Department of Chemistry

IIT Kharagpur

Biochemical Techniques Laboratory (CY49006)

	Name of the Experiments
1	Agarose Electrophoresis of Food Dyes
2	Protein Structure Analysis
3	Protein Denaturation Studies
4	Ion Exchange Chromatography
5	Enzyme Kinetics and Inhibition Studies
6	Determination of the pI of an Amino Acid

Experiment No. 1
Agarose Electrophoresis of Food Dyes

Introduction

Molecules can be separated by chromatography based on the polarity (hydrophobic or hydrophilic) of the molecule and the polarity of the solvent. Some molecules actually have a net charge or can become charged in a solvent at a specific pH. Molecules with a net charge can be separated in an electric field by a procedure known as **electrophoresis**. Electrophoresis is a common laboratory technique used for the separation of macromolecules such as proteins, DNA, and RNA.

The mobility of a molecule during electrophoresis depends on the net charge of the molecule, the intensity of the electrical field, and how freely the molecule can pass through the pores (sieving action) within the gel matrix. Electrophoresis is nearly always carried out in a gel. Gels serve as molecular sieves that assist the separation of molecules. Molecules with a net positive charge (cationic) will migrate to the negative (black) electrode while molecules with a net negative charge will migrate to the positive (red) electrode. If the voltage drop across the gel is increased (electrical field is strengthened) the molecules will migrate more quickly.

Experiment

Commercial Food dyes will be provided to you and an unknown sample. You are to determine the components of the unknown sample from the electrophoresis pattern obtained.

The experiment will be conducted at two pH values to evaluate the dye characteristics.

Procedure

Preparation of gel

Weigh out 0.50 grams of agarose and add to a 250-mL Erlenmeyer flask (conical flask) containing 25 mL of your assigned buffer. Place the flask containing the undissolved agarose on a hot plate and swirl until the agarose is completely dissolved. Your solution should be clear. Pour the solution into the gel-bed while it is still molten (it will solidify when cooled to about 40°C). Pour the gel to a depth of 3 to 5 mm.

While the solution is still molten, place the comb in the **middle slot** which is used to form wells for loading samples once the gel has set. The comb used in this experiment produces 8 wells.

Leave the gel at room temperature for at least 20 minutes to fully gel.
During this time set up the electrophoresis unit. Obtain additional buffer, of the same pH, that will be placed in the reservoir of the electrophoresis device.

Loading the gel

After the gel has cooled, remove the comb from the gel by pulling the comb steadily and slowly straight up from the horizontal gel. Pour enough of the reservoir buffer into the chamber to just cover the gel. There has to be sufficient reservoir buffer that the gel is actually submerged and the sample wells are full of buffer.

- Use a clean micropipette tip for each dye.
- Using micropipettes load 15µl of each of the 4 dyes into 4 separate wells formed within the gel. Do not puncture the bottom of the well with your micropipette and inject sample under the gel.
- Load 15 µl of the unknown mixture into another well.

Running the gel

1. Place the plastic safety cover over the electrophoresis apparatus. Attach the electrode wires from the power supply to the electrodes of the reservoir.
2. Turn on the power supply and set the voltage to 150 volts.
3. Allow the electrophoresis to proceed for 15-20 minutes.
4. Shut the power supply off, remove the electrode wires and inspect the gel without removing it. Decide if you want to let it run longer or not. If not, then remove the gel and rinse it briefly with water.
5. Examine the gel on a white-light box if required.

Results

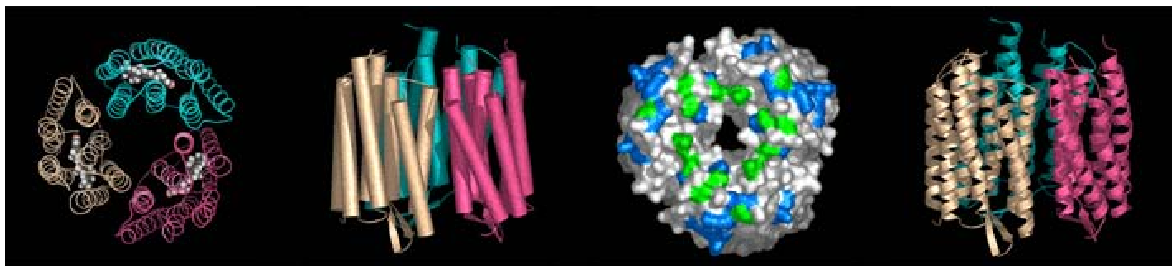
1. Study the gel pattern and sketch it. Label the diagram clearly.
2. Determine the relative movement (R_f) of the bands by determining the ratio of the distance of the centre of the band to the total length of the gel.
3. Based on the information about the structure of the known dyes, plot a graph of the R_f value vs the charge/mass ratio. From this information determine the charge/mass ratio of the unknown dye.

