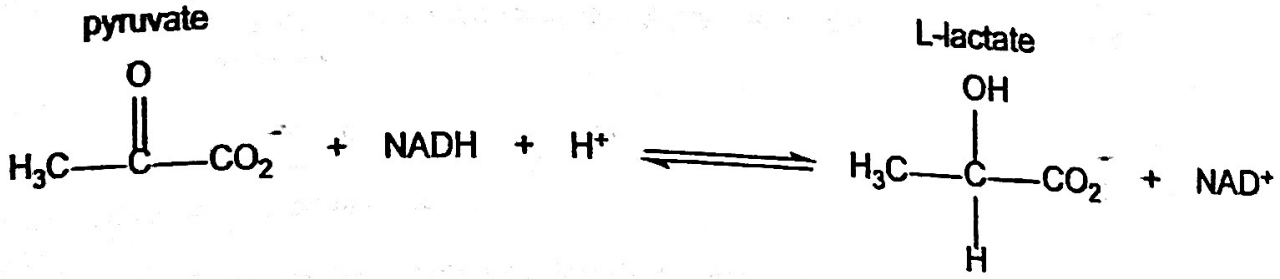
Lactate Dehydrogenase: Isolation, Purification and Kinetics

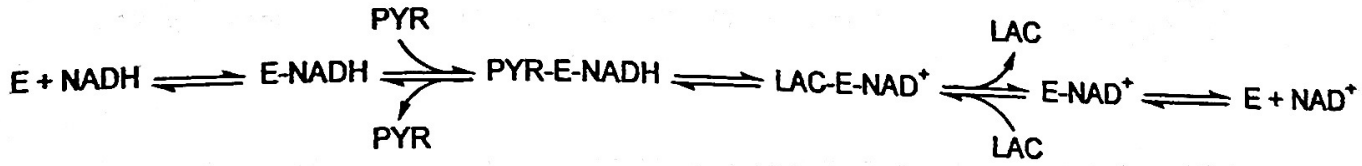
Introduction

Lactate dehydrogenase (LDH) is a tetrameric enzyme which catalyzes the reduction of pyruvate by reduced nicotinamide adenine dinucleotide (NADH). In mammals, five isoenzymes are known; they consist of the various possible combinations of 4 subunits of the "H" and/or "M" type. The isoenzymes differ in molecular weight, isoelectric pH and K_m for substrates even though they catalyze the same reaction. The "M_4" type of LDH is found to predominate in muscle; it is characterized by a low K_m for pyruvate, and thus promotes glycolysis by allowing removal of pyruvate as fast as it is formed. The H_4 LDH, found in heart (an actively respiring tissue) has a high K_m for pyruvate; this results in pyruvate being funneled directly into the citric acid cycle for total oxidation, rather than sidetracking it into lactate. In emergencies, where the O_2 supply is not sufficient, the enzyme allows glycolysis to occur to produce the necessary energy.

All isozymes of LDH catalyze the following reaction:



Kinetic studies indicate that there is a compulsory binding order:



The rate of the reaction can be followed by monitoring the absorbance of NADH at 340 nm (the oxidized form, NAD^+, does not absorb at 340 nm).

In this experiment, LDH will be extracted from bovine heart muscle and partial purification will be accomplished by successive ammonium sulfate precipitations. This crude LDH preparation will be further purified by Affi-Gel Blue affinity chromatography. The increase in specific activity will be used as an indication of the degree of purification achieved. Furthermore, the K_m and V_{max} of LDH for pyruvate will be determined, and the type of inhibition produced by an unknown inhibitor and its K_i will be ascertained.

Experimental Procedure

Day 1 Extraction and Partial Purification of LDH

As a class, carry out the following:

1. Homogenize a mixture of 125 g of minced heart muscle and 400. mL of ice cold 0.0500 M sodium phosphate, pH 7.00 in a Waring blender with four, 30 sec bursts at 60 volts. Between each burst, place blender on ice for 30 sec.

