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Title: METABOLIC RESPONSES OF THE BURROWING MUD SHRIMP, CALLIANASSA CALIFORNIENSIS, TO ANOXIC CONDITIONS

Abstract approved: Redacted for privacy

Austin W. Pritchard

Callianassa californiensis (Dana), a burrowing crustacean inhabitant of estuarine mudflats along the Pacific coast, is subjected to severely hypoxic interstitial water twice each tidal cycle.

A preliminary approach was made to understanding the metabolic mechanisms employed by Callianassa during periods of anoxic stress. Percent glycogen in the hepatopancreas and chela muscle, blood lactic acid, and blood glucose were determined for animals subjected to periods of anoxia. Blood lactate and glucose analyses were performed on animals at various intervals during a post-anoxia recovery period.

Callianassa was found to accumulate the anaerobic end product, lactic acid, at a steady rate during anoxia. The blood glucose concentration was shown to increase with initiation of anoxia until about 12 to 14 hours when it tended to reach an equilibrium. Glycogen

utilization did not appear to be important during short periods of anoxic stress, but for periods longer than 12 to 14 hours, glycogenolysis became important in maintaining glucose substrate for glycolysis. During the post-anoxia recovery period, the accumulated blood lactate was oxidized and blood glucose was reduced to the normal concentration. The possibility of the existence and role of free blood oligosaccharides as a source of glucose during anoxia was discussed.

Metabolic Responses of the Burrowing Mud Shrimp, Callianassa californiensis, to Anoxic Conditions

by

Dan Lee Hawkins

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Typed by Gwendolyn Hansen for Dan Lee Hawkins

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INTRODUCTION

The physiology of crustacean respiratory and general metabolism has been examined in detail only during the past twenty years. Aspects of respiratory and carbohydrate metabolism have been reviewed for crustaceans by Wolvekamp and Waterman (1960), Vonk (1960), Scheer and Meenakshi (1961), Lockwood (1967), and Huggins and Munday (1968).

In 1928 Meyerhof and Lohmann (as cited in Huggins and Munday, 1968) first demonstrated the overall conversion of glycogen to lactate in crustacean muscle. However, questions were raised as to the nature of metabolic pathways involved in oxidation of carbohydrates in crustaceans when Scheer and Scheer (1951), Scheer, Schwabe, and Scheer (1952), and Kermack, Lees, and Wood (1954) failed to find expiration of labeled carbon dioxide following injection of ^{14}C-glucose into lobsters.

More recent work, however, has provided considerable evidence that glycolysis is the main route of glucose degradation in crustaceans. Hu (1958) isolated a number of glycolitic intermediates from the crab, Hemigrapsus nudus, injected with labeled glucose. Hochachka, Teal, and Telford (1962) showed that administration of various glycolytic substrates and hexosemonophosphate pathway intermediates to

homogenates of lobster hepatopancreas stimulated increased oxygen uptake, indicating the presence of the corresponding pathways. McWhinnie and Kirchenberg (1962) and McWhinnie and Corkill (1964) used various metabolic inhibitors to study carbohydrate metabolism at different stages of the molt cycle in the crayfish, Orconectes virilis. They found evidence to suggest that glycolysis may be more important during premolt, while the pentose phosphate shunt is the main route during intermolt in this species. Puyear, Wang, and Pritchard (1965), using a radiorespirometric technique, demonstrated that the glycolytic, glucuronate and pentose phosphate pathways are operative as catabolic pathways for glucose utilization in the intermolt crayfish, Pacifastacus leniusculus. Evidence is presented which suggests that glucose is oxidized predominantly via glycolysis, very little being catabolized through the pentose phosphate or glucuronate pathways. With the crabs, Cancer magister and Hemigrapsus nudus, Meenakshi and Scheer (1961) found no evidence that the pentose phosphate shunt is used to oxidize glucose but have demonstrated that glycolysis is operative. In work with Carcinus maenas, Huggins (1966) furnished further evidence for glycolysis in crab tissue. Data is also presented which suggests that pyruvate tends to be transaminated or reduced rather than oxidized via the Krebs cycle. Direct evidence of the presence of the glycolytic enzymes has also been established in various crustaceans by Allison and Kaplan (1964) and Boulton and Huggins (1970).

Aside from investigations establishing the main pathways of glucose degradation, most studies of crustacean respiratory physiology have dealt with the influence of various environmental parameters on metabolic rate (Wolvekamp and Waterman, 1960; Prosser and Brown, 1961; Lockwood, 1967). Although the respiratory response to wide ranges of oxygen tension has been investigated for numerous crustaceans, few correlations have been made between availability of environmental oxygen and metabolic adaptations. Most studies of this nature with invertebrates have been conducted with mud-dwelling worms and clams, aquatic snails and insects, parasitic helminths, and nematodes (Beadle, 1961; Awapara and Simpson, 1967).

Not many studies of crustacean metabolism under anaerobic conditions have been made. Although a general lack of tolerance to anoxic conditions by decapods has been noted by several authors (von Brand, 1946; Wolvekamp and Waterman, 1960; Lockwood, 1967), Teal (1959) observed that certain marsh crabs (Uca pugnax, Uca pugilator, and Eurytium limosum) maintain normal rates of oxygen uptake with the oxygen tension less than three percent of air saturation. Uca pugnax was reported to accumulate lactate with a corresponding disappearance of glycogen during anoxia (Teal and Carey, 1967). Thompson and Pritchard (1969) measured the metabolic responses of two mud-dwelling thalassinid shrimp, Callianassa californiensis and Upogebia pugettensis, to decreasing oxygen tension. Both shrimp

exhibit low metabolic rates, regulation with critical oxygen tensions less than 50 mm Hg, and survival in anoxia for at least three days. However, Upogebia has the greater metabolic rate, higher critical oxygen tension, and is less tolerant of anoxia than Callianassa. Sampling of interstitial and burrow water for oxygen content as well as general observations of the ecology of the shrimp permitted close correlation of the metabolic requirements and availability of oxygen in the environment.

Thompson and Pritchard (1969) also observed a compensatory increase in metabolic rate following 12 and 36 hours of anoxia for Callianassa and Upogebia suggesting that anaerobic metabolism might be employed during anoxia. The purpose of this study was to investigate the metabolic mechanisms used by Callianassa californiensis during anoxic stress. Emphasis was placed on the nature of the anaerobic pathway as indicated by the metabolic products accumulated. Changes in levels of blood lactic acid and blood glucose were studied during a post-anoxia recovery period.

MATERIALS AND METHODS

Collection and Maintenance of Animals

The study was carried out at the Oregon State University Marine Science Center in Newport, Oregon, and at the main campus in Corvallis, from June, 1969, to August, 1970.

All shrimp used in the experiments were collected from mud flats on the south shore of Yaquina Bay directly east of the Marine Science Center laboratories. The vertical distribution of Callianassa californiensis Dana (Stevens, 1928) ranges from about plus three foot to zero tide level in substrates that are predominantly sand. The sandy nature of the substrate does not permit burrows to remain intact during ebb tide and Callianassa californiensis (hereafter, Callianassa) are generally found in the interstitial spaces and collapsed burrows, about two to three feet below the surface. The shrimp were most effectively obtained using a "clam gun," an aluminum tube (4 x 34 inches) with one end closed off except for a small hole serving as an outlet for the displaced air. After the tube is pushed down into the substrate, the small hole is blocked and the gun is withdrawn bringing with it a column of sand. Best results are achieved if this process is repeated two or three times in the same hole.

