Exercise 2. Production of an Amino Acid Hydrophobicity Scale Using Paper Chromatography

A. Introduction

Amino acids are the building blocks of proteins. The structure of a protein, the structure and chemistry of its active site, and ultimately the specific function of a protein are all determined by the sequence of amino acids in the protein's polypeptide chain. Therefore, we must understand amino acid biochemistry if we are to understand protein structure and function.

One of the most basic and important chemical characteristics of an amino acid is its polarity, that is how relatively polar or nonpolar it is. An amino acid's polarity determines how it interacts with surrounding water and how it interacts with other amino acids in the protein, and in this way serves as a major molecular force that determines how a protein folds into its stable three dimensional conformation. In fact, the single most important factor that determines the final conformation of a protein is the requirement that the interior core of the protein be densely packed and very hydrophobic. Therefore, as a protein folds, regions of the polypeptide chain that contain a relatively large number of hydrophobic amino acids tend to fold to the interior core of the protein, and regions of the polypeptide chain that have more polar amino acids tend to be displaced to the exterior of the protein, where they interact with the aqueous surroundings.

The figure to the right shows a model of a protein with the amino acids color-coded to show their relative degree of polarity. The more red the color, the more polar the amino acid. Blue depicts the nonpolar amino acids and green indicates moderately polar/nonpolar amino acids. The model shows that the more polar red, orange and yellow colored amino acids tend to be found near the hydrophilic surface of the protein while the more hydrophobic blue amino acids tend to be buried in the core of the protein.

Image Source: Biochemlabsolutions.com
Scientists express an amino acid's polarity in terms of its **hydrophathy** or **hydrophobicity**, that is, the relative degree to which an amino acid is hydrophobic. The hydrophobicity of each of the 20 commonly-occurring amino acids has been determined using a variety of methods, and scientists have ranked the amino acids according to their hydrophobicities in a variety of hydrophobicity scales. The specific objective of this week's experiment is to independently determine the hydrophobicity of 18 of the 20 commonly-occurring amino acids using paper chromatography, build our own hydrophobicity scale and compare it to other scales produced by other groups of scientists.

### B. The Amino Acids

#### Amino Acid Structure

There are 20 different amino acids commonly found in proteins. The general structure of these amino acids is shown in the figure to the right. The figure shows that a free amino acid has a central α-carbon covalently linked to four different molecular groups: an α-amino group (\(\text{NH}_3^+\)), an α-carboxylic acid group (\(\text{CO}_2^-\)), a single hydrogen atom, and a variable side group (R group).

The α-amino (\(\text{NH}_3^+\)) group is a base and is positively charged at a neutral pH, and the α-carboxylic acid (\(\text{CO}_2^-\)) group is an acid and is negatively charged at a neutral pH. Thus, at a neutral pH, as is found in the cell, all free, individual amino acids have at least one functional group that is positively charged and one that is negatively charged, making all free amino acids soluble, to some degree, in water.

The R group is unique to each amino acid and accounts for each amino acid's unique chemistry. The 20 different R groups of the commonly-occurring amino acids differ in molecular size, functional group composition, and polarity. Polarity is one of the most basic and important
characteristic of the amino acid R groups, so much so that the 20 amino acids have been divided into four groups based on the polarity of their R groups.

**Four Groups of Amino Acids**

The chart to the right shows the 20 commonly-occurring amino acids divided into the four major amino acid groups: There are 8 nonpolar amino acids, 7 uncharged, polar amino acids, 2 negatively-charged acidic amino acids and 3 positively-charged basic amino acids.

These groups are defined by the chemical characteristics of their R groups:

The nonpolar amino acids have R groups composed of atoms linked through nonpolar covalent bonds - they lack a polar functional group.

The uncharged, polar amino acids have R groups that contain at least one polar functional group that is uncharged at a neutral pH.

The negatively-charged basic amino acids contain at least one carbocyclic acid group that is negatively charged at a neutral pH.

The positively-charged acidic amino acids contain at least one amino group that is positively charged at a neutral pH.

