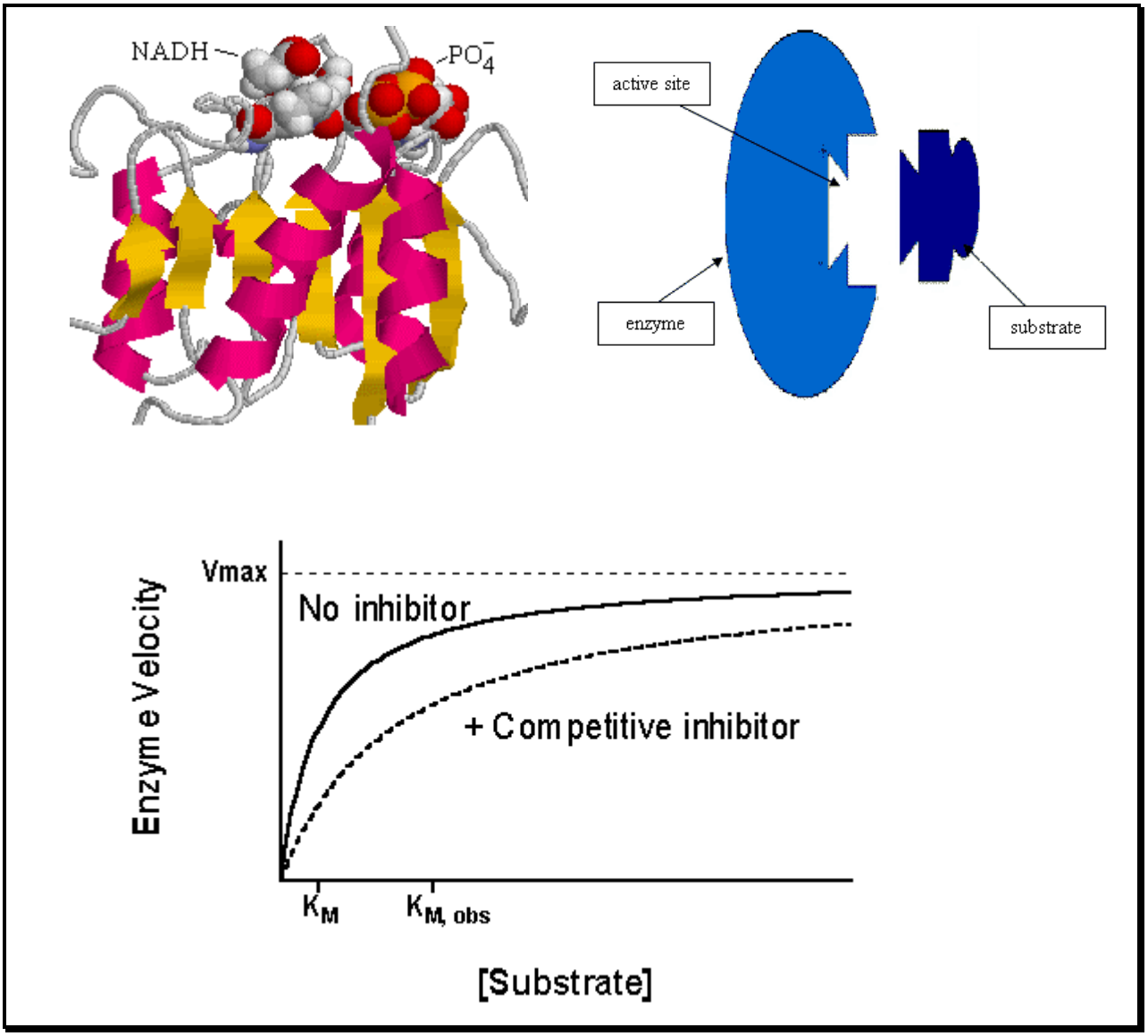
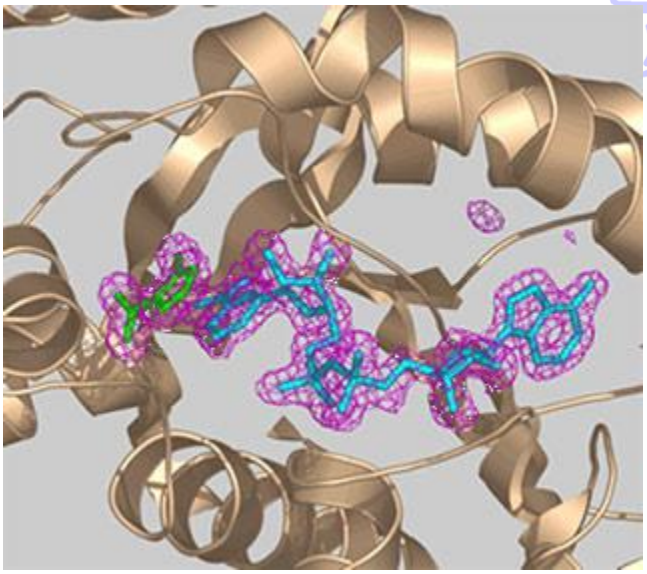
Biochemistry Laboratory Manual



Chemistry Department
Peru State College
Peru, NE 68421
Fall 2013

Semester Schedule and Point Allocation per Lab

Date:	Name of Laboratory	Points
Aug 23-25	Introduction: Calculations handout	10
Sept 1	Use of Pipettors – Acurracy vs Precision	20
Sept 8	Isolation of Casein	25
Sept 15	Acid-Base Behavior of an Amino Acid	25
Sept 22	Protein Determination: Bio-Rad	30
Sept 29	Size Exclusion Column Chromatography	30
Oct 6	Isolation & Characterization of LDHase	75
Oct 13	- Protein Deter. of LDHase	
Oct 20	- SDS-PAGE of LDHase	
Oct 27	- Kinetics of LDHase	
Nov 3	LAB EXAM & Independent Research on LDHase	50
Nov 10	Independent Research on LDHase	-
Nov 17	Independent Research on LDHase	-
Nov 24	Fall Break	
Dec 1	Independent Research on LDHase	
Dec 1 – 2 – 8 - 9 (?)	Independent Research Report & Poster Presentation on LDHase	100
	Total Points	365



Structure of Lactate Dehydrogenase (LDHase)

New drugs for malaria are being developed at the Department of Biochemistry, by Professor Leo Brady's group, at the University of Bristol. The crystal structure of the cofactor NAD (blue) and an inhibitor (green) bound to lactate dehydrogenase from Plasmodium falciparum, the parasite responsible for the most lethal form of malaria.

Note to Student,

Welcome to the Laboratory Manual for Chemistry 431. In the annual process of selecting laboratory textbook for this course I have never been completely satisfied with available titles. Therefore, I have undertaken the task of writing a manual that will directly apply to this laboratory section. Throughout the semester, if you find any component of this manual requiring some revision, please let me know, your information will help improve this manual.

This manual was written with the assumption that you the student is proficient or knowledgeable in the following areas: (Remember this is your 5th chemistry course at least)

1) All safety rules and regulations of the laboratory.
2) Proper cleaning of glassware. (without use of acid cleaning solution)
3) Proper disposal of broken glassware.
4) Proper disposal of chemical waste.
5) Use of equipment such as:
    a. Meltpoint apparatus
    b. Volumetric Pipets
    c. Burettes
    d. Organic glassware kits
    e. FTIR, UV-VIS, chromatography
    f. And many others
6) Use of Merck Index and CRC Handbook of Chemistry and Physics.

You will find this laboratory course to be different from all others that you have taken.

1) Primarily you will find yourself relying on information about science you may have learned in a different area.
2) You will be making your own solutions, and they will be in small quantity.
3) Beginning in November you will, by definition, be undertaking a project of your own design. Your work will be selectively different from all other past, present, and future students.

I hope you find this course to be rewarding and this manual two be a helpful guide.

Dr. Dennis W. Welsh
Peru State College
Peru, NE 68421

Peru State College Laboratory
Safety & Laboratory Information

1.) Read the experiment before coming to the laboratory session. It is important that you have a firm grasp of this material as the time available for you to carry out the experiment is limited. Lack of preparation will include but not be limited to the lose of points.

2.) No unauthorized experiments are allowed.

3.) To do an experiment, have at least one other person in the laboratory with you.

4.) Keep your desk and apparatus neat and clean. Do not discard any chemicals down the sink unless instructed to do so. At the end of the laboratory period, clean and put away all apparatus, and wash and dry the desktop. This will be graded.
You will need to provide dishwashing soap and a towel for your lab drawer.

5.) Never leave spilled chemicals or water on a desktop or floor. Clean up immediately.

6.) Wash your hands thoroughly when exiting and entering the lab. Biochemical experiments are very sensitive to contamination & the chemicals can harm you without any symptoms being apparent.

7.) Record your observations and results directly in your notebook immediately after you obtain them. **Do not** put them on odd pieces of paper.

8.) The chemical reagents necessary for the experiments will be made available in labeled bottles placed in the hoods or in the appropriate location.
   a.) Read the label carefully before removing the material.
   b.) **Never return** unused chemicals to the stock bottles. This may lead to contamination of the entire supply of reagent.
   c.) Be careful not the mix stoppers or caps.
   d.) Return reagent bottles to proper place.
   e.) If a solution should be kept cold make sure and place the container in the refrigerator.

