Chapter 3

Chemical Properties of Amino Acids and Identification of Unknown Amino Acids

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Contents

Introduction

Two labs, Chemical Properties of Amino Acids, and Identification of Unknown Amino Acids, were designed to illustrate the physical and chemical properties of amino acids that determine the shapes and biological activities of proteins. The labs are the first two in a series that is closely coordinated with lectures in Cellular Biochemistry—a sophomore level core curriculum course required of University of Oregon biology majors. The course is preceded by a course in Genetics and Evolution and one in Molecular Biology, and it is followed by a course in Cellular Physiology. This set of four courses, each with an associated laboratory course, presents students with the key principles and relationships that underlie all of biology.

The Cellular Biochemistry laboratory series was created originally by W.R. Sistrom. The two exercises presented here are new, and were created during a general revision whose goal was to focus students' intellectual effort on the lab exercise itself, instead of on the lab report. That is, we wanted students to be intellectually engaged while working on the lab—not only while writing the report sometime later. In the case of these two new labs, we also wanted to address specific aspects of amino acid structure and ionization that students consistently find difficult.

The method of presenting these lab exercises is as important as their content. We use a weekly lab lecture (1 hour) given to the full class of approximately 250 students to set the tone for each exercise by introducing the relevant concepts and techniques. The exercise itself is done during a three hour lab period in which 26-30 students and two teaching assistants participate. The lab work is structured to create a cooperative environment that encourages both questions from individuals and group work. The text of the lab manual is designed to provide continual intellectual challenge, rather than passive data collection.

Materials

Chemical Properties of Amino Acids

For each lab section (26-30 students):

A complete set of models of amino acids (CPK) plus spare parts
Demonstration of column chromatography equipment—including samples of column matrix that students can see and touch.

For each pair of students:

CPK space-filling components—enough to build glycine in three ionization states, plus alanine, isoleucine and serine in a single ionization state (pH 6)
- Nitrogen, tetrahedral (3)
- Nitrogen, trigonal (3)
- Carbon, tetrahedral (10)
- Carbon, trigonal (4)
- Hydrogen (31)
- Oxygen, double-bonded (8)
- Oxygen, single-bonded (5)

Identifying Unknown Amino Acids

For each lab section:

Demonstration of titration equipment
Equipment and materials for electrophoresis of amino acids
- Horizontal mini-gel apparatus, modified for paper electrophoresis of amino acids
- Electrophoresis paper, Whatman 3MM
- Stock solutions of amino acids
  - Valine (0.5M)
  - Arginine (0.5M)
  - Aspartate (0.5M)
  - Lysine (0.5M)
- P_{20} micropipettors
- Electrophoresis buffers
  - pH 2 (0.5M phosphoric acid)
  - pH 6 (0.5M sodium phosphate)
  - pH 11 (0.5M sodium carbonate)

Set of index cards, labeled from A to Q, to represent the complete set of possible unknowns. You will need enough sets of cards so that each pair of students (lab partners) will be able to draw six different unknowns.

Notes for Instructors

Lab Structure

Each lab exercise consists of three parts: a pre-lab assignment, a group of lab activities, and a brief report.

1. Pre-lab assignment The pre-lab assignment is used to focus students' thinking on the important concepts connected with the lab exercise. Instead of emphasizing technical issues, pre-lab assignments require students to construct a framework for understanding before coming to lab. Pre-lab exercises are due at the beginning of the lab period, and are checked and returned during the period. This procedure allows instructors to discover quickly what students don't understand, and to address those problems during the lab session.

2. Lab activities The lab exercise is divided into several distinct activities, each of which focuses on a particular concept. As part of each activity, students must answer questions that require specific predictions or applications of concepts. These questions (set in italics) are an important tool for monitoring understanding. Teaching assistants can use the questions to engage individual students in discussions of the lab material, and also to assess the progress of the group as a whole.

3. Lab report The lab report is short and focuses on applications of concepts learned during the lab. If there is time, students are encouraged to complete the lab report during the lab period. This often leads to group interactions and problem solving that can be monitored by the teaching assistants.

The Role of the Lab Instructors

It is important to recognize that this lab format demands very active participation by the lab instructors. We spend a great deal of time making sure that the teaching assistants understand the material, and training them to interact effectively with the students. The teaching assistants must be sufficiently confident to elicit questions from students and to probe comprehension. This involves circulating through the lab room, and engaging individual students in specific discussions that get at key ideas. These discussions should be encouraging, but they should also be very clear — so that students can recognize misconceptions, and instructors can identify common problems that should be discussed by the group as a whole.

Organizing the Logistics of the Unknown Amino Acids Activity

You will need to plan carefully to organize the flow of information and people during the unknown amino acids activity. Some students will be confused by the structure of the activity (e.g., index cards representing unknowns, experimental results collected from the instructor, interpreting test results).

When a group of students is ready (i.e. they have completed lab activities 1 through 5), they can pick up a group of unknowns from a teaching assistant. Before asking for test results (they are only allowed three results per unknown) they should think about their strategy for discriminating between the possible amino acids based on the tests they have at their disposal (titration, solubility, gel filtration, sulfur test, electrophoresis, formaldehyde derivitization, and presence of conjugated rings). After collecting each experimental result they should carefully narrow the list of possible amino acids for that unknown.

We photocopy and hand out small unlabeled graphs representing the results from the titration and electrophoretic tests (see Appendices C and D). This forces students to interpret the test results and then apply them to their list of possible amino acids.

Based on their physical and chemical properties, several of the amino acids will not be positively identifiable given the data that students have available to them. Specifically they will not be able to differentiate between leucine and isoleucine, or serine and threonine. Be sure to emphasize that their goal should be to draw appropriate conclusions based on the data they have.

Chemical Properties of Amino Acids

Pre-lab Assignment

1. Read the lab exercise, focusing on the Overview section, and the introductory material for each activity.
2. Make amino acid cards.
3. Answer the questions below.

Making Amino Acid Cards

Using 3∞5 cards, cut up notebook paper, or whatever else is handy, make a card for each amino acid. The card should include the name of the amino acid and the chemical structure of its side-chain. You are welcome to put additional information about the amino acids on the back of the card.

You will be using these cards for several weeks. Please remember to bring them with you to the lab periods.

Questions

1. Given the following chain of amino acids:

   Val–Cys–Asp–Leu–Ala–Arg–Phe–Glu–Trp

   a. identify the largest and smallest amino acids,
   b. identify the amino acids with ionizable side chains,
   c. identify the amino acids whose side chains are non-polar.

2. Draw glycine, lysine, and glutamic acid below. How many ionizable groups does each contain (circle them)? For each amino acid, number the ionizable groups from most acidic (tends to give up protons easily, number 1) to most basic (tends to hold protons tightly, numbers greater than 1).

   Glycine Lysine Glutamic Acid

Chemical Properties of Amino Acids

Objectives

1. To understand the general structure of an amino acid.
2. To distinguish amino acids from one another on the basis of several characteristics (size, shape, charge, hydrophobicity).
3. To understand how the charge on an amino acid is determined by the pH of its environment.

Overview

Last term you learned how genetic information encoded in DNA is translated into the sequence of amino acids corresponding to proteins. This term will focus on proteins — their structures and functions in cells. First we need to look closely at the structure and chemistry of the building blocks of proteins, amino acids.

There are 20 different amino acids that can be linked together in linear sequences to form proteins. Proteins are usually 100–1,000 amino acids long. The amino acids have different physical and chemical properties and it is these properties that determine the 3-dimensional shape and biological activity of the folded protein.

The focus of this lab will be to gain insight into the properties of amino acids. You will build models of amino acids, learn about their charge characteristics, and then use this information to understand how ion exchange chromatography can be used to separate amino acids. Next week you will apply what you learn today to help you identify unknown amino acids.

Activity 1: Amino Acid Structure

All of the 20 amino acids share some structural and chemical features, but each amino acid is distinguished from the others by the properties of one part of the molecule, the side chain (R). The common region of an amino acid (the body) contains a central carbon atom, called the alpha carbon (C_α), to which are attached an amino group (NH_2), a carboxyl group (COOH), and a hydrogen atom (H). See Figure 3.1.

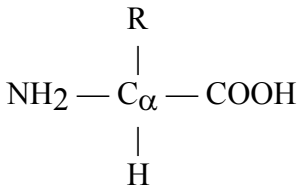

Figure 3.1. The structure of an amino acid in a vacuum.

We will be using space filling models (CPK) to represent molecular structure. Developed by Corey, Pauling, and Koltun, space-filling models represent the actual volume that is occupied by a molecule in space. By representing the electron cloud, CPK models provide a good picture of the exterior, but not the interior, of the molecule.

The model pieces follow the standard color convention for atoms.

Hydrogen -white
Carbon - black
Nitrogen - blue
Oxygen - red
Sulfur - yellow

Build a model of the body of an amino acid (everything except the R group).

Because there are four different groups attached to the alpha carbon atom, amino acids have a tetrahedral shape and there are two different chemical structures that are possible for each amino acid. Position the tetrahedron you built so that it is oriented with the hydrogen atom pointing straight up toward you. Starting with the carboxyl group and moving in a clockwise direction, determine the order in which the R and amine groups are attached to the α-carbon.

If the order is carboxyl group, followed by R group, followed by amino group, then you have built an L-amino acid. An easy way to remember this is the abbreviation CORN (Carboxyl, R group, Nitrogen containing amino group). If the order of the side groups is carboxyl, followed by amino, followed by R, then you have constructed a D-amino acid.

Is the amino acid you have built in the L or D configuration? Build the other configuration to help you recognize that their structures are mirror images.

Since only L-amino acids are found in naturally occurring proteins, we will model only L-amino acids from now on in this lab exercise.

If you have not already done so, make your space filling model a glycine (abbreviated Gly, or G), by adding a single hydrogen as the R group.

Are there L and D isomers of the amino acid glycine?

Now let's get a little fancier. Build additional space-filling models of alanine (Ala, A) and isoleucine (Ile, I). Notice how the amino acids you have built differ from one another.

To further understand the similarities between amino acids see if you can change your space-filling alanine model to serine (Ser, S).

In cells, the native forms of most proteins have a globular shape, in which the polypeptide chain folds into a tightly packed structure. In this globular structure, some of the amino acids are found in a non-aqueous environment (inside the glob), and others are found in an aqueous environment (on the outside of the glob).

