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The Function of the Extracellular Hemoglobin
of the Opheliid Polychaete
Euzonus mucronata (Treadwell)

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A THESIS

Presented to the Department of Biology and the
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in partial fulfillment of the
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Master of Science
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Terwilliger, R.C., N.B. Terwilliger, E. Schabtach and
L. Dangott (1976) Erythrocrucorins of Euzonus
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extracellular hemoglobin Comp. Biochem. Physiol.
57A; 143-149

Haynes, J.K., D.R. Wilson, M. Price, L.J. Dangott,
P. Mied and D.A. Powers (1976) Structural and
functional studies of the isohemoglobins from
Fundulus heteroclitus Bio Bull 151; 413

TABLE OF CONTENTS

| <u>SUBJECT</u> | <u>PAGE</u> |
|--------------------------------|-------------|
| Introduction | 1 |
| Material and Methods | 5 |
| Results. | 10 |
| Discussion. | 13 |
| Conclusion. | 24 |
| Figures. | 25 |
| Bibliography | 46 |

LIST OF FIGURES

| DESCRIPTION OF FIGURE | PAGE |
|--|------|
| Figure 1. Oxy and deoxy-hemoglobin absorption spectra of <u>Euzonus mucronata</u> combined pigments. . . . | 26 |
| Figure 2. Oxygen equilibrium curve of <u>Euzonus mucronata</u> combined hemoglobins. | 28 |
| Figure 3. Hill plot of oxygen equilibrium of combined pigments of <u>Euzonus mucronata</u> | 30 |
| Figure 4. Effect of pH on the oxygen binding of <u>Euzonus mucronata</u> combined hemoglobins | 32 |
| Figure 5. Effect of specific ions on the oxygen binding of <u>Euzonus mucronata</u> combined hemoglobins. | 34 |
| Figure 6. Effect of temperature on the oxygen affinity of <u>Euzonus mucronata</u> combined hemoglobins | 36 |
| Figure 7. Relationship of size and metabolic rate in <u>Euzonus mucronata</u> | 38 |
| Figure 8. Effect of carbon monoxide-blocked hemoglobin on the respiration of <u>Euzonus mucronata</u> | 40 |
| Figure 9. Percent decrease in respiration rate of <u>Euzonus mucronata</u> as caused by carbon monoxide-blocked hemoglobin | 42 |

LIST OF TABLES

| DESCRIPTION OF TABLE | PAGE |
|--|------|
| Table I. Absorption maxima and extinction coefficients of <u>Euzonus mucronata</u> combined hemoglobins. | 43 |
| Table II. Metabolic rates of <u>Euzonus mucronata</u> | 44 |
| Table III. Oxygen binding characteristics of selected Annelid worms | 45 |

INTRODUCTION

Euzonus mucronata, the blood worm, is abundant in the sandy beaches along the West coast of Oregon. This member of the family Opheliidae was first described by Treadwell (1914) in Southern California and is now known to occur along the entire West coast of North America. The worm lives in strictly defined zones high in the intertidal and occurs in population densities from 2,500 to 3,000 worms per square foot (McConnaughey and Fox 1949). Although the animals are found in such great abundance and have been shown to be an important factor in the chemical and physical turnover of the sandy beach, few studies have been done on the physiology of the animal.

Fox in 1948 in describing biochromes from the worm showed the presence of hemoglobin. McConnaughey and Fox (1949) described further the anatomy and biology of Euzonus. The larval development of E. mucronata was described by Dales(1949). Eikenberry(1966), describing the behavior of the worm, showed that the animal makes a diurnal migration vertically through the sand column. This pattern of activity was shown to be stimulated by the change in nature of the beach sand during a tidal cycle. It was proposed that the purpose of this activity is to remove

the worm from the upper layers of sand which are exposed to surf action and agitation, conditions that could lead to the worms possible dislodgement. This interesting observation was followed by work of Ruby and Fox(1976). They showed that E. mucronata was able to live for up to 20 days under complete anoxia. It was proposed that this ability to survive anaerobically would allow the animal to escape into anoxic layers of sand when the tide is high. When the tide recedes the worms could then return to the aerated surface layers. One of the most obvious characteristics of this worm is the presence of high concentrations of hemoglobin which can be observed through the body wall. However, Ruby and Fox(1976) present little evidence for the role of the hemoglobin in the respiratory physiology of this worm.

Recently, there is increasing interest in the physiologic function of the invertebrate hemoglobins(for review see Mangum 1977). Studies have been done on the role of the coelomic cell hemoglobins in annelid respiration (Mangum 1974, Wells 1975, Hoffman and Mangum 1970) as well as the function of the high molecular weight extracellular pigments(Johnson 1941, Mangum 1974, Mangum et al 1975). The role of the extracellular hemoglobins is believed to be one of oxygen transport and the intracellular annelid hemoglobins are thought to function in oxygen transport and

also in oxygen storage. One strategy of oxygen transport proposed by Manwell(1960) is the presence of an oxygen transfer system between the circulating vascular hemoglobins and the higher affinity coelomic cell hemoglobins. Oxygen equilibria of the vascular and the coelomic cell hemoglobins of Pista pacifica and Thelepus crispus give evidence for the possibility of such oxygen transfer systems in Terebellids(Terwilliger 1974, Garlick and Terwilliger 1975).

Euzonus mucronata has a high molecular weight extracellular hemoglobin(Terwilliger 1976). It has been shown that the hemoglobin is heterogeneous consisting of a naturally occurring dimer, 6×10^6 molecular weight, that comprises approximately 15% of the molecular population as well as the usual 3×10^6 molecular weight hemoglobin found in other annelid species(Waxman 1971, Weber 1970). No work has been reported, however, on the function of these hemoglobins.

The purpose of this thesis is to 1. Examine the oxygen binding properties of the hemoglobin of Euzonus under a variety of conditions ie. ions, pH and temperature 2. To observe the metabolic requirements of the worms under conditions the animal might encounter in its environment 3. To analyze the possible function of the pigment in reference to the metabolic needs of the worm. The respiratory

strategies of Euzonus are compared with those known for other annelid species.

MATERIALS AND METHODS

Euzonus mucronata Treadwell was collected from Lighthouse Beach, two miles south of Charleston, Oregon and identified according to Smith and Carleton (1975). The animals were bled immediately after cleaning the body surface or kept in moist sand with daily flushes of sea water until use.

Extraction of hemoglobin was accomplished by cutting the worms and allowing them to bleed into ice-cold 0.1 ionic strength Tris-HCl buffer (pH 7.0) 0.1M in NaCl, 0.01M in MgCl₂. The solution was centrifuged at 12,000g (4°C) to remove debris and treatment of the supernatant with 60% ammonium sulfate precipitated the pigment. The precipitated hemoglobin was resuspended in the extraction buffer and chromatographed on a column of Sepharose 4B (1.8 x 75cm) which was in equilibrium with the same buffer. Fractions were collected, combined and further purified by passing the solution through Millipore filters (0.45 μ pore size) before use. All experiments were performed on combined monomers and dimers.

Absorption maxima of oxy, deoxy and carbonmonoxy hemoglobin were measured on a Zeiss PMQ-II spectrophotometer. Deoxygenated hemoglobin was prepared by adding

sodium dithionite (hydrosulfite) to the hemoglobin solution. Carbonmonoxy hemoglobin was prepared by reacting concentrated sulfuric acid with formic acid and bubbling the liberated carbon monoxide gas through the solution of deoxy-hemoglobin.

Oxygen equilibrium curves were determined on freshly prepared combined hemoglobins by the spectrophotometric method of Benesch et al(1965). Samples to be used in experiments measuring the effect of pH or salt were dialyzed against several changes of the appropriate buffer overnight. pH was measured before and after each experiment with a Corning Model 12B Research pH Meter. Absorption spectra were made before and after each experiment to determine the amount of met-hemoglobin formation. Isosbestic points were monitored during the experiments.

Metabolic rates were measured with a Gilson Differential Respirometer Model GRP-14. Animals to be used for experiments were kept in glass culture dishes in running sea water for 2 days prior to each experiment to allow the worms to evacuate any sand from their guts. Experimental worms were dried with Kimwipes and weighed. One worm was placed in each reaction vessel and acclimated to the experimental temperature for 2 hours prior to the experimental run. Readings were taken every hour for up to three hours. The reaction vessels were allowed to equilibrate to

atmospheric conditions between each measurement.