9.) Leave any food, beverages outside the lab. Never drink water or eat ice in or from the laboratory. Never taste a chemical. **Never mouth pipette**.

10.) Know the properties (i.e., flammability, toxicity, and reactivity) of the chemicals used. All hazardous chemicals must be handled in the fume hood.

11.) Do not use cracked or broken glassware.

12.) Consult the teaching assistant before using any unfamiliar equipment.

13.) Do not remove any chemicals or equipment from lab without specific permission from Dr. Welsh.

14.). Learn proper exit routes from the building for a fire or other emergency. Know where fire extinguishers, first-aid kits, showers and eye wash stations are located in the laboratory and/or building and be familiar with the guidelines for their use.

15.) Report any injury, no matter how slight, to your instructor.

16.) If you have any health conditions, which may impact your presence or work in the lab, inform Dr. Welsh.

17.) Never pour water into conc. acid. Pour the acid slowly into the water with stirring. (In a fume hood).

Biochemistry Laboratory

Calculations Handout

The biochemistry laboratory requires the use of many different chemicals and reagents. As with any laboratory the proper preparation of these reagents are vital to the success of experiments. The difference in the biochemistry laboratory from your previous experiences is that biochemicals are generally expensive and used in very small quantities.

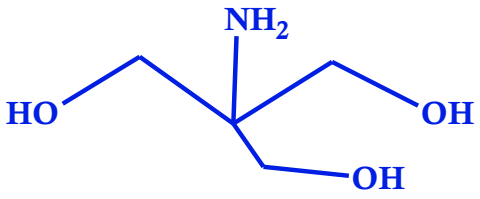
This handout is designed to test you ability to properly prepare solutions (buffers) that we will be using this semester. Show your calculations.

EXAMPLE: BSA (Bovine Serum Albumin) is the most abundant protein found in cattle. BSA has a molecular weight of 66000 g/mol. Describe in detail how to prepare 100 mL of a 0.3 mg/mL solution of BSA.

ANSWER: I would measure 30 milligrams of BSA and place it into a 100 mL volumetric flask. I would then fill the volumetric flask to the mark with water.
Note: Concentration expressed as mg/mL does not require the use of the molecular weight.

Questions #1: Describe in detail how to prepare a 250 mL solution of 0.1 M acetic acid from 12 M concentrated acetic acid.

Questions #2: Tris (or TRIZMA) is an abbreviation of the chemical:
[Tris(hydroxymethyl)aminomethane]
or
2-amino-2-(hydroxymethyl)-1,3-propanediol



Tris has a molecular weight of 121.1 g/mol and when dissolved in H_2O will result in a basic solution of pH ≅ 10.5

a. Circle the removable protons on the figure of trizma above.

b. Describe in detail how to prepare 1 L of 0.5 M Tris buffer solution at pH 7.5. Do not forget to use either HCl or NaOH to adjust the pH to 7.5.

c. How would you arrive at the same results with the Henderson-Hasselbach equation?

Question #3: We will need a 0.05 M Tris-HCl (pH 7.5) buffer solution for the column chromatography lab. Describe in detail how to prepare 2L of this buffer by diluting the buffer in question #1.

Question #4: We also will need a 0.1 M NaCl in 0.05 M Tris-HCl (pH 7.5) buffer solution for the column chromatography lab. YES, two chemicals in the same solution. Describe in detail how to prepare 2L of this buffer again using the buffer in question #1.

Question #5: Describe in detail how to prepare 5 mL of a 15% (v/v) glycerol solution containing all of the following protiens: 3 mg/mL "Blue Dextran", 8 mg/mL myoglobin, and 0.3 mg/mL DNP-aspartate.

Calculation and Solution Preparation

You will be responsible for making the following solutions. You MUST show me your calculations before beginning to prepare your solutions.

You will be graded on your:

Calculations
Accurate solution preparation
Timely solution preparation
Proper labeling of solution (ID of solution, Your Name, Date) See example below.
Proper storage of solution (throughout the semester)
Clean laboratory

Group A	**Group B**	**Group C**
250 mL of 0.5 M Tris HCl – pH 7.	100 mL of 10% (w/v) NaOH	100 mL of 0.5% (w/v) $CuSO_4 \bullet 5H_2O$
50 mL of 2% (w/v) BSA	250 mL of 0.01 M HCl	250 mL of 5 M HCl
50 mL of 0.01% (w/v) Tyrosine	500 mL of 0.25 M KOH	200 mL of 0.01 M Sodium Citrate – pH 6.6
50 mL of 2% (w/v) Proline	100 mL of 0.3 mg/mL BSA	100 mL of Million reagent (Casein Lab)
550 mL of 2% (w/v) Gelatin	250 mL if 0.3 mg/mL Ovalbumin	500 mL of 0.1 M acetic acid
Degases Distilled H_2O	1 L of 200 mM Tris HCl – pH 8.6	50 mL of 2% (w/v) Glycine

Example Label

2% Gelatin
(Refrigerate)

D. Welsh
Biochem. Lab. 8-17-07

Use of Pipettors
(Accuracy vs Precision)

INTRODUCTION

Success in the biochemistry lab will come with your ability to choose and use various devises for measuring volumes. Accuracy and precision is fundamental in the field of biochemistry because reproducibility is a must and measured volumes are extremely small. You are already family with how to read accuracy of simple laboratory glassware (i.e. graduated cylinder) and the more complicated buret. You are now going to begin use with a new measuring device known as an adjustable pipette.

As you learn to use these adjustable pipettes, remember the goal is to strive for two things:

Accuracy – the relation between the volume you dispense and the volume you wanted to dispense.

Precision – the relation of the reproducibility of your measearment.

EXAMPLE

If you are supposed to dispense 100 µL of water into 5 test tubes, and what you actually dispensed is 89.9 µL, 90.0 µL, 89.8 µL, 89.9 µL, then your volumes are very inaccurate but very precise.

Many of the mechanical pipettes (including the Oxford brand) are famous for their precision, but their accuracy depends greatly on proper technique and how often they are calibrated.

Determining accuracy of a Pipette.

It is very easy to establish the accuracy of a pipettor. Simple pipette a known volume of degassed water onto a balance and weight it. With the digital top loading balance in our laboratory, this will take only a few minutes and could save you hours of confusion later.

How to use a Pipette.

The directions of the Oxford adjustable pipette are included and should be thoroughly read. To operate the pipette you will be using the "FORWARD MODE"

Safeguards for a pipette.

1) These measuring pipettes are very expensive and should only be used by properly trained persons.
DO NOT ALLOW NON-BIOCHEMISTRY STUDENTS TO USE THESE PIPETTES.

2) If at anytime a pipette is dropped or for any reason you suspect the pipette is not functioning properly **INFORM YOUR INSTRUCTOR IMMEDIATELY**.
You will then be asked to re-determine to accuracy of your pipette.

3) Keep the pipette clean and unclogged at all times. If you feel excessive resistance when you depress or release the plunger the pipette is clogged and needs to be cleaned, **INFORM YOUR INSTRUCTOR IMMEDIATELY**.

Experimental Procedure

In this experiment you will learn to use the Oxford adjustable pipettes of various sizes and measure their accuracy, precision, and calibration.

Pre-Lab Questions

1) FIll in the blanks _____ μL = 250 mL

2) What should 250 μL of water weigh? _____grams

3) Is the following set of measurements precise? YES NO

Is the following set of measurements accurate? YES NO

Using a 200 μL to 1 mL pipette attempting to measure 500 μL the mass of water equaled: (note the grams measurement)

0.551 g 0.525 g 0.448 g 0.476 g 0.502 g Average = 500.4 g

4) Why should you only use degassed water for this experiment?

Procedure:

1) Acquire the proper of the Oxford pippetor and appropriate tip for a 150 μL volume.

2) Set the pipettor to 150 μL.

3) Place a weighing boat or watch-glass on the balance and tare the weight to zero.

4) Draw up the designated volume according to the "forward" mode directions and dispense it onto the weighing boat or watch-glass. Record the weight of the water to four significant figures.

5) Repeat this procedure three more times.

6) Calculate the % error between the average of the four trials and the true value

$$\% \text{ error} = \frac{|\text{Average weight} - 0.150\ \text{g}|}{0.150\ \text{g}} \times 100 = \_\_\_\_\_$$

(Remember the goal is < 1% (as per manufacture specifications)

7) Calculate the mean deviation for the four trials:

$$\text{Mean deviation} = \frac{\sum \left| x_i - x_{average} \right|^2}{4} = \_\_\_\_\_$$

8) Repeat steps 1-7 for the volumes of 45 μL and 645 μL.
9) Repeat steps 1-7 for the assignment volumetric flask, graduated cylinder, and volumetric pipette.
10) Identify the most accurate and precise methods used.

