Exercise and anaerobic metabolism

Lactate concentrations in blue crabs (*Callinectes sapidus*)

BACKGROUND

During periods of intense activity, oxygen demand to fuel aerobic (oxygen-requiring) metabolic pathways may exceed oxygen supply via the cardio-respiratory system. At this point, tissues resort to anaerobic (non-oxygen requiring) pathways to generate ATP for cellular work. Metabolic reactions that occur in the absence of oxygen are collectively referred to as "fermentation" processes. One of the most common types of fermentation is lactic acid fermentation (Fig. 1). The first step of lactic acid fermentation is glycolysis, a metabolic pathway in which a 6-carbon molecule of glucose is broken down into two 3-carbon molecules of pyruvic acid. The pyruvic acid produced by glycolysis is further modified through a redox reaction to produce lactic acid. The latter step is catalyzed by the enzyme lactate dehydrogenase, and is necessary to regenerate NAD electron acceptors so that glycolysis can continue in the absence of oxygen.

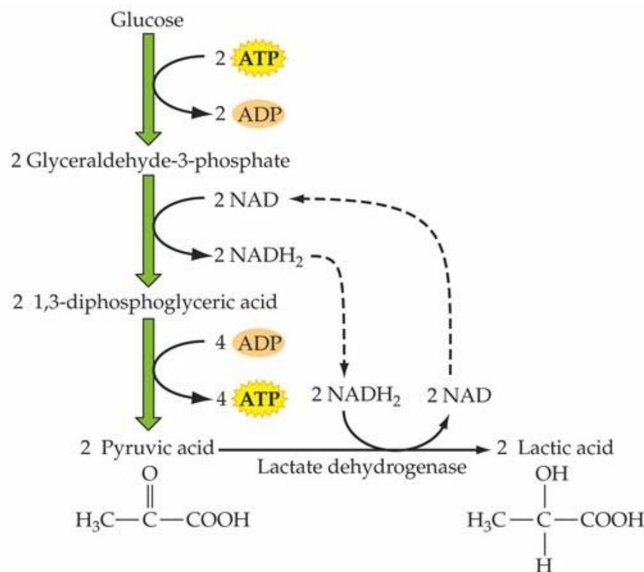


Figure 1. Lactic acid fermentation

Lactic acid (as the name suggests) is an acid, i.e. it readily donates protons to surrounding tissues and fluids. When lactic acid loses a proton, it forms a compound called lactate. Increases in lactate concentration of blood and tissues during exercise is typically associated with a decrease in pH (acidosis) and muscle discomfort, although a direct link between lactate and acidosis has not been proven. Nevertheless, lactate concentrations are a good indicator of flux through the anaerobic metabolic pathway of lactic acid fermentation, and can provide insight into the physiological status of an animal during periods of intense activity.

OBJECTIVES

1) Learn the principles of spectrophotometry

2) Use spectrophotometry to determine lactate concentrations hemolymph of blue crabs

3) Compare lactate concentrations for resting and active blue crabs

EQUIPMENT AND SUPPLIES

Solutions	*Hemolymph collection*	*Assay*
crab hemolymph (*on ice!*)	ice (in cooler)	spectrophotometer
lactate standards (5, 10, 15)	syringe and needle (*on ice*)	cuvettes
Reagent 1 (R1)	centrifuge tube (*on ice*)	pipettes and pipette tips
Reagent 2 (R2)	centrifuge	parafilm
TRIS buffer		

PROCEDURES

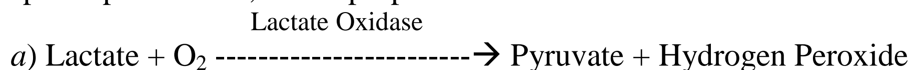
The TA will assign you a crab treatment group (*Resting* or *Active*).

- If you are assigned to the *Resting* group: do not disturb your crab until it is time to take hemolymph!

- If you are assigned to the *Active* group: 1) remove crab from water and dry off carapace, 2) use SuperGlue to attach string to the carapace and let the glue dry completely, 3) suspend the crab in water column by attaching string to wood board above tank, 4) induce crab to swim by prodding it lightly with a stick, 5) keep your crab swimming for at least 30 minutes!!!

While your crab is either resting or swimming, prepare the spectrophotometer and work on generating your calibration curve (see below)

You will be determining lactate concentrations in samples using a commercially available standardized lactate assay kit (Pointe Scientific Inc.) and spectrophotometric techniques. The lactate assay kit consisted of two reagents (R1 and R2). The assay works as follows: Lactate reacts with an enzyme (lactate oxidase) in reagent R1 to form pyruvate and hydrogen peroxide (*a,* below). The enzyme in reagent R2 (peroxidase) then catalyzes a reaction that ultimately results in formation of a purple dye from hydrogen peroxide and other compounds in the R2 solution (*b,* below). The color intensity of the resulting solution is measured using the spectrophotometer, and is proportional to the amount of lactate that was present in the sample.