Which part of a folded protein (inside or outside) would most likely contain the amino acids you have modeled (Gly, Ala, Ile, Ser)? Why?

Activity 2: Why Amino Acids are Charged

Today you will learn how to distinguish certain amino acids on the basis of charge. The charge on amino acids arises from the fact that they contain groups that are either weakly acidic or weakly basic — that is, groups that are capable of releasing or binding protons (H^+). Since protons are charged (+1), it follows that the loss or gain of protons is accompanied by a change in charge.

All amino acids contain at least one acidic and one basic group — the α–carboxyl and α–amino groups, respectively. Some amino acids also contain an additional acidic or basic group in their side chain. In this exercise, we are focusing on free amino acids. This means that the amino acid is *not* bonded to other amino acids as it would be in a polypeptide chain, and that the α–carboxyl and α–amino groups as well as side chain groups, are ionizable. Therefore, we will consider the charges on all of the ionizable groups.

What happens to the charges on the α–amino and α–carboxyl groups if an amino acid is part of a polypeptide chain?

To distinguish certain amino acids from each other, you will take advantage of the fact that amino acids of different overall charge vary in their ability to bind to negatively charged material.

You will also exploit the fact that the charge on an amino acid is influenced by the hydrogen ion concentration (pH) of the surrounding solution. To make sense of this, you will want to remember what you learned about acids and bases in chemistry. If you, like many people, were mystified by this subject, take heart! Here's an outline of the key ideas. There are really just a few essential ones.

Review of acid-base chemistry

First, let's consider an aqueous solution of a weak acid (HA—that rather bland species that we will use to represent the more interesting acids you will encounter in real life). The acid, of course, will dissociate into a hydrogen ion and a conjugate base (A-), as shown in the reaction below:

$$HA \rightleftharpoons H^+ + A^-$$
$$\text{acid} \rightleftharpoons H^+ + \text{base}$$

The extent of dissociation of this acid corresponds to the ratio of [base] to [acid] when the system has reached equilibrium. For our purposes, the important thing to realize is that the extent of dissociation tells us how charged or uncharged the population of acid molecules is. In this particular example, if [base] / [acid] is large, then most of the molecules bear one negative charge. If [base] / [acid] is small, then most of the molecules have no charge.

What is an expression for the fraction of total molecules that is negatively charged?

The expressions below do not represent the fraction of negatively charged molecules. What is wrong with each of them?

$$\frac{[A^-]}{[HA]}$$

$$\frac{[A^-]}{[HA]\,[H^+]}$$

Effect of pH on the fraction of charged molecules

Clearly, the fraction of charged molecules would change if we were to alter the concentration of one of the components in the dissociation reaction. In particular, let's think about what would happen if we changed the concentration of hydrogen ions. You might wonder how we could do this, but remember that we can add or subtract hydrogen ions from the outside. For instance, we could easily increase the hydrogen ion concentration by adding protons in the form of a strong acid such as HCl. On the other hand, we could decrease the hydrogen ion concentration by adding OH- ions (in the form of NaOH, for instance) that remove protons by binding them very tightly to form water.

This situation — in which the protons of the system come from multiple sources — is analogous to real life inside a cell. The nice thing is that by simply looking at the dissociation reaction above, you can tell how the charge on a molecule will respond to changes in the hydrogen ion concentration.

When $[H^+]$ increases, what happens to the fraction of total molecules that is negatively charged Why? What happens if $[H^+]$ decreases? Why?

Relation between equilibrium constants and the strength of acids

Now, all of this probably seems fairly obvious, and you may be wondering how it's going to help you distinguish one amino acid from another. Hang on, there's something interesting we have to consider. That is, the ease with which the extent of dissociation responds to changes in hydrogen ion

concentration is not the same for all acids. In fact, it varies dramatically — and you have probably heard of this characteristic in discussions of strong and weak acids.

For a strong acid, it's really hard to change the extent of dissociation because a very high concentration of hydrogen ions is required to convert A^- to HA. For a weak acid, it's much easier because a lower concentration of hydrogen ions will do. Now, we're getting somewhere! We can use the differences in the way various acids respond to changes in pH to recognize the acids. What we are really saying is that the extent to which an acid is dissociated at a particular concentration of hydrogen ions (pH) will tell us whether we've got a strong acid or a weak acid.

Now, happily for us, other people have already measured the tendency of the world's known acids to dissociate, and have given us this information in the form of dissociation constants (K_{diss}). The dissociation constants are simply the equilibrium constants (K_{eq}) for the dissociation of these acids.

The dissociation constant for our generic acid would be written;

$$K_{diss} = \frac{[H^+][A^-]}{[HA]} \quad \text{or} \quad \frac{[H^+][base]}{[acid]}$$

Now rewrite this expression to represent the dissociation of acetic acid (H_3CCOOH).

Let's consider some acid groups that occur in a real amino acid, aspartate (also called aspartic acid, and abbreviated Asp or D). You will notice that the side chain of aspartate looks like acetic acid.

Build a space filling model of aspartic acid and identify the ionizable groups.

There are three ionizable groups in aspartate: the α–carboxyl and the α–amino groups, that are parts of the core structure, plus the carboxyl group that is part of the side chain. Table 3.1, below, gives the dissociation constants for these groups.

Table 3.1. The dissociation constants for aspartic acid.

Ionizable Group	K_{diss}
α–COOH	$\approx 10^{-2}$
α–NH_3^+	$\approx 10^{-10}$
side-COOH	$\approx 10^{-4}$

Which of the groups listed in the table is the strongest acid?

Which is the weakest acid?

Is any of the groups such a weak acid that you would be tempted to call it a base?

What determines whether a molecule is considered an acid or a base?

Assuming that the pH inside the cell is 7, what is the concentration of hydrogen ions surrounding this aspartate molecule? Remember, pH = – log [H^+].

Now let's figure out how charged each of these groups would be if it were part of an aspartate molecule inside a cell. Let's focus on the α-carboxyl group first.

Write the expression for the dissociation of Asp α-COOH.

Rearrange the equation to solve for the ratio [base] / [acid].

At pH = 7 what is the value of this ratio?

Which form of Asp α-COOH will predominate at pH 7?

What fraction of Asp α-COOH groups would be charged at pH 7?
At what pH would 1/2 of the Asp α-COOH groups be charged?
Put you results into Table 3.2.

Table 3.2. Data sheet for dissociation and charge in an aspartate molecule.

Ionizable Group	K_{diss}	Extent of Dissociation at pH 7: [conj. base] / [conj. acid]	Fraction Charged at pH 7	pH at which 1/2 groups are charged
α–COOH	$\approx 10^{-2}$			
α–NH_3^+	$\approx 10^{-10}$			
Side-COOH	$\approx 10^{-4}$			

Now let's consider the α-amino group. Start with an expression for the dissociation of α-NH_3^+. The important thing is to identify the acidic and basic forms of the amino group.

Which form of the group corresponds to HA, the conjugate acid?
Which corresponds to A, the conjugate base?
Which is the charged form in this case, the conjugate acid or the conjugate base?
Complete Table 3.2 by filling in the results for the side chain carboxyl group.
Now you are ready to consider the behavior of the aspartic acid molecule as a whole.
What would be the average charge (sign and magnitude) for each of the groups, if aspartic acid were inside a typical cell?
What would be the average net charge (sign and magnitude) for the molecule as a whole under these conditions?

The charge on amino acids is important for their biological function. The distribution of charged and uncharged side chains in a polypeptide plays a big role in determining the 3-dimensional shape of the protein. The charge (or lack of charge) on a side chain is also essential for the function of active sites in enzymes, pores created by membrane proteins, and proteins that regulate gene expression.

Charge can be used to separate certain amino acids. The next section will explore a separation method that exploits differences in charges on amino acids.

Activity 3: Using Charge Differences to Separate Amino Acids

In this exercise you will learn how amino acids can be separated by ion exchange chromatography. Most amino acids have an overall positive charge (that is, they are cations) below pH 4, and an overall negative charge (they are anions) above pH 8. Generally, the α–carboxyl group of an amino acid dissociates (giving up a proton) around pH 2. The α–amino group dissociates around pH 9. Because some amino acids have additional ionizable groups in their side chains, the net charge at a particular pH varies among amino acids. These differences can be exploited to allow certain amino acids to stick to an ion exchange column, while others pass through it.

Ion exchange chromatography is a technique in which mobile ions (amino acids in this case) dissolved in water or buffer are reversibly bound to a charged stationary phase called the exchanger (Figure 3.2 and 3.3).

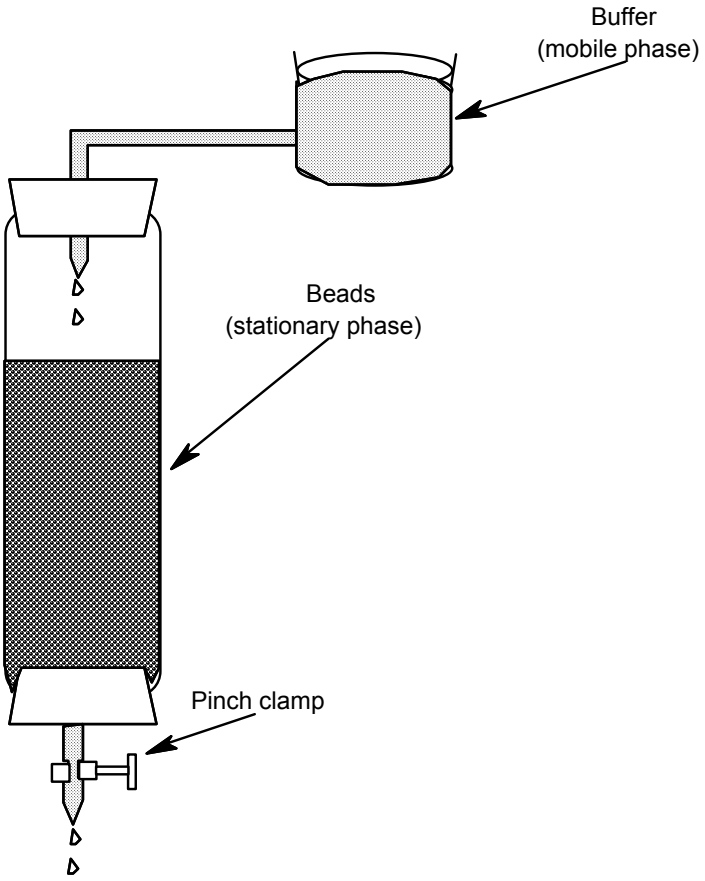

Figure 3.2. A simple column for ion exchange chromatography.