The smallest amino acid, glycine, has a single H atom as its R group. While this chart places glycine in the uncharged, polar group, many protein scientists consider it a nonpolar amino acid.
C. Amino Acid Hydrophobicity

Amino Acid Hydrophobicity Scales

Scientists express an amino acid’s polarity in terms of its **hydropathy** or **hydrophobicity**, that is, the relative degree to which an amino acid is hydrophobic. Amino acid hydrophobicity is usually expressed as a number, and the amino acids are ranked in a scale called an **amino acid hydrophobicity scale**.

Many amino acid hydrophobicity scales have been developed. Five of the traditionally more popular are shown in the table to the right. Each scale was determined using different theoretical and empirically-derived criteria. The **Eisenberg and Weiss scale**, for example, is based on the calculation of hydrophobic dipole moments of regions of a polypeptide chain and the calculation of the free energy required to move an amino acid residue from the interior to the surface of a hydrated protein. The **Engleman, Steits and Goldman scale** is based on the determination of energies involved in the partitioning of amino chains between aqueous solutions and membranes. The **Kyte-Doolittle scale** is a type of consensus scale based on a combinations of experimental observations from other studies. The **Hopp-Woods scale** is really a **hydrophilicity scale** based on the water solubulity of individual amino acids. The **Janin scale** is based on a statistical measure of the tendency for a residue to be found inside of a protein rather than on its surface.

As can be seen in the table, there are differences in the relative degree of hydrophobicity of each amino acid among the scales. For example, while isoleucine has the greatest hydrophobicity in the Eisenberg and Weiss scale (0.73) and the Kyte and Doolittle scale (4.5), phenylalanine has the highest hydrophobicity in the Engleman scale (3.7). Despite these differences, there are some common trends in the distribution of the amino acids within these scales. Nonpolar amino acids generally have the greatest hydrophobicity, and the charged amino acids generally have the lowest hydrophobicity. However, there is overlap in some

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Group</th>
<th>Eisenberg and Weiss</th>
<th>Engleman et al.</th>
<th>Kyte and Doolittle</th>
<th>Hoop and Woods</th>
<th>Janin</th>
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<tr>
<td>Ile</td>
<td>Nonpolar</td>
<td>0.73</td>
<td>3.1</td>
<td>4.5</td>
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<td>0.7</td>
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<td>Phe</td>
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<td>Val</td>
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<td>2.6</td>
<td>4.2</td>
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<td>0.6</td>
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<td>0.3</td>
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<td>2.0</td>
<td>2.5</td>
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<td>0.9</td>
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<tr>
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<td>Unch/Polar</td>
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<td>-0.7</td>
<td>-0.4</td>
<td>-0.2</td>
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<td>0.6</td>
<td>-0.8</td>
<td>0.3</td>
<td>-0.1</td>
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<td>His</td>
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<td>-0.1</td>
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<td>-3.5</td>
<td>3.0</td>
<td>-0.7</td>
</tr>
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<td>Asn</td>
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<td>0.2</td>
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<td>-3.5</td>
<td>0.2</td>
<td>-0.7</td>
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<td>Asp</td>
<td>Charged</td>
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<td>-9.2</td>
<td>-3.5</td>
<td>3.0</td>
<td>-0.6</td>
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<td>3.0</td>
<td>-1.8</td>
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<td>Arg</td>
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<td>-12.3</td>
<td>-4.5</td>
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</table>

Five popular hydrophobicity scales. The amino acids are ranked according to the Eisenberg and Weiss scale.

Image Source: Stephen Gallik

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D. Paper Chromatography

Introduction

In this week’s lab, we are going to create our own hydrophobicity scale using paper chromatography. Chromatography is a method of separating the chemical components of a mixture based on the differential solubilities of the components in two solvents or phases, a stationary phase and a mobile phase. Many different types of chromatographic methods are in practice; two major types are partition chromatography, where the stationary phase is a thin film of water adsorbed on the surface of an inert matrix, and adsorption chromatography, where the stationary phase is a solid adsorbent.

