The Detection of Proteins and Carbohydrates – A Laboratory Experiment

Introductory Background

Proteins are ubiquitous in nature. They are present in cell membranes, hair, fingernails, muscle – in other words, are structural. Proteins are also non-structural – functional -- such as hormones, e.g., growth hormone, follicle stimulating hormone, transport proteins, e.g., albumins, transferring, oxyphysin, and enzymes, e.g., lactate dehydrogenase, alcohol dehydrogenase, creatine kinase, to name a few.

When a biochemist describes proteins, he or she talks about protein structure in four levels:

1) Primary structure – what is the sequence of the amino acids?
2) Secondary structure – how does the protein chain fold on itself?
3) Tertiary structure – how does the folded protein RE-fold onto itself?
4) Quaternary structure – how many different proteins are there in the fully functional protein?

Let’s start at an intermediate point as we’ve already discussed amino acids and proteins in class.

Amino Acids

Any time one deals with biology or chemistry, one must eventually contend with and understand amino acids, to learn their structures and to learn a few of the functions and essentiality of the amino acids. There are 20 amino acids we will study: glycine (gly), alanine (ala), valine (val), leucine (leu), isoleucine (ile or ileu), praline (pro), phenylalaninie (phe), tyrosine (tyr), tryptophan (trp), serine (ser), cysteine (cys), cystine (cys-cys), threonine (thr), methionine (met), aspartic acid (aspartate; asp), asparagine (asn), glutamic acid (glutamate; glu), glutamine (gln), histidine (his), lysine (lys) and arginine (arg).

Of these 20 amino acids, 8 are essential (humans require them in their diets as humans lack the enzymes to synthesize them from scratch) and 2 are semi-essential (required for growth by the young human). The essential amino acids are phe, val, trp, thr, ile, met, lys, leu. The semi-essential amino acids are his and arg. A helpful mnemonic to remember these is: PVT TIM HALL, where the first letter of each amino acid makes up this mnemonic.

Amino acids are the building blocks of proteins. In order for the amino acids to link together to form the numerous proteins necessary to keep a human functioning, they form a special bond between each other: the peptide bond. The peptide bond is formed
between the carboxyl group of the first amino acid and the amino group of the second amino acid to form a dipeptide. The peptide bond is unique in that it appears to be a single bond, but has the characteristic of a double bond, i.e., it is a rigid bond. This kind of bond only occurs between amino acids. As the amino acid chain increases in length, the next amino acid adds onto the previous carboxyl group by its amino group. By convention, the left amino acid in a peptide or protein is always #1; is the free amino end or the N-terminus. The farthest amino acid residue to the right is the amino acid in the protein that has the highest number and, as a general rule, is the free carboxyl end or the C-terminus. In some cases, the –OH may be replaced with an NH₂, making it an amide. When dealing with peptides, there is always one LESS peptide bond than there are amino acid residues in the protein, i.e., a tripeptide has 2 peptide bonds and three amino acids; a hexapeptide has five peptide bonds and six amino acids, ad nauseum.

Hierarchy of Peptide/Protein Structure

The sequence[s] of the amino acids held together by peptide bonds ONLY is called the primary structure of a protein.

The secondary structure of proteins is determined by how the amino acid sequence (primary structure) folds upon itself and bonds with hydrogen bonds, i.e., non-covalent attractive forces. A protein that coils on itself in a right handed turn is called an α-helix. This is the first of three types of secondary structure of proteins. The α-helix permits tissues to stretch a bit, like hair. H bonds that form the helix are between the carbonyl oxygen and the amino hydrogen. Only a PORTION of a protein is in alpha-helix, NOT the whole protein.

The second of the secondary structures of proteins is called the pleated sheet or, sometimes, the β-pleated sheet. The story goes that Linus Pauling had already worked out the α-helix in his lab and, while recovering from surgery, solved the β-pleated sheet by folding his hospital bed sheet like a fan (like we made in kindergarten). The pleated sheet runs in the anti-parallel organization, i.e., the peptide chains making up the sheet are running in opposite directions to each other. Pleated sheets tend to make proteins that do not "give", e.g., silk. It doesn't seem to be of great importance to other proteins.

The last secondary structure about which we have interest is the thermodynamic random coil. Although we call this a random coil, nature tells us that there is a reason for every structure. We call it random as we have not worked out the "code" of this structure. In addition, if we denature this structure, the protein loses its function.

The tertiary structure of a protein is, for all intents and purposes the three dimensional shape of the protein brought about by interaction forces of ionic, hydrophobic and covalent disulfide links of the one protein chain. Two examples include the β-chain of hemoglobin and the myoglobin molecule.