Continuous recordings of oxygen consumption in a closed container were made using a YSI Oxygen Electrode Model 5720 with a YSI Model 54 ARC Oxygen Meter and Model 80A Single Channel Recorder. In these experiments one hundred worms were cleaned as described above in the individual respiration experiments, weighed and acclimated to filtered sea water at the experimental temperature overnight. All one hundred animals were then placed in a black vessel containing filtered sea water at the same temperature. The vessel was saturated with air and sealed without trapping any air bubbles with a rubber stopper fitted with the oxygen electrode. A magnetic stirring bar, present in a separate compartment so as to not physically disturb the worms, was used to keep the water well circulated. The worms were then allowed to deplete the oxygen in the vessel until the oxygen was exhausted. Worms were then removed from the vessel and placed in running sea water overnight to allow recovery from any effects of accumulated waste products and re-equilibrate to high ambient pO_2 s. The same worms were then exposed for 15 minutes to water through which CO had been bubbled. The worms were again placed in the respiration chamber and allowed to deplete the oxygen until a ppm oxygen concentration of zero was reached. Spectrophotometric measurements of blood samples drawn

from individual worms at the end of the experiment were performed to determine if the hemoglobin had been blocked by the carbon monoxide. To be certain that the cytochrome enzymes of the worms oxidative respiratory chain had not been blocked by the CO, the worms were retained after the experiment for a period of three weeks during which time hemoglobin samples were analysed for CO. No worms died during this period although the hemoglobin remained in the carbonmonoxy form. Oxygen consumption rates were calculated ($\mu\text{l/gm wet weight/hr}$) from the graph and plotted against oxygen concentration (ml/liter). Straight lines were fitted to the data by least squares method.

Hemoglobin content was measured in worms whose gut contents had been voided. The animals were bled into buffer, 0.1 ionic strength Tris-HCl (pH 7.0) 0.1M in NaCl, 0.01M in MgCl_2 , and centrifuged at high speed and the supernatant collected. The pellet was resuspended and extracted repeatedly until no red color could be seen in the supernatant. The supernatants were pooled and the volume recorded. The optical density of the oxy-hemoglobin solution was measured at 540nm and the hemoglobin concentration determined by the protein extinction coefficient for oxy-hemoglobin $\epsilon_{540}^{\text{mg/ml}} = 0.602$.

Water content of the worms was measured in order to determine the relationship between wet weight and dry weight. Worms were cleaned as previously described and frozen in weighed flasks. The worms were then freeze-dried and the drying vessels re-weighed. Calculations from the wet weight and dry weight yield water content.

RESULTS

Absorption maxima and extinction coefficients for Euzonus hemoglobin in the oxy, deoxy and carbonmonoxy forms are listed in Table I. They are similar to absorption maxima of other hemoglobins. The flattened deoxy-hemoglobin peak between 562 and 550nm characteristic of other annelid hemoglobins is present (figure 1).

Metabolic rates at ambient pO_2 are listed in Table II. There does not appear to be any unusual temperature influence on metabolic rate of this worm. Q_{10} of adult worms (greater than 0.15gm wet weight) for the temperature interval between $10^{\circ}C$ and $20^{\circ}C$ is 2.04. Figure 7 shows the relationship of log body weight (grams) to log oxygen uptake ($\mu l/gm$ wet weight/hr) at $15^{\circ}C$. Regression analysis gives the slope of the line as 0.42.

At $10^{\circ}C$ the worms metabolic rate is decreased 29% by the blocking of the hemoglobin with carbon monoxide (figure 8). Below external pO_2 of $1ml O_2/liter$ ($10^{\circ}C$) the metabolic rate is decreased by 21%. These data are expressed in figure 9 in which percent difference refers to the percent of the animals respiration contributed to by the oxygen transported by the hemoglobin.

Six determinations of hemoglobin content show that an average adult worm (0.15gm wet weight) has approximately 6.3mg hemoglobin. This is equivalent to 42mg Hb/gm wet weight worm ie. $4.05\% \pm 0.99$ S.D. of the animals total wet weight. The mean of 24 determinations show that E. mucronata is 22.3% water by weight. Thus the hemoglobin concentration is 188mg Hb/gm dry weight ie. 18.16% of the animals dry weight.

Euzonus mucronata hemoglobin shows an asymmetric sigmoid binding curve with a P_{50} equal to 2.57mm Hg ± 0.41 S.D. and an 'n' value of 2.36 at 20°C in a 0.1M Tris-HCl buffer (pH7.0) 0.1M in NaCl, 0.01M in MgCl₂ (figure 3).

The effect of pH on oxygen binding is shown in figure 4. There is essentially no Bohr effect over the pH range 6.5 to 8.0 at 20°C. The value for the pH interval 6.5 to 8.0 is -0.09. There is an apparent decrease in oxygen affinity over the pH range 8.0 to 8.5.

Figure 5 shows the effect of certain ions on the oxygen affinity and cooperativity of Euzonus hemoglobin. Both the P_{50} and the 'n' value do not change over the ion concentration ranges tested. All experiments were carried out in 0.1M Tris-HCl pH 7.0 at 20°C.

Increasing the temperature from 10°C to 25°C decreases the affinity of the pigment for oxygen while the 'n' value remains unchanged. The overall heat of oxygenation for the

combined pigments is -12.08 Kcal/mole over the range of 10°C to 25°C (figure 6).

DISCUSSION

The Opheliid polychaete Euzonus mucronata Treadwell is an inhabitant of the sandy beach and lives in strictly demarcated zones around mid-high water (McConnaughey and Fox 1949). This habitat is characterized by heavy surf action during high tide, exposure to drying, fresh water runn-off with rain or cliff seepage at low tide, as well as considerable temperature fluctuation both seasonally and daily. Behavioral studies of the worm show that the animals diurnal migration vertically through the sand column is in response to the incoming and the outgoing tides (Eikenberry 1966). During the period of the low tide the worms are found in the upper layers of well ventilated sand with most of the population no more than 10cm below the surface. The presence of the worm can be detected at low tide by numerous tiny holes in the surface of the sand. These holes are caused by the collapse of the worms numerous tunnels as they wind beneath the surface. Observations by Dales (1967) indicate that the worms form a small funnel at the surface and expose their posterior ends in what is thought to be respiratory posture. This behavior is not confirmed by McConnaughey and Fox (1949) or myself. The worms position is different, however, when the tide is high. According to

Eikenberry (1966) the thixotropic quality of the sand caused by the returning tide stimulates the worm to dig downwards and thereby escape probable dislodgment by the pounding, grinding surf. However, the animal is escaping from a situation of physical abrasion and possible dislodgment in the upper surface layers to that of oxygen depletion in the layers below. (Gordon 1960) has shown from direct subsurface sampling of interstitial water on sandy beaches that whereas at low tide there is uniform oxygen saturation to depths of at least 20cm (the extent of the authors sampling), at high tide the beach sand is virtually anaerobic below 5cm. His findings show that on the rising tide water percolates downward through the interstices causing the uniform distribution of oxygen. Gordon (1960) points out, however, that this oxygen is gone at depths of 5cm after only 15 minutes.

It seems likely that for an animal to maintain oxidative metabolism under these conditions it must have physiological and/or biochemical adaptations to allow its existence under fluctuating oxygen conditions. Ruby and Fox (1976) investigated the capability of E. mucronata to survive anoxia and found the accumulation of end products (succinate and propionate) of proposed anaerobic pathways after 72 hours of anoxia. Their observations that the worm can survive for several weeks in sealed anaerobic flasks

led the authors to conclude that Euzonus can survive anoxia, as experienced in the sediments, for extended periods. However, little was given the possible role of the hemoglobin in the animal.

Euzonus mucronata contains large quantities of an extracellular hemoglobin (Terwilliger et al 1976). There is no evidence for any coelomic cell hemoglobin as has been reported for another Opheliid polychaete Travisia pupa (Manwell 1960). Anatomical work by McConnaughey and Fox (1949) show that the worm has a closed circulatory system including a large blood sinus covering the gut region. Vessels from the circulatory system run into the paired gills that are along the body wall of the animal. The extracellular hemoglobin can be seen in the branchiae giving them a bright red color. The unusually large quantities of hemoglobin in this worm would lead one to expect it to have a functional role in the worms respiratory physiology.

Euzonus hemoglobin has a very high oxygen affinity for an annelid extracellular hemoglobin. When tested in Tris-HCl buffer (pH 7.0) 0.1M in NaCl, 0.01M in MgCl₂ at 20°C, the pigment has a P₅₀ of 2.5mm Hg and a Hill coefficient, 'n', of 2.4 showing strong cooperativity in oxygen binding. Oxygen binding curves also show slope heterogeneity which is characteristic of many high molecular hemoglobins (Weber 1971). The pigment also exhibits no measurable

Bohr effect nor any significant salt effects when tested in the presence of a number of specific ions. The values for P_{50} and 'n' fall within the ranges of other annelid extracellular hemoglobins, yet the pigment is unusual in its insensitivity to pH and salt along with its high oxygen affinity (Table III).