Paper chromatography is a type of partition chromatography in which the stationary phase of the system is water permanently bound to the cellulose fibers of chromatography paper. The mobile phase is usually a relatively nonpolar solvent that is allowed to migrate through the paper. A number of different nonpolar solvents can be used, each solvent likely giving slightly different results. In this week’s experiment, 70% 1-propanol is the mobile phase (fig. 1). The system, then, is designed to compare the solubility of solutes in water to their solubility in a more nonpolar solvent. Thus a simple method like paper chromatography could be used to measure amino acid solubility in nonpolar solvents and produce an amino acid hydrophobicity scale.

charge on the C ring of the molecule. This charge, however, is pH dependent. At a pH of 1.0, ring C carries the positive charge and the molecule is pigmented. At a pH of 4.5 and higher, however, the the C-ring is hydrated, the positive charge is neutralized, and the anthocyanins lose their pigmentation and become colorless.

The pH-dependent chemical reaction of anthocyanins and the anthocyanin absorption spectrum is shown in the figure to the right. Clearly, the spectrum shows its pH dependency. At a pH of 1.0, due to the positive charge on the C ring, anthocyanins strongly absorb light between 460 and 550 nm (long blue, cyan and green light) and have an absorption maximum of about 520nm. Thus, at a pH of 1.0, anthocyanins are colored; they transmit violet, short blue and red light to the eye. However, at a pH of 4.5, due to the loss of the positive charge in
the C ring, anthocyanins absorb no light in the visible range. At this higher pH they transmit all wavelengths of visible light equally to the eye, and thus are colorless.

**How it Works**

Small samples of the substances being studied are applied near one end of the chromatography paper and allowed to dry. The end of the paper containing the dried samples is then submerged into a shallow pool of the mobile phase, and the mobile phase is given time to migrate up the paper.

As the mobile phase migrates, it reaches the dried samples. At that point, each sample is partitioned between the two phases to various extents, depending on their solubility in each of the two solvents. The more soluble a sample is in the nonpolar mobile solvent, the greater its tendency to migrate with the mobile solvent. The more soluble a sample is in water, the greater its tendency to remain stationary in the aqueous phase. Thus, the amount of sample migration along the chromatography paper ultimately depends on differences in solubility of each sample in water versus the nonpolar solvent.
Collecting the Data.

The raw data collected from the chromatogram is migration distance data, usually measured in mm or cm. From this raw data, an index of solubility, known as relative mobility, is calculated. Known as the retention factor (Rf) among chemists, relative mobility is the ratio of the distance the experimental solute migrates to the distance the solvent migrates along a chromatographic medium:

\[ \text{Rf} = \frac{D_{\text{sample}}}{D_{\text{solvent}}} \]

where \( D_{\text{sample}} \) is the migration distance of the sample and \( D_{\text{solvent}} \) is the migration distance of the mobile phase.

Since the \( D_{\text{sample}} \) is never greater than the \( D_{\text{solvent}} \), the Rf is always a value between 0.0 and 1.0. The greater the Rf, the greater the relative solubility in the nonpolar mobile phase. Using this as a measure of hydrophobicity, the greater the Rf, the greater the hydrophobicity.

E. This Week’s Experiment

Introduction, The Question Being Asked and The Hypothesis

Introduction and Question: An amino acid’s hydrophobicity determines how it interacts with surrounding water and with other amino acids in the protein, and in this way serves as a major molecular force that determines how a protein folds into its stable 3-D conformation. Scientists have used a variety of complex empirical and theoretical methods to determine the hydrophobicity of each of the 20 commonly-occurring amino acids and produced through these methods a variety of hydrophobicity scales. Pliska et al, 1981, determined the relative mobility of each of the amino acids using a thin layer chromatography system and related relative mobility to amino acid hydrophobicity. Similar to thin layer chromatography, paper chromatography is a simple and inexpensive method of determining relative mobilities of amino acids in a two-phase chromatographic system and might also be used to relate relative mobility to hydrophobicity and create an amino acid hydrophobicity scale. The question being asked is "What are the relative mobilities of free amino acids using a paper chromatography?"
system and how does an amino acid hydrophobicity scale produced by such a method compare to scales produced by other methods?"

