Experiment I: Tripeptide Sequence Determination (2 weeks)

Experiment V: Purification of LDH from chicken (4 weeks)
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Experiment I - Tripeptide Sequencing

(Experiment I due 2/23)

I. INTRODUCTION

"The anatomical dissection of proteins requires three attributes: courage, a faith in the concept that proteins are basically constructed in the form of polypeptide chains, and a mastery of chromatography and related techniques." (Greenstein and Winitz, Chemistry of the Amino Acids, 1961.)

Today, the polymeric form of proteins is an established fact. Amino acids joined together by amide linkages, or peptide bonds, make up the immense class of biologically important molecules known as proteins, peptides, and polypeptides (these terms are used rather nonspecifically, although generally protein refers to molecules of 50 or more amino acids, peptide refers to molecules with fewer than 50, and polypeptide encompasses molecules of 10 to 100 amino acids.)

In this experiment, we hope to be able to impart to you some understanding of the chemical and physical properties of peptides. We ask you to characterize the tripeptide (three amino acids) by determining its amino acid composition, its amino acid sequence, and obtaining and interpreting its UV spectrum.

Acid Hydrolysis

Amide bonds, like esters, can be hydrolyzed in the presence of either strong acids or strong bases. Acid is the method of choice for peptide bond hydrolysis because racemization at the α-carbon (via an enolate intermediate) readily occurs under basic conditions.

Total acid hydrolysis cleaves all peptide bonds in a protein, and under the proper protective conditions leaves the amino acids intact. Analysis of the hydrolysate gives the amino acid composition for the peptide or protein under investigation. The hydrolysate is fractionated on an ion-exchange column and the amino acids are quantified by reaction with ninhydrin. Reaction of an amino acid with ninhydrin yields a purple or blue derivative, the concentration of which can be determined spectrophotometrically. (The mechanism of the ninhydrin reaction can be found in Solomons, Organic Chemistry, Sixth Edition, pp. 1155–1156 or in Jones, Organic Chemistry, Third Edition, pp. 1286–1287.)

For purposes of this lab, qualitative identification of the amino acids released by hydrolysis will suffice. This can be best accomplished by thin layer chromatography (TLC) on cellulose plates, followed by visualization with ninhydrin spray. Each amino acid, depending on the chemical characteristics of its side chain, will have a different mobility on the thin layer
plate. Hydrophobic amino acids such as leucine will migrate the farthest, while charged acidic or basic residues will remain close to the origin. Identification of unknowns can be made by comparison of mobility with known amino acid standards. Each amino acid is characterized by its $R_f$, which is the distance the solvent front travels divided by the distance the amino acid travels.

**The Edman Degradation**

The sequential Edman degradation is an important tool in the determination of protein sequence. Developed in 1948 by the Swedish biochemist Pehr Edman, the method has been improved to the point where it is possible to sequentially remove and identify sixty or more amino acids from the amino-terminal (N-terminal) end of a protein. (The N-terminal end is that which has the free amino group.) With this capability, the classical method of proteolytic enzyme cleavages, peptide mapping, and fluorodinitrobenzene (FDNB) labeling, followed by endless peptide isolation and amino acid analysis, are no longer necessary. (Stent and Calendar, *Molecular Genetics, Second Edition*, pp. 93-100 provides a nice description of Sanger’s classic determination of the sequence of bovine insulin, a feat that relied upon the FDNB method.)

A sequence determination begins with limited fragmentation of the protein under investigation. A variety of methods that yield small numbers of specific peptide fragments are listed below:

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</tr>
<tr>
<td>Staphylococcal protease</td>
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The peptide fragments obtained can then be fractionated by either gel filtration or ion-exchange column chromatography. The purified peptides are then subjected to automated Edman degradation—and with modern instrumentation less than a microgram of each peptide fragment is required for a complete sequence determination!

An example of this kind is illustrated by the determination of the amino acid sequence of bovine parathyroid hormone (BPTH). This important hormone, responsible in part for calcium regulation in the body, was known to be an 84-amino acid peptide. An Edman degradation from the amino-terminal end of the hormone removed and identified the first 54 residues in the molecule. At this point, it was recognized that all of the five arginine residues in BPTH were accounted for. The last was at position 52. Succinylation of BPTH blocked the amino terminus and the lysine ε-amino groups (thus rendering lysine resistant to trypsin digestion). Trypsin,
which cleaves peptide bonds on the carboxyl side of the basic amino acids lysine and arginine, would now only attack at the arginine residues. Tryptic digestion of the molecule yielded four peptides, the largest of which comprised residues 53 to 84. This fragment was isolated on a gel filtration column and sequenced by Edman degradation. Thus, the total amino acid sequence of the hormone was deduced from two long Edman degradations, one enzyme digest, and a single isolation step. The work was done in a matter of weeks, which favorably compares with the ten years it took Sanger to sequence insulin (which consists of two polypeptide chains—the first having 21 amino acids, the second 30—connected by two cystine linkages).

The mechanism of the Edman degradation is as follows. First, phenylisothiocyanate (PITC) is coupled to the amino group of the N-terminal residue to yield the phenylthiocarbamyl (PTC)-peptide derivative:

\[
\text{PTC} + \text{Tripeptide} \rightarrow \text{PTC-tripeptide} \quad \text{pH 9.5, 50 °C}
\]

(Although stereochemistry is not indicated in the drawing above, naturally occurring peptides would contain amino acids of the L-configuration.) The reaction is carried out in a volatile buffer at pH 9.5. If the pH falls below 8.5, the amino groups become largely protonated, and thus are incapable of acting as nucleophiles toward the PITC. Above pH 10, the PITC is unstable, breaking down to form aniline (PhNH₂) and carbon oxysulfide (COS). The aniline then reacts with remaining PITC to form diphenylthiourea. Edman has determined that essentially complete reaction of N-terminal amino acids with PITC occurs after 30 min at 50 °C.

Following the coupling reaction, extraction with toluene removes excess PITC, diphenylthiourea, and the organic components of the coupling buffer. The PTC-peptide remains behind, often precipitated on the glass, or in suspension in a small volume of the aqueous phase. After removal of the aqueous solvent, the peptide is ready for the cleavage reaction. Treatment with trifluoroacetic acid (TFA) results in protonation of the amide, rendering the peptide susceptible to nucleophilic attack. The positioning of the sulfur atom in the PTC-peptide makes it possible for the sulfur to attack the carbonyl of the first peptide bond, forming a five-membered cyclic thioester (called a thiazolinone derivative) and releasing the amino group of the second amino acid:

The residual peptide and the thiazolinone derivative are separated by extraction with butyl
chloride. The peptide can then be subjected to further Edman degradation releasing the second, third, etc., amino acids.

The initially formed thiazolinone derivative is unstable and must be converted to the phenylthiohydantoin (PTH) derivative before it can be identified. This is accomplished by incubation in 1N HCl at 80 °C for 10 min:

Once the PTH-amino acid derivative is formed, it may be identified by thin layer chromatography, gas chromatography, mass spectroscopy, or high-pressure liquid chromatography.

All parts of the Edman procedure are carried out in an inert atmosphere. Atmospheric oxygen can desulfurize both the PTC-peptide and the thiazolinone derivative. In either case, an adduct incapable of cyclization is formed and further degradation is impossible

**N-Terminus Analysis with Dansyl Chloride**

Dansyl chloride reacts with amino groups according to the following reaction:

The dansylated product is highly fluorescent and is stable to the conditions of acid hydrolysis. A quantity of dansylated amino acid as small as 10 pmol (1 pmol = 10^{-12} mol) can be detected visually on a TLC plate. The procedure involves labeling the terminal amino groups of the peptide with dansyl chloride, followed by complete hydrolysis of the peptide to release the dansylated terminal amino acid. (All other positions in the peptide simply yield the corresponding free amino acid.) The dansylated amino acid can then be identified by TLC chromatography.
II. DETAILED PROCEDURES—Work in groups of three

**Strategy:** Each group will be given an unknown tripeptide supplied as an aqueous solution of approximately 10 mg/mL. Acid hydrolysis and subsequent chromatography on cellulose-TLC will reveal the amino acid composition of the peptide. Two cycles of an Edman degradation and subsequent analysis of the PTH-amino acids by TLC on silica gel will identify the first and second amino acid in the sequence. Analysis of the aqueous residue from the second Edman cycle by TLC on cellulose will confirm the identity of the third amino acid, the carboxy-terminal (C-terminal) residue. Dansylation of the N-terminal amino acid, followed by hydrolysis and TLC, will provide the final check of the sequence.

