Hemerythrin Bohr shift in the sipunculid, *Themiste pyroides*

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Abstract: The sipunculid, *Themiste pyroides* is found intertidally to subtidally from Southern British Colombia to Northern Mexico. An avid burrower in rock nooks and crevices, this worm is prone to hypoxic environments, particularly during low tide. *T. pyroides* uses the oxygen-transport protein, hemerythrin to transport oxygen from the tentacles to the vascular system and into the coelom. Because hemerythrin changes to colorless when deoxygenated, the degree of saturation could be measured using spectrophotometry. This study looks at three replicates of purified *T. pyroides* hemerythrocytes and the degree of saturation with pH, to assess the possibility of a Bohr shift. Conclusively, no Bohr shift can be identified at this time due to the variation in the number of cells. A study looking at absorbency of multiple hemerythrocyte concentrations would serve worthwhile to eliminate this variation. Also, future studies could be done on assessing the presence of a Root effect.

Keywords: Themiste pyroides, hemerythrin, Bohr shift, Root effect

INTRODUCTION

Themiste pyroides and its environment:

The sipunculid *Themiste pyroides* is found intertidally to subtidally from Southern British Columbia to Northern Mexico (Lamb 2005). Their abundance in crevices (e.g. boring clam burrows, etc.) and between tight-fitting rocks showcases their ability to burrow, conforming their body shape to their surroundings through the use of strong lateral and circular muscles (Kozloff 1993). When burrowing to deeper layers of sand and mud the supply of oxygen can get very low, as temporary layers of sand and pebbles subsequently prevent aerated water from reaching the animal. Also, during low tide *T. pyroides* may be cut off from a supply of fresh oxygenated water due to their sessile nature (Newell 1973, Cutler 1994). How is *T. pyroides* able to increase efficiency of oxygen uptake and usage during hypoxia? This question forms the basis for my study of oxygen usage in *T. pyroides*.

Respiration and hemerythrin function in *T. pyroides:*

T. pyroides respires mainly by extending an introvert with tentacles into aerated water. Oxygen diffuses through the tentacles, passes into the vascular system, and finally reaches the coelom (Mangum & Kondon 1975, Wells 1982). Along this route, oxygen transport occurs via an iron-containing protein known as hemerythrin. Hemerythrin is present as a cellular constituent in the coelomic fluid, vascular system, and muscles of all sipunculids (Edmonds 1976, Rajulu 1976, Terwilliger et al. 1985, Long et al. 1992). The iron active site of deoxygenated hemerythrin is ferrous, but when oxygenated the iron is oxidized to a ferric state. This simple oxidation reaction allows for a colorimetric change (i.e. deep violet-pink when completely oxygenated and colorless when deoxygenated)

that is visible to the naked eye and that has different spectral properties (Klotz et al. 1957, Garbett et al. 1969, Joshi & Sullivan 1973, Klippenstein 1980).

Oxygen affinity in hemerythrin and the Bohr shift:

The degree of oxygenation partial pressure can also be termed, the degree of affinity. The affinity of hemerythrin for oxygen can be affected by multiple physiological characteristics such as oxygen tension and pH. Hemerythrin's oxygen consumption decreases as oxygen tension in the surrounding water falls, suggesting that the pigment has a maximum efficiency at a maximum oxygen tension (Peebles & Fox 1933, Edmonds 1976). Manwell (1960) showed different oxygen affinities in coelomic and vascular hemerythrin. These differences in affinity help transport oxygen from the environment to the vascular system and into the coelom. Because *T. pyroides* encounters hypoxic environments for short periods of time, some hypothesize that pH plays an important role in the transport of oxygen due to its ability to alter affinity (Wells et al. 1982, McMahon 1988).

When blood becomes more acidic, the result is a decreased affinity for O_2 due to a subsequent decrease in pH. The Bohr shift describes this scenario by suggesting that when environmental pH drops, a particular protein (in this case, hemerythrin) requires higher pO_2 to become equally saturated as the protein at a higher pH. This means the protein will release O_2 showing a decreased affinity for oxygen at lower pH (Moyes & Schultz 2006). Based on these observations, could the different types of hemerythrin show a Bohr effect? As of 1976 no sipunculid hemerythrin had been reported showing a Bohr effect. However, in 1982, Wells and colleagues documented the occurrence of a modest Bohr effect in *Xenosiphon mundanus*. In this experiment I varied pH

environment to evaluate whether *T. pyroides* shows a Bohr shift in its coelomic hemerythrin.

