Laboratory 13
Analysis of Proteins in Different Animal Organs, Part C

I. Introduction

In the first part of this experiment, you made a tissue homogenate or an acetone powder suspension of a certain animal organ, and measured the protein concentrations of the suspension using the Coomassie Plus protein reagent. In the second part of this experiment, you ran a set of SDS-PAGE gels to separate and compare the proteins in the different organs, and learned how to analyze SDS PAGE Gels.

In this lab, you will

- analyze your gels in order to characterize the protein composition of the different organ samples
- measure the activity of an enzyme called L-lactate dehydrogenase in the various acetone powder suspensions. This enzyme plays are major role in intermediary metabolism but is found in quite different concentrations in different organs.

II. Pre-Lab Preparation

Read the Introduction, Background Information, and Experimental Procedures for this experiment. Please also review the Background Information in **Laboratory 11** and **Laboratory 12** on SDS-polyacrylamide gel electrophoresis. Also, review the material from **Laboratory 5** on Enzymes and the measurement of enzyme activity. After preparing for the lab, you should be able to answer the following questions. **Record the answers to these questions in your lab notebook before the lab.**

A. Why were molecular mass markers be included in the new gels of the proteins from the tissue extracts?

B. Which tissues would you expect to contain the most complex mixtures of proteins? Which should contain simpler combinations of proteins?

C. Suppose you find a band in acetone powder sample with a molecular mass of 66,000. Is this protein bovine serum albumin?

D. Many proteins in a cell have similar sizes. If you find a band with a molecular weight of 42,000, how do you know if it represents one protein or several different proteins?

E. If you find a protein with a molecular mass of 24,000 in different samples, how could you determine if they are the same protein?

F. What is the reaction catalyzed by L-lactate dehydrogenase?

G. In what metabolic pathway does this reaction occur?

H. How will this reaction be measured in this laboratory?

I. What is the definition of specific activity?

J. What is the importance of determining the specific activity of a certain enzyme in a particular extract?

K. Which tissue(s) would you expect to contain the highest specific activity of L-lactate dehydrogenase?

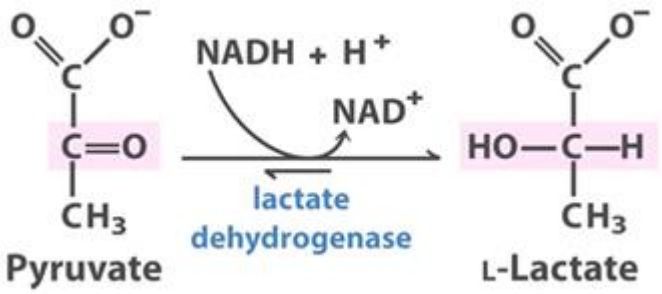
III. Background Information

A. Measurement of L-Lactate Dehydrogenase Activity

1. The L-Lactate Dehydrogenase Reaction

In this experiment, you will measure the activity of the enzyme L-lactate dehydrogenase in the acetone powder suspensions that were made last week. Since the cells in these different organs vary both morphologically and physiologically, you would expect them to contain different amounts of this particular enzyme. L-Lactate dehydrogenase is an example of a NAD^+-dependent dehydrogenase that catalyzes an oxidation/reduction reaction using the coenzyme NAD^+ as the electron carrier. In this type of reaction, an organic substrate is oxidized and the electrons are passed to NAD^+ as the electron acceptor, leading to the formation of NADH. Many enzymes that catalyze this type of reaction can also work in reverse direction, and so they can catalyze the reduction of an organic substrate using NADH as the electron donor, leading to the formation of NAD^+.