**First Week:**

1. Set up the HCl hydrolysis (Part A). Your T.A. will remove the hydrolysis tubes from the oven after 16 hr and save them for next lab period.

2. Carry out 2 Edman cycles (Part B) and save the dried PTH-amino acids (step B.15, first and second cycles) and C-terminal residue (step B.11, second cycle). Store under nitrogen in the freezer.

3. Perform the dansyl end group analysis (Part C). Your T.A. will also remove these hydrolysis tubes from the oven after 16 hr and save them for you.

**Second Week:**

1. Dry down HCl hydrolysates from Parts A and C (do this first; it takes time). Activate the silica gel plates.

2. Run TLC of your HCl hydrolysate from A.6, the free amino acid from B.11, second cycle, and the aqueous product from C.6 on cellulose plates with the appropriate standards (Part D).

3. Run TLC of PTH derivatives from B.15 on fluorescent silica gel plates with the appropriate standards (Part E).

4. Run TLC of your dansyl-derivative from C.5 on non-fluorescent silica gel plates with the appropriate standards (Part F).

5. Take the UV spectrum of your peptide (Part G).
A. Acid Hydrolysis

Materials: 6N HCl
hydrolysis tube

Method:

1. Add 0.5 mL 6N HCl to a hydrolysis tube containing 3.0 μmol of the peptide. The peptide is supplied as an aqueous solution containing approximately 10 mg/mL of peptide. Determine how many μmol/mL this is (based on an average amino acid molecular weight of 110 g/mole), and hence the volume of solution that you will need to add to the hydrolysis tube.

2. Evacuate the hydrolysis tube with vacuum pump.

3. While under vacuum, seal the tube with a torch.

4. Place the sealed tube in the hydrolysis oven at 105 °C. Your gracious T.A. will remove your tube after 16 hr and store it in the freezer.

(2nd week:)

5. Open the hydrolysis tube by scoring with a file and snapping the top off—once again your T.A. will be of assistance here.

6. Transfer the HCl solution to a conical tube and dry completely with nitrogen (heating in the 80 °C water bath while drying will hasten the procedure). The resulting dry residue of the three amino acids will be analyzed by cellulose chromatography (Part D).

B. Edman Degradation (Please do all work in the hood)

Materials: Phenylisothiocyanate (PITC)
Coupling buffer (0.4M dimethylallylamine in 3:2 (v/v) pyridine:water, pH 9.5)
Toluene
Trifluoroacetic acid (anhydrous)
1-Chlorobutane (butyl chloride)
Ethyl acetate
1.0 N HCL
Conical test tubes (12 or 15 mL), stoppers

Method: All steps are to be carried out in the hood!

Generation of the thiazolinone derivative:

1. To 3.0 μmol of peptide in a conical test tube, add 0.5 mL of coupling buffer. (Remember, the peptide is supplied as a 10 mg/mL solution.)
2. **Carefully** add 50 μL of PITC. **DANGER:** PITC, in addition to having an unpleasant odor, is toxic!!

3. Briefly, flush test tube with nitrogen (approx. 5 sec) and quickly stopper.

4. Incubate at 50 °C for 25 min. Check occasionally to see that solution is a single phase. If two phases form, vortex briefly.

5. Extract the solution once with 1.0 mL of toluene. (Add toluene, vortex until phases are well mixed, let layers separate fully, remove the organic layer—on top!—and discard into the non-halogenated waste jar.)

6. Dry down the aqueous phase completely with nitrogen (again, heating to 80° while blowing nitrogen across surface will accelerate the process). **Note:** Your sample, even when dry, may well end up as a viscous oil, rather than a solid.

7. Add 250 μL of TFA to the test tube. Flush briefly with nitrogen and stopper. Make sure all peptide has been dissolved. **DANGER:** TFA is volatile and causes burns.

8. Incubate the tube for 5 min at 50 °C.

9. Completely flush the sample with nitrogen to remove the TFA. The sample should be completely dry at this point.

10. Add 0.4 mL of butyl chloride. A solid, which contains residual dipeptide, may be deposited on the sides of the tube, or it may appear as a very fine precipitate. For some tripeptides, it is nearly impossible to see this solid until the butyl chloride is removed. To ensure that this solid separates from the liquid, centrifuge the reaction tube for 30 sec. The butyl chloride liquid, which contains the thiazolinone of the N-terminal amino acid, should then be transferred to a second conical tube.

11. The solid material remaining in the centrifuge tube contains the residual dipeptide and should be thoroughly dried under a gentle stream of nitrogen. Eventually (see step 16) you will perform a second Edman degradation on this sample.

**Conversion of the thiazolinone to the phenylthiohydantoin (PTH) derivative:**

12. Dry down the butyl chloride extract in the second conical tube with nitrogen.

13. Add 0.3 mL 1N HCL to the tube, flush with nitrogen, and stopper.
14. Incubate at 80 °C for 10 min.

15. Extract twice with 1.0 mL of ethyl acetate. Combine these two 1 mL extracts in a clean conical tube and flush with nitrogen to remove the ethyl acetate. You will now have the phenylthiohydantoin (PTH) amino acid derivative. Store dried residue in a desiccator at -20 °C until next week. You will analyze this PTH-derivative by TLC on silica gel (Part E). (The aqueous layer from the ethyl acetate extractions can be discarded in the aqueous waste container.)

Second cycle:

16. Cycle the residual dipeptide (from step 11) back through steps 1-15. Save both the second PTH-amino acid (step 15) and the third (C-terminal) residue (“solid material” from step 11). The free amino acid will be analyzed in part D, the PTH-derivative in Part E.

C. N-Terminal Dansylation and Hydrolysis

**Materials:**
- Dansyl chloride (5 mg/mL in acetone)
- 0.2 M NaHCO$_3$ buffer
- 6N HCl
- Hydrolysis tube

**Method:**
1. Add 3.0 μmol of the peptide to a conical tube containing 0.5 mL of the NaHCO$_3$ buffer.
2. Add 200 μL of the dansyl chloride solution, vortex briefly, and incubate for 1 hr at 50 °C. Be sure the sample is securely stoppered.
3. After the reaction is complete, flush with nitrogen to dryness. Again, gentle heating will hasten the process.
4. Add 0.5 mL of 6 N HCl and transfer entire mixture to a hydrolysis tube. Label the tube, and give the tube to your T.A., who will seal it and place it in the 105°C oven for 16 hr.

(2nd week:)
5. Open the hydrolysis tube as per A.5. Divide the contents into two clean conical tubes (250 μL of solution into each tube). One of the halves should be dried down and analyzed on a non-fluorescent silica TLC plate. This will tell you the identity of your dansylated N-terminus (Part F).
6. The other half should be dried down and analyzed on a cellulose TLC plate (Part D).
THIN LAYER CHROMATOGRAPHY OF AMINO ACIDS

NOTE: Demonstrations and detailed advice on TLC procedures will be given in the laboratory. The general setup is shown below.

![Diagram of TLC setup with cover, tank, plate, and solvent](image)

D. Cellulose TLC to Separate Free Amino Acids

Materials: Eastman cellulose thin layer plates
           1 μL micropipettes
           Thin layer tank
           TLC solvent: 5:1:1 butanol:water:acetic acid
           Ninhydrin spray reagent
           Amino acid standards (10 mg/mL in water)

Method:
1. With a pencil, draw a faint line about 2.5 cm from the bottom of the plate. Mark positions, equally spaced, for spotting of the samples, of which there are eight (5 standards and 3 unknowns).

2. Add 30 μL of water to each of your three samples—the residue from the HCl hydrolysis (step A.6), the C-terminal amino acid from the Edman procedure (step B.11, second cycle), and the residue from the dansylation (step C.6). Note: Due to the presence of the dansylated N-terminal amino acid in the residue from step C.6, this sample may not completely dissolve.

