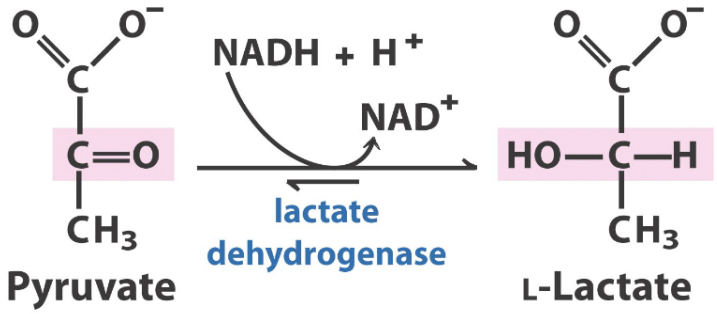
BCH 372
Modern Concepts in Biochemistry Laboratory

Laboratory 4
Assay for L-Lactate Dehydrogenase

The purpose of this laboratory session is to learn how to measure the activity of L-lactate dehydrogenase (LDH) and to define some of the basic factors that affect the rate of the reaction. As noted earlier, L-lactate dehydrogenase catalyzes the reduction of pyruvate to form L-lactate in the presence of NADH. It also catalyzes the oxidation of L-lactate to form pyruvate in the presence of NAD^+ as an electron acceptor.



While the reaction can be measured in either direction, it is more convenient to assay LDH activity in the direction of L-lactate oxidation because NADH absorbs light at 340 nm and NAD^+ does not. The reaction thus can be measured as an <u>increase</u> in A_{340}. For this experiment, you will be provided with a stock solution of a purified sample of LDH from bovine heart. You will determine the activity of this solution first. You will then determine the substrate specificity of the reaction and measure the effects of pH on its activity using buffers.

I. PreLab Preparation

Before the lab, please read Chapter 4 in the lab manual **Experiments in Biochemistry: a hands-on approach** by S. O. Farrell and L. E. Taylor. You do not need to understand all of the details related to enzyme purification for this lab session, so concentrate on sections 4.1, 4.4, 4.6, and 4.8. Pay particular attention to the calculations shown in Practice Session 4.2 on page 97 and to the additional calculations on page 99 of the lab manual. Students sometimes find the use of the terms rate, activity, and specific activity confusing and have difficulty doing the appropriate calculations. The section on pages 8-12 at the end of this handout summarizes the calculations that will be done throughout the semester. At this point in the course, you only need to pay attention to those related to **initial velocity** and **enzyme activity**.

II. Laboratory Procedures

A. Measurement of L-Lactate Dehydrogenase (LDH) Activity

The purpose of this part of the experiment is to learn how to measure the activity of LDH using a standardized protocol. The standard protocol or assay involves combining a buffer (0.15 M CAPS, pH 10.0), a solution of L-lactate (the primary substrate), a solution of NAD^+ (the electron acceptor), and the enzyme L-lactate dehydrogenase (LDH). An alkaline pH buffer favors the reverse (L-lactate -----> pyruvate) reaction. While the amounts of the buffer, L-lactate, and NAD^+ will usually be the same, the source and the volume of the enzyme solution can be varied. The reaction will be followed by measuring an increase in absorbance at 340 nm as NAD^+ is reduced and as NADH is formed. You will start with step 4 of the experiment described on page 102 of the lab manual.

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

2. You will be provided with the following stock solutions for the LDH assay:

   Assay Buffer Solution = 0.15 M CAPS, pH 10.0
   NAD^+ = 6 mM
   L-lactate = 150 mM, pH 7.0
   stock bovine heart LDH solution = an ammonium sulfate preparation of bovine heart LDH which was diluted about 1/200 in 0.1 M KPO_4 buffer, pH 7.5

3. To set up a standard assay, add 1.9 ml of the Assay Buffer to a 4.5 ml methacrylate cuvette. Then add 0.5 ml (500 µl) of the NAD^+ solution and 0.5 ml (500 µl) of the L-lactate solution. Note that the total volume at this point is **2.9 ml**. It will be easiest if you use a 5 or 10 ml pipet to add the buffer and a P-1000 micropipetter to add the NAD^+ and L-lactate solutions. Be sure to use a clean tip for each solution so you do not contaminate the stocks.