Experiment No. 2

Protein Structure Analysis

Introduction

PyMOL is a powerful molecular visualization package from DeLano Scientific, LLC. PyMOL is increasingly being used for very advanced molecular graphics applications in research laboratories and in the pharmaceutical industry. You will use PyMOL to view molecular structures and to make distance measurements.



Display a Molecule

PyMOL reads .pdb files, which come from the Protein Data Bank (PDB). These files are used to describe the 3D structure of any molecule, not just proteins but also nucleic acids (like RNA and DNA) and even small molecules.

Click on Start to go the Programs menu where you will find PyMOL. Start the program by double clicking on the icon. TWO windows will open up – one with a command line prompt and the other a viewer window.

PyMOL allows the user to easily manipulate the orientation of the molecule with respect to the viewer. The easiest way is to use the mouse to do this. Move the molecule around to see how the structure rotates in the window.

Distance Measurements (To find the distance use the measurement wizard)

1. Access the wizard by clicking on the Wizard menu and then choose the Measurement option.
2. You will be prompted in the upper left corner of the molecule-display region to select the first atom of interest. To select an atom, as shown in the Mouse region of the display, left click on the atom in the molecular display part of the window. When you have successfully selected an atom, a little pink square will appear on that atom.
3. Next choose the second atom. Left-click on another atom in the molecule to complete the measurement.
4. You will see a yellow dashed vector drawn between the two atoms you selected and a measurement in Angstroms drawn above it.

The view menu lists the various objects that are displayed at a given time. In our display at present there is only one molecule, so there is just one named object.

The view menu shows five popup menus to the right of each listed object: In order, let's call these the A, S, H, L, and C(rainbow) menus. If you left-click on these and drag down, you will be able to choose various options from the pop-up menu that comes up.

- The A (Action) menu allows the user to control the view of the molecule. The main option you might use is zoom, which resets the size of the molecule to fit in the display
- The S (Show) menu shows different modes of viewing a molecule. The lines and spheres options are the ones that we will use at first.
- The H (Hide) menu hides the different modes. So if you have both lines and spheres modes turned on (using the S menu), clicking H and then selecting one of the modes turns one of them off.
- The L (Labels) menu controls different ways of labeling the molecule.
- The C (Color/rainbow) menu chooses different styles of coloring the different elements in the molecule.

You will be given a protein file (PDB file) e.g.1CRN

1. Determine the number of each amino acid type present in the protein and record your results in a table. Calculate the percentage occurrence of each residue type in the protein.

   To select a specific residue name in the command line - write:

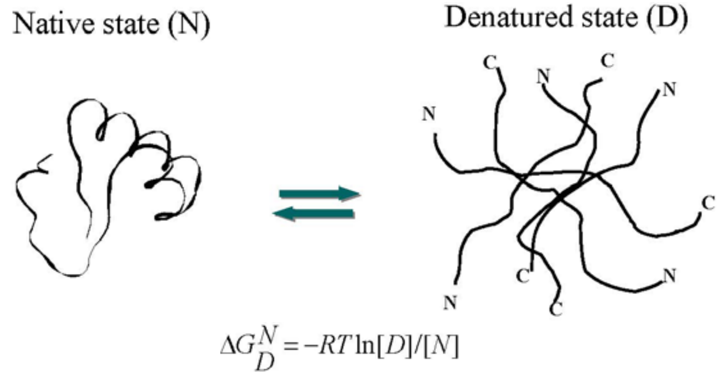
   - Select resn ALA (if you want to select ALA residues only)
   - You can then color them differently to help you count them more easily – use the C menu on the right side panel to color it.