3. Spot approximately 0.5 μL of each of the eight samples onto the plate in the order alanine, glycine, leucine, phenylalanine, tyrosine, and then the three unknowns. Spot no sample closer than 2.5 cm from either side edge of the cellulose plate.
4. Place the plate in the appropriate chromatography tank, with the origin at the bottom. Double check that you have the correct tank before putting your plate inside! Run the plate until the solvent front is approximately 3 cm from the top.

5. Using a hair-dryer, carefully dry the TLC plate, and spray it with the ninhydrin reagent. So that the color may develop, let the plate stand at room temperature for 30 min.

6. Identification of the amino acids is made by comparison of their $R_f$ values (distance spot travels divided by the distance the solvent front travels) with those of the standards.

E. **Fluorescent Silica TLC to Identify PTH-Derivatized Amino Acids**

**Materials:**
- Silica gel thin layer plates (glass-backed, with fluorescent indicator)
- Micropipettes, 1 μL
- Thin layer tank equilibrated with 98:2 CHCl$_3$:EtOH
- PTH-amino acid standards (10 mg/mL in ethyl acetate)
- Ethyl acetate
- UV light source (short wavelength)

**Method:**

1. Obtain a **fluorescent** silica gel plate and activate it by heating it for 15 min in the 105 °C oven. Quenching of the fluorescent indicator by the PTH group on your plate will allow you to visualize your amino acid derivatives. Make sure you don’t use a non-fluorescent silica plates.

2. With a **pencil**, draw a faint line about 2 cm from the bottom of the plate. Mark seven positions, equally spaced, for spotting the samples (5 standards, 2 unknowns).

3. Dissolve your samples (first and second Edman products from B.15) in 20 μL of ethyl acetate.

4. Spot approximately 1 μL of each of the seven samples in the order PTH-ala, PTH-gly, PTH-leu, PTH-phe, PTH-tyr, PTH-unknown amino acid 1, PTH-unknown amino acid 2. Spot no sample closer than 0.5 cm from either edge of the plate.

5. Run thin layer plate until solvent front has advanced at least half way to the top, remove the plate from the TLC tank, and let it air dry.

6. Examine the plate under UV light. The phenylthiohydantoin derivatives will appear as dark spots—circle them with a pencil. The unknown amino acids are identified by comparison of their $R_f$ values with those of the standards.
F. Non-fluorescent Silica TLC to Identify Dansyl Derivative

**Materials:**
- Silica gel TLC plates (**non-fluorescent**)
- Micropipettes, 1 μL
- TLC tank equilibrated with solvent
- TLC solvent: CHCl₃:EtOH:HOAc 180:20:1
- Dansylated standards (1 mg/mL in butanol)
  - Butanol
  - Pyridine
  - UV light source

**Method:**
1. Obtain a **non-fluorescent** silica gel plate and activate it by heating it for 15 min in the 105 °C oven.
2. With a **pencil**, draw a faint line about 2 cm from the bottom of the plate. Mark six positions, equally spaced, for spotting the samples.
3. Add 50 μL of pyridine to your dansylated-residue from C.5. Note that due to the presence of the two free amino acids, the sample may not all dissolve.
4. Spot approximately 1 μL of each of the six samples in the order dansyl-ala, dansyl-gly, dansyl-leu, dansyl-phe, dansyl-tyr, dansyl-unknown. Spot no sample closer than 0.5 cm from either edge of the plate.
5. Run the solvent front at least half way up the thin layer plate.
6. Air dry the plate and examine under UV light. Here the dansyl-derivatives themselves are highly fluorescent, and hence can be detected in minute quantities. (Note that the dansyl standard solutions are ten times less concentrated than the PTH standards.) Circle the spots with a pencil. The unknown amino acids are identified by comparison of their R<sub>f</sub> values with those of the standards.

G. UV Absorption Spectra

**Materials:**
- Solution of 1.0 mg of the peptide dissolved in 3 mL H₂O.

**Method:**
The spectrum of your peptide (from 200 to 400 nm) should be recorded during the second laboratory period. An instructor will supervise the use of the UV-visible spectrophotometer. The spectra of free tyrosine, phenylalanine, and tryptophan are provided on p. 15 of this lab manual. Note that each of these spectra is labeled with the amino acid concentration, an absorbance scale, and a wavelength scale.
III. WRITE-UP

The following should be included in your notebook:

1. Of course, note the letter that corresponds to your particular unknown tripeptide. Include this identifier in all lab protocols, notes, and observations.

2. Deduce the sequence of your tripeptide (insofar as is possible) and explain your reasoning. Clearly state the results from each of your TLC plates and include tracings or photocopies of your plates. Include a table of R_f values for all of the amino acids and derivatives. Note any uncertainties in your assignment of the sequence and state how you would resolve them.

3. With reference to your table of R_f values, discuss the relative extent of migration of the amino acids and derivatives on the various TLC plates. Is there a general principle that governs the mobilities you observed?

4. Discuss the UV spectrum of your tripeptide. (Include the spectra for the three aromatic amino acid standards from p. 15 in your notebook.) What can you conclude from the spectrum of your tripeptide alone? What could you conclude if, in addition to the spectrum, you also had an approximate molecular weight for the peptide, and extinction coefficients for each of the aromatic amino acids—which, incidentally, can be calculated from the spectra appended! (Hint: you will want to consider Beer’s law: absorbance = εLc, where ε is the extinction coefficient in M^{-1}cm^{-1}, L is the sample path length in cm, and c is the concentration of the sample in M.) If you tripeptide contains no aromatic amino acids, please answer the above questions in hypothetical mode!

5. In part A.1 of this lab you used an estimated molecular weight to calculate the concentration of your tripeptide. How good was this estimate? Now that you know its actual molecular weight, re-calculate the stock concentration of your tripeptide in μmol/mL and molarity (Remember that you were given the concentration in mg/mL.)
Mastery of a protein purification scheme is a rite of passage for all biochemists. The techniques employed in protein purification utilize important biochemical ideas about the structure, catalytic activity, and the optical and electronic properties of proteins. The common themes that emerge in purification strategy are outlined below.

1. **Devise an assay for your protein that is simple, quick and specific.** The presence of an enzyme is typically assayed by observation of the formation of a specific product or the depletion of a specific reactant. This method, which gives both qualitative and quantitative information, is the one that you will employ in this protein isolation. If the protein being purified is not an enzyme, it sometimes can be detected by measurement of a unique spectral characteristic, such as the 410 nm absorption of a heme protein or 615 nm absorption of a blue copper protein.

2. **Production of a clear solution from something alive.** This one step has been the nemesis of many novice biochemists. From bacterial cell paste, human blood serum, bovine brain, or chicken muscle, you must obtain a solution that will flow through columns, dialyze without precipitating and, in general, be well behaved. Extra time spent here to make your solution as pristine as possible will always save you time and heartache later.

3. **Isolate the protein you want from those you don't want.** In order to do this, it helps to know something about the protein you're isolating, such as its molecular weight, isoelectric point, solubility, color, or functional activity. As you're precipitating, dialyzing, and running your solution through columns, it's crucial to monitor your progress by using your assay from #1 above.

4. **Evaluation of the purity and percent yield.** Usually this involves the quantification of your data from #1 above—perhaps a translation of an absorption reading into the activity of the protein per mg. This is usually charted for each step in the purification scheme. An unequivocal standard of purity is a single band on a polyacrylamide gel.

We will carry out all of these steps for the enzyme lactate dehydrogenase (LDH) from chicken. LDH is a tetramer of 35 kD subunits. Two different types of subunit, known as M(uscle) and H(ear), exist. (In general, enzymes that catalyze the same reaction, but differ in structure, are referred to as “isozymes.”) Overall, five different tetrameric assemblies of LDH are possible: $M_4$, $M_3H_1$, $M_2H_2$, $M_1H_3$, and $H_4$. These differences in subunit composition lead to functionally important differences in pyruvate binding affinities and allosteric inhibition by pyruvate.
The muscle enzyme (M₄) plays a critical role in anaerobic glycolysis by coupling the reduction of pyruvate to lactate with the oxidation of NADH to NAD⁺. The product NAD⁺ can then again participate in glycolysis. The product lactate is a dead end in muscle metabolism and must be first transported to the liver where it is converted by liver LDH (also M₄) back into pyruvate, and ultimately into glucose through the gluconeogenic pathway. In a sense, LDH buys time by sustaining glycolysis during periods of stress and shifting the metabolic burden from the muscle to the liver. The heart isozyme (H₄) functions in vivo by oxidizing lactate to pyruvate, which can then be funneled into the citric acid cycle.