Selection of the animals to be used was made at the collecting

site. Only males estimated to weigh from three to six grams were used. Animals judged to be in postmolt were excluded. Male Callianassa are easily discerned by their abdominal appendages; they do not have the first two pair of pleopods. These appendages are present in the females but are modified for carrying developing eggs prior to hatching. Males are also the larger of the two sexes and their large chela is a much lighter color than the pink chela of the female. The individual stages of the molt cycle were not determined, but a large percentage of the postmolt animals could be recognized and culled according to three criteria: softness of exoskeleton, lighter color, and possession of soft, white setae.

Immediately after collection the shrimp were taken to the laboratory in plastic buckets containing about one inch of substrate and about six inches of water from their environment. The shrimp and substrate were then transferred to a three-gallon glass aquarium which was placed in a large water table maintained at 10° to 15°C with running seawater piped into the laboratory from the bay. The water inside the aquarium was continuously circulated by slowly running unfiltered seawater into it. The seawater in the laboratory was found to fluctuate between 25‰ and 35‰ (mean of 31.9‰) for a similar period of time in 1966 (Thompson and Pritchard, 1969). No attempt was made to feed the animals as they are detritus-feeders and obtain sufficient organic material by sifting through the sandy substrate

(Mac Ginitie, 1934). Observations of fecal material in the hind-guts and aquarium for a group of animals kept for a month in the manner as described above support the conclusion of Thompson and Pritchard (1969), that the animals can obtain sufficient food to maintain themselves under these conditions. No animal used in the experiments was maintained in the lab longer than two days. However in studying tissue glycogen one experiment was conducted using animals which had been in the laboratory for 10 days. The results of this experiment (14 hours of anoxia) were so variable that the data was not used.

The animals used for experiments in Corvallis were transported in 7 x 7 x 10 inch styrofoam containers with substrate and about three inches of water. These containers were placed in constant temperature rooms at 10°C and aeration resumed.

Experimental Protocol

Experiments were executed both at the Marine Science Center and at the main campus in Corvallis. The procedure followed at each location was essentially the same with two major exceptions: (1) all experimental work was done in the 10°C constant temperature room at Corvallis while at Newport a circulating water table was used as a means of controlling the temperature; (2) in Corvallis liquid nitrogen was employed to quick-freeze all samples of tissue and blood. At Newport glycogen extraction from the tissue samples and protein

precipitation from the blood samples prior to freezing were carried out immediately. All analytical measurements were conducted on the main campus.

Seawater used in all experiments had been passed through a synthetic filter (5μ clearance) and an ultraviolet sterilizing device. The water was collected directly from the filter during high tides and the salinity in parts per thousand (‰) read from a constant recording hydrometer described by Thayer and Redmond (1969). The range in salinity used was 28‰ to 34‰ with a mean of 32.7‰.

Prior to the experiment the shrimp were starved for two to five hours by being placed in another aquarium with only aerated, filtered seawater, the same as that to be used in the experiment. In preparation of the seawater for the experiment, two groups of flasks containing filtered seawater of known salinity were set up. Water of one group (the control) was aerated with compressed air for two hours. Simultaneously anoxic seawater was prepared by bubbling nitrogen gas through the water of the second group. A micro-Winkler determination on the latter group showed only a small amount of oxygen remaining in the water (0.00 cc/l--0.02 cc/l) which was considered negligible and was probably consumed in the experiment. A micro-Winkler sample at the termination of the experiment showed no detectable oxygen.

Museum specimen jars (0.4 l) were used as chambers for both

control and stressed animals. At zero time jars for animals to be stressed were filled with anoxic seawater. Care was taken to avoid aeration. Two shrimp were then placed in the jar and the jar was sealed with a piece of Parafilm and a screw cap. For the control animals, two shrimp were placed in each jar which had been filled about three-quarters full with saturated seawater. The top of the jars were then covered with Parafilm to prevent evaporation and an aerating stone was inserted through a small hole in the Parafilm to maintain the saturated condition. All jars were then placed either in the water table or on a shelf in the constant temperature room.

At each sampling time six controls and six stressed animals were sacrificed and blood, chela muscle and hepatopancreas samples taken. Just prior to preparing the experimental chambers, pre-stress values were obtained from six animals sacrificed directly from the aquarium. In experiments designed to follow recovery after anoxia, samples of stressed and control animals were taken after thirteen hours. The remaining stressed animals were then transferred to aerated water and samples taken as before at $1\frac{1}{2}$, 4, and 10 hours during the recovery period.

To prevent contamination of the blood sample by seawater draining into the collection vessel, the tail was blotted dry and the entire animal was wrapped in a Kimwipe during collection. Approximately 0.5 ml of blood was removed from each shrimp by puncturing the

exoskeleton just in front of the anus with a capillary tube, and allowing the blood to drain into a chilled vessel. Blood samples to be frozen and used later were collected in 1.0 ml plastic vials, capped, and dropped into liquid nitrogen. They were then stored at -80°C until used for analyses. For samples to be deproteinated immediately, 0.2 ml aliquots were transferred to a 1.0 ml polystyrene microfuge tube containing 8% $HClO_4$.

Tissue samples were either dissected and digested in 30% KOH immediately or the whole animal was frozen in liquid nitrogen and the tissue dissected and processed later upon thawing. In either case the tissue was excised in the same manner. Muscle from the carpus of the cheliped was removed, blotted with a damp towel, and placed in KOH. The animal was next bisected directly posterior to the carapace, the hepatopancreas removed and treated in the same way as muscle.

Samples of blood and tissue were also obtained from shrimp directly at the collecting site. A five liter Dewar thermos containing liquid nitrogen was carried to the mud flats at low tide to freeze the blood and tissue samples. As soon as a male, intermolt shrimp was dug from the substrate, its blood sample was taken and frozen as previously described. The whole shrimp was then put into a 50 ml, plastic, snap-cap vial and put into liquid nitrogen. Later the vials were transferred to a -80°C freezer and stored until used for analyses.

Determination of Blood Lactic Acid

Blood L-(+)-lactic acid was determined enzymatically at 340 mμ, per Sigma Technical Bulletin No. 826-UV. Sigma reagent kits and individual reagents were used for all L-(+)-lactate analyses.

The method employed is based on the following reversible reaction catalyzed by the enzyme, lactic dehydrogenase:

$$\text{Pyruvate} + \beta\text{-NADH} \rightleftharpoons \text{L-(+)-lactate} + \beta\text{-NAD}$$

To determine L-(+)-lactate the reaction is run left to right by making β-nicotinamide-adenine dinucleotide (β-NAD) present in excess. The amount of lactate oxidized and β-NADH produced is followed spectrophotometrically by measuring the optical density at 340 mμ, the wavelength of the maximum peak of absorption for β-NADH. The amount of β-NADH formed is a measure of the amount of L-(+)-lactate originally present. The reaction does not go to completion unless the pyruvate is removed. Hydrazine, incorporated into the buffer, forms a hydrazone with pyruvate and removes it from the reaction mixture.

Aliquots of 2.0 ml whole blood were added to 0.4 ml 8% $HClO_4$ in polystyrene microfuge tubes to precipitate blood proteins. The protein-free supernatant was obtained by spinning these tubes for 15 minutes at "full speed" in an International clinical centrifuge. The samples were then frozen at -12° C until used for analyses.

In preparation of the reaction mixture, 2.0 ml of glycine buffer

(pH 9.2, containing hydrazine), 4.0 ml glass distilled water, and 0.1 ml lactic dehydrogenase (in $(NH_4)_2SO_4$ suspension) was added to vials containing 10 mg of β-NAD. One such vial was prepared for each two samples to be analyzed. Each vial was inverted several times and the contents of all vials combined in a flask.