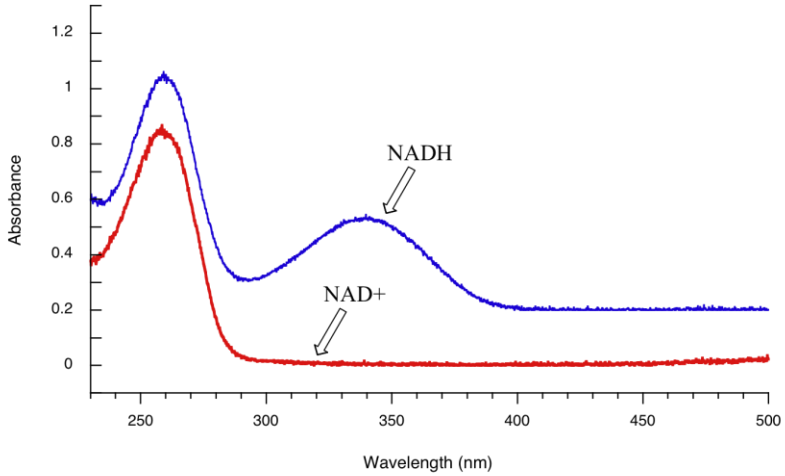
L-lactate dehydrogenase catalyzes the interconversion of pyruvate and L-lactate. The reaction is shown in the next figure.



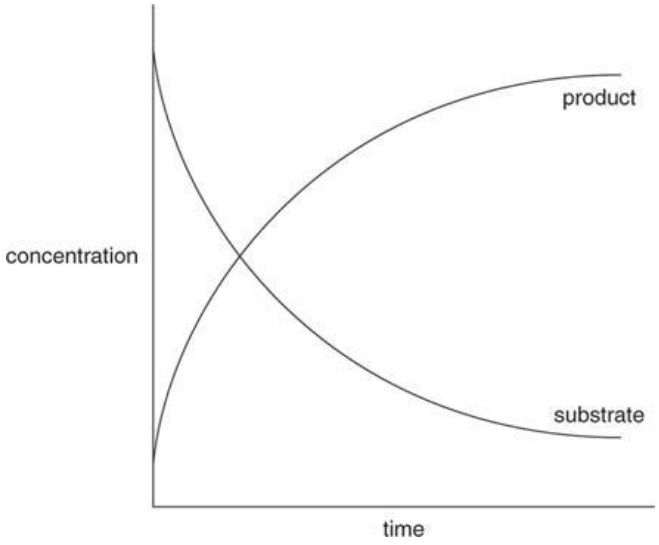
L-lactate dehydrogenase plays a key role in the metabolism of carbohydrates such as D-glucose. D-glucose is usually metabolized by the glycolytic pathway to pyruvate. In the absence of oxygen, the pyruvate is then converted to L-lactate as part of a process of lactate fermentation. This commonly occurs in muscle tissue during active exercise as way of continuing to provide ATP for muscle contraction. L-lactate formed by muscle is then released into the circulatory system, where it is transported to other aerobic tissues such as liver. In the liver, the L-lactate is converted back to pyruvate, which is then degraded to carbon dioxide through the tricarboxylic acid cycle.

2. Measurement of L-Lactate Dehydrogenase Activity

Reactions involving NAD^+ and NADH can be easily measured in a spectrophotometer because, while NAD^+ does not absorb light at 340 nm, NADH does. This is shown in the next figure.



The reaction catalyzed by L-lactate dehydrogenase can be measured in either direction. That is, one can combine the enzyme, the reduced substrate (L-lactate), and NAD^+, and then follow the increase in absorbance at 340 nm as the product NADH is formed. Alternatively, one can combine the enzyme, the oxidized substrate (pyruvate), and NADH, and then follow the decrease in absorbance at 340 nm as the substrate NADH is removed. Typical reaction plots are shown in the following figure:



In either case, there is an initial linear phase in which absorbance increases or decreases at a constant rate. This is followed by a change in the rate as the reaction slows and eventually approaches a plateau. The linear phase with NAD^+-dependent dehydrogenases is often quite short: only 1-3 minutes. In this experiment, you will measure the activity of L-lactate dehydrogenase in the direction of NADH oxidation: that, NADH will be removed from the reaction as pyruvate is reduced to L-lactate. The reason for doing this is that crude homogenates and acetone powders commonly contain many enzymes that can reduce NAD^+ to NADH and so tend to give very high background values.