Insert

OXFORD Adjustable pipette Handout

Here

Isolation and identification of Casein

INTRODUCTION

Milk contains members of each of the five basic food groups: vitamins, minerals, carbohydrates, proteins and lipids. Milk is nutritionally complete and consumed in many different forms. The majority of milk that humans consume comes from cattle. The composition of cattle milk is

Component	Water	Protein	Fat	Carbohydrate	Mineral
% Composition	87.1%	3.4%	3.9%	4.9%	0.7%

Milk contains three different forms of globular proteins: caseins, lactalbumins and lactoglobulins. Globular proteins fail to interact with themselves and form colloid suspensions more easily than fibrous proteins, the other general class of protein. Therefore globular proteins can be more easily purified. In this experiment, casein will to be extracted from the fresh milk. Casein is a mixture of three similar proteins, called the alpha, beta, and kappa caseins. Casein is a phosphoprotein, a protein that has phosphate groups as members of its amino acid side chains. The primary difference in the three forms of casein is the number of phosphate groups contained in the protein. The protein exists in micelles in the form of a calcium salt. Calcium caseinate has an isoelectric point at a pH = 4.6. The salt is insoluble at a lower pH; therefore, lowering the pH of milk from its normal value of 6.6 to below 4.6 by the addition of acid will neutralize the casein salt. Casein precipitates out of the acidic milk. This principle is illustrated when milk sours.

Therefore, the precipitation of casein is a result of two phenomenons:

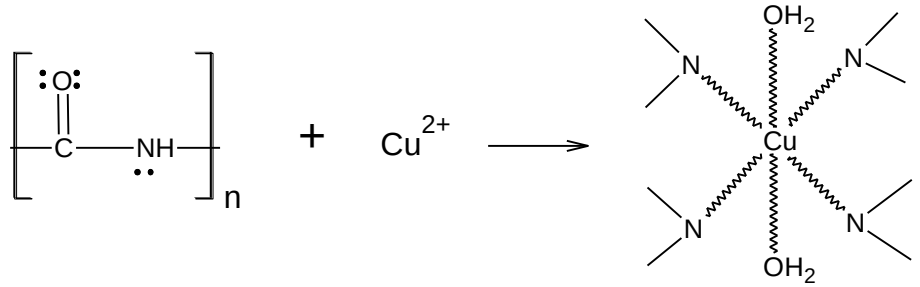
1) The lowering of the pH changes an organic salt to a less soluble organic molecule.
2) The lowering of the pH to the isoelectric point minimizes the charges on the protein, thus lowering the interaction of the protein with the polar water molecules.

In the first part of the experiment, you will be isolating casein by lowering milk pH to 4.6. The fat that precipitates along with casein can be removed by dissolving it in alcohol.

In the second part of the experiment, you are going to prove the precipitated product is a protein. The identification will be achieved by performing some important chemical tests.

The Biuret Test

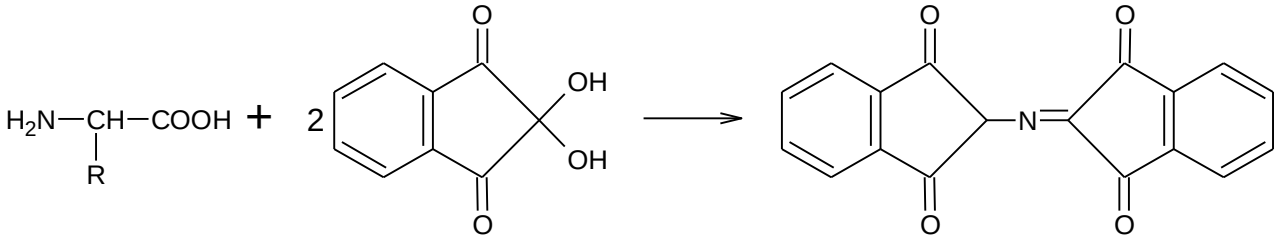
This is one of the most general tests for proteins. When a protein reacts with copper (II)sulfate, a positive test is the formation of a copper complex which has a violet color.



Protein Blue color Protein-copper complex (violet color)

The Ninhydrin Test

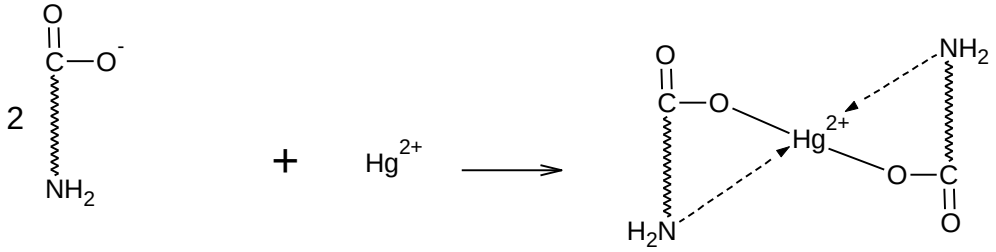
Amino acids with a free $-NH_2$ group and/or proteins with amino group side chains react with ninhydrin to give a purble-blue complex.



+ RCHO + CO_2 + $3H_2O$

Million Test

Heavy metal ions precipitate proteins from solution. Although many di- and tri-valent ions can cause precipitation, Hg^{2+}, Cd^{2+} and Pb^{2+} are infamous toxins to human. They can cause serious damage to proteins by denaturing them. This can result in death. The precipitation is a result of proteins becoming cross-linked by the heavy metals. The Million's test (shown below) is specific for the hydroxy-phenyl group of tyrosine and will result in a red precipitate.



insoluble precipitate

Xanthoproteic Test

This is a characteristic reaction of proteins that contain phenyl rings. Concentrated nitric acid reacts with the phenyl ring to give a yellow-colored aromatic nitro compound (ortho position). Additional alkali at this point will deepen the color to orange.

Example:

Tyrosine + Nitric Acid ⟶ *o*-nitro-tyrosine + H_2O

PROCEDURE

Part A – Isolation of Casein

1. To a 250-mL Erlenmeyer flask, add approximately 50 g of 2% milk. Record the mass to the nearest $1/100^{th}$ of a gram. Heat the flask in a water bath while stirring the milk. When the milk temperature has reached 40°C, remove the flask from the water bath, and add 10 drops of glacial acetic acid while stirring.

2. Filter mixture by pouring it through 4 layers of cheesecloth held in a 100-mL beaker. Rinse the Erlenmeyer flask with 0.1 M acetic acid if necessary. Remove most of the liquid from the solid (casein and fat) by squeezing the cloth gently. Discard the filtrate.

3. Place the solid into a 100-mL beaker and add 40-mL of 95% ethanol. After stirring the mixture for 5 min., allow the solid to settle. Carefully decant the liquid containing the fat into a beaker. Discard the liquid.

4. To the residue, add 15-mL of 95% ethanol and10-mL diethylether. After stirring the mixture 5 min., collect the solid by vacuum filtration using pre-weighed filter paper.

5. Allow the casein to dry, weigh it, and calculate the percentage of casein in the milk.

Part B – Chemical Analysis of Proteins

1. For the chemical analysis prepare the following solution:

Casein Solution – 0.2 g mixed with 10 mL liquid (you decide the best liquid from the list below):

a) distilled H_2O
b) 0.01 M HCl,
c) 0.01 Sodium Citrate – pH 6.6

The biuret test – Place 15 drops of each of the following solutions in six clean, labeled test tubes.

2% glycine	2% gelatin	2% bovine serum albumin (BSA)
0.01% tyrosine 2%	proline	casein solution

To each of the test tubes, add 5 drops of 10% NaOH solution and 2 drops of 0.5% $CuSO_4$ solution. Mix well and record your observations.

The ninhydrin test – Place 15 drops of each of the following solutions in six clean, labeled test tubes.

2% glycine	2% gelatin	2% bovine serum albumin (BSA)
0.01% tyrosine	2% proline	casein solution

To each of the test tubes, add 5 drops of ninhydrin reagent* and heat the test tubes in a boiling water bath for about 5 min. Record your observations.

The Million test – Place 15 drops of each of the following solutions in six clean, labeled test tubes.

2% glycine	2% gelatin	2% bovine serum albumin (BSA)
0.01% tyrosine	2% proline	casein solution

To each of the test tubes, add 5 drops of Million's reagent** and heat the test tubes in a boiling water bath for about 2 min. Record your observations.

The xanthoproteic test – Place 15 drops of each of the following solutions in six clean, labeled test tubes.

2% glycine	2% gelatin	2% bovine serum albumin (BSA)
0.01% tyrosine	2% proline	casein solution

To each of the test tubes, add 10 drops of concentrated HNO_3 (UNDER THE HOOD) with swirling. Heat the test tubes in a boiling water bath for about 2 min. Record your observations.

* Ninhydring reagent = 200 mg ninhydrin in 200 mL n-butanol (water saturated)
** Million reagent = 2.2% $Hg(NO_3)_2$ in 5.3 M HNO_3

PRE-LAB QUESTIONS

1) Casein has an isoelectric point of 4.6. What is the charge of casein in its native environment, milk?

2) How do you separate the fat from the protein in the casein precipitate?

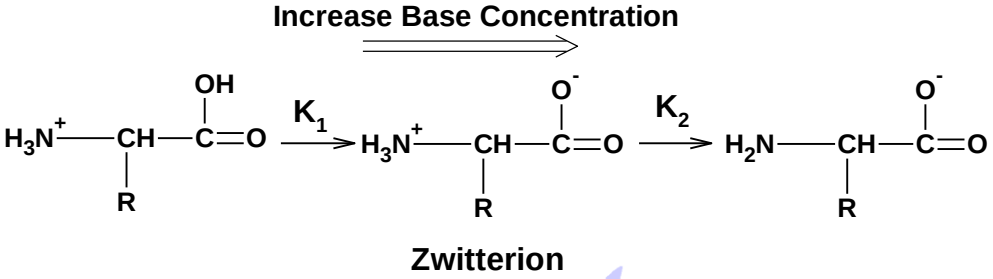
3) To make the casein solution in Part B which liquid have you chosen? Explain your answer.