An aliquot of 2.8 ml reaction mixture was added to each reaction tube and incubated at 37°C for five to ten minutes. The protein-free supernatants were thawed on ice and 0.2 ml added to each reaction tube. Duplicate determinations were made on each blood sample. An equal amount of 8% $HClO_4$ was added to one tube as a blank. The optical density at 340 mμ was recorded after 30 minutes using either a Beckman Model DU-2 ultraviolet spectrophotometer or a Beckman DU monochromator (catalog no. 2800) with a Gilford attachment. All reactions were considered 98% complete at 30 minutes if the optical density did not change more than .001 unit per minute after 30 minutes. If samples were expected to contain more than 60 mg/100 ml blood, 0.1 ml aliquots of protein-free supernatant and 0.1 ml distilled water were added to the 2.8 ml of reaction mixture.

This method does not require a calibration curve if a narrow band instrument such as the Beckman DU-2 and quartz cuvettes with one centimeter lightpath are used. Lactic acid concentration may be calibrated directly from the optical density.

Determination of Blood Glucose

Levels of blood glucose were measured colorimetrically using Glucostat, a prepared enzymatic reagent developed by Teller (1956) and produced by the Worthington Biochemical Corporation. Glucostat is specific for β-D-glucose and makes use of the following coupled enzyme reactions:

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{glucose}} H_2O_2 + \text{gluconic acid}$$

$$H_2O_2 + \underset{\text{reduced}}{\text{chromogen}} \xrightarrow{\text{peroxidase}} \underset{\text{oxidized}}{\text{chromogen}} + H_2O$$

A blood supernatant was first prepared by adding 1.0 ml 1.8% $Ba(OH)_2 \cdot 8H_2O$ to 0.1 ml whole blood in 1.9 ml distilled water. After mixing, 1.0 ml 2.0% $ZnSO_4 \cdot 7H_2O$ was added with more mixing. The preparation was then placed on ice until the precipitate could be spun down at 1000 x g for five minutes in a Sorvall Superspeed RC2-B refrigerated centrifuge. Duplicate determinations were made on each blood sample.

After centrifugation 2.0 ml portions of each blood supernatant was placed in a separate test tube. Equal aliquots of distilled water and 15 mg% or 30 mg% standards were placed in additional tubes for a reagent blank and standard. At timed intervals 2.0 ml of freshly prepared Glucostat reagent was added to each tube and the reaction allowed to proceed at room temperature. After ten minutes two drops

of 4 N HCl was added to each tube to stabilize the color. After five minutes the color, which was stable for several hours, was ready to read at 415 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer.

Determination of Glycogen

The method used for glycogen determination in tissues is a modification of that employed by Montgomery (1957). It involves the phenol-sulfuric acid procedure for the determination of alkali-soluble polysaccharides ("glycogen") in tissues and is not affected by amino acids or proteins unless the latter contain carbohydrate constituents. The phenol-sulfuric acid procedure was selected because it is simple, rapid, sensitive (to about 5 μg of glycogen), reproducible, and employs a reagent which is inexpensive and stable on storage (Montgomery, 1957).

Prior to tissue dissection a pre-weighed graduated centrifuge tube containing 3.0 ml 30% KOH was set up for each tissue sample. Immediately following dissection the tissue was transferred to the KOH and the wet weight of the sample obtained by subtraction. A marble was placed on the top of each tube and the tissues were digested (with occasional shaking) for one to one and a half hours in a boiling water bath.

After cooling the tubes, enough 95% ethanol was added to each digest to bring the meniscus to the 7.0 ml mark. Just after the heat

was taken away, the tubes were placed back into the boiling water bath for three minutes and watched carefully. They were then cooled rapidly and spun at "full speed" in an International clinical centrifuge (head #221) for 10-15 minutes to get the glycogen pellet. The supernatant was decanted and the pellet redissolved in about 3.0 ml of water. Ethanol was again added to the 7.0 ml mark and the precipitation procedure repeated. The supernatant was decanted and the tubes drained at a 45° angle for ten minutes. The glycogen pellet was then thoroughly dissolved in water and the tube filled to 10.0 ml. For the final dilution one milliliter portions of each solution were added to 9.0 ml of water in a test tube.

For triplicate determinations of each sample, three drops of 80% phenol were added to each of three 2.0 ml aliquots of the unknown solution in 16 x 150 mm test tubes. Next 5.0 ml of concentration H_2SO_4 was delivered to each tube from an automatic pipette syringe. The stream of H_2SO_4 was directed into the center of the liquid to obtain good mixing, which was further ensured by shaking after the addition of the acid. A yellow-orange color was produced in the reaction, the temperature of which rose to approximately 110° C. After 60 minutes at room temperature the optical density was measured at 490 mμ on a B & L Spectronic 20 spectrophotometer. The color obtained is stable for several hours. A reagent blank and standards were run with each series.

In order to reduce variation encountered in a set of colorimeter tubes, only one tube was used for the samples. In between reading each sample, a pasteur pipette connected to an aspirator was used to aspirate the solution from the tube.

RESULTS

Effect of Anoxia on Blood Lactate and Blood Glucose

When Callianassa were deprived of oxygen they accumulated blood lactic acid at a steady rate. Lactic acid was produced at a rate of about 9.4 mg/100 ml per hour (Figure 1). After 30 hours of anoxia the blood lactate level was 290.86 ± 19.66 (6)[1] mg/100 ml while 5.47 ± 0.98 (6) mg/100 ml was the level for the control group at this time. The values for the control groups remained relatively constant throughout the experiment. The mean for 40 control animals sampled throughout the study was 7.16 ± 0.47 (40) mg lactate/100 ml of blood.

Samples were also taken from the sea water in the experimental jars at 30 hours to determine if lactic acid was excreted. No lactate was detected either because there was none present or because it was diluted by the large volume of water in the jar (approximately 385 ml).

Larger individual variation was observed in blood glucose values as compared to lactic acid. According to several authors (Kleinholz and Little, 1949; Scheer and Scheer, 1951; McWhinnie and Saller, 1960; Dean and Vernberg, 1965; Telford, 1968a) variation in blood glucose concentration among individuals or even within the same individual