Extracellular annelid hemoglobins generally have low oxygen affinity, normal Bohr effect, moderate cooperativity and an increase in affinity brought about by salts (Mangum 1976). However, there is no strict rule and many worm hemoglobins are found either lacking a Bohr effect or possessing high oxygen affinity. Eunice aphroditis hemoglobin has a P_{50} of 18.9mm Hg at pH 7.0 and 20°C with a strong Bohr effect (Bannister 1976) yet the high molecular weight hemoglobin of Amphitrite ornate has a P_{50} of 10mm Hg and no Bohr effect at 20°C (Mangum et al 1975). The extracellular hemoglobin of the Terebellid Pista pacifica, on the other hand, shows moderate cooperativity, low affinity and no Bohr effect above pH 8.0. This increase in affinity at pH 8.0 is thought to correspond to the dissociation of the molecule into subunits (Terwilliger 1974). Further examples of low affinity hemoglobins are Eupolytmia crescentis and Nephtys hombergi with P_{50} s of 36mm Hg (10°C, no Bohr effect) and 10.2 -16.8mm Hg (20°C) respectively (Manwell 1959, Weber 1971b).

In contrast, Arenicola marina is reported to have an oxygen affinity of 2.0mm Hg, 'n' value of 2.5-4.7 as well as a strong Bohr effect and salt effect (Weber 1970, 1971b, 1972). Alma emini the swamp worm, possesses an extracellular hemoglobin with a P_{50} of 2.6mm Hg at pH 7.4, 20°C, a moderate Bohr effect and only slight cooperativity (Mangum et al 1975).

Whereas Euzonus mucronata shares certain characteristics with those worms discussed above, it is of interest to note that no worm discussed which had been tested for all three factors on binding share the absence of both Bohr and salt effect along with a high degree of cooperativity and a very high oxygen affinity. The oxygen binding properties of this hemoglobin under the conditions investigated are more similar to a myoglobin than a hemoglobin. Myoglobins, in general, have very high oxygen affinity, no salt or Bohr effect and no cooperativity (Rossi-Fannelli and Antonini 1958).

The unusually high oxygen affinity of Euzonus hemoglobin under all conditions investigated suggest that it could function as an oxygen transport pigment at high ambient pO_2 s. Experiments with carbon monoxide-blocked hemoglobin show that the pigment is responsible for 29% of the oxygen consumed at 10°C (figure 9). Objections have been made as to the validity of oxygen consumption studies with

carbon monoxide poisoning. Hoffman and Mangum (1970) point out that high concentrations of CO would probably be oxidized by the cytochromes with a consequent elevation of measured oxygen consumption. In this regard, spurious data would lead to an underestimate of the role of hemoglobin in oxidative metabolism.

It is more difficult to understand with certainty the functional role the pigment plays as the external oxygen tension nears anoxia or during complete anoxia. As seen in figure 9, even when the external oxygen tension decreases to 1ml O₂/liter, the hemoglobin contributes about 21% of the total oxygen consumed. The very high oxygen affinity of this pigment would allow it to load oxygen even at very low oxygen tensions depending on the diffusion gradient. Although the diffusion gradient across the epithelium has not been measured, histological evidence suggests that the diffusion pathway of gases is short and that loading of the pigment is possible at low ambient pO₂s. There is, however, a critical internal oxygen tension, 7mm Hg, below which the hemoglobin will not be able to load completely and the relative amount of oxygen transported would steadily decrease. At the point where there is no external oxygen available to the worm any oxygen bound to the hemoglobin would be utilized without replacement at the gills. Calculations show that Euzonus at 15⁰C would deplete its hemoglobin bound

oxygen after approximately 45 minutes at a constant rate of oxygen consumption measured at high external O_2 tension. This situation would seem disadvantageous to an actively digging animal.

Another strategy available to the worm is that as the O_2 tension decreases in the external environment, there is a concomitant decreasing demand for oxygen by the worm. The relation of oxygen consumption of the worm to oxygen tension is linear with no apparent regulation (figure 8). These results are similar to those found for Glycera dibranchiata (Hoffman and Mangum 1970). These authors report that ideally, the rate of release of stored oxygen should be based on oxygen consumption rates under conditions of continuously decreasing oxygen supply. Under these circumstances oxygen would be consumed by Euzonus much more slowly at low ambient pO_2 . To determine the rate of oxygen consumption at low oxygen tensions and the length of time that a given oxygen supply can be utilized is a problem, however, most methods commonly used to measure respiration rely on the presence (or disappearance) of oxygen. In order to measure respiration at low oxygen tensions or during anoxia it is necessary to measure this rate by some other criteria. For lack of any other method, respiratory rate at anoxia has been estimated by extrapolating the line in figure 8 to zero oxygen concentration. The

metabolic rate determined in this way is approximately $12\mu\text{l O}_2/\text{gm wet weight /hr}$. The hemoglobin of Euzonus can bind about $53\mu\text{l O}_2/\text{gm Hb}$ at full saturation. Using the extrapolated metabolic rate from above and assuming that the hemoglobin is saturated, the hemoglobin bound oxygen would last approximately 4.25 hours at a constant rate of consumption. Unfortunately, we do not know what happens to metabolic rate or rate of change of metabolic rate under anoxia or near anoxia. If oxygen consumption further decreases as the hemoglobin bound oxygen is utilized these calculations can be underestimates and the period of aerobic metabolism could be extended even longer. To understand more realistically the availability of hemoglobin bound oxygen requires that we know the venous saturation of the hemoglobin at that critical partial pressure of oxygen at which the diffusion gradient prohibits loading. These values are not known. Catheterized samples from the dorsal vessel of Glossoscolex giganteum, the giant earthworm, show that the pigment is only 74% saturated. The pigment has a P_{50} of 7.0mm Hg at 20°C . The authors explain that the dorsal vessel is a mixture of blood from the gas exchange areas and venous blood from the tissues (Johansen and Martin 1966). This could also be true for Euzonus, however the small size of the worm prohibits sampling of post and pre-branchial blood. If such were the case, it is likely that

the amount of reserve oxygen available for turnover would be reduced.

It is possible that the unusually high oxygen affinity of this pigment allows the hemoglobin to function in facilitating the diffusion of oxygen from the environment to the respiring tissues of the worm. It is known that hemoglobin solutions facilitate oxygen diffusion through Millipore filters (Scholander 1960) and the possibility that this diffusion enhancement occurs in a biological system is likely (Wittenberg 1965). It would make good sense for an actively respiring animal in an environment of declining oxygen to facilitate the diffusion of what oxygen is available and extend the period that the animal can draw on high energy producing aerobic pathways. The lack of heterotropic effects, pH and ions, may also be important in the function of this pigment. It would be advantageous for this animal to have a hemoglobin without a Bohr effect. According to a review by de Zwaan (1975) it is becoming more apparent that many invertebrates may rely upon anaerobic energy pathways even during periods of oxygen availability. Chen and Awapara (1969) point out that Rangia, a bivalve, may derive most of its energy from anaerobic pathways. End products of these pathways accumulate in the animal and although lactate may only be a relatively minor end product respiratory pigments in the system will be subjected to an

unstable ionic environment. Decrease in the pH decreases the affinity of many hemoglobins. If end products are accumulating in Euzonus (Ruby and Fox 1976) as the worm digs its way lower into the sand a decrease in oxygen affinity, causing an increased unloading, would not give the animal the advantage of the high affinity pigments ability to deliver as much oxygen and extend the availability of an aerobic energy supply during these periods of high activity and low oxygen.

Mangum (1977) has introduced the idea that a respiratory molecule may function in an unstable ionic environment without changing its loading or unloading characteristics by the action of opposing effects of salts and pH. In the estuarine crab, Callinectes sapidus, she points out that in a decreasing salt environment the binding curve of hemocyanin would normally be shifted to the right resulting in loss of effective range of oxygen turnover. It is the possible deaminations of amino acids, however, in response to osmotic stress that would lead to an increase in pH that would cause a Bohr effect pigment to shift its binding curve to the left. Because the effects of salt and pH are opposite and simultaneous the binding of the pigment is stabilized in an unstable ion environment. Although it is not known if this theory can be applied to annelids, Mangum (1977) interprets Webers results (1971) on Arenicola

cristata as a possible enantiostatic system. Effectively these same stable properties can be maintained by a pigment that has no response to either salt or pH. Whereas one system is finely tuned by opposing forces to maintain stability, the other system maintains the same stability of function by insensitivity to either of the usually opposing forces. The result is the same.

CONCLUSION

The functional characteristics of the combined extracellular hemoglobins of Euzonus mucronata have been studied. The very high affinity of the hemoglobin for oxygen ($P_{50} = 2.5$ mm Hg in 0.1M Tris-HCl (pH 7.0) 0.1M in NaCl, 0.01M in $MgCl_2$ at $20^{\circ}C$) and a high degree of cooperativity ('n' = 2.4) along with the large quantities of the pigment present in the animal would suggest a transport function in vivo. There are no apparent effects of pH on binding over the range of pH tested nor are there any changes in binding characteristics brought about by selected salts over a range of concentrations. Respiratory rates for Euzonus mucronata have been measured and fall within ranges recorded for other annelid species. There are no unusual temperature effects on respiration rate. Respiration experiments in a closed vessel show that Euzonus mucronata decreases its respiratory rate under conditions of declining oxygen concentration. The importance of this 'oxy-conformity' along with the unusual binding characteristics of the combined hemoglobins are discussed in relation to the possible functional role of the pigment.