2. Divide the homogenate equally among three 250 mL polycarbonate bottles. Centrifuge the homogenate at 14,000 rpm, 15 min, 4 °C.

The class will divide up into three groups (of ~6 students each). Each group should obtain 1/3 of the bovine heart muscle homogenate in a 400 mL beaker on ice. In your ~6 person group:

3. Measure the volume of the supernatant with a graduated cylinder. Keep sample on ice as much as possible during the measuring process.

4. Calculate the amount of ammonium sulfate needed to adjust supernatant to 40.0% saturated aqueous ammonium sulfate. This will entail adding 0.242 g ammonium sulfate for every mL of supernatant. Weigh out the required amount of ground, solid, ammonium sulfate.

5. With supernatant in the 400 mL beaker on ice, slowly add ground solid ammonium sulfate (a little at a time) while stirring until all ammonium sulfate has dissolved. Use a stirrer with a magnetic stir bar.

6. Once all ammonium sulfate has dissolved, allow supernatant to stand on ice for 15 min. Start chilling down two clean 250 mL polycarbonate bottles on ice.

7. Divide supernatant equally into the two 250 mL polycarbonate bottles and centrifuge at 13,000 rpm, 15 min, 4 °C. Carefully decant the supernatant into a clean, pre-chilled 400 mL beaker that is on ice. Measure the volume of the supernatant with a graduated cylinder, keeping the supernatant on ice as much as possible. Return supernatant to 400 mL beaker on ice.

8. Adjust the supernatant to 65.0% saturated aqueous ammonium sulfate by slow, gradual addition of ground solid ammonium sulfate. Use a stirrer with a magnetic stir bar. You will need 0.166 g ammonium sulfate per mL of supernatant. After all of the ammonium sulfate is added, allow mixture to stand on ice for 15 min.

9. Centrifuge at 13,000 rpm for 15 min, 4 °C in two 250 mL polycarbonate centrifuge bottles. Carefully decant the supernatant and discard it.

The class will divide into six groups (of ~3 students)

10. Resuspend each pellet in an equal volume of fresh 65.0% sat'd aqueous ammonium sulfate.

11. Swirl to break down the pellets. This sample is your **"LDH suspension"**.

12. **Measure and record the volume of your LDH suspension.** Label and store in refrigerator at 4 °C until the next lab period.

Day 2

A. Preparation of Affi-Gel Blue (AGB)

1. Transfer 1.00 mL of uniform Affi-Gel Blue suspension to thick-walled centrifuge tube.
2. Centrifuge in clinical centrifuge at maximum rpm for 5 min.
3. Carefully remove buffer with Pasteur pipet leaving 0.500 mL of packed AGB in centrifuge tube. Place in ice bath.

B. Batch Purification of LDH

NOTE: Always use different pipet tip for each different **ENZYME** solution.

1. Take 0.400 mL of **LDH suspension** and add 1.60 mL of 0.050 M phosphate buffer, pH 7.50 (1–5 dilution of LDH suspension). Place in ice bath. (**B-1 solution**)
2. Transfer 1.00 mL of this LDH solution to centrifuge tube containing AGB. Vortex gently.
3. Allow mixture to stand in ice bath for 5 min, vortexing gently once per min.
4. Centrifuge for 5 min at maximum rpm.
5. Carefully separate supernatant with Pasteur pipet and discard.
6. Add 2.00 mL of 0.0500 M phosphate buffer, pH 7.50, vortex and centrifuge for 5 min at max rpm. Remove supernatant and discard.
7. Repeat step 6.
8. Add 2.00 mL of Specific elution buffer (2.00 mM NADH / 0.200 M NaCl / 0.0500 M phosphate buffer, pH 7.50) to washed AGB from step 7 and vortex.
9. Allow mixture to stand at room temperature for 15 min. Vortex once per min.
10. Centrifuge for 5 min at max rpm.
11. Carefully separate supernatant with a Pasteur pipet and place in test tube in ice bath. **Affi-Gel Blue purified LDH solution. (B-11 solution)**