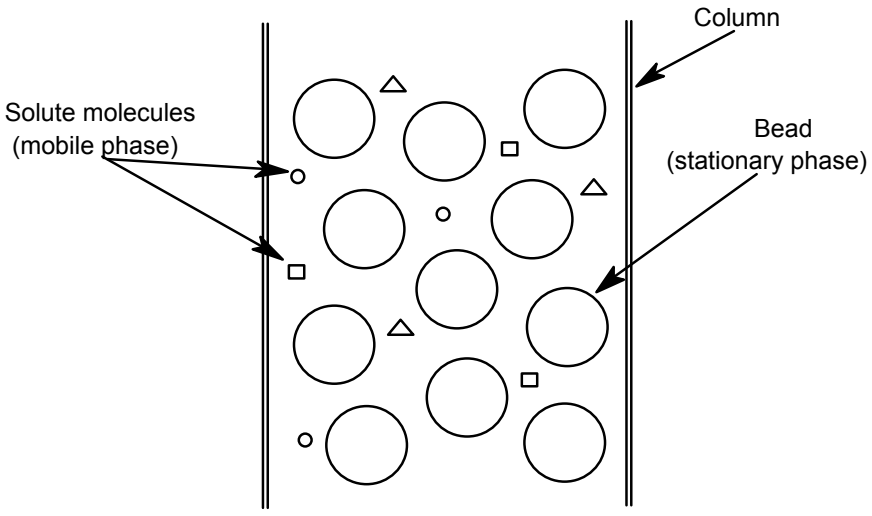

Figure 3.3. An enlargement of the stationary and mobile phases in a chromatography column.

The stationary phase is made of beads whose surface carries either a strong acidic group or a strong basic group. The groups attached to the beads determine the fixed charges on the column material. Through electrostatic interactions, the beads bind oppositely charged mobile ions (Figure 3.4).

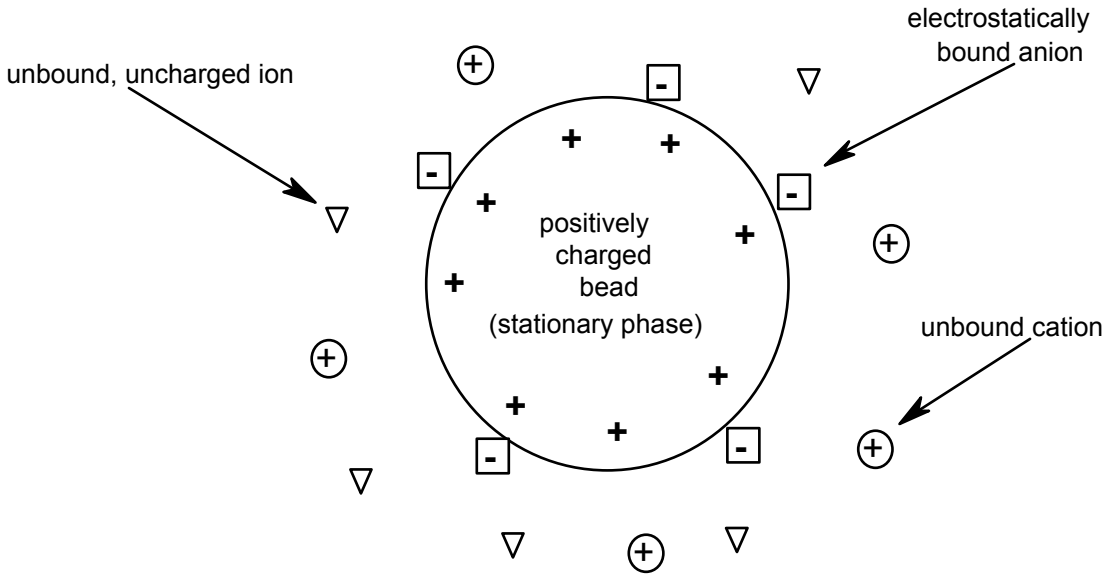

Figure 3.4. A close-up view of a stationary phase bead, showing its charge-charge interaction with dissolved solutes in the mobile phase.

Ion exchange beads are named according to the charge of the ions that they bind. Cation exchangers have negative charges linked to an inert material such as polystyrene. The negative charges allow binding by mobile cations (+). An example is sulfonic acid groups coating polystyrene beads.

Anion exchangers have positive charges linked to an inert material, and they bind mobile **anions** (–). An example is quaternary ammonium groups coating polystyrene beads.

The ion exchange column is created by filling a glass cylinder with beads to which ionic groups have been attached (Figure 3.5a). A mixture of various solute molecules is brought into the ion exchange medium by simply allowing a solution of them to flow through the column. Solutes entering the column may be negatively charged, positively charged, or uncharged (Figure 3.5b). Solute molecules that have a charge opposite to that of the stationary beads bind tightly to the beads, whereas neutral solute molecules or those with an identical charge do not bind (Figure 3.5c,d). The bound solute molecules can be released by changing their charge — as a consequence of changing the pH of their environment (Figure 3.5 e, f).

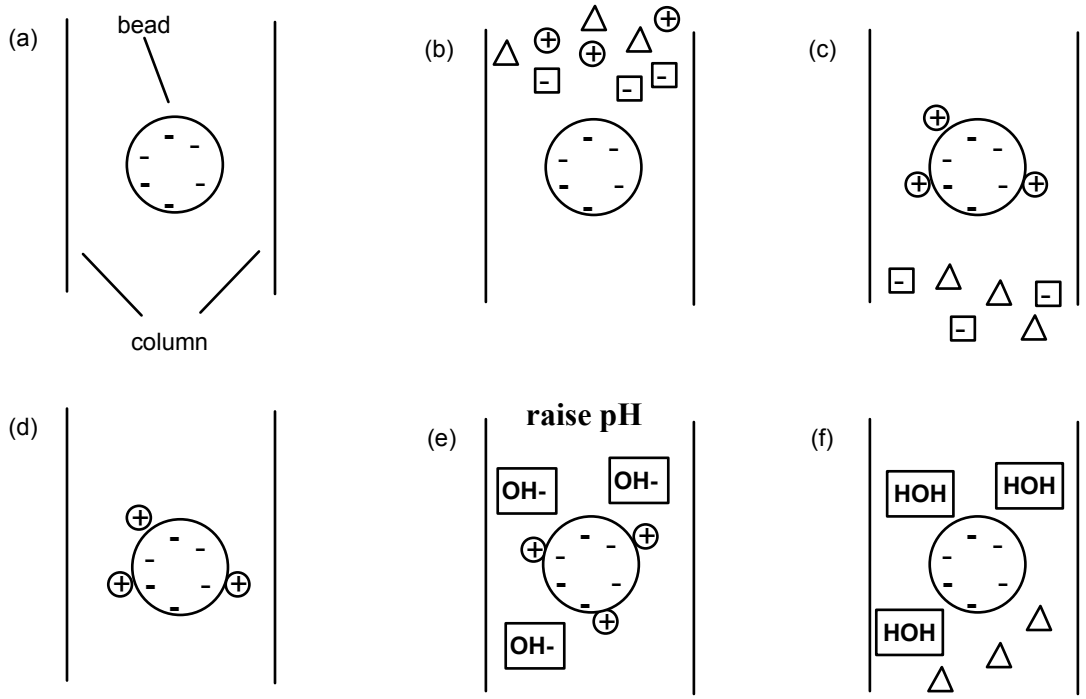

Figure 3.5. The process of ion exchange at a single bead. The individual steps are explained in the text.

Ionization Diagrams

To understand ion exchange chromatography, and the charges of amino acids in general, it is useful to draw ionization diagrams. These are representations of the different ion states of a molecule. Here is an ionization diagram for glycine.

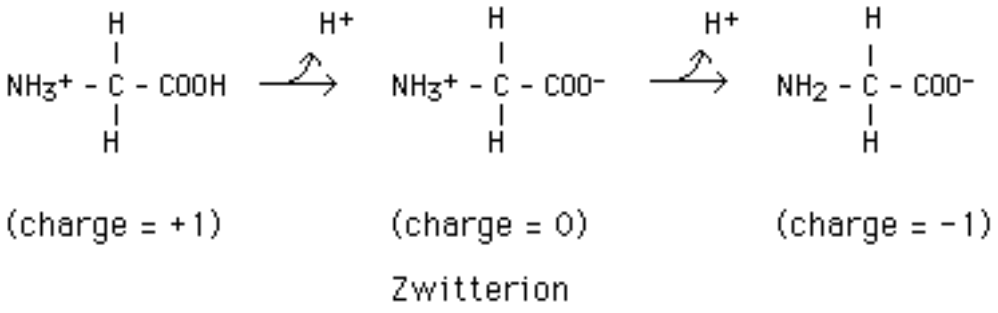

Figure 3.6. Ionization diagram for glycine.

Now model the ionization states of aspartic acid. How many ionization states do you think there will be? Arrange the molecules on your desk from the form found in the lowest pH environment, on your left, to the form found in the highest pH environment, on your right. Estimate the net charge of the aspartic acid molecule in each state.

Compare the net charges on glycine and aspartic acid at a range of pH values and fill in Table 3.3 below.

Table 3.3. A comparison of the net charges on glycine and aspartic acid under several pH conditions.

	Glycine				Aspartic Acid			
	α-COOH	α-amino	Side group	Net Charge	α-COOH	α-amino	side group	Net Charge
PH 3								
PH 7								
PH 11								

Glycine K_{diss} α-carboxyl $\approx 10^{-2}$

α-amino $\approx 10^{-10}$

Design an ion exchange column protocol to separate a mixture of glycine and aspartic acid. List the exchanger type (anion / cation) you would use and describe the steps you would follow to do the separation.

Report: Chemical Properties of Amino Acids

Activity 1: Amino Acid Structure

1. Explain why you might be able to utilize only about half of the mass of synthetically produced amino acid supplements?
2. The combination of amino acids to form a dipeptide is a dehydration reaction. Draw any two amino acids separately and then draw the structures of two different dipeptides formed from them.

