear to serve as the primary food reserve. The heteronemertean Micrura leidyi has been shown to store glycogen, but in lesser amounts than do parasitic helminths (Reid 1950). During aerobic starvation M. leidyi does not consume its glycogen reserves, but during anaerobic starvation its glycogen is rapidly depleted. This may be an adaptation which allows the animal to survive short periods of low oxygen availability.

Other members of the order Heteronemertea which have been briefly studied with regard to food reserves are: Lineus sanguineus, L. ruber, and Basiodiscus delineatus. Based on histochemical observations, fat appears to be the principle food reserve, but small amounts of glycogen have also been detected in the gastrodermis, parenchyma, and musculature (Jennings 1960, Jennings and Gibson 1969, Gibson 1974). Among the Palaeonemerteans, Cephalothrix biocula and C. linearis have been shown to store only very small amounts of glycogen (Jennings and Gibson 1969).

In the order Hoplonemertea, which includes Carcinonemertes, the food reserves of six species from four genera have been studied. Again these observations are based on histochemical studies only. Paranemertes perigrina has been shown to contain a small amount of glycogen in the distal half of the gastrodermal columnar cells, body

wall musculature, and in the parenchyma adjoining the gastrodermis (Gibson 1970). Otoyphlonemertes affinis, O. erneba, and O. lactea all store small amounts of glycogen as particulate deposits in the gastrodermis (Gibson 1974). Prostoma rubrum contains small amounts of glycogen in the gastrodermis with larger deposits in the gonads (Jennings and Gibson 1969). Extensive deposits of glycogen have been observed in the distal region of the gastrodermis in Amphiporus lactiflores (Jennings and Gibson 1969). Smaller amounts were found in the parenchyma, musculature, and gonads. In all six of these heteronemerteans fats appeared to be stored in larger quantities than glycogen.

Most physiological studies of nemerteans have dealt with free-living, carnivorous species. The only symbiotic nemertean in which food reserves have been studied is Malacobdella grossa. This worm is a commensal in the mantle cavity of various bivalve molluscs. It feeds on the plankton brought in by the ciliary currents of the host (Gibson 1972). It shows several structural modifications coincident with its life style. Most obvious is the development of a posterior sucker for attachment. There has also been a loss of eyes, simplification of the nervous system, increased number of gonads, and a reduction of the proboscis. However, according to Gibson and Jennings (1969), the adoption of an entocommensal life style does not appear to have affected the food reserves of M. grossa as compared to the free-living nemerteans. Fat again appears to be the principle food reserve. Glycogen is stored as small granules in the parenchyma, musculature, and gastrodermis, and in larger quantities in the gonad endothelium. It should be realized, however, that during times when

the host valves are closed M. grossa could be exposed to anoxic conditions. During these periods the host itself probably obtains energy anaerobically by glycolysis, and therefore depends on glycogen as a food reserve. It would seem that under these conditions fat reserves would be of little value to M. grossa.

There have been to date no published reports concerning modifications in food reserves or other physiological modifications which may be coincident with the symbiotic life style of Carcinonemertes.

MATERIALS AND METHODS

Specific identification of the nemertean used in this study was based on a comparison of the size of the proboscis armature of experimental animals taken from Cancer magister with the proboscis armature size of worms taken from Hemigrapsus nudis. Measurements obtained are reported in Table I along with values reported by other authors for worms taken from other crab species. These data show that no significant difference could be found between the proboscis length of worms taken from C. magister and H. nudis in the present study. The mean length of the basis and the stylet correlate well with the values reported by Wickham in his description of Carcinonemertes errans Wickham, 1978; so this is considered to be the species used as the experimental animal in this study. To further insure that only C. errans was being used for the glycogen and metabolic inhibitor studies, juvenile worms were always collected from male C. magister since Wickham (1978) reports C. errans to be specific for that host. This also insured that only juveniles were involved because the worms do not mature on male crabs (Humes 1942, Kuris 1978).