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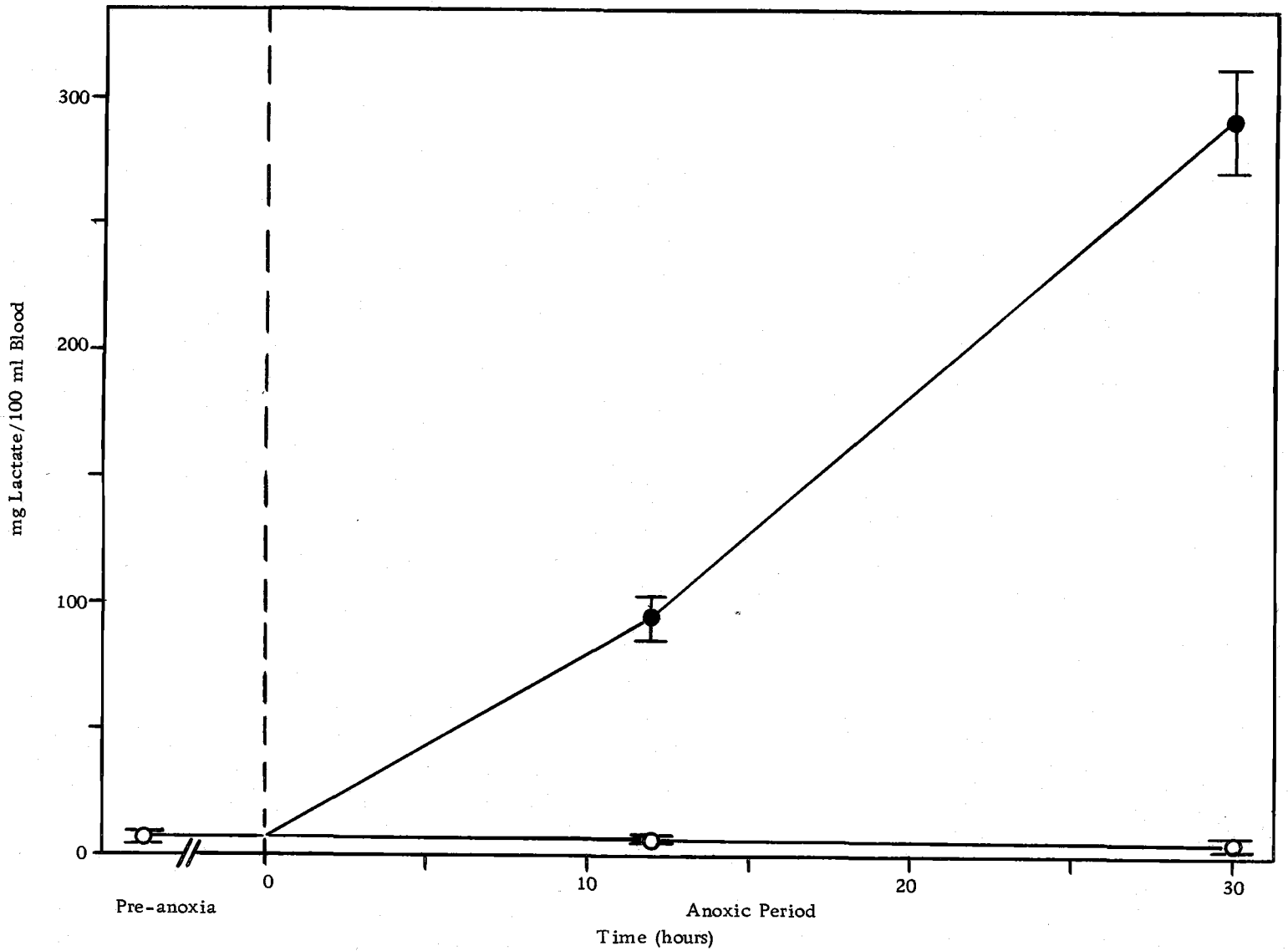
[1] Values are given as: Mean ± S.E. (N); where S.E. is standard error and N is the number of animals sampled.


300
200
100
0
mg Lactate/100 ml Blood
0
10
20
30
Pre-anoxia
Anoxic Period
Time (hours)

over different intervals of sampling is not uncommon for crustaceans. Blood glucose concentration is seen to increase over the first 13 hours of anoxia to 42.40 ± 2.15 (5) mg/100 ml as compared to the control group (Figure 2). After 13 hours the blood glucose no longer increases but levels off and tends to slowly decrease. T-test analysis showed that there is no significant difference between the means of experimental and control groups at 30 hours as the variation in response between individuals is large. The mean value of blood glucose for all 29 control animals sampled was 14.96 ± 1.43 (29) mg/100 ml. This value is among the higher ones in a table of "fermentable reducing substances" presented in the review by Florkin (1960) of crustacean metabolism.

Effect of Anoxia on Tissue Glycogen

Glycogen content of the hepatopancreas and chela muscle were determined after 14 hours and 31 hours of anoxia. Two 14-hour anoxia exposure experiments were performed, approximately one year apart. After 14 hours of anoxia t-test analysis showed there was no significant difference between the means of the control and experimental groups, either for individual tissues or for combined values (Table 1). However, there are consistent trends which should be noted. The overall level of glycogen is considerably lower in May 1970, compared with July 1969. I can offer no explanation for this other than the

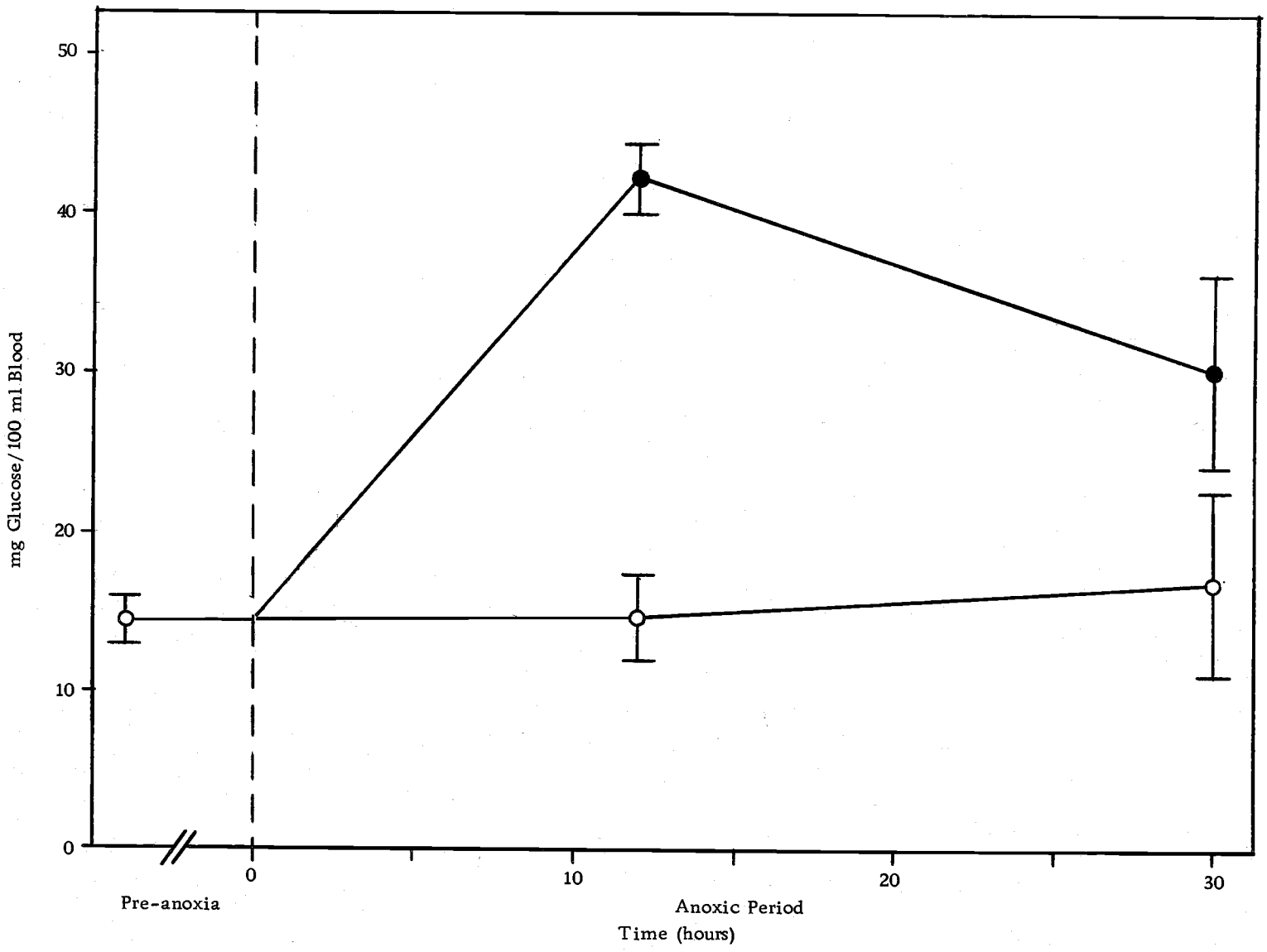

50
40
30
20
10
0
mg Glucose/100 ml Blood
0
10
20
30
Pre-anoxia
Anoxic Period
Time (hours)

