

THE QUANTITATIVE ROLE OF HEMOGLOBIN IN
OXYGEN UPTAKE AND OXYGEN STORAGE IN THE
PONDEROUS ARK CLAM, NOETIA PONDEROSA

A Thesis

Presented to

The Faculty of the Department of Biology
The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of
Master of Arts

by

Lewis Edward Deaton

1974

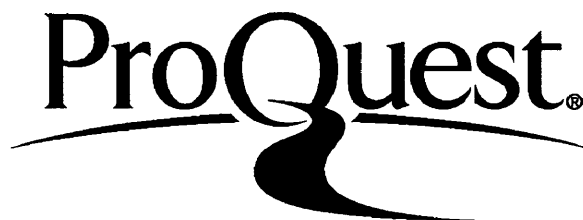
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the requirements for the degree of

Master of Arts

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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to Dr. Charlotte P. Mangum for her constant guidance and enthusiasm during the course of this study. I am also grateful to Drs. Stewart A. Ware and Robert E. L. Black for their careful reading and critical review of the manuscript. I am also indebted to the Virginia Institute of Marine Science Eastern Shore Laboratory staff for assistance in collection of the animals and to Mr. Glen Bean for aid in design and construction of equipment. I also wish to thank several graduate students whose efforts facilitated completion of this study: Ken Bullock, Marvin Freadman, and Larry Hirsch.

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ABSTRACT

The quantitative role of hemoglobins in oxygen uptake of the arcid clam Noetia ponderosa was investigated at 10° C and 23° C at various ambient oxygen levels. At 23° C, the hemoglobins are responsible for 20-52% of oxygen uptake and 60-65% at 10° C, depending on ambient PO₂. Oxygen extraction from the incurrent ventilatory stream is 55-75%, depending on ambient PO₂. Mean blood volume is 60% of wet weight of the animal.

The hemoglobins seem to function as oxygen transport substances at all ambient oxygen levels encountered by the animal in its environment. They may also serve as a limited oxygen store, allowing the animal to maintain aerobic respiration when it is unable to ventilate.

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INTRODUCTION

The quantitative function of hemoglobin in aerobic respiration at various ambient PO_2 's is known in several species of annelids and larval insects. The values for participation in oxygen uptake by extracellular hemoglobins are generally in the range of 25-55% at summer temperatures and slightly less at lower temperatures (summarized by Hoffmann and Mangum, 1970). Cellular hemoglobins in annelids, which occur only in poorly circulated coelomic fluids, are responsible for 15-30% of oxygen uptake (Hoffmann and Mangum, 1970; Mangum et. al., 1975). The quantitative roles of hemoglobins found in members of other phyla, including the cellular pigments found in the bloods of the more primitive families of lamellibranch molluscs, are unknown.

Aspects of gas exchange and ventilation in lamellibranches are best understood in species which are members of the less primitive families that contain no oxygen transport substances in their bloods. Rates of oxygen consumption ($\dot{V}O_2$) at similar temperatures in large bivalves show great variability. For example, at 20° C, Van Dam found that $\dot{V}O_2$ is 70 $\mu l O_2/gm$ wet weight-hr in the swimming scallops Pecten irradians and Pecten grandis. In Pecten jacobaeus $\dot{V}O_2$ is 12-28 $\mu l O_2/gm$ wet weight-hr and in Pecten varius, 37-71 $\mu l/gm$ wet weight-hr (summarized by Ghiretti, 1966). The oyster Crassostrea virginica consumes 15.5 $\mu l O_2/gm$ wet weight-hr (Nicol, 1960). $\dot{V}O_2$ of the sedentary mussel Mytilus edulis (1 gm dry weight) at 23° C is 600 $\mu l/gm$ -hr (Widdows, 1973). At 20° C,

the acclimated $\dot{V}O_2$ of Mya arenaria is 600 $\mu\text{l}/\text{gm}$ dry weight-hr which decreases to 300 $\mu\text{l}/\text{gm}$ -hr at 10° C (Kennedy and Mihursky, 1972).

In lamellibranches, rates of oxygen extraction from the ventilatory stream are generally low. Estimates made by measuring the oxygen content of continuous syringe samples from the excurrent range from 0.5-13% at ambient PO_2 's approaching air saturation (Hazelhoff, 1938; Van Dam, 1954; Bayne, 1967). While the rate in Mytilus edulis (measured directly with an oxygen electrode in the excurrent) is generally 5-10% under normoxic conditions, it increases to 30% at low PO_2 's (<30 mm Hg) when the animal regulates oxygen uptake (Bayne, 1971b). Mangum and Burnett (1974), who made continuous measurements with an oxygen microelectrode placed in situ at the excurrent, found considerable short term (<5 min.) variation in rates of oxygen extraction by molluscs. Average values computed from integrals of the recorder traces for 15 minute periods are less than 20% for Mercenaria mercenaria, 5% for Rangia cuneata, less than 5% for Mya arenaria, and 10-13% in Dinocardium robustum at incurrent PO_2 's of 140-159 mm Hg; and higher rates were found at intermediate and low oxygen levels.