Figure 1. Optical scans of oxy and deoxy-hemoglobin from Euzonus mucronata showing characteristic absorbance of combined pigments. Scans performed on a Perkin Elmer Model 124 Double Beam Grating Spectrophotometer.

Figure 1

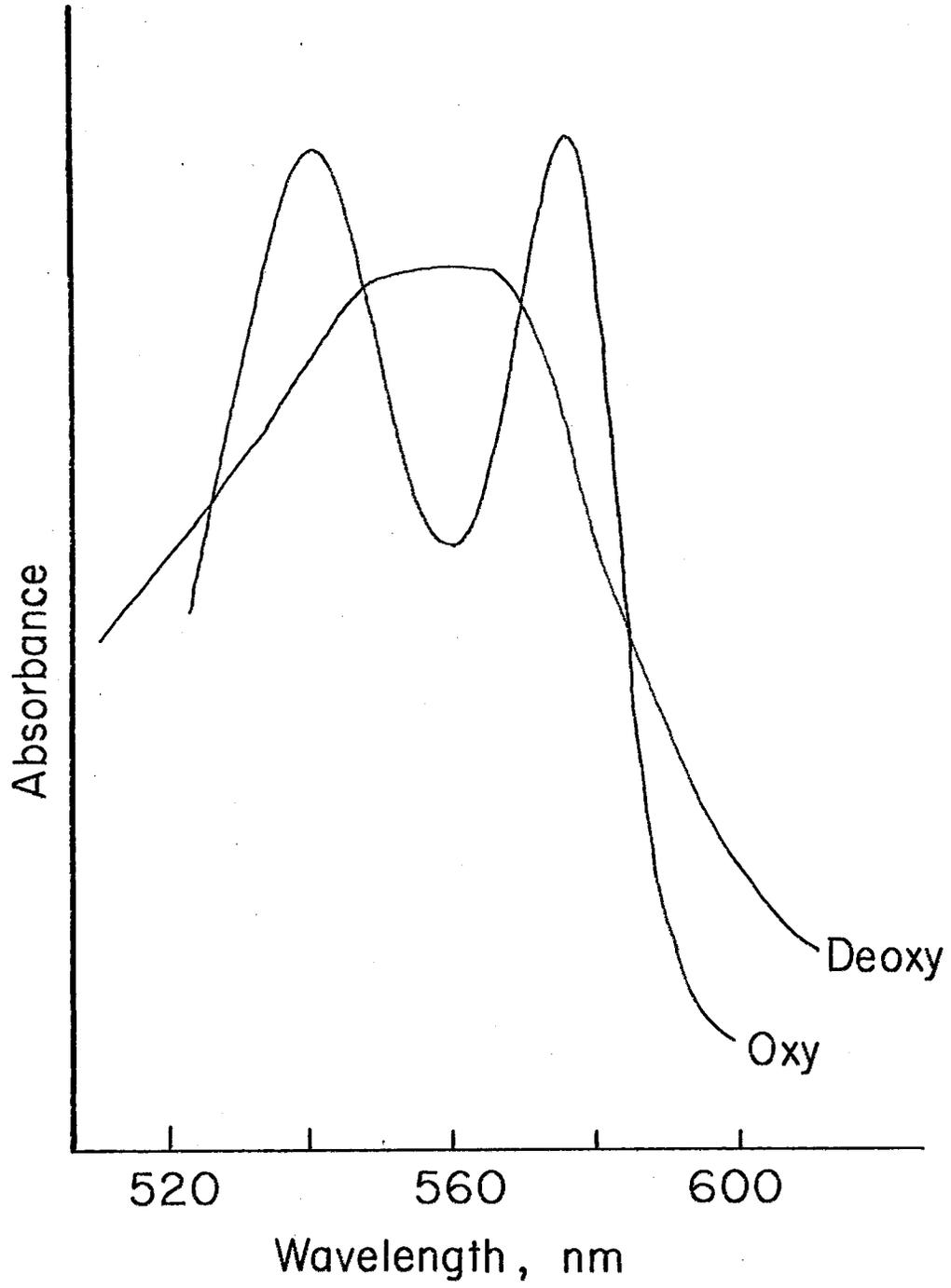


Figure 2. A typical oxygen saturation curve for Euzonus mucronata combined hemoglobins in 0.1M Tris-HCl (pH 7.0) 0.1M in NaCl, 0.01M in MgCl₂ at 20°C.

Figure 2

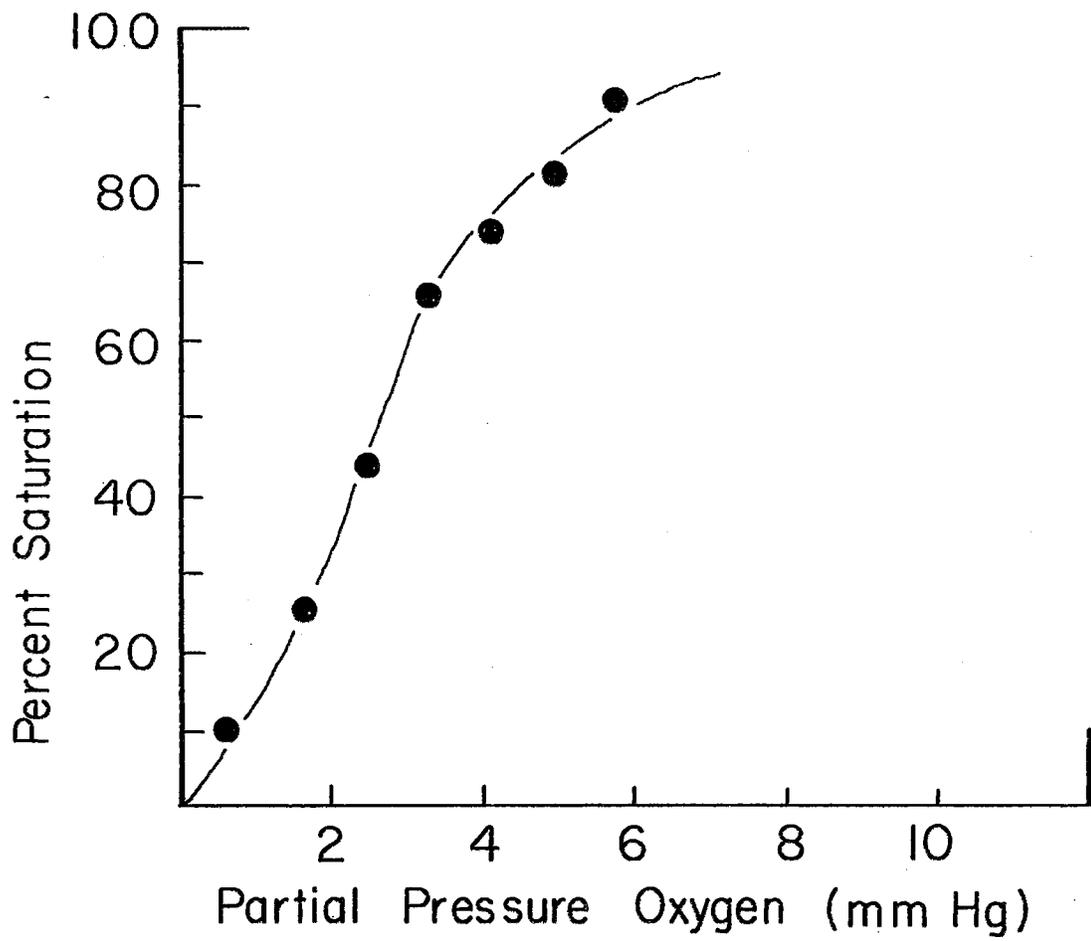


Figure 3. Hill plot of oxygen binding data for Euzonus mucronata in 0.1M Tris-HCl (pH 7.0) 0.1M in NaCl, 0.01M in $MgCl_2$ at 20°C. The graph displays the results of three separate experiments. Straight line determined by least squares method.

