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THE RESPIRATORY RESPONSE OF *BUSYCON CANALICULATUM* (L.) TO SEASONAL VARIATION OF WATER TEMPERATURE, SALINITY, AND OXYGEN

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

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by

Harry Gregory Polites Jr.

1987

APPROVAL SHEET

This thesis is submitted in partial fulfillment of

the requirements for the degree of

Master of Arts

Author

Approved, July 1987

Charlotte Mangum

Gregory Capelli

Robert Black

DEDICATION

This work is dedicated to my mother and father.

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ABSTRACT

Busycon canaliculatum (L.) is an osmoconformer distributed on the eastern coast from Cape Cod to Florida in an estaurine environment that exposes its respiratory protein, hemocyanin, to significant thermal and ionic fluctuations. In vivo studies of the oxygenation properties of this respiratory protein demonstrated both a positive and negative Bohr shift and cooperative oxygen binding that was sensitive to salinity. Also, the pH dependence of oxygen affinity was strongly influenced by temperature. This investigation attempted to define the physiological relevance of the in vivo oxygenation properties.

The ecological range of B. canaliculatum appears to be controlled by the limits of the osmoregulatory and oxygen transport systems. The reduction of cooperative oxygen binding and elevation of oxygen affinity with salinity, restrict the function of the oxygen transport system and also limit penetration of the very low salinity areas of the estuary. The sharp increase in oxygen affinity and the progressive influence of the negative Bohr shift in arterial circulation as temperature drops effectively limit locomotor activity to transitional periods between summer temperature and winter lows. In the fluctuating ionic and thermal environment of the estuary, B. canaliculatum adjusts to changes by regulating ventilation, possibly heart rate, and the balance of primary and secondary sites of oxygen uptake.

THE RESPIRATORY RESPONSE OF *BUSYCON CANALICULATUM* (L.) TO SEASONAL VARIATION OF WATER TEMPERATURE, SALINITY, AND OXYGEN

INTRODUCTION

The Molluscs phylum display unmatched adaptive diversity. A primitive body design radiated into seven distinct classes but even distantly related molluscs have maintained a fundamental arrangement of body elements. Despite a common body form, the Mollusca have evolved a diversity of physiological systems that prevent predictions about one group from knowledge of another. The diversity of respiratory strategies exhibited among molluscs illustrates an amazing degree of exploitation of a unique set of metabolic and structural potentials.

The most common respiratory protein in the molluscan phylum is hemocyanin which occurs dissolved in the blood of many species of cephalopods, gastropods, bivalves, and polyplacophorans. In vitro work on molluscan hemocyanin by Redfield, Coolidge, and Hurd (1926) and Redfield and Ingalls (1933), revealed moderate oxygen cooperativity of oxygen binding and a reverse Bohr shift. In addition to the gastropod Busycon canaliculatum (L.), the chelicerate arthropods (e.g. Limulus polyphemus) are the only other marine animals known to have an oxygen transport protein with a negative Bohr shift within physiological pH limits. The physiological relevance of the negative Bohr shift has been demonstrated only in L. polyphemus. As L. polyphemus migrates into the estuaries in summer it encounters hypoxic and hyposaline water. In response, blood pH drops allowing blood oxygen affinity to rise, thus supplying more oxygen to the tissues (Mangum, et al. 1976). B. canaliculatum has a similar ecological range in the estuary and is found in both polyhaline and euhaline waters from Cape Cod to Florida. This environment presents daily and annual fluctuations in salinity of 18 to

$36^{o}/_{oo}$ and a temperature range from $6^{o}C$ to $26^{o}C$. The *in vitro* biochemical properties of oxygenation of *B. canaliculatum's* hemocyanin have been investigated by Mangum and Lykkeboe (1979) covering the thermal and ionic ranges encountered in the estuary. They found a hemocyanin with a strong negative Bohr shift from pH 6.6 to 7.9 which was replaced by a small positive Bohr shift above pH 7.9. Also, cooperativity of oxygen binding was strongly influenced by the levels of inorganic ions and drops sharply at low ($18^{o}/_{oo}$) salinity and below blood oxygenation levels of 40%. *In vitro* oxygen affinity and the Bohr shift were significantly altered by changes in ionic and thermal variables encountered in the estuary. Between $22^{o}C$ and $10^{o}C$ oxygen affinity (P_{50}) increases four fold at pH 7.9 and high salinity ($35^{o}/_{oo}$). The ionic and thermal sensitivity of this hemocyanin suggested that operation of the oxygen transport system could limit the ecological distribution of *B. canaliculatum*.

This study intended to determine the *in vivo* operating conditions for hemocyanin oxygenation and define which of the *in vitro* biochemical characteristics of oxygenation were physiologically relevant. Another important question was the dynamics of the *B. canaliculatum* respiratory system and the interaction of respiratory, excretory, and cardiovascular systems to cope with environmental ionic and temperature variations.

We initially examined *B. canaliculatum's* respiratory physiology at summer temperatures ($21\text{-}24^{o}C$) and both high salinity ($31\text{-}34^{o}/_{oo}$) and low salinity ($18\text{-}20^{o}/_{oo}$). Typical temperatures for fall and spring conditions ($10^{o}C$) and winter conditions ($6^{o}C$) combined with high and low salinity were subsequently investigated for as many respiratory variables as possible.

METHODS AND MATERIALS

Animal capture and maintenance

B. canaliculatum was obtained from the York River Seafood Co. in the lower Chesapeake Bay and from trapping specimens near Wachapreaque, Virginia. Magahales (1948) investigated trapping methods and specific bait preference for B. canaliculatum populations in Beaufort, North Carolina. Her conclusions were consistent with our experience trapping the Virginia species. B. canaliculatum was attracted to the traps with any type of decomposing meat but the most successful bait was crushed Mercenaria.

After capture, B. canaliculatum was maintained in recycled, carbon filtered seawater (31-34$^{o}/_{oo}$ or 18-20$^{o}/_{oo}$) at room temperature (21-24^{o}C). Salinity was measured with a refractometer. Attempts to feed B. canaliculatum in the laboratory were unsuccessful and in the first week of acclimation there was a significant decline in mucous secretion. Mucous production by the pedal muscle and the hypobranchial organ facilitates feeding, locomotion, and cleans the mantle cavity and gill surface. After seven days of acclimation to laboratory conditions, mucous production stabilized at reduced levels. Since mucous production is metabolically expensive for gastropods (Denny, 1980) and starvation can significantly alter metabolism we attempted to record physiological data within seven to ten days after capture.

Oxygen consumption

Prior to each measurement, the shells were scrubbed to remove epipytes, then coated with wax to prevent oxygen uptake by epibiota

(Kushins and Mangum, 1971). Individual specimens were placed in a glass respiratory vessel with a stirrer and Yellow Springs Instrument Co. (YSI) polarographic oxygen electrode inserted in the top with an air tight seal. The depletion of oxygen from air-saturated levels was monitored versus time with a YSI Model 54 oxygen meter. Oxygen concentration was measured until there was no detectable oxygen in the water. The specimens were dried to a constant weight at 60°C without their operculums. Oxygen consumption rates were computed at fifteen minute intervals with an Olivetti Underwood Programma 101.

Oxygen extraction

Individual specimens were suspended with a clamp device in a non-circulating aquarium. The dissolved oxygen level in water entering the mantle cavity (inhalant current) was measured with a YSI polarographic oxygen electrode. Measurements of oxygen tension from water leaving the mantle cavity (exhalant current) was taken with a hypodermic microelectrode (Beckman) and the signal was amplified with a Beckman model 160 physiological gas analyzer. Oxygen levels in the water supplying the mantle cavity and respiratory organ were gradually lowered by bubbling nitrogen gas in the aquarium. Occasionally measurements of exhalant oxygen levels were interrupted by secretion of mucus into the mantle cavity or body movements. At each ambient oxygen level (P_{O2}) individual measurements were repeated until consistent data were obtained covering the cyclical fluctuations of excurrent P_{O2}. Oxygen extraction rates were determined by measuring the area under a continuous recording of exhalant water oxygen levels.