Activity 2: Why Amino Acids are Charged

3. List the seven amino acids with ionizable side chains and approximate (to the nearest 0.1 charge unit) the net charge on each amino acid when the environment is at pH = 7.

Activity 3: Using Charge Differences to Separate Amino Acids

4. Design a protocol for separating valine, histidine and lysine using ion exchange chromatography. Describe the exchanger type you would use, and the steps you would follow to do the separation, including which amino acids would bind and which would pass through the ion exchange medium at each step.

Identification of Unknown Amino Acids

Pre-lab Assignment

1. Complete the activities and questions in Appendix 1.
2. Read the lab exercise, focusing on the Overview section, and the introductory material for each section.
3. Complete the questions below.

Questions

1. Use the charges you calculated on the last page of Appendix 1 to plot the characteristics of alanine and aspartic acid over a range of pH values. Connect the points by *estimating* what the charge should be at pH values where you lack data.
   Of course, it's possible to calculate the charge at any pH, but if you understand the relationships presented in the appendix you should be able to make adequate estimates quickly and easily.

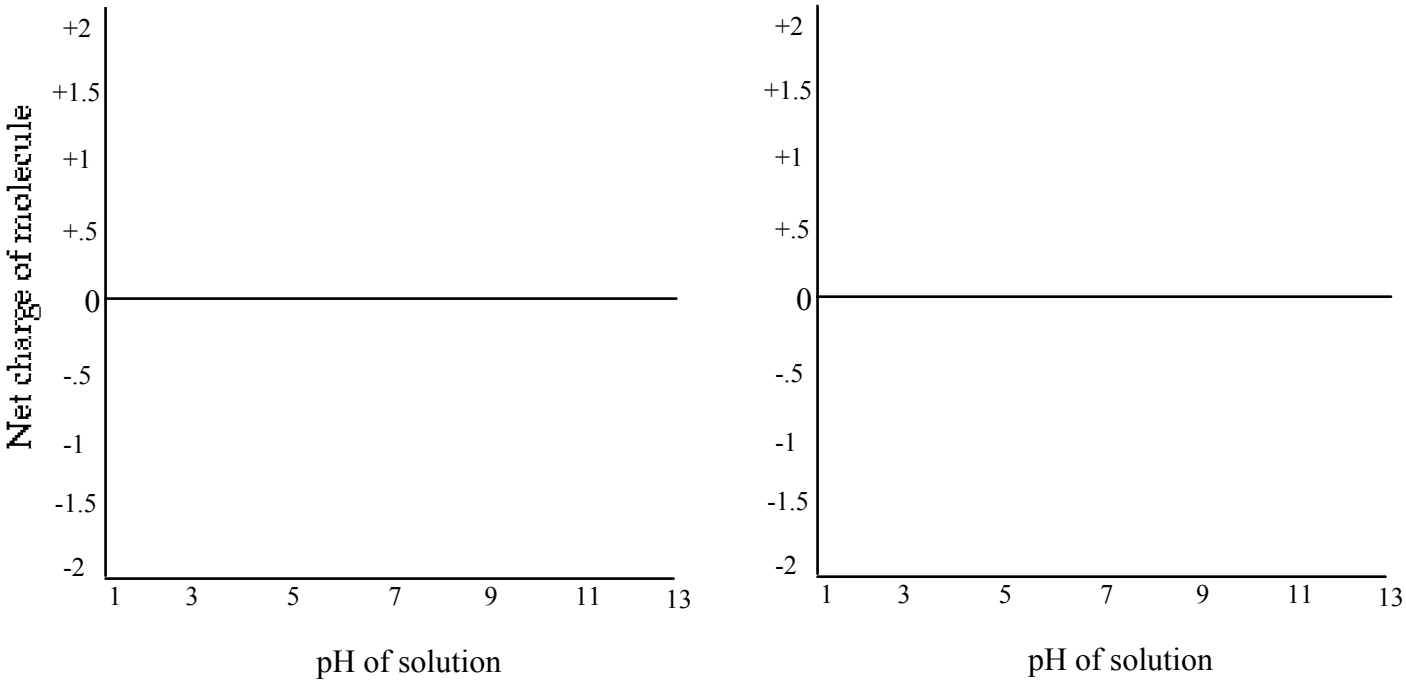

Figure 3.7. Axes for the predicted titration curves of alanine (left) and aspartate (right).

Identification of Unknown Amino Acids

Objectives

1. Group amino acids into categories based on biologically important properties.
2. Apply your understanding of amino acid ionization to titration and electrophoretic data.
3. Design an experimental protocol to identify unknown amino acids.

Overview

In this lab exercise, you are going to be a biochemical detective. You will continue to explore the properties of amino acids, as you did last week, and then you'll use your sleuthing skills to identify unknowns.

To help you learn about the biologically important properties of amino acids, you will be introduced to some techniques that can be used to measure them. The properties we'll be concerned with today include: electrical charge, interaction with water (hydrophobicity), and size.

You will use results from simple chemical tests of unknown amino acids to learn to separate the amino acids. It will be up to you, super-sleuth, to decide which properties distinguish certain amino acids from others. By going through the process of deciding which properties are relevant, choosing the appropriate technology to measure them, and then interpreting the results, you'll become a skilled amino acid tracker!

Activity 1: Using Titration to Determine the Relation Between Charge and pH

Curves similar to those you drew in the pre-lab can be constructed experimentally for unknown amino acids by titrating them with a strong base (e.g. NaOH) while measuring the pH of the solution. We are interested in changes in the net charge of the amino acid as hydrogen ions are added to, or subtracted from the system. Because the different amino acids have different numbers and kinds of ionizable groups, their titration curves can serve as "fingerprints" to identify them. Look back at the curves you drew in the pre-lab. You wouldn't have any trouble distinguishing alanine from aspartic acid on the basis of these curves, would you?

Look at the experimentally derived titration curve for alanine. Copy the graph onto the axis in Figure 3.8. Compare it to the curve you estimated in your pre-lab. How well did you do?

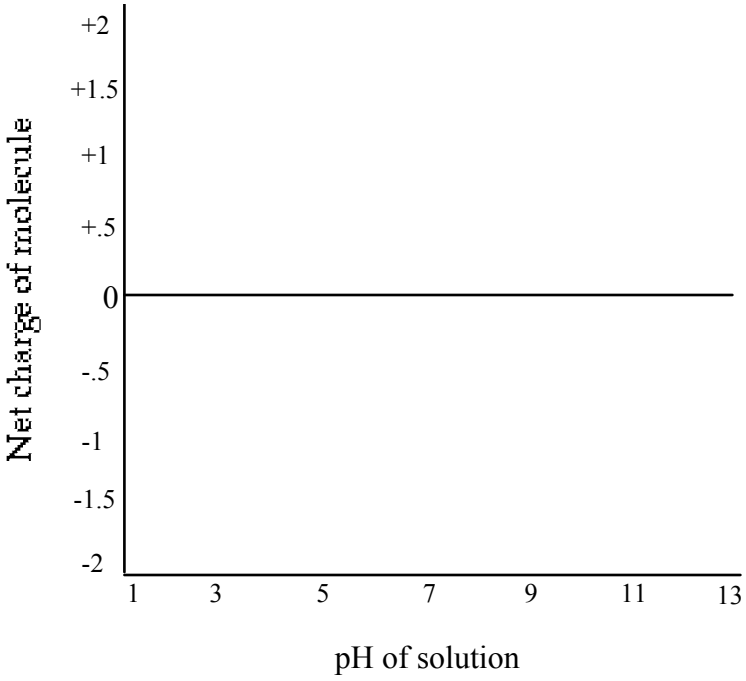

Figure 3.8. Axis for the experimentally derived titration curve for alanine.

Draw the forms of alanine, directly on the graph in Figure 3.8, that would predominate at pH 1, 6, and 12.

Label the point(s) on the graph where $[COO^-] = [COOH]$ *and* $[NH_3^+] = [NH_2]$.

What is the pK_a *for the* α*-carboxyl group of alanine?*

What is the pK_a *for the* α*-amino group of alanine?*

Based on what you've learned by studying alanine and aspartic acid, predict the titration curve for lysine in Figure 3.9. You may look up K_{diss} *or* pK_a *values, if you want to. Don't worry about being too precise. Plot a few key points on the graph, and then estimate the shape of the curve that connects them.*

How many steep places will the graph have, and where they will be? What do these steep places mean?

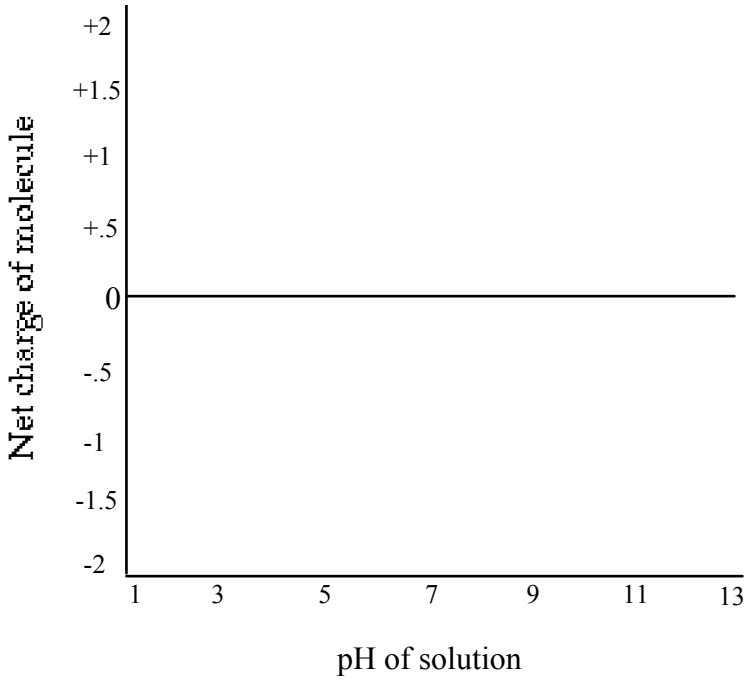

Figure 3.9. Axis for the prediction of the lysine titration curve.

On the appropriate parts of the graph, draw the four forms of lysine that differ from each other.

Now, check your prediction against the experimentally derived data.