Collections of worms to be used for glycogen assay were made every other month beginning June 1978 until February 1979. Worms to be used for studies with metabolic inhibitors were collected in July 1979. Observations of living organisms were made on specimens collected over the entire study period, and these specimens were also used for histological studies. All worms collected from C. magister

TABLE I

MEASUREMENTS (μm) OF COMPONENT PARTS OF PROBOCSIS ARMATURE
OF VARIOUS SPECIES OF CARCINONEMERTES

Nemertean	Host	Basis length	Stylet length	Reference
<u>C. carcinophila</u>	several portunids	25-30	8-12	Humes 1942
<u>C. mitsukurii</u>	several species of <u>Charybdis</u>	27	8	Humes 1942
<u>C. coei</u>	<u>Charybdis natator</u>	22.7	8.7	Humes 1942
<u>C. epialti</u>	<u>Pugettia producta</u>	27-33	12-15	Coe 1902
<u>C. epialti</u>	<u>Hemigrapsus oregonensis</u>	31.63 $\pm$ 0.50	10.58 $\pm$ 0.20	Wickham 1978
<u>C. errans</u>	<u>Cancer magister</u>	35.22 $\pm$ 0.33	11.01 $\pm$ 0.16	Wickham 1978
experimental worms	<u>Cancer magister</u>	35.73 $\pm$ 1.08	11.00 $\pm$ 0.36	Wolgamott present study
comparison worms	<u>Hemigrapsus nudis</u>	35.80 $\pm$ 0.94	11.26 $\pm$ 0.34	Wolgamott present study

were taken off crabs obtained from the holding tanks at the Oregon Institute of Marine Biology, Charleston, Oregon, with the exception of the October 1978 collection made from crabs taken by a private fisherman at Newport, Oregon. Worms collected from H. nudis were taken from crabs captured near the Coast Guard facility at Charleston, Oregon. Only juvenile C. errans removed from male C. magister were used in the glycogen and metabolic inhibitor studies.

Worms were either removed from the crab immediately after collection of the crab and fixed in 100% ETOH or in formal-alcohol (1 part formalin - 9 parts 95% ETOH), or were transported alive, in jars of sea water, to the laboratory and maintained at 10 C until needed. In a few cases live crabs were transported to the laboratory and maintained at 10 C with worms being removed as needed.

Observations and measurements of individual worms were made using a Nikon binocular phase-contrast microscope equipped with an ocular micrometer. Observations of groups of living worms were made by placing the worms in filtered sea water in 60X25mm glass stender dishes.

Localization of Glycogen

Juvenile C. errans, fixed in formal-alcohol, were dehydrated, cleared, and embedded in paraffin. Sections (6 μ m) were cut and affixed to glass slides coated with Haupt's solution. The paraffin was removed with xylene and the sections were brought to water. Sections were then stained using the Periodic Acid-Schiff (PAS) reaction carried out according to the method of Galigher and Kozloff (1971). In some cases a fast green counter-stain was used.

Glycogen was removed from control sections by incubating with the enzyme amylo- α -1,4- α -1,6-glucosidase (Sigma) for thirty minutes at room temperature. These sections were then oxidized with periodic acid and stained in the same way as sections of experimental tissue.

Quantitative Determination of Glycogen

To prevent the loss of glycogen from the tissues, juvenile worms were fixed and stored in 100% ETOH at 10 C until they could be assayed. To extract glycogen, alcohol-fixed worms (usually 10 per sample) were placed in concavity slides (36mm dia.X5mm deep) and the alcohol was evaporated under a 60 watt light bulb. The dry worms were crushed, mortar and pestle fashion, with a glass rod; 0.5 ml cold water was added and the worms were crushed further. The samples were then incubated 20 minutes (at 10 C) with occasional stirring and crushing. Previous experiments showed that fixing the worms in acidified alcohol or increasing the incubation time did not increase the final yield of glycogen.