2. Identify the helices and sheets present in the protein. Determine the percentage of residues present as helices and sheets and calculate the percentage present as random structure.
3. Calculate the distances from the N-terminus to the C-terminus of each helix and determine the pitch
4. Calculate the distances from the N-terminus to the C-terminus of each strand and determine the average distance between the C_α atoms for each strand
5. Construct a helical wheel of the helices present to determine the amphipathic nature of the helix if present.
6. From the number of aromatic residues present in the protein estimate the extinction coefficient of the protein.

Experiment No. 3

Protein Denaturation Studies

Disruption of the noncovalent interactions will lead to unfolding of the protein with reversible or irreversible denaturation. It is believed that hydrophobic collapse is a key driving force for protein folding resulting in the formation of a hydrophobic core with the polar surface interacting with the solvent.



Fundamental constraints of protein structures, φ and ψ angles dictate possible polypeptide backbone conformations. Both local and nonlocal interactions play a role in protein folding. Apart from these hydrophobic interactions and hydrogen bonding play an important role.

Factors that cause denaturation are (i) Temperature and (ii) Denaturants such as acid, urea and Guanidinium Hydrochloride (GuHCl)

In this experiment you will follow the effect of urea (up to 8 M) on protein unfolding by viscosity and fluorescence measurements. Unfolding may also follow a two-state mechanism.
You will use two different proteins for the two sets of experiments.

For viscosity measurements use the viscometer (5ml/10ml) and add the denaturant – mix thoroughly and then measure the time taken. Calculate the viscosity by comparing with the viscosity for water.
For fluorescence measurements, you need a much smaller volume of sample – you will do this experiment with a different protein. The excitation wavelength used will be 295 nm with emission set at 350nm for Tryptophan residues in proteins.
Mix specific proportions of solutions indicated and record your measurements.

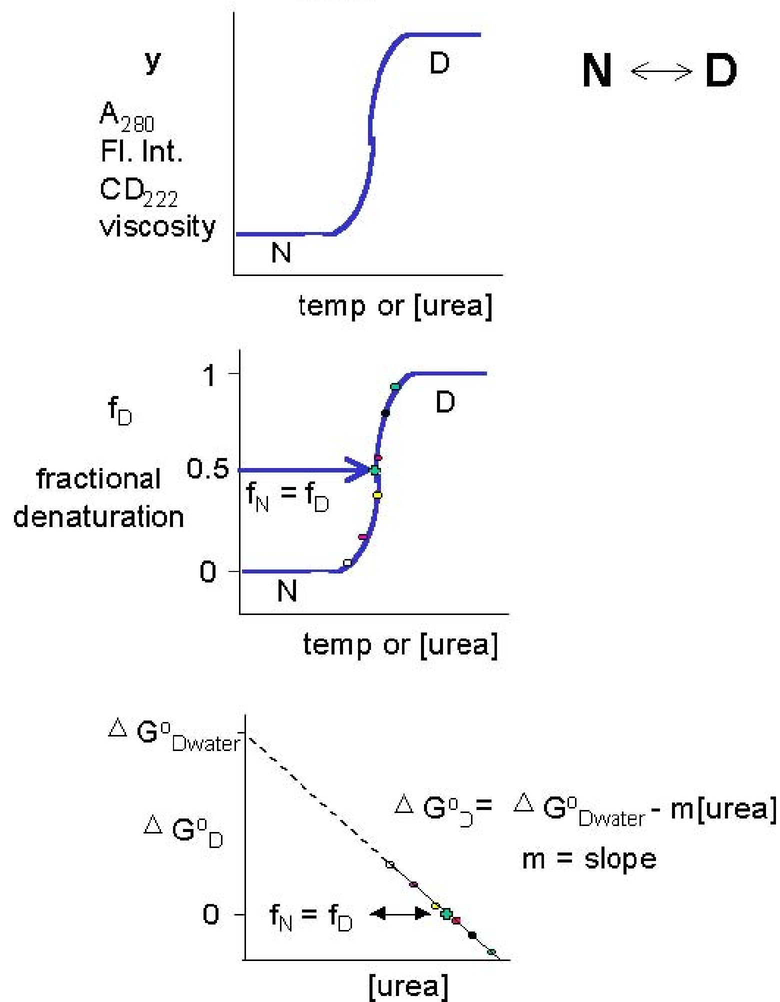
A sample table is given below – note that the volumes used are the same in each case (the x and y values will differ based on the experiment being performed)