Ventilation

A thermistor flowmeter described by LaBarbera and Vogel, (1976) was used to determine exhalant water flow rates. The thermistor flowmeter was fitted with one of three different size plastic apertures corresponding to the approximate diameter of the animal's exhalant current siphon. A variable speed pump created a controlled flow of water around the thermistor flowmeter and attached in line was a Gilson Instruments manometric flowmeter which calibrated the thermistor flowmeter's signal. Data was collected at various ambient oxygen levels as described above for measuring oxygen extraction. The same problems in making continuous records were encountered. Body movement and secretion of mucus interrupted the recording of data and these distortions were eliminated from the data set.

Blood gases and pH

Blood samples were taken with a one or ten milliliter syringe fitted with an 18 or 25 gauge needle. Unpaired samples were collected from the pedal, ventricle, and nephridial sinuses rapidly (thirty seconds) after removal of the specimens from the aquarium. Paired samples of pre-branchial and post-branchial vessels were taken anaerobically after quick removal of the shell with hammer claws. B. canaliculatum's quick withdraw reflex complicated the sampling by this procedure and required two individuals to co-ordinate rapid blood sampling. In addition, the open circulatory system allows shunting of large volumes of blood between the many blood sinuses and body organs. Speed in blood sampling and attention to removing only blood volumes corresponding to the vessel's size was carefully practiced for accuracy in data collection.

Blood P_{O2} and pH were measured with a Radiometer Corp. BMS-1 blood gas analyzer. pH of blood samples was determined with a Fisher model 540 digital pH meter.

Blood salts and ions

Blood samples were taken from the pedal gland with a syringe as described above for sampling blood gases. The blood osmolarity was measured by the Osmette freezing point osmometer and free chloride levels were analyzed with a Buchler-Cotlove chloridometer. Circulating ammonia values were measured by the phenol hypochlorite method (Solorzano, 1969; Gravitz and Kleye, 1975). Henry and Mangum (1980) described the method used for determining total ninhydrin positive substances. A Technicron auto amino acid analyzer was used to measure pedal muscle free amino acid levels as described by DuPaul and Webb (1970). For free amino acid analysis freshly dissected tissue was blotted and extracted with 80% ethanol for two days then dried at 60°C to a constant weight.

Hemocyanin concentration and oxygen carrying capacity

Oxygen carrying capacity of the blood was initially measured from pedal blood samples with the Lexington (Lex-O_2-Con-TL) oxygen concentration analyzer. However irreproducible measurements on identical samples forced us to calculate oxygen carrying capacity from measurements of hemocyanin concentration determined by the absorbance at 345 nm and the extinction coefficient given by Nickerson and Van Holde (1971).

Statistics

Differences in physiological parameters were tested for significance using an independent sample t-test.

RESULTS

Behavior and locomotion

While trapping B. canaliculatum, we observed that it was subtidal and migrated vertically with the upper limits of the tidal zone in the estuary. B. canaliculatum was rarely observed in shallow water or exposed to the air but occurred in dense concentrations along oyster reefs in association with dark organic substrates. There appeared to be no interspecific competition with B. carica which prefers sandy substrata.

During the winter months, when the water temperature drops below 10°C , B. canaliculatum is believed by local observers to move offshore to deeper water (>10 M), or burrow and "hibernate" in the mud of the estuary. In the laboratory we observed that at 10°C, locomotion was reduced with the margins of the mantle containing blue oxygenated blood. As temperature dropped from 10°C to 6°C there was a dramatic change in the animals behavior and locomotion. At 6°C, the animals retreated more than halfway into their shells. This blocked ventilation of the gills and the superficial blood sinuses in the foot became swollen and exhibited a darker blue tone. The exhalant opening was still maintained for excretion. Individuals exhibited little locomotion at $\leq$6°C and were assumed this corresponded to the period of winter "hibernation" referred to by local observers. Inactive or "hibernating" individuals resumed locomotion within twelve hours after exposure to temperatures >8°C. Individuals survived sudden changes in salinity (maximum change of 16°/$_{oo}$ in <1 minute) but exposure to acute temperature increases $\geq$10°C produced high mortality ($\geq$50%). Thermal stress (10°C temperature

increase) encountered during summer trapping as the animals were transported by boat back to the lab appeared to decrease the animals' ability to acclimate to new salinity. To keep mortality $\leq$50% during the summer, acclimation to new salinities had to be done in steps of 8-10$^{o}/_{oo}$ allowing three days for acclimation at each step.

In the normal mode of locomotion the shell is carried high upon the dilated pedal muscle (Figure 1). The siphon projects anteriorly over the foot and typically arches from side to side sampling water currents with its chemically sensitive osphradium organ. This organ is the first component in the mantle cavity to contact the inhalant water current. The major mass of the foot projects anteriorly during locomotion ahead of the shell body channeling water currents dorsally into the mantle cavity. At irregular intervals, the foot muscle tenses, extending the shell further off the foot and enlarging the mantle cavity. This is interpreted to be a "deep breathing" response and allows a large volume of water to flush the mantle cavity and possibly enhance circulation of oxygenated water.

Normal B. canaliculatum's shell and body posture appears to reduce hydrodynamic drag and enhance passive circulation of water through the mantle cavity. The streamlining of the shell and body posture may decrease the energy demand of locomotion. The only significant area of drag is assumed to be behind the coils of the shell which are held posteriorly during locomotion. The mantle cavity exhalant aperture is anterior to this area of drag. Thus, passive water flow may enhance active ventilation by generating negative pressure at the exhalant aperture and positive pressure at the inhalant aperture. B. canaliculatum is not a rapidly moving animal but tidal currents in the

estuary can produce significant water current speeds. Whether B. canaliculatum orients the shell and body to oppose the water currents during locomotion or strong tidal currents was not determined in this study.

Blood circulation

In B. canaliculatum there are three major circulatory divisions: arterial, venous, and renal (figure 2 and 3). These two figures which can be overlaid to produce a composite circulatory scheme were derived from personal observations and descriptions for related species by Dakin (1912) and Pierce (1950).

Arterial

The auricle of the heart receives blood from two different veins, the nephrio-cardiac vein (NCV), which bypasses the gills, and the efferent branchial sinus (EBS), supplying freshly oxygenated blood from the gills. A single aorta (Ao) from the ventricle divides after a short distance into the posterior aorta (A. post.) and anterior aorta (A. ant.). The cephalic branches of the anterior aorta lead to the head, foot, and mantle. The visceral or posterior aorta (A. post.) supplies the digestive gland, stomach and gonads. While the major arterial routes run parallel to the water current in the mantle cavity, the siphonal branches and secondary branching networks of the mantle cavity arteries, orient blood flow in counter-current arrangements. It is possible that this generates secondary sites for oxygen uptake at the mantle, foot, and siphon after blood leaves the gill.

Venous

The center of the venous system is the renal sinus system (RSS), which forms a long loop behind the pericardium and underneath the renal organs and pallial cavity (figure 3). Deoxygenated, venous blood from the foot, siphon, and mantle cavity return to cephalic sinus, which drains blood to the renal sinus. Deoxygenated blood returning from the posterior viscera pool drains into the visceral vein (VV) connected to the renal sinus system. Blood in the mantle cavity can return directly to the gill bypassing the kidney (MR).

Renal

The kidney has two sources of venous blood: 1) the highly branched network of the renal sinus system and, 2) a direct vessel from the cephalic sinus. Venous blood exits the kidney via two routes, 1) the nephrio-cardiac vein connected directly to the heart and 2) the renal sinus vessel supplying the hypobranchial gland and the gill with deoxygenated blood.

Physiological responses to estuarine environmental conditions: effect of changes in salinity and temperature.

Osmoregulation and ion balance

Typical of marine and estuarine gastropods (Todd, 1964), B. canaliculatum demonstrated no ability to osmoregulate at various temperatures (6, 10, and 21-24°C) or salinities (high salinity = 915mOsm, low salinity = 466mOsm) (Table I). While blood osmotic values were consistently higher than those for seawater the difference was not

statistically significant until low temperature at 6°C.

Total blood chloride was analyzed at one temperature (21-24°C) and high (535,545 mEq) or low (271 mEq) salinities (Table I). Chloride ion concentrations at both salinities were not significantly different from environmental values.