C. **LDH Assay**

1. Dilute 1–5 solution of LDH suspension (B-1) 1–40 with 0.0500 M phosphate buffer, pH 7.50 (100 µL of 1–5 plus 3.90 mL of buffer). Place this solution on ice. (**C-1 solution**)

12 - Add 1 mL of specific elution buffer to AGB and do step # 9 but 9 min instead - vortex 9 times then # 10-11

2. Dilute Affi-Gel Blue purified LDH solution (B-11) 1→10 with 0.0500 M phosphate buffer, pH 7.50 (100 µL of AGB purified LDH solution plus 0.900 mL of buffer). Place this solution on ice. (C-2 solution) → 100 µL of b-11 (0.1 mL) 900 µL of buffer

3. LDH Assay Procedure (see page 34 for spectrophotometer settings)

a. Zero spectrophotometer with deionized water at 340 nm.

b. Place 1.00 mL of deionized water, 1.00 mL of 0.100 M phosphate buffer, pH 7.00, 0.700 mL of 0.500 mM pyruvate, and 0.200 mL of 1.50 mM NADH in a glass cuvette. All these solutions are kept at room temperature.

c. Cover with parafilm, mix by inversion and observe A_{340} (~0.6 - 0.55).

d. Use an automatic pipet to add 100. µL (0.1 mL) of C-1 solution, mix assay solution rapidly by inversion and immediately place cuvette in Biospec-1800 in kinetics mode to measure initial rate. See p. 34 for parameters (LDH).

e. Repeat b-d using 100. µL (0.1 mL) of C-2 solution (instead of 100. µL of C-1 solution). → should be YAG1 data

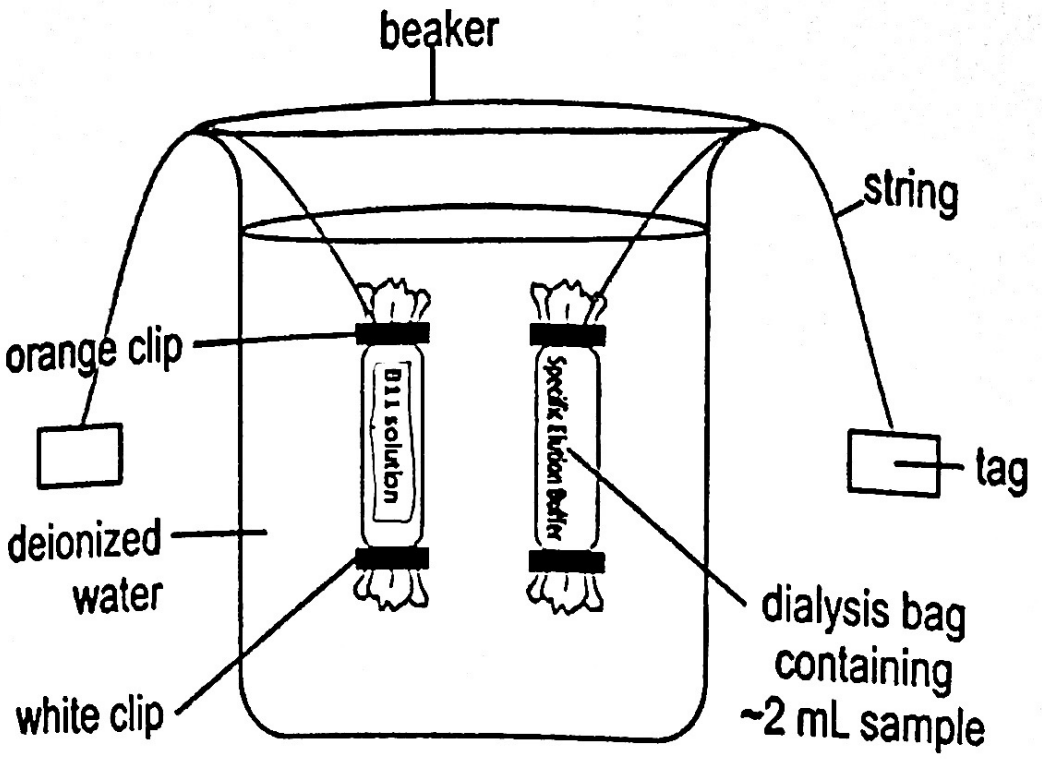
f. Dilute C-1 and C-2 further if rate is too fast to read accurately. It is too fast to read accurately if the absorbance drops to zero before the run ends.

g. Cover test tube containing remainder of B-1 solution with parafilm and place in beaker in refrigerator. You will determine the protein concentration in this solution next lab period.

