INTRODUCTION

The primary importance of amino acids in cell structure and metabolism lies in the fact that they serve as building blocks for proteins. They may also serve as constituents of a variety of other molecules of biological interest. An understanding of the properties of amino acids is thus vital to an understanding of protein structure and function.

Amino acids occurring in nature have the general structure shown below:

$$\begin{align*}
\text{H} & \\
\text{R} & \\
\text{N}^+ - \text{C-COO}^- \\
\text{H}_2N' - \text{C-COO}^- \\
\text{H}_2O & \\
\end{align*}$$

With one exception, proline, all are amino acids are $\alpha$-amino acids. All except glycine have an asymmetric carbon atom. Perhaps the most important property of amino acids is their ability to be linked together in what is known as the peptide bond (as shown in the example below on the right of the two-amino acid peptide, dipeptide).

The amino acids commonly found in proteins can be classified into four general groups based upon the properties of the R- groups (nonpolar: alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, and methionine; polar, uncharged: glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; polar, negatively charged [acidic]: glutamic acid and aspartic acid; polar, positively charged [basic]: lysine, arginine, and histidine).

The classification given here is the most general type of classification. Various texts will classify the amino acids according to slightly different criteria. For example, phenylalanine, tyrosine, and tryptophan are often classified together in a separate category known as aromatic.

Ionic Properties of Amino Acids

Look at the ionization states of a generic amino acid:

$$\begin{align*}
\text{H} & \\
\text{R} & \\
\text{N}^+ - \text{C-COO}^- & \approx \text{H}_2N' - \text{C-COO}^- \approx \text{H}_2N - \text{C-COO}^- \\
\text{H}_2O & \\
\text{H}_2O & \\
\text{H}_2O & \\
\end{align*}$$

The carboxyl group can be considered a weak acid and the amino group can be considered a weak base. The $\text{pK}_a$ for the carboxyl group of an amino acids is generally between 2.0 and 2.5 while the $\text{pK}_a$ of the amino group is between 9.0 and 10.0. If the R group is nonpolar, at low pH (high H$^+$ concentration) both the carboxyl and amino groups are protonated, giving the amino acid a net positive charge. As the pH is raised, the carboxyl group's hydrogen is lost (deprotonation) and the charge on the molecule becomes neutral. This form is known as the zwitterion; the pH where all of the amino acid molecules in a sample have no net charge is called the isoelectric point, $\text{pI}$.

In addition to the general charge considerations just mentioned, it should be noted that certain R-groups may also contribute to the net charge of an amino acid (and in fact are the major factors in the net charge on a protein). In such cases, the ionization of the amino acid will be described by three $\text{pK}_a$ values instead of two (as shown for aspartic acid below).
The second molecule in the scheme is the zwitterion or isoelectric form (the form with a charge of zero) and the pI is usually going to be about halfway between the pK values of the isoelectric form and the previous form (pK2 and pK1 in this example). Please refer to one of the references for the structures of the side chains for the other amino acids (i.e. Stryer, Biochemistry, 4th edition, p.20 or Lehninger, Principles of Biochemistry, 2nd edition, p. 115).

Solubility Properties of Amino Acids
The ionization of the carboxyl and \( \alpha \)-amino groups give amino acids a strong dipolar character and thus an appreciable solubility in water. Generally, the lowest solubility for a given amino acid will occur when the pH of the medium equals the isoelectric point (i.e. pH = pI). The solubility is also dependant upon the nature of the R-groups. In general, the solubilities of the various classes of amino acids are as follows:

- polar (acidic or basic) > polar uncharged > nonpolar

Within each class, the solubility will decrease as the polarity of the amino acid decreases. Alanine will therefore be more soluble than valine, and valine more soluble than leucine.

Partition Chromatography
In this experiment we will be studying the properties of amino acids and identifying an unknown amino acid or acids using partition chromatography. Partition chromatography is based on the partition principle which states that an amino acid will distribute itself between two immiscible liquids according to its partition coefficient. For example, if your two liquids are water and n-butanol, some of the amino acid will dissolve in water and some in n-butanol. (The proportions depend on the structure of the amino acid) Because each amino acid has a different partition coefficient under standard conditions, they can be separated by many successive partitions. When done in a separatory funnel, this is a time consuming process; but many partition steps may be carried out using a column or plate of hydrated-inert substance. We will be using microcrystalline silica gel plates (basically hydrated sand, \( \text{SiO}_2 \cdot \text{H}_2\text{O} \)). The associated water is called the stationary phase while the solvent is referred to as the mobile phase. If the amino acid is more strongly attracted to the water, it will stay near the point of application. Amino acids more strongly attracted to the solvent will move away from the point of application as the solvent moves up the plate. Since both phases are liquid, this type of chromatography is referred to as liquid/liquid partition chromatography. Since it is performed on a thin layer of material, it is known as thin layer chromatography (TLC).

In ideal partition chromatography the only factor that affects the mobility of a compound is its relative solubility in the two phases. In reality, other factors such as adsorption will also influence the rate of migration of a compound subjected to TLC. It is important to remember that the partition of a compound between two immiscible solvents is unaffected by the presence of other substances.