Tertiary structure, put another way, is the manner in which the R groups assist the protein in secondary structure formation to fold, twist, bend, kink, AGAIN, upon itself. Water
soluble proteins fold so that hydrophobic R groups are tucked inside the protein and hydrophilic R groups are on the outside of the protein. WHY? This way, the protein may interact with the solvent (water) and not precipitate or otherwise be inactivated. Water insoluble proteins fold so that hydrophilic R groups are tucked inside the protein and hydrophobic R groups are on the outside of the protein. WHY? This is so that a protein, e.g., an ion channel in a cell membrane, may insert itself in a non-polar environment so that polar particles may be transported into or out of regions compartmentalized from each other.

Ionic interactions also stabilize tertiary structures. Where, though, are the ionic groups? They are the R groups! The carboxyl groups on asp and glu; the epsilon amino group on lys; the guanidino group on arg; the imidazole ring on his.

Disulfide bonds assist in tertiary structure by allowing the protein chain to interconnect itself and introduce a hair-pin into its structure – just like how straight hair is curled and curly hair is straightened out.

The last structure of proteins in which we have interest is called the **quaternary structure**: the organization of two or more protein chains to bind together in such a manner as to give the group of proteins a single function, e.g., the tetramer of hemoglobin. The 4 proteins are held together by salt links, hydrophobic and hydrophilic interactions. In hemoglobin, disruption of these forces (to form deoxy hemoglobin) cause the hemoglobin molecule to become smaller than oxy-hemoglobin.

**Groups of Proteins**

**Fibrous proteins** include:

1) Collagens: connective tissue; after it's boiled, the soluble part is called gelatin (Bill Cosby sells this as JELLO™)
2) Elastins: in stretching tissues
3) Keratins: water-proofing proteins
4) Myosins: in muscle
5) Fibrin: blood clotting protein

**Globular proteins** include:

1) Albumins: water soluble; transporters and increase blood osmotic pressure
2) Globulins: saline soluble; transporters and antibodies
3) Enzymes: biological reaction catalysts

**Enzymes**

Enzymes have specific functions and are categorized into 6 activities according to the Enzyme Commission (E.C.) – all but 2 enzymes that are currently known are proteins – the other two are ribozymes (RNA with enzyme activity):
1) Oxidoreductases: catalyze redox reactions -- involve NAD and FAD
2) Transferases: catalyze group transfers
3) Hydrolases: use water to lyse bonds
4) Lyase: nonhydrolytic and non-oxidative group removal
5) Isomerases: catalyse isomerization reactions
6) Ligase: catalyzes reactions requiring ATP hydrolysis

Note that enzymes end with “ase” in their names.

Some proteins are soluble in water (albumins) or in saline (globulins). All proteins may be precipitated by heavy metals, e.g., Hg, Pb, Au, acids, e.g., HNO₃, HCl, or any compound which will increase solute-solute interactions and decrease solute-solvent interactions. Proteins are dissolved by strong bases such as NaOH or KOH, making them particularly dangerous to have spilled in a person’s eyes.

**Carbohydrates**

Carbohydrates are generally seen as sources of quick energy. They consist of carbon, hydrogen and oxygen. In the old days, they were named carboHYDRATES as the ratio of hydrogen to oxygen was thought to be 2:1. We now know differently, although the name has stuck throughout time. There are three categories of carbohydrates in which we have interest: monosaccharides, disaccharides and polysaccharides.

**Mono-saccharides**, single sugars, include glucose, fructose and galactose. **Di-saccharides**, double sugars, include lactose and maltose. **Poly-saccharides**, many sugars, include glycogen (animal storage form of glucose) or starch (plant storage form of glucose) or cellulose (fiber based on β-glucose).

Carbohydrates are polyhydroxy aldehydes or ketones. Classically, carbohydrates are carbon compounds which contain hydrogen and oxygen in a ratio of 2:1. The empirical formula of carbohydrates, with a few exceptions, is Cₓ(H₂O)ᵧ. Each saccharide ends in “ose” in its name. Monosaccharides are named by their functional group, e.g., aldehyde (aldo) or ketone (keto) and with the number of carbons (hex or pent) per each chain, e.g., glucose is an aldohexose, fructose is a ketohexose, ribose is an aldopentose.

Note that sugars that have a free –OH group on the first carbon of the pyran or furan rings are reducing sugars. Reducing sugars reduce Benedict’s solution (an alkaline Cu²⁺ solution) and Tollen’s reagent (a solution of ammoniacal Ag⁺) while the reducing sugars are oxidized – review your redox reactions from CHEM 101 (soon to be CHEM 121). A positive Benedict’s test turns the bluish solution to a red-orange mixture/precipitate; a positive Tollen’s test leaves a silver mirror on the bottom and sides of a test tube.