Color intensity of the solution is measured by the spectrophotometer as Absorbance at 550 nm. In other words, the spectrophotometer measures how much light (at a wavelength of 550 nm) is absorbed by the purple solution. Samples that contain high concentrations of lactate will appear dark purple and have high Absorbance values. Samples with very little lactate will appear light purple and have low Absorbance values. The first step for this experiment is to generate a calibration curve to relate Absorbance values recorded by the spectrophotometer with known lactate concentrations. To do this you will need to create lactate standards of various concentrations (5, 10, and 15 mmol/L), perform the lactate assay with samples from each standard, and then record Absorbance values of standards with the spectrophotometer. Once the calibration curve is generated, you can use the regression equation for the curve to estimate lactate concentration in crab hemolymph based on Absorbance values.

Prepare the spectrophotometer

1) Set the wavelength on the spectrophotometer to 550 nm

2) Set the display mode to Transmittance by pressing the mode control key.

3) Place black box in the cuvette holder and close lid. If display does not read 0.00% T, press the 0%T button.

4) Fill a clean square cuvette with TRIS buffer and place in the cuvette holder. If display does not read 100% T, press 0A/100%T button. Set display mode to Absorbance by pressing the mode control key. If the display does not read 0.00 A, press the 0A/100%T button again.

Mix lactate standards

Use the stock solution of 15 mmol/L and TRIS buffer to make 1 ml standards with concentrations of 5, 10, and 15 mmol/L.

$V_1C_1 = V_2C_2$

where: V_1 = volume of starting solution needed to make the new solution
C_1 = concentration of starting solution
V_2 = final volume of new solution
C_2 = final concentration of new solution

Standard concentration (mmol/L)	15 mmol/L Stock solution (mls)	TRIS buffer (mls)	Final volume (mls)
5			1.0
10			1.0
15			1.0

Run lactate assay on standards

Now that you have created your standard solutions with known lactate concentration, perform the lactate assay using reagent R1 and reagent R2. General procedures used for the assay are represented below:

		room temp		37°C	
Sample +	Reagent 1	-------------→	Reagent 2	--------------→	Measurement of
6 ul	300 ul	2 minutes	200 ul	5 min	absorbance (550 nm)

You will run this assay once for each lactate standard. Compare your Absorbance values with those obtained by other groups. Absorbance values for each standard concentration (5, 25, or 50 mmol/L) should be similar between groups.

1) Pipette 300 ul of reagent R1 into a clean square cuvette

2) Pipette 6 ul of 5 mmol/L standard solution into the cuvette containing reagent R1, allow to stand for at least 2 minutes

3) Pipette 200 ul of reagent R2 into the cuvette containing R1 and standard.

4) Add 2500 ul (i.e. 2.5 ml) of TRIS buffer to the cuvette. Place parafilm over the top of the cuvette and gently invert the cuvette twice to mix contents. Transfer cuvette into holder in water bath (at 37°C) for 5 minutes.

5) Remove cuvette from water bath, carefully wipe all water off the sides of the cuvette, and place cuvette in spectrophotometer to measure Absorbance. Record the Absorbance value for 5 mmol/L lactate standard in Table 1 of the Worksheet.

6) Repeat steps 1 – 4 using the 10 mmol/L standard and then the 15 mmol/L standard.

7) Enter data in an Excel worksheet, values for lactate standards in column A and Absorbance values in column B.

8) Use the Insert→Chart→"X-Y scatter" command to generate a graph that plots values for lactate standards (Y axis) against their corresponding absorbance values (X axis).

9) Use the Insert→Function→ "LINEST" command (under the Statistical category) to calculate the slope (m) and Y-intercept (b) values for the linear regression equation [$Y = m(X) + b$] that describes the relationship between lactate concentration (Y) and absorbance values read by the spectrophotometer (X).

> **Note:** The LINEST function will initially only return one number – the slope (m) value. To get the Y-intercept value (b), highlight the cell that contains slope value and the adjacent cell to the right of it. Press F2, and then press CTRL+SHIFT+ENTER. The Y-intercept value (b) should now appear in the cell to the right of slope value (m).

10) Enter your slope (m) and Y-intercept (b) values on the Worksheet. You can now use the equation $[\text{Lactate}] = m(\text{Absorbance}) + b$ to estimate lactate concentrations of crab hemolymph samples based on Absorbance values that you obtain for these samples using the lactate assay and spectrophotometry.

Obtain hemolymph from crab

1) Use a cooled needle and syringe to collect ~ 0.5 ml of hemolymph from blue crabs. Hemolymph may be collected by inserting needle at the base of the rear-most appendage and pulling back on the syringe plunger. Your TA will demonstrate!
Note: The reason we keep the syringe and needle cool is to prevent the hemolymph from clotting.