4) Write the Lewis structures for the reaction shown for the xanthoproteic test in the introduction of this laboratory write-up.

Acid-Base Behavior of an Amino Acid

INTRODUCTION

Amino acids serve as a basic unit for molecules that are important in biochemistry such as peptides or enzymes. They have both a basic amino group, NH_2, and an acidic carboxylic acid group, COOH. If the amino acid is dissolved in water, it largely exists as the zwitterion, containing both positive and negative charges. If the zwitterion is treated with acid, that is, a solution containing H^+, the H^+ will add to the COO^- to form COOH. Similarly, treating the zwitterion with base will result in the loss of the removable proton attached to the NH_3^- group to form NH_2, The following pH-dependent equilibrium can be drawn



Zwitterion

In this experiment you will be studying the equilibrium reaction outlined above. You will be given an unknown animo acid in water at pH ≅ 2. you will then very slowly add a basic solution to it, while observing the changes in pH. This is due to the fact that when you have added enough of the base to combine with all the available H^+, the pH of the solution will change rapidly.

SUPPLIES

Unknown amino acid solution (??? M), 400 mL beaker, 100 mL graduated cylinders, 50 mL burets, ring stand, buret clamp, pH standards, 0.20 M KOH solution.

PROCEDURE

1. Record the code letter for your unknown solution.
2. Place 40 grams of your amino acid sample into a 400 mL beaker.
3. Place the beaker on the stirring hotplate, add a magnetic stir bar, and begin gentle stirring. DO NOT turn on the heat!
4. Clamp the pH electrode to the ring stand with the tip submerged in the solution in the 400 mL beaker. Be sure the electrode is clear of the magnetic stir bar. You may have to add 50-100 mL of distilled H_2O to make sure the electrode tip is under to solution. Add 5 N HCl until pH is between 1.7 –1.9.
5. Record the initial pH of your solution and the initial reading of your buret (which doesn't have to be 0.0 mL but it helps).
6. One person should be collecting buret volume readings while the partner watches the change in pH of your solution. Record buret reading for each change in pH in your notebook. You do not have to take a reading for each pH unit shown, but try not to skip more than two units.

   Initially, the volume of KOH needed to cause a change in pH will be fairly large (over 1 mL). As the titration continues, the volume of KOH solution needed to cause pH changes will become less and less. Near the equivalence point, a single drop may change the pH dramatically. As the volume of KOH needed to change the pH decreases, it is STRONGLY recommended that you proceed drop by drop. You may increase the amount added each time as the pH changes become less (after passing the equivalence point). Continue this procedure until you reach pH 13.0.

DATA COLLECTION

Collect data in a table formate.

INTERPRETATION

Plot pH versus mmoles of KOH solution added. Use pH as the dependent variable (Y-axis) and mmloes of KOH as the independent variable (X-axis).

The first equivalence point in the data marks the end of the region in which the H^+ is being removed from the acidic group, COOH. You may or may not see a second equivalence point. At the first equivalence point, the number of moles of KOH added is exactly equal to the number of moles of amino acid present in your sample. You will need to calculate the molality of your amino acid solution, that value will be compared to actual value.

Concentration of KOH solution _____

Volume of KOH solution added to 1st equivalence point _____

Volume of KOH solution added to 2nd equivalence point _____

Moles of KOH used _____

Molality of amino acid present _____

Actual Molality (for office use only) _____

Unknown sample code _____

Identity of unknown _____

Actual Identity of unknown (for office use only) _____

PROCESSING DATA

1. Submit a copy of the titration data and a graph of the titration curve.
    a. Label the equivalence point(s) and pK_a(s) on your graph.
    b. Explain the meaning of the equivalence point.
    c. Explain the process of titration.
2. What is a Zwitterion? pKa?
3. At the equivalence point of the titration, what is the relationship of moles of KOH and moles of the amino acid?

References:

Your Textbook, specifically page 64

Pre-Lab Questions:

1) If the equilibrium constant, Ka, for the ionization of the carboxylic acid group is 1×10^{-3}, what is the pKa?

2) If in a solution of alanine, the number of negatively charged carboxylate groups (COO^-) is 1×10^{20} at the isoelectric point, what is the number of positive charged amino groups, MH_3^+?

Amino Acid	α-carboxylic acid	α-amino	Side chain	Possible Unknown
Alanine	2.35	9.87		✓
Arginine	2.01	9.04	12.48	✓
Asparagine	2.02	8.80		✓
Aspartic Acid	2.10	9.82	3.86	✓
Cysteine	2.05	10.25	8.00	✓
Glutamic Acid	2.10	9.47	4.07	✓
Glutamine	2.17	9.13		
Glycine	2.35	9.78		✓
Histidine	1.77	9.18	6.10	✓
Isoleucine	2.32	9.76		
Leucine	2.33	9.74		
Lysine	2.18	8.95	10.53	✓
Methionine	2.28	9.21		✓
Phenylalanine	2.58	9.24		
Proline	2.00	10.60		✓
Serine	2.21	9.15		
Threonine	2.09	9.10		✓
Tryptophan	2.38	9.39		
Tyrosine	2.20	9.11	10.07	✓
Valine	2.29	9.72		

Methods for Determine Protein Concentration

Below is a list of assays for the determination of protein concentration in a solution. This list includes the sensitivity range, volume/amount of sample needed, subjective comments on accuracy and convenience, and major interfering agents. Procedural details, equipment requirements, and references are outlined in the individual assay documents.

The criteria for choice of a protein assay are usually based on convenience, availability of protein for assay, presence or absence of interfering agents, and need for accuracy. For example, the Lowry method is very sensitive but is a two step procedure that requires a minimum of 40 minutes incubation time. The Bradford assay is more sensitive and can be read within 5 minutes, however proteins with low arginine content will be underestimated. Generally, estimates are more accurate for complex mixtures of proteins. Estimates of concentration of pure proteins can be very inaccurate depending on the principle of the assay, unless the same pure protein is used as a standard. Criteria will be discussed in the individual documents.

General Reference: Stoscheck, CM. Quantitation of Protein. *Methods in Enzymology 182:* 50-69 (1990).

Absorbance assays

- Absorbance at 280 nm
  - Range: 20 micrograms to 3 mg
  - Volume: Depends on cuvette - volumes range from 200 microliters to 3 ml or greater
  - Accuracy: Fair
  - Convenience: Excellent, if equipment available
  - Major interfering agents: Detergents, nucleic acids, particulates, lipid droplets

Colorimetric assays

- Modified Lowry
  - Range: 2 to 100 micrograms
  - Volume: 1 ml (scale up for larger cuvettes)
  - Accuracy: Good
  - Convenience: Fair
  - Major interfering agents: Strong acids, ammonium sulfate
- Biuret
  - Range: 1 to 10 mg
  - Volume: 5 ml (scale down for smaller cuvettes)
  - Accuracy: Good
  - Convenience: Good
  - Major interfering agents: Ammonium salts
- Bradford assay
  - Range: 1 to 20 micrograms (micro assay); 20 to 200micrograms (macro assay)
  - Volume: 1 ml (micro); 5.5 ml (macro)
  - Accuracy: Good
  - Convenience: Excellent
  - Major interfering agents: None

Absorbance Assay (280 nm)

Considerations for use

Absorbance assays are fast and convenient, since no additional reagents or incubations are required. No protein standards need be prepared. The assay does not consume the protein. The relationship of absorbance to protein concentration is linear. Because different proteins and nucleic acids have widely varying absorption characteristics *there may be considerable error*, especially for unknowns or protein mixtures. Any non-protein component of the solution that absorbs ultraviolet light will interfere with the assay. Cell and tissue fractionation samples often contain insoluble or colored components that interfere. The most common use for the absorbance assay is to monitor fractions from chromatography columns, or any time a quick estimation is needed and error in protein concentration is not a concern.

Principle

Proteins in solution absorb ultraviolet light with absorbance maxima at 280 and 200 nm. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm. Secondary, tertiary, and quaternary structure all affect absorbance, therefore factors such as pH, ionic strength, etc. can alter the absorbance spectrum.

Equipment

In addition to standard liquid handling supplies a spectrophotometer with UV lamp and quartz cuvette are required.

Procedure

Conduct steps 1-4 for a very rough estimate. Conduct all steps if nucleic acid contamination is likely.

1 Warm up the UV lamp (about 15 min.)
2 Adjust wavelength to 280 nm
3 Calibrate to zero absorbance with buffer solution only
4. Measure absorbance of the protein solution
5. Adjust wavelength to 260 nm
6. Calibrate to zero absorbance with buffer solution only
7. Measure absorbance of the protein solution

Analysis

★ *Unknown proteins or protein mixtures.* Use the following formula to roughly estimate protein concentration.

Path length for most spectrometers is 1 cm.

Concentration (mg/ml) = Absorbance at 280 nm divided by path length (cm.)

Pure protein of known absorbance coefficient. Use the following formula for a path length of 1 cm.