4. Since you do not know how much activity is present in the stock solution of LDH, you will probably need to try several different volumes in order to find a volume that gives a reasonable rate of reaction. **The final total volume in the cuvette should always be 3.0 ml (3000 µl)**, so you can add up to **100 µl (0.1 ml)** of the enzyme solution. You will make up the difference (up to 100 µl) with water.

5. Start by trying to detect LDH activity with a small volume of the stock enzyme solution. After adding the CAPS buffer, NAD^+, and L-lactate to the cuvette, add **90 µl** of water. Place a piece of Parafilm over the top of the cuvette and invert the solution several times to mix everything together. Insert the cuvette into the spectrophotometer and set the instrument to zero absorbance.

6. Then remove the cuvette from the spectrophotometer and add **10 µl** of the stock enzyme solution. Rapidly place a piece of Parafilm over the top of the cuvette and invert the solution several times to mix everything together. Place the cuvette back in the instrument and read the absorbance at 340 nm. Take readings at 15 second intervals for a total of 3 minutes (180 seconds). Record the absorbance values in your lab notebook.

7. If the reaction occurs at a reasonable rate - an increase in A_{340} of about 0.1 or 0.2 per minute - repeat the assay two more times, so that you have a total of **three (3) replicate assays**.

8. If the rate of the reaction with 10 µl of the stock enzyme solution is too slow, try adding a larger volume of stock enzyme such as 25 µl or 50 µl to the reaction. **Be sure to reduce the amount of water, so that the final total volume in the cuvette is 3.0 ml in each case**.

9. If the rate of the reaction with 10 µl of the stock enzyme solution is too fast, make an additional 1/10 dilution of the enzyme solution by adding 100 µl of the enzyme solution to 900 µl of 0.1 M KPO_4 buffer, pH 7.5, in a microcentrifuge tube. Then assay 10, 25, or 50 µl volumes of the diluted enzyme using the standard protocol. Again, the total volume should be **3.0 ml**. Once a suitable volume of the enzyme solution has been identified, do **three (3) replicate assays**.

10. Once you have obtained three good replicate assays of the LDH activity in the stock enzyme solution, plot the results on a piece of graph paper as shown in Figure 4.3 on page 97 of the lab manual. Note that Absorbance (at 340 nm) is plotted on the Y axis as a function of Time in Seconds on the X axis. Make a separate graph for each of your three reactions. You can also make graphs using Excel. Be sure to do this as XY scatterplots, again with A_{340} on the Y axis and Time in Seconds on the X axis. While you may find that a reaction continues linearly for the full three minutes, it is more likely that it will start off at a linear rate but then slow down. This is commonly observed with NAD^+-dependent dehydrogenases.

11. You should only use the initial linear rates of reaction in all of your calculations. If you make the rate plots on graph paper, use a ruler to identify the **linear portion** of the rate curve for each reaction. Draw the best-fit straight line to as many points in the linear region as you can. The line does not have to go through the origin because some additional absorbance at 340 nm may be introduced when the enzyme solution is added. If you make the graph in Excel, you can fit a linear trendline to the data points. You may need to select which data points to use in order to ensure that you only have a line through only the linear region of the rate plot.

B. Calculation of the Initial Velocity and Activity of the LDH Reaction

1. Use the linear part of each rate curve to determine the initial velocity (V_o) or rate of the reaction. This value corresponds to the slope of the straight line. You can calculate this V_o value by taking the difference between any two absorbance values within the linear portion of the curve and then dividing by the difference in time between these values.

$$V_o = \frac{A_{340}\text{ (time 2)} - A_{340}\text{ (time 1)}}{\text{time 2 (seconds)} - \text{time 1 (seconds)}}$$

$$V_o = \frac{\Delta A_{340}}{\text{second}}$$

If you are making graphs with Excel, you can use the equation for the trendline that you fit to those points within the linear region. Be careful, however, to fit the line to only those data points in the linear region.

2. Then convert the initial velocity for each reaction to a change (Δ) in the absorbance at 340 nm per minute (ΔA_{340}/minute) as follows:

$$V_o = \frac{\Delta A_{340}}{\text{second}} \times \frac{60\text{ seconds}}{\text{minute}}$$

$$= \frac{\Delta A_{340}}{\text{minute}}$$

3. Then determine the **average** initial velocity as ΔA_{340}/min. That is, calculate the mean of the three replicate assays with a particular volume of the stock enzyme solution or a dilution of it.