Figure 3

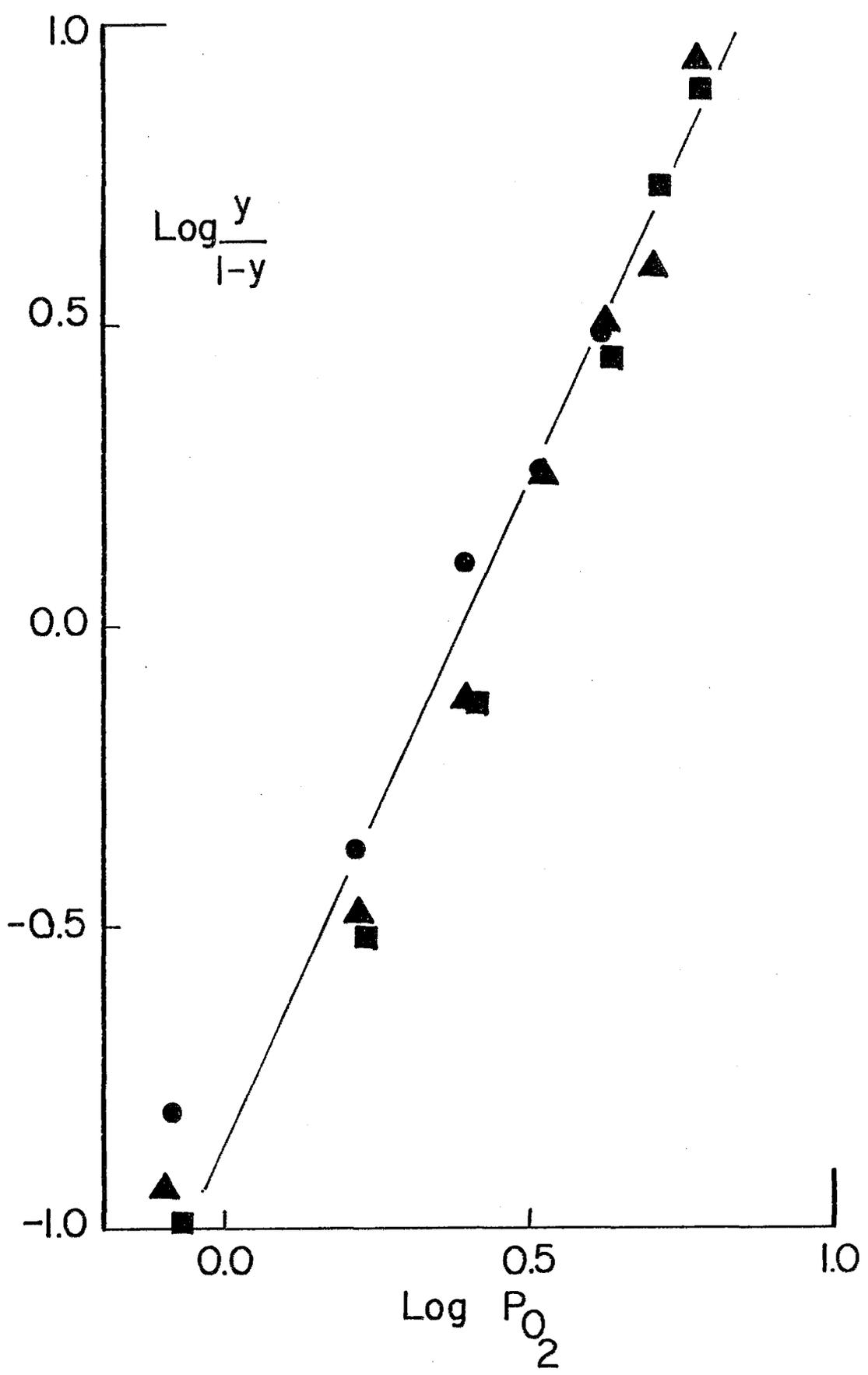


Figure 4. The effect of pH on the oxygen affinity and cooperativity of Euzonus mucronata combined hemoglobins at 20°C. All experiments were performed in 0.1M Tris-HCl (pH 7.0) 0.1M in NaCl, 0.01M in MgCl₂. Each point represents the mean of four samples.

Figure 4

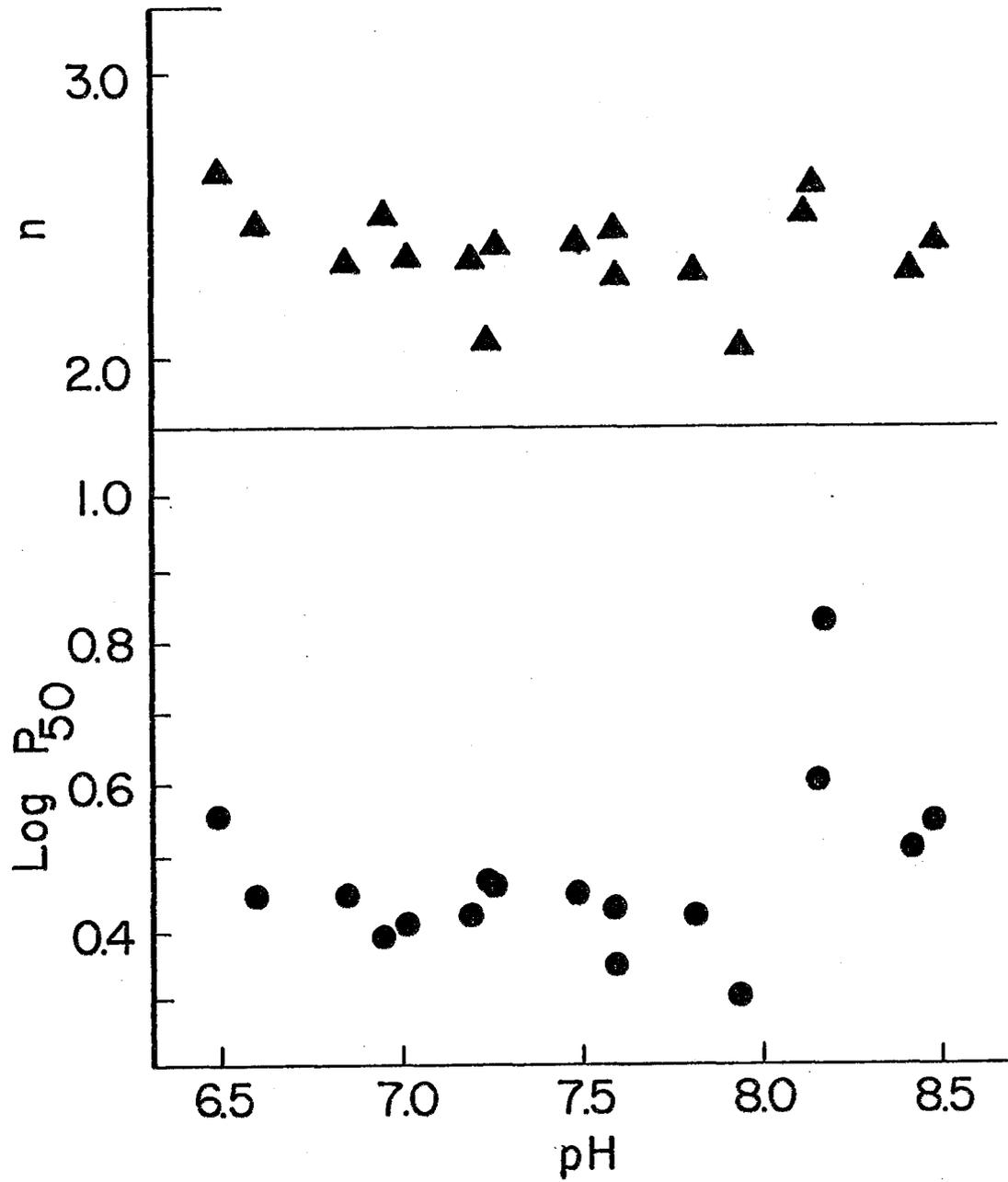


Figure 5. The effect of specific ions on oxygen binding characteristics of Euzonus mucronata hemoglobin. All experiments performed in 0.1M Tris-HCl (pH 7.0), 0.1M in NaCl, 0.01M in $MgCl_2$ at 20°C. Each point represents the mean of four samples. ●, NaCl; ■, $CaCl_2$; ▲, $MgCl_2$.