MATERIALS & METHODS

Organism collection and set-up:

Themiste pyroides were collected from Middle Cove, Cape Arago near Charleston, Oregon. The sipunculids were placed in a screened, lidded plastic container in fresh running seawater and were allowed to acclimate for five weeks before experimentation.

Coelomic hemerythrocyte extraction and preparation:

All materials involved (e.g. microcentrifuge tubes, needles, syringes, etc.) during the extraction of hemerythrocyte samples were kept cold on ice. For the following procedure, 3 replicates were completed using similarly sized worms. A 25-gage needle was used to extract 0.2 mL of coelomic hemerythrocyte from the posterior end of the trunk of an individual *Themiste pyroides*. The needle was inserted only a couple millimeters into the coelomic cavity of the worm to prevent interaction with gonad and the ventral nerve cord. In a microcentrifuge tube, 200 _L hemerythrocyte sample was extruded into 900 _L chilled Lou's anticoagulant buffer (HAC). This was repeated a second time in a separate microcentrifuge tube to allow for enough volume during pH manipulation. Each sample was vortexed and 20 _L was aliquoted into a microcentrifuge tube for counting cells. The remaining hemerythrocyte samples were centrifuged for 5 minutes at 16,000 rpm. The supernatants were removed and placed into microcentrifuge tubes, and the hemerythrocyte samples were resuspended using 900 _L HAC.

Hemerythrocyte samples were centrifuged again for 5 minutes at 16,000 rpm, after which supernatants were tossed and hemerythrocytes were resuspended with 900 _L HAC.

Determination of maximum absorbency and deoxy-oxyhemerythrin characterization:

A hemerythrocyte sample was taken from one worm and two samples were made: initial concentration and 50% concentration. An absorbance spectrum was made using a spectrophotometer from 320-790 nm. The maximum absorbency (440nm) was determined by the highest absorbance reading for each concentration (Fig 1-2).

In a 3 mL cuvette, 895 _L hemerythrocyte sample were extruded and absorbance readings were taken at 440 nm using a schimadzu spectrophotometer. A plastic loading tip was mounted on an N_2 cylinder and the cylinder was turned on slightly above 0.0 mmHg. The tip was inserted into the parafilm-covered cuvette until the solution was colorless, indicating deoxygenation of the hemerythrocyte. Immediately after deoxygenation, an absorbance reading was taken. Three replicates were completed to derive average absorbency of oxygenated and deoxygenated hemerythrocytes. These values served as high and low ends, respectively, of absorbance during pH analysis.

Spectrophotometer assay of pH manipulated hemerythrocytes:

In a 3 mL cuvette, 795 _L chilled phosphate buffer (pH 4.7) was combined with 200 _L chilled hemerythrocyte sample. A 5 mm stir bar was placed in the cuvette, and the solution was mixed for 10 seconds on a stir plate. The sample was scanned at 440nm, and the absorbency was recorded for time zero. Absorbance values were taken at 5 and 8 minutes with continuous stirring between readings. This procedure was repeated for the same hemerythrocyte sample with eight other phosphate buffers (pH 5.3, 5.9, 6.2, 6.7, 7.4, 7.8, 8.4, and 9.0) and was replicated for each hemerythrocyte sample from the other

two worms. Control absorbance readings of each phosphate buffer and HAC were taken using the same method. Hemerythrocytes were counted for each worm using a hemocytometer.

RESULTS

Peak absorbance at 440 nm of initial hemerythrocyte and 50% hemerythrocyte samples were 0.40 and 0.20 respectively (Fig 1-2). During pH trials, the ranges in absorbance were 0.12-0.22, 0.16-0.21, and 0.17-0.23 for worms 1-3 respectively (Fig 3-5). *T. pyroides* 1 showed a peak absorbance at pH 5.9 and 8.4 with a minimal absorbance at pH 5.3 and 9.0 (Fig 3). *T. pyroides* 2 showed a peak absorbance at pH 6.7 and 8.4 with a minimal absorbance at pH 7.4 (Fig 4). *T. pyroides* 3 showed a peak absorbance at pH 5.9 and 8.4 with a minimal absorbance at pH 9.0 (Fig 5). Average *T. pyroides* hemolymph absorbance peaked at pH 5.9 and 8.4 with a minimal absorbance at pH 7.4 and 9.0 (Fig 4). Fully oxygenated hemolymph had an absorbance of 0.23 while deoxygenated hemolymph had an absorbance of 0.07. The concentration of cells in each worm was 7.14×10^6, 1.28×10^7, and 1.18×10^7 cells/mL for worms 1-3 respectively.