Table 1. Percent glycogen (mean ± S. E.) in the hepatopancreas and chela muscle of Callianassa californiensis subjected to anoxic conditions for 14 hours (10° C). Values are given for experiments performed in July, 1969, and May, 1970.[a]

Tissue	Animals	Hepatopancreas	Chela Muscle	Hepatopancreas and Muscle Combined[b]
July, 1969				
Initial Sample	6	.721 ± .167	.384 ± .074	.590 ± .121
Control, 14 hrs.	6	.789 ± .224	.568 ± .066	.682 ± .140
Exptl., 14 hrs.	6	.743 ± .166	.604 ± .067	.691 ± .121
May, 1970				
Control, 14 hrs.	6	.482 ± .094	.406 ± .063	.433 ± .073
Exptl., 14 hrs.	6	.371 ± .091	.532 ± .084	.457 ± .077

[a] Percent glycogen = mg glycogen in tissue/mg wet weight tissue x 100

[b] Hepatopancreas and Muscle Combined = mg glycogen in hepatopancreas + mg glycogen in muscle/mg wet weight of hepatopancreas + mg wet weight muscle x 100

obvious possibility of differing nutritional condition.

The data in Table 2 reveal that after 31 hours of anoxia there is considerable glycogen utilization in both the hepatopancreas and muscle. The means for the experimental groups were significantly lower than the control groups ($P < 0.05$) for each tissue and for the two tissues combined. Even though the differences are statistically significant, there is considerable individual variation as shown by the large standard errors. In view of the fact that only five experimental animals were sampled at 31 hours, more samples should be taken in order to predict the actual degree of response to a relatively long period of anoxic stress.

The range of mean concentrations of glycogen in the hepatopancrease for control groups was from .482 ± .094 to 1.012 ± .211 percent of the wet weight. The range for the chela muscle was from .384 ± .074 to .854 ± .174 percent of the wet weight. These values agree with the average of .80 percent for hepatopancreas of the crab Cancer pagurus reported by Renaud (cited by Vonk, 1960). Jungreis (1968) gives an anverage concentration in the hepatopancreas of the crayfish Orconectes virilis of .70 percent. Meenakshi and Scheer (1961) report an average of .76 percent for the hepatopancreas of Hemigrapsus nudus and .30-1.2 percent for Cancer magister.

A graphic presentation of the amount of tissue glycogen depletion

Table 2. Percent glycogen (mean ± S. E.) in the hepatopancreas and chela muscle of Callianassa californiensis subjected to anoxic conditions for 31 hours (10°C). [a]

Tissue	No. Animals	Hepatopancreas [c]	Chela Muscle [c]	Hepatopancreas [c] and Muscle Combined [b]
Initial Sample	6	1.012 ± .211	.854 ± .174	.952 ± .191
Control, 31 hrs.	6	.915 ± .188	.787 ± .143	.868 ± .164
Exptl., 31 hrs.	5	.343 ± .124	.360 ± .110	.354 ± .114

[a] Percent glycogen = mg glycogen in tissue/mg wet weight tissue x 100

[b] Hepatopancreas and Muscle Combined = mg glycogen in hepatopancreas + mg glycogen in muscle/mg wet weight of hepatopancreas + mg wet weight muscle x 100

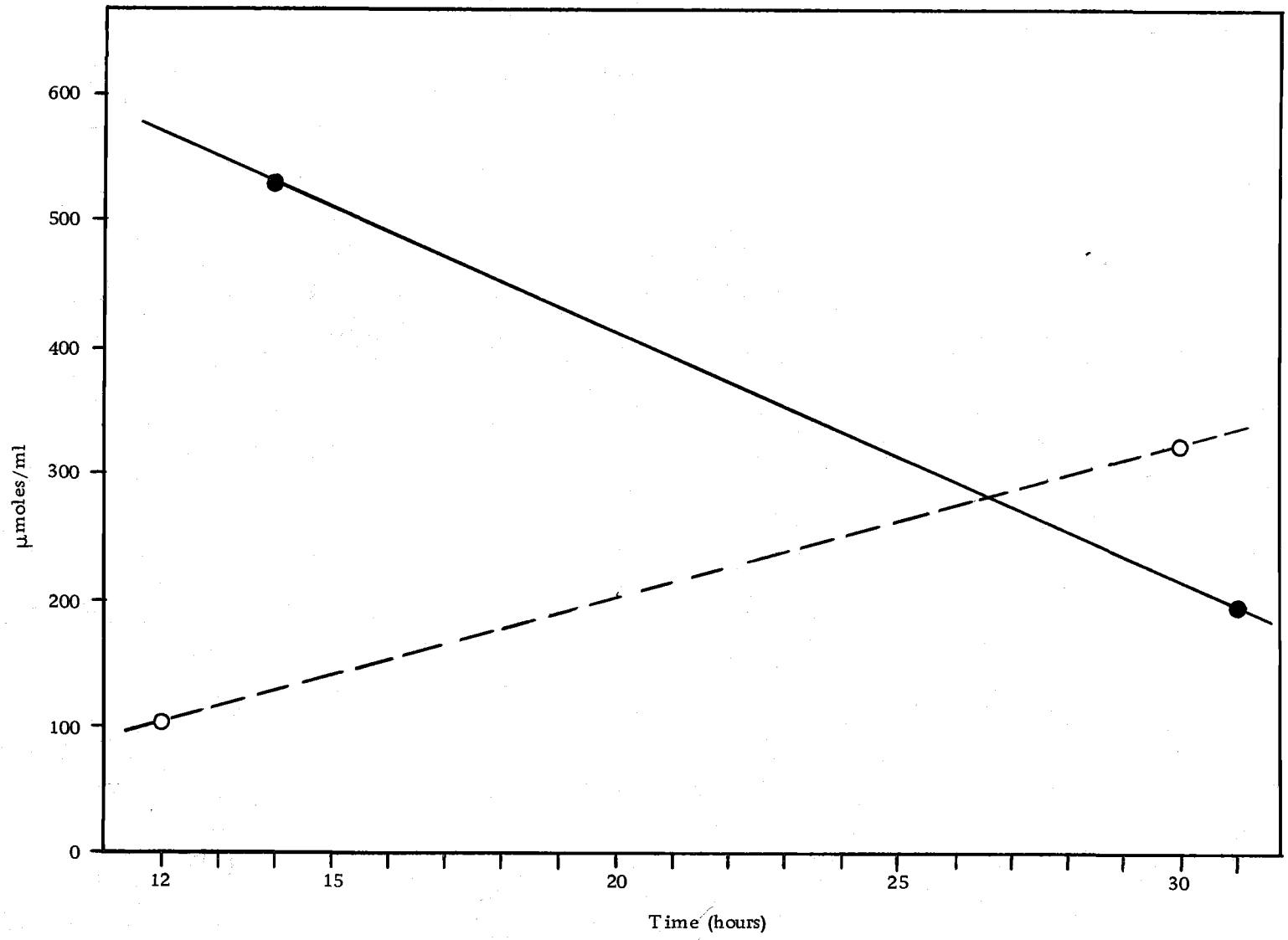
[c] Means for control and experimental groups significantly different at the 5% level as determined by t-test analysis