The change in absorbance at 340 nm can be used to calculate the activity of L-lactate dehydrogenase in nmoles/min ml. The molar extinction coefficient (**E**) for NADH is 6.22 x 10^3. That is, a 1.0 M solution will give an absorbance in a 1 cm light path of 6220. A 1.0 mM solution will give an absorbance of 6.22. Since a 1.0 mM solution contains 1 mmol/liter or 1 μmole/ml, an absorbance of 6.22 in a 1.0 ml reaction mixture corresponds to 1 μmole. An absorbance of 0.00622 corresponds to 1 nmole.

Suppose you set up a 1.0 ml reaction mixture with appropriate substrates and 50 μl (0.05 ml) of enzyme solution. You then observe that when NADH is added to the mixture, the absorbance at 340 nm decreases at a rate of 0.0478/min. From this, you can then calculate that the activity is:

$$\frac{0.0478\ A_{340}}{\text{min x 0.05 ml enzyme}} \times \frac{1\ \text{nmole}}{0.00622\ A} = \frac{154\ \text{nmole}}{\text{min ml enzyme}}$$

Suppose that instead you set up a 3.0 ml reaction mixture using the same reagents. If you then observe that the absorbance at 340 nm decreases at a rate of 0.0478/min, you can calculate that the activity is:

$$\frac{0.0478\ A_{340}}{\text{min x 0.05 ml enzyme}} \times \frac{1\ \text{nmole}}{0.00622\ A} \times 3\ \text{ml} = \frac{462\ \text{nmole}}{\text{min ml enzyme}}$$

The rate is now three times as fast because the total volume is three times larger and so three times NADH is removed. It is important to be careful to distinguish between the total volume of the reaction mixture (1.0 or 3.0 ml) and the volume of the enzyme used (0.05 ml or 50 μl).

3. Determination of Specific Activity

Cells contain hundreds of proteins, but the concentrations of these proteins vary dramatically from organ to organ. One way to compare the concentrations of a particular enzyme is in terms of their specific activity. Specific activity is usually defined as the amount of activity (nmoles/min or μmoles/min) per mg of cellular protein. An enzyme that occurs at a high concentration in a certain organ will have a high specific activity, but an enzyme that occurs at a low concentration will have a low specific activity.

In **Laboratory 4**, you measured the activity of the enzyme β-galactosidase (lactase) using ONPG as the substrate. Suppose you determine the activity of this enzyme in extracts of the two individuals, one of whom is lactose-tolerant and one of whom is lactose-intolerant. The extract from the first individual contains an activity of 246 nmoles/min ml of extract, while the extract from the second individual contains an activity of 37 nmoles/min ml of extract. While this certainly supports the idea that lactose tolerance depends on having a high level of β-galactosidase, you have no way of knowing that the extracts were prepared in exactly the same way. To solve this problem, you can determine the protein concentration of each extract in mg/ml and then express the activity in nmoles/min mg protein. For example, suppose the protein concentration of the extract from the first individual is 10.3 mg/ml and that from the second individual is 27.3 mg/ml. The specific activities are thus:

individual 1: $\frac{246\ \text{nmoles}}{\text{min ml}} \times \frac{1\ \text{ml}}{10.3\ \text{mg protein}} = \frac{23.9\ \text{nmoles}}{\text{min mg}}$

individual 2: $\frac{37\ \text{nmoles}}{\text{min ml}} \times \frac{1\ \text{ml}}{27.3\ \text{mg}} = \frac{1.35\ \text{nmoles}}{\text{min mg}}$

Thus, while the first individual has about 6.6 times as much activity as the second individual (246/37), the specific activity is actually 17.7 times higher. The importance of the enzyme is even more obvious. For this experiment, you will carry out a similar calculation to compare the L-lactate dehydrogenase activities in the different acetone powder suspensions.