Figure 5

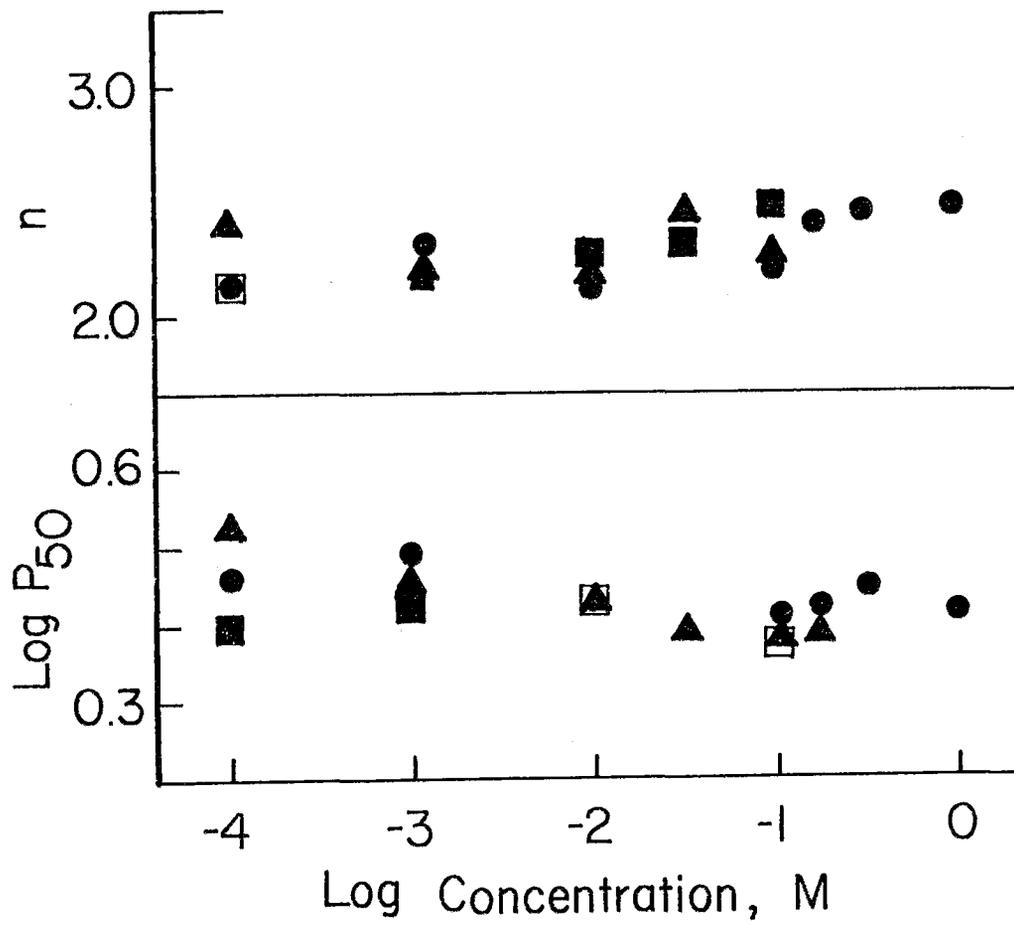


Figure 6. The effect of temperature on the oxygen affinity of Euzonus mucronata combined hemoglobins. Temperature: 10-25^oC. All experiments performed in 0.1M Tris-HCl (pH 7.0) 0.1M in NaCl, 0.01M in MgCl₂.

Figure 6

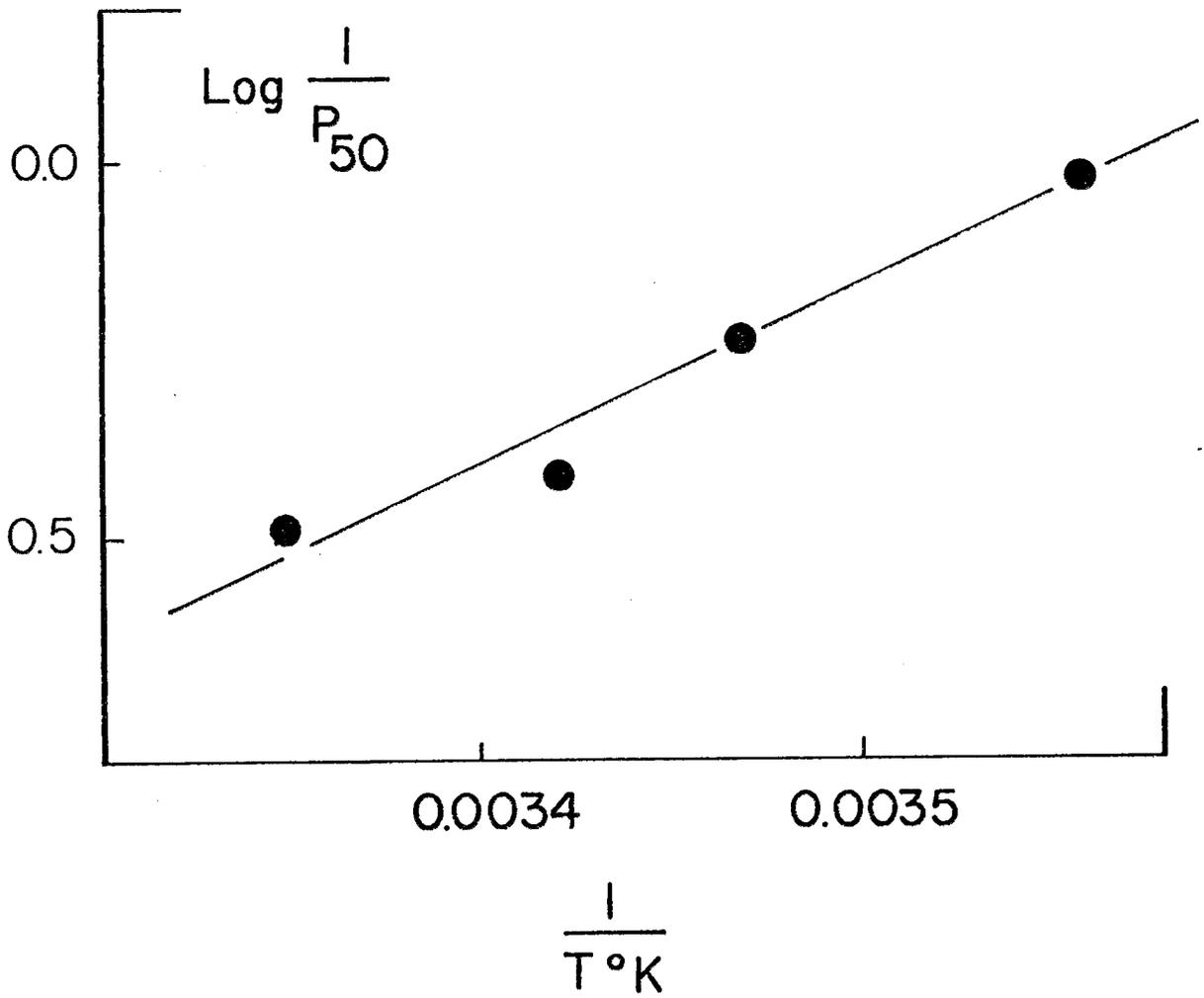


Figure 7. The relationship of size and metabolic rate of Euzonus mucronata, log-log plot. Experimental data obtained at 15^oC.

Figure 7

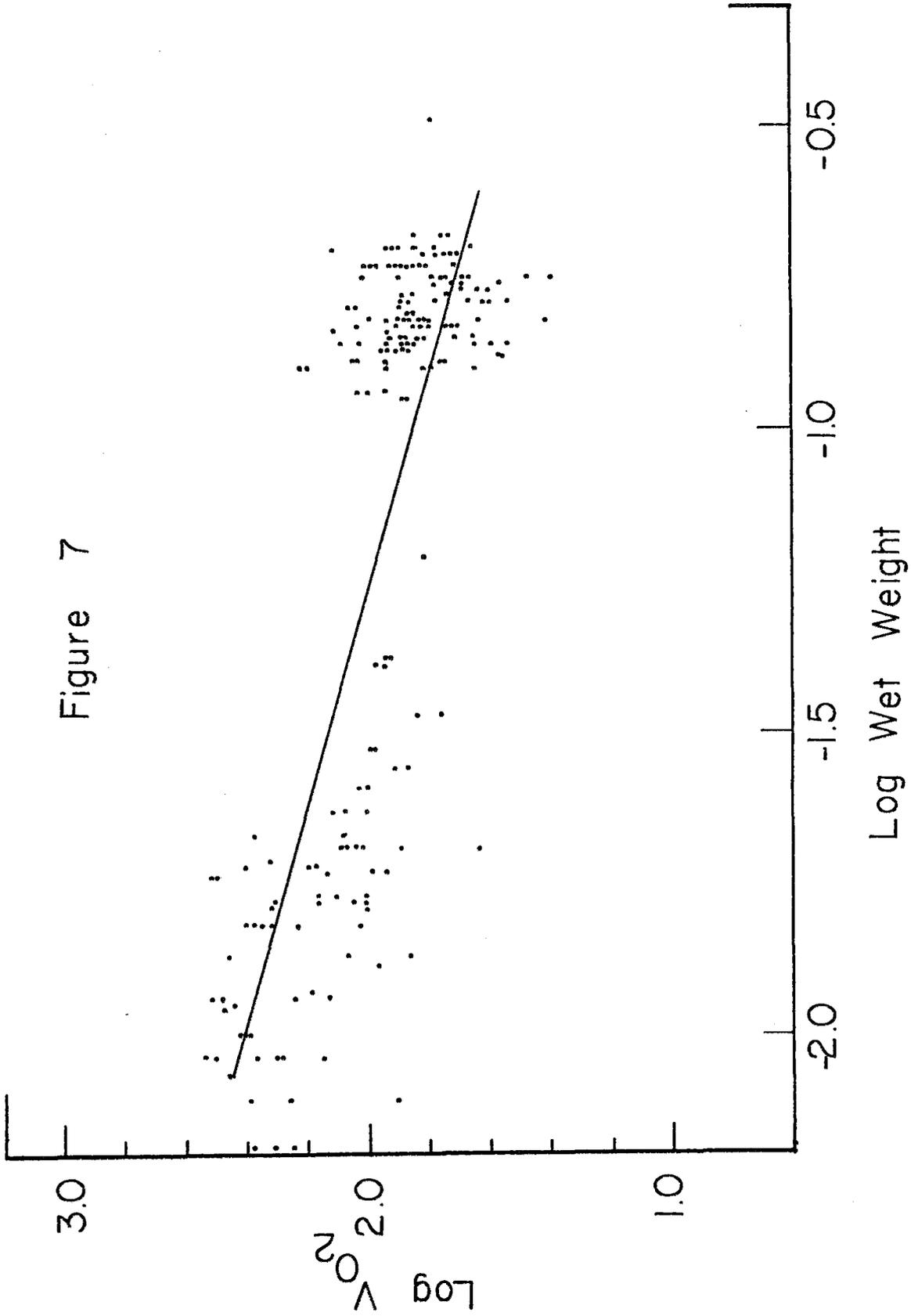


Figure 8. A summary of respiration experiments under conditions of declining oxygen concentration at 10°C . ● represents oxy-hemoglobin; Δ represents carbonmonoxy-hemoglobin. Lines fitted to data by least squares method.