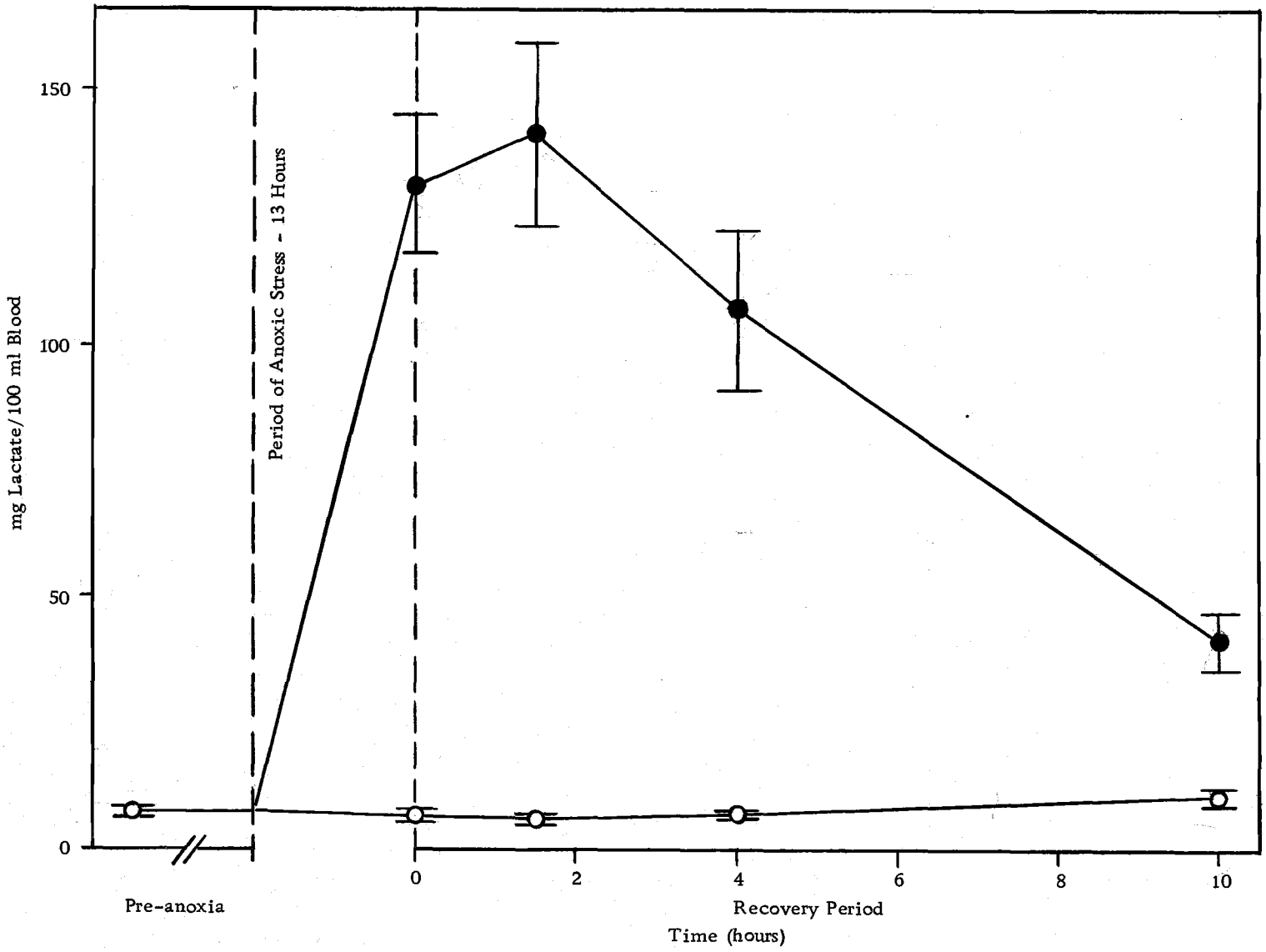
(hepatopancreas and muscle combined) and blood lactic acid accumulation during the last 18 hours of a 30-hour anoxic test is illustrated in Figure 3. The data are taken from the last column of Table 2. In converting percent glycogen to μmoles/ml, glycogen is treated as glucose equivalents. Tissue glycogen is hydrolyzed at the rate of about 19 μmoles glucose/ml per hour while lactic acid is accumulated at a rate of about 12 μmoles/ml per hour in the blood.

