THE EFFECT OF pH ON OXYGEN BINDING BY HEMOCYANIN

BACKGROUND READING
Animal Physiology by Hill, Wyse & Anderson, 2004: pp. 577–593; Fig. 23.22.

PRE-LAB
(Due at the start of lab)

** In your lab notebook, draw a flow chart of the basic procedure for the experimental procedure you will be using in today’s lab, including preparing the hemocyanin solution, deoxygenating the solution, and reoxygenating the solution. Make sure that your flow chart includes what needs to happen before you go from deoxygenating to reoxygenating and what needs to happen before you finally open the tonometer to room air.

** On a separate piece of paper to be handed in, please answer the following questions. These must be word-processed

1. What are three ways in which arthropod hemocyanin differs from vertebrate hemoglobin?

2. Would you expect crab blood to exhibit a sigmoidal equilibrium curve? Why or why not?

3. What is p50? What are the units of p50? What is p50 a measure of?

4. When comparing your results to literature values, you discover that the p50 that you measured for Dungeness crab hemocyanin was, on average, twice as high as that reported in the literature. What are two biologically-based plausible reasons for this difference? Cite where in your book (page number) or in your lecture notes (date of lecture) you obtained this information.

ANIMALS AND EQUIPMENT

Living material
Crabs of the genus Cancer.

Equipment
Syringe and needles; centrifuge tubes; ultracentrifuge; hand homogenizer; flasks; crustacean ringers; 1N HCl & 5N NaOH; pH meter; glass pipets; tonometers; N2 gas; spectrophotometer; cuvettes; ice; light shields.

STANDARD PROCEDURE

Preparation of Hemocyanin (one preparation for each lab)
1. Extract crab blood by poking a syringe needle into the blood sinus at the base of the legs and slowly drawing up 6-12 ml of blood. *Don't keep the blood in the syringe for very long because it will start to coagulate*

2. Quickly empty the blood into a plastic centrifuge tube and let it develop a clot. This will take 5-7 minutes.
3. Gently break up the clot with a hand homogenizer if necessary, then centrifuge the tube at 15,000g for 25 minutes to separate the fluid from the clot. For some hemocyanin, filtering through cheesecloth is a better method. You should wind up with over 50% of your original volume.

**While the blood is centrifuging ...**

*Preparation of Buffers*

Make up the pH solutions with which you will later dilute the plasma fraction. Start with about 40 ml of crustacean ringers. Check that the pH of this solution is approximately 7.5 (instructions for using the pH meter will be given in lab). Divide the solution in half and add 1N HCl or 5N NaOH to one of the aliquots to adjust the pH to either 7.2 or 8.0, respectively. Use a pH meter to check the pH as you add acid or base. Ringers is an effective buffer, so don't be surprised if you need to add a fair number of drops to alter the pH; but be careful, because the pH can change very quickly.

*Preparation of Hemocyanin Solutions*

The goal here is to prepare a dilution of the hemolymph. The first dilution will be at a pH of about 7.5 while the second one (later in the lab) will be either acidic or basic.

- Obtain one tonometer and add 0.25 ml* of the prepared hemocyanin to each.
- Add 4.75 ml* of buffer at the desired pH.

* These volumes are approximate and may change depending on the species and condition of the animal

*Generation to O2 Binding Curves*

1. Close off the top of the tonometer flask by carefully wedging a spectrophotometer cuvette into the mouth of the flask with a rubber tubing gasket to hold it snugly in place (see diagram below).

2. Close the stopcocks on the flask. **Be careful to hold the stem of the stopcock while you turn the stopcock to avoid popping the stopcock off the flask.** The tonometer (flask + tube) should be air-tight and water-tight so that you can turn it upside down and no fluid leaks out. Check for air leaks (seen as bubbles) from the valves as well. If there are leaks, obtain another tonometer.
3. Set the wavelength on the spectrophotometer to 335 nm. Zero the spectrophotometer with a cuvette containing the pH-adjusted Ringers.

4. Measure the absorbance of the hemocyanin solutions within the tonometer. Since the tonometer will prevent you from closing the cuvette chamber, cover the chamber with the light shield. This is the absorbance of the hemocyanin pigment when fully oxygenated. Ideally it will be around 0.3-0.4, which is a reflection of the concentration of hemocyanin in the solution. If the initial absorbance is too low, add more blood in 0.1 ml increments (record how much more blood you add because the final volume of the fluid may be important in later calculations). If the initial absorbance is too high, add more buffer.