Concentration = Absorbance at 280 nm divided by absorbance coefficient

★ *Unknowns with possible nucleic acid contamination.* Use the following formula:

Protein concentration (mg/ml) = (1.55 x A280) - 0.76 x A260)

Comments

Cold solutions can fog up the cuvette, while warm solutions can release bubbles and interfere with the readings. For concentrated solutions (absorbance greater than 2) simply dilute the solution.
Absorbance coefficients of some common protein standards:

Bovine serum albumin (BSA) = 63 | Bovine, human, or rabbit IgG: = 138 | Chicken ovalbumin: = 70

References

- Layne, E. Spectrophotometric and Turbidimetric Methods for Measuring Proteins. *Methods in Enzymology 3:* 447-455. 1957.
- Stoscheck, CM. Quantitation of Protein. *Methods in Enzymology 182:* 50-69. 1990.

Hartree-Lowry and Modified Lowry Protein Assays (For future reference)

Considerations for use

The Lowry assay (1951) is an often-cited general use protein assay. For some time it was the method of choice for accurate protein determination for cell fractions, chromatography fractions, enzyme preparations, and so on. The bicinchoninic acid (BCA) assay is based on the same principle and can be done in one step, therefore it has been suggested (Stoscheck, 1990) that the 2-step Lowry method is outdated. However, the modified Lowry is done entirely at room temperature. The Hartree version of the Lowry assay, a more recent modification that uses fewer reagents, improves the sensitivity with some proteins, is less likely to be incompatible with some salt solutions, provides a more linear response, and is less likely to become saturated. The Hartree-Lowry assay will be described first.

Principle

Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion. Monovalent copper ion and the radical groups of tyrosine, tryptophan, and cysteine react with Folin reagent to produce an unstable product that becomes reduced to molybdenum/tungsten blue.

Equipment

In addition to standard liquid handling supplies a spectrophotometer with infrared lamp and filter is required. Glass or polystyrene (cheap) cuvettes may be used.

Procedure - Hartree-Lowry assay

Reagents

Reagent A Consists of 2 g. sodium potassium tartrate, 100 g. sodium carbonate, 500 ml 1N NaOH, H_20 to one liter (that is, 7mM Na-K tartrate, 0.81M sodium carbonate, 0.5N NaOH final concentration). Keeps 2 to 3 months.

Reagent B Consists of 2 g. sodium potassium tartrate, 1 g. copper sulfate ($CuSO_4 \bullet 5H_20$), 90 ml H_20, 10 ml 1N NaOH (final concentrations 70 mM Na-K tartrate, 40 mM copper sulfate). Keeps 2 to 3 months.

Reagent C Consists of 1 vol Folin-Ciocalteau reagent diluted with 15 volumes water.

Assay

1. Prepare a series of dilutions of 0.3 mg/ml bovine serum albumin in the same buffer containing the unknowns, to give concentrations of 30 to 150 micrograms/ml (0.03 to 0.15 mg/ml).
2. Add 1.0 ml each dilution of standard, protein-containing unknown, or buffer (for the reference) to 0.90 ml reagent A in separate test tubes and mix.
3. Incubate the tubes 10 min in a 50 °C bath, then cool to room temperature.
4. Add 0.1 ml reagent B to each tube, mix, incubate 10 min at room temperature.
5. Rapidly add 3 ml reagent C to each tube, mix, incubate 10 min in the 50 °C bath, and cool to room temperature. Final assay volume is 5 ml.
6. Measure absorbance at 650 nm in 1 cm cuvettes.

Analysis

Prepare a standard curve of absorbance versus micrograms protein (or *vice versa*), and determine amounts from the curve. Determine concentrations of original samples from the amount protein, volume/sample, and dilution factor, if any.

Procedure - Modified Lowry (room temperature) (For future reference)

Reagents

1. **Copper reagent** Dissolve 20 g. sodium carbonate in 260 ml water, 0.4 g. cupric sulfate (penta hydrated) in 20 ml water, and 0.2 g. sodium potassium tartrate in 20 ml water. Mix all three solutions.
2. Prepare 100 ml of a **1% solution** (10 g./100 ml) of **sodium dodecyl sulfate (SDS).**
3. Prepare a **1 M solution of NaOH** (4 g./100 ml).
4. For the **2x Lowry concentrate** mix 3 parts copper reagent with 1 part SDS and 1 part NaOH. Solution is stable for 2-3 weeks. Warm the solution to 37 ºC if a white precipitate forms, and discard if there is a black precipitate. Better, keep the three stock solutions, and mix just before use.
5. Prepare **0.2 N Folin reagent** by mixing 10 ml 2 N Folin reagent with 90 ml water. Kept in an amber bottle, the dilution is stable for several months.

Assay

1. Dilute samples to an estimated 0.025-0.25 mg/ml with buffer. If the concentration can't be estimated it is advisable to prepare a range of 2-3 dilutions spanning an order of magnitude. Prepare 400 □L each dilution. Duplicate or triplicate samples are recommended.
2. Prepare a reference of 400 □L buffer. Prepare standards from 0.25 mg/ml bovine serum albumin by adding 40-400 □L to 13 x 100 mm tubes + buffer to bring volume to 400 □L/tube.
3. Add 400 □L of 2x Lowry concentrate, mix thoroughly, incubate at room temp. 10 min.
4. Add 200 □L 0.2 N Folin reagent very quickly, and vortex immediately. Complete mixing of the reagent must be accomplished quickly to avoid decomposition of the reagent before it reacts with protein. Incubate for 30 min. more at room temperature.
5. Use glass or polystyrene cuvettes to read the absorbances at 750 nm. If the absorbances are too high, they may be read at 500 nm.

Comments

Recording of absorbances need only be done within 10 min. of each other for this modified procedure, whereas the original Lowry required precise timing of readings due to color instability. This modification is less sensitive to interfering agents and is more sensitive to protein than the original. As with most assays, the Lowry can be scaled up for larger cuvette sizes, however more protein is consumed. Proteins with an abnormally high or low percentage of tyrosine, tryptophan, or cysteine residues will give high or low errors, respectively.

References

- Lowry, OH, NJ Rosbrough, AL Farr, and RJ Randall. *J. Biol. Chem. 193:* 265. 1951.
- Oostra, GM, NS Mathewson, and GN Catravas. *Anal. Biochem. 89:* 31. 1978.
- Stoscheck, CM. Quantitation of Protein. *Methods in Enzymology 182:* 50-69 (1990).
- Hartree, EF. *Anal Biochem 48:* 422-427 (1972).

Biuret Protein Assay(For future reference)

Considerations for use

The principle of the Biuret assay is similar to that of the Lowry, however it involves a single incubation of 20 min. There are very few interfering agents (ammonium salts being one such agent), and Layne (1957) reported fewer deviations than with the Lowry or ultraviolet absorption methods. However, the Biuret consumes much more material. The Biuret is a good general protein assay for batches of material for which yield is not a problem. The Bradford assay is faster and more sensitive.

Principle

In alkaline conditions substances containing two or more peptide bonds form a purple complex with copper salts in the reagent.

Equipment

In addition to standard liquid handling supplies a visible light spectrophotometer is needed, with maximum transmission in the region of 450 nm. Glass or polystyrene (cheap) cuvettes may be used.

Reagent

Biuret reagent, consisting of 2.25 g. NaK tartrate, 0.75 g. $CuSO_4$•5 H_2O, 1.25 g. KI. Dissolved all in order in 100 ml 0.2 M NaOH (0.8 g/100 ml). Bring volume to 250 ml with distilled H_2O. Discard if a black precipitate forms.

Assay

1. Warm up the spectrophotometer 15 min. before use.
2. Dilute samples to an estimated 1 to 10 mg/ml with buffer. Add 1 ml to each assay tube. Duplicate samples are recommended, and a range of dilutions should be used if the actual concentration cannot be estimated.
3. Prepare a reference tube with 1 ml buffer.
4. Prepare standards from 10 mg/ml bovine serum albumin. Range should be from 1 to 10 mg protein.
5. Add 9 ml Biuret reagent to each tube, vortex immediately, and let stand 20 min.
6. Read at 550 nm.

Analysis

Prepare a standard curve of absorbance versus micrograms protein (or *vice versa*), and determine amounts from the curve. Determine concentrations of original samples from the amount protein, volume/sample, and dilution factor, if any.

Comments

The color is stable, but all readings should be taken within 10 min. of each other. As with most assays, the Biuret can be scaled down for smaller cuvette sizes, consuming less protein. Proteins with an abnormally high or low percentage of amino acids with aromatic side groups will give high or low readings, respectively.

References

- Gornall, AG, CS Bardawill, and MM David. *J. Biol. Chem. 177:* 751. 1949.
- Layne, E. Spectrophotometric and Turbidimetric Methods for Measuring Proteins. *Methods in Enzymology 10:* 447-455. 1957.
- Robinson, HW and CG Hogden. *J. Biol. Chem. 135:* 707. 1940.
- Slater, RJ (ed.). *Experiments in Molecular Biology.* Clifton, New Jersey: Humana Press, 1986. P. 269.
- Weichselbaum, TE. *Am. J. Clin. Pathol. Suppl. 10:* 40. 1946.

Bradford protein assay (For this Lab)

Considerations for use

The Bradford assay is very fast and uses about the same amount of protein as the Lowry assay. It is fairly accurate and samples that are out of range can be retested within minutes. The Bradford is recommended for general use, especially for determining protein content of cell fractions and assaying protein concentrations for gel electrophoresis.