If you are still having problems, practice drawing titration curves for a few other amino acids and see if you can predict their general shapes. You need to be comfortable with the interpretation of titration curves because you may decide to use them later in the lab when you are collecting data on your unknown amino acids.

Titrations of Derivitized Amino Acids

By altering a chemical group so that it is no longer able to dissociate, you can modify the titration curve of the amino acid. For example, you could take advantage of the fact that under certain conditions, formaldehyde (HCHO) reacts with α-amino groups, and with the side chain amino group in lysine to form derivatives in which the amino group is no longer readily ionizable.

To help you understand the change in the titration curve of derivatized amino acids, we will look at the effect of derivatization with formaldehyde of the α-amino group on glycine. After derivatization, glycine has only a single remaining ionizable group (see Figure 3.10).

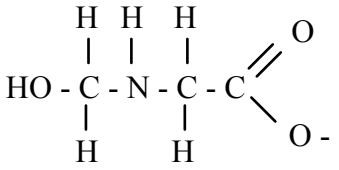

Figure 3.10. The chemical structure of glycine after formaldehyde derivitization.

How would the titration curve of derivatized glycine differ from that of the native glycine shown below? Draw you prediction on the same axes in Figure 3.11.

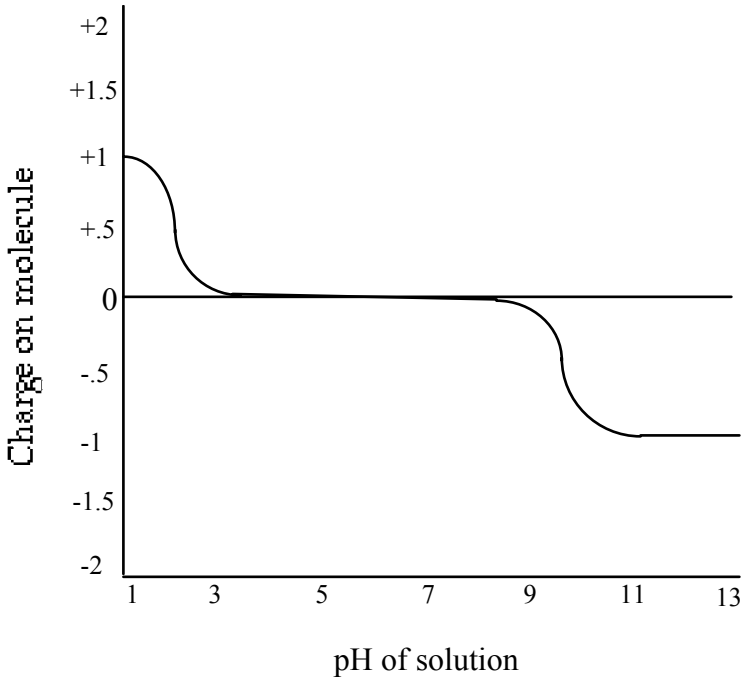

Figure 3.11. Axis for the prediction of the titration curve for derivitized glycine.

Check your results by comparing them with your lab partner's and with the experimentally derived curve.

Why would lysine be easy to identify using this technique?

Can you identify the other amino acid that would be immediately recognizable if it's derivitized form was titrated?

Activity 2: Measurement of Charge by Electrophoresis

Electrophoresis is the movement of charged molecules in an electric field. You used the same basic principle last term to move negatively charged DNA fragments through a gel. Electrophoresis without a gel can also be used to distinguish molecules simply on the basis of differences in the sign or magnitude of their charge, without regard to differences in mass. Molecules carrying a net positive charge migrate toward the negative pole of an electric field, whereas molecules carrying a net negative charge move toward the positive pole.

Like titrations, electrophoresis in solutions with different pH values can be used to classify amino acids. This is because the change in charge caused by a change in pH shows up readily as altered electrophoretic mobility (how the amino acids move in an electrical field). For today's exercises we will be using pH values of 2, 6 and 11. Based on your practice with amino acid ionization states, you should be able to predict the net charge on the molecules and how they ought to migrate in an electric field at these pH values. Since the amino acids, themselves, are invisible, we will stain them after the run to see how far they've gotten. We'll use ninhydrin, a reagent that reacts with the α-amino group to give a purple color.

Because the electrophoresis run requires some time, it will be started early to ensure you'll have the results before the end of the lab period.

To make sure that you understand how to interpret electrophoretic data you should practice by predicting the movement of amino acids under the experimental conditions in Table 3.4.

Table 3.4. Data sheet for the electrophoretic movement of several amino acids at various pH conditions.

	Net Charge at pH:			Predicted Movement at pH:			Observed Movement at pH:		
Amino Acid	2	6	11	2	6	11	2	6	11
Glutamic Acid									
Lysine									
Glycine									
Histidine									

The electrophoretic data for these amino acids is posted in the front of the lab room so you can check your predictions. Remember, this type of data could be important when you are working to solve your unknowns.

Activity 3: Distinguishing Amino Acids by Solubility Differences

An important property that distinguishes some amino acid side chains from others is their relative solubility in water versus a less polar solvent. Simply by looking at the structures of various side chains, you have probably already guessed that some of them ought to dissolve easily in water, whereas others should not. Side chains that are not soluble in water ought to dissolve, instead, in less polar solvents, such as benzene or ethanol.

As you learned in chemistry, the key to predicting solubility from structure lies in knowing the properties of particular chemical groups. Charged groups are highly soluble in water because, as ions, they interact very favorably with a surrounding shell of polar water molecules. Polar groups —

for our purposes, those containing oxygen or nitrogen atoms — also interact favorably with water, and are, therefore, water-soluble. On the other hand, hydrocarbons (CH_2 groups) are insoluble in water because the interactions between water and CH_2 are not particularly favorable. Given the choice, the hydrocarbon units prefer to stay together in a pure solid or liquid — minimizing their unfavorable interactions with water. The larger the overall structure of the hydrocarbon is (that is, the larger the number of CH_2 units it contains), the greater is its preference for itself over water. This is because large hydrocarbons encounter more water molecules than do small hydrocarbons.

Based on these ideas, you should be able to make good guesses as to the solubility of particular amino acid side chains. Many of them contain both polar and non-polar (hydrocarbon) components. You can estimate the overall solubility of the side chain by considering the relative proportions of both kinds of groups. For instance, the side chains of both serine and tyrosine contain one hydroxyl group, obviously a polar component, but the solubilities of these two side chains in water is very different.

Is tyrosine or serine more soluble in water? Why?

Give several examples of amino acids whose side chains are water soluble and amino acids whose side chains are water insoluble.

These observations suggest that a handy way to distinguish one amino acid from another would be to compare their solubilities in water and in ethanol. Of course, such solubilities are determined by the sum of the contributions made by each part of the amino acid — not just the side chain.

How does the body (everything except the side group) of an amino acid affect it's solubility in water? How does the body affect it's solubility in ethanol?

In fact, it turns out that all *free* amino acids are more soluble in water than they are in ethanol because the side chain makes a relatively small contribution to the overall solubility. Nonetheless, we can detect the effects of different side chains by comparing the ratios of solubility in water to solubility in ethanol for each amino acid. These ratios form a clear pattern showing that certain amino acids prefer water over ethanol by a much larger margin than do other amino acids. Thus, the value of the water/ethanol solubility ratio can be used to decide whether a particular amino acid side chain is soluble in water (hydrophilic) or insoluble in water (hydrophobic).

The data that will be available for your unknown amino acids will put them into the three broad categories shown in Table 3.5.

Table 3.5. Solubility classes of amino acids for use when solving your unknowns.

Solubility class	Relative solubility H_2O/EtOH	Example amino acids
Low	10 - 200	leucine, tyrosine
Medium	200 - 2000	methionine, valine
High	2000 - 8000	alanine, threonine

What do larger numbers mean? What do smaller numbers mean?

It will be helpful later, when you are working on your unknowns, if you have tried to group the amino acids into solubility classes. Start by placing the examples listed above and then, based on the structure of the side chains, try to group the rest of the amino acids. Don't hesitate to ask for help if you are having trouble.

Activity 4: Distinguishing Amino Acids by Size Differences

Gel Filtration

The experimental set-up for gel filtration looks similar to ion exchange chromatography because it uses a column packed with small beads, through which a solution is poured. There are important differences between the two types of columns, however, allowing the gel filtration column to separate molecules on the basis of size, not charge.

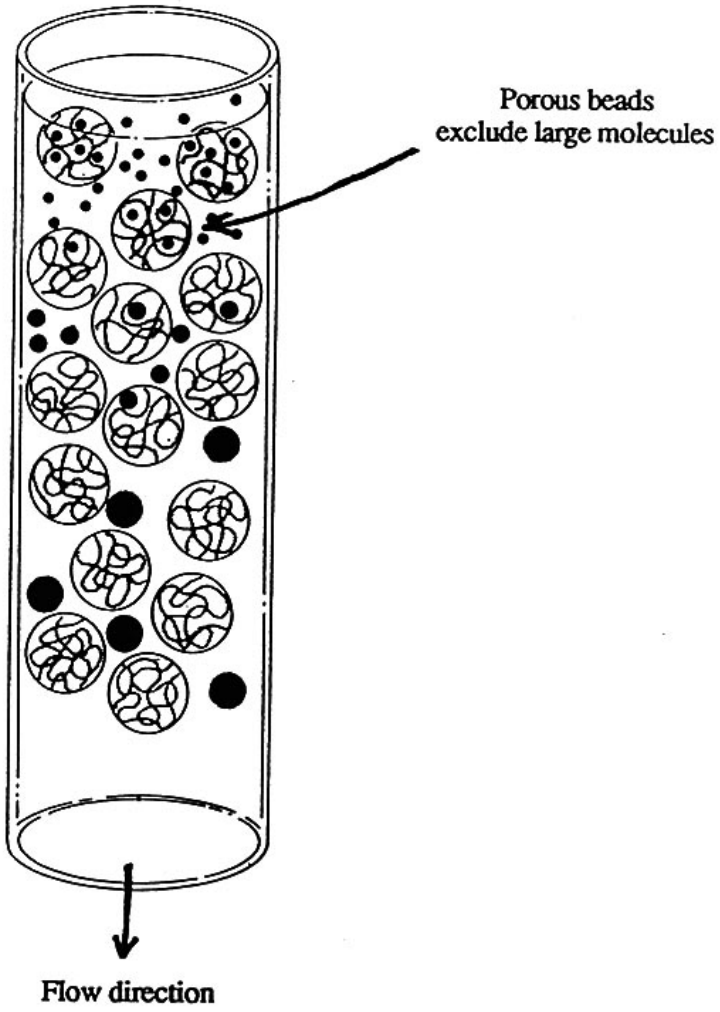

Figure 3.12. A schematic representation of a gel filtration column.