One hundred microliter aliquots of the extract were assayed for glycogen quantitatively using a modification of the enzymic fluorometric micro-method of Nahorski and Rogers (1972). This method is based on the enzymatic conversion of glycogen to glucose by the enzyme amylo- α -1,4- α -1,6-glucosidase and subsequent determination of glucose. In the method, the glucose concentration is determined by converting it to 6-phosphogluconate with the enzymes hexokinase and glucose-6-phosphate dehydrogenase and measuring the change in fluorescence as NADP is converted to NADPH. The reactions involved are as

follows:

Because one molecule of NADP is converted to one molecule of NADPH for every molecule of glucose-6-phosphate reacted, and since the amount of glucose-6-phosphate reacted depends on the initial glycogen concentration in this system, the change in fluorescence of the solution can be related to the total glycogen content of the solution when all reactions are allowed to go to completion. Using this method glycogen can be determined in amounts as low as 200 ng (Nahorski & Rogers 1972).

One slight modification of Nahorski and Rogers' method was employed. In order to compensate for smaller cuvette size used in this study (75 X 10 mm Kimex tubes) the volume of the tris buffer, in which the last two reactions are carried out, was increased to 1.66 ml and the volume of MgCl solution was increased to 0.82 ml. All solutions were made with double glass-distilled water. Fluorescence was measured using a Turner model 110 fluorometer.

Before assaying unknown solutions a standard curve was constructed by assaying solutions of known concentration of oyster glycogen (Sigma) and plotting the fluorescence change vs. the concentration. A line was then fitted to these data using linear regression (Fig. 1). The glycogen content of the unknown extracts of juvenile C. errans could then easily be determined. Fluorescence change of the unknown extract was observed and a linear estimate of glycogen concentration for that fluorescence value was calculated.

Figure 1. Standard curve; relation of NADPH fluorescence and glycogen concentration. Each point is the mean fluorescence change $\pm$ two standard errors.

To determine the difference in glycogen usage during aerobic and anaerobic conditions the following experiment was designed: Animals were incubated at 10 C in 125 ml side-arm flasks containing 30 ml filtered sea water. The flasks were stoppered with single-holed rubber stoppers fitted with glass tubes (3 mm dia.) which extended to just above the water surface. These served as inlet tubes for gas; gas escaped out the side-arm. Aerobic cultures were supplied with a continuous flow of air. For anaerobic incubations the flasks were continuously flushed with nitrogen at a rate of approximately 25 ml/min. Complete anaerobiosis was achieved in approximately 8.5 hours. This was determined by removing 0.5 ml water samples from one flask at half-hour intervals and analyzing with a Yellow Springs Instruments model 53 oxygen monitor. Worms were removed from the flasks after various lengths of incubation, fixed in 100% ETOH, and subsequently analyzed for glycogen content.

Determination of Effects of Metabolic Inhibitors

The effects of iodoacetate on survival of juvenile C. errans was determined both aerobically and anaerobically at 10 C. Ten worms were placed in each of two depressions on a spot plate which already held 0.5 ml filtered sea water containing 1.0 M and 0.5 M iodoacetate respectively. A control with 0.5 ml filtered sea water containing no iodoacetate was used. The pH of the water was 7.9. Plates containing aerobic cultures were placed in double side-arm spinner flasks with the stirrer removed and left open to the air. For anaerobic cultures the plates were placed in double side-arm spinner flasks which were then flushed with nitrogen for 3 hours with occasional

agitation to stir the solutions. The flasks were then sealed and maintained at 10 C. After 48 hours, percent survival was determined by counting the number of worms still alive. Worms were considered to be dead when no ciliary movement could be detected and when they did not respond to mechanical stimulus. Three repetitions of the experiment were performed. An additional anaerobic control entailed exposing 10 worms aerobically to 0.5 M iodoacetate for one hour, then washing three times with filtered sea water and placing the worms in a spot plate with 0.5 ml clean filtered sea water. The culture was then placed in a spinner flask, flushed with nitrogen, and maintained in the same way as other anaerobic cultures.