Figure 8

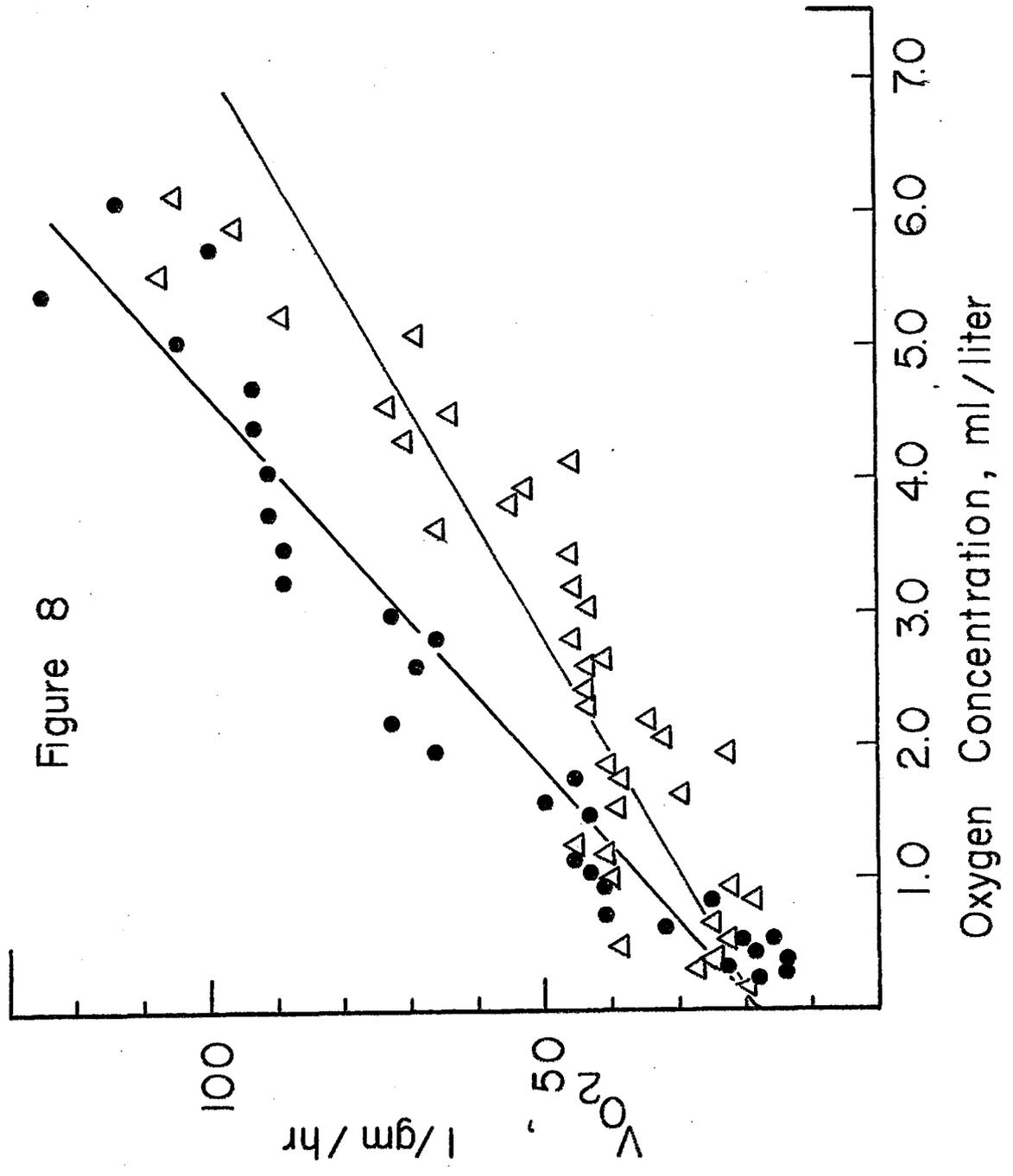


Figure 9. Percent depression of metabolic rate caused by carbonmonoxide-blocked hemoglobin at 10°C. Each point is the mean of five separate experiments. The vertical bars represent one standard deviation.

Figure 9

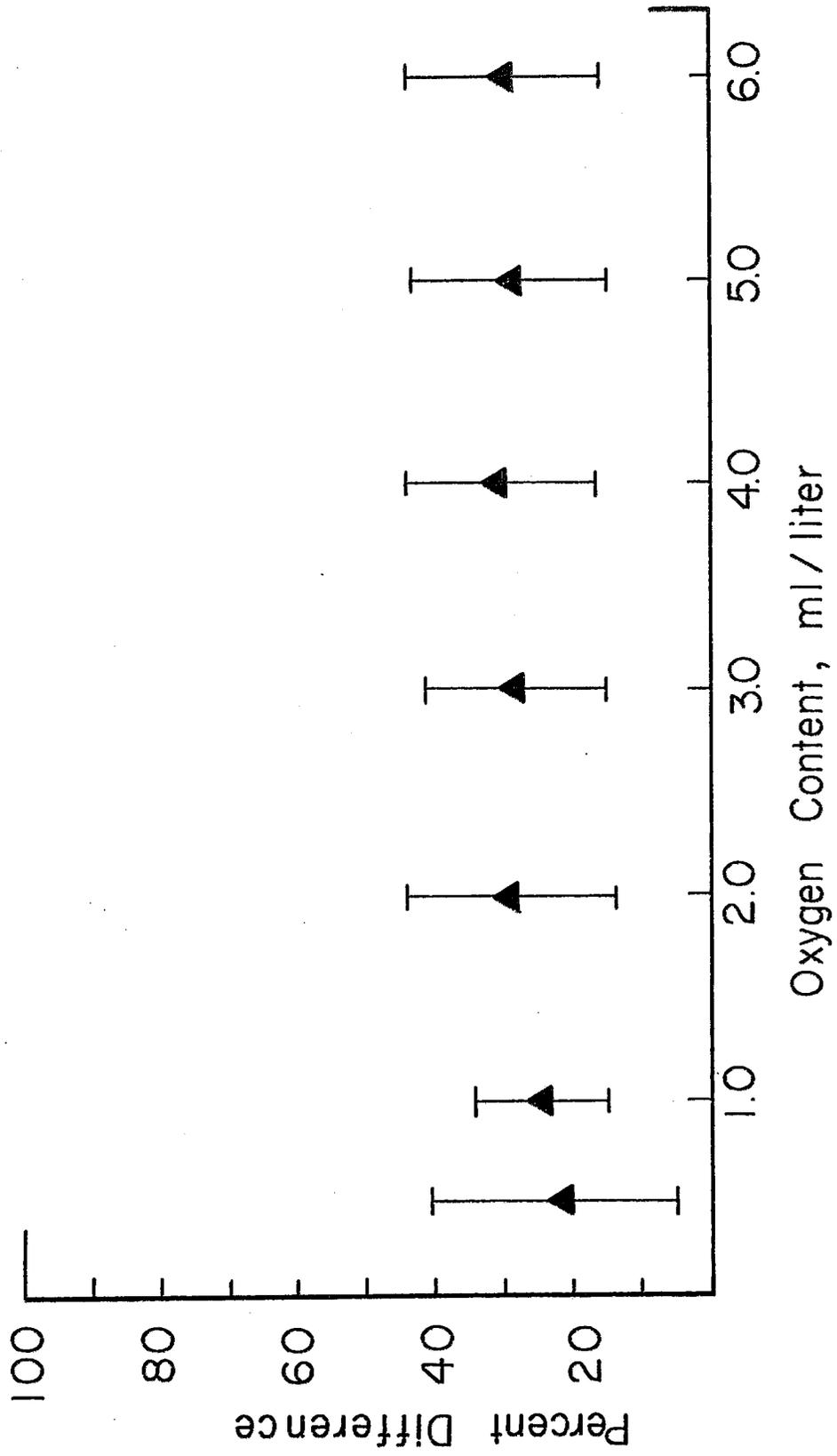


TABLE I

| Liganded state of pigment | Maximum Absorbance(nm) | One Percent Extinction Coefficient |
|------------------------------|------------------------|---------------------------------------|
| oxy | 575 | 6.10 |
| oxy | 539 | 6.02 |
| oxy | 415 | 49.36 |
| deoxy | 558 | 5.46 |
| deoxy | 429 | 46.59 |
| carboxy | 568 | 5.04 |
| carboxy | 536 | 5.48 |
| carboxy | 419 | 63.78 |