Assay materials including color reagent, protein standard, and instruction booklet are available from Bio-Rad Corporation (included in your notebook). The method described below is for the "standard Procedure" with sensitivity to about 20 to 200 micrograms protein. Microtiter plate protocols are also described in the flyer that comes with the kit.

Principle

The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

Equipment

In addition to standard liquid handling supplies a visible light spectrophotometer is needed, with maximum transmission in the region of 595 nm, on the border of the visible spectrum (no special lamp or filter usually needed). Glass or polystyrene (cheap) cuvettes may be used, however the color reagent stains both. Disposable cuvettes are recommended.

Procedure

Reagents

Bradford reagent Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman #1 paper just before use.

(Optional) 1 M NaOH (to be used if samples are not readily soluble in the color reagent).

The Bradford reagent should be a light brown in color. Filtration may have to be repeated to rid the reagent of blue components. The Bio-Rad concentrate is expensive, but the lots of dye used have apparently been screened for maximum effectiveness. "Homemade" reagent works quite well but is usually not as sensitive as the Bio-Rad product.

Assay

1. Warm up the spectrophotometer 15 min. before use.
2. Dilute samples with buffer to an estimated concentration of 20 to 200 micrograms/ml
3. Prepare standards containing a range of 20 to 200 micrograms protein (albumin or gamma globulin are recommended) to a standard volume (generally 1 ml or less).
4. Prepare unknowns to estimated amounts of 20 to 200 micrograms protein per tube, same volume as the standards.
5. (optional) Add 0.25 ml 1 M NaOH to each sample and vortex.
6. Add 5 ml dye reagent and incubate 5 min.
7. Measure the absorbance at 590 nm.

Analysis

Prepare a standard curve of absorbance versus micrograms protein (or *vice versa*), and determine amounts from the curve. Determine concentrations of original samples from the amount protein, volume/sample, and dilution factor, if any.

Comments

The dye reagent reacts primarily with arginine residues and less so with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues. Obviously, the assay is less accurate for basic or acidic proteins. The Bradford assay is rather sensitive to bovine serum albumin, more so than "average" proteins, by about a factor of two. Immunoglogin G (IgG - gamma globulin) is the preferred protein standard. The addition of 1 M NaOH was suggested by Stoscheck (1990) to allow the solubilization of membrane proteins and reduce the protein-to-protein variation in color yield.

References

- Bradford, MM. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry 72:* 248-254. 1976.
- Stoscheck, CM. Quantitation of Protein. *Methods in Enzymology 182:* 50-69 (1990).

Experiment #5

Bradford protein assay

Background: Refer to previous material for background information on the Bradford (& other) protein assays.

Procedure:

Bradford reagent Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml 95% ethanol, add 100 ml 85% (w/v) phosphoric acid. Dilute to 1 liter when the dye has completely dissolved, and filter through Whatman #1 paper just before use.

Assay **(See attached table)**

1. Warm up the spectrophotometer 15 min. before use.
2. In seven 16 X 150 mm tubes, place the following amounts of a 0.3 mg/mL BSA solution: 0.0, 0.05, 0.10, 0.20, 0.30, 0.40, 0.50 mL.
3. In four tubes, place the following amounts of 0.3 mg/mL ovalbumin solution: 0.05, 0.10, 0.30, 0.50 mL.
4. In three tubes, place dilutions of your unknown sample. The three dilutions will guarantee that one of the absorbance values will be with the standard absorbance range.
   (See instructor for appropriate dilutions)
5. Add water to each tube from steps 2, 3, and 4 to bring the final volume to 1 mL.
6. Add 5 ml Bradford reagent and vortex: avoid excessive foaming.
7. After 5 min to 1 hour (but no longer), read the absorbance at 595 nm.

Report

1. On a sheet of graph paper (or on a computer graphing program), plot a standard curve of A_{595} (y-axis) vs. μg BSA (x-axis). If using a computer graphing program make sure to plot an x-y scatter type graph.

2. Overlay a graph of the A_{595} (y-axis) vs. μg ovalbumin (x-axis). Do the graphs overlay? If not propose an explanation why they do not.

3. What is the experimentally determined concentration of your unknown protein solution based on the BSA standard curve?

4. You analyzed three unknown samples (one sample with three dilutions). Which of these three diluted samples gave you the most reliable protein concentration, and why?

Bradford Protein Assay Table

Math Setup – for graph

Tube #	1	2	3	4	5	6	7	8	9	10	11	12	13	14
μg BSA	0 μg						150 μg							
μg ovalbumin											150 μg			

Measurements

volume BSA [0.3 mg/mL]	0	0.05 mL	0.10 mL	0.20 mL	0.30 mL	0.40 mL	0.50 mL							
volume ovalbumin [0.3 mg/mL]								0.05 mL	0.10 mL	0.30 mL	0.50 mL			
volume unknown [??? mg/mL]														
volume H_2O	1.00 mL	0.95 mL	0.90 mL	0.80 mL	0.70 mL	0.60 mL	0.50 mL	0.95 mL	0.90 mL	0.70 mL	0.50 mL			
Abs_{595}														

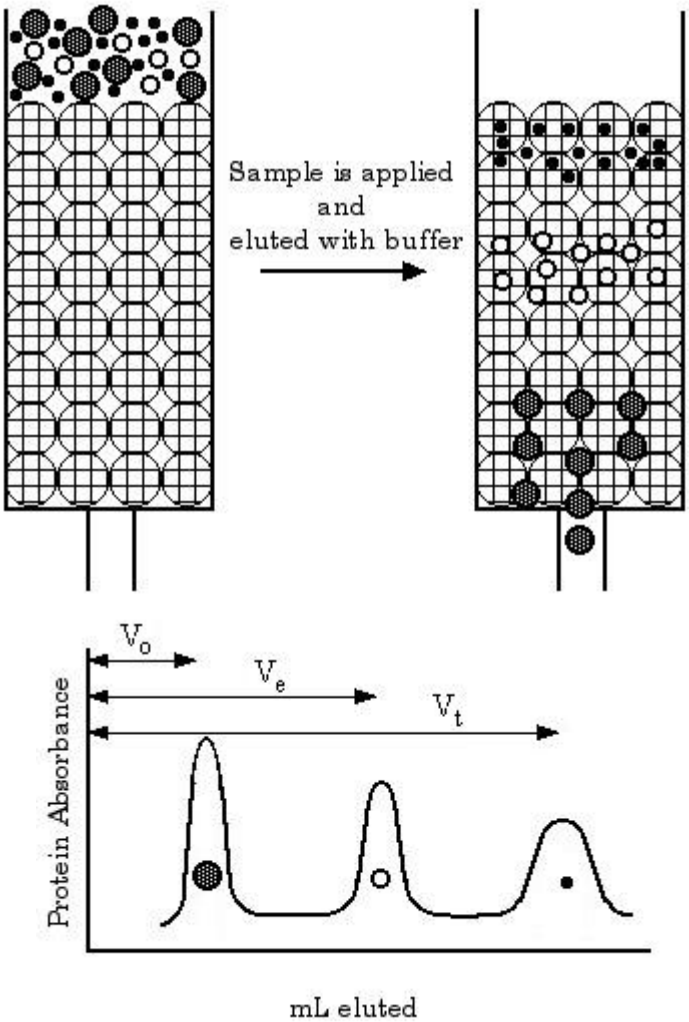
Final Volume of all tubes should be 1.00 mL

Gel filtration Chromatography

Introduction

Gel-filtration chromatography is a form of column chromatography in which molecules are separated on the basis of their molecular mass or, more properly, their Stokes radius. The Stokes radius is the effective radius a molecule has as it tumbles rapidly in solution. A long, extended molecule has a larger Stokes radius than a compact molecule of the same molecular mass. The stationary phase in gel-filtration chromatography consists of fine beads that contain pores of controlled size. The space between the beads is referred to as the void space (V_0).

The sample is applied in a narrow band at the top of the column and then it is washed through the column by the mobile phase. Large molecules in the sample that cannot pass through the pores of the beads are excluded from the beads and are restricted to the outer voids. They elute from the column after an amount of the mobile phase equal to V_0 has passed through the column. Molecules that are much smaller than the pores equilibrate with the entire liquid volume and elute at a volume equal to V_t. Molecules that are small enough to pass through some of the pores of the beads, however, elute at various volumes (V_e), depending on how small they are and what fraction of the pores of the beads are accessible to them. Such molecules are referred to as partially included. Molecules in the sample can be separated in order of their size by collecting fractions as the mobile phase is eluted through the column, with the largest molecules eluting first and the smallest last.



Experiment #6

Molecular Weight Estimation by Exclusion Chromatography

Background: The value K_d will be calculated in this experiment for standard proteins and for an unknown protein.

$$K_d = \frac{(V_e - V_o)}{V_i}$$

V_e = elution volume for the protein of interest

V_o = void volume (i.e. volume required to completely exclude "Blue Dextran")

V_i = inner volume = $V_t - V_o$

V_t = total volume (i.e. volume required to completely exclude "DNP-Aspartate")

Note: V_t is not the same as the volume of gel matrix

The high-molecular weight, blue-colored substance, Blue Dextran, is completely excluded from the beads; therefore, its elution time or volume from a column gives a measure of V_o. In contrast, a small molecule, DNP-aspartate, enters all solvent spaces, and thus its elution volume gives a measure of V_i. Molecules of intermediate size are able to penetrate the gel particles to some extent and so are eluted from the gel at intermediate positions. The concepts may be expressed quantitatively by the distribution coefficient, K_d.