	Protein soln	Denaturant solution	Viscosity/fluorescence
1	Water (for blank reading)		
2	x ml	y ml of 0 M	
3	x ml	y ml of 1 M	
4	x ml	y ml of 2 M	
.	…	…	

Determine the ΔG for unfolding from the equation:

$\Delta G = -RT\ln K = -RT\ln[(yN - y)/(y - yD)]$
where K is the equilibrium constant, y is the observed value of the parameter used to follow unfolding, yN and yD are the values of y characteristic of the native and denatured conformations respectively (these are functions of urea concentration: y = a + b[urea]).

Plot ΔG as a function of denaturant concentration and extrapolate to 0M urea concentration to determine the ΔG of unfolding in water.



Experiment No. 4

Ion Exchange Chromatography

Introduction:

Ion exchange chromatography is a separation technique used for purification or analysis of molecules based their charge. The method can be used to separate charged molecules from uncharged ones or it can separate molecules of different charge from one another.

Principle of the method:

Ionizable chemical groups are immobilized on a solid support such as cellulose or agarose. The support, or resin, is usually maintained in a column. Molecules of opposite charge can bind the column by electrostatic interaction while uncharged residues will pass through. Once bound to the column, molecules can be released with salt (NaCl is commonly used, but other salts can be used also). The salt ions compete for interaction for the column, and the molecule of interest is released. Hence the term "ion exchange".

Molecules having different charges can be separated from one another by gradually increasing the salt concentration. This is achieved with a gradient of increasing salt concentration in the solution being passed through the column. Lower charged groups are released at low salt concentrations because they are weakly bound. Highly charged molecules are more tightly bound and require higher salt concentration to release them. Thus molecules are released from the column according to the magnitude of their charge.

It should be noted that pH of the column buffers can have a profound effect on ion exchange chromatography. Both the ion exchange resin and the molecule binding to it are charged molecules with a defined pKa. If the pH is on one side of the pKa, the molecule will be uncharged, and it will be charged on the other. Also, the difference between the pH and the pKa will determine how much of the resin is ionized that in turn will determine how tightly other molecules will bind.

The charged resin can be of two types: cation exchangers and anion exchangers. The name of the resin refers to the molecules being exchanged, **not** the molecule bound to the resin. Cation exchangers bind positively charged molecules and anion exchangers bind negative ones.

In this experiment, you will separate adenosine 5'-monophosphate (AMP) and adenosine 5'-triphosphate (ATP). These compounds will be separated by chromatography on diethylaminoethyl (DEAE) cellulose. The mixture of compounds will be loaded onto the column, and eluted with a NH_4Cl/NH_3 gradient.

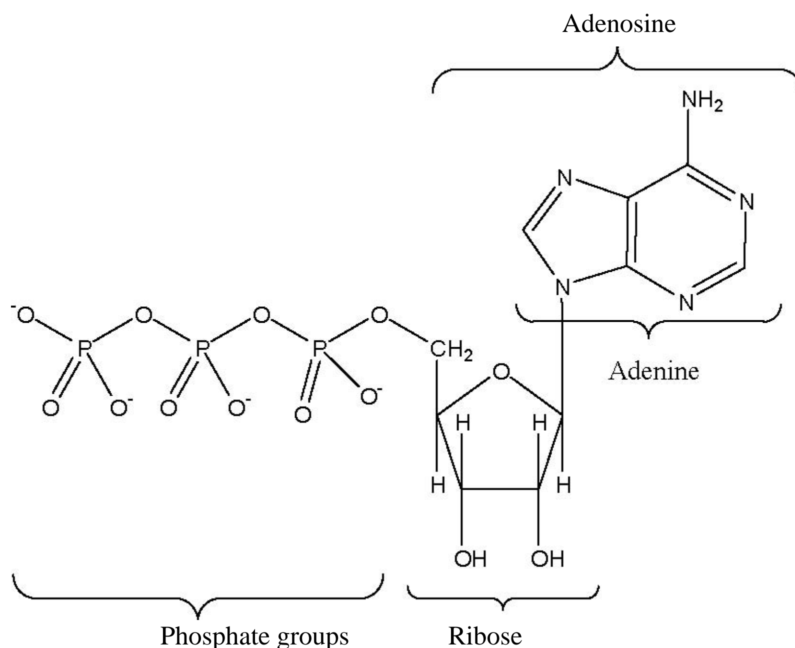
Reagents and Materials:
Compound mixture: AMP and ATP
Buffer: 0.25 M NH_4Cl/NH_3, pH 9.0