DISCUSSION

I hypothesized that *Themiste pyroides* hemerythrin would show a Bohr shift, expressed as a reduced affinity in a lower pH environment (Moyes & Schutlz 2006). However, this study poses a predicament, in which I need more data collection in order to carefully accept or refute this hypothesis. I can, however, discuss my results in the context of how they would assist future research on this topic. I can also speculate as to the idea of *Themiste pyroides* having a Root effect in their hemerythrin.

Absorbance values are largely dependent on the number of cells binding oxygen in the sample. With each oxygen molecule that hemerythrin binds, a resultant color change occurs (Klotz et al. 1957). The intraspecific variation in absorbance in this study may be attributable to differences in the number of cells. Worm 1 had a value of cells/ml about half that found in the other two worms. The high saturation of cells could explain the fairly stable absorbance values in worms 2 and 3 (Fig 4-6) and the observed sensitivity in absorbance for worm 1 across pH treatments (Fig 3). In future studies I would suggest gaining a set of absorbance values over a range of hemerythrocyte concentrations. This would include a pilot study to determine the most appropriate concentration of cells that will obtain an absorbance range that allows maximum variation. Once this number of cells is determined, all manipulated samples would need to fall within this range. Without this control, the variation in my data could be due either to pH or to the inconsistent number of cells and their absorbance properties.

A common minimal absorbance at pH 9.0 and peak absorbency within seawater pH (i.e. 7.8-8.0) for all three worms indicate a curiosity worth exploring in future experiments (Fig 3-5) (Burnett et al. 2002). Although the difference in number of cells creates a potential source of variation, this trend was true across all three worms. It would be interesting to see if these trends repeated themselves upon making constant the cell concentrations. Furthermore, instead of assessing whether there is a Bohr effect it would be more appropriate to assess the possibility of a Root effect. Incidentally, the methods of this experiment were organized in a way to look at a Root effect not a Bohr shift. The Root effect is a subsequent reduction in carrying capacity due to a reduction in

pH (Moyes & Schultz 2006). Because I never measured pO_2 directly I would have never been able to say that the changes were changes in affinity; instead, they are changes in O_2 saturation in the hemerythrin molecules.

There are several areas of error in this study. Deoxygenated hemolymph absorbance values would be even closer to 0 if they were truly deoxygenated. Using the N_2 gas wasn't enough to change hemerythrin to completely colorless, as a faint pink was noticed after 20 minutes. This bit of error should be expected when using this technique. Since absorbance is dependent on the amount of cells in solution, error when pipetting could play a tremendous role. This can change the concentration and subsequently could account for some of the variation in my data. Given this source of error, it would be interesting to look at whether there is a Bohr shift in vascular hemerythrin. Because an affinity difference exists between vascular and coelomic hemerythrin (Manwell 1960), life in hypoxic environments might increase the likelihood that a Bohr shift plays a role in vascular hemerythrin, where a majority of the oxygen uptake from the environment occurs (Kozloff 1993).

LITERATURE CITED

Burnett, L; Terwilliger, N; Carroll, A; Jorgensen, D and D Scholnick (2002). Respiratory and Acid-Base Physiology of the Purple Sea urchin, *Strongylocentrotus purpuratus*, During Air Exposure: Presence and Function of a Facultative Lung. Biological Bulletin **203**: 42-50.

Cutler, EB (1994) The Sipuncula: their systematics, biology, and evolution. Cornell University Press: Ithaca, New York.

Edmonds, SJ (1976) Respiration in sipunculans. In: Proceeding of the international symposium on the biology of the sipuncula and echiura II – physiology and biochemistry. p. 3-9.

Garbett, K; Darnall, DW; Klotz, IM and RJP Williams. (1969) Spectroscopy and Structure of Hemerythrin. Archives of Biochemistry and Biophysics. **103**: 419-434.

Joshi, JG and B Sullivan (1973). Isolation and preliminary characterization of Hemerythrin from *Lingula unguis*. Comp. Biochem. Physiol. **44B**: 857-867.

Klippenstein, GL; Van Riper, DA and EA Oosterom. (1972) A Comparative Study of the Oxygen Transport Proteins of *Dendrostomum pyroides*. The Journal of Biological Chemistry. **247**(18): 5959-5963.

Klotz, IM; Klotz, TA and HA Fiess. (1957) The Nature of the active Site of Hemerythrin. Archives of Biochemistry and Biophysics. **60**: 284-299.

