BCH 372
Modern Concepts in Biochemistry Laboratory

# Laboratory 7
# Ammonium Sulfate Fractionation of L-Lactate Dehydrogenase, Part A

The purpose of this laboratory session is to begin the purification of L-lactate dehydrogenase (LDH) from the crude extract prepared earlier. In this week's lab, you will fractionate the proteins in the crude extract by precipitating different sets of proteins with the salt Ammonium Sulfate (AS). At each step along the way, you will measure the LDH activity of the fractions using the standard protocol that you have used during the past few weeks. Some of the assays will be done this week and some of the assays will be done next week. You will also save a portion of each fraction for a protein assay that will be done as part of next week's lab. At the end of these two lab periods, the solution with the most LDH activity will be saved for use in various types of liquid chromatography and gel electrophoresis in the coming weeks. The flow chart on the next page summarizes the experimental work for this week. This work is based on Experiment 4a of the lab manual.

## 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. Pay particular attention to sections 4.2, 4.3, 4.4, and 4.5. Work through Practice Sessions 4.1 and 4.2 so you understand the calculations that will be done. Look also at the Summary of Basic Calculations Used in the Measurement and Purification of L-Lactate Dehydrogenase which was provided as part of the handout for **Laboratory 4**.

# Flow Chart for Laboratory 7

**crude extract** (about 100 ml)
**(10,000 x g supernatant fraction)**

1. add Ammonium Sulfate to give 40% saturation;
2. centrifuge at 10,000 x g for 15 minutes

**40% AS pellet** **40% AS supernatant** -----> 1. assay for LDH;
2. freeze 0.5 ml for protein assay
**(fraction 40S)**

1. resuspend in 5 ml of 0.05 M $KPO_4$, pH 7.0;
2. assay for LDH;
3. freeze rest for protein assay **(fraction 40P)**

1. add Ammonium Sulfate to give 65% saturation;
2. centrifuge at 10,000 x g for 15 minutes

**65% AS pellet** **65% AS supernatant** -----> 1. store at 4°C for next week
**(fraction 65S)**

1. resuspend in 5 ml of 0.03 M bicine, pH 8.5;
2. store at 4°C
**(fraction 65P)**

## II. Laboratory Procedures

Because this experiment involves several centrifugation steps and multiple enzyme assays, it will be important for the students in each group to work together. It may be helpful to have one student do the ammonium sulfate precipitations and centrifugations and another do the enzyme assays. At the end of the lab, however, each student must have a complete record of everything that was done.

**NOTES:**

1. **You must measure the volume of each fraction as you go along and save a portion of each fraction for LDH and protein assays.**

2. **You must keep your enzyme solutions cold in an ice bucket to prevent denaturation of proteins.**

3. **You must record all of your data carefully and label your graphs because there will be many different LDH assays.**

4. **You must calculate the average initial velocity ($V_o$) of each fraction in $\Delta A_{340}$/min, the average initial velocity in µmoles/min, and the activity in µmoles/min ml.**

### A. Precipitation of Proteins with 40% Ammonium Sulfate

The purpose of this part of the experiment is to selectively remove some of the proteins in the crude extract (the 10,000 x g supernatant fraction) by precipitation with enzyme-grade Ammonium Sulfate.

1. Retrieve your bottle of crude extract from the refrigerator. Invert it several times to make an even suspension and pour it into a graduated cylinder. Measure the volume again.

2. Calculate the amount of Ammonium Sulfate that you will need to be added to create a 40% saturated solution. You will need **0.242 g for each ml** of the 10,000 x g supernatant fraction or crude extract.

3. Weigh out the Ammonium Sulfate on a top-loading balance. If necessary break up any lumps of material.

4. Transfer your crude extract to a 250-400 ml beaker. Place the beaker in an ice bucket on a stir plate. Add enough ice to the ice bucket around the beaker to keep the solution cold. Place a stir bar in the beaker and turn on the stirrer. **Be sure the heater is off.**

5. Slowly add the Ammonium Sulfate powder to the solution while it is stirring slowly. You should add the salt over a period of about 10-15 minutes.

6. When all the salt has been added**, turn off** the stirrer and allow the solution to sit on ice for 15 minutes.

7. Transfer the solution to a clean 250 ml plastic centrifuge bottle. Prepare another 250 ml centrifuge bottle with an equal volume of water to use as a balance.

8. With the instructor's help, centrifuge the samples at 10,000 x g (10,000 rpm) for 15 minutes in the SLA-1500 rotor in the Sorvall-RC5B refrigerated centrifuge in CLCC 329. We will do the samples for all of the groups at one time.