Ion exchange columns are filled with charged beads, which bind molecules of the opposite charge. The beads in a gel filtration column are *not* charged. Instead, they are porous, or sponge-like, so that small molecules get caught inside them, whereas large molecules stay outside (Figure 3.12). As a result, small molecules take longer than big molecules to pass through the column. Think of the beads as ping pong balls with small holes in them. Molecules that are smaller than the holes will diffuse into the ping pong balls, and spend time there. Molecules that are larger than the holes can't waste time "exploring" the inside of the ping pong balls, and, therefore, travel quickly through the column (Figure 3.13).

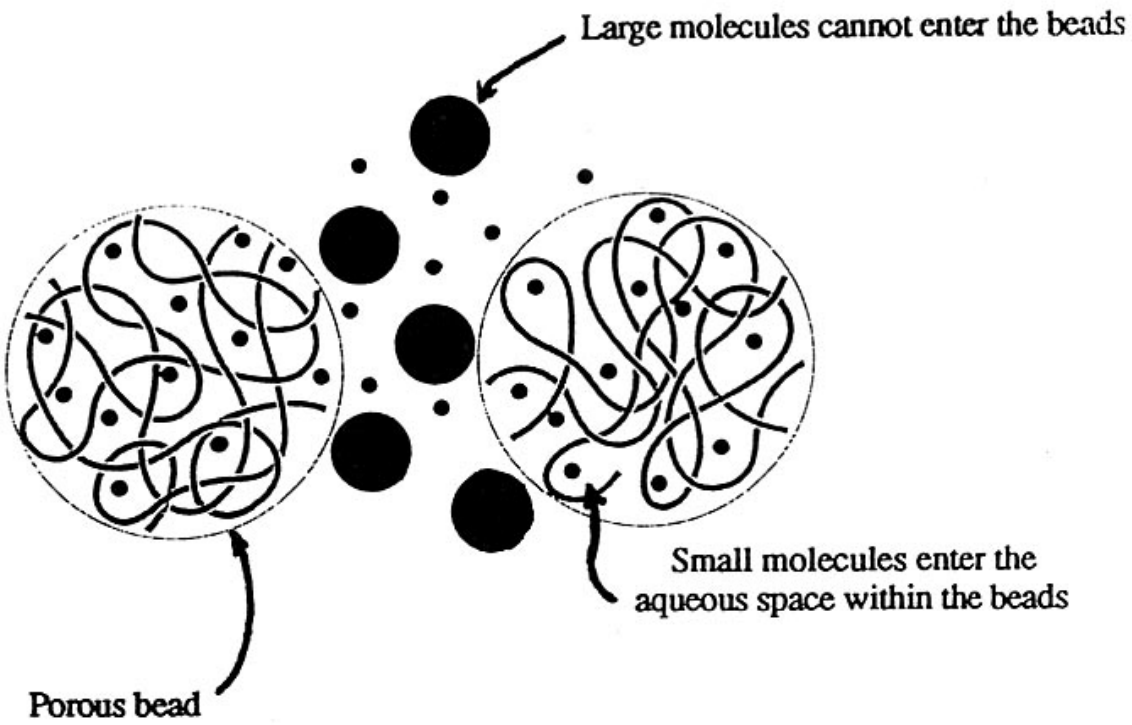

Figure 3.13. An enlargement of the beads and solutes within a gel filtration column.

By choosing beads with appropriately sized holes (or pores) in them, the gel-filtration column can be designed to separate molecules in different size ranges. You will have access to data from a specially designed column capable of resolving amino acids based on the size classes described in Table 3.6.

Table 3.6. Sizes classes of amino acids determined by gel filtration.

Gel Filtration Fraction	Molecular Weight Range	Example Amino Acid
1	>170	tyrosine
2	145-165	methionine
3	130-140	leucine
4	100-125	valine
5	<90	glycine

The results of gel filtration of your unknowns later in the lab may help you in their identification. Before you start calculating the exact molecular weight for each amino acid, you should take a look at the examples given for each size class. You will probably be able to assign amino acids to particular size classes simply by comparison with the example amino acids.

Activity 5: Other Tests for Distinguishing Amino Acids

Identification of Amino Acids Containing Sulfur

A chemical test to confirm the presence of sulfur in a compound is procedurally quite simple. First the molecule is "disassembled", then it is reacted with lead acetate, $Pb(CH_3COO)_2$. A black precipitate indicates the presence of sulfur.

Which amino acids contain sulfur?

Using Absorption Spectra to Indicate the Presence of Conjugated Rings

Organic compounds that contain alternating single and double bonds within a ring structure absorb certain wavelengths of light energy. Theses amino acids can be easily distinquished by their absorption spectra.

Which amino acids contain conjugated ring structures?

Activity 6: Design a Protocol to Identify Unknown Amino Acids

Now for the fun part! In this exercise, you will use the characteristics you have studied to help you identify unknown amino acids. You will select six amino acids from the full group of possible amino acids, and then try to identify them.

The unknown amino acids are the products of acid-catalyzed hydrolysis of a polypeptide. Under the hydrolysis conditions, tryptophan would have been destroyed, and the amide side chains of asparagine and glutamine would have been hydrolyzed to free carboxyl groups. Consequently, asparagine would have been converted to aspartic acid, and glutamine would have been converted to glutamic acid. Your job is to identify six unknown amino acids from the total of 17 that could potentially be in this mixture.

To make this more challenging and realistic, you will be allowed to collect only three (3) test results for each unknown. This resembles real life in the lab — where you never have enough materials to carry out every conceivable test! The tests and results should be recorded directly on your unknown cards to help you keep track of them. When you are ready to run a test, write the name of the test on the card and then give the card to a TA. The TA will write the result on the card and return the card to you. To use your resources efficiently, you should plan carefully and request only tests that clearly distinguish between amino acids that you consider reasonable possibilities.

As you will undoubtedly find, there will be some amino acids that are easy to identify and others that are more difficult, or downright impossible. In completing this assignment, you need to focus on correctly interpreting the data you collect. Do not be discouraged if you are not able to identify each unknown unambiguously. Be sure to record all of the candidate amino acids that are consistent with your test results. You may then list other tests, or combinations of tests, that you think could distinguish them.

Before you begin, develop a format for tracking your results and deciding on candidates for the different unknowns. Your lab report will involve showing how each test result eliminates or includes amino acids from the list of possible unknowns.

When you are ready, come to the front of the lab and choose your unknowns. Good Luck.

Lab. Report: Amino Acid Identification

State your results and justifications in summary form. The information should be concise, well organized, and complete enough to convince others of the logic behind your identifications.

APPENDIX 1: A Review Of The Relationship Between pH and pK

As you learned in the last lab, all *free* amino acids have at least two groups that have the potential to be charged. Some amino acids also have side chains with dissociable groups. If these groups give up their protons easily, we call them acids; if, instead, they take up protons, we call them bases.

The loss or gain of protons from any of these groups changes the net charge of the amino acid as a whole. As you remember from last week, the loss or gain of protons can be influenced by manipulating the pH of the solution. Titration of a solution containing an amino acid can be used to follow the way the molecule responds to changes in pH. As a "warm-up" for the titration (which will include many values of pH), you will estimate the behavior of two amino acids (alanine and aspartic acid) at four different pH values.

You will need to recall the expression for the equilibrium constant for dissociation of a weak acid:

$$Kdiss = [H+] \cdot \frac{[A-]}{[HA]} \qquad \text{(equation 1)}$$

which, if rearranged, is:

$$\frac{1}{[H+]} = \frac{1}{Kdiss} \cdot \frac{[A-]}{[HA]} \qquad \text{(equation 2)}$$

What we are concerned with right now is the relationship between pH and the ratio of charged to uncharged molecules.

Starting from equation (2), derive this relationship for yourself.

pH = (equation 3)

At what pH would half of the acid molecules be dissociated?

Now let's consider the charge on two groups found in amino acids.

What would the average charge on a *carboxyl* group be at the pH where this group is half *dissociated?*

What would the average charge be on an amino group at the pH where this group is half dissociated?

Now go on to two real amino acids, alanine and aspartic acid. For each of them, fill in Table 3.7 for each ionizable group. You need only the relationship you have derived in equation 3, and the information given in the chart.

Table 3.7. Exploring the relationship between dissociation constant and pK_a for groups.

	Group	K_{diss}	pH when $[A^-] / [HA] = 1$	sign and magnitude of average charge when $[A^-] / [HA] = 1$
Alanine	α-COOH	$\approx 10^{-3}$		
	α-NH_3^+	$\approx 10^{-9}$		
Aspartic acid	α-COOH	$\approx 10^{-2}$		
	α-NH_3^+	$\approx 10^{-9}$		
	side-COOH	$\approx 10^{-4}$		

The pH at which a group is half dissociated is called the pK_a of the group.

Write an expression for the relationship between the pK_a and the dissociation constant of a group.

What is the relationship between the affinity of a group for protons, and its pK_a?

A high K_{diss} value indicates (high or low) affinity for protons.

It takes a (higher or lower) concentration of protons to protonate a group whose pK_a is low.

Now consider each of the amino acids as a whole molecule.

Use the K_{diss} values from the chart, and your understanding of the relationships among K_{diss}, pH and charge, to draw the predominant form of alanine and aspartic acid that would exist in each of the solutions represented in Figure 3.14. Also indicate the net charge (sign and magnitude) of the amino acid for each pH.

Alanine

pH 1 pH 5 pH 7 pH 11

Net
Charge _____ _____ _____ _____

Aspartic acid

pH 1 pH 3 pH 7 pH 11

Net
Charge _____ _____ _____ _____

Figure 3.14. Space for drawing ionization forms of amino acids under various pH conditions.

Acknowledgements

This lab was developed jointly by Sam Donovan, Carl Stiefbold, and Karen Sprague in the Department of Biology at the University of Oregon. We would like to acknowledge Bill Sistrom's contribution as a founder of the Biochemistry lab sequence, Vicki Chandler as an important user and reviewer of the labs, and the many teaching assistants and students who made useful comments during the development of the lab exercises. Support for this project was provided by a grant from the Howard Hughes Medical Institute.