Pedal blood ammonia levels were not altered significantly by changes in salinity but did rise as temperature dropped from 21-24°C to 10°C or 6°C (Table I).

Nitrogen excretion and amino acid balance

Ammonia concentration dropped sharply as the blood crossed the gill. Measurements were taken at one temperature and variation in salinity did not significantly affect results (Table II).

Ammonia and free amino acid excretion values (Table II) were not significantly altered by salinity as determined by paired measurements.

Total amino acid composition of pedal muscle cells exhibited a small increase (20%) as salinity increased from low $18^{o}/_{oo}$ to high $33^{o}/_{oo}$ with major changes in the levels of: alanine, taurine, and ornithine (Table III).

Blood pH

Unpaired pedal blood pH values were obtained from animals acclimated to high salinity ($31\text{-}34^{o}/_{oo}$) or low salinity ($18\text{-}20^{o}$/oo) at three different temperatures (Table IV). At both salinities blood pH rose slightly (0.1 pH units) as temperature dropped. The change in blood pH was the largest as temperature dropped from 22°C to 10°C then only small changes were seen in blood pH at the lowest acclimation

temperature. This relationship was consistent at both salinities.

Blood pH levels were measured at four different sites in the circulatory system, pre-branchial, post-branchial, pedal muscle, and kidney, (Table IV). Paired observations were taken from as many selected sites as possible to enhance comparative analysis. B. canaliculatum was much more active at high temperatures (22-24°C) than at 10°C and its quick withdrawal reflex prevented simultaneous sampling of blood from all four sites of one individual.

At high temperature (22-24°C) and salinity (31-34°/$_{oo}$) blood pH values showed a large difference (0.19-0.34 pH units) as blood left the gill and followed the circulatory path returning to the pre-branchial sinus. Lower salinity amplified by nearly 80% the acid shift of blood pH during circulation.

Simultaneous blood samples were successfully obtained from animals acclimated to lower temperature (10°C). Surprisingly, the blood pH values at both high and low salinity did not drop during circulation but wobbled within the range 0.13-0.2 pH units.

Individuals were held overnight (16 hours) in sealed jars to simulate hypoxic conditions. The mean pedal blood pH of this group dropped to 7.647 ($\pm$0.031, N=5) but after the measurements, no individuals survived. Build up of acidic metabolites in non-filtered water may have been the major factor causing high mortality in hypoxic conditions. Carbon filtered, circulating water depleted of dissolved oxygen would have been a better simulation of hypoxic conditions. Alternatively, true hypoxic conditions maybe lethal for B. canaliculatum due possibly to the negative Bohr shift of its respiratory pigment.

Blood oxygen

Circulating hemocyanin in B. canaliculatum acclimated to summer condition (21-24°C) and high salinity, delivered 55% of its bound oxygen to cells (Table V). Low salinity decreased by only 5% the quantity of oxygen unloaded during circulation as determined by pre-branchial and post-branchial blood oxygen measurements. After crossing the gill, the percent oxygenation of hemocyanin was reduced at low salinity by 9%, even though blood oxygen levels were held constant.

Reduced salinity had a more significant influence on oxygen levels sampled at distant locations from the gill in the circulatory path. Samples from the foot showed a 50% drop in both the blood oxygen and the percent oxygenation of hemocyanin at low salinity. However, returning venous blood at the pre-branchial site indicated blood oxygen levels and percent oxygenation of hemocyanin fell to the same values at both salinities.

Lower temperatures (10 and 6°C) drastically altered the pattern of blood oxygen observed at summer temperatures. At both high and low salinities and 10°C, hemocyanin was almost fully oxygenated at the gill (98-99% HcO_2) and delivered only 11-12% of the bound oxygen during circulation, one-fourth the quantity observed at summer temperatures (Table V). After leaving the gill, blood oxygen levels were significantly elevated at low salinity in comparison to high salinity. For both salinities at 10°C, the percent oxygenation of hemocyanin was the same. High salinity, pedal blood oxygen levels at 10°C were higher than blood leaving the gills, but low salinity values were not successfully sampled.

Post-branchial and pedal blood oxygen levels at 10°C for both

salinities are within the range of cooperative oxygen binding by hemocyanin (Mangum and Lykkeboe,1979). Returning venous blood dropped to the same oxygen level, regardless of salinity, and this venous reservoir was four times as large as high temperature values. Samples of only foot blood at 6°C, high salinity also showed complete oxygenation P_{O2}=70.2 9mmHg (n=5), 99.0% oxyhcy.

Oxygen consumption in declining ambient oxygen

For all the conditions outlined in Table VI, there was a rapid decline in oxygen consumption rates from air saturation to approximately 120 mmHg. Above 120 mmHg ambient oxygen an impairment of locomotion and contractile reflex was considered to be abnormal behavior and oxygen consumption measurements were not used for comparing the influence of salinity or temperature. Therefore, oxygen consumption rates were compared at two ambient oxygen levels (40 and 100 mmHg).

Oxygen consumption was regulated as ambient oxygen declined below 120 mmHg (Figure 4). At 10-20 mmHg ambient oxygen, regulation was diminished and oxygen consumption rates fell until oxygen was depleted. Once regulation of oxygen consumption was initiated, the majority of the animals maintained a constant rate oxygen consumption to the end of the recording, or 10-20 mmHg ambient oxygen. Also, regulation of oxygen consumption was not always continuous and 17.6% of the individuals demonstrated cycling regulation, marked by a periodic shutdown. Thus, B. canaliculatum regulated oxygen consumption over the ambient oxygen range of 20-120 mmHg (Figure 4).

Oxygen consumption: influence of temperature and salinity

The standard errors for measurement of oxygen consumption rates were large (Table VI), but the trends in unpaired data were confirmed with several paired observations of oxygen consumption rates in declining oxygen.

At ambient oxygen levels of 40 and 100 mmHg, oxygen consumption rates declined sharply with temperature from summer conditions (21-24°C) to winter temperatures (6°C) (table VI). The temperature coefficients (Q_{10}) for total oxygen metabolism are 2.0 for the interval 10-22°C and 14 for 6°C to 10°C. At 100 mmHg ambient oxygen, the difference between high and low salinity rates of oxygen consumption increased as temperature dropped. A difference due to salinity at 22°C of 8.8% increased to a 22.5% difference at 6°C. But at 40 mmHg ambient oxygen, the degree of influence of salinity on oxygen consumption rates was the opposite to that found at 100 mmHg. At summer temperature (24°C), lower salinity produced a 20% decrease in the rate of oxygen uptake. As temperature drops to 10°C and 6°C, salinity has a decreasing influence (5.5% or less) on altering oxygen consumption rates.

Ventilation and oxygen extraction

Ventilation measurements, (Table VII) relied on an approximation of the exhalant diameter. Ventilation and oxygen extraction were measured simultaneously in declining oxygen at summer temperatures and high salinity (Figure 5). The ventilatory and oxygen extraction rates had parallel responses as oxygen declined. Figure 6 can be produced by overlaying figure 4 of oxygen consumption in declining ambient oxygen and figure 5 for ventilation and oxygen extraction. From 120 to 90 mmHg

ambient oxygen, the ventilation and oxygen extraction values dropped and this response corresponded to the rapid drop in oxygen consumption. Between 100 mmHg and 10mmHg, ventilation and oxygen extraction increased by the same magnitude and oxygen consumption values demonstrated strong regulation.

Oxygen extraction had the same complex response to declining ambient oxygen at both high and low salinities (Table VII). Oxygen extraction declined with ambient oxygen from 120 to 90 mmHg. As ambient oxygen declined further, oxygen extraction increased then plateaued from 60 to 20 mmHg. A 28% increase in oxygen extraction rates due to low salinity at 120mmHg was reduced to an average 5-9% increase thru the region of strong regulation of oxygen consumption (20-100mmHg). Below 20 mmHg, oxygen extraction declined quickly and low salinity values were progressively higher.