The particulars to our application of the steps outlined above are as follows:

1. **Assay:** In this prep, we will use two complementary assays; first, an enzyme assay specific to LDH and, second, a so-called Bradford assay that quantitates by visible absorption the total amount of protein present, and is not specific for LDH. By calculating the ratio of the results from these two assays (i.e. the amount of LDH to the amount of total protein), we will be able to quantitate the purification of LDH from chicken heart and muscle.

   a. **Enzyme Assay:** We will monitor the catalysis of the following reaction by our LDH enzyme.

   ![Chemical Reaction](image)

   Even after hours of incubation, no products are formed when just pyruvate and NADH are mixed in buffer. Add just a tad of LDH, and the reaction takes place immediately. Since the NADH reactant (not the NAD⁺ product) absorbs light at 340 nm, the course of the reaction can be monitored by the decrease in the 340 nm absorption as reactant is converted to product. Our assay will monitor the drop in OD₃₄₀ as a function of time when an aliquot containing an unknown amount of LDH is added to a mixture of pyruvate and NADH. The slope of a plot of OD₃₄₀ vs. time can be related to the amount of enzyme present. We will use this assay to monitor the purification procedure and to measure the kinetics during Week 3.

   b. **Bradford Assay:** The enzyme assay will be complemented by an assay for total protein which makes use of the reaction between proteins and Coomassie blue, a coumarin dye. By monitoring the blue absorption and comparing it to standards, you can determine the total amount of protein present. Again, this assay is not specific for LDH, but rather measures total protein.
2. **Purification**: The enzyme will be isolated from chicken muscle, heart, or liver.

   a. The chicken will be sacrificed and dissected. The muscle, heart, or liver tissue will be homogenized in a blender to break the myofibrils and release LDH and the rest of the soluble proteins into the buffer. Centrifugation of this murky extract at moderately high speeds sediments the nuclei, mitochondria, membranes and other insoluble material. The remainder of the purification is an attempt to separate the LDH contained in the clear supernatant from the other soluble proteins.

   b. The remainder of the purification scheme used here will consist of only two steps. The first is a classical differential precipitation by ammonium sulfate to partially purify the LDH and reduce the load of protein that enters the second step. This latter step consists of specific binding to and selective elution from an affinity column. The column material is constructed of insoluble polysaccharide beads to which AMP has been covalently attached via a six carbon-long “arm.” AMP acts as a structural analogue for NADH. The structures of both are shown below for comparison.

![AMP-SEPHAROSE and NAD⁺](image)

LDH, like most dehydrogenases, possesses a strong binding affinity for NAD⁺ and its AMP analogue. Dehydrogenases are thus selectively retained on the column when the extract is poured over the polysaccharide beads. This process by itself is not sufficient to completely purify the LDH since it is still contaminated by other dehydrogenases that are also bound to the column. The final purification is effected by eluting just the LDH from the column with a solution of a special adduct of NADH and pyruvate which is an intermediate of the LDH reaction. The adduct binds to the LDH selectively, displacing it from the AMP and allowing the LDH adduct complex to be eluted from the column.

c. We will be able to chart the progress of our purification by several complementary methods. First, we will calculate the unit activity per mg protein by combining our enzyme assay results (NADH absorption vs. time) with our Bradford assay results (Coomassie dye). The total of these assays is to determine the specific activity of our LDH. **Specific Activity is defined as units of activity per mg of protein where 1.0 unit of activity is that amount causing transformation of 1 μmol of substrate per minute at 25°C** under conditions where the enzyme is saturated with both pyruvate and NADH.
d. Next, we will run a denaturing polyacrylamide gel electrophoresis (SDS-PAGE) on sample aliquots that have been collected along the way. Under the influence of an electric field, a SDS-denatured protein will migrate in the gel according to its molecular weight. A protein mixture will be resolved on the gel into numerous bands, which can be stained with a protein specific dye. As the purification scheme progresses, the number of bands should decrease.

e. Finally, you will have purified LDH suitable for experimentation. Kinetic analyses and calculation of $k_{cat}$ and $K_M$ will be performed for the enzyme.
Experiment V: Lactate Dehydrogenase, Week 1: Extraction & Initial Purification

SPECIFIC PROCEDURES

NOTE: ALL LDH SOLUTIONS SHOULD BE KEPT ON ICE TO PROTECT YOUR PROTEIN FROM DENATURATION AND TO INHIBIT ENDOGENOUS PROTEASES!

SECOND NOTE: You MUST record the total volume of all solutions from which an aliquot is taken!

Preparation of the Extract. Each group should weigh a 100 mL plastic beaker containing 50 mL of 50 mM potassium phosphate buffer (PB). After chilling the beaker and contents to 0 °C, place approximately 25 cm$^3$ of tissue—either heart, liver, or muscle—from the chicken into the beaker to bring the total volume to about 75 mL. Weigh the beaker/buffer/tissue and determine the weight of tissue taken. From this weight, you will eventually calculate the weight-percent of LDH in your tissue.

First mince the tissue with a single-edge razor blade and then homogenize it in 50 mL of buffer in the Waring blender. Blend for four 30-second bursts, separated by 1-minute intervals in which the blender jar is chilled on ice to keep the whole extract cold. Split the homogenate exactly equally between two chilled centrifuge tubes. (You can increase your yield by rinsing the residual goop out of the blender with a little cold PB, but don’t use more volume than will fit into the two centrifuge tubes.) Thoroughly rinse the blender jar with distilled water when you are finished so as not to contaminate the next group's preparation. When everyone has completed this step, the tubes will be centrifuged at 17,000 x g for 20 minutes.

Ammonium Sulfate [(NH$_4$)$_2$SO$_4$] Precipitation. During the centrifugation, each group should prepare for the ammonium sulfate precipitation by rinsing a 100 mL beaker with PB and adding a clean stirring bar. The supernatant from the centrifugation should be decanted away from the pellet in one smooth motion into this beaker. Remove an aliquot of approximately 0.5 mL of this homogenate (aliquot #1)—remember: record the total volume of the supernatant solution!—for later assay. Weigh out 0.35 g of ammonium sulfate per mL of supernatant, and add this salt gradually to the supernatant with continuous stirring. When all of the ammonium sulfate has been added, continue stirring for 10 more minutes. Divide the protein suspension exactly between the two centrifuge tubes, and centrifuge at 17,000 x g for 20 minutes. This step “salts out” the LDH and some other proteins from solution. The pellet will contain the LDH, while the supernatant will contain other proteins not currently of interest.
Dialysis. While the tubes are spinning, each group should prepare dialysis tubing. Take one section of tubing from the beaker provided. Rinse the tubing thoroughly inside and out with distilled water. Place a dialysis clip as near as possible to one end of the tubing. Store the tubing in a beaker of water until ready to use so as to prevent it from drying out.

When the centrifugation is complete, remove an aliquot (aliquot #2) of the supernatant—did you record the total volume of the supernatant solution?—for later analysis, and then pour off the supernatant as before, this time retaining the pellets. The pellets should be resuspended in 10 mL of cold PB. A Pasteur pipette can be used to encourage the pellet to dissolve. Remove an aliquot (aliquot #3)—surely, you have already recorded the total volume of the solution!—and transfer the remainder of the solution to your dialysis tube. Don't allow the contents to warm up. Place a dialysis clip at the free end of the tubing as before. Attach a piece of thread to one of the clips, and to the thread attach a piece of tape with your group number, group initials, and the date. Submerge the bag in the large beaker of PB provided for dialysis.

Dialysis tubing keeps the large protein molecules inside the bag and allows solvent and small molecules to travel through the small pores in the tubing. This step effectively removes salt and other small molecules. Your T.A. will change the external PB at least two times over the next week so as to thoroughly dialyze away the residual ammonium sulfate. When you return next week, your protein will be happily dissolved in pure PB.