Experimental Procedure:

1. Prepare a column of DEAE-cellulose by placing a filter paper at the bottom of a column to serve as a plug. Add a slurry of DEAE-cellulose equilibrated in 0.05M buffer. The final height of DEAE-cellulose in the column should be between 7 to 8 cm.
2. Prepare 10ml solutions of eluting buffer from the stock 0.25M solution. The concentrations should range from 0.05M to 0.25M in increments of 0.05M.
3. Drain the column to just above the top of the resin (do not let resin go dry). Add 1 ml of the compound mixture and allow it to run into the column.
4. Cap the column, and start collecting effluent.
5. Have a test-tube rack ready with 20 numbered test-tubes. Collect ~3 ml of effluent in each tube.
6. Measure the absorbance of each tube at 260 nm.

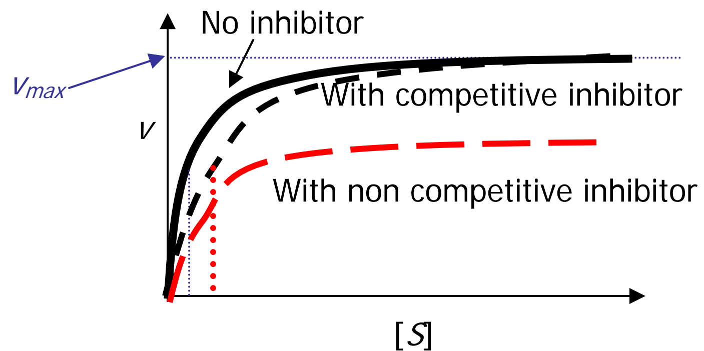
Data Analysis:

1. Tabulate absorbance of column eluent at 260 nm vs fraction number.
2. Graph your data, plotting absorbance vs fraction number.
3. Draw the structural formulae of the predominant chemical species for the compounds separated in this experiment. Ionic charges in the species must be clearly labeled.



Experiment No. 5
Enzyme Kinetics and Inhibition Studies

Enzymes are protein catalysts that, like all catalysts, speed up the rate of a chemical reaction without being used up in the process. The study of the rate at which an enzyme works is called **enzyme kinetics**. Enzyme kinetics as a function of the **concentration of substrate [S]** available to the enzyme is shown in the figure.



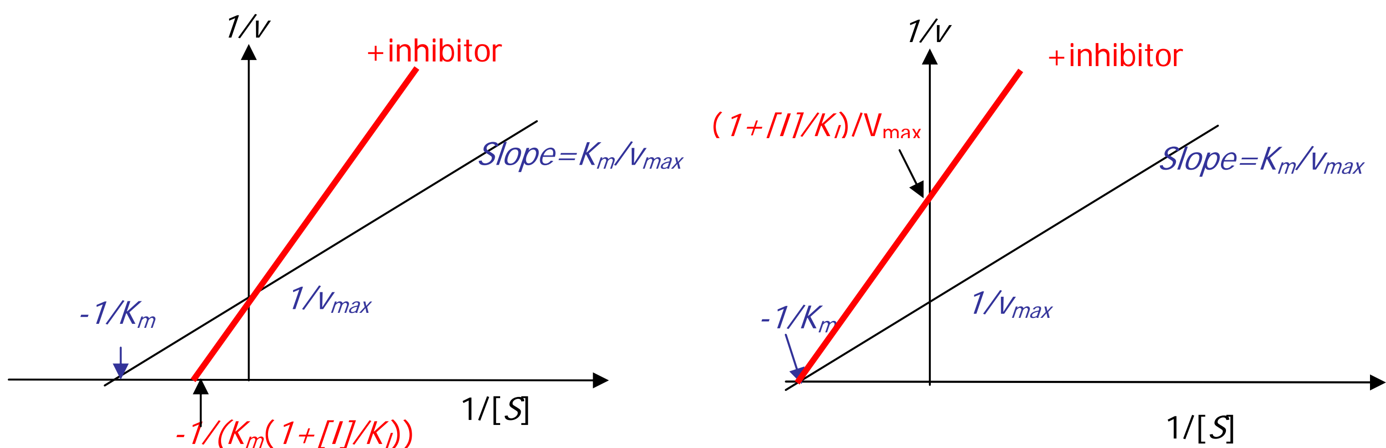
Plotting $\mathbf{V_i}$ as a function of **[S]**, we find that