APPENDIX A

Key to Amino Acid Properties

	Titration groups	Relative Solubility	MW-class*	Presence of Sulfur	formalde-hyde rxn**	E-phoresis pH 2,6,11***	Presence of conj. ring	unknown code
Alanine	2	High	89–5	–	1	+,0,–	–	
Arginine	3—12.3	High	174–1	–	1	+,+,0	–	
Aspartic Acid	3—3.9	High	133–3	–	1	+,–,–	–	
Cysteine	3—8.3	High	121–4	+	1	+,0,–	–	
Glutamic Acid	3—4.0	High	147–2	–	1	+,–,–	–	
Glycine	2	High	75–5	–	1	+,0,–	–	
Histidine	3—6.0	High	155–2	–	1	+,+,–	+	
Isoleucine	2	Low	131–3	–	1	+,0,–	–	
Leucine	2	Low	131–3	–	1	+,0,–	–	
Lysine	3—10.8	High	146–2	–	2	+,+,–	–	
Methionine	2	Medium	149–2	+	1	+,0,–	–	
Phenylalanine	2	Low	165–2	–	1	+,0,–	+	
Proline	2	Low	115–4	–	0	+,0,–	–	
Serine	2	High	105–4	–	1	+,0,–	–	
Threonine	2	High	119–4	–	1	+,0,–	–	
Tyrosine	3—10.1	Low	181–1	–	1	+,0,–	+	
Valine	2	Medium	117–4	–	1	+,0,–	–	

* Size categories: molecular weight range (number of AAs in each class)

1: >170 (2)
2: 145–165 (5)
3: 130–140 (3)
4: 100–125 (5)
5: <90 (2)

** Formalyde reaction - number of groups derivitized

*** Net charge on molecule at the pH values

APPENDIX B

Experimentally Derived Curves Corresponding to Figures 8, 9, and 11.

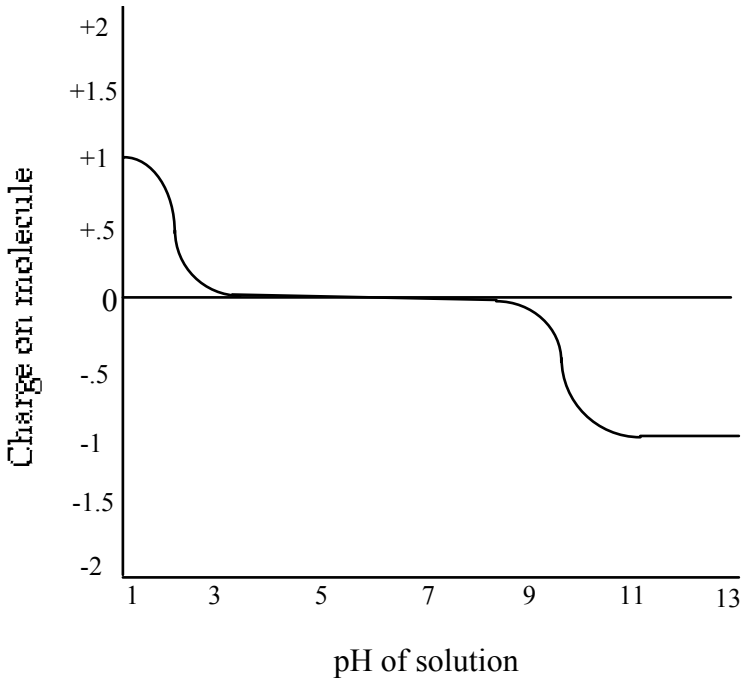

Figure 3.15. Experimentally derived titration curve for alanine. Corresponds to Figure 3.8 in text.

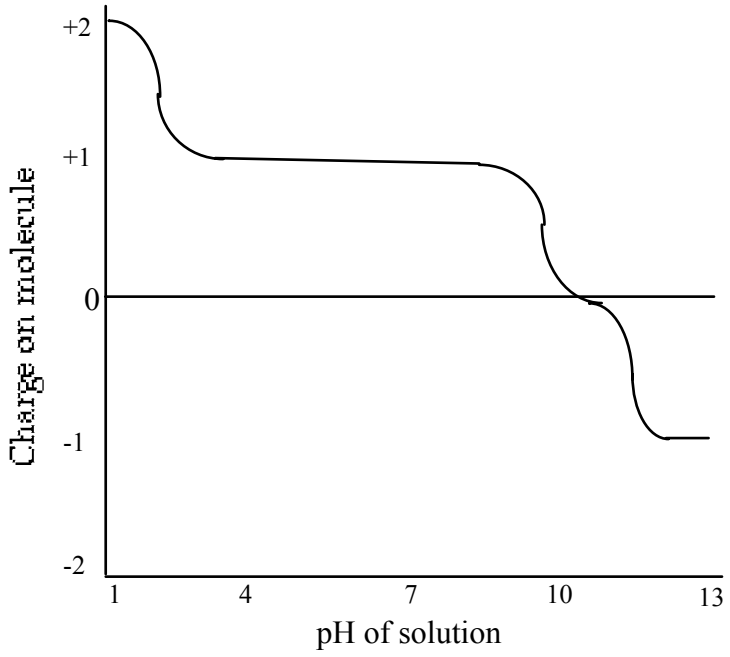

Figure 3.16. Experimentally derived titration curve for lysine. Corresponds to Figure 3.9 in text.

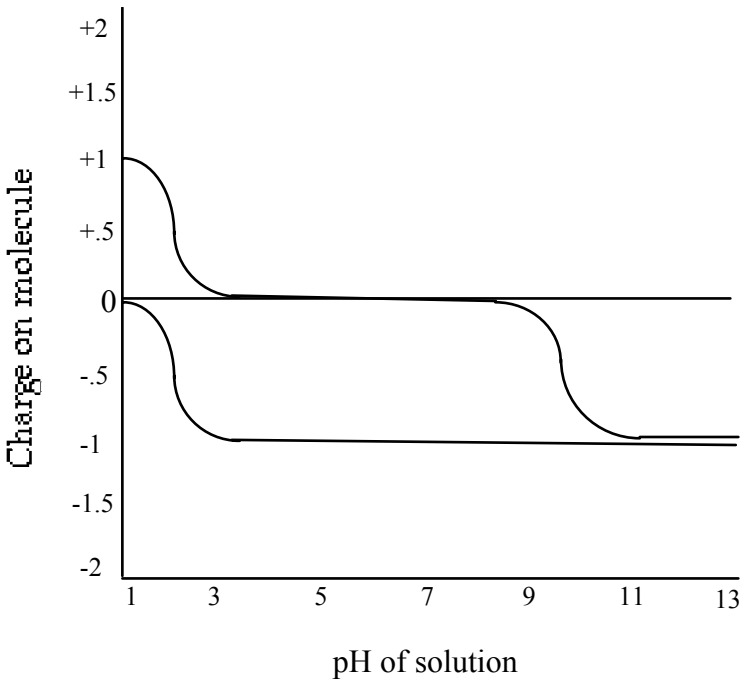

Figure 3.17. Experimentally derived titration curve for alanine. Corresponds to Figure 3.11 in text.

APPENDIX C
Titration Curves to be Handed Out as Clues.

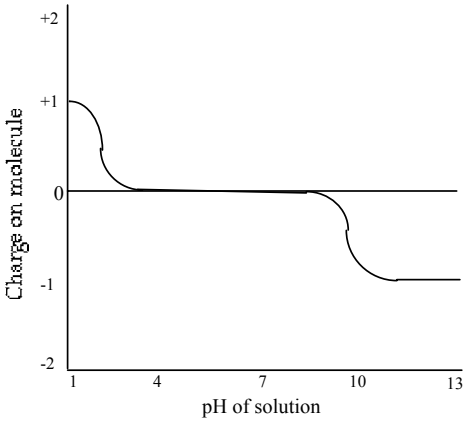

Figure 3.18. Titration curve for alanine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, and valine. Also titration curve for *derivatized form* of proline.

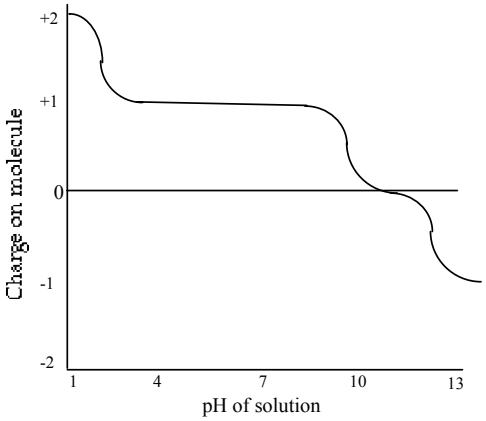

Figure 3.19. Titration curve for argainine.

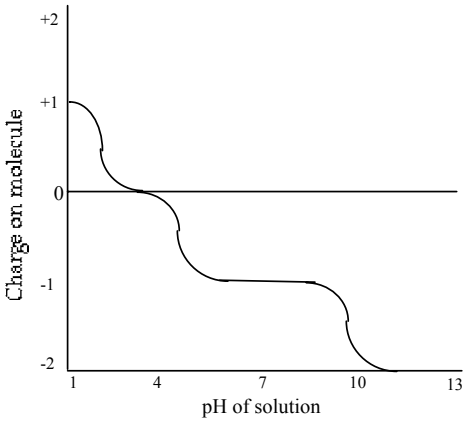

Figure 3.20. Titration curve for aspartic acid and glutamic acid.

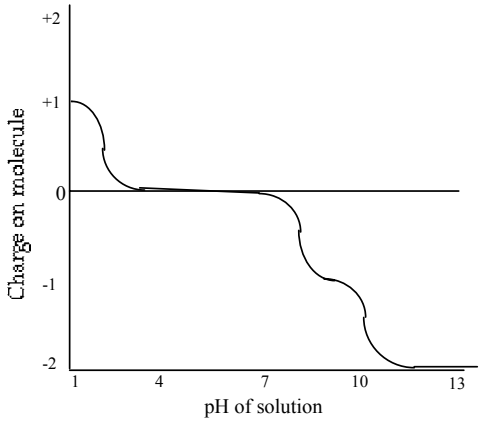

Figure 3.21. Titration curve for cystine.