IV. Experimental Procedures

This experiment involves only two steps, which can be done in either order.

The following is a flow chart for this laboratory session:

Analysis of Proteins in Acetone Powder Suspensions
(Section IVA)
↓
↓
Measurement of L-lactate Dehydrogenase Specific Activity (Section IVB)

A. Analysis of Proteins in Acetone Powder Suspensions

The objective of this part of the experiment is to complete the analysis of the SDS-polyacrylamide gels.

1. Retrieve you gels from last week's lab. The gels will have been destained by the instructor and stored in water.

2. Take the gels over to the digital imaging system, and with the instructors help, photograph the gels on the white light box. Save and print out a copy of each gel.

3. Look first at the lane with the Precision Plus Protein Standard. Identify each band and measure the distance from the bottom of the well to the center of each protein band.

4. Use a piece of semi-log graph paper to construct a standard curve for the 4-15% gradient polyacrylamide gel. Plot mobility (in cm) on the X axis and log molecular mass on the Y axis. **You should include the results from both gels and put the data for the Precision Plus Protein Standard on the same graph.**

5. Now look at the lanes with the samples from the various animal organs. For each lane in which there are well-defined protein bands, **number the bands sequentially from the top of the gel downwards**. Then measure the distance from the bottom of the well to the center of each protein band and express the mobility of the protein in cm. Then using the standard curve, estimate the molecular mass of each of the proteins. **Note: the only thing that you know about these proteins is their size. Even though a protein may have the same mobility as one of the standard proteins, it is not the same protein!**

6. Compile the results as a series of tables on a piece of paper and include it in your lab notebook. For each organ, indicate the number of different proteins, the mobility of each protein, and the apparent molecular mass. You can use the results from the lanes that were loaded with samples of that particular organ suspension. Use the following chart as a guide.

organ	protein	mobility in cm	apparent mass
PK			
CB			
BP			
CL			
PH			
PB			

7. Compare the proteins from the different animal organs. Are there some proteins that appear to be common to all tissues? Are there specific proteins that appear to be unique to a particular tissue? It is important to remember that in a cell, there may be many proteins of a similar size, so that a band at 35,000 daltons may actually represent a mixture of several proteins.

B. Measurement of L-lactate Dehydrogenase Specific Activity

The objective of this part of the lab is to measure the L-lactate dehydrogenase activity in the organ acetone powder suspensions. Each group should use the same suspension it made in **Laboratory 11** and used for the protein assay. The LDH assays will be done by setting up a reaction mixture in a cuvette containing buffer, substrate, and enzyme, and then initiating the reactions by addition of NADH. The total volume will be 3.0 ml in each case. **The decrease in absorbance at 340 nm will be used to determine the enzyme activity**.

1. Get your remaining tissue or organ sample out of the freezer box and allow it to thaw.

2. Turn on the Genesys 20 spectrophotometer and allow it to warm up for 15 minutes. Set the wavelength of the spectrophotometer to 340 nm.

3. Add the following solutions to a 4.5 ml plastic cuvette in the order given:

0.1 M potassium phosphate buffer, pH 7.0	2.5 ml
0.1 M sodium pyruvate	0.3 ml
(water + enzyme)	0.1 ml
total	2.9 ml

   For the (water + enzyme), you will **add a combination of water and enzyme to a total volume of 0.1 ml** or 100 µl. For the first reaction, just add 100 µl of water so that this reaction can serve as control. Mix the solution by covering the cuvette with piece of Parafilm and inverting it several times. Place the cuvette in the spectrophotometer and set the instrument to zero absorbance.

4. Remove the cuvette from the instrument. To start the reaction, add 0.1 ml (100 µl) of 7.0 mM NADH to give a total volume of 3.0 ml and start the stopwatch. Rapidly mix the solution again and place the cuvette back in the spectrophotometer. Record the initial absorbance at 340 nm at 30 second intervals for 5 minutes.