Ventilation in most lamellibranch molluscs serves both nutritional and respiratory functions. Crassostrea virginica ventilates at a rate of 10 l/hr (Nicol, 1960). The rate for Mercenaria mercenaria is 5 l/hr (Hamwi and Haskin, 1969); and 28 ml/gm wet weight-hr for Mytilus californianus (Fox et. al., 1937). The queen scallop, Chlamys opercularis ventilates at 6 l/gm dry weight-hr (McClusky, 1973). At 15° C, Mytilus

edulis ventilates at 2 l/gm dry weight-hr (Bayne, 1971b).

While oxygen transport by the cellular hemoglobins under normoxic conditions has been demonstrated in Noetia ponderosa (Freadman and Mangum, 1975), the magnitude of the transport function at lower oxygen levels is unknown. N. ponderosa has a relatively high hemoglobin concentration in the blood (Freadman and Mangum, 1975), which suggests the possible function of the blood as an oxygen store. The objectives of this study are to investigate the oxygen uptake of N. ponderosa at various oxygen levels, determine the quantitative role of hemoglobin in the oxygen uptake of the animal, and determine the ventilation and oxygen extraction rates. This study is intended to clarify the functions of the respiratory pigment in the blood of Noetia ponderosa.

MATERIALS AND METHODS

Noetia ponderosa was collected with an oyster dredge at 3-5 meters depth in the vicinity of Wachapreague, Virginia. In the laboratory they were maintained in natural sea water (28-35 o/oo salinity) at 17-19° C. The water in the aquaria was vigorously aerated and recirculated through spun glass and charcoal filters.

Oxygen level in the habitat

Measurements of oxygen content of the water at the site of animal collection were made with a Yellow Springs Instrument Co. Model 5418 submersible oxygen electrode.

Survival during anoxia

Two clams were placed in a 4 liter bottle of sea water and the oxygen partial pressure reduced to less than 10 mm Hg by bubbling washed nitrogen gas through the water. The bottle was then sealed with glass plates and Vaseline, placed in the dark, and subsequently examined at 24 hour intervals. In addition, clams were placed singly in 500 ml vessels containing deoxygenated sea water and a layer of mineral oil was floated on the top to minimize diffusion of oxygen into the vessel. When the experimental animals no longer adducted the valves in response to mechanical stimulation, they were removed and placed in an aerated sea water aquarium for a further 24 hours. Failure to close the valves and putrefaction were used as indicators of death.

Ventilation rate

Ventilation rates of animals burrowed into sand were measured directly with a thermistor flow meter. The design and circuitry for the flow meter are modification of those specified by Heusner and Enright (1966), Lowe and Trueman (1973), and Vogel (1974, personal communication). The flow meter consists of a Fenwal GB31P2 thermistor probe wired into a wheatstone bridge circuit so that the thermistor is heated a few degrees above ambient temperature by the power supply. Cooling of the thermistor by flowing water results in current imbalances in the bridge, which are measured as deflections of a Linear Instruments Corp. pen recorder. The flow meter probe was placed in a tank of water and calibrated by allowing water to flow from a burette through a tube whose diameter approximated the gap between the edges of the mantle of N. ponderosa past the thermistor tip.

Oxygen extraction

Noetia ponderosa was allowed to burrow into sand in aerated aquaria. While the animals were ventilating, an oxygen hypodermic microelectrode held by a micromanipulator was lowered into the exhalant current of an animal as close as possible to the exposed edges of the mantle without inducing valve closure. The oxygen content of the exhalant current was recorded with a Beckman Model 160 Physiological Gas Analyzer and a Linear Instruments Corp. Model 112 recorder. A Yellow Springs Instrument Co. Model 5420 oxygen electrode was placed near the incurrent of the animal

and the ambient oxygen concentration recorded with a Yellow Springs oxygen meter and recorder. The ambient oxygen concentration was successively lowered at intervals of 30 minutes by introduction of washed nitrogen gas. At each ambient PO_2 , an animal was allowed to ventilate for 30 minutes. Since the short term variability noted in other species (Mangum and Burnett, 1974) was not found here, values for excurrent PO_2 were taken directly from the recorder trace.

Quantitative role of hemoglobin

Clams were exposed to one of the two experimental temperatures (10 and 23° C) for at least one week. The shell was then coated with wax to prevent interference of epibionts in the determination of $\dot{V}O_2$ (Kushins and Mangum, 1971). None of more than 40 animals showed adverse effects from the wax coating. Next the clam was placed in a respiration vessel containing air saturated sea water and a Yellow Springs Model 5420 oxygen electrode was inserted into the top of the vessel to seal it from the atmosphere. The vessel was then darkened and placed in a constant temperature water bath in the dark. The oxygen concentration of the water in the respiration vessel was monitored continuously with a Yellow Springs Model 54 oxygen meter and Model 80 laboratory recorder until the measurable oxygen content was depleted.