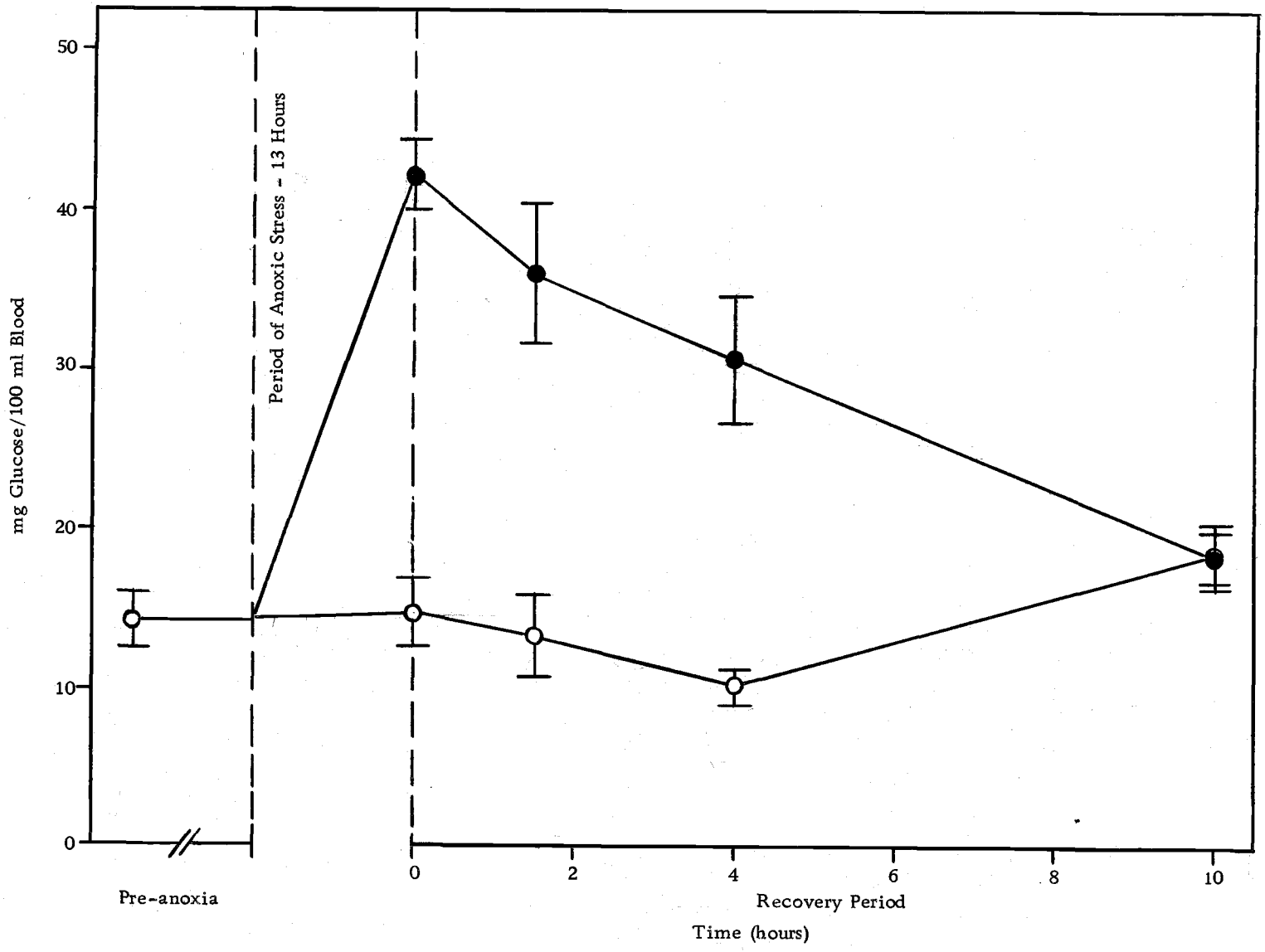
Post-Anoxia Recovery Period

Experiments were conducted in which shrimp, stressed for 13 hours in anoxic conditions, were gently transferred to saturated sea water, and levels of blood lactate and glucose measured at intervals during a ten hour recovery period. A plot of the blood lactate values is presented in Figure 4. During the initial interval of the recovery period the blood lactate concentration shows an increase over the immediate post-stress value. The standard errors, however, overlap considerably. Over the interval from one and a half to ten hours of recovery, lactate steadily decreases to a level of 41.45 ± 6.27 (5) mg/100 ml at ten hours, where it is still significantly higher than the mean of the control group (10.10 ± 1.77) (6) mg/100 ml) at the 0.01 level of probability.

The levels of blood glucose following a 13 hour period of anoxia are illustrated in Figure 5. Immediately following the stress, the


600
500
400
300
200
100
0
µmoles/ml
12
15
20
25
30
Time (hours)


150
100
50
0
mg Lactate/100 ml Blood
Period of Anoxic Stress - 13 Hours
0
2
4
6
8
10
Pre-anoxia
Recovery Period
Time (hours)


mg Glucose/100 ml Blood
50
40
30
20
10
0
Period of Anoxic Stress - 13 Hours
Pre-anoxia
0
2
4
6
8
10
Recovery Period
Time (hours)

blood glucose level shows about a 300% increase above pre-anoxia levels. During the recovery period blood glucose decreases at about 4.67 mg/100 ml per hour until it approximates the control group mean at ten hours.

The pre-anoxia values of Figures 4 and 5 are taken as the means of all the control samples for the recovery experiment. The original pre-anoxia samples were frozen in liquid nitrogen, but thawed overnight when the Dewar flask containing them leaked.

Field Samples

Sampling of shrimp directly in the mudflats was carried out as previously described (see Methods). Tissue samples from six shrimp were obtained and processed for glycogen content in September, 1969. An average of .541 ± .168 percent wet weight was found for the hepatopancreas, .441 ± .124 percent for the muscle, and .544 ± .140 percent for the hepatopancreas and muscle combined.

Blood samples from six animals were taken and assayed for lactic acid and glucose in May, 1970. The mean value for blood lactate was 7.24 ± 1.48 (6) mg/100 ml and is not significantly different from the mean presented above for 40 control animals (7.16 ± .47 (40) mg/100 ml). The mean value of blood glucose for field samples was 5.12 ± 0.72 mg/100 ml which is considerably less than 14.96 ± 1.43 (29) mg/100 ml, the mean of 29 animals used as controls in the laboratory.

DISCUSSION

Glycolysis, generally resulting in pyruvate or lactate end products, is a well documented pathway of anaerobic metabolism in vertebrates. The existence of glycolysis has been established in Crustacea. A rapid rate of lactic acid accumulation in the blood during anoxia furnishes evidence that glycolysis is also the major anaerobic pathway in Callianassa and that lactate is the principal end product. It has been shown that another decapod, Uca pugnax, of Georgia salt marshes, produces lactic acid at a constant rate during anaerobiosis (Teal and Carey, 1967). However the mean value of blood lactate for 40 control Callianassa (7.16 ± 0.47 (40) mg/100 ml) was higher than the 2.7 mg lactate/100 g of whole animal tissue reported by Teal and Carey (1967) for Uca pugnax. Production of lactate during anaerobic conditions has been shown in three intertidal barnacles (Barnes, Finlayson, and Piatigorsky, 1963).

The presence of end products other than lactic acid was not examined in this study. However, the production of other fermentation products, such as the acetic and propionic acids found in snails by Mehlman and von Brand (1951), is not ruled out. The possibility of other end products is consistent with the findings of von Brand (1946), who concludes that invertebrates generally metabolize glycogen to a mixture of acids.

The observed hyperglycemia in Callianassa during anoxia (Figure 2) probably provides an increased supply of substrate for glycolysis during the first 13 hours of anoxia. Anoxic hyperglycemia seems to be a general response in Crustacea. Kleinholz and Little (1949) reported a three-fold increase in the fermentable blood sugar of the spider crab Libinia emarginata with asphyxiation. An increase in fermentable substances of the blood was also produced by asphyxia in Astacus trowbridgii and Callinectes sapidus (Kleinholz, Havel, and Reichart, 1950). In 1935 Roche and Dumazert (cited in Kleinholz and Little, 1949) were able to elicit an increased glycemic level in Cancer pagurus by removing the animals from sea water for one hour.

The hyperglycemia incurred during anoxia might be attributed to glycogenolysis in view of the wide occurrence of this process in Crustacea. Recent support for glycogenolysis in the crayfish, Astacus astacus, has been reported for in vivo experiments with normal animals (Zandee, 1966). The results of the present study (Table 1), however, reveals that no significant amount of glycogen was mobilized in Callianassa during the first 14 hours of the stress period. Two possible explanations of these findings will be presented. Both explanations depend on reports of free oligosaccharides, namely maltose, maltotriose, maltotetraose, and a galactan derivative in the blood of several decapods (Hu, 1958; Meenakshi and Scheer, 1961; Scheer and Meenakshi, 1961; McWhinnie and Saller, 1960; and Dean

and Vernberg, 1965). Telford (1965, 1968a, b, c) has also reported the occurrence of fructose, trehalose, and galactose in addition to the maltose oligosaccharides in crustaceans.

In the first explanation it is suggested that as glucose units are hydrolyzed from glycogen and passed into the blood, free maltose or oligosaccharides from the blood might be synthesized into glycogen, resulting in no net decrease of glycogen. This first possibility is based on pathways of sugar metabolism in crustaceans proposed by Scheer and Meenakshi (1961) and Meenakshi and Scheer (1961). Their evidence suggests that maltose oligosaccharides of the blood are intermediates in glycogenesis, and any oxidation of maltose or its oligosaccharides takes place through glycogen synthesis, and subsequent phosphorolysis, glycolysis, and the Krebs cycle.

A second possibility is that a considerable portion of the increased blood glucose levels result from the direct hydrolysis of maltose or oligosaccharides in the blood. Although Scheer and Meenakshi (1961) reported that no maltase had been found in crustacean blood, Handel (1968) has reported indirect evidence for maltase and sucrase in the blood of insects. She observed the oxidation of injected maltose and sucrose to carbon dioxide in homogenates of gut-free mosquito abdomens at a rate equivalent to that which injected glucose was oxidized. She concluded that the hydrolysis takes place in the extracellular space because the rate of hydrolysis of sucrose and

maltose *in vitro* by hemolymph of the honeybee was sufficient to explain the utilization of these sugars when injected *in vivo* into the honeybee. Measuring changes in blood glucose and reducing sugars of *Homarus americanus*, Telford (1968a) demonstrated that stress (constant handling in air for five minutes or storage in a commercial pound) caused a greater increase in blood glucose than reducing substances. Using paper chromatography he found that the levels of blood glucose increased at the expense of certain free oligosaccharides in the blood. Telford concluded that, although glucose could be released into the blood while some other reducing substance was withdrawn, new glucose was derived directly from reducing substances in the blood either in part or *in toto*.