The effect of the following metabolic inhibitors on oxygen consumption by juvenile C. errans was determined: 0.5 mM iodoacetate, an inhibitor of glycolysis, and 0.5 mM sodium cyanide, an inhibitor of electron transport. Oxygen consumption was measured on a Yellow Springs Instruments model 57 oxygen meter equipped with the YSI 5739 dissolved oxygen probe and attached to a pen recorder. Twenty-five juvenile worms, previously acclimated to 10 C, were placed in a glass metabolic chamber containing filtered sea water and supplied with a magnetic stirrer. The probe was then inserted into the chamber and the entire assembly was submerged in a water bath adjusted to 10 C. The volume of the chamber, with probe inserted, was 6.34 ± 0.04 ml. The oxygen consumption of the control was determined by measuring the O_2 uptake of twenty-five uninhibited worms. The metabolic chamber was then opened and the water re-saturated with O_2 by bubbling air through it. Enough stock solution of the inhibitor was then added to give the appropriate concentration. The electrode was then reinserted and the

chamber submerged in the water bath. Fifteen minutes were allowed for temperature stabilization before oxygen consumption was measured again. The percent difference between the oxygen consumption of the control and inhibited worms was then calculated. In this way the effect of the inhibitor could be measured on the same twenty-five worms that were used to obtain the control value.

RESULTS

Localization of Glycogen:

Juvenile C. errans collected from under the abdominal flap of male C. magister were shown to contain glycogen by the Periodic Acid-Schiff (PAS) method. The glycogen was localized primarily in the columnar cells of the gastrodermis (Fig. 2). These cells stained bright red in sections of experimental tissue. In control sections, where glycogen was removed enzymatically, the columnar cells stained only faintly pink. Glycogen was also found, in smaller but easily recognizable amounts, as granules scattered throughout the parenchyma and body wall musculature.

Quantitative Determination of Glycogen:

Juvenile C. errans removed from under the abdomen of male C. magister and fixed immediately were found to contain on the average $1.73 \pm 0.06 \mu\text{g}$ glycogen per 10 worms. The glycogen content was shown to be quite constant and varied only slightly with season (Fig. 3). When worms were incubated in filtered sea water (changed every 2-4 days) in the laboratory at 10 C for thirty days there was no significant change in glycogen content. During this time the worms did not feed on particulate matter. No food substance was ever observed in the gut of a juvenile worm.

In order to determine if worms were capable of using their glycogen when exposed to anoxic stress the oxygen saturation of their media was slowly lowered to zero over a period of approximately 8.5

Figure 2. Tissue sections of juvenile C. errans stained by the PAS method.

Figure 3. Seasonal variation in glycogen content of juvenile C. errans (± 2 standard error).

hours. During the first few hours of decreasing O_2 the glycogen content of the worms rapidly increased to a maximum of $2.92 \pm 0.1 \mu\text{g}$ per 10 worms after four hours incubation (Fig. 4). This is a 60% increase in glycogen content. At that point the oxygen saturation of their environment had dropped from 100% to approximately 9%.

After eight days of complete anaerobiosis the glycogen content had dropped to $2.65 \pm 0.09 \mu\text{g}$ (Fig. 5). No data on glycogen content after longer anaerobic incubations are available. However, worms were shown to be capable of surviving complete anaerobiosis for at least 21 days.

Effects of Metabolic Inhibitors:

The effect of iodoacetate on survival of worms at 10 C both aerobically and anaerobically is shown in Figure 6. When worms were placed in filtered sea water containing 0.5 mM iodoacetate and incubated aerobically, 80.67% of the worms remained alive after 48 hours. When the incubation was carried out anaerobically only 46.67% survived after 48 hours. When the concentration of iodoacetate was increased to 1.0 mM, 76.67% of the worms survived the aerobic incubation while only 0.67% survived the anaerobic incubation. In an additional experiment where worms were exposed to 0.5 mM iodoacetate for one hour then washed and placed in clean filtered sea water containing no iodoacetate and incubated anaerobically, five out of ten worms were dead in 48 hours. In the control, where no iodoacetate was used, there was 100% survival both aerobically and anaerobically.

It was shown that 0.5 mM iodoacetate does not significantly affect the average O_2 consumption of juvenile C. errans within one

Figure 4. Effect of decreasing environmental oxygen on glycogen content of juvenile *C. errans* (± 2 standard error).

Figure 5. Glycogen content of juvenile *C. errans* during long term anaerobic incubation (± 2 standard error).