Before you leave, make sure you have:

1. recorded in your notebook the identity (i.e. heart, liver, muscle) and weight of the tissue used.
2. recorded in your notebook the weight of (NH₄)₂SO₄ employed for precipitation.
3. recorded in your notebook the volume of the solutions from which aliquots #1, 2, and 3 were taken.
4. collected three labeled aliquots. Store these in the freezer until next week. You will then perform enzyme assays, Bradford assays, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on the (thawed) samples.
5. a sample of crude LDH dialyzing in the cold room. This sample is precious and should be treated with respect, tenderness, and affection.
Experiment V: Lactate Dehydrogenase, Week 2: Affinity Chromatography

In this second week of the laboratory, you must complete three separate tasks. (Division of labor among the various members of the group is essential.) During today's lab, you must:

1. Set up and run the AMP-Agarose affinity column—a description of this step is given in the LDH Lab Overview (p. 18). (Note: Some years AMP-Agarose is used, as opposed to the AMP-Sepharose indicated in the Overview. This change has no effect; the resin—sepharose or agarose, which are both polysaccharides—merely serves as an inert matrix to which the AMP is attached.)

2. Assay for the total protein content in the various fractions and batches.

3. Assay for the LDH activity in the various fractions and batches.

SPECIFIC PROCEDURES

A. Purification of LDH on an AMP-Agarose Affinity Column (requires preternatural dexterity and extraordinary poise)

Since this portion of the experiment must be run before the other two sections can be completed, start it immediately.

1. Carefully remove the top clip from your dialysis bag containing your protein from last week and transfer your LDH solution into a chilled centrifuge tube. It requires some dexterity to avoid spilling the contents of the bag and thereby running out of things to do. Centrifuge your sample at 34,000 x g for 15 min and CAREFULLY transfer the supernatant to a plastic conical tube—do not let ANY of the pellet, which contains column-clogging gunk, contaminate the supernatant. The supernatant, of course, contains the LDH and will be loaded onto the column. To make sure that this solution is absolutely clear, filter the supernatant through a 0.45 μM syringe filter into a clean conical tube. Take a 0.5 mL aliquot of the dialyzed and filtered LDH solution (aliquot #4) and set it aside (recording, as always, in your notebook the total volume of the solution whence came this aliquot).

2. The basic setup of the affinity column and its operation will be demonstrated to you in lab. Follow the instructions in parts a.-f. below. DO NOT let your column run dry. NOTE: you should record the volume of every batch of eluent you collect.

   a. Your column will be pre-equilibrated with PB. When you are ready to start your column, remove the clip and let the excess PB flow out by gravity. Remember: DO NOT let your column run dry. You will want to carefully watch every step so that you can stop the flow when the liquid level is just above the column bed

   b. When the PB level is just above the beads, stop the flow and carefully load the LDH (your LDH-containing supernatant from part 1) onto your column. Immediately place a conical tube labeled “load and wash” under your column and
start to collect the eluent. When your LDH has been entirely loaded (again, **DO NOT** let your column run dry), carefully rinse down the sides of the column with 3 mL of PB and allow this buffer to pass through the column. This rinse will ensure that all of your protein has been loaded onto the column.

c. When the rinse has been drawn into the column, carefully load 10 mL of additional PB, and continue to collect the eluent into the “load and wash” conical tube. Repeat this process with another 10 mL of PB, again collected in the same “load and wash” conical tube. These washes should remove any proteins from the column that haven't bound to the AMP-Agarose.

d. Now, you will elute the LDH. Make sure the level of PB is within 1 cm of the top of the column bed and add 10 mL of NAD-pyruvate adduct, collecting the eluent in a fresh tube. Repeat this process two more times. Collect the eluent in three ≈10 mL batches labeled “adduct 1,” “adduct 2,” and “adduct 3.”

e. Clean your column of residual protein by running 20 mL (2 x 10 mL) of 8 M urea/2 M NaCl solution through it. Collect the eluent into a tube labeled “urea wash.” *Other people will use your column material in the future, so although your precious LDH should be eluted at this point, please **DO NOT** let your column run dry.*

f. As a final wash and re-equilibration for the next group, pass 50 mL (5 x 10 mL) of PB through the column. You may discard this eluent. When this wash is nearly complete, “turn off” the column by securely clamping the outlet tube at the bottom and covering the top.

**REMEMBER:** you should record the volume of every batch of eluent you collect—that is, the volume of the “load and wash,” the three adduct-eluted batches, and the “urea wash.”

3. As described in parts B. and C. below, your column batches will be assayed both for total protein content and for LDH activity:

a. Once the batches from the NAD-pyruvate elution containing high LDH activity have been identified, they should be pooled for dialysis against PB to rid the solution of NAD-pyruvate adduct. Record the total volume of the pooled solution, and make sure this pooled sample is again analyzed for LDH activity and total protein before leaving it to dialyze.

b. Protein from the initial wash (step c.) and the urea/salt wash (step e.) should also be assayed using the Bradford Reagent and the LDH Activity Assay. Again, note total volumes of these solutions

**B. Bradford Protein Assays (requires unusually compulsive analytical technique)**

We will use a dye-binding assay known as the Bradford Assay to determine the concentration of protein in our various samples. A protein is added to a solution containing the
dye, Coomassie Brilliant Blue. When the dye binds to proteins, its visible spectrum changes and an absorption maximum appears at 595 nm. We will estimate the total amount of protein in each aliquot by measuring the absorbance at this wavelength (also referred to as optical density at 595 nm, or OD$_{595}$) and comparing it that of standards.

**Basic Procedure:** The volume of protein will vary for different samples as described below. Accurately pipette “y” mL of a protein sample into a plastic cuvette containing $(0.1 – y)$ mL of PB. Add 2.0 mL of dye reagent. (The total volume in every sample should thus always be 2.1 mL.) After about 2–5 minutes, read the OD$_{595}$. **Make sure that all of your experimental absorbance readings are well within the absorbance range of your standard curve (see point 1, below). Outside of this range, useful data are not obtained.** You may have to vary the volume of protein solution (or dilute it in PB) to get a reading within this range.

1. In the first series of experiments, run a standard curve containing at least six points ranging from roughly 2 μg to 50 μg of the BSA standard. (Your lab instructor will inform you of the concentration, in mg/mL, of the BSA standard.) Also, measure the absorbance of a “0 mg/mL” sample (i.e., just PB, no protein) and subtract the absorbances of all protein-containing Bradford measurements from this “zero” value. From the resulting data, make a graph of OD$_{595}$ vs. amount of BSA. (Your plot may show some curvature.) Rerun any suspicious points. The total amount of protein in each of the samples below can be directly determined by reference to this standard curve.

2. In the second experiment, you will be measuring the protein concentrations of your aliquots 1–4. Again, the volume of protein sample (y) should be chosen to give OD$_{595}$ readings well within the bounds of your standard curve. Start by assaying 5 μL of each aliquot, and then increase or decrease the volume as necessary. Always note the volume used! (Note: the P20 pipetmen will not accurately pipette less than 2 μL. If your aliquots contain a large amount of protein, you may need to dilute a sample before running the Bradford assay—this is best done in a microfuge tube.)