Sea water containing carbon monoxide to block the hemoglobin was prepared by vigorously shaking a one liter reagent bottle of sea water to which 4 ml (18% saturation at 23° C and 14% at 10° C) of carbon monoxide

gas had been added. After exposure to aerated sea water for 48 hours following the control measurement of $\dot{V}O_2$, the clam was placed in a sealed, darkened respiration vessel containing carbon monoxide sea water for one hour. The animal was observed in dim light every 15 minutes to ascertain whether it was ventilating, and if it was it was placed in a respiration vessel containing sea water with carbon monoxide (1 ml/l). The depletion of oxygen was again recorded. At the conclusion of the experiment, the animal was removed from the respiration vessel and a small hole chipped between the valves at the mid-posterior region of the shell. A glass syringe with a 24 gauge needle was inserted and a 1 ml blood sample withdrawn. Hemoglobin in the blood sample was extracted into distilled water, centrifuged at high speed (Sorvall SS-1), and the supernatant solution examined with a Beckman DK-2A spectrophotometer. Complete shift of the α and β absorbance bands from the positions characteristic of oxyhemoglobin, 578 nm and 544 nm to those of carbon monoxy-hemoglobin, 570 nm and 540 nm was the criterion for success of hemoglobin blockage.

After several trials indicated that the hemoglobins were not fully blocked at lower carbon monoxide levels, the high concentration of 4 ml/l was used. These concentrations of carbon monoxide may cause an elevation of tissue $\dot{V}O_2$ due to oxidation of carbon monoxide compounds by the cytochromes (Black and Tyler, 1962). Although the carbon monoxide : oxygen partition coefficient for Noetia ponderosa hemoglobin is only slightly lower than that of Hemoglobin A (L. J. Parkhurst, personal communication),

the carbon monoxide compound should form in vivo at considerably lower carbon monoxide levels than 4 ml/l. However, ventilation periods were very brief at low carbon monoxide levels, which may explain the failure. Therefore, our estimates of the quantitative role of hemoglobin may be minimized at ratios of carbon monoxide : oxygen below about 4-5 : 1 or $PO_2 = 30$ mm Hg, where oxidation of carbon monoxide heme proteins occurs (Black and Tyler, 1962).

These experiments were also conducted on the clam Mercenaria mercenaria, which has no hemoglobin, to determine whether the carbon monoxide concentrations used had any effect on cellular level respiration. Entire animals and excised gill tissues were used.

$\dot{V}O_2$ of each animal in control and experimental measurements was plotted as a function of PO_2 , and a linear regression line calculated for the data points (mean $r^2 = 0.644$ at $23^\circ C$ and 0.722 at $10^\circ C$). The difference between the lines for the same animal with functional and non-functional hemoglobin was determined at various ambient oxygen levels and expressed as a percentage of the value for functional hemoglobin. These percentages (transformed to arc-sin) for each oxygen concentration at each experimental temperature were treated as single samples, and the significance of the means and 95% confidence intervals determined by Student's t.

Blood volume

Blood volume was determined by the method of Chapman (1967). A 0.1 ml blood sample was drawn into a glass syringe through a small hole chipped

in the mid-posterior margin of the valves and diluted to 10 ml with distilled water. The sample was centrifuged at high speed and the optical density of the supernatant hemoglobin solution determined at 540 nm with a Bausch and Lomb Spectronic 20 colorimeter. The valves of the animal were pried open and the mantle fluid allowed to drain out. The adductor muscles were then cut and the soft parts removed from the shell and weighed. During the dissection, blood was collected in a small beaker. The soft parts were then minced in a Waring blender, washed with distilled water and added to the beaker with the drained blood. An equal volume of distilled water was added to the minced tissues and blood and the preparation centrifuged. The supernatant hemoglobin solution was collected and diluted with distilled water to the volume necessary to match the diluted 0.1 ml sample in optical density. Blood volume was calculated according to the expression:

$$\text{blood volume} = \frac{(0.1 \text{ ml}) (\text{total diluted volume from tissues})}{10 \text{ ml}}$$

To ensure that the hemoglobin extracted in this way was not altered during the procedure, in which case absorbance at 540 nm would not be an accurate measure of the original hemoglobin content, absorbance of the extract was also determined with a Beckman DK-2A spectrophotometer and Hufner's quotient, $\frac{K_{\text{max II}}}{K_{\text{min}}}$ (Heilmeyer, 1943), calculated.

RESULTS

Absorption spectra and bands

The peaks of oxy-hemoglobin absorbance on a trace made by a Beckman DK-2A spectrophotometer are located at 544 nm and 578 nm in addition to the Soret peak at 414 nm; carbon monoxy-hemoglobin absorbs at 540 nm and 570 nm while deoxy-hemoglobin absorbs at 560 nm. In the Hartridge Reversion Spectroscope oxy-hemoglobin absorbs at 541.1 nm and 575.6 nm, carbon monoxy-hemoglobin absorbs at 538.0 nm and 569.0 nm, and deoxy-hemoglobin absorbs at 558.0 nm. The relative heights of α and β peaks and other properties of the visible spectrum do not differ from those of Hemoglobin A. This is of interest only because the intact cells appear to be much darker than mammalian erythrocytes.

Oxygen content of the habitat

The oxygen content of the water at the site of animal collection in early September was 102 mm Hg at the bottom and 111 mm Hg just beneath the surface of the water column. The salinity was 33 o/oo and the temperature 23.5° C.

Survival during anoxia

Tolerance of low oxygen (0-10 mm Hg) water at 23° C was quite variable (Table 1), but the length of survival at that temperature is truly remarkable, even for lamellibranch molluscs. Twenty-two days elapsed before half of the test group was dead.