4. Next, express the average initial velocity in terms of the actual number of µmoles of NADH formed per minute. The molar extinction coefficient for NADH is 6220 A_{340} M^{-1} cm^{-1} (refer back to Laboratory 3). This means that a 1.0 M solution of NADH in a 1 cm light path cuvette will have an A_{340} of 6220. Absorbance is usually expressed on a 1.0 ml basis, so an absorbance of 6220 also represents what would be observed with a 1 mmole/ml solution. Since the total volume of the reaction mixture in the cuvette was actually 3.0 ml, the total amount of NADH formed per minute is

$$V_o = \frac{XXX\ \Delta A_{340}}{\text{minute}} \times 3.0\text{ ml total} \times \frac{1\text{ mmole}}{6220\ A_{340}} \times \frac{1000\ \mu\text{moles}}{1\text{ mmole}}$$

$$= \frac{XXX\ \Delta A_{340}}{\text{minute}} \times \frac{3000\ \mu\text{moles}}{6220}$$

$$= \frac{XXX\ \mu\text{moles}}{\text{minute}}$$

As shown on page 99 of the lab manual, 3000/6220 = 0.48, so

$$V_o = \frac{XXX\ \Delta A_{340}}{min} \times 0.48 = \frac{XXX\ \mu moles}{minute}$$

0.48 is a simple conversion factor that allows you to easily convert a ΔA_{340}/min value to a µmole/min value. While using this number is simple, you always need to remember what it means. It would have a different value if you had a total reaction volume of 1.5 ml or if the molar extinction coefficient of the light absorbing chemical had a value of 4569.

5. Finally, calculate the **activity** of the stock LDH solution in CAPS buffer at pH 10. **Activity is defined as the rate of the reaction (µmoles/min) per ml of the enzyme solution.** For example, if you find that 10 µl of the stock LDH enzyme solution gives a rate of 0.247 µmoles/min, the activity is

$$\frac{0.247\ \mu moles}{min \times 10\ \mu l\ enzyme} \times \frac{1000\ \mu l}{ml} = \frac{24.7\ \mu moles}{min\ ml\ enzyme}$$

Note that you must be careful to distinguish between the total volume of the reaction mixture (3.0 ml or 3000 µl in this case) and the volume of enzyme added to the reaction mixture (in this example, 10 µl). Be sure to use the volume of enzyme you actually used in your three replicate assays.

6. If you found it necessary to make a dilution of the stock enzyme, be sure to correct for the dilution factor (1/10) by multiplying the activity rate by 10. For example, if you used 50 µl of a 1/10 dilution and found the rate to be 0.192 µmoles/min, the activity would be

$$\frac{0.192\ \mu moles}{min \times 50\ \mu l\ enzyme} \times \frac{1000\ \mu l}{ml} \times 10 = \frac{38.4\ \mu moles}{min\ ml\ enzyme}$$

7. Finally, express the activity in units/ml of enzyme. **In the case of L-lactate dehydrogenase, 1 unit of LDH activity is defined as the amount of enzyme needed to form 1 µmole of NADH/min.** So in the case of the calculations shown in step 5 and step 6, the activities would be 24.7 units/ml and 38.4 units/ml, respectively.

This is a way of saying how much activity is present in the stock enzyme solution. If you had a total of 350 µl of the stock enzyme and found the activity was 38.4 units/ml, you would have a total of

$$350\ \mu l \times \frac{1\ ml}{1000\ \mu l} \times \frac{38.4\ units}{ml}$$

= 13.44 units of LDH altogether.

C. Specificity of the L-Lactate Dehydrogenase Reaction

The purpose of this part of the experiment is to determine the specificity of the reaction with respect to its substrates. As part of this section, you will measure the rate of the reaction with D-lactate (the enantiomer of L-lactate) as the substrate and with $NADP^+$ (the modified form of NAD^+) as the electron acceptor. NADPH has an extra phosphate group but still absorbs light at 340 nm.