**Hypothesis:** Amino acid relative mobilities determined using a paper chromatography system can be used to produce a satisfactory amino acid hydrophobicity scale that compares well with other hydrophobicity scales.

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**Specific Objective of the Experiment and Predictions**

**Specific Objective:** The specific objective of this week's experiment is to determine the relative mobilities of 18 commonly-occurring amino acids, use the relative mobilities as an index of amino acid hydrophobicity, create an amino acid hydrophobicity scale based on these relative mobilities and compare the scale to the scales produced by other groups of scientists.

**Predictions:** An amino acid hydrophobicity scale constructed from amino acid relative mobilities determined through paper chromatography will compare well to other amino acid hydrophobicity scales.

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**Experimental Design**

This is an observational study. As such, there are no treatment or control groups. Eighteen of the 20 commonly-occurring amino acids will be studied. Each pair of students will collect data on triplicate samples of 3 of the 18 amino acids. Once the raw data are collected, each pair of students will calculate the relative mobility (Rf) of each sample, then calculate the mean Rf of each of the 3 amino acids. Once these calculations are made, the data from the entire class will be compiled to create a class amino acid hydrophobicity scale for all 18 amino acids. The scale will be compared to other scales using linear correlation.

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**Equipment & Materials**

- Stock solutions of 18 different amino acids (3 per student pair)
- A stock of 70% 1-propanol to serve as the mobile phase
- A solution of ninhydrin in acetone to stain the chromatogram
- A 15 cm X 20 cm piece of chromatography paper
- A large jar to serve as the chromatography vessel
- A large petri dish to serve as the vessel lid
- A porcelain depression dish to hold you amino acide samples
- A specialized capillary pipettor to apply samples to the paper
Experimental Protocol

Part 1: Prepare the Chromatogram

A. Preparation of the Mobile Phase

1. Carry your chromatography jar over to one of the fume hoods and add 20 ml of the mobile phase into your chromatography jar. The mobile phase for today's experiment is 70% 1-propanol. 1-propanol is a primary alcohol with the empirical formula CH3CH2CH2OH. It is fully miscible in water and all common solvents.

2. Place the jar at a convenient spot on the lab bench surrounding the room. You should place it in a position that will allow you to remove the lid and work with the jar without moving the jar. Once you have positioned the jar, cover it with the over-sized petri dish cover.

B. Preparation of the Stationary Phase

1. Obtain one sheet of chromatography paper from the instructor's bench. The paper measures 15 cm (h) x 20 cm (w). With a pencil, write your initials in the upper right hand corner of the paper.

2. Using a plastic ruler, draw a light pencil line across the paper 15 mm from the bottom edge, then place 9 small pencil dots at 2 cm intervals along this line (see figure).

![Diagram showing 9 pencil dots at 2 cm intervals along a line 15 mm from the bottom edge.]
C. Collect Samples of 3 Amino Acids.

Each lab group is assigned 3 amino acids to analyze.

1. Using a wax pencil, label depressions of a spot plate A - C.

2. Using the pasteur pipets that come with each amino acid, half-fill each of the labeled depressions with the amino acids your are assigned.

3. Label a fourth depression D and half-fill depression D with water.

D. Apply samples of each Amino Acid to the Chromatography Paper.

1. Using a capillary pipet calibrated for 2 μl, apply 2 μl samples of your first amino acid (amino acid A) to each of the first three pencil dots. Clean the pipette by pulling distilled water from depression D into the pipet several times. Expel the rinse water onto a piece of filter paper.

2. With your cleaned pipette, apply 2 μl samples of your second amino acid (amino acid B) to each of the second three pencil dots, then clean the pipette with water as you did above.

3. Now apply 2 μl samples of your third amino acid (amino acid C) to each of the last three pencil dots. Clean the pipette a final time, then put it away.