Table 1. Survival of Noetia ponderosa during anoxia ($PO_2=0-10$ mm. Hg) at 23° C. •

Day	Number dead	Cumulative per cent
1	1	5.5
2	2	11.1
4	3	16.1
9	4	22.2
17	5	27.8
18	6	33.3
20	7	38.9
22	9	50.0
23	10	55.5
27	12	66.7
28	14	77.8
29	17	94.4
30	18	100.0

Ventilation rate

The results of measurements on six animals at various PO_2 's are shown in Fig. 1. Ventilation rates are about 30 ml/gm wet weight-hr or 100 ml/gm dry weight-hr at all PO_2 's. Bayne (1971b) found ventilation rate in Mytilus edulis to vary directly with declining oxygen concentration; and this relationship has also been observed in Mya arenaria (Lowe and Trueman, 1972).

Oxygen extraction

Oxygen extraction rates are shown as a function of PO_2 in Fig. 1. The rates increase slightly with declining PO_2 from 60% near air saturation to a maximum of nearly 75% at the intermediate PO_2 of 70 mm Hg; they then decrease with further reduction in PO_2 to about 55% at 20 mm Hg. A single data point obtained at 10 mm Hg suggests a further decline. Animals only rarely closed their shells in response to low PO_2 . The oxygen extraction rate does explain the $\dot{V}O_2$ of the animal above 30 mm Hg. The unusually high rates must be due to the functioning of hemoglobin in gas exchange.

Quantitative role of hemoglobin

Six experiments were conducted at each temperature (10 and 23° C). At 140 mm Hg, $\dot{V}O_2$ is 316 μ l/gm dry weight-hr at 23° C and 148 μ l/gm dry weight-hr at 10° C (Fig. 2). Q_{10} for the temperature range 10-23° C is 1.72, which is similar to the value (1.83) for large Mya arenaria obtained by Kennedy and Mihursky (1972). The per cent differences

Fig. 1. Oxygen extraction rate (\pm S.E.) (squares), and Ventilation rate (\pm S.E.) (circles) of Noetia ponderosa at 23° C at various ambient oxygen levels. Oxygen extraction, N=9; ventilation, N=6.