3. In the third series of experiments, you will measure the protein concentration of the eluent batches. Once the adduct-elution batches with the highest LDH activity have been pooled (part C.2.c. below), perform a final Bradford assay on the pooled solution. **Please do not throw anything away until you are sure that you should.**

C. **LDH Assays (requires a facile mind and great wit—and an intimate familiarity with the proper use of a Pipetman)**

LDH activity will be assayed by adding protein samples to 50 mM PB buffer, pH 7.5, containing 0.12 mM NADH and 0.33 mM pyruvate—saturating concentrations for each of these substrates. The absorbance change at 340 nm is then monitored. Note: the change in OD$_{340}$ for conversion of NADH to NAD$^+$ is $6.22 \text{ OD}_{340}/(\text{mM NADH} \rightarrow \text{NAD}^+)$

1. To determine the background rate of NADH oxidation, run one control with 1.5 mL of assay buffer containing no enzyme. This rate should be essentially zero.
2. Activity Assay:

a. **Aliquot 4.** Measure the OD$_{340}$ using 1.5 mL of assay buffer, and then add directly to that cuvette 10 μL of enzyme extract. Mix and reinsert into the spectrophotometer. Record the OD vs. time. If the change in OD occurs too quickly to measure (*i.e.*, if a nice, linear drop in OD is not observed), dilute 10 μL of enzyme into 90 μL of PB—it's easiest to prepare the diluted sample in a microfuge tube. Add 10 μL of this diluted enzyme to 1.5 mL of assay buffer. Try various dilutions until linear kinetics are obtained. Using two dilutions of the enzyme, show that the rate of change of OD with time is proportional to the amount of enzyme added.

b. **Aliquots 1, 2, and 3.** With the information from aliquot 4 as a guide, assay the amount of enzyme in the remaining three aliquots taken during the purification.

c. **Column Batches.** Assay the LDH activity of the several column batches: the load and wash batch, the three adduct-elution batches, and the final urea wash batch. As noted above, you should pool the adduct-elution batches with the highest activity and dialyze these in preparation for week 3. You should also measure the LDH activity in this final pooled solution.

**Before you leave,** make sure you have performed the following:

1. **Bradford assay:**
   a) standard curve
   b) aliquots 1–4
   c) column batches (5), plus pooled LDH

2. **LDH assay:**
   a) aliquots 1, 2, and 3
   b) aliquot 4 (two different concentrations)
   c) column batches (5), plus pooled LDH

3. **Dialysis:**
   pooled LDH adduct-eluted batches
Experiment V: Lactate Dehydrogenase, Week 3: Kinetics

Today, you will analyze the kinetic properties of your purified LDH.

THEORY

As you are well aware, LDH catalyzes the following reaction:

\[
\begin{align*}
\text{CH}_3\text{C}-\text{COO}^- + \text{NADH} + \text{H}^+ & \rightleftharpoons \text{CH}_3\text{C}-\text{COO}^- + \text{NAD}^+ \\
\text{pyruvate} & \rightleftharpoons \text{L-lactate}
\end{align*}
\]

Below is a simplified reaction scheme that permits the kinetics to be expressed in the Michaelis-Menten form. The NADH concentration will be held constant (and saturating), while that of pyruvate will be varied. As suggested by the scheme, NADH does in fact rapidly bind to LDH prior to pyruvate.

\[
\begin{align*}
\text{NADH} & \quad k_1 \\
\text{E} - \text{H}^+ + \text{PYR} & \quad \text{E} - \text{H}^+ \\
& \quad k_{-1} \\
& \quad \rightarrow \\
\text{k}_{\text{cat}} \\
\text{k}_{\text{cat}} & \quad \text{E} + \text{LAC} + \text{NAD}^+ \\
& \quad \text{PYR}
\end{align*}
\]

Analysis of this kinetic scheme yields the familiar equation:

\[
\text{initial velocity (} v_i \text{)} = \frac{k_{\text{cat}} [E_0] [\text{PYR}]}{K_{M^{\text{pyr}}} + [\text{PYR}]}
\]

SPECIFIC PROCEDURES

The following reagents should be mixed in a cuvette:

- 1400 µL 50 mM phosphate buffer, pH 7.5
- 50 µL NADH Stock Solution (4 mM). The final concentration of NADH in the approximately 3 mL of assay solution will thus be approximately 0.13 mM. This is a saturating concentration of NADH.
Pyruvate solution. The initial pyruvate concentration in the cuvette should vary between 1 mM and 0.05 mM; you will be provided with a 30 mM stock solution. Calculate how much of this stock solution you will need to add to the cuvette to achieve the desired pyruvate concentrations (run about 8 different concentrations). The useful data will be collected below about 0.2 mM, so don't run too many points above this concentration.

50 - y μL  50 mM phosphate buffer. That is, add enough buffer so that the total volume in the cuvette is now exactly 1.5 mL.

Cover the cuvette with a square of parafilm, mix the solution thoroughly, and place the cuvette into the spectrophotometer. Measure the ΔOD₃₄₀ for a minute or two before adding enzyme.

Initiate the reaction by addition of 10 μL of enzyme solution (your dialyzed POOL sample, which of course should be removed from the dialysis tubing and kept on ice throughout the afternoon). Experiment with enzyme dilutions until reasonable kinetics are obtained with 1 mM pyruvate (but on the fast side of reasonable—do you see why?). In all subsequent reactions, use the same dilution of enzyme. Note that the total volume of solution in the assay mix is 1510 μL. At low pyruvate concentrations, your kinetic runs may well show some curvature due to depletion of the pyruvate substrate. In such cases, determine initial velocities by using only the linear data at the beginning of the runs.

Before you leave the lab, make sure that your data make sense! If you have data points that do not make sense (e.g., did you added a lower substrate concentration and measure a higher initial velocity??), you probably made a pipetting or dilution error somewhere. In consultation with your instructor, re-run data points as necessary.
Experiment V: Lactate Dehydrogenase, Week 4: Gel Electrophoresis

In this laboratory we will employ the high-resolution analytical tool of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to the samples derived from our first two weeks of work, and will attempt to establish:

1) the effectiveness of the various purification steps,
2) the purity of the LDH, and
3) the LDH subunit molecular weight.

THEORY

Electrophoresis is a method whereby charged molecules in solution, chiefly proteins and nucleic acids, migrate in response to an electric field. Their rate of migration, or mobility through the electrical field depends on the strength of the field, on the net charge, size and shape of the molecules, and also on the ionic strength, viscosity, and temperature of the medium in which the molecules are moving. The technique is used here as an analytical tool to measure the number of different protein species that are present in the different samples collected during the LDH purification. Ideally, one will see the number of bands decrease during the course of purification, from many, many different proteins in the crude cell extract to pure LDH monomer in the adduct-eluted fractions.

We will run the electrophoretic system that is most widely used at present, the Laemmli system sodium dodecyl sulfate-polyacrylamide gel electrophoresis or SDS PAGE. As shown in the reaction scheme on the following page, polyacrylamide gel is formed by polymerization of acrylamide with the crosslinking agent N,N'-methylene bisacrylamide (bis); the polymerization is initiated by the addition of ammonium persulfate (APS) and catalyzed by N,N,N',N'-tetramethylethylenediamine (TEMED). Today, we will utilize a “disc-gel,” in which discontinuities in pH, ionic strength, buffer composition, and gel concentration are employed to enhance resolution. (The precise reasons for this enhancement are complicated, and the interested reader is directed to the references at the end of the handout.) To achieve the discontinuity, a “stacking” gel is poured directly on top of a “resolving” gel. The stacking gel contains less polyacrylamide and has a lower pH and ionic strength than does the resolving gel—that is, the two gels are discontinuous. The low polyacrylamide concentration of the stacking gel results in large pores and hence relatively free migration of the charged molecules, whereas the extensive crosslinking within the resolving gel results in small pores, which retard the migration of molecules according to their molecular weight. Thus, the large and small proteins have different mobility through the resolving gel and can be separated (see figure on p. 34).
The mobility of the protein in the electric field as a function of molecular weight can be analyzed quantitatively. In our experiments, we will denature the proteins with the anionic detergent, sodium dodecyl sulfate (SDS), as well as a disulfide reducing agent, β-mercaptoethanol. To insure full denaturation, the samples will also be heated in a boiling water bath. Under these conditions, we can expect most proteins to unfold. The exposed protein backbone becomes wrapped in the anionic detergent, which imparts a net negative charge to the protein in direct proportional to the protein’s molecular weight (approximately one molecule of SDS binds per two amino-acid residues in a protein). Since a given unit of polypeptide backbone treated with SDS carries the same charge, all proteins treated with SDS have the same charge density (the charges of the side groups are assumed to be negligible compared to the charges from the SDS). The ion front of chloride ions will be marked with a “tracking dye,” bromophenol blue, included in the sample buffer. The proteins mobility is measured by calculating its \( R_f \) (the “ratio of fronts”—i.e., the distance from the top of the gel to the polypeptide divided by the distance from the top of the gel to the dye front). Proteins of known molecular weights are run as standards, and the logs of their molecular weights (logM) are plotted versus their \( R_f \) values to generate a standard curve. The \( R_f \) of the unknown polypeptide is measured, and the corresponding logM is read directly from the standard curve.