D. Dialyze sample in two **separate bags**. Add the remaining B-11 solution to one of the bag and add 2.00 mL specific elution buffer to the other bag against 800. mL of deionized water at 4 °C. Dialysate will be changed before next lab. NADH must be removed as it interferes with protein assay.

1. Cut two, 3-inch pieces of dialysis tubing and place in 200. mL of deionized water for ~10 min.

2. Prepare the two samples for dialysis as shown below. Place clips to give "tight" dialysis bags. ***Tight dialysis bags are important to ensure that neither sample becomes diluted by osmosis or "lost" due to leakage out the ends of the dialysis tubing.***



3. Use a **clean** Pasteur pipet to transfer each sample.

4. Cover dialysis beaker with sealwrap and place in cold room. Save LDH, B.I.

E. Using the rate (ΔA_{340}/min) you observed for the C1 solution in part C3, calculate the dilution of the original AS suspension of LDH required to give a rate of 0.28 A_{340}/min under the same saturating conditions.

$$\text{dilution required} = \frac{\text{(rate observed)(dilution made)}}{\text{(rate required)}} = \frac{\times (200)}{0.28}$$

Day 3

A. Protein determination:

1. Transfer dialyzed samples to new, clean test tubes (provided) using clean Pasteur pipet and clearly label the tubes.
2. Bradford assay procedure:
   a. Label 10 clean test tubes. Add the protein solutions as shown below.

Standard Curve

Test tube number	*1	2	3	4	5	6	7	8	9	10
BSA concentration (mg/mL)	0	0.125	0.250	0.500	0.750	1.00	___	___	___	___
BSA volume (mL)	0	0.100	0.100	0.100	0.100	0.100	___	___	___	___
DI water (mL)	0.100	0	0	0	0	0	___	___	___	___
1 →25 dilution of B-1 solution (mL)	___	___	___	___	___	___	0.100	___	___	___
1 →50 dilution of B-1 solution (mL)	___	___	___	___	___	___	___	0.100	___	___
Dialyzed, affi-Gel Blue (B11) (mL)	___	___	___	___	___	___	___	___	0.100	___
Dialyzed, specific elution (mL)	___	___	___	___	___	___	___	___	___	0.100
Bradford assay reagent (mL)	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
*Test tube #1 is a blank for tubes 2-10.										

Test tube #1 serves as a blank for tubes 2-10

b. Mixing content in each test tube by vortexing
c. Let the test tubes sit at room temperature for 5 minutes.
d. Set the wavelength to A_{595}. Zero spectrophotometer with test tube #1 and measure for test tubes 2-10.

A. Enzyme Kinetics

Note: the purpose of steps B1 and B2 is to find a dilution of enzyme that will give a rate somewhere between approximately 0.25-0.30 ΔA_{340}/min for use in Part B3 enzyme kinetics work. If the slope is less than approximately 0.25, the signal-to-noise is too low to easily see kinetic inhibition by your unknown inhibitor in part B5. If the slope is much more than approximately 0.30, the NADH will become depleted before the spectrophotometer run ends (during runs containing higher concentrations of pyruvate), which throws off the slope that will be reported by the spectrophotometer.