Kozloff, Eugene N. (1993). Seashore Life of the Northern Pacific Coast: An Illustrated Guide to Northern California, Oregon, Washington, and British Columbia. University of Washington Press: Seattle, WA.

Lamb, A (2005). Marine Life of the Pacific Northwest: A Photographic Encyclopedia of Invertebrates, Seaweeds and Selected Fishes. Harbour Publishing: BC Canada.

Long, RC; Zhang, JH; Kurtz Jr., DM; Negri, A; Tedeschi, G; and F Bonomi (1992). Myohemerythrin from the sipunculid, *Phascolopsis gouldii*: purification, properties and amino acid sequence. **1122**: 136-142.

Mangum, CP and M Kondon. (1975) The Role of Coelomic Hemerythrin in the Sipunculid Worm *Phascolopis gouldi*. Comp. Biochem. Physiol. **50A**: 777-785.

z, C. (1960) Histological Specificity fo Respiratory Pigments-II. Oxygen Transfer Systems Involving Hemerythrins in Sipunculid Worms of Different Ecologies. Comp. Biochem. Physiol. **1**: 277-285.

McMahon, BR. (1988) Physiological Responses to Oxygen Depletion in Intertidal Animals. Amer. Zool. **28**: 39-53.

Moyes, CD and PM Schultze 2006. Principles of Animal Physiology. Pearson Education: Benjamin Cummings, San Francisco, CA.

Newell, RC (1973). Factors Affecting the Respiration of Intertidal Invertebrates. Amer. Zool. **13**: 513-528.

Peebles, F and DL Fox (1933). The structure, function and general reactions of the marine sipunculid worm, *Dendrostoma zostericola*. Bull. Scripp. Inst. Oceanog. **3**(9): 201-224.

Rajulu, GS (1976) A Biochemical study of the coelomic fluid of two sipunculan worms *Sipunculus robustus* and *Themiste signifer*. In: Proceeding of the international

symposium on the biology of the sipuncula and echiura II – physiology and biochemistry. p. 39-49.

Terwilliger, NB; Terwilliger, RC; and E. Schabtach (1985). Chapt. 7: Intracellular Respiratory Proteins of Sipuncula, Echiura, and Annelida. Blood Cells of Marine Invertebrates: Experimental Systems in Cell Biology and Comparative Physiology **6**: 193-225.

Wells, RMG. (1982) Respiratory characteristics of the blood pigments of three worms from an intertidal mudflat. New Zealand Journal of Zoology. **9**: 243-248.

APPENDIX

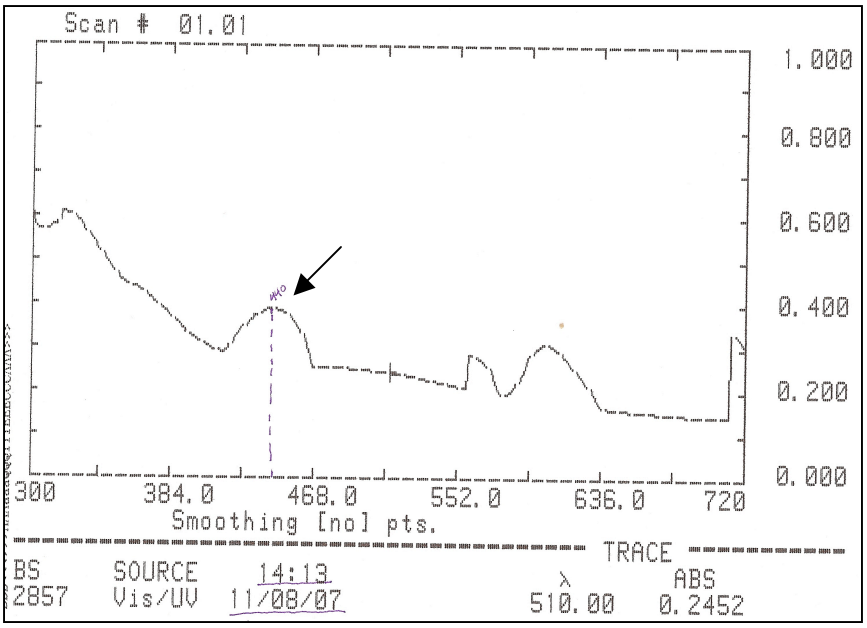

Figure 1: Absorbance spectrum of initial hemolymph concentration. Maximum absorbance is indicated by an arrow at 440 nm.