4. Allow the samples to dry.

Part 2: Run the Chromatogram

1. Gently roll the paper into a cylinder (sample side out), then loosely staple the ends together.

2. With the spots towards the bottom and without moving your chromatography jar, gently and in one smooth motion place the chromatogram cylinder into the middle of your chromatography jar containing the mobile phase. Cover immediately and do not disturb.

3. Ideally, we would like to give the organic organic time to rise to within about 10 mm (½") from the top of the paper. However, we only have about 60 - 70 minutes to allow
the chromatogram to develop. This will be enough time for the mobile phase to migrate to within about 8 cm of the top of the paper. This is the best we could do.

4. When the solvent front has migrated to within about 8 cm of the top of the chromatogram, carefully remove the paper from the jar without moving the jar, immediately remove the staples and accurately trace the solvent front lightly with a pencil. (Once the solvent dries, your trace will be the only way of knowing exactly where the solvent front was.)

Part 3. Dry and Stain the Chromatogram

1. Hang the chromatogram under the hood and let the chromatogram dry for 10 minutes.

2. Using a large pair of forceps, grasp the dried chromatogram at its edge and dip it into the ninhydrin staining solution, remove it quickly, let it drip back into the staining solution, then re-hang the chromatogram under the hood.

3. Allow the chromatogram to dry under the hood for 2 minutes, then place it in a 105 degree oven. As the chromatogram heats in the oven, the amino acid spots will begin to appear. When the stain is fully developed, remove it from the oven.

Part 4. Collect the Raw Data

A. The Microsoft Excel® Worksheet

Before you begin, open the Microsoft Excel® Worksheet that goes with this experiment and make sure you have it saved on your computer. Read the instructions carefully. Go to the tab labelled My Raw Data. Table #1 contains cells into which you will enter your raw data. There are also cells reserved for a variety of calculations, such as the Rf of each sample and the Mean Rf and standard deviation for each amino acid. The table is NOT programmed for you. You will need to program these cells and calculate these numbers before you leave lab today. Your instructor will provide you with instructions on how you can program the cells to make these necessary calculations.

Tips:

- to calculate Mean: =AVERAGE()
- to calculate Standard Deviation: =STDEV()

When you are ready, proceed to collect and record your raw data.
**B. Collect and Record the Raw Data**

1. Accurately trace the outer edge of each amino acid spot with a pencil line. Then, estimate the center of each spot and mark it with a pencil point.

2. For each amino acid sample applied to the chromatography paper, collect two pieces of raw data and record the data in Table 1:
   a) Measure the distance the sample migrated from the origin in mm ($D_{\text{sample}}$). To do this, measure from the origin to the center of the spot.
   b) Measure the distance the mobile solvent migrated from the origin at that point in mm ($D_{\text{solvent}}$). Make a separate measurement of $D_{\text{solvent}}$ for each amino acid.

As you enter the data, the Rfs, Means and standard deviations will be calculated for you.

**C. Save the Excel Worksheet**

When all of the data have been collected & recorded, save the Microsoft Excel© worksheet to your computer. When you save the file, it is recommended you change the filename to `YourName_CellBiologyOLM_Lab02_2011.xlsx`.

**D. Compiling the Data**

Now that you have collected, recorded and saved your data, your instructor will instruct you to transfer your mean Rf for each of your 3 amino acids in the pooled class data table at the instructor’s computer. Your instructor will then compile the data from all groups in your section to create a hydrophobicity scale of 18 amino acids that will be shared with you. Once the data are pooled, return to your Excel worksheet and click on the tab labelled **Section Hydrophobicity Scale**. Follow the instructions on the worksheet to record your class hydrophobicity scale and compare it to the scales reported by others.

**E. Homework Assignment**

Your instructor will likely follow today's work with a homework assignment. Make sure you understand the assignment before you leave lab today.
F. Clean Up

Once you saved the Excel table to your computer and entered your data in the instructor’s computer, today’s experiment is complete. Before you shut down, you should make sure the Excel file is saved to your computer. You can logout of the lab manual. Before you leave you must clean up your place so it looks the way it did when you walked into lab today.

F. Bibliography

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