Procedure:

Reagents **Note: All buffers must be degassed before use**

1. **Sephadex G-75**, hydrated in 0.05 M Tris-HCl (pH 7.5)
2. **Elution buffer:** 0.1 M NaCl in 0.05 M Tris-HCl (pH 7.5)
3. **Separation mixture:** Measure 3 mg "Blue Dextran", and 0.3 mg DNP-aspartate, 8 mg unknown protein. Dissolve the above solutes in 1.0 mL elution buffer which also contains 15% (v/v) glycerol.
4. **Chromatographic column:** 1.5 × 50 cm

Experiment

1. The column should be vertical. After setting up the column, confirm this by looking at it from two directions. See Figure 1.
2. Prevent air bubbles from forming in the column bed, and pack the matrix material uniformly with no troughs or channels. The bed material should never be allowed to go dry. If the column has bubbles or does go dry it should be repacked
3. Your sephadex G-75 has been stored in the appropriate buffer containing $CuSo_4$ so bacterial contamination does not occur.
4. Pour the blue Sephadex G-75 into your column allowing the liquid to exit the column at the bottom. Continue to add Sephadex mixture until your column until it contains a well packed continues bed of resin 5 cm from the top.
5. Wash your column with elution buffer until the blue color has been removed from your column.
6. You are now ready to apply the separation mixture to the top of the column. It is often helpful to cut a filter paper wafer just a bit smaller than the inside diameter of the column, and insert this into the liquid above the column bed. Let the wafer settle evenly on top of the Sephadex. Now when the sample is applied, it will not disturb the column.
7. Let the buffer level drop so that the top of the gel bed (filter paper) is exposed.
8. Fill a pipet with 0.50 mL of the mixture.
   Carefully place the sample onto the filter paper.

   Let the sample flow into the bed.

9. Add buffer on to the top of the column as needed to maintain a fairly constant flow rate. *Important: Do not disturb the top of the gel bed, and do not allow the liquid level to drop below the top of the gel bed.*
10. Collect the eluting buffer into a graduated cylinder until your blue dextran is about to elute (there should be no protein in this initial volume). This volume will be important in your calculations
11. Begin collecting 4-mL fraction into labeled test tubes. When all of the color has been eluted from the column, shut off the flow of buffer.
12. Read the absorbances of the test tube fractions at the appropriate wavelengths.

Blue Dextran (blue)	650 nm
DNP-aspartate (yellow)	440 nm
Unknown (not visible)	280 nm

Report

1. On a single graph, plot the absorbance of each fraction versus the collected volume for each fraction. Do not forget the amount of buffer collected in the graduated cylinder.

2. Determine the midpoint of each peak on your graph.

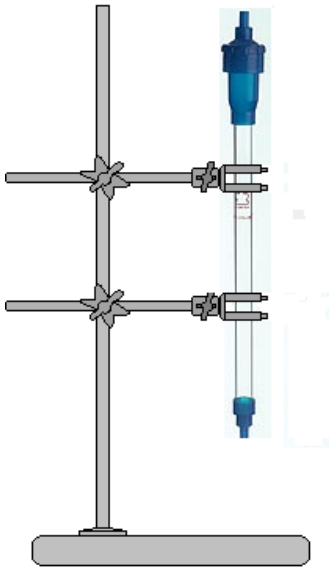
3. Calculate the K_d for your unknown from your data.

Protein	Molecular Weight	K_d
Trypsin inhibitor (pancreas)	6,500	0.70
Trypsin inhibitor (lima bean)	9,000	0.60
Cytochrome c	12,400	0.50
a-Lactalbumin	15,500	0.43
a-Chymotrypsin	22,500	0.32
Carbonic anhydrase	30,000	0.23
Ovalbumin	45,000	0.12

Source: Andrews, P. Estimation of molecular size and molecular weights of biological compounds by gel filtration. In *Methods Biochem. Anal*. (D. Glick, ed.) Interscience, New York (1970)

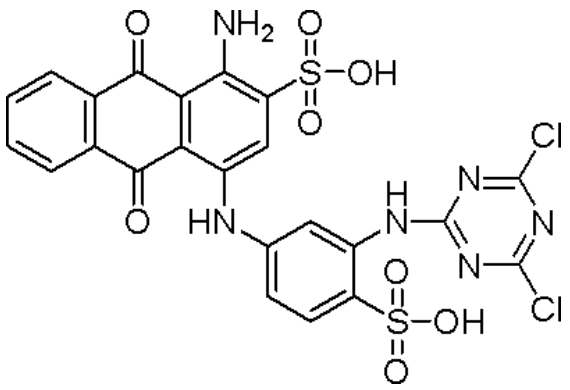
4. Using the above information, prepare a graph of $\log_{10}$ MW vs K_d for the known proteins. Interpolate from the graph and determine the MW of the unknown protein. I am not asking that you identify the actual name of the protein.

Figure 1



Isolation of Lactate Dehydrogenase by Affinity Chromatography

Reactive Blue 4 Agarose



Procedure:

Preparation of Chicken Breast Homogenate

1) Cut tissue into dice size cubes, weight and record mass.
2) Place cubes in blender and add 2x volume of:

   20 mM Tris/HCl – pH 8.6 w/
   1 mM 2-mercaptoethanol
   1 mM PMSF (phenylmethylsulfonyl fluoride)

3) Blend until tissue has been homogenized but with limited foaming.
4) Place homogenate in four 50-mL falcon tubes, **balance to within 0.01g**.
5) Let solutions set on ice for 15 min.
6) Centrifuge for 40 min @ 3000 rpm.

Affinity Chromatography

Collect all eluents for future analysis. Label as indicated to avoid confusion.

1) Load column with Reactive Blue 4 agarose hydrated in:

. 20 mM Tris/HCl – pH 8.6

2) Wash column with 30 mL:

   20 mM Tris/HCl – pH 8.6 w/
   1 mM 2-mercaptoethanol

3) Load 30 – 100 mL of chicken homogenate. **(Label = flow through)**

4) Wash column and collect 5 mL fractions until eluent's Abs280 is low with:
   (Label = 1Ea, 1Eb, 1Ec, ……)

   20 mM Tris/HCl – pH 8.6 w/
   1 mM 2-mercaptoethanol
   1 mM PMSF (phenylmethylsulfonyl fluoride)

5) Wash column and collect 5 mL fractions until eluent's Abs280 is low with:
(Label = 2Ea, 2Eb, 2Ec, ……)

20 mM Tris/HCl – pH 8.6 w/
1 mM 2-mercaptoethanol
1 mM lithium lactate
1 mM NAD^+

6) Wash column and collect 2 x 10 mL fractions with:
(Label = 3Ea, 3Eb)

10 mM Tris/HCl – pH 8.6 w/
0.5 mM 2-mercaptoethanol

7) Wash column and collect 5 mL fractions until eluent's Abs280 is low with:
(Label = LDH_1, LDH_2, LDH_3, ……)

10 mM Tris/HCl – pH 8.6 w/
0.5 mM 2-mercaptoethanol
1 mM NADH

8) Pool selected fractions.

9) Clean column with 25 mL:

20 mM Tris/HCl – pH 8.6 w/
1 mM 2-mercaptoethanol

Future studies with this isolated enzyme include:

Protein determination
SDS-PAGE
Enzyme Kinetics
Independent research

ELECTROPHORESIS

Electrophoresis is the migration of charged molecules in solution in response to an electric field.

SDS-PAGE

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

Separation of Proteins under Denaturing conditions

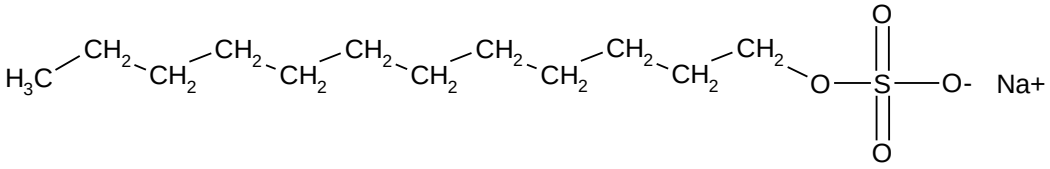
The Matrix

The separation platform is a "sandwich" of highly cross-linked gel matrix of polyacrylamide between two thin plates of glass.

An electric field is applied with the cathode at the bottom and the anode at the top of the sandwich

The Samples

Sodium dodecyl sulphate (SDS) is an anionic detergent which helps denature proteins by binding to the hydrophobic side chains of the amino acid residues - and SDS binds to proteins fairly specifically in a 2:1 ratio (2 SDS molecules per 3 or 4 amino acids in polypeptide chain)



In so doing, SDS confers a negative charge to the polypeptide in proportion to its length - ie: the denatured polypeptides become "rods" of negatively charged clouds with equal charge per unit length.

It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size: this is done with 2- mercaptoethanol or dithiothreitol.

In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

More than one sample can be applied to a sandwich

Typical Samples

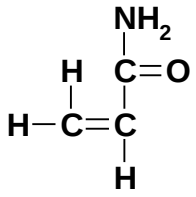
Protein Mixtures Purified Protein (you hope) Molecular Weight Standards

What do you get?