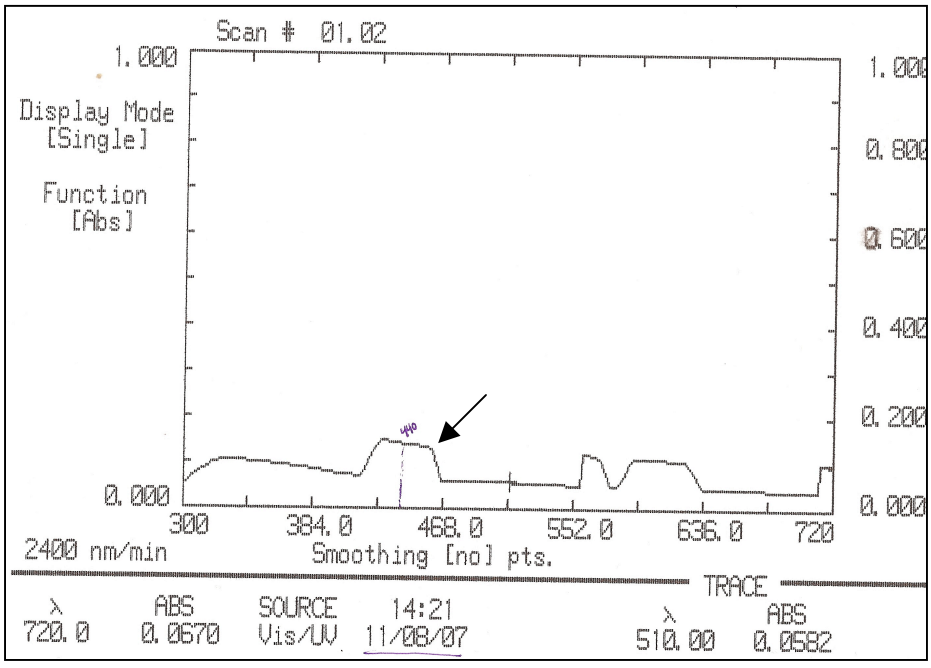

Figure 2: Absorbance spectrum of 50% hemolymph concentration. Maximum absorbance is indicated by an arrow at 440 nm.

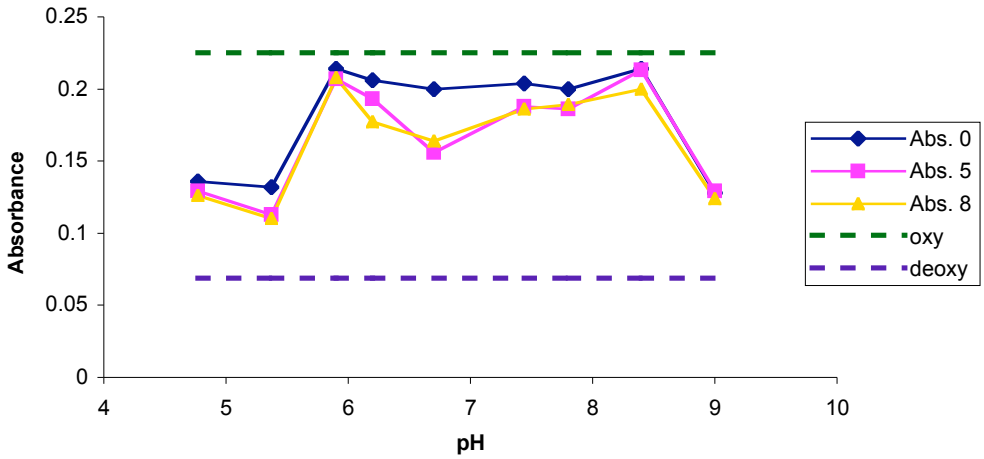

Figure 3: *Themiste pyroides* #1, absorbance of hemerythrocytes when manipulated in nine different pH solutions at three different time points (0, 5, and 8 minutes). 'Oxy' represents absorbance of fully oxygenated hemolymph while 'deoxy' represents absorbance of deoxygenated hemolymph.