2) Quickly empty the blood into a cooled microcentrifuge tube. Keep on ice until you ready to use.

Run lactate assay on hemolymph sample

You will use the same assay to determine lactate concentration in crab hemolymph that you used to determine lactate concentration in the standard solutions. General procedures used for the assay are repeated below:

		room temp		37ºC	
Sample +	Reagent 1	--------------→	Reagent 2	---------------→	Measurement of
6 ul	300 ul	2 minutes	200 ul	5 min	absorbance (550 nm)

You will run this assay TWICE (i.e. in duplicate) for your crab hemolymph sample as a quality control measure.

1) Pipette 300 ul of reagent R1 into a clean square cuvette

2) Pipette 6 ul of supernatant from crab hemolymph into the cuvette containing reagent R1, allow to stand for at least 2 minutes

3) Pipette 200 ul of reagent R2 into the cuvette containing R1 and supernatant.

4) Pipette 2500 ul (i.e. 2.5 ml) of TRIS buffer into the cuvette. Place parafilm over the top of the cuvette and gently invert the cuvette twice to mix contents. Transfer cuvette into holder in water bath (at 37°C) for 5 minutes.

5) Remove cuvette from water bath, carefully wipe all water off the sides of the cuvette, and place cuvette in spectrophotometer to measure Absorbance. Record the Absorbance value for crab hemolymph in the Worksheet.

6) Repeat steps 1 – 4 and record Absorbance value in Worksheet.

7) Calculate lactate concentration in crab hemolymph using the regression equation calculated derived from standard lactate samples and their corresponding Absorbance values. Enter values for lactate concentration in the Worksheet.

WORKSHEET

Students:____

Resting or Active Crab:________________________________

Calibration curve

Table 1

Standard concentration (mmol/L)	Absorbance reading
5	
10	
15	

Enter these values in an Excel file, generate a calibration curve, and calculate regression equation describing the linear relationship between lactate concentration and Absorbance. The equation should follow the formula Y = mX + b, where

Y = lactate concentration in mmol/L
m = slope of the regression equation (calculated in Excel)
X = Absorbance value (recorded using spectrophotometry)
b = Y-intercept value (calculated in Excel)

Insert your values for *m* and *b* here: *m* = ____________ *b* = ____________

Write your regression equation here: ____

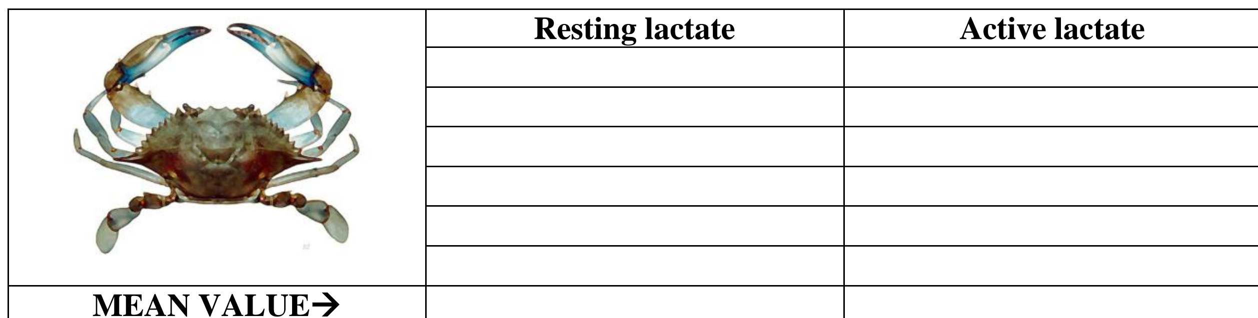
Attach to this Worksheet a print-out of your calibration curve generated in Excel.

Lactate concentration in crab hemolymph

Enter your Absorbance values here: Replicate 1 = ____________ Replicate 2 = _____________

Enter the mean value for lactate in your crab hemolymph sample here : _____________
[(Replicate 1 + Replicate 2)/2]

Share your data (mean value for lactate) with the rest of the class so that you can compare lactate concentration in resting and active crabs. Enter data from your classmates in the Table below:

	**Resting lactate**	**Active lactate**
MEAN VALUE→		

Questions

What is the main benefit of anaerobic metabolic pathways? What are the drawbacks?

Name some situations an animal might encounter for which rapid ATP production via anaerobic metabolism would come in handy.

How might by-products of anaerobic metabolism (fermentation) disrupt homeostasis?

Could a physiological disturbance like lactate accumulation or acidification affect an animal's behavior? If so, how and why?

Is fermentation the only metabolic pathway being utililized by the crab during exercise? What other pathways might operate during periods of burst exercise?

How might the crab rid its body of excess lactate following a bout of exercise?