1. Set up a new reaction by adding 1.9 ml of the Assay Buffer to a 4.5 ml methacrylate cuvette. Then add 0.5 ml (500 µl) of the 6 mM NAD^+ solution and 0.5 ml (500 µl) of the 150 mM **D-lactate** solution. Note that the total volume at this point is **2.9 ml**. Again, it will be easiest if you use a 5 or 10 ml pipet to add the buffer and a P-1000 micropipetter to add the NAD^+ and D-lactate solutions. Be sure to use a clean tip for each solution.

2. Then add the amounts of water and stock enzyme which you found in Part A gave you a reasonable rate of reaction and which you used for your replicate assays. Place the cuvette back in the instrument and read the absorbance at 340 nm at 15 second intervals for a total of 3 minutes. Record the absorbance values in your lab notebook. If there is no increase in A_{340}, record the rate of the reaction as zero. If there is an increase in A_{340}, repeat the assay two more times so that you have three replicate assays.

3. Now set up a new reaction by adding 1.9 ml of the Assay Buffer to a 4.5 ml methacrylate cuvette. Then add 0.5 ml (500 µl) of the 6 mM **$NADP^+$** solution and 0.5 ml (500 µl) of the 150 mM L-lactate solution. Note that the total volume at this point is **2.9 ml**. Again, it will be easiest if you use a 5 or 10 ml pipet to add the buffer and a P-1000 micropipetter to add the NAD^+ and D-lactate solutions. Be sure to use a clean tip for each solution.

4. Then add the amounts of water and stock enzyme which you found in Part A gave you a reasonable rate of reaction and which you used for your replicate assays. Place the cuvette back in the instrument and read the absorbance at 340 nm at 15 second intervals for a total of 3 minutes. Record the absorbance values in your lab notebook. If there is no increase in A_{340}, record the rate of the reaction as zero. If there is an increase in A_{340}, repeat the assay two more times so that you have three replicate assays.

5. Calculate the activities of the LDH with these substrates as described in Part B and compare to those found with the standard combination.

D. Effect of pH on the Rate of the L-Lactate Dehydrogenase Reaction

The purpose of this part of the experiment is to determine the effect of pH on the rate of the L-lactate dehydrogenase reaction. Each group will determine the activity of the enzyme with the two buffers at different pHs. The class will then pool the data so that the results with all of the buffers can be determined.

1. Set up a new series of assays for LDH **using the volume or dilution of the stock enzyme found in Part A to give a reasonable rate of reaction**. Use 150 mM L-lactate and 6 mM NAD^+ as the substrates. In this case, instead of adding 1.9 ml of CAPS, pH 10 as the buffer, you will add 1.9 ml of one of your buffers instead. Again, the total volume of the reaction mixture should be 3.0 ml.

2. Do the LDH assay **three times** with each of your buffer solutions. Again, plot the data, determine the initial velocities (V_o), and calculate the average initial velocity of the reaction for that buffer as ΔA_{340}/min.

3. For each buffer, convert the initial activity from a ΔA_{340}/min to μmol/min as described above. Again, the conversion factor of 0.48 takes into account the facts that 1) the molar extinction coefficient of NADH is 6220 M^{-1} cm^{-1}, 2) the path length of light through a standard cuvette is 1.0 cm, and 3) the total volume of each reaction is 3.0 ml (0.003 L).

E. Calculation of LDH Activities at Different pHs

1. Express the initial velocity with each buffer as units of **activity** as described in Part B.

2. **Exchange contact information with the other groups in the class, so you can make arrangements for exchanging data for the purposes of pooling.**

3. Make a graph in which you plot the LDH activity (in units/ml) with each buffer as a function of pH. What does the graph look like? Draw a best fit line through the data points to determine the pH optimum of LDH. Why do you think that the use of different buffers to achieve the various pH values might affect the results?

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Basic Calculations Used in the Measurement and Purification of L-Lactate Dehydrogenase

There are a number of simple calculations that are used throughout the course to study the activity of L-lactate dehydrogenase from an animal tissue and to follow its purification. The following is a quick summary of the formulas used in these calculations. WWW, XXX, YYY, and ZZZ just represent numbers found in an experiment. More detailed descriptions of each calculation are then given in the rest of this handout. All of the calculations are also described in the lab manual.