**DEOXYGENATION**

To completely de-oxygenate the hemocyanin, we will bubble nitrogen gas (N\textsubscript{2}) over the hemocyanin solutions. This process is a bit touchy because we want to remove all the O\textsubscript{2} but it’s possible that too much N\textsubscript{2} will permanently alter the O\textsubscript{2} binding curve. To avoid this potential complication, we have come up with the procedure below.

1. Open both valves of the tonometer.
2. Open the nitrogen tank flow valve as instructed by the prof.
3. Connect the nitrogen tank hose to one valve of the tonometer for about 2 minutes.
4. **CLOSE THE NITROGEN VALVE.**
5. Close the tonometer inlet valve. Remove the nitrogen hose from the tonometer inlet valve first; wait until no hissing can be heard, then close the other valve.
6. Rotate the tonometer so that the hemocyanin solution is spread as a thin film around the bulb. (Rotate for 2-3 minutes and do not warm the hemocyanin with your hands; recall the temperature effect. Minimize contact with the tonometer during these manipulations).
7. Insert the wiped cuvette end of the tonometer into the spectrophotometer and read the optical density of the sample. (Do not adjust any settings; simply read the optical density scale.). It is always good practice to repeat absorbance measurements to obtain duplicate or triplicate readings of the absorbance.
8. Record the absorbance value obtained in your notebook and then REPEAT the flush-rotate-read sequence until two consecutive readings are identical or very similar. It may take 6 or more repeats. What does it indicate when two consecutive readings are identical?

**OXYGENATION**

To generate an O\textsubscript{2} binding curve (= O\textsubscript{2} dissociation curve) for the hemocyanin, we will add back small amounts of normal air using a syringe, and measure the optical density after allowing time for the uptake of oxygen by the hemocyanin. Our goal is to bring the oxygen level back up to the original level. The procedure is as follows:

1. Open the intake and exhaust valves. Using the syringe provided, inject 0.5 - 2 ml of air into the tonometer. Close both valves after injection. The amount of air added will depend on how readily the Hc binds oxygen.
2. Rotate the tonometer for 5 minutes and read the optical density in duplicate.
3. Repeat steps 1-2 until the absorbance reading matches the fully oxygenated reading. This should be reached in about 10 readings but the exact amount will vary depending on the amount of hemocyanin in the tonometer. If you reach it in the first or second reading, either the valve was left open or there was a leak. If this is the case, you will need to get a new tonometer and repeat the experiment.

4. When you think that you have reached complete saturation, remove the cuvette from the tonometer and pass the hemocyanin solution back and forth to ensure complete re-equilibration with air. Determine the final optical density. Use this value as the fully oxygenated value in your calculations below.

**Data Analysis**

To compare your data with literature experiments, you'll need to manipulate your data such that you can graph % saturation versus pO2. Recall that the data you have now would only allow you to graph absorbance versus ml air, making it difficult to compare your data with that of your classmates and impossible to compare it with literature values. Below we describe how to perform these conversions.

The partial pressure of oxygen in the tonometer is a function of the total amount of air in the tonometer (vol. air), the volume of the tonometer, the volume of hemocyanin used, the barometric pressure, and the percentage of oxygen in the air (21%). Don’t forget to subtract out the water vapor in the air before calculating the partial pressure of oxygen.

- \( \text{PO}_2 = \frac{(\text{total amount of air added})*(0.2094 \times [\text{barometric pressure-pH2O}])}{(\text{volume of tonometer - volume of fluid within tonometer})} \)

The % saturation for a given volume of air is the fraction of the range of optical density values encompassed by the distance from the deoxygenated optical density to the optical density at full saturation. The total range of optical density value is the difference between the optical density for deoxygenated hemocyanin and the optical density for hemocyanin equilibrated with air. The portion of the range filled with oxygen is then the difference between the optical density for deoxygenated hemocyanin and the optical density measured for the particular volume of air. Use the final absorbance value, after opening the tonometer to air, for the fully oxygenated value.

- \( \% \text{ sat.} = \frac{[(\text{absorb. of deoxygenated hemocyanin}) - \text{absorb. after partial re-oxygenation})]}{[(\text{absorb. of deoxygenated hemocyanin}) - \text{absorb. of fully oxygenated hemocyanin})]} \times 100 \)

Calculate the PO2 and % saturation for each volume of air and prepare a plot of % saturation vs. PO2. Determine the P50 for the hemocyanin solution used.

- Repeat the procedure on the other sample at the different pH. Make sure that the sample volumes that are degassed and the length of degassing are the same for all samples because this will facilitate any calculations you may wish to do.
POST-LAB
(Due next week in lab)

The post-lab this week is to write an abstract for the study you did.