Both mean and maximum ventilation rates at summer temperatures (21-24°C) were reduced with salinity at 80-100 mmHg ambient oxygen (Table VII). The maximum ventilation rate dropped 17% with salinity and mean values dropped by 41%. Ventilation was not a constant function and therefore mean values were questionable measurements for quantitative comparisons. Maximum rates of ventilation were a more accurate basis for correlation with respiratory demand.

Hemocyanin concentration and oxygen carrying capacity

In gastropods, hemocyanin concentration and oxygen carrying capacity are extremely variable and unrelated to weight, sex, reproductive activity, or nutritional status (Betzer and Pilson, 1974). Redfield and Ingalls (1926) found over a two fold variation in oxygen

carrying capacity.

Our values for oxygen carrying capacity (Table V) are associated with the dormant-winter phase. These values are only half those reported by Redfield, Coolidge, and Hurd (1926) and about half those found in Busycon carica (Freadman and Mangum, unpublished).

While obtaining blood samples from the kidney and pre-branchial areas we observed the blood in the kidney was much darker and 2-4 times more concentrated than pedal muscle samples. Another study (Mangum 1979) addressed the hypothesis that seawater is mixed in the foot with blood for hydrostatic muscle control and the blood is reconcentrated in the kidney for oxygen transport from the gill to the tissues.

DISCUSSION

The blood ion data verified that B. canaliculatum is an osmoconformer, thus exposing its respiratory protein to ionic fluctuations associated with estuarine salinity variations. Blood pH of B. canaliculatum is relatively insensitive to changes in blood ionic concentrations. Therefore, maintenance of its hemocyanin's respiratory function by altering the acid-base status of the blood was not an effective compensatory mechanism. This relationship correlates with the cellular free amino acid pool and blood ammonia levels, which changed very little with salinity. As in L. polyphemus, (Mangum et al.,1976) levels of cellular free amino acids and blood ammonia suggested that at low salinity there was no increase in the deamination of cellular free amino acid pools to raise blood pH and supply ammonium ion to serve as a counter-ion for osmoregulation.

While blood pH is relatively stable for the thermal and ionic variations encountered in the estuary, there are large changes in blood pH along the circulatory path. Through the arterial circulation hemocyanin oxygenation operates with a small positive Bohr effect, but in the venous route blood pH falls to where the negative Bohr effect occurs. The negative Bohr shift appears to be confined to areas of poor circulation and low blood oxygen levels, such as the cephalic and renal sinus which supply the kidney. Functionally, the negative Bohr shift maintains a venous reserve for blood returning through inefficient circulatory sinuses to the kidney and hypobranchial gland. The venous reserve may be important for insuring oxygen supply to the kidney and hypobranchial gland. Alternatively, the venous reserve could function

during the initial stage of aerobic metabolism after extended periods of anaerobic metabolism. Anaerobic metabolism operates during hypoxic conditions and may increase during periods of high metabolism, such as capturing and opening oyster shells. This hypothesis may explain why there was not an initial increase in oxygen uptake after brief exposure to hypoxic conditions. In either case, more data is needed to determine whether blood pH can fall below 7.4 without being lethal.

At summer temperatures the performance level of the oxygen transport system is reduced with salinity. The salinity influence was magnified at low ambient oxygen, where B. canaliculatum strongly regulates oxygen consumption. At low salinity, B. canaliculatum's ventilation rate drops, allowing longer time for the equilibration of blood and water at the gill. This compensation is also reflected in increased oxygen extraction from water leaving the mantle cavity. The adjustment of ventilation and oxygen extraction to low salinity suggests that heart rate would be reduced by the same increment to maintain blood oxygen levels. B. canaliculatum strongly regulated heart rate in response to declining ambient oxygen (deFur and Mangum, 1979) and it is possible that heart rate could be adjusted with salinity. The net result, at low salinity, is the maintenance of blood oxygen levels at the gill even though the percent hemocyanin oxygenation is reduced by the loss of cooperativity. There is no attempt to elevate blood oxygen at the gills by either reducing heart rate more than ventilation, or by raising ventilation to compensate for reducing hemocyanin oxygenation as salinity dropped.

At summer temperatures, changes in salinity dramatically alter blood oxygen levels and hemocyanin oxygenation in the foot of B.

canaliculatum. This is attributed to either changes in the supply of oxygen from secondary sites of oxygen uptake or the alteration of the metabolic demand by the tissues. Measurements of isolated tissues acclimated to low and high salinity demonstrated no significant change in metabolism except for the pedal musculature, which reduced the oxygen consumption rate with salinity (Polites and Mangum, 1980). Therefore, as ventilation of the mantle cavity drops with salinity, there is a reduction in oxygen uptake at secondary sites (mantle, siphon and foot) and blood oxygen levels drop more rapidly during circulation. Blood oxygen levels ultimately fall to the same level, regardless of salinity, to maintain a venous reserve. However, reduced oxygen uptake from secondary sites at low salinity results in lower blood oxygen levels along the circulatory pathway thus reaching the levels for the venous reserve much sooner in the circulatory pathway.

The role of secondary sites in total oxygen uptake appear to be reduced not only with salinity but also with lower environmental oxygen. Obviously the efficiency of oxygen absorption at secondary sites would decline with lower oxygen gradients. In addition, the reduction in cooperative oxygen binding by hemocyanin with salinity lowers both the degree of blood oxygenation and the oxygen gradient driving oxygen uptake across poor exchange surfaces primarily the mantle cavity and pedal muscle.

At summer temperatures, the correlation between lower salinity and decreased oxygen consumption was more apparent at low ambient oxygen levels. This suggests that changes in salinity have a greater influence on the primary oxygen uptake system (gill and heart). The contribution of secondary sites to total oxygen consumption is believed to decrease

with ambient oxygen by means of a decrease in the oxygen gradient between the blood and seawater.

As temperature dropped from 22°C to 6°C there was a corresponding drop in oxygen uptake. A gradual reduction from 22°C to 10°C was followed by a rapid drop from 10°C to 6°C which was observed at both salinities and all ambient oxygen levels. The reduction in oxygen consumption from 22-10°C is correlated with changes in the biochemical properties of hemocyanin oxygenation. At lower temperatures, blood pH wobbled during circulation and was clearly operating within the pH range of the negative Bohr shift. The increased oxygen affinity and negative Bohr shift with decreasing temperature, apparently elevated blood P_{o2} levels. Oxygen transport by the respiratory protein via the primary respiratory system appeared to be progressively more inefficient as temperature dropped. Blood oxygen levels at 10°C actually rose during circulation from the gill to the mantle and foot, illustrating the increased contribution of secondary sites in oxygen transport to deep tissues (stomach, gonads, and kidney).

The postulated shift in the balance from primary to secondary oxygen supply sites as temperature drops, is also believed to alter the influence of salinity and ambient oxygen on total respiratory demand. At 10°C, high ambient oxygen levels (100 mmHg), the reduction in cooperativity with salinity appears to reduce total oxygen uptake at secondary sites. Low salinity values for pedal blood at 10°C would help substantiate this conclusion. As ambient oxygen drops at 10°C, the difference in oxygen consumption rates due to salinity diminish with the loss of cooperativity of oxygen binding at secondary sites. At 10°C, increased post-branchial blood oxygen levels with lower salinity

suggested respiratory adjustments to maintain the same degree of hemocyanin oxygenation. If ventilation is reduced with salinity at summer temperatures, then one could postulate that heart rate slows to a greater degree at low salinity and lower temperatures allowing longer equilibration of fluids. Measurements of ventilation and heart rate at 10°C would substantiate this hypothesis.

At lower winter temperatures (6°C), oxygen consumption drops dramatically, suggesting that the negative Bohr shift eliminates the primary oxygen transport system and reduces the efficiency of secondary sites for oxygen exchange. Heart rate also stops (deFur and Mangum,1979) as the primary oxygen transport system apparently shuts down. While the animal is fully contracted into its shell at 6°C, restricting fluid flow across the mantle, oxygen uptake at secondary sites was still possible at the exposed epithelium of the foot and mantle margin. Changes in salinity and ambient oxygen levels may have their effects solely on blood that was oxygenated at secondary sites. At 6°C, and high ambient oxygen levels, reduction in total oxygen consumption with salinity could be correlated exclusively with reduction in cooperative oxygen binding of hemocyanin at secondary sites. But, as ambient oxygen drops, cooperative binding of oxygen is reduced and oxygen consumption rates are not influenced by salinity.