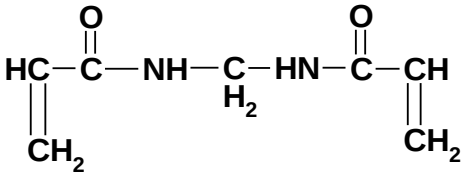
You get an idea of the number of proteins in each sample and the molecular weight of each protein.

Polymerization & Cross-Linking of Acrylamide

Commonly contains a three-dimensional matrix of randomly cross-linked polymers of acrylamide and cross-linking agent N, N' –methylene bisacrylamide

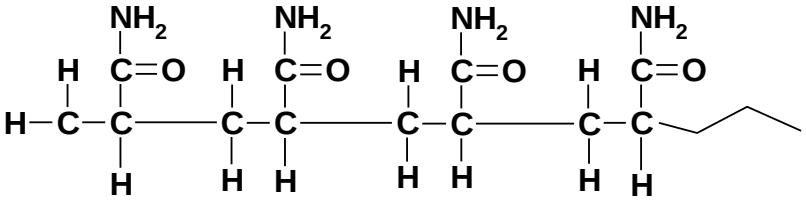


Acrylamide



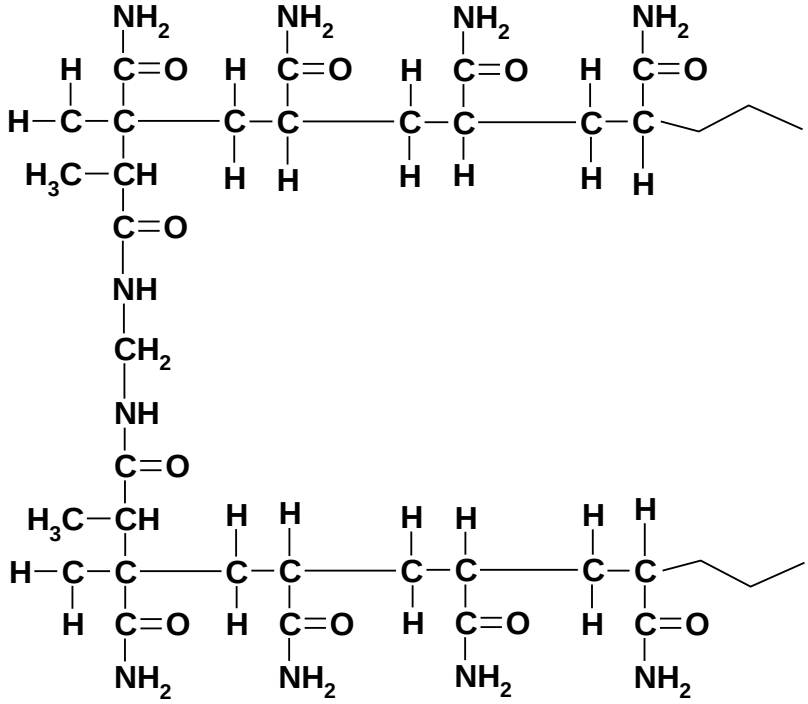
N, N' –methylene bisacrylamide

The individual residues of acrylamide will form together to make a long polymer.



Acrylamide polymer

These long chains of acrylamide will be cross-linked with N, N' –methylene bisacrylamide to produce the final matrix



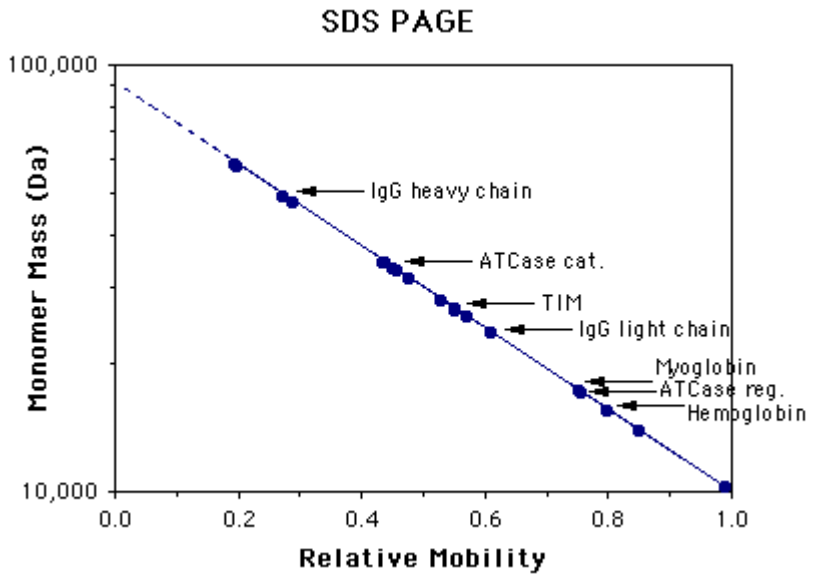
The extent of cross-linking can be controlled by the ratio of Acrylamide to N, N' –methylene bisacrylamide

Electrophoresis Behavior of Proteins on an SDS-PAGE gel

The proteins will be separated with smallest molecular weight traveling faster than larger molecular weights.

The M.W. (logarithmic scale) is plotted vs relative mobilities on SDS-PAGE. The proteins for figure below and their subunit molecular masses:

Protein	Subunit M.W. (Da)
IgG (heavy chain)	49,200
ATCase (catalytic subunit)	34,300
TIM (triosephosphate isomerase)	26,600
IgG (light chain)	23,400
Myoglobin	17,200
ATCase (regulatory subunit)	17,000
Hemoglobin	15,500



SDS-PAGE Procedure

Mini-PROTEAN3 Cell Set-Up

1) Wash all glass plates to be used with both soapy water and extensive amounts of methanol. A chem-wipe will be required with the methanol and no streaks should be visible.
2) Assemble the Mini-PROTEAN3 Cell as described in the manual.
3) You are going to produce a discontinuous Laemmli Buffer Gel. The concentrations of Acrylamide my change but it is recommended that you use 10% for the resolving gel and 4% for the stacking gel. See chart below and loading picture on next page.

Percent Gel	**Degased DI H_2O (mL)**	**30% Acrylamide/Bis (mL)**	**Gel Buffer* (mL)**	**10% w/v SDS (mL)**
4%	6.1	1.3	2.5	0.10
5%	5.7	1.7	2.5	0.10
6%	5.4	2.0	2.5	0.10
7%	5.1	2.3	2.5	0.10
8%	4.7	2.7	2.5	0.10
9%	4.4	3.0	2.5	0.10
10%	4.1	3.3	2.5	0.10
11%	3.7	3.7	2.5	0.10
12%	3.4	4.0	2.5	0.10
13%	3.1	4.3	2.5	0.10
14%	2.7	4.7	2.5	0.10

*Resolving Gel Buffer = 1.5 M Tris-HCl pH 8.8
Stacking Gel Buffer = 0.5 M Tris-HCl pH 6.8

4) Mix the appropriate reagent for your resolving gel as listed above. Avoid foaming of solution. **NOTE: acrylamide is a neurotoxin in its aqueous form, you should wear gloves for this step.**
5) Add 50 μL 10% APS and 10 μL TEMED to your gel mixture. These combined reagents are the gel catalysts. You must now work quickly but introduce no air bubbles.
6) Add the gel mixture between the Mini-PROTEAN sandwich to within 1 cm of the comb height with a plastic pipette. Let excess gel mixture remain in pipette as an indicator of when your gel has set.
7) Add enough butanol (water saturated) to just cover your gel. Pipette slowly in order to not disturb the gel top. This step will even the gel top and protect the gel from oxygen.
8) When the gel is completely solid remove the butanol with pieces of filter paper. Rinse gel top with water and again remove with filter paper.
9) Mix the appropriate stacking gel. Add to top of to within 1 cm of top of glass, then inset the comb without adding air bubbles.
10) Allow gel to solidify.
11) Remove comb at an angle.

SAMPLE PREP. & ADDITION

1) Your should prepare the following samples: 10 μg of LDH, 25 μg Chicken homogenate, 10 μg of BSA and 10 μg (μL) protein standards.
2) Each of your samples should be pipetted into a small eppendorf tube and diluted with 3x the volume of sample buffer. The protein standards are already prepared in sample buffer so no dilution is required. Boil all samples 10 minutes in a boiling water bath.
3) Load your sample into a well in the gel without overflowing.
4) When all sample are loaded assemble the Mini-PROTEAN apparatus, run the sample at 200 volts until the blue dye just leaves the gel.

GEL STAINING

1) Remove gel from sandwich and place in a tray with staining solution. Let the gel stain overnight.
2) Destain your gel with the destaining solution until protein bands appear on a clear background.
3) Store your gel in water after destaining is complete.

Write-Up

1) Have a drawing of your loading pattern placed in your notebook like the one below. We will also try to photocopy your finished gel for your report.

2) Identify the lanes.

3) Comment on the purity of the pure LDHase sample.

4) Comment on the purity of the chicken homogenate sample.

5) Identify each protein in the standards lane, as per identity and molecular weight.

6) Make a graph of the log MW versus relative mobility (Rf) for the standard protein.

7) Use this graph to determine the MW of your pure LDHase.

8) Does your LDHase MW agree with the known value?