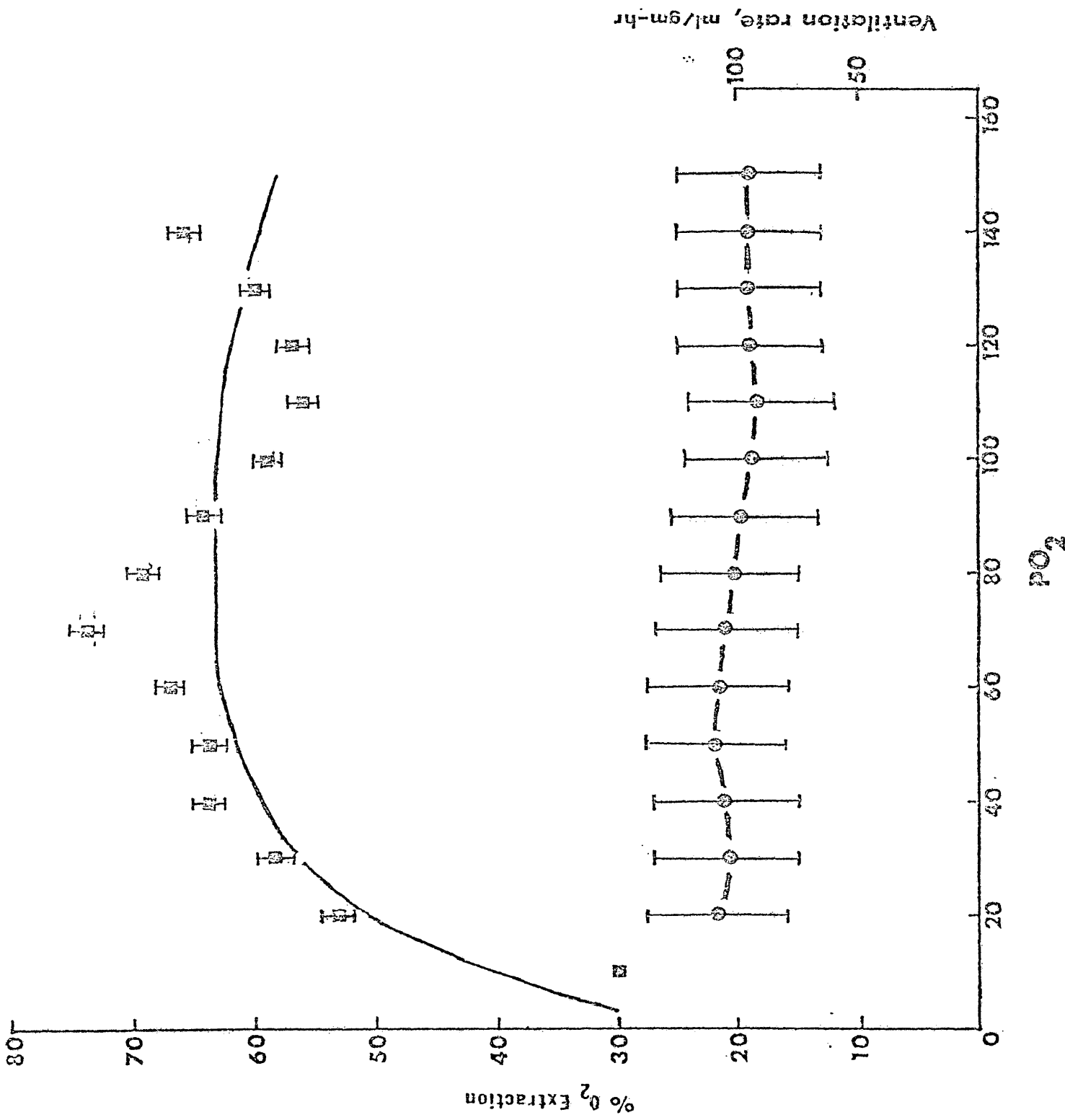
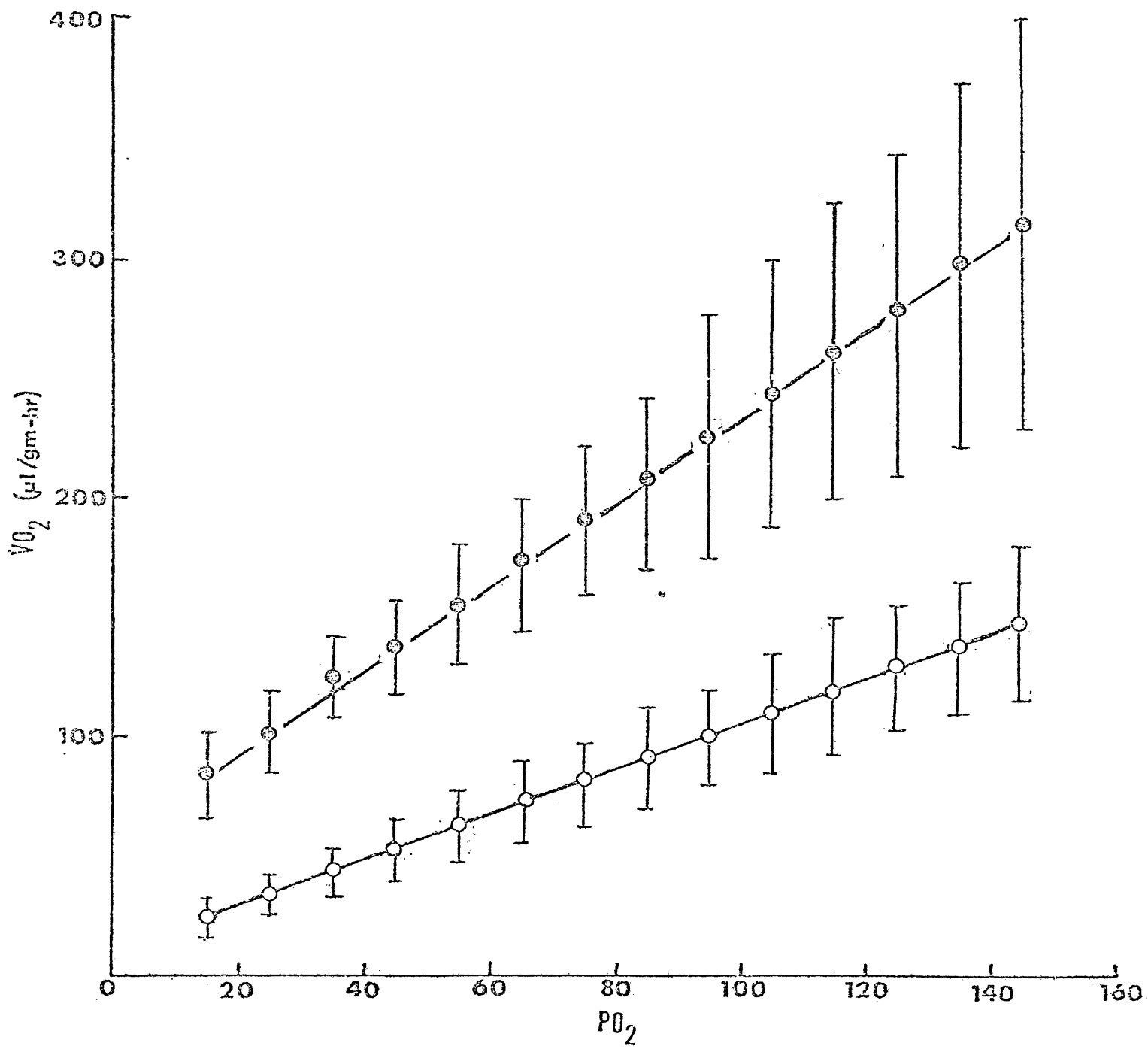


Fig. 2. $\dot{V}O_2$ (\pm S.E.) of Noetia ponderosa at 10° C (open circles) and 23° C (solid circles) at various ambient oxygen levels.