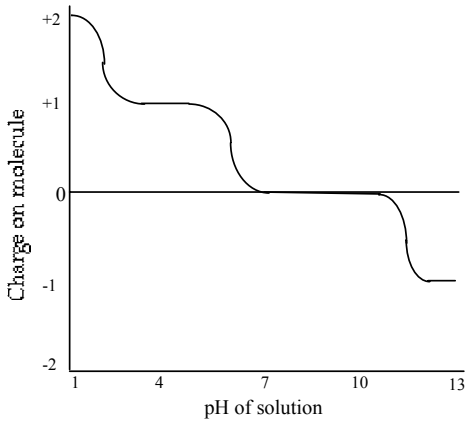

Figure 3.22. Titration curve for histidine.

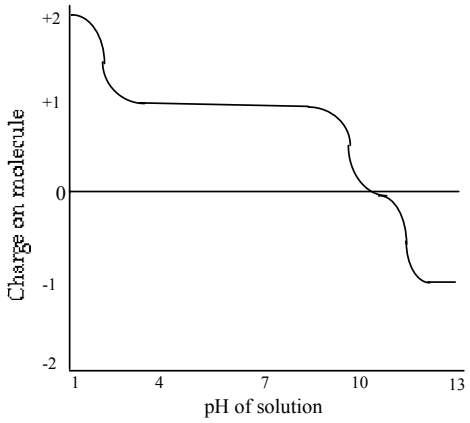

Figure 3.23. Titration curve for lysine.

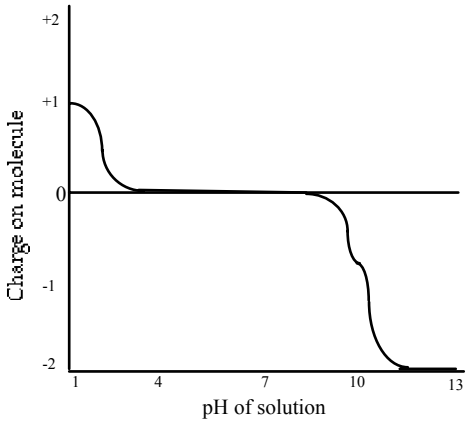

Figure 3.24. Titration curve for tyrosine.

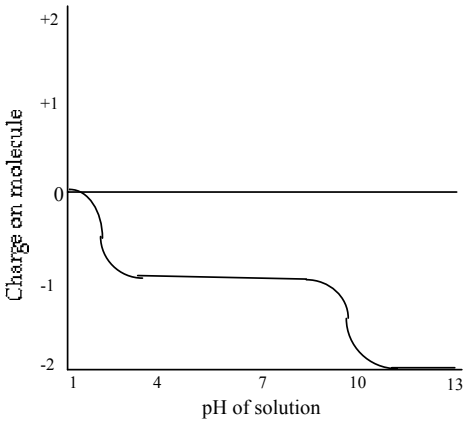

Figure 3.25. Titration curve for the *derivatized forms* of alanine, glycine, isoleucine, leucine, methionine, phenylalanine, serine, threonine, and valine.

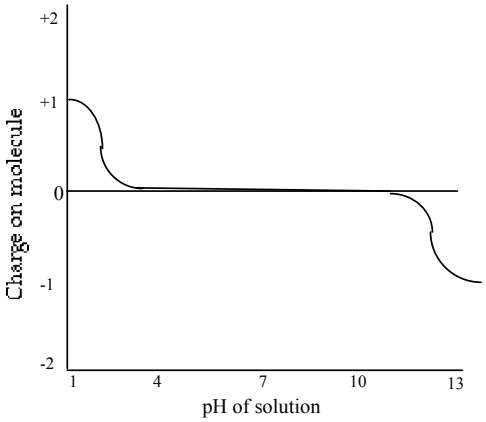

Figure 3.26. Titration curve for *derivatized form* of arganine.

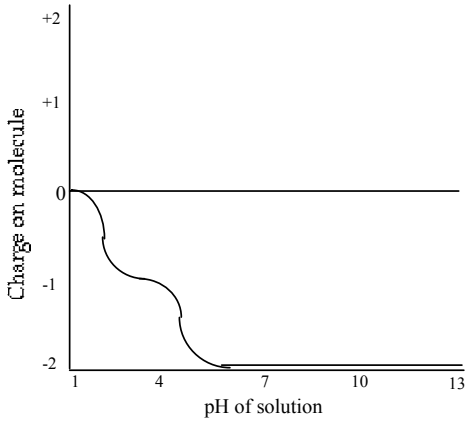

Figure 3.27. Titration curve for *derivatized form* of aspartic acid and glutamic acid.

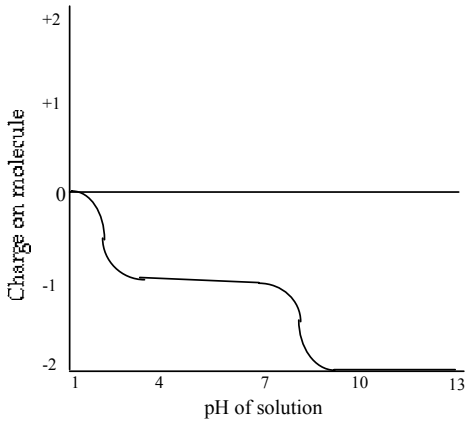

Figure 3.28. Titration curve for *derivatized form* of cystine.

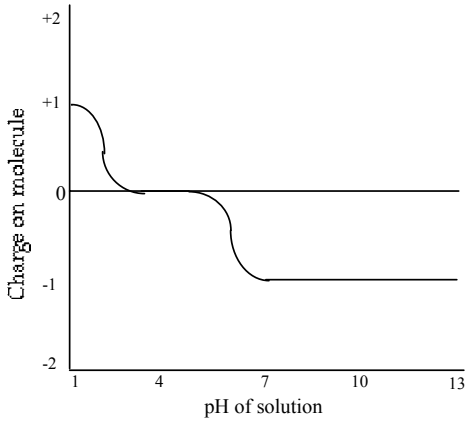

Figure 3.29. Titration curve for *derivatized form* of histidine.

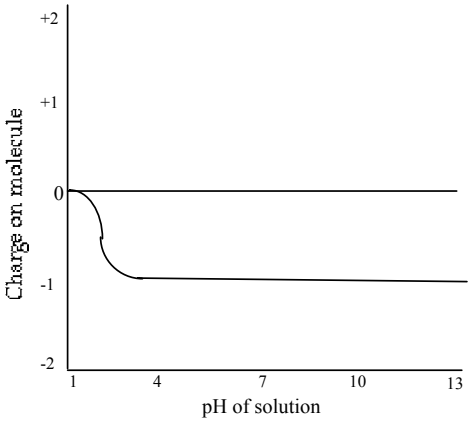

Figure 3.30. Titration curve for *derivatized form* of lysine.

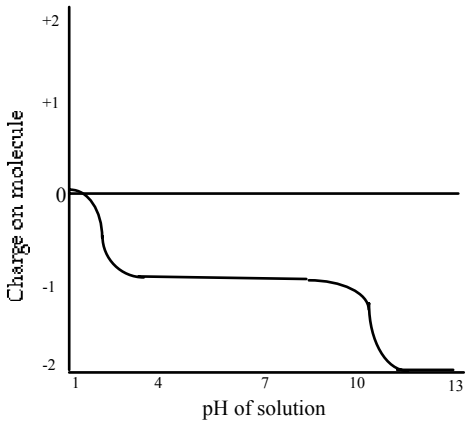

Figure 3.31. Titration curve for *derivatized form* of tyrosine.

APPENDIX D

Electrophoresis Results to be Handed Out as Clues.

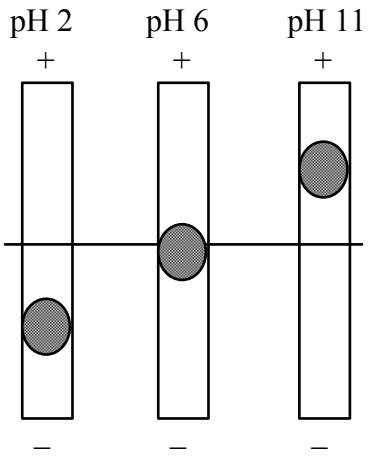

Figure 3.32. Electrophoresis results for alanine, cystine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine.

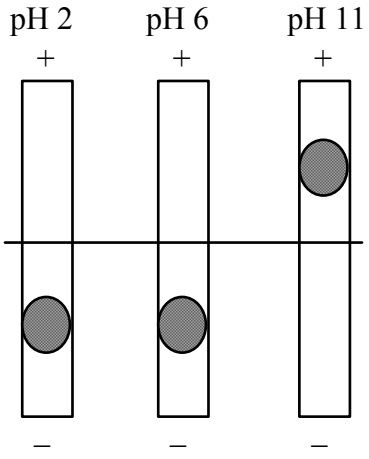

Figure 3.33. Electrophoresis results for lysine and histidine.

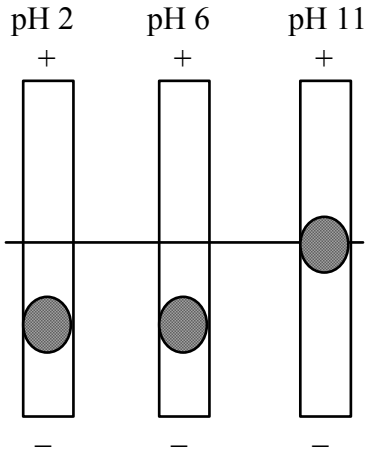

Figure 3.34. Electrophoresis results for arginine.

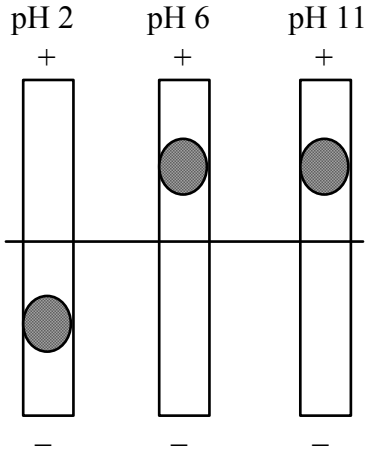

Figure 3.35. Electrophoresis results for aspartic acid and glutamic acid.