- At low values of **[S]**, the initial velocity,$\mathbf{V_i}$, rises almost linearly with increasing **[S]**.
- The asymptote represents the maximum velocity of the reaction, designated $\mathbf{V_{max}}$
- The substrate concentration that produces a $\mathbf{V_i}$ that is one-half of $\mathbf{V_{max}}$ is designated the Michaelis-Menten constant, $\mathbf{K_m}$(named after the scientists who developed the study of enzyme kinetics).

$\mathbf{K_m}$ is (roughly) an inverse measure of the affinity or strength of binding between the enzyme and its substrate. The lower the $\mathbf{K_m}$, the greater the affinity (so the lower the concentration of substrate needed to achieve a given rate).

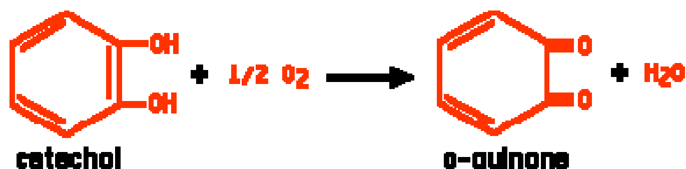
Enzymes can be inhibited **competitively**, when the substrate and inhibitor compete for binding to the same active site or **noncompetitively**, when the inhibitor binds somewhere else on the enzyme molecule reducing its efficiency.

In the presence of a competitive inhibitor (left panel), it takes a higher substrate concentration to achieve the same velocities that were reached in its absence. So while V_{max} can still be reached if sufficient substrate is available, one-half V_{max} requires a higher [S] than before and thus K_m is larger. With noncompetitive inhibition (right panel), enzyme rate (velocity) is reduced for all values of [S], including V_{max} and one-half V_{max} but K_m remains unchanged. A "double-reciprocal" or Lineweaver-Burk plot is usually plotted to determine the kinetic parameters V_{max} and K_m



When a slice of apple is exposed to air, it quickly turns brown. This is because the enzyme **o-diphenol oxidase** catalyzes the oxidation of phenols in the apple to dark-colored products.

The $\mathbf{V_{max}}$ and the Michaelis-Menten constant ($\mathbf{K_m}$) for this enzyme are to be determined.



- With **catechol** as the substrate. The enzyme converts it into **o-quinone**, which is then further oxidized to dark products.
- when it acts in the presence of a **competitive inhibitor**
- when it acts in the presence of a **noncompetitive inhibitor**

Procedure:

- Grind up pieces of apple and centrifuge the resulting soup.
- The supernatant is your enzyme preparation.
- Because of the speed with which colored products are formed, we can use the intensity of the color as a measure of product formation.

No Inhibitor

1. Set up five tubes (Tubes A to D) with different concentrations of catechol (the substrate).
2. Add a fixed amount of enzyme preparation to Tube A and measure the change in absorbance (**O**ptical **D**ensity) at 540 nm at 1 minute intervals for several minutes.
3. Record the average **change** in OD_{540} per minute ($\Delta\ OD_{540}$). Since the OD is directly proportional to the concentration of the products, we can use it as a measure of the rate or velocity of the reaction ($\mathbf{V_i}$).
4. Repeat with the other three tubes.

Effect of para-hydroxybenzoic benzoic acid and phenylthiourea

Repeat the above procedure adding a fixed amount of a solution of any one of the above compounds to each of the four tubes.

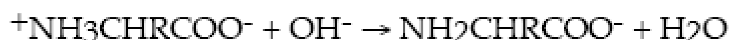
Plot Lineweaver-Burke plots to determine the kinetic parameters in each case.