The behavior and the ecological limits of B. canaliculatum in the estuary appear to be influenced by the physiological features of the osmoregulatory and oxygen transport systems. While there must be other systems that interact to limit ecological distribution, this hemocyanin's sensitivity to ionic and thermal changes appears to control its ecological range. The reduction of cooperative oxygen binding and

elevation of oxygen affinity with salinity, restrict the function of the oxygen transport system and also limit penetration of the very low salinity areas of the estuary (<18 $^{o}/_{oo}$). Also, the sharp increase in oxygen affinity and the progressive influence of the negative Bohr shift in arterial circulation as temperature drops effectively limit locomotor activity to transitional periods between summer temperature (<24°C) and winter lows (>10 C).

In the fluctuating ionic and thermal environment of the estuary, B. canaliculatum has not developed cellular, metabolic mechanisms to stabilize blood osmotic levels. Compensatory mechanisms to changes in salinity and temperature are limited to regulation of ventilation, possibly heart rate, and the balance of primary and secondary sites of oxygen uptake. This strategy appears to focus on supplying the demands of aerobic metabolism and allows rapid adjustments to daily fluctuations in salinity and temperature. Osmoregulation by changing the free amino acid pool to control cell volume is not a rapid enough process to stabilize blood ionic concentrations in an environment of daily fluctuations in salinity (Henry and Mangum, 1980).

The potential of secondary sites for oxygen uptake has been demonstrated in Modiolus demissus, a bivalve filter feeder without a respiratory protein. It can oxygenate its blood to the same degree as bivalves with a respiratory protein by increasing ventilation rates, enlarging gill surface area, and decentralizing oxygen exchange sites (Booth and Mangum, 1978). M. demissus short-circuits its branchial system by having its tissues, that are in contact with seawater, extract oxygen more efficiently than the gill. The gill's oxygen uptake and transport function was significant only in low ambient oxygen

conditions.

Reliance of the respiratory system on secondary sites of oxygen uptake is a passive, non-energy demanding, compensatory response to environmental changes, but for B. canaliculatum was not a totally effective mechanism to overcome the thermal and ionic biochemical properties of hemocyanin oxygenation.

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Table I

Temperature (°C)	21		10	6
Osmolality (mOsm)				
Seawater	915	466	971	546
Blood	918	468	977	560
	±2.2(11)	±1(6)	±2.5(7)	±1.4(11)*
Chloride (mM)				
Seawater	554	271	--	--
Blood	529	257	--	--
	±3.3(9)	±1(8)		
Pedal blood ammonia (μm)				
High salinity (31-34°/oo)	123		203	187
	±16(7)		±18(18)	±14(5)
Low salinity (18-20°/°o)	129		--	--
	±8(18)			

* P< 0.05

Blood osmotic and ionic properties of B. canaliculatum (mean values ± S.E. (n))

Table II

Branchial blood ammonia (μM)	19°C 20 °/oo	19°C 33°/oo
pre-branchial	89±12.5 (6)	91±10 (10)
post-branchial	36±7.9 (6)	3±6 (11)

	Ammonia excretion (μM/gm dry wt-hr)	Free amino acid excretion (μM leucine/gm dry wt-hr)
Paired observations on intact animals		
15°C 18°/oo	0.54±0.05 (7)	0.33±0.5 (7)
15°C 30°/oo	0.49±0.06 (7)	0.36±0.41 (7)

Nitrogen excretion in B. canaliculatum (mean ± S.E. (n))

Table III

	$18^{o}/_{oo}$		$33^{o}/_{oo}$		
	μM/gm	% total	μM/gm	%total	ΔμM/gm
taurine	114.2	46.8	94.1	31.7	-20.1
aspartic acid	26.1	10.7	28.1	9.5	2.0
threonine	7.2	3.0	3.6	1.2	-3.6
serine	13.5	5.5	11.7	3.9	-1.8
glutamic acid	24.5	10.0	18.3	6.2	-6.0
proline	19.3	7.9	17.4	5.9	-1.9
glycine	3.1	1.3	10.4	3.5	7.3
alanine	15.0	6.2	57.6	19.4	42.6
valine		0	7.1	2.4	7.1
methionine	3.8	1.6		0	-3.8
isoleucine	1.3	0.5	4.9	1.7	3.6
leucine	1.8	0.7	10.5	3.5	8.7
tyrosine	-	0	4.8	1.6	4.8
phyenylalanine	2.2	0.9	2.0	0.7	-0.2
ornithine	1.7	0.7	20.1	6.8	18.4
lysine	6.8	2.8	6.1	2.1	-0.7
histidine	3.6	1.5	-	0	-3.6
TOTAL	243.9	100.1	296.7	100.1	52.8

The concentration (μM/gm dry wt) and percent composition of free amino acids in pedal muscle fibers of B. canaliculatum acclimated to high and low salinity at 22-24°C. Analysis performed by R.P. Henry.

Table IV

Temp. (°C)	22-24		10		6	
Salinity	High*	Low*	High	Low	High	Low
pedal blood pH	7.85 ±0.009 (24)	7.90 ±0.03 (7)	7.93 ±0.04 (10)	8.00 ±0.03 (7)	7.95 ±0.04 (5)	8.00 ±0.06 (7)
Paired blood pH values						
pre-branchial	7.85 ±0.02 (4)	7.70 ±0.03 (4)	7.86 ±0.02 (4)	7.97 ±0.03 (4)		
post-branchial	8.04 ±0.02 (4)	8.04 ±0.03 (4)	7.93 ±0.02 (4)	7.82 ±0.04 (4)		
pedal muscle	--	--	8.06 ±0.03 (4)	7.95 ±0.03 (4)		
kidney			7.87 ±0.02 (4)	7.82 ±0.04 (3)		

*High salinity = 31-34 °/oo
*Low salinity = 18-20 °/oo

Blood pH in B. canaliculatum (mean ± S.E.(n))

Table V

Temp. (°C)	22-24		10	
Salinity	High*	Low*	High	Low
pre-branchial pO2/%oxy-hemcy.	2.7 /22.5 ±0.5 (7)	2.08 /18.8 ±0.3 (5)	19.3 /87.0 ±0.3 (4)	19.5 /87.5 ±3.1 (5)
post-branchial	26.9 /80.0 ±3.9 (7)	25.1 /71.3 ±7.4 (6)	38.5 /98.0 ±5.7 (4)	55.5 /99.0 ±6.5 (3)
pedal muscle	10.3 /50.0 ±3.9 (8)	3.85 /26.0 ±1.2 (4)	62.0 /99.0 ±6.7 (8)	

Pedal blood oxygen carrying capacity (ml/100ml) = 1.39±0.074(11)

Blood oxygen levels (mmHg) and percent oxy-hemocyanin (calculated from blood pH Table IV, blood pO_2 and O_2 binding data reported by Mangum and Lykkeboe, 1979) in B. canaliculatum at ambient oxygen level of 100 mmHg (mean P_{O2} ± S.E. (n) / %oxy-hemocyanin).

Table VI

Ambient oxygen	40 mmHg		100 mmHg	
Salinity	High	Low	High	Low
Temperature				
6°C	5.5 ±1.6 (6)	5.4 ±1.2 (8)	11.5 ±0.6 (6)	8.9 ±2.3 (8)
10°C	21.8 ±2.4 (13)	22.9 ±2.9 (15)	40.6 ±3.1 (13)	35.3 ±4.5 (15)
21-24°C	30.4 ±20.4 (18)	24.2 ±7.1 (16)	63.3 ±11.7 (18)	57.8 ±12.2 (16)

Oxygen consumption rates (μl oxygen/dry gm hr) in declining ambient oxygen for B. canaliculatum (mean rate ± S.E. (n)).