TABLE II

| Temperature | V_{O_2} (l/gm/hr) | N* |
|-------------|-------------------------|-----|
| 10°C | 48.71 ± 19.18 S.D. | 149 |
| 15°C | 71.13 ± 23.49 S.D. | 117 |
| 20°C | 99.33 ± 40.36 S.D. | 82 |

*N denotes sample size

TABLE III

| Species | P ₅₀ (mm Hg) | n | pH | Bohr | Temp | Source |
|------------------------------|----------------------------|-----------|-----------|------|------|----------------------------|
| <u>Polychaetes</u> | | | | | | |
| <u>Eunice aphroditois</u> | 18.9 | 3.0 | 7.0 | **** | 20°C | Bannister 1976 |
| <u>Amphitrite ornata</u> | 10 | 1.3-1.6 | 7.0 | 0 | 20°C | Mangum <u>et al</u> 1974 |
| <u>Pista pacifica</u> | 25 | 1.5 | 7.0 | 0 | 20°C | Terwilliger 1974 |
| <u>Thelepus crispus</u> | 32 | 1-1.6 | 7.0 | ++ | 20°C | Garlick & Terwilliger 1975 |
| <u>Eupolyomia crescentis</u> | 36 | 1.06 | 7.0 | 0 | 10°C | Manwell 1959 |
| <u>Nephtys hombergii</u> | 10.2-16.8 | 1.2-1.8 | | + | 20°C | Weber 1971b |
| <u>Arenicola marina</u> | 1.4-2.5 | 2.12-4.70 | 7.4 | +++ | 20°C | |
| <u>Oligochaetes</u> | | | | | | |
| <u>Alma emini</u> | 2.6 | 0.97-1.44 | 7.4 | + | | Mangum <u>et al</u> 1975 |
| <u>lumbricus terrestris</u> | 3.9-6.5 | 4.6-5.4 | 7.4-7.9 | + | | Waxman 1975 |
| <u>Tubifex tubifex</u> | 0.67-2.29 | 1.95-2.9 | 6.02-8.56 | + | | Scheler 1960 |

* +++ denotes strong Bohr effect
 ++ denotes moderate Bohr effect
 + denotes slight Bohr effect
 0 denotes no Bohr effect

BIBLIOGRAPHY

- Bannister, J.V., W.H. Bannister and A. Anastasi (1976).
Isolation, characterization and oxygen equilibrium of
an extracellular hemoglobin from Eunice aphrodotois
(Pallas). Biochem. J. 159, 35-42
- Benesch, R., G. MacDuff and R.E. Benesch (1965). De-
termination of oxygen equilibria with a versatile new
tonometer. Analyt. Biochem. 11, 81-87
- Chen, C. and J. Awapara (1969). Effect of oxygen on the
end products of glycolysis in Rangia cuneata. Comp.
Biochem. Physiol. 31, 395-401
- Dales, R.P. (1967). Annelids, Hutchinson University
Library, London
- de Zwaan, A. and T.C.M. Wijsman (1975). Anaerobic
metabolism in bivalvia (mollusca); Characteristics of
anaerobic metabolism. Comp. Biochem. Physiol. 54B,
313-323

- Eikenberry, A.B. (1966). A study of the vertical and horizontal migrations of Euzonus mucronata (Treadwell) 1914, on Pacific coast beaches with regard to environmental factors. Masters Thesis, University of the Pacific, Stockton, California.
- Fox, D.L. and B.H. McConnaughey (1949). A biochemical study of the marine annelid Thoracophelia mucronata Treadwell. J. mar. Res. 7, 567-585
- Garlick, R.L. and R.C. Terwilliger (1974). Coelomic cell hemoglobin of the Terebellid polychaete Thelepus crispus Johnson Comp. Biochem. Physiol. 47B, 543-553
- Garlick, R.L. and R.C. Terwilliger (1975). The quaternary structure and oxygen equilibrium properties of the vascular hemoglobin of the Terebellid polychaete Thelepus crispus Johnson. Comp. Biochem. Physiol. 51A, 849-857
- Gordon, M.S. (1960). Anaerobiosis in marine sandy beaches. Science, N.Y. 132, 616-617
- Hoffman, R.J. and C.P. Mangum (1970). The function of coelomic cell hemoglobin in the polychaete Glycera dibranchiata. Comp. Biochem. Physiol. 36, 211-228

- Johansen, K. and A.W. Martin (1966). Circulation in the giant earthworm Glossoscolex giganteus. II. Respiratory properties of the blood and some patterns of gas exchange. J. exp. Biol. 45, 162-172
- Johnson, M.L. (1941). The respiratory function of the hemoglobin of the earthworm. J. exp. Biol. 18, 266-277
- Jones, J.D. (1971). Comparative Physiology of Respiration Ed. Arnold, London, Publishers
- Mangum, C.P., B.R. Woodin, C. Bonaventura, B. Sullivan and J. Bonaventura (1975). The role of coelomic and vascular hemoglobin in the annelid family Terebellidae. Comp. Biochem. Physiol. 51A, 281-294
- Mangum, C.P., G. Lykkeboe and K. Johansen (1975). Oxygen uptake and the role of hemoglobin in the East African Swampworm Alma emini. Comp. Biochem. Physiol. 52A, 477-482
- Mangum, C.P. and D. Towle (1977). Physiological adaption to unstable environments: Inconsistency of the internal milieu may be a regulatory mechanism. Amer. Sci. 65, 67-75

- Mangum, C.P. (1976). In Adaptation to Environment, Editor: R.C. Newell, p 191. Butterworth & Co. (Publishers)
- Manwell, C. (1959). Alkaline denaturation and oxygen equilibrium of annelid hemoglobins. J. cell. comp. Physiol. 53, 61-74
- Manwell, C. (1960a). Histological specificity of respiratory pigments. I. Comparisons of the coelom and muscle hemoglobins of the polychaete Travisia pupa and the echiuroid Arhynchite pugettensis. Comp. Biochem. Physiol. 1, 267-276
- McConnaughey, B.H. and D.L. Fox (1949). The anatomy and biology of the marine polychaete Thoracophelia mucronata (Treadwell) Ophelidae. Univ. Calif. Publs. Zool. 47, 319-340
- Rossi-Fanelli, A. and E. Antonini (1958). Studies on the oxygen and carbon monoxide equilibria of human myoglobin. Arch. Biochem. Biophys. 77, 478-492
- Ruby, E.G. and D.L. Fox (1976). Anaerobic respiration in the polychaete Euzonus (Thoracophelia) mucronata. Mar. Biol. 35, 149-153

- Scheler, W. (1960). Lictabsorption and sauerstoffbindskurve de Haemoglobins von Tubifex tubifex; Biochem. Zeitschr. 332, 336-375
- Scholander, P.F. (1960). Oxygen transport through hemoglobin solutions. Science, 585-590
- Smith, R.I. and J.T. Carleton (1975). Editors: Lights Manual. 3rd edn, p 151. University of California Press.
- Terwilliger, R.C. (1974). Oxygen equilibria of the vascular and coelomic hemoglobins of the terebellid polychaete Pista pacifica. Evidence for an oxygen transfer system. Comp. Biochem. Physiol. 48A, 745-755
- Terwilliger, R.C., N.B. Terwilliger, E. Schabtach and L. Dangott (1976). Erthrocrucorins of Euzonus mucronata (Treadwell). Evidence for a dimeric annelid extracellular hemoglobin. Comp. Biochem. Physiol. 57A, 143-149
- Waxman, L. (1971). Properties of Arenicola hemoglobin. J. Biol. Chem. 246, 7318-7327
- Waxman, R. (1975). The structure of Annelid and Molluscan hemoglobins. J. Biol. Chem. 250, 3790-3795

- Weber, R.E. (1970). Relations between functional and molecular properties of annelid hemoglobins. Interactions between haems in the hemoglobin of Arenicola marina L. Comp. Biochem. Physiol. 35, 179-189
- Weber, R.E. (1971b). Oxygenational properties of vascular and coelomic hemoglobins from Nephtys hombergii (Polychaeta) and their functional significance. Neth. J. Sea Res. 5, 240-251
- Weber, R.E. (1972). On the variation in oxygen binding properties of hemoglobins of lugworms (Arenicolidae, Polychaete). Proc. 5th Eur. Mar. Biol. Symp. Piccin. Publ., Padova, 231-243
- Wells, R.M.G. and R.P. Dales (1975). Haemoglobin function in Terebella lapidaria L. an intertidal terebellid polychaete. J. mar. biol. Ass. U.K. 55, 211-220

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