A. Basic Formulas for Calculations

1. initial velocity (V_o) = __XXX___ µmoles/min

V_o = $\frac{V_o \text{ (run 1)} + V_o \text{ (run 2)} + V_o \text{ (run 3)}}{3}$

= average ΔA_{340}/min

average ΔA_{340}/min x 0.48 = __ XXX___ µmoles/min

2. enzyme activity = __XXX___ µmoles/min ml

activity = $\frac{\text{__YYY__ µmoles}}{\text{min x __ZZZ__ µl enzyme}} \times \frac{1000\ \mu l}{ml}$

= $\frac{\text{__XXX__ µmoles}}{\text{min ml enzyme}}$

3. total enzyme units = __XXX___ µmoles/min

total units = $\frac{\text{__YYY__ µmoles}}{\text{min ml enzyme}} \times$ _ZZZ__ total ml of enzyme fraction

= $\frac{\text{__XXX__ µmoles}}{\text{min}}$

4. percent recovery = $\frac{\textbf{total units in fraction K}}{\textbf{total units in first fraction}} \times$ 100%

5. protein concentration = ____WWW_____ mg/ml

6. specific activity = __XXX ___ µmoles/min mg

sp. act. = $\frac{\text{__YYY__ µmoles}}{\text{min ml}}$ x $\frac{\text{ml}}{\text{WWW mg protein}}$

= $\frac{\text{__XXX__ µmoles}}{\text{min mg}}$

7. purification factor = $\frac{\text{specific activity in fraction D}}{\text{specific activity in first fraction}}$

purification factor = $\frac{\text{__XXX_____ µmoles/min mg}}{\text{__YYY_____ µmoles/min mg}}$

= **N fold**

B. Descriptions of Calculations

1. Initial Velocity

The rate of the L-lactate dehydrogenase reaction is expressed in terms of the initial velocity (V_o) in the presence of L-lactate and NAD^+. In the reaction, L-lactate is oxidized to form pyruvate and NAD^+ is reduced to form NADH. This results in an increase in absorbance at 340 nm since NADH absorbs light at this wavelength and NAD^+ does not. The initial velocity is determined by plotting the absorbance of a standard 3.0 ml reaction mixture at 340 nm as a function of time in seconds. The best straight line is fitted to the initial set of data points and the slope of the line is expressed as ΔA_{340}/min. This slope is the initial velocity (V_o) since it represents the amount of product formed per unit of time. See Figure 4.3 in the lab manual on page 97. Because there will be some variation in the initial velocity, LDH reactions are normally run in triplicate and the average (mean) of the three values is used.

$$V_o = \frac{V_o \text{ (run 1)} + V_o \text{ (run 2)} + V_o \text{ (run3)}}{3}$$

V_o = _____ ΔA_{340}/min

The initial velocity can be converted to an actual amount of product in µmoles/min by using the absorption characteristics of the NADH. The molar extinction coefficient of NADH at 340 nm is 6220. This means that a 1.0 M solution of NADH will have an absorbance of 6220 in a 1.0 cm light path. Since the total volume of the reaction mixture is 3.0 ml (0.003 liter), you can convert ΔA_{340}/min to µmoles/min as follows:

$$V_o = \frac{\_\_\_\_\_\ \Delta A_{340}/\text{min}}{(6220\ M^{-1}\ cm^{-1})\ (1\ cm)} \times \frac{10^6\ \mu\text{moles}}{\text{moles}} \times 0.003\ \text{liter}$$

$$= \_\_\_\_\_\ \Delta A_{340}/\text{min} \times 0.48$$

$$= \_\_\_\_\_\ \mu\text{moles/min}$$

This is shown on page 99 of the lab manual. You can also think about the calculation in the following way. Since a 1.0 M solution of NADH has an absorbance of 6220, a 1.0 mM solution of NADH will have an absorbance of 6.220 and a 1.0 μM solution will have an absorbance of 0.00622.

$$V_o = \frac{\_\_\_\_\_\ \Delta A_{340}/\text{min}}{\text{liter} \times 0.00622\ A_{340}} \times \frac{1\ \mu\text{mole}}{} \times 0.003\ \text{liter}$$

$$= \_\_\_\_\_\ \Delta A_{340}/\text{min} \times 0.48$$

$$= \_\_\_\_\_\ \mu\text{moles/min}$$

Suppose you measure the initial velocity of a crude homogenate and find the ΔA_{340}/min to be 0.135/min, 0.159/min, and 0.146/min. The average ΔA_{340}/min is thus 0.147 ΔA_{340}/min. This can be converted to 0.147 ΔA_{340}/min x 0.48 = 0.0704 μmoles/min.