Figure 6. Percent survival of juvenile *C. errans* incubated with iodoacetate in anaerobic and aerobic conditions (± 2 standard error). Dark stippling = anaerobic; light stippling = aerobic.

hour after introduction of the iodoacetate (Fig. 7). However, if these data are presented to show O_2 uptake during the first half hour after introduction of the iodoacetate and O_2 uptake during the second half hour, we see that there is a significant increase in O_2 consumption during the first half hour followed by a decrease in the second half hour.

Sodium cyanide, which inhibits electron transport, was shown to reduce oxygen consumption by juvenile C. errans (Fig. 8). At a concentration of 0.1 mM, NaCn caused a 37.14% decrease in O_2 uptake as compared to the uninhibited control.

Figure 7. Effect of iodoacetate on oxygen consumption by juvenile C. errans (± 2 standard error).

Figure 8. Effect of sodium cyanide on oxygen consumption by juvenile C. errans (± 2 standard error).

DISCUSSION AND CONCLUSIONS

The Periodic Acid-Schiff (PAS) staining technique as used in this study shows the presence and locality of glycogen in tissue sections. The technique is limited in that positive color reactions occur when any compounds having free reactive -amino alcohol and/or 1,2 glycol groups are present after treatment with periodic acid (Thompson 1966). Not all of the substances meeting these requirements are glycogen or even carbohydrates. But when the appropriate controls are used the degree of specificity of the method is increased. If tissue sections are stained by the PAS method and then compared to similar control sections which were treated with a hydrolytic enzyme specific for a particular carbohydrate, then those areas which are PAS positive in the experimental tissue section but not in the control tissue section can be said to contain the particular carbohydrate. This approach was used. It showed that glycogen is present in juvenile C. errans, concentrated for the most part in the gastrodermis (Fig. 1), an observation not inconsistent with reports for other nemertean species (Appendix).

Glycogen exists in two physical states in animals (Von Brand 1966). Desmoglycogen is bound to proteins or other cell components and is extractable only by alkali treatment. Lyoglycogen is "free glycogen"; it is not bound to other compounds and is more readily extracted from tissues than is desmoglycogen. In this study the amount of free glycogen contained in juvenile C. errans was determined quan-

titatively. Glycogen was extracted from the tissues of alcohol-fixed worms by crushing them in cold water. This extraction technique was chosen because it extracts only the free glycogen; desmoglycogen is left behind. Free glycogen is metabolically more active than is desmoglycogen (Strang and Bachelard 1971). For this reason, fluctuations in the free glycogen content should indicate the worms use of stored carbohydrate. Undoubtedly, higher yields of glycogen would have been obtained had the samples been heated during extraction, or had one of the more drastic extraction procedures been used (e.g. hot alkali extraction).

My data show that the free glycogen content of the worms remains unchanged for long periods of aerobic maintenance. This indicates that the worms either do not use glycogen under normal aerobic conditions - glycogen would then be in a stable state; or the worms replace glycogen at the same rate that it is being used for metabolic activities - glycogen would then be in a state of dynamic equilibrium. I have also shown that blocking glycolysis with iodoacetate has little effect on the aerobic survival of juvenile C. errans. This implies that the worms are not dependent on normal glycolysis during aerobic maintenance. Therefore, the worms must be obtaining energy from some other source; either by obtaining energy from carbohydrate through some alternative pathway, or by depending on some other substrate. The latter seems most probable at this point. Microvilli have been shown in large numbers on the epidermis of juvenile Carcinonemertes (David Metge, California State College, Stanislaus, personal communication; David Cook, Portland State University, personal communication). This suggests an absorptive function for the epidermis. Pamela Roe,

et al (1980) have shown Carcinonemertes to be capable of active uptake of amino acids from surrounding water. Degens, et al (1964) have shown free amino acids to be present in waters of the Pacific Ocean off California in concentrations ranging from 16 - 125 $\mu\text{g/l}$. Free sugars were also found in concentrations ranging from 14 - 35 $\mu\text{g/l}$. The highest concentrations were found close to the water-sediment interface which is where the crab host, and therefore Carcinonemertes are located. Amino acid and sugar concentration within the boundary layer of water immediately surrounding the crab (where Carcinonemertes lives) could conceivably be even higher due to release of organic compounds by the microorganisms which live as cohabitants with Carcinonemertes on the exoskeleton of the crab.