1. Determine the appropriate dilution of your AS suspension required. Start by making the dilution you determined you would need in part "E" on Day 2. You may wish to use a two-step dilution procedure if the total dilution is greater than 1→100 (such a dilution might also be expressed as 1:100 or 1/100) and round the number off. For example, if your CALCULATED dilution factor from Part E above was 483, round to 480. Using 0.100 M phosphate buffer, pH 7.00, make a 1 to 10 dilution of a 0.100 mL sample of your ammonium sulfate suspension of LDH(*). Take 0.100 mL of this solution and dilute with 4.70 mL of pH 7.00 buffer (1 to 48 dilution) to give 4.80 mL of a 1 to 480 dilution of your ammonium sulfate suspension of LDH. This dilution might be expressed in your notebook or in a protocol as follows:

6.41

LDH Suspension $X\frac{1}{10}X\frac{1}{48} = Day3\ B1\ Solution$

2. Measure the INITIAL RATE of the solution in part B1 above (using the assay procedure described in step B.3.b. below). If the rate is between 0.25 and 0.30 ΔA_{340}/min, you do not need to continue adjusting the dilutions. Proceed to step B.3. If your rate was not between 0.25 and 0.3, use the INITIAL RATE you obtained to calculate a corrected dilution factor. If you used a two-step dilution procedure you can use your 1 to 10 dilution (*) and further adjust the magnitude of the second dilution based on the corrected dilution factor to give the desired LDH stock solution. Measure the INITIAL RATE of this solution to confirm that it is within the desired range. $\frac{1}{10} \rightarrow \frac{1}{30}$ [0.1, 0.9 buffer] [0.3, 0.7 buffer] 3a

3. Make a **minimum** of 3 mL of LDH stock solution for kinetic runs. 100µl → 2.9

   a. Using 0.100 M phosphate buffer, pH 7.00, dilute your original ammonium sulfate suspension of LDH to give a rate of 0.25-0.30 ΔA_{340}/min. **Keep solution on ice.**

   b. Assay procedure to check LDH dilution

      1. Blank spectrophotometer with deionized water at 340 nm.

      2. Place 1.00 mL of deionized water, 1.00 mL of 0.100 M phosphate buffer/pH 7.00, 0.700 mL of 0.50 mM pyruvate, and 0.200 mL of 1.50 mM NADH in cuvette. All these solutions are kept at room temperature.

      3. Mix by inversion and observe A_{340} (~0.50-0.65).

      4. Using an automatic pipet, add 100. µL of the solution from 3a. A → 0.453

      5. Mix rapidly by inversion and immediately place cuvette in Biospec-1800 in kinetics mode to measure initial rate. See p. 34 for parameters (LDH).

4. Determination of V_{max} and K_m for pyruvate.

   a. Blank initially with deionized water.

   b. Make mixtures in the cuvette.

   c. Use automatic pipet to add 0.10 mL (1000µl) of LDH last.

   d. Use the following protocol for solution preparation.

RUN #	1 (200µl)	2	3	4	5	6
mL of 1.50 mM NADH	0.200	0.200	0.200	0.200	0.200	0.200
mL of 0.50 mM pyruvate	0.100 (100µl)	0.200	0.300	0.500	0.800	1.10
mL of 0.100 M phosphate pH 7.0	1.00	1.00	1.00	1.00	1.00	1.00

(1000)

mL of deionized water	1.8	1.5	1.4	1.2	0.90	0.60
mL of LDH stock solution (add last)	0.100	0.100	0.100	0.100	0.100	0.100

100 µl

5. Use the following protocol to determine unknown inhibitor type and K_i.

RUN #	7	8	9	10	11	12
mL of 1.5 mM NADH	0.200	0.200	0.200	0.200	0.200	0.200
mL of 0.5 mM pyruvate	0.100	0.200	0.300	0.500	0.800	1.10
mL of 0.10 M phosphate pH 7.0	1.00	1.00	1.00	1.00	1.00	1.00
mL of deionized water	1.50	1.40	1.30	1.10	0.800	0.500
mL of 5.0 mM unknown inhibitor	0.100	0.100	0.100	0.100	0.100	0.100
mL of LDH stock solution (add last)	0.100	0.100	0.100	0.100	0.100	0.100

The concentration of NADH, pyruvate and inhibitor are their stock concentrations. These are not their final cuvette concentrations.