2. Enzyme Activity

The rate of the LDH reaction will, of course, depend on the amount of enzyme added to the reaction mixture. Enzyme activity is normally expressed in terms of the initial velocity of the reaction per ml of solution. Suppose that you measured the initial velocity of the reaction described above on using 10 μl of a crude homogenate. The enzyme activity of the solution is thus:

$$\frac{0.0704\ \mu\text{moles}}{\text{min} \times 10\ \mu\text{l}} \times \frac{1000\ \mu\text{l}}{\text{ml}} = \frac{7.04\ \mu\text{moles}}{\text{min ml}}$$

This is important because different solutions will have different amounts of LDH in them. If it took 50 μl of enzyme solution to get this same velocity, the activity of the solution would be only

$$\frac{0.0704\ \mu\text{moles}}{\text{min} \times 50\ \mu\text{l}} \times \frac{1000\ \mu\text{l}}{\text{ml}} = \frac{1.41\ \mu\text{moles}}{\text{min ml}}$$

In the lab manual, 1 unit of activity is defined as the amount of the amount of enzyme that converts 1 μmole of L-lactate to pyruvate/min (or that reduces 1 μmole of NAD^+ to NADH/min). We would therefore say that the first solution has 7.04 units of enzyme/ml and the second solution only 1.41 units of enzyme/ml.

3. Total Units and Percent Recovery

During the purification of LDH from beef heart, some of the activity is lost along the way as fractions are discarded or as the enzyme is denatured. To monitor the recovery of the enzyme, the amount of enzyme in each fraction is expressed in terms of total units. The total number of enzyme units is simply the enzyme activity in (μmoles/min ml or units/ml) multiplied by the volume of each fraction. If the crude homogenate has 7.04 units/ml and a total volume of 110 ml, the total amount of activity is:

$$\frac{7.04 \text{ units}}{\text{ml}} \times 110 \text{ ml} = 774 \text{ units}$$

Suppose that much of the LDH in this crude homogenate is precipitated with ammonium sulfate and recovered in a fraction that has 66.5 units/ml and a total volume of 4.8 ml. The total number of units in this fraction is:

$$\frac{66.5 \text{ units}}{\text{ml}} \times 4.8 \text{ ml} = 319 \text{ units}$$

The percent recovery of the activity in this fraction compared to the crude homogenate is:

$$\frac{319 \text{ units in ammonium sulfate fraction}}{774 \text{ units in crude homogenate}} \times 100\% = 41.2\%$$

4. Protein Concentrations and Specific Activity

Most of the fractions obtained during the purification of LDH contain many different proteins. The total amount of protein is determined using a protein standard curve and is expressed in mg/ml. See pages 70-72 in the lab manual. The specific activity of LDH is the amount of activity per unit of protein. It is expressed as μmoles/min mg or units/mg. See pages 94-95 in the lab manual. Suppose that you determine that the crude homogenate has 7.04 units/ml. If the protein concentration of this fraction is 1.2 mg/ml, then the specific activity is

$$\frac{7.04 \text{ units}}{\text{ml}} \times \frac{\text{ml}}{1.2 \text{ mg}} = \frac{5.87 \text{ units}}{\text{mg}}$$

If the ammonium sulfate fraction has 66.5 units/ml and 6.5 mg/ml of protein, then the specific activity of this fraction is

$$\frac{66.5 \text{ units}}{\text{ml}} \times \frac{\text{ml}}{6.5 \text{ mg}} = \frac{10.2 \text{ units}}{\text{mg}}$$

5. Fold Purification

The extent of purification is expressed in terms of the increase in specific activity that occurs as extraneous proteins are removed from fractions containing LDH. It is expressed as the ratio of the specific activity in the fraction of interest to the initial specific activity. In the example shown above, the fold purification is

$$\frac{10.2 \text{ units/mg}}{5.87 \text{ units/mg}} = 1.74 \text{ fold}$$

See page 95 in the lab manual.