Table VII

Percent oxygen extraction

Ambient oxygen (mmHg)	High salinity (31-34°/oo)	Low salinity (18-20°/oo)
120	65.0	93.0
110	59.0	78.0
100	54.0	63.0
90	56.6	56.5
80	66.0	72.0
70	72.5	79.0
60	82.5	92.0
50	85.0	91.0
40	98.0	89.0
30	85.0	89.0
20	87.0	89.0
18	77.5	---
16	---	87
12	62.5	77.5
10	60.0	---
8	56.5	---
6	---	70

Ventilation rates (ml/gm wt hr) and ambient oxygen 100mmHg

Salinity	Maximum rate	Mean ±S.E. (n)
31-34°/oo	3.25	2.85±0.2 (9)
18-20°/oo	2.68	1.69±0.3 (8)

Oxygen extraction and ventilation rates for B. canaliculatum (temperature =21-24°C)

Figure 1. Body and shell posture during locomotion for *B. canaliculatum*

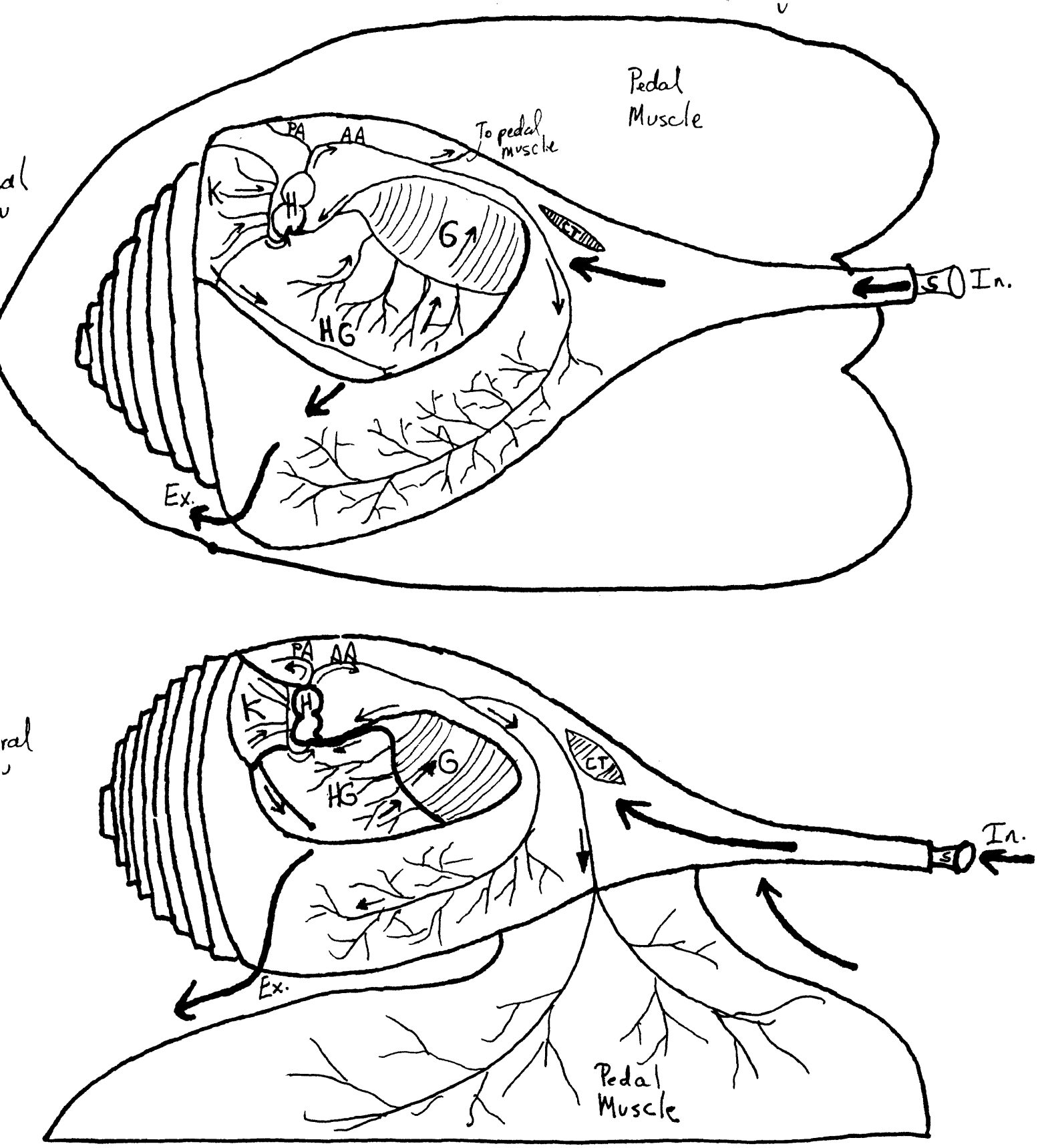

Pedal
Muscle
PA
AA
To pedal
muscle
K
H
G
HG
CT
S
In.
Ex.
PA
AA
K
H
G
HG
CT
S
In.
Ex.
Pedal
Muscle

Figure 2. Arterial circulation in B. canaliculatum

FIGURE 2

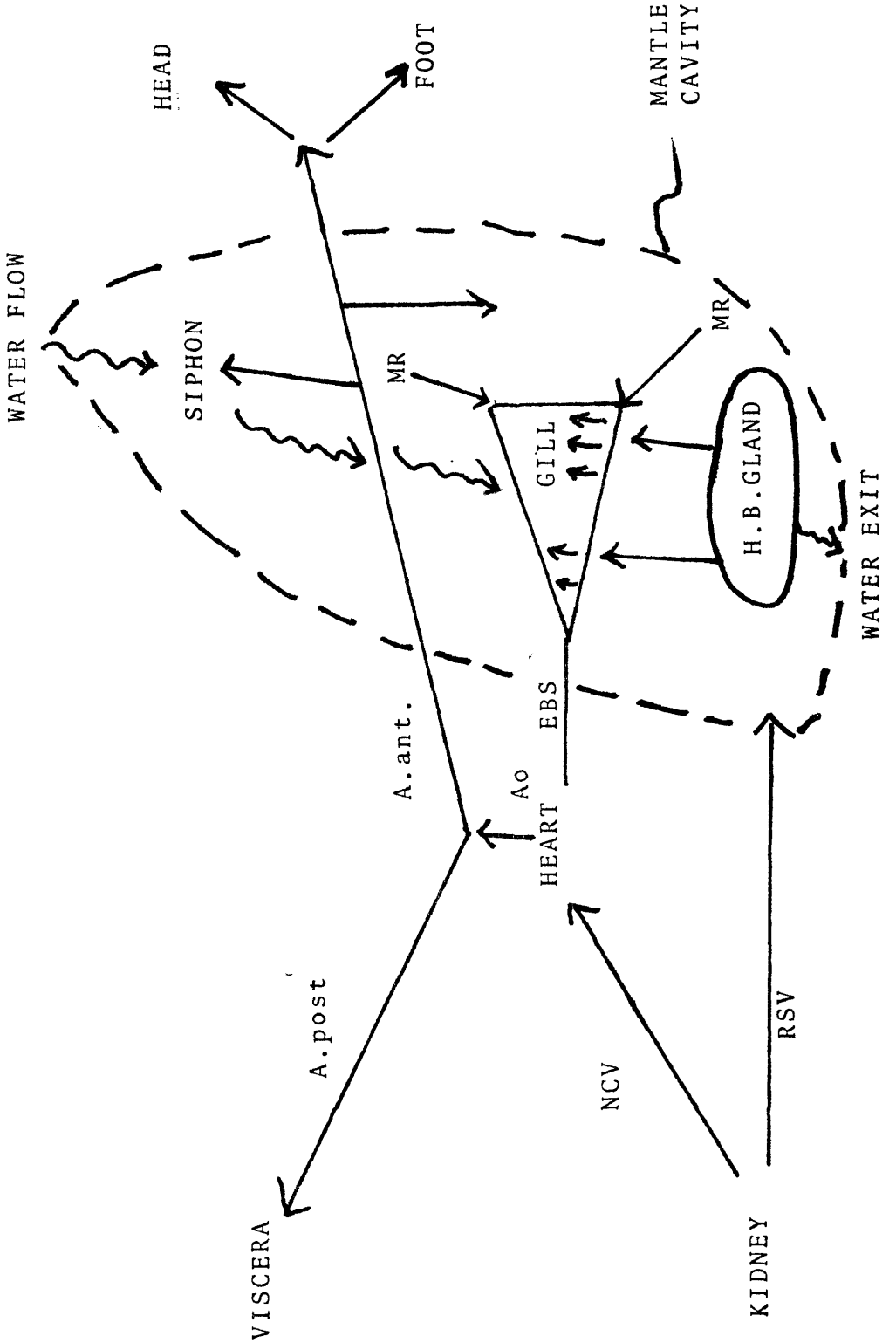

WATER FLOW
SIPHON
HEAD
FOOT
MANTLE CAVITY
MR
MR
GILL
H.B.GLAND
WATER EXIT
EBS
A.ant.
Ao
HEART
A.post
NCV
RSV
VISCERA
KIDNEY