9. At the end of the centrifugation, pour off the supernatant fraction into a graduated cylinder. Measure its volume and transfer the liquid to a clean flask. This is the 40% Ammonium Sulfate supernatant fraction or **fraction 40S**.

10. Resuspend the 40% Ammonium Sulfate pellet in 5 ml of 0.05 M $KPO_4$ buffer, pH 7.0. Add the buffer to the material in the centrifuge bottle and draw the liquid up and down through a pipet to make an even suspension. Transfer the suspension to a 15 ml plastic centrifuge tube and measure its volume. This is the 40% Ammonium Sulfate pellet fraction or **fraction 40P**.

B. Assays of the 40% Ammonium Sulfate Fractions for LDH Activity

The purpose of this part of the experiment is to determine how the LDH enzyme activity in the crude extract was distributed between the 40% Ammonium Sulfate supernatant fraction (**fraction 40S**) and the 40% Ammonium Sulfate pellet fraction (**fraction 40P**).

1. Assay the 40% Ammonium Sulfate supernatant fraction for L-lactate dehydrogenase activity using the standard protocol. It will be best to start with the same volume that you found to be most suitable for the crude extract last week. If necessary, use a smaller or larger volume or make a dilution of this fraction with 0.05 M $KPO_4$ buffer, pH 7.0.

2. Once a suitable volume of the enzyme solution has been identified, do three replicate assays. Plot the data, determine the initial velocities ($V_o$), and calculate the average initial velocity of the reaction. Calculate the activity of the fraction in μmoles/min ml.

3. Transfer 0.5 ml of 40% Ammonium Sulfate supernatant fraction to a 1.5 ml microcentrifuge tube. Label the tube with your group number and **fraction 40S**. Place the tube in the designated rack for storage at -20$^{o}$C. Keep the rest of fraction 40S on ice for use in part C.

4. Assay the 40% Ammonium Sulfate pellet fraction for LDH activity using the standard protocol. Because this fraction should have relatively little activity, start with 50 μl. If necessary, use a smaller or larger volume to get a reasonable rate of reaction.

5. Once a suitable volume of the enzyme solution has been identified, do three replicate assays. Plot the data, determine the initial velocities ($V_o$), and calculate the average initial velocity of the reaction. Calculate the activity of the fraction in μmoles/min ml.

6. Place the 15 ml centrifuge tube (labeled with your group number and **fraction 40P**) in the designated rack for storage at -20$^{o}$C.

C. Precipitation of Proteins with 65% Ammonium Sulfate

The purpose of this part of the experiment is to precipitate an additional set of proteins from the 40% Ammonium Sulfate supernatant fraction by increasing the ammonium sulfate concentration. This should include the proteins with LDH activity.

1. Calculate the amount of ammonium sulfate that will need to be added to raise the ammonium sulfate concentration in the remaining 40% Ammonium Sulfate supernatant fraction to achieve a 65% saturated solution. You will need **0.166 g for each ml** of the 40% Ammonium Sulfate supernatant fraction.

2. Weigh out the ammonium sulfate on a top-loading balance. Again, break up any lumps in the powder.

3. Transfer the rest of the 40% Ammonium Sulfate supernatant fraction to a 250-400 ml beaker. Place the beaker in an ice bucket on a stir plate. Add enough ice to the ice bucket to keep the solution cold. Place a stir bar in the beaker and turn on the stirrer. **Be sure the heater is off.**

4. Slowly add the ammonium sulfate to the solution while it is stirring slowly. You should add the salt over a period of about 10-15 minutes. When all the salt has been added, turn off the stirrer and allow the solution to sit on ice for 15 minutes.

5. Transfer the solution to a clean 250 ml plastic centrifuge bottle and prepare a suitable balance bottle.

6. With the instructor's help, centrifuge the samples at 10,000 x g (10,000 rpm) for 15 minutes in the SLA-1500 rotor in the Sorvall-RC5B refrigerated centrifuge in CLCC 329. We will do the samples for all of the groups at one time.

7. At the end of the centrifugation, pour off the 65% Ammonium Sulfate supernatant fraction into a clean graduated cylinder. Measure its volume. Then transfer the liquid to a plastic bottle for storage in the refrigerator at 4$^{o}$C until next week. Label the bottle with your group number and **fraction 65S.** You will measure the LDH activity in this fraction next week.

8. Resuspend the 65% Ammonium Sulfate pellet in 5 ml of 0.03 M bicine buffer, pH 8.5. Add the buffer to the material in the centrifuge bottle and draw the liquid up and down through a pipet to make an even suspension. Transfer the suspension to a 15 ml plastic centrifuge tube and measure its volume. This is the 65% Ammonium Sulfate pellet fraction or **fraction 65P**. Store this fraction in the refrigerator at 4$^{o}$C until next week.