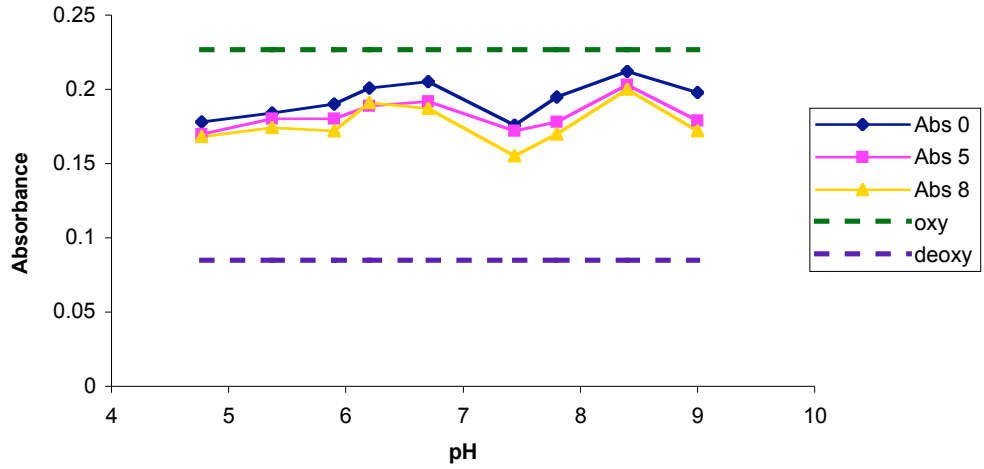

Figure 4: *Themiste pyroides* #2, absorbance of hemerythrocytes when manipulated in nine different pH solutions at three different time points (0, 5, and 8 minutes). 'Oxy' represents absorbance of fully oxygenated hemolymph while 'deoxy' represents absorbance of deoxygenated hemolymph.

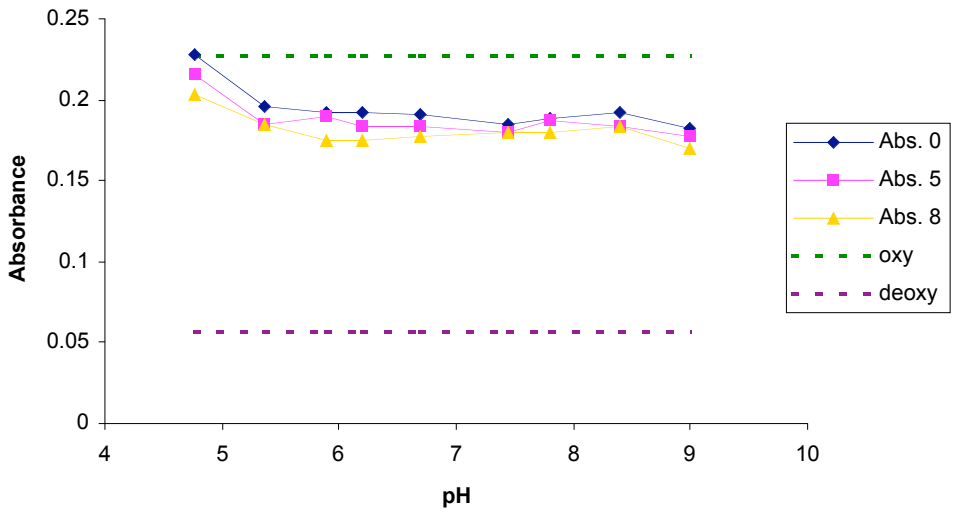

Figure 5: *Themiste pyroides* #3, absorbance of hemerythrocytes when manipulated in nine different pH solutions at three different time points (0, 5, and 8 minutes). 'Oxy' represents absorbance of fully oxygenated hemolymph while 'deoxy' represents absorbance of deoxygenated hemolymph.