As already mentioned, glycogen content of juvenile C. errans does not change during aerobic maintenance, and aerobic glycolysis can be circumvented to supply resting aerobic metabolism. One might then wonder why measurable quantities of glycogen are present in the worms at all. The answer, based on my experimental data, is that the worms are dependent on glycogen as an energy source during anaerobic maintenance. It was shown that the anaerobic survival of the worms is greatly reduced after exposure to iodoacetate, a metabolic inhibitor which blocks glycolysis at the level of triose-phosphate oxidation. This evidence suggests that juvenile C. errans do use the process of glycolysis to release energy from stored glycogen during anaerobiosis, and that they are dependent upon this process as an energy source during periods of anoxia.

The current study has also shown that juvenile C. errans can rapidly increase their glycogen content when the oxygen tension of

their environment is lowered (Fig. 3). In this study the oxygen in the environment was lowered from 100% saturation to approximately 9% saturation in four hours. During this same period of time the glycogen content of the worms increased by approximately 60%. After this time the oxygen saturation continued to decrease to zero, and the glycogen content of the worms began to decrease slowly over the next eight days. These findings show that decreasing oxygen tension leads to a shift in the metabolism of the worm such that glycogen is rapidly produced until some maximum glycogen content is achieved or until glycogen can no longer be produced due to lack of oxygen. After this point the worms become dependent upon anaerobic glycolysis as an energy source until oxygen again becomes available.

The ecological significance of the ability to rapidly produce glycogen when the oxygen tension is slowly reduced is clear when one realizes that C. errans probably is periodically exposed to anoxic conditions. The juvenile worms on male crabs are located, for the most part, around the reproductive appendages under the abdominal flap, and since the abdominal flap is held tightly against the sternum, it seems likely that an oxygen-poor microhabitat may periodically exist due to oxygen consumption of the worms themselves as well as oxygen consumption by other organisms which also live on the exoskeleton of the crab. This would be particularly true after a molt, when the crab buries itself in the sandy bottom. The habitat would be the same on non-reproductive females; however, it would be slightly different on ovigerous females. The adult worms cause extreme fouling of the egg mass within which they live (Wickham 1979b). This fouling could result in periodically-reduced oxygen tensions within the egg mass.

The data on the effect of metabolic inhibitors on oxygen consumption presented here show two things. First, the lack of an inhibition of oxygen consumption by iodoacetate shows the worms either do not use aerobic glycolysis or they possess some alternative pathway which allows them to survive even when normal glycolysis is blocked. This is because blocking glycolysis with iodoacetate severely impedes the breakdown of carbohydrates. If the endogenous respiration were dependent on carbohydrates, the result would be a decreased oxygen consumption (Webb 1966). Second, the large inhibition of oxygen consumption as a result of exposure to 0.1 mM sodium cyanide suggests that C. errans possesses a respiratory chain which, at least in part, resembles that typically found in aerobes.

It may be possible to arrive at one further conclusion, deductively, using the data presented in this thesis. Several authors (Halton 1967, Jennings 1971, von Brand 1966) have suggested that a correlation may exist between the amounts and nature of food reserves of an animal and its mode of life. It appears that parasitic animals tend to store more glycogen than their non-parasitic relatives, regardless of the availability of oxygen to the parasite. For example, Jennings (1971) has shown that free-living turbellarians have food reserves typical of free-living predators (i.e. fats with small amounts of glycogen), but parasitic and endocommensal turbellarians (e.g. Syndesmis franciscana) store large amounts of glycogen even though their habitat is far from anaerobic. By comparing the anaerobic survival of the parasitic Carcinonemertes errans with the anaerobic survival of the free-living nemertean Micrura leidy, it can be seen