Explain what you observe. What type of inhibitors are the two compounds?

Experiment No. 6

Determination of the pI of an Amino Acid

Amino acids are the building blocks of **proteins**. Almost all proteins consist of various combinations of the same 20 amino acids. Amino acids are compounds containing both an amine group, $-NH_2$, and a carboxylic acid group, -COOH. In addition there is an "R" group which is different for each amino acid.



In this experiment the titration of the amino acid provided will be carried out in two ways. Method I will involve titration with NaOH alone and Method II will involve titration with HCl and NaOH.

You will determine the pK values and from the information obtained determine the pI of the amino acid given.

Standardization of NaOH (~1 N)

Pipette 25 ml of standard oxalic acid solution into a clean dry conical flask, add 2 drops of phenolphthalein indicator solution, and titrate with NaOH solution to the pink endpoint.

Standardization of HCl solution (~1 N)

Pipette exactly 5.00 ml of the HCl solution into a clean conical flask, add 2 drops of phenolphthalein indicator solution, and titrate to the pink endpoint with standard NaOH.

Preparation of amino acid solution: Prepare 100 ml of a 0.1 M solution of the amino acid considering an approximate molecular weight 110.

Before beginning, calibrate the pH meter using standard buffer solutions of pH 4 and pH 7, or pH 7 and pH 10.

Method I:

Protonation of the amino acid carboxylate group

Transfer exactly 10 ml of the amino acid solution to a clean beaker. Calculate the number of moles of amino acid in the beaker. Calculate the volume of standard HCl needed to provide this same number of moles of HCl. Add this volume of HCl solution to the beaker using a graduated burette. The amino acid carboxylate group should now be protonated.

Amino acid titration

Fill a burette with standard NaOH solution. Rinse and dry the pH electrode and submerge it in the solution containing protonated amino acid.

RECORD THE INITIAL pH OF THE SOLUTION.

Initiate the pH titration by adding 0.5 ml of NaOH solution, stirring the solution thoroughly, and reading the pH. After each pH reading, record the total added volume of NaOH and the pH. Continue adding NaOH 0.50 ml at a time until the total added volume is about 80% of the total required to titrate the -COOH proton. At this point, add NaOH in smaller increments of first 0.20, then 0.10 ml. Continue adding NaOH incrementally until you are past the -COOH equivalence point (pH is approximately 8 or greater). Go back to adding NaOH 0.5 ml at a time until you have added 80% of the amount required to titrate the $-NH_3+$ proton. At this point, add smaller increments as above until you are past the equivalence point (pH is approximately 12).

Repeat the titration at least once.

Method II:
Transfer exactly 25 ml of a 0.1 M amino acid solution to a clean, 100ml beaker. Place a thoroughly rinsed teflon-coated stirring bar into the beaker and place the beaker on a magnetic stirrer. Insert the rinsed electrode of the pH meter into the solution and **record the starting pH**. Obtain ~50 ml of 1 N HCl. With the stirrer turned on, add 0.2 ml HCl using an appropriate pipettor, wait for the pH value to stabilize on the pH meter and record the pH. Again add 0.2 ml HCl while stirring, record the pH and so on. A graph of pH versus milliliters HCl added should be made during the experiment as well as recording the data in tabular form. Continue the titration until the solution reaches a pH of 1.5. Remove the electrode from the solution, wash, and insert it into a beaker of distilled water. Rinse the electrode.

Transfer exactly 25 ml of the same amino acid solution into a clean, 100 ml beaker. Place a teflon coated stirring bar into the beaker and place the beaker on the magnetic stirrer. Insert the rinsed electrode of the pH meter into the solution and **record the starting pH**. Obtain ~50 ml of 1N NaOH. With the stirrer turned on, add 0.2 ml NaOH using an appropriate pipettor, wait for the pH value to stabilize on the pH meter and record the pH. Again add 0.2 ml NaOH while stirring, record the pH and so on. A graph of pH versus milliliters NaOH added should be made during the experiment as well as recording the data in tabular form. Continue the titration until the solution reaches a pH of 12.0. Remove the electrode from the solution, wash and insert it into a beaker of distilled water.

Plot a graph for each case and determine the pI of the amino acid provided.