![Graph](image)

The center of this region can be well approximated by a straight line, which we shall do. The molecular weight range over which this approximation holds depends on the pore size, and thus on the polymer and crosslinker concentrations. These must be adjusted to fit the proteins under study in any given experiment.

**SPECIFIC PROCEDURES**

To begin, you will pour the gel and prepare the protein samples. Both should progress simultaneously, so organize yourselves. Each group will have a gel to itself, and each electrophoresis box runs two gels simultaneously. Since two gels are cast on a single gel-casting stand, you should coordinate the pouring and especially the sample loading with your companion group. Samples should be loaded into the sample wells in the gels immediately prior to connecting to the power supply to avoid diffusion of samples.

A. Assembling the Gel Apparatus:
   The glass plates for the gels must be scrupulously clean for the gel to form properly, and they are provided as such by your TA. Handle them carefully by the edges so that you don’t get fingerprints (wearing gloves will help with this) on them. Follow the instruction sheet (to be
handed out in lab) for the Bio-Rad Mini-Protean 3 assembly.

1. Place a Short Plate on top of a Spacer Plate.
2. Slide the plate sandwich into the Casting Frame, short plate forward. Make sure both plates are flush at the bottom.
3. Lock the pressure cams by pushing the “wings” fully outward.
4. Place the cassette into the Casting Stand by means of the top spring lever. Make sure all is snug against the gray rubber gasket in the bottom of the stand.

B. Pouring the Gel:

As discussed above, the gel will consist of both a resolving gel (bottom) and a stacking gel (top). Both the resolving and stacking solutions contain acrylamide, which is a potent neurotoxin: once you start working with the gel solutions you must WEAR GLOVES AT ALL TIMES. Polymerization begins when ammonium persulfate (APS) is added to the gel solution; therefore, make sure that you are completely ready to pour the gel immediately after addition of the APS.

1. Resolving Gel - 12% acrylamide. We have prepared for you 10 mL of “Solution A”:
   - 12% 37.5:1 Acrylamide:Bis (w/v)
   - 375 mM Tris-HCl, pH 8.8
   - 0.1% Sodium dodecyl sulfate (SDS) (w/v)

   When you are ready, add:
   - 5 µl TEMED
   - 50 µl freshly dissolved 10% APS

   Immediately mix the complete solution by gently inverting the tube a few times, and pour between the plates; pouring is easier than it looks, but tip the plates if you wish. Pour to a height 1” from the top of the Spacer glass plate—that is, to a point even with the top of the “green wings.” (To help you decide when the gel has polymerized, monitor the excess gel solution left in the tube.) Carefully add a layer of t-amyl alcohol without disturbing the gel surface—this will promote a smooth interface between the stacking and resolving gel. When you are certain the gel has polymerized (35 to 45 minutes) tilt the apparatus to pour off the alcohol. (Make sure that you don’t disturb the gel next to yours when you do this; remove your cassette from the stand if necessary.) Rinse the gel surface by with ddH2O, and remove excess water fairly thoroughly (kimwipe).

2. Stacking Gel - 4% acrylamide. We have prepared for you 10 mL of “Solution B”:
   - 4% 37.5:1 Acrylamide:Bis (w/v)
   - 126 mM Tris-HCl, pH 6.8
   - 0.1% SDS (w/v)

   When you are ready, add:
   - 12 µl TEMED
   - 60 µl 10% APS from above

   Mix as before, and pour as before to the top of the short plate. Insert the comb slowly, displacing excess acrylamide solution onto a paper towel to contain the mess. Allow 30 minutes for polymerization, observing the left-over mixture saved in the centrifuge tube.
When the stacking gel has polymerized, slowly remove the comb from the gel, pulling straight upwards to avoid disturbing the sides of the wells. Remove your gel sandwich from the stand, and rinse the wells a few times under distilled H$_2$O. Pour off the rinse each time. Place your gel sandwich into the electrophoresis cell, following the Bio-Rad instructions.

1. Place the sandwich into the Electrode Assembly, with the short plate facing in. Once both groups have done this (or using a “dummy” sandwich if you are alone),
2. Slide the two gels + the electrode assembly together, as one snugly held array, into the Clamping Frame.
3. Pressing down on the complete array, close the two cam levers on the frame.
4. Lower the entire inner assembly into the Mini Tank. Fill the inner chamber with 125 mL of Running Buffer. Fill the outer chamber with 200 mL Running Buffer. Running buffer should now fill each of your sample wells.

C. Protein Sample Preparation, to be done during the two polymerization periods:

You will load eight samples onto your gel: the four aliquots, the three dialyzed column fractions (load and wash, adduct eluted, and urea wash), and a standard solution containing several proteins of known molecular weight. 50 µg of protein should be loaded into each lane of the gel—from your Bradford data, calculate the volume of each sample required to give the 50 µg of protein (time spent doing these calculations before you come to lab will reduce the time spent in lab); your lab instructor will inform you of the concentration of the molecular weight standards. In ten labeled microfuge tubes, combine the requisite volume of each of your protein samples with enough distilled H$_2$O to yield a final volume of 25 µL. If the concentration of a sample is less than 2 µg/µL, just use 25 µL of the sample. Then add 25 µL of (two times concentrated) sample buffer to each tube. The sample buffer contains β-mercaptoethanol, which is odoriferous and poisonous. WEAR GLOVES (and work in the fume hood if you wish) unless you want to be smelling rotten eggs for the rest of the day. After capping and mixing each sample, immerse them in boiling water for 2–3 minutes.

D. Sample Application:

When both groups using a single box have positioned their gels in the electrophoretic cell and prepared the protein solutions, layer 20 µL of each sample into the wells using a micropipettor and the special elongated tips; the sample buffer contains glycerol so that the samples will sink to the bottom of the wells. The plastic loading guide does help. Change tips after each sample addition. So as to orient the gels once they have been stained, load the molecular weight standards in the well farthest to the left. Load the all the lanes without delay (to keep mixing and diffusion to a minimum), but neatly and carefully to minimize cross-contamination.

E. Running and Staining:

When all the samples have been layered, the cell lid will be closed and power applied. Gels are run at 200 volts: amperage will range from 90 mA (for 2 gels) at the start to 60 mA near the end. It is interesting to watch carefully for a few minutes to see those proteins stack! Run the gels until the Bromphenol blue dye has reached the bottom green strip (about 35 minutes). Disassemble the gel sandwich and carefully place your gel in the Coomassie blue staining
solution (your instructor will show you how to do this). Give the gel (in a clearly labeled container) to your TA, who will (after an overnight staining incubation) destain it, and email an image of the gel to you for your notebook and lab write-up.

F. Molecular weight standards:

97,400 Phosphorylase B
66,200 Bovine serum albumin
45,000 Ovalbumin
31,000 Carbonic anhydrase
21,500 Soybean trypsin inhibitor
14,400 Lysozyme

G. Solution Compositions:

1. Resolving gel (Solution A), premixed for you. Just add APS and TEMED.
   3.4 mL dd H₂O
   4.0 mL 30% Acrylamide/Bis-acrylamide mixture
   2.5 mL 1.5M Tris, pH=8.8 with HCl
   0.1 mL 10% SDS

2. Stacking gel (Solution B), also premixed for you. Just add APS and TEMED.
   6.1 mL ddH₂O
   1.3 mL 30% Acrylamide/Bis-acrylamide mixture
   2.5 mL 0.5 M Tris pH 6.8
   0.1 mL 10% SDS

3. Sample buffer (Premixed. This is “twice concentrated.” Use 1:1 with samples.)
   20% Glycerol (v/v)
   4% SDS (w/v)
   150 mM Tris-HCl, pH 6.8
   0.2% Bromophenol blue (w/v)
   8% β-mercaptoethanol (v/v)

4. Running buffer
   25 mM Tris Base
   0.1% SDS (w/v)
   200 mM Glycine
   (Buffer pH~8.3 Do not adjust. Solution must be free of Cl⁻ to allow stacking.)