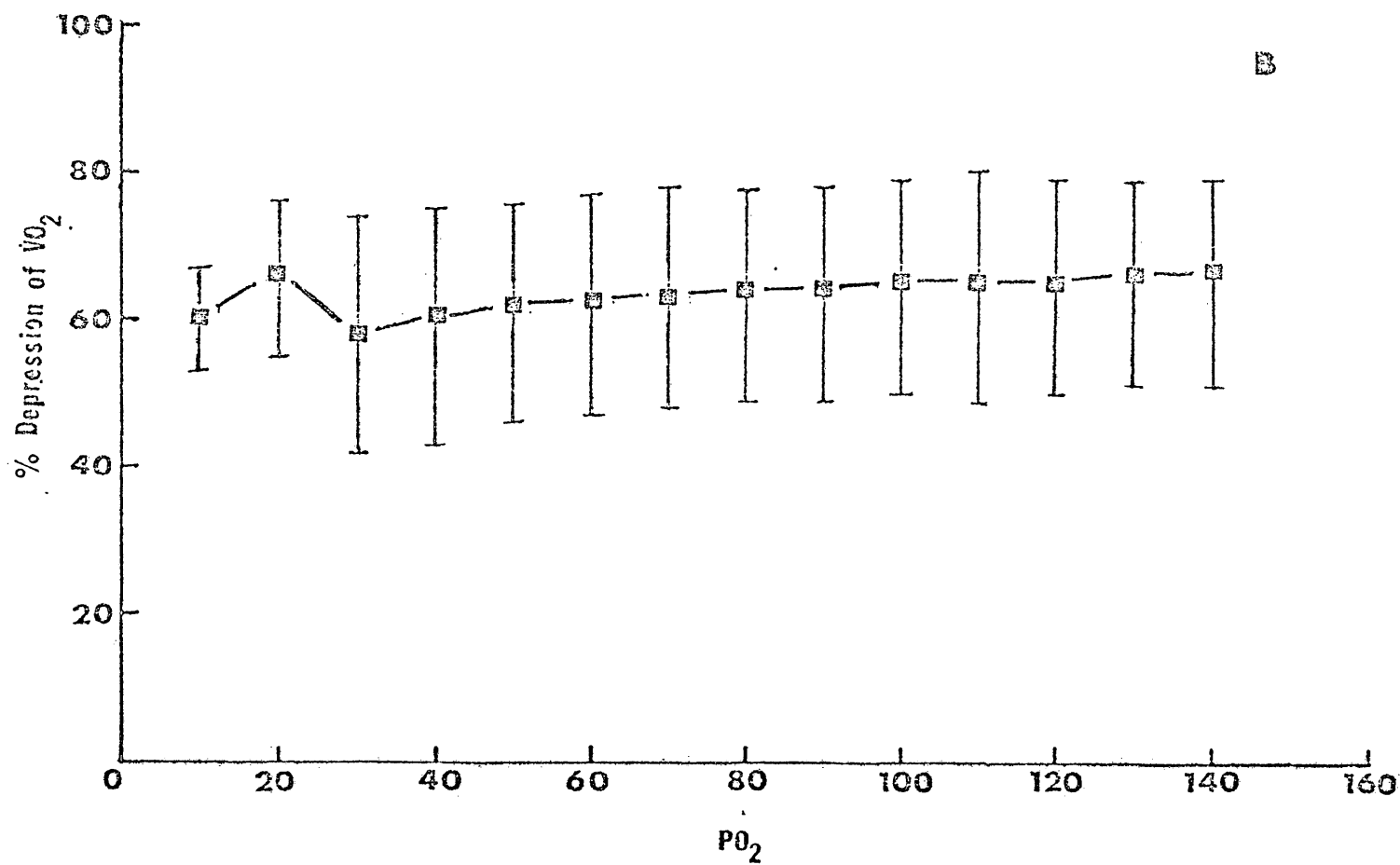
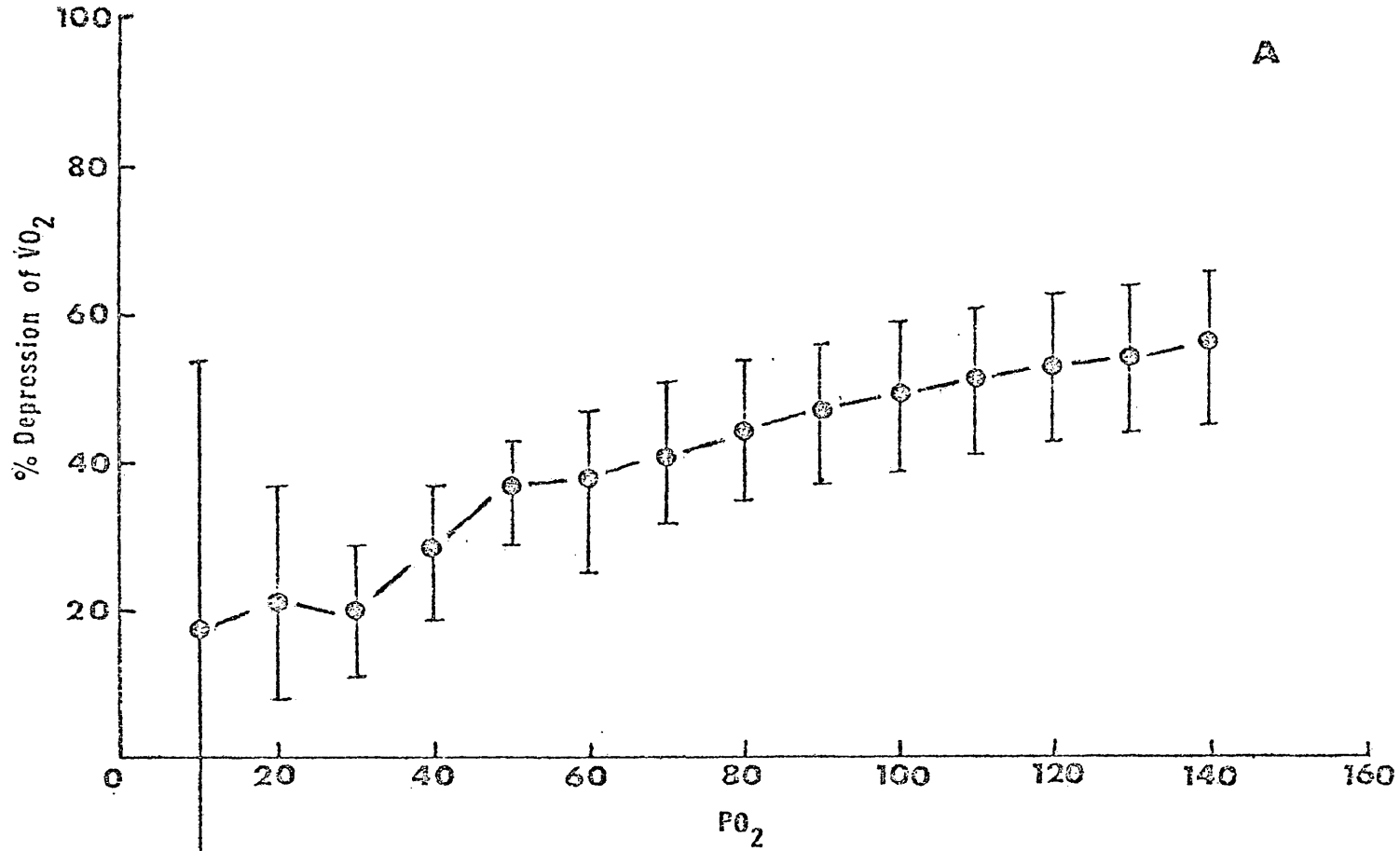