As stated in your Writing Assignment Guidelines handout, an abstract:

- should be a single paragraph that summarizes the findings concretely. The abstract should not contain abbreviations that will be recognized only by an expert on the subject, and it should not contain citations of the literature.

Refresh your memory of the content and tone of scientific abstracts by reviewing the three abstracts you studied in the Paper Dissection lab.

The abstract you write for this post lab will be shorter than published abstracts but should otherwise be similar.

Specifically, the abstract for this post lab should:

- make sense without reading the entire manuscript.
- start with the central question or objective of the study.
- contain a brief mention of your methods. Thus, you don’t need to go into detail; focus on the important things like the method you used (you used a tonometer), the variable tested (pH … what pHs?), the species of animal used, etc.
- contain a summary of your major results. These should include both qualitative and quantitative results. The qualitative results should mention the shape of the curves and how they shifted in response to changes in pH. The quantitative results should, at the very least, state the p50s for each curve. Thus, you’ll need to analyze your data as described in this lab handout and obtain the p50 values from the curves you generate.
- contain a summary of your major conclusions.
- do all of the above in a single paragraph
- do all of the above in 75-100 words

RESEARCH PAPER

**NOTE: If you are going to write this paper up for credit, you need to incorporate data from additional lab groups so that you have at least 4 animals for each pH. This will make your analysis much more meaningful and fun. Your professor will facilitate data exchange among lab groups.**

The first step in turning this study into a research paper (or even deciding if you want to go through the trouble) is determining what your data are telling you and how to best present them. Some questions to consider when thinking about how to analyze and present these results are:

- Is it important to show raw data or should you use averages? Why or why not?
- What is the best way to show the trends that you want to emphasize? Look in your assigned reading for some effective ways to present these types of data.
- You measured absorbance as an indicator of % saturation. How will you go about converting the absorbencies to % saturation?
- You added in volumes of air during the re-oxygenation step, yet O2 data is generally presented as pO2 (see figures in your text). How do you go about making this conversion?
- How should you deal with any outliers in the data set?
- You should have enough data points to analyze your data statistically. What aspects of your data would be logical to compare? What test(s) will you use? How will you present the statistical results? To answer these questions, you need to figure out what you want to know.
Some questions to consider when thinking about how to pitch you study to readers:

- Is the hemocyanin that you examined typical of crustacean hemocyanin?
- Are the conditions to which we subjected the animal's blood physiologically relevant? If not, why have we done this experiment?

Discussions should definitely include a comparison with previous studies. In addition, consider the additional questions generated by your work on the blood of this one species of crustacean.

**RECORD DATA IN A TABLE SIMILAR TO THIS ONE** => Use data to calculate pO2 & % Sat

<table>
<thead>
<tr>
<th>Amount of air injected</th>
<th>Cumulative volume of air in tonometer</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>_____ ml</td>
<td>_____ ml</td>
<td>_____</td>
</tr>
<tr>
<td>_____ ml</td>
<td>_____ ml</td>
<td>_____</td>
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<tr>
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</tr>
<tr>
<td>_____ ml</td>
<td>_____ ml</td>
<td>_____</td>
</tr>
<tr>
<td>_____ ml</td>
<td>_____ ml*</td>
<td>_____ ʃ</td>
</tr>
</tbody>
</table>

* This last value will be the volume of the air in the tonometer because it represents the volume after you have opened the tonometer to room air.

ʃ Use this final value for the absorbance of the fully-oxygenated Hc solution.

Additional data required for calculations:

- pH of solution ________
- volume of hemocyanin ________ ml
- volume of tonometer ________ ml
- barometric pressure ________ mm Hg
SAMPLE DATA TABLE AND RESULTS
(pO2 and %Sat calculated using equations given)

<table>
<thead>
<tr>
<th>BP</th>
<th>DeOx Abs</th>
<th>Sat Abs</th>
<th>Cum. Vol Air</th>
<th>Abs. Vol tonometer</th>
<th>Vol. hemocy</th>
<th>PO2</th>
<th>% sat</th>
</tr>
</thead>
<tbody>
<tr>
<td>754.38</td>
<td>0.241</td>
<td>0.81</td>
<td>0</td>
<td>0.26</td>
<td>40</td>
<td>5</td>
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<td>40</td>
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<tr>
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<td>40*</td>
<td>0.81</td>
<td>40</td>
<td>5</td>
<td>180.53</td>
</tr>
</tbody>
</table>

* This last value is where the tonometer has been opened to room air. Thus, this is the fully saturated value where the cumulative volume of air is the same as the tonometer volume.