Blood glucose concentration did not continue to increase during the last 18 hours of anoxia but remained at about the same level or tended to decrease slightly (Figure 2). It appears that after about 13 hours of anoxia an equilibrium is established between the rate of blood glucose production and the rate at which tissues absorb blood glucose for use in glycolysis. The glycogen concentration in the muscle and hepatopancreas displays a significant decrease during this same period (Table 2). This suggests that during a longer period of anoxic stress the blood oligosaccharides used, either for direct glucose production or for a glucose-oligosaccharide exchange in glycogen, are diminished to a base level. Although this investigation presents no

direct evidence concerning the existence or utilization of free blood oligosaccharides in Callianassa, their possible significance during anoxia is worthy of further investigation.

Longer periods of anoxic stress are associated with considerable decline in tissue glycogen (Table 2). As indicated in Figure 3 blood lactic acid build-up during the last half of the 30-hour anoxic stress does not match the glycogen disappearance--i.e., 19 μmoles of glycogen were hydrolyzed per milliliter per hour, giving an expected lactic production of 38 μmoles per milliliter per hour; however the observed rate of accumulation in the blood was only 12 μmoles per milliliter per hour. Several possibilities might account for the discrepancy: accumulation of end-products other than lactic acid; excretion into the medium; or tissue storage.

As previously mentioned, no attempts were made in this study to identify other possible end-products of anaerobic glycolysis, although the existence of such products, especially particular amino acids, is certainly a possibility. Awapara and Simpson (1967), for example, have noted the importance of transamination of carbohydrate intermediates in maintaining the amino acid pool of marine invertebrates. A mud-dwelling marine clam, Rangia cuneata, accumulates small quantities of lactate while its main anaerobic products are succinate and alanine (Stokes and Awapara, 1968). Huggins (1966) discusses the importance of transamination reactions in the

intermediary metabolism of the shore crab Carcinus maenas. Moreover, amino acids are known to play an important role in osmoregulation of brackish-water crustaceans (Lockwood, 1967).

Attempts to find lactate in the sea water surrounding the animals failed, either because there was none excreted or because the lactate was diluted beyond the sensitivity of the analysis.

The possibility of lactic acid storage in the tissue was not investigated. Large quantities of acid metabolites could have a drastic effect on cellular pH and the osmotic balance of the cells. Beadle (1961) and von Brand (1946) have noted that intracellular accumulation of anaerobic end products often results in paralysis in many invertebrates. Paralysis or lethargy was not noted for any of the Callianassa stressed in anoxic conditions, but decreased activity was observed in these animals relative to the controls, in agreement with the observations of Thompson and Pritchard (1969). Decreased activity as an adaptation to temporary oxygen deficiency should be interpreted with caution, as Beadle (1961) warns that this "closing of the throttle" might be the consequence of anaerobic end-product accumulation in the tissue. Wieser and Kanwisher (1959) showed that slow-moving animals (nematodes and mites) living among intertidal algae remain beneath the fronds at low tide. In the laboratory they are paralyzed by lack of oxygen but can recover after 16 hours of anoxia when exposed to oxygen. The paralysis is attributed to build-up

of end-products in the tissue.

Re-establishment of aerobic conditions following a period of anaerobiosis generally results in oxidation of fermentation products and an increase in oxygen consumption referred to as an oxygen-debt. In some species, where there is no rise in oxygen consumption during the recovery period, as in Schistosoma mansoni (Bueding, 1949) and Arenicola marina (Dales, 1958), there is usually a loss or excretion of the end products. Other examples exist in snails, however, where the oxygen-debt during recovery is in excess of that needed to oxidize all the end products accumulated (von Brand, McMahon, and Nolan, 1955). In the present study decline in blood lactic acid (Figure 4) during the recovery period can be associated with the post-anoxia increased oxygen consumption found for Callianassa by Thompson and Pritchard (1969). Quantitative estimations of oxygen-debt are difficult to interpret (Prosser, 1961) because the percentage paid off depends on whether the end products are completely oxidized, partially converted to carbohydrate, or partly excreted. The fate of anaerobic end-products during and after anoxia is not completely understood for Callianassa and the experiments with post-anoxia respiration (Thompson and Pritchard, 1969) were only preliminary. Hence no attempt was made to calculate the percentage of the oxygen-debt paid back during recovery.

The gradual decline in blood glucose to control levels during

the recovery period (Figure 5) is a generally accepted response in crustaceans. In 1924 Hemmingsen (cited by Kleinholz and Little, 1949) observed a similar response for Astacus when a concentrated free glucose solution was injected.

More recently in vivo studies by Meenakshi and Scheer (1961) with crabs suggest a possible explanation for the decline in blood glucose. When the blood of Cancer magister was sampled at intervals after injection with ^{14}C-glucose, the label was found in maltose after two hours, in maltotriose and maltotetraose after six to nine hours, and in all three in maximal amounts after twelve hours. Considering the results of Meenakshi and Scheer (1961) and the lack of glycogen depletion during the first 14 hours of anoxia for Callianassa, it is interesting to speculate that the decrease in blood glucose during recovery corresponds to glucose utilization in polymerization reactions yielding free blood oligosaccharides. Such a mechanism would build up oligosaccharide reserves used during the short period of anoxic stress and would be consistent with the hypothesis mentioned previously concerning the source of glucose during brief periods of anaerobiosis.

The importance of mild stress as it affects blood sugar values in lobsters has recently been investigated (Telford, 1968a; Stewart, Dingle, and Odense, 1966). Mild handling and shipping of lobsters elicited at least a three-fold increase in blood glucose. The state of

hyperglycemia in animals that were shipped was maintained up to three weeks in some individuals. In Callianassa the large difference for blood glucose observed between laboratory controls (14.96 ± 1.43 mg/100 ml) and field samples (5.12 ± 0.72 mg/100 ml) can probably be attributed to stress also. The shrimp inevitably received some handling prior to the experiments. All Callianassa used received as close to identical treatment as possible. Hence, even though the absolute values may be somewhat high, the relative response between controls and experimentals is considered most important in this study.

Callianassa californiensis, a burrowing crustacean inhabitant of mudflats along the Pacific coast, is subjected to severely hypoxic interstitial water twice daily for a maximum of 18 hours in a 24-hour period (MacGintie, 1935). Callianassa has shown several adaptive respiratory responses (Thompson and Pritchard, 1969) among which was the ability to survive approximately 6 days of anoxia. In the present study a preliminary approach has been made to an understanding of the metabolic mechanisms employed by Callianassa during anoxic stress. Callianassa has been found to accumulate lactic acid in the blood during anoxia, reaching a level more than 300 percent of normal (pre-stress) animals after 30 hours of anoxic stress. Hyperglycemia also occurs, and both blood lactate and blood glucose return to normal levels after 10 hours of post-anoxia recovery. During shorter periods of anoxia (12-14 hours) mobilization of glycogen is not

significant, but in the latter half of the 30-hour tests considerable amounts of glycogen are utilized. The amount of lactate appearing in the blood during this period is less than expected from the depletion of glycogen. The question of lactate excretion or storage in tissues during long anoxia stress periods should be further studied in Callianassa.

One of the most intriguing questions posed by this study is that concerning the existence and possible role of free oligosaccharides in the blood of Callianassa. The author is hopeful that the discussion of these substances might spur further investigation into their significance during short periods of anoxia, comparable to that encountered during a tidal cycle.

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