between animals with and without functional hemoglobin are shown in Fig. 3. Each data point is significantly different from zero except at 10 mm Hg and 23° C. Hemoglobins are responsible for half or more of the total oxygen uptake at 10° C and for from 20-52% at 23° C. Extrapolation of the curve in Fig. 3A to 159 mm Hg yields an estimate of 60% of $\dot{V}O_2$ at 159 mm Hg, still considerably smaller than the 86% change in oxidation of hemoglobin in post- and prebranchial blood (Freadman and Mangum, 1975). The discrepancy may be due in part to the elevation of $\dot{V}O_2$ by high carbon monoxide, but must also be due to uptake of oxygen at sites other than the gill.

The ventilatory current in Noetia ponderosa enters ventrally, passes anteriorly and then across the gill, and exits posteriorly only after flowing past the visceral mass and the mantle. Oxygen entry into the tissues on the surface of the visceral mass must be direct--this fraction of $\dot{V}O_2$ would not be reflected in measurements on blood. In addition, oxygen taken up by the blood which pulses through the mantle tissue may be delivered before it reaches the hepatic sinus and therefore this fraction would not be expressed in the measurements of Freadman and Mangum (1975).

Ambient PO_2 has little effect on hemoglobin function at 10° C, but the two vary directly at 23° C. The decrease at low oxygen levels becomes significant (Fig. 3A) in the interval 50-60 mm Hg. Respiratory pigment function in Noetia ponderosa is greater at the lower temperature, possibly because its oxygen affinity changes very little while $\dot{V}O_2$

Fig. 3. Per cent depression ($\pm 95\%$ C.I.) of VO_2 in Noetia ponderosa at 23° C (3A) and 10° C (3B) following exposure to carbon monoxide.



decreases, or perhaps because of higher per cent oxygenation at the gill (Freadman and Mangum, 1975).

The effect of carbon monoxide on $\dot{V}O_2$ of excised gill tissues of Mercenaria mercenaria is shown in Table 2. $\dot{V}O_2$ is elevated by 29-44% following exposure to carbon monoxide. Three intact Mercenaria showed no significant difference between control and experimental $\dot{V}O_2$. The mean $\dot{V}O_2$ for these animals at oxygen levels near oxygen saturation is 40 $\mu\text{l/gm dry-weight-hr}$.

Blood volume

The mean blood volume of nine animals (23.10-51.01-gm wet weight) is 23.3 ml (\pm 2.9 S.E.), or 60.2% (\pm 3.2 S.E.) of wet weight. Blood volume in molluscs varies from 40-80% of the body weight of the animal (Martin and Johansen, 1965). The mean blood volume of Margaritana margaritifera is 49% of body weight and that of Mytilus californianus 50.8 of body weight (Martin et. al., 1958).

A value of 1.55 was obtained for Hüfner's quotient for a diluted hemoglobin solution extracted from minced tissues. This is within the range of 1.54-1.57 for quotients of absorption spectra of hemoglobin solutions extracted from whole blood obtained by heart puncture.