5. Stain
   0.1% Coomassie blue (w/v) in 4:1:5 (v/v/v) methanol, acetic acid, H₂O

6. Destain
   4:1:5 (v/v/v) methanol, acetic acid, H₂O
The write-up for experiment V is due on Monday, April 27. It is a long and complicated write-up. To make the process less daunting, the write-up is broken into different parts that can be completed independent of one another (weeks 1–2, 3, and 4). It is highly recommended that you complete the sections as you go—and not wait until the end of the four weeks to write everything up.

LDH Week 1 and 2 Write-up Instructions and Hints

Note: When using data gathered by your lab partner(s), you must include your own calculations. Please see the course’s “Organization Details” handout under “Intellectual Responsibility,” and if you are unclear on these intellectual-responsibility requirements, ask.

Preparation of the tables described below and answers to questions 1-3:
Your Weeks 1–2 write-up should include tables with data for each of the solutions from which you took aliquots 1–4 and each of your batches from the affinity column. As a reminder, your column batches include one from the load and wash; one from each of the three LDH fractions eluted with the adduct; one from the pooled LDH fractions; and one from the urea wash—six samples in total. The tabular data should contain:

I) the total units of LDH (units = μmol/min)
II) the total milligrams of protein
III) the specific activity of LDH. (units/mg)

I. Total Units of LDH:
   Note: If you’re so inclined, feel free to save some number-crunching time and set up an Excel spreadsheet to carry out these calculations. You must, however, show one sample calculation with all of the relevant units in your notebook.

Step 1: First, calculate the total units of LDH in the cuvette. A unit of LDH will turn over one μmol of substrate per minute. MAKE SURE THAT YOUR UNITS WORK OUT!

Helpful hints and reminders:
- OD is unitless
- the conversion factor is 6.22 OD_{340}/(mM NADH → NAD^+)
- 1 mM = 1 μmol/mL
- your total volume of solution in the cuvette was 1510 μL

Step 2: Calculate the total units of LDH in the entire sample.
Your table of data (or spreadsheet) will look something like this:

<table>
<thead>
<tr>
<th>Aliquot</th>
<th>dilution factor (if any)</th>
<th>ΔOD_{340}/min</th>
<th>cuvette volume</th>
<th>units assayed</th>
<th>volume of sample assayed</th>
<th>total sample volume</th>
<th>total units in sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<tr>
<td>L&amp;W</td>
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<td>A1</td>
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<td>A2</td>
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<td>A3</td>
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<tr>
<td>Urea</td>
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<tr>
<td>POOL</td>
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</tr>
</tbody>
</table>

This procedure needs to be adjusted if you had to dilute the aliquot in order to get a reasonable ΔOD_{340}/min. In that case, you need to include a dilution factor (10, 50, 100) if the activity of the neat aliquot was too high. Of course, if no dilution was made, the dilution factor in the above table would be “1.”

II) Total milligrams protein from Bradford assays

Remember that this assay is designed to measure total protein concentration and is not specific for LDH. One compares the OD_{595} of the aliquots to the standard curve to determine the micrograms protein in the cuvette. (To generate your standard curve, you may fit your data to derive an equation using a computer graphing program; or you may simply graph your data on paper, draw a line through it, and determine protein concentrations from the line by inspection.) Dividing the micrograms of protein in the cuvette by the small volume of aliquot assayed yields the concentration of protein in that aliquot. To calculate the total micrograms protein in the entire biological sample, one needs to multiply this concentration by the volume of the sample. Again, remember to take into account any dilution factors that were made.

**MAKE SURE THAT YOUR UNITS WORK OUT!**
Your table of Bradford data should look something like this. Again, please show at least one sample calculation with all of the relevant units:

<table>
<thead>
<tr>
<th>Aliquot</th>
<th>dilution factor (if any)</th>
<th>volume of sample assayed</th>
<th>OD₅₉₅</th>
<th>total protein assayed</th>
<th>conc. of protein in sample</th>
<th>sample volume</th>
<th>total protein in sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>Column</td>
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<td>L&amp;W</td>
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<tr>
<td>Urea</td>
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<tr>
<td>POOL</td>
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</tr>
</tbody>
</table>

III. Calculate the specific activity of LDH
For each aliquot and column fraction, divide the Total Units of activity by the total milligrams of protein.

**Question 1:** How does the specific activity of your column-purified enzyme compare with that given in the “Handy LDH information” below? **Answer:** It is likely lower. Why?

**Question 2:** Do the data make sense—for example, is the enzymatic activity where you expect it to be?

**Question 3:** How well did your purification work? **Discuss the efficacy of each step.**

**Handy LDH Information**
(From the 2000 Sigma Chemical Company Catalog)
LDH Week 3 Write-up Instructions and Hints

Generate a Lineweaver-Burk plot by plotting $1/V_{\text{initial}}$ vs. $1/[S]$. For the latter number, remember that a change of 6.22 OD$_{340}$/min corresponds to 1 mM/min. Your plot will provide $K_{M}^{\text{pyr}}$ and $V_{\text{max}}$. For this plot, please use a graphing program (Excel will do). Report the equation for the line and the square of the correlation coefficient ($r^2$). If you are a graphing-program whiz, you may, of course, fit your $V_{\text{initial}}$ vs. [S] data directly to the Michaelis-Menten equation (equation 27 on page 219 of Stryer); you will get the same answers, just without a linear fit.

**Question 4**: How does your $K_{M}^{\text{pyr}}$ compare with the literature value (0.32 mM for the $M_4$ isozyme, 0.08 mM for the $H_4$ isozyme)? Suggest an explanation for any discrepancy.

Using your Bradford data on the pooled, purified LDH and the molecular weight per subunit from your gel (see below), and assuming that your enzyme is completely pure, determine the $k_{\text{cat}}$ for a LDH subunit from the equation $V_{\text{max}} = k_{\text{cat}}[E_T]$. Also determine the $k_{\text{cat}}$ using the known subunit molecular weight of 35,500. **Note**: $[E_T]$ is, of course, the enzyme concentration in the cuvette for each of your kinetic runs in week 3, not in your stock solution (again, you may assume that the stock is pure LDH). Remember, the enzyme concentration was the same in every assay—only pyruvate concentration varied.

**Question 5**: How does your value for $k_{\text{cat}}$ compare with the literature value (approximately 500 s$^{-1}$ per subunit for both the $M_4$ and $H_4$ isozymes)? Suggest an explanation for any discrepancy.

Also, provide a recalculation of the specific activity of your LDH from your $k_{\text{cat}}$ value (just to be sure that you have become a kinetics expert). How is this done? $k_{\text{cat}} = (\text{molecules substrate turned over/s})/\text{molecule LDH} = \mu\text{mol substrate turned over/s} / \mu\text{mol LDH} \Rightarrow (\mu\text{mol substrate turned over/s})/\text{mg LDH} \Rightarrow (\mu\text{mol substrate turned over/min})/\text{mg LDH} = \text{units/mg LDH} = \text{specific activity.}$ For the purposes of the above calculation you may assume each subunit of LDH is catalytically identical and hence may simply use the subunit molecular weight to convert from moles of LDH into mass of LDH.

**Question 6**: How does this recalculated value compare with that you determined in question III (p. 37)—that is, with the value you determined directly from your Bradford and kinetic assays? Account for any discrepancy.
LDH Week 4 Write-up Instructions and Hints

Analyze your gel and answer the questions below. Include a copy of your SDS-PAGE gel and a standard curve derived from it in your lab notebook (the curve may be generated by hand, on graph paper, or using a graphing program). The molecular weights of the standards are given in the lab handout. Remember, for your standard curve plot log M vs. Rf.

**Question 7:** What is the molecular weight of your LDH subunits? How pure does your “purified” enzyme seem to be? Based on your estimated purity—emphasis on “estimated”—calculate your total yield of purified LDH, as well as the yield of LDH per gram of chicken tissue. Comment on the seven lanes derived from the four aliquots (1–4) and three column samples (load and wash, pooled adduct-eluted LDH, urea wash)—i.e. does the preponderance, or dearth, of bands in each lane make sense? That is, you should cross-reference your answer to your tables from last week.

**References**