With a given chromatography system and solvent, the movement of a compound relative to the movement of the solvent front (the front border of the solvent's motion) is a reproducible (repeatable) quantity depending upon the compound, the solvent, and the support matrix (like silica gel). In the case of paper or TLC, the movement of a compound is conveniently expressed as an Rf value:

\[
R_f = \frac{\text{distance moved by compound}}{\text{distance moved by solvent}}
\]

For example, note the system shown on the left. Two compounds are fractionated using TLC. Each compound was applied to a spot at the origin and subsequently fractionated by placing the plate into tray containing solvent so that the solvent level is initially below the origin line. Compound A moves a distance of "a" units, compound B moves a distance of "b" units, and the solvent moves "c" units. The Rf for each compound is given by:
A: \( Rf = a/c \)  

B: \( Rf = b/c \)

In this case, compound B is more attracted to the solvent than compound A as indicated by a larger Rf value.

**What is the limiting value Rf?**

**Ninhydrin Assay**

Amino acids are almost invisible to the naked eye so you won’t see anything when you apply the sample to the TLC plate (except in some cases you’ll notice a very slight color). This is especially true after developing the TLC plate, therefore we’ll need a system for detecting the amino acid. Typically, ninhydrin is the reagent used for detecting and quantifying amino acids and peptides. Ninhydrin is a powerful oxidizing agent that oxidizes amino acids to yield ammonia, carbon dioxide, the corresponding aldehyde, and a reduced form of ninhydrin called hydrinatin.

![Chemical reaction diagram]

The reaction is a two stage system. In the first stage, ninhydrin reacts with the amino acid breaking off both the \( \alpha \)-amino and \( \alpha \)-carboxyl groups to yield \( \text{CO}_2 \) and \( \text{NH}_3 \) along with an aldehyde composed of the amino acids R group and the reduced ninhydrin (hydrinatin). In the second stage unreacted ninhydrin reacts is joined with hydrinatin via nitrogen from the previously produced ammonia to produce a blue to purple colored product. The intensity of the product is proportional to the amount of amino acid originally present (i.e. the more amino acid you have the darker the spot will be).

**REFERENCES**

Lehninger, Nelson, and Cox; *Principles of Biochemistry, 2nd ed.*; 1993.

Stryer; *Biochemistry, 4th ed.*; 1995.
EXPERIMENTAL PROCEDURE

Objectives
1. To compare the movement of known amino acids in different solvent systems on thin layer chromatography plates and relate the movement to the chemical properties of the amino acids.
2. To determine the identity of an unknown amino acid using Rf values.

Preparation of TLC Plates
PRECAUTION: TLC plates should be handled only by their edges because fingerprints can be detected by ninhydrin giving a false result of masking a true one.

1. By very gently using a pencil, (the resin rubs off the plate rather easily) mark a baseline on the TLC plate 1.0cm from the bottom of the plate. Mark spots on this line 1.0cm apart (and 1.0cm from the plate edges).
2. Transfer 1.0μL of amino acid solution onto the plate at a pencil mark and allow it to dry. Repeat this once more for that same amino acid on the same spot (you're loading the spot with a reasonable amount of the amino acid). Repeat this for each of the remaining amino acids (including the unknown). Make note of which amino acid is at which spot.
3. Mark the solvent system in an upper corner (A, B, or C as designated below).

Chromatography
You will prepare three chambers; each containing one of three solvent systems indicated below. The volume of solvent in the chamber should be such that the level is below 1cm (the base line on the TLC plate). Determine and note the pH of each solvent system.

A: propanol : 0.1M phosphate buffer (pH = 7.0) 7:3 (v:v)
B: propanol : formic acid : water 20:1:5 (v:v:v)
C: propanol : NH₄OH 7:3 (v:v)

1. Carefully place the spotted plate into the solvent chamber so that the solvent level is just below your baseline. Close the lid on the chamber.
2. Allow the chromatogram to develop until the solvent front is 0.5cm from the top edge of the plate (about 90 min).
3. Remove the chromatogram from the chamber and IMMEDIATELY mark the solvent front all the way across the plate with a pencil. Allow the chromatogram to dry completely.
4. Spray the plate lightly but thoroughly with ninhydrin solution under a fume hood and allow to dry. If the spots don't develop, incubate at about 60°C for 5 min.

Data Treatment
The following is for each plate in each solvent system. Note the color of each spot. Draw a small line across each spot in the middle of the spot. Measure and record the distance from that line to the baseline. Measure and record the distance of the solvent front line directly above the spot to the baseline. Determine the Rf values for each amino acid from the distance data. Compare the spots and Rf values for the unknown amino acids with those for the known amino acids to determine the identity of the unknown amino acid. You may need to know the pK values of each amino acid to decide how soluble they should be in the different solvents.
QUESTIONS FOR DISCUSSION

1. What steps might be taken in order to improve the resolution and accuracy in the determination of Rf values?

2. What does the term Rf refer to?

3. Many procedures for TLC use butanol instead of propanol. Why? Which kinds of amino acids would you expect to have the highest Rf values in butanol? Would you expect these Rf values to be as high as in propanol? Why?

4. What are the identities of the amino acid(s)?

5. In what pH range(s) would each amino acid used be a good buffer?

6. Proline stains a different color in ninhydrin than other amino acids. Why? Draw the reaction of ninhydrin with proline.