The longevity of an oxygen store can be estimated from the time required by animals to deplete the volume of oxygen stored in the blood when there is no external oxygen supply. Assuming that the rate of oxygen uptake from an external source is the same as that from an internal

Table 2. Effect of carbon monoxide on $\dot{V}O_2$ of Mercenaria mercenaria gill tissue at 23° C.

Experiment	Control $\dot{V}O_2$	Experimental $\dot{V}O_2$	$\dot{V}O_2$	Per cent Elevation
1	42	54	12	28.6
2	61	80	19	31.1
3	39	56	17	43.6

source, the period can be read directly from the recorder traces of oxygen depletion of animals with carbon monoxyhemoglobin. Using data of Freadman and Mangum (1975), the oxygen store in Noetia ponderosa is:

$$(4.53 \text{ ml O}_2/100 \text{ ml}) \left(\frac{86.5\% + 53\%}{2} \right) (23.3 \text{ ml}) (1 \times 10^{-2})$$

↑
 oxygen carrying
 capacity of
 blood

↑ ↑
in vivo in vivo
 oxygenation oxygenation
 of Hb in of Hb in
 postbranchial prebranchial
 blood blood

mean
 blood
 volume

The value obtained from the calculation is 0.74 ml O₂. From the recorder traces, that volume would support aerobic respiration for an average of 67 minutes at 23° C.

DISCUSSION

Despite its moderately high oxygen affinity, the hemoglobin in Noetia ponderosa seems to function as an oxygen transport substance at ambient PO_2 's of from 10-20 to 159 mm Hg. There is no evidence that hemoglobin in Noetia is a special adaptation to high environmental temperature. Indeed, the increased participation of the pigment in oxygen transport at lower temperatures contrasts with a slight opposite trend for annelid hemoglobins and a strikingly different finding for sipunculid hemerythrin (Hoffmann and Mangum, 1970; Mangum and Kondon, 1974; Mangum et. al., 1975) even though the species have similar geographic ranges. This result must be explained in part by the unusually low temperature sensitivity of oxygen affinity ($\Delta H = -2.12$ Kcal/mole) in Noetia (Freadman and Mangum, 1975).

The habitat of the animals used in these experiments is probably never depleted of oxygen. In general, the PO_2 of subtidal waters on the outer coast of Virginia is believed to be high unless the surface layer is dilute and the water column highly stratified. This characteristic is not true of the habitat where N. ponderosa was found, where the salinity is rarely lower than 28 o/oo. Late summer is the time of year that stratification would result in oxygen depletion of the water if it ever occurs. Our measurements show that the water is at 60-70% air saturation even at this time of year. Presumably, tidal action and the absence of a pronounced halocline prevent stratification of these shallow waters, although exceptional situations are known (Menzies et. al., 1968).

Prediction of the longevity of the oxygen store in N. ponderosa by treating oxygen depletion as a first order reaction (Hoffmann and Mangum, 1970) results in a value of 70 minutes, which agrees well with the value (67 min) obtained from recorder traces of oxygen depletion by animals exposed to carbon monoxide. A hemoglobin oxygen store however, is useful only to an animal which is periodically exposed to low oxygen levels for relatively short periods. It would not support aerobic respiration for the long periods (4-8 weeks) for which subtidal estuarine populations encounter low oxygen levels in the summer. Oxygen storage may be important to Noetia ponderosa when it is impossible for the animals to ventilate during massive freshwater influxes or periods of extreme turbidity. It may also be of value in more southerly populations, which occur intertidally.

The high oxygen extraction rates are extremely interesting, especially in comparison with the very low rates found in other species of lamelli-branches. The values in Fig. 1 resemble those in aquatic species with gas exchange organs that do not serve a nutritional function. Among the molluscs, they are similar to oxygen extraction rates in gastropods, which are not filter feeders (Hazelhoff, 1938; Petersen and Johansen, 1973; Mangum and Burnett, 1974). The trophic function of the arcid gill has not been studied, however. In contrast, the ventilation rate of N. ponderosa is low compared to other large bivalves.

The slight increase in oxygen extraction is not accompanied by a pronounced increase in ventilation rate when the ambient PO_2 declines from

159 to 80 mm Hg. This result must reflect some form of compensation by the circulatory system.

The primary function of the cellular hemoglobins of Noetia ponderosa is facilitation of gas exchange at all oxygen levels. They probably also serve as an oxygen store during occasional periods when the animal cannot ventilate. The presence of the pigment results in a high oxygen extraction efficiency which allows Noetia to conserve energy by means of a relatively low ventilation rate.

SUMMARY

1. Hemoglobins of Noetia ponderosa are responsible for 20-52% of total oxygen uptake at 23° C and 60-65% at 10° C, depending on ambient PO₂.
2. Oxygen extraction from the ventilatory stream is 55-75%, depending on ambient PO₂. Ventilation rate is 160 ml/gm dry weight-hr.
3. Mean blood volume is 23 ml, or 60% of wet weight.
4. Hemoglobins function as oxygen transport substances under both high and low oxygen conditions in the environment. They may function as an oxygen store for brief periods when the animal cannot ventilate.

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