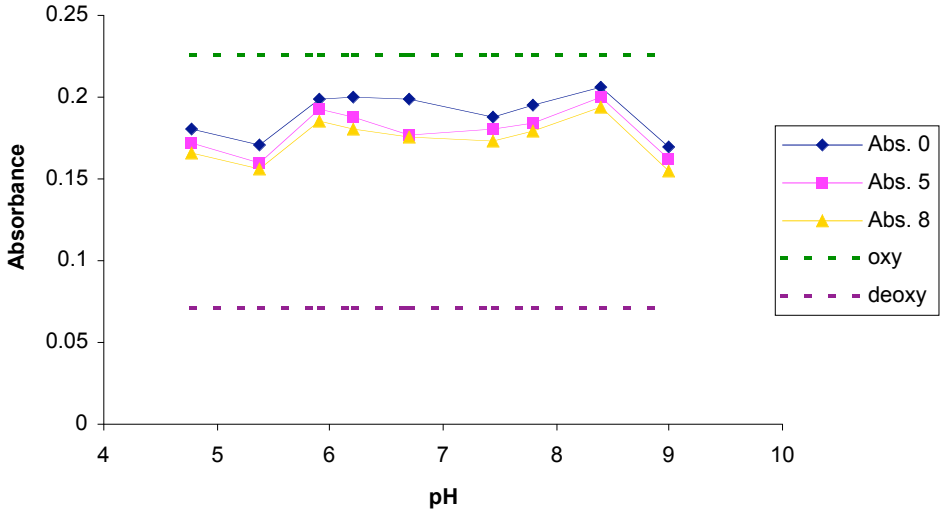

Figure 6: Average absorbance (for all three worms) of hemerythrocytes when manipulated in nine different pH solutions at three different time points (0, 5, and 8 minutes). 'Oxy' represents average absorbance of fully oxygenated hemolymph while 'deoxy' represents average absorbance of deoxygenated hemolymph.

Megan Copley – MMP Final Project Protocol – Fall 2007

Question: Does the hemerythrin of the sipunculid, *Themiste pyroides*, have a Bohr shift?

1. Preparation of coelomic hemerythrin samples: This portion is intended to prepare hemerythrin samples for spectrophotometric analysis. Be careful in pipetting as equal concentrations between samples are a key importance.

Keep everything on ice or at 5ºC!

- Chill a 1ml syringe and 25G needle.
- Chill dated and labeled microcentrifuge tubes, five for each worm:
    - Hemerythrocyte stock
    - Hemerythrocytes 1 (for pH manipulations)
    - Hemerythrocytes 2 (for pH manipulations)
    - Supernatant – negative control to ensure good hemerythrocyte collection
    - Cell count

*NOTE: There are two 'hemerythrocyte' microcentrifuge tubes to allow for enough volume when doing the pH manipulations.

- Add 900 _L chilled Lou's anticoagulant (HAC) to each 'hemerythrocyte' tube (1 & 2). Keep them on ice.
- Draw 0.2 mL of hemolymph from the posterior tip of *Themiste pyroides'* trunk using a chilled syringe, and extrude immediately into the chilled 'hemerythrocyte stock' tube.
- Pipette 200 _L of hemolymph from the 'hemerythrocyte stock' tube and extrude into 'hemerythrocyte' tube 1. Repeat with 'hemerythrocyte' tube 2.
- Close tubes, vortex, put back on ice.
- Aliquot 20 _L of each sample into 2nd chilled tube, 'cell count'.
- Centrifuge hemerythrocyte samples (980 _L) 5min, 1,600 rpm. Keep centrifuge cold with a bag of ice.
- Save supernatant to 3rd chilled tube, 'supernatant', without disturbing pellet.
- Add 900 _L HAC to 'hemerythrocyte' tube with pellet and resuspend cells.
- Centrifuge samples 5 min, 1,600 rpm
- Toss supernatant, save pellets.
- Resuspend pellet in 900 _L cold HAC.
- Keep samples chilled on ice!

*Repeat this procedure for as many worms as you want to test. I had 3 worms.
*You will want to have a separate purified sample for the oxy-deoxy background value. This can be done following the same procedure listed above, but you only need one microcentrifuge of sample instead of two.

2. Spectrophotometric creation of an oxy-deoxy background value:
This portion is intended to create a standard background absorbance value that will allow for a qualitative comparison of hemerythrin O_2 binding properties at different pH treatments.

- Set schimadzu spectrophotmeter to 440 nm.
- Auto Zero (no cuvette).
- In cuvette pipette 995 _L oxy-hemerythrocyte sample.
- Place cuvette in spectrophotometer, record the absorbance.
- Cover cuvette with parafilm (TIGHTLY) and poke a small hole for the N_2 tip to fit in.
- On the tip of the N_2 cylinder place a plastic loading gel tip (if it doesn't fit snug, use a bit of parafilm.
- Set N_2 cylinder to 0.0 mmHg. The pressure needs to be just right so that the solution doesn't bubble out, but that it has a steady input of N_2.
- Bubble N_2 into the sample until colorless.
- Place cuvette in spectrophotometer (with parafilm cover) and record an absorbance. This value represents the deoxy-hemerythrocyte absorbance value.

3. Spectrophotometric study of absorbency at different pH:
SAMPLES: (3 Worms, 9 pH levels, HAC and phosphate buffer controls, supernatant controls)

- Set schimadzu spectrophotometer to 440nm
- Auto Zero (no cuvette)
- In cuvette, mix
  - 795 _L Phosphate buffer (pH 4.8, 5.3, 5.9, 6.2, 6.7, 7.4, 7.8, 8.4, or 9.0)
  - 200 _L hemerythrocyte sample
- Scan sample.
- Record absorbency value.
- Place a 5 mm stir bar into the cuvette and place on a stir plate for 5 minutes.
- Remove stir bar and scan sample. Record value.
- Place 5 mm stir bar into the cuvette and place on stir plate for 3 minutes.
- Remove stir bar and scan sample. Record value.
- Repeat until all 9 phosphate buffers have been read for all hemerythrocyte replicates.