Figure 3. Venous and renal circulation in B. canaliculatum

FIGURE 3

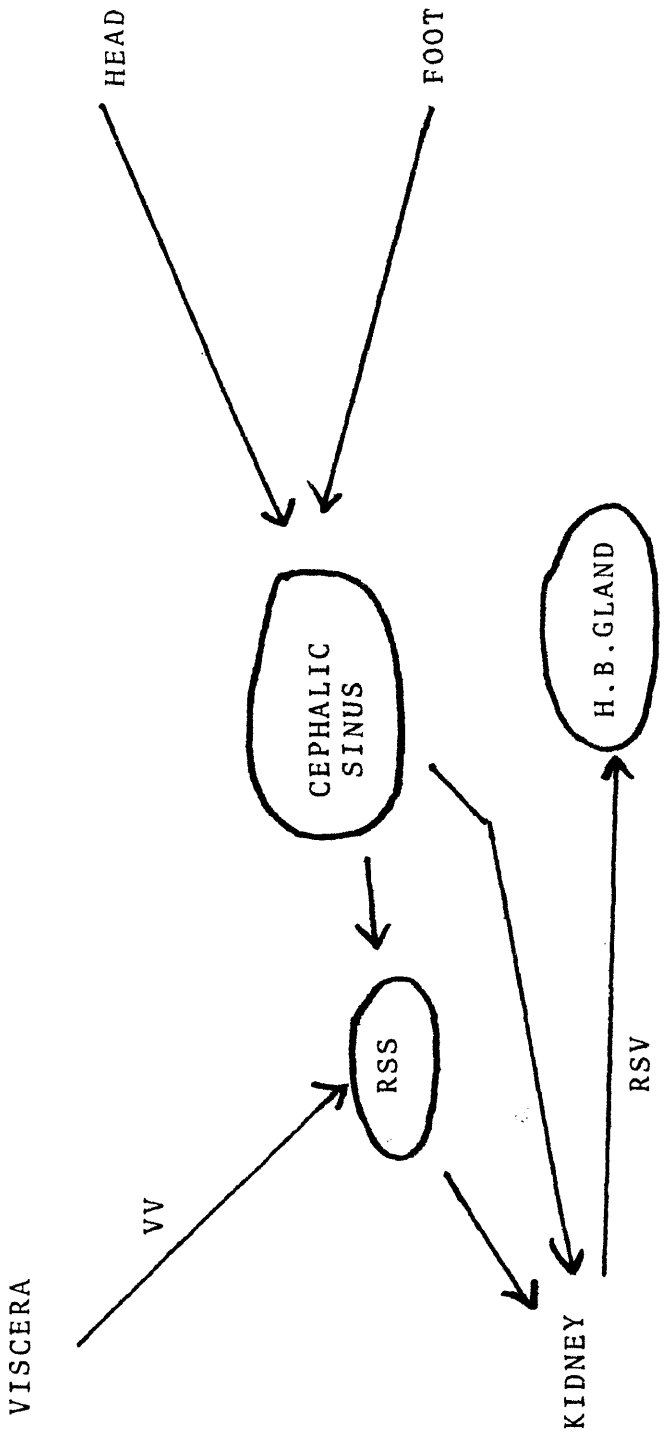


Figure 4. Oxygen consumption in declining ambient oxygen for B. canaliculatum, (21-24°C, 31-34°/oo)

FIGURE 4

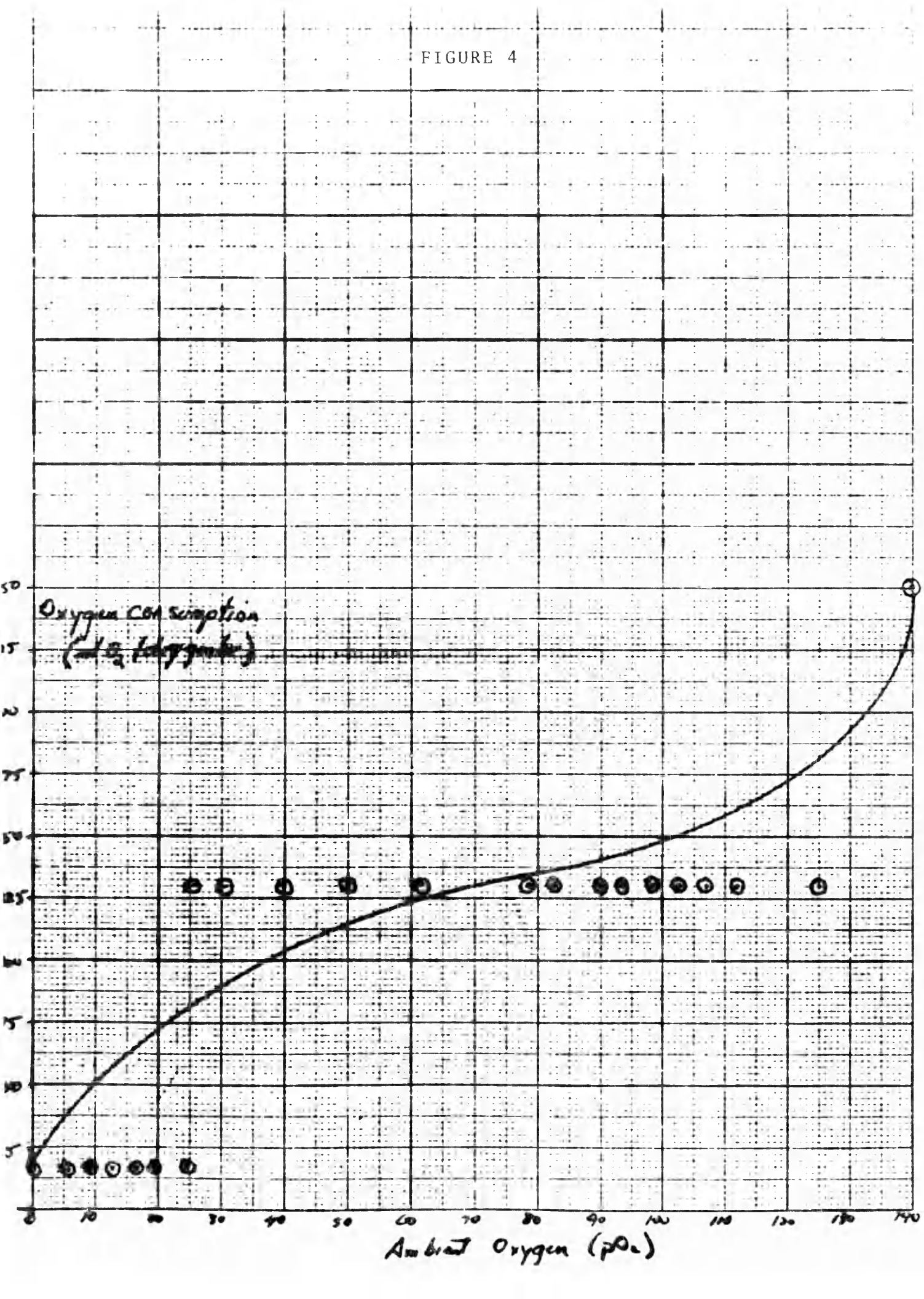


Figure 5. Simultaneous ventilation and oxygen extraction in declining ambient oxygen for B. canaliculatum, ($21^{o}C$, $34^{o}/_{oo}$)