The next group of carbohydrates that we're interested in are the disaccharides. We are interested in only three of them:
1) Maltose is also known as malt sugar.
2) Lactose is also known as milk sugar.
3) Sucrose is also known as table sugar.

Maltose consists of two glucose molecules bonded together; lactose consists of one molecule of galactose and one molecule of glucose -- pretty clever considering that young animals living on mother's milk use the glucose for quick energy and send the galactose to their livers where it will stored for future energy needs as glycogen -- bonded together; sucrose consists of one molecule of glucose and one molecule of fructose bonded together.

The bonds that hold these sugars together are called glycoside bonds. An oxygen atom between the first and 4th carbons of each respective glucose molecule (see above) connects the two glucose molecules linked together in maltose. Since the linkage is from an -OH group on the left glucose molecule that is in the \( \alpha \) configuration, this is called an \( \alpha \) 1 to 4 link, or \( \alpha 1 \rightarrow 4 \) link. Since the linkage between the galactose molecule and the glucose molecule starts in the \( \beta \) configuration and is also between the 1st and 4th carbons via an oxygen atom, this is called a \( \beta \) 1 to 4 link, or \( \beta 1 \rightarrow 4 \) link. The linkage between the glucose and fructose molecules in sucrose occurs through the 1st and 2nd carbons of glucose and fructose, respectively. This is an \( \alpha \) 1 to 2 link, or \( \beta 1 \rightarrow 2 \) link. Remember, also, that there are NO carbons in the actual glycoside bond: ONLY an oxygen atom links the monosaccharides together to “make” the di-saccharide.

The polysaccharides are the next carbohydrates of interest. We are interested in three polysaccharides: starch, glycogen and cellulose.

Starch consists of two forms of complex carbohydrates: amylose and amylopectin:

<table>
<thead>
<tr>
<th>Amylose</th>
<th>Amylopectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starch is found in PLANTS</td>
<td>The more abundant form in starch</td>
</tr>
<tr>
<td>Is the less abundant form</td>
<td>Forms BOTH ( \alpha 1 \rightarrow 4 ) and ( \alpha 1 \rightarrow 6 ) links</td>
</tr>
<tr>
<td>Forms ( \alpha ) helix</td>
<td>Similar to glycogen due to the branching caused by the ( \alpha 1 \rightarrow 6 ) links</td>
</tr>
<tr>
<td>Iodine &quot;crawls&quot; into the helix and forms inclusion compounds which turns a dark blue</td>
<td></td>
</tr>
<tr>
<td>Amylose is hydrolyzed by amylase in our mouths</td>
<td>Has lesser helix amount, hence less iodine binding; the color obtained is a red-violet color</td>
</tr>
</tbody>
</table>

Glycogen is found in ANIMALS, specifically in the skeletal muscle and liver of animals. It is also found in fetal hearts and fetal lungs. Fetal hearts run off glycogen while adult hearts run off lipids. The glycogen in fetal lungs is necessary to form surfactant to make oxygen passage into the body from the lungs easier when the fetus is in room air rather than the womb.
Glycogen branches due to the same sort of linkages found in amylopectin (α \(1\rightarrow 6\) links). Having both α 1 to 4 and α 1 to 6 links lets the glycogen molecule become very dense and be very efficient for storage. Approximately 1/3 of the weight of the human liver is glycogen. The branches that are formed are then "de-formed" by an enzyme called the "debranching enzyme" when glycogen is needed for energy. Although glycogen has some helix, it is more like amylopectin: it forms less inclusion compounds with iodine. The color obtained is amber red and may be stabilized with the addition of the dihydrate of calcium chloride. Each glycogen molecule contains approximately 100,000 molecules of glucose per molecule of glycogen.

Cellulose, our last carbohydrate, is a bit different. In order to hold the glucose molecules together, they are linked by β 1→ 4 linkages. Humans can not metabolize these β links, while ruminants can. Ruminants digest cellulose because they have bacteria in their stomachs that contain the enzyme cellulase that hydrolyzes these links. Cellulose is the most abundant carbohydrate on earth; humans utilize it as dietary fiber. BTW: fiber is what we ingest – roughage is what we excrete.

**Experimental: Supplies**

<table>
<thead>
<tr>
<th>Casein</th>
<th>6 M NaOH</th>
<th>Egg Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO₄ solution</td>
<td>Concentrated HNO₃</td>
<td>Urea</td>
</tr>
<tr>
<td>Disposable test tubes</td>
<td>Disposable pipets</td>
<td>Beaker for water bath</td>
</tr>
<tr>
<td>Vortex</td>
<td>Evaporating dish</td>
<td>Ignition tube</td>
</tr>
<tr>
<td>Glucose</td>
<td>Fructose</td>
<td>Sucrose</td>
</tr>
<tr>
<td>Lactose</td>
<td>Benedict’s Reagent</td>
<td>Tollen’s Reagent</td>
</tr>
<tr>
<td>Bunsen burner and tubing</td>
<td>Ring stand</td>
<td>2 rings -- identical</td>
</tr>
</tbody>
</table>

**GOGGLES!!!!!!!!!!!!!!!**

Preparation of Benedict’s Reagent: add 173 grams of sodium citrate and 100 grams of sodium carbonate to 800 mL warm water. Mix well, then filter. Bring the volume up to 850 mL with water. At the same time, add 17.3 grams copper sulfate to 100 mL water. Add enough water to this last solution to make up to 150 mL total volume. Slowly mix the two solutions together with stirring. Dispense as directed, below.

