Total Protein Content

Introduction

Recent studies have determined that the proteins found in saliva have the potential as biomarkers that may be in clinical and diagnostic utilities (Yoshizawa, 2013). This lab investigation will introduce a technique to identify and measure the protein content found in saliva.

Concepts:

SEE'd Standards: CHEM1.6, 1.7, 2.1, 2.3, and 4.2

- Function of Macromolecules and Structure
- Molecular Structure and Desired Properties
- Distribution and Proportion in Solution
- Properties of Substances in Reactions
- Effect of Frequencies of Energy on Matter

Background

Current research involves the search and identification for biomarkers in saliva that may be used in the diagnosis and management of a variety of diseases and disorders. Blood testing is most commonly used to measure normal biological processes, diseases, and the response of the body to medicines. Many of the antibodies, microbes, DNA and RNA markers, lipids and proteins that are tested in blood, are also found in saliva (Matse, J. H., et.al, 2013).

Scientific investigation into the proteins found in saliva have resulted in connections of protein levels or activity to oral cancers, breast cancer (Streckfus, C, 2005), diabetes (Genco, RJ, 2005), leukemia (Li, Y., et.al., 2004) and immune deficiency disorders (Malamud, D, 2008).

To enhance our ability to use the biomarkers in saliva, it is necessary to have tools to measure and quantify the proteins found therein. It is necessary to understand the structures of proteins, how they react with other molecules, and how these reactions can be used to make measurements to determine the amount or types of proteins are present in saliva.

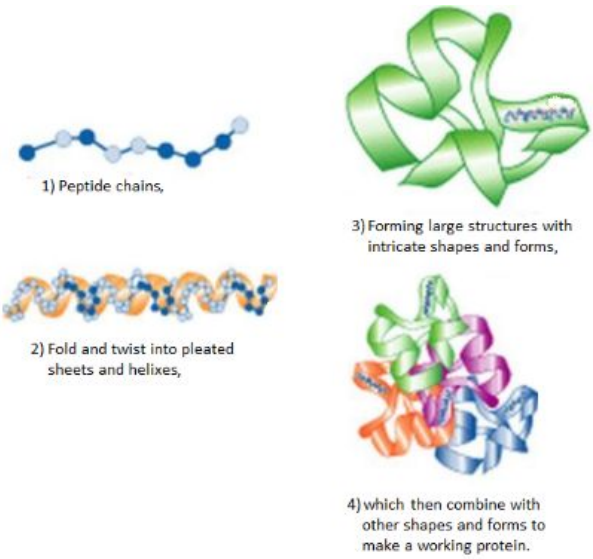
Proteins:

Saliva is filled with thousands of protein types, each at a different concentration with a particular shape and function.

In this investigation you will learn to use a few of the chemist's tools to identify, measure and explore the functions