Table _____ Determination of Suitable Acetone Powder Concentration for LDH Assay

µl organ suspension	0	25			
µl water	100	75			
Time (seconds)	Absorbance (A_{340})				
0					
30					
60					
90					
120					
150					
180					
210					
240					
270					
300					
Is the change in absorbance within the range of 0.05 to 0.10 A per minute?	*Yes*	*Yes*	*Yes*	*Yes*	*Yes*
	No	*No*	*No*	*No*	*No*

***Continue until the change in absorbance is within the acceptable range (0.05 to 0.10 A per minute over a period of 2-3 minutes).*

5. Use a piece of linear graph paper to plot the results. Graph A_{340} on the Y axis as a function of time in minutes on the X axis. Draw a smooth curve through the data points. For the first control reaction, you will probably find that there is little change in absorbance, so you can just draw a simple straight line through the points.

6. Rinse out the cuvette and set up a new reaction as described above. In this case, however, add 25 µl of the organ acetone powder suspension. To make the total sample volume up to 100 µl, add 75 µl of water. As before, set the instrument to zero absorbance before adding the NADH solution. Add 100 µl of NADH to start the reaction, and follow the absorbance at 340 nm at 30 second intervals for up to five minutes.

7. You may find that the A_{340} decreases very rapidly, a sign of a high level of activity, or very slowly, a sign of a low level activity. You may also find that the change in the absorbance slows down with time. The ideal reaction is one that gives a consistent change in absorbance in the range of 0.05 to 0.10 A per minute over a period of 2-3 minutes.

8. If the rate of change in absorbance is too rapid to measure accurately, rinse out the cuvette and set up a new assay with a smaller volume of the acetone powder suspension. Add a sufficient amount of water so that the total volume of (enzyme + water) is 100 µl. For example, if you use only 10 µl of acetone powder suspension, add 90 µl of water. Run the assay and record the A_{340} at 30 second intervals for up to five minutes. If the reaction is still too rapid, make a 1/10 dilution of acetone powder suspension by combining 100 µl of the original suspension with 900 µl of 0.1 M potassium phosphate buffer, pH 7.0.

9. If the rate of change in absorbance is too slow, rinse out the cuvette and set up new assay with a larger amount of enzyme. You can use up 100 µl of the acetone powder suspension.

10. Once you have found a suitable dilution or volume of the acetone powder suspension to use, carry out three (3) replicate assays. Enter the data in the following table.

Table _____ LDH Assay

	Absorbance (A_{340})		
Time (seconds)	Replicate 1	Replicate 2	Replicate 3
0			
30			
60			
90			
120			
150			
180			
210			
240			
270			
300			

11. Make a separate graph of the results of each of your good assays and draw a straight line through as many of the data points as possible. **Insert a copy of these graphs after this page in your lab manual.** Using this line, calculate the change in absorbance (ΔA_{340}) per minute. Then subtract the change in absorbance at 340 nm that was seen with just water in the control reaction.

12. Average the three values and then calculate the amount of activity in ΔA_{340}/min ml. Use the conversion factor of 0.00622 A_{340}/nmole to express the activity of the tissue homogenate or acetone powder suspension in nmoles/min ml. Be sure to multiply by 3 since the total reaction volume was 3.0 ml.

13. Now, using the protein concentration of your organ acetone powder suspension you found in **Laboratory 10**. Calculate the specific activity of L-lactate dehydrogenase, which will have units of nmoles/min mg protein.

14. Add your data to the class summary chart shown below.

Summary of L-lactate Dehydrogenase Activities

tissue suspension	protein conc. (mg/ml)	LDH activity (ΔA_{340}/min ml)	LDH activity (nmole/min ml)	specific activity (nmol/min mg)
PK	_____	_____	_____	_____
CB	_____	_____	_____	_____
BP	_____	_____	_____	_____
CL	_____	_____	_____	_____
PH	_____	_____	_____	_____
PB	_____	_____	_____	_____