Preparation of Tollen’s Reagent: mix equal parts of 10% AgNO₃ with 10% NaOH. Add enough ammonia to dissolve the Ag₂O precipitate that forms. Dispense as directed, below.

**CAUTION!!!!! This solution (Tollen’s Reagent) MUST be acidified and completely and correctly disposed of after use: it is HIGHLY explosive when dried out!**
Char test: Place about a pea-sized bit of casein in an evaporating dish and burn it with the Bunsen burner. What does the odor remind you of? What do you think it smells like?

Xanthoproteic reaction: Add 10 gtt concentrated nitric acid to a third of a pea sized amount of egg albumin in 30 drops of water. Heat this tube to boiling in your hot water bath. Did it turn yellow?

Now add 12 gtt 6 M NaOH to your mixture and vortex it. Did it turn orange?

If it turned orange, this is a positive test. This test tests for the presence of aromatic (contains a benzene ring for our purposes) amino acids.

Biuret test: Obtain 5 test tubes. Label them 1-5. Leave the #1 tube empty for now. Into each of the next 4 tubes add solid egg albumin in the following manner:

<table>
<thead>
<tr>
<th>Approximate Sample Size of Egg Albumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 2</td>
</tr>
<tr>
<td>About a quarter the size of a small pea</td>
</tr>
</tbody>
</table>

Now, into each of the 5 tubes, add 20 gtt water and vortex to mix. Add 20 gtt 6 M NaOH to each of the five tubes and re-vortex. Add 3 gtt of the CuSO₄ solution to each of the 5 tubes and re-vortex. Record your observations (look at the color[s] and intensities):

<table>
<thead>
<tr>
<th>Biuret Test Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1</td>
</tr>
</tbody>
</table>

Note (and remember) that your first tube (tube #1) has NO albumin in it.
Urea hydrolysis: Place about a half cm of urea in the bottom of an ignition tube (hold the tube with a three fingered clamp to the ring stand – “aim” it away from people) and place a piece of moistened RED litmus paper folded in a “V” shape in the neck of the tube. Heat it gently with your Bunsen burner (the urea will bubble). CAREFULLY waft the odor towards you. What is the gas that is emitted?

What color did the litmus paper turn?

Experimental: Carbohydrate Methods

Benedict’s Test

Obtain 4 test tubes. Label them. Place about a quarter the size of a small pea of glucose, fructose, sucrose or lactose into the appropriately labeled tube, e.g., tube #1 has glucose, tube #2 has fructose, ad nauseum, in it. Add about 20 gtts water to each of the 4 tubes. Get your water bath boiling. When the bath is boiling, add 20 drops of Benedict’s reagent to each of the four tubes and heat them to boiling, as well. Let boil for 5 minutes. Record your results below (orange is positive, blue is negative for reducing sugars):

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Fructose</th>
<th>Sucrose</th>
<th>Lactose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tollen’s Test

Obtain 4 test tubes. Label them. Place about a quarter the size of a small pea of glucose, fructose, sucrose or lactose into the appropriately labeled tube, e.g., tube #1 has glucose, tube #2 has fructose, ad nauseum, in it. Add about 30 gtts of Tollen’s Reagent to each of the 4 tubes. Vortex immediately, place on the lab bench and do not disturb for at least 15 minutes at room temperature. Observe and record the order of mirroring – or lack thereof – in the table below:

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Fructose</th>
<th>Sucrose</th>
<th>Lactose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Which sugars are reducing sugars?

What was the order of mirroring, i.e., which tube mirrored first, last, never?

When you have completed your testing, dump the solutions down the sink with lots of water. Rinse your tubes out with water and put them in the rack for your professor to dispose of.

Questions

1) Using some source, e.g., library, web, find and draw the structures of the 8 essential amino acids, below.

2) Using some source, e.g., library, web, find and draw the structures of the 2 semi-essential amino acids, below.

3) Using some source, e.g., library, web, find and draw the structures for 6 monosaccharides.
4) Based upon your results in the Tollen’s test, explain what happened in each tube based on the carbohydrate chemistry of each saccharide you used.