that the above hypothesis may be true for nemerteans as well. Reid (1950) has shown that M. leidy does not use glycogen during aerobic starvation, but when exposed to anaerobic conditions the worm rapidly consumes the glycogen present in its tissues and is incapable of surviving anaerobiosis for more than seven days. On the other hand, juvenile C. errans were shown in the present study to survive at least 21 days of anaerobiosis by depending on their glycogen reserves. Because M. leidy is much larger than juvenile C. errans one would expect the weight specific metabolic rate of M. leidy to be lower than that of juvenile C. errans. If both animals are dependent upon anaerobic glycolysis as an energy-gaining pathway during periods of anoxia, then one would expect the larger animal to have the longest anaerobic survival, given the same carbohydrate reserve per unit weight. In this case the smaller animal survives much longer than the larger animal. This is of course a comparison of only one parasitic form with one free-living form and cannot be considered absolute proof. But the comparison shows that glycogen storage may be related to mode of life in nemerteans as it is in other worms.

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APPENDIX

FOOD RESERVES IN NEMERTEANS

Class Anopla

Order: Palaeonemertea

Family: Cephalothricidae

Cephalothrix biocula and C. linearis

Fat forms the only significant food reserve.

It occurs in droplets varying in diameter from 1-5 μ , in gastrodermal columnar cells and to a lesser extent in the parenchyma. Amount present decreases with starvation.

Glycogen is found only in very small amounts in the gastrodermis as minute particles, also scattered through out parenchyma (Jennings and Gibson 1969)

Order: Heteronemertea

Family: Baseodiscidae

Baseodiscus delineatus

Fat deposits in distal half of gastrodermal columnar cells occurring as droplets 5-6 μ in diameter.

Glycogen appears as small granules scattered throughout gastrodermis. Can also be found in parenchyma of freshly collected animals (Gibson 1974).

Family: Lineidae

Lineus sanguineus

Principal food reserve is fat stored in droplets 2.5 μ in diameter in gastrodermis and parenchyma. Small amounts of glycogen, in well fed individuals, as tiny granules scattered throughout gastrodermis, parenchyma and musculature (Jennings and Gibson 1969).

Lineus ruber

Fat is principal food reserve as intracellular globules up to 5 μ diameter in parenchyma and to a lesser extent in columnar cells of gastrodermis. Glycogen in tiny granules throughout parenchyma, musculature, and columnar cells of gastrodermis (Jennings 1960).

Class Enopla

Order: Hoplonemertea

Family: Amphiporidae

Amphiporus lactifloreus

Fat is principal food reserve, mostly in columnar cells of gastrodermis.

Extensive amounts of glycogen in distal region of gastrodermis and lesser amounts in parenchyma, musculature, and gonads (Jennings and Gibson 1969).

Family: Ototyphlonemertidae

Ototyphlonemertes affinis, O. erneba and O. lactea

Principal food reserve is fat which fills the intestinal columnar cells as globules 1-4 m in diameter. Lesser amounts in body wall musculature and anterior proboscis epithelium. Glycogen stored as small particulate deposits confined to gastrodermis, mainly restricted to the distal half of columnar cells (Gibson 1974).

Family: Tetrastemmatidae

Prostoma rubrum

Fat in columnar cells of the gastrodermis in droplets 3-5 m in diameter. Lesser amounts in gonads.

Glycogen in same sites with larger amounts in gonads (Jennings and Gibson 1969).

Family: Emplectonematidae

Paranemertes perigrina

Principal food reserve is fat, mostly in gastrodermal columnar cells. No fat deposits in parenchyma, epidermis, foregut, blood system, or body wall musculature. Occasional deposits in anterior proboscis.

Small amounts of glycogen as tiny granules in distal regions of gastrodermal columnar cells, body wall musculature, and parenchyma adjoining the gastrodermis (Gibson 1970).

Order: Bdellonemertea

Family: Malacobdellidae

Malacobdella grossa

Principally fat in droplets 3-4 m diameter, mostly in gastrodermal columnar cells. Also in parenchyma and epithelia of other structures such as pharynx and proboscis.

Glycogen in endothelia of gonads and small granules throughout parenchyma, musculature, and gastrodermis (Gibson and Jennings 1969).