FIGURE 5

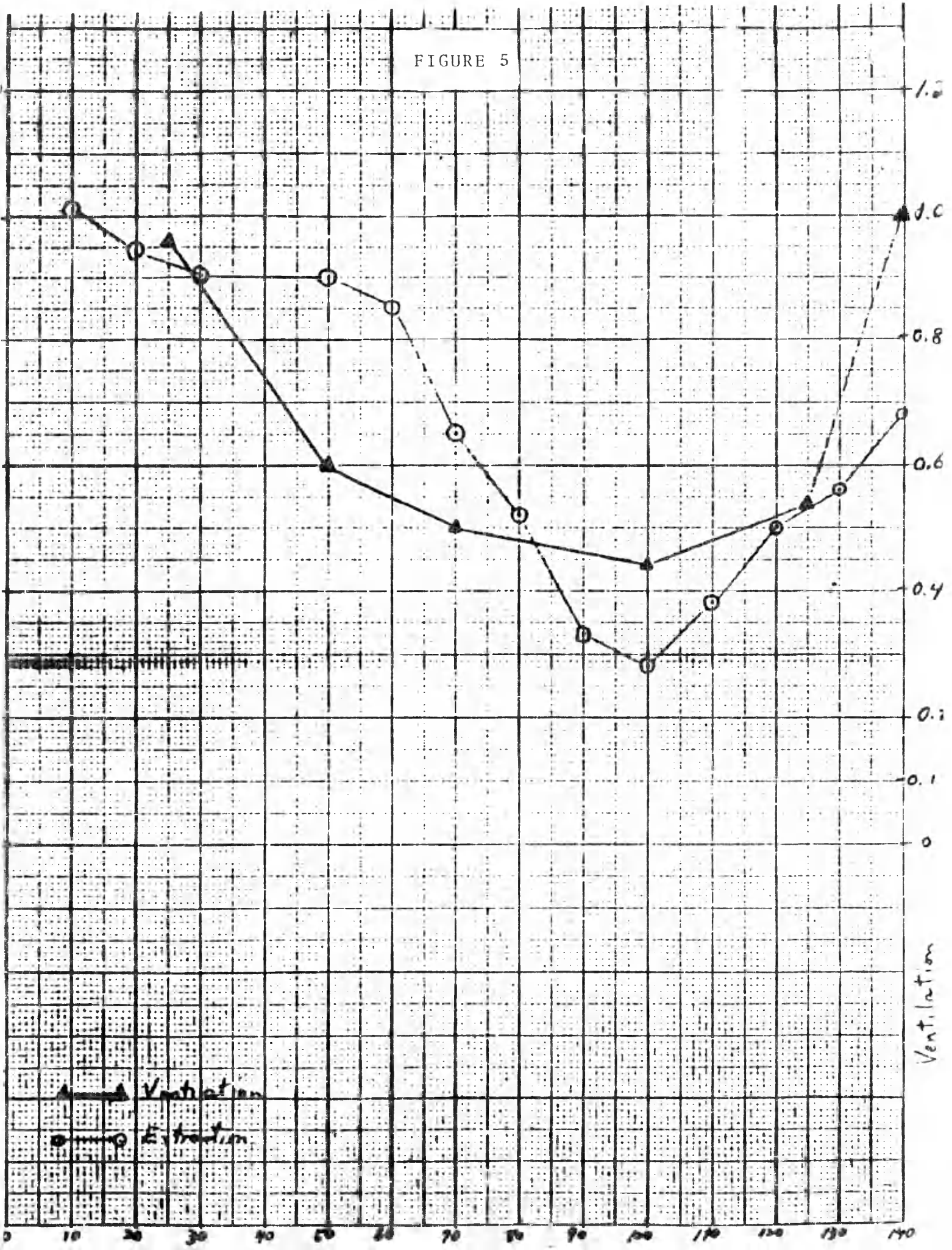
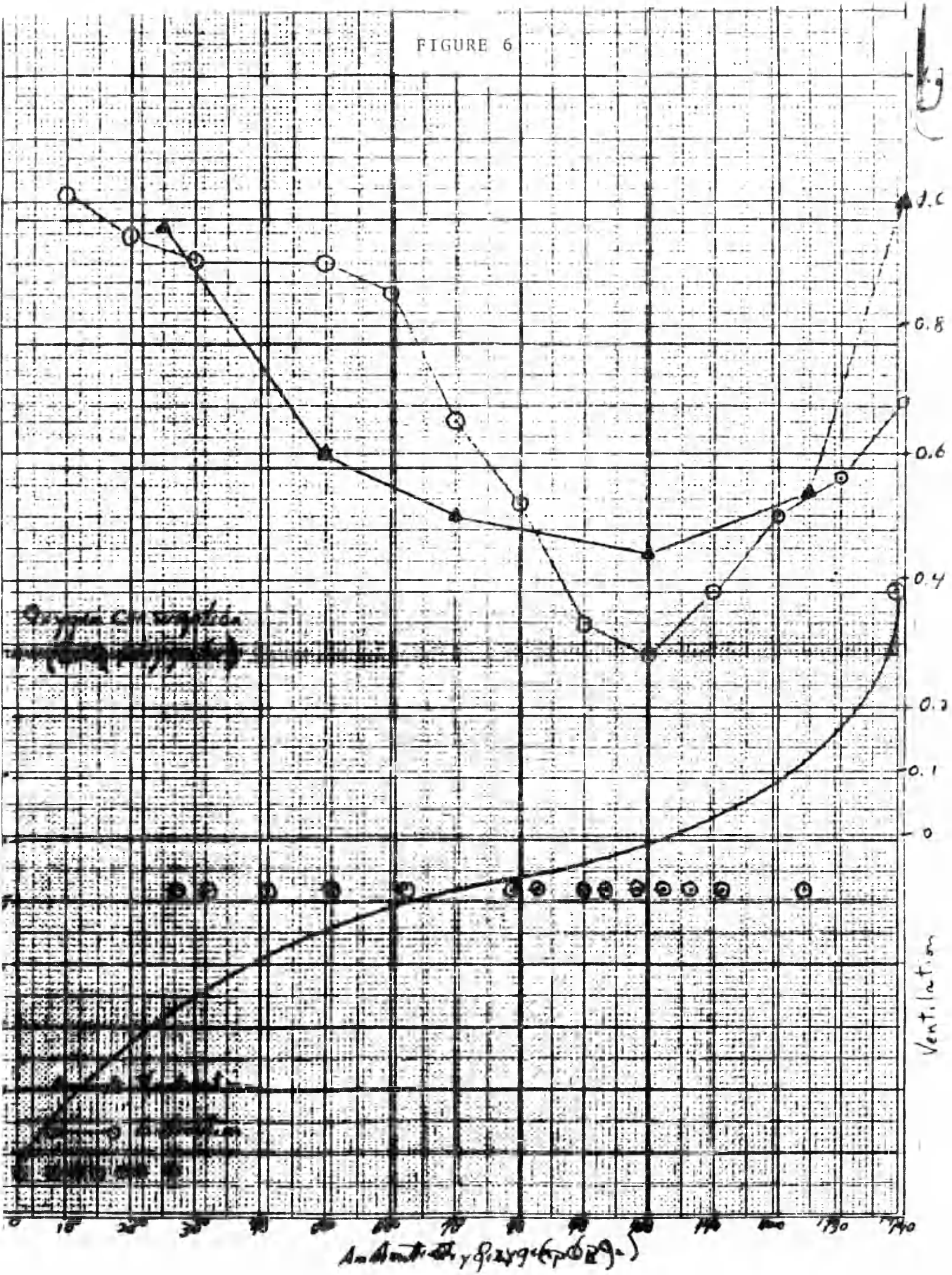

1.0
0.8
0.6
0.4
0.2
0.1
0
Ventilation
Inhalation
Exhalation
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140

Figure 6. This figure is produced by overlaying fig 4 and 5. It compares ventilation, oxygen extraction and comsumption in declining ambient oxygen at $24^{o}C$ and $34^{o}/_{oo}$

FIGURE 6



VITA

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