*The stirring of the sample is essential, as the hemocytes like to settle at the bottom of the cuvette so they need resuspension.

CONTROLS:

- In cuvette, mix
  - 795 _L Phosphate buffer (pH 4.8, 5.3, 5.9, 6.2, 6.7, 7.4, 7.8, 8.4, or 9.0)
  - 100 _L HAC buffer of 'supernatant' sample
- Scan sample.
- Record values

*These controls should register with almost 0.0 absorbance.

4. Hemocytometer counts of blood cells:

- Place special coverslip on the hemocytometer.
- Pipette 10 _L of your GENTLY agitate 'cell count' sample
- Touch the tip of the pipette to the junction of the coverslip and the hemocytometer, where there is a groove.
- Gently squeeze out the suspension, allowing the liquid to run under the coverslip by capillary action. Be sure you have not flooded the hemocytometer, as that will change the depth of liquid between the coverslip and the hemocytometer.
- Place hemocytometer on the stage.
- Turn on the microscope light.
- Count a grid.
- Calculate the number of cells.
- Repeat for all cell count samples.

5. Determination of maximum absorbency: using a prepared sample, following the outline in the first portion of this procedure, you can make a 100% and 50% concentrated sample of hemerythrocytes and scan from 350-720 nm to see where the peak absorbance is. This is how the 440 nm value was determined for the phosphate and oxy-deoxy absorbance determinations.

List of Animals:

- fresh *Themiste pyroides* worms

List of Materials:

- 1mL syringes with 25G needles, chilled on ice
- Gloves
- Microcentrifuge tubes, chilled on ice
- Ice bucket with ice
- Ice-chilled microcentrifuge
- Spectrophotometer with computer, paper and working printer
- Plastic spectrophotometer 3.0 mL cuvettes
- Parafilm
- 10 _L pipettor
- 1 mL pipettor
- A data sheet for recording.
- Hemocytometer
- Compound Microscope
- Plastic tip for N_2 cylinder.
- 5 mm stir bar
- Stir bar remover stick.
- Stir plate

List of Chemicals:

- N_2 (enough for 3 trials of prepared hemerythrocytes).
- Lou's Anticoagulant Buffer (HAC)
- Stock Phosphate Buffers (Monobasic, Dibasic)
- Working Phosphate Buffers (pH 4.7, 5.3, 5.9, 6.2, 6.7, 7.0, 7.5, 7.8, 8.4, 9.0). Enough for 3 samples and 3 controls.

*See attached handout-for how to mix these chemicals.

Literature Cited:

Garbett, K; Darnall, DW; Klotz, IM and RJP Williams. (1969) Spectroscopy and Structure of Hemerythrin. Archives of Biochemistry and Biophysics. **103**: 419-434.

Klippenstein, GL; Van Riper, DA and EA Oosterom. (1972) A Comparative Study of the Oxygen Transport Proteins of *Dendrostomum pyroides*. The Journal of Biological Chemistry. **247**(18): 5959-5963.

Klotz, IM and TA Klotz. (1955) Oxygen-Carrying Proteins: a Comparison of the Oxygenation Reaction in Hemocyanin and Hemerythrin with That in Hemoglobin. Science. **121**(3145): 477-480.

Klotz, IM; Klotz, TA and HA Fiess. (1957) The Nature of the active Site of Hemerythrin. Archives of Biochemistry and Biophysics. **60**: 284-299.

McMahon, BR. (1988) Physiological Responses to Oxygen Depletion in Intertidal Animals. Amer. Zool. **28**: 39-53.

Mangum, CP and M Kondon. (1975) The Role of Coelomic Hemerythrin in the Sipunculid Worm *Phascolopis gouldi*. Comp. Biochem. Physiol. **50A**: 777-785.

Manwell, C. (1960) Histological Specificity fo Respiratory Pigments-II. Oxygen Transfer Systems Involving Hemerythrins in Sipunculid Worms of Different Ecologies. Comp. Biochem. Physiol. **1**: 277-285.

Wells, RMG. (1982) Respiratory characteristics of the blood pigments of three worms from an intertidal mudflat. New Zealand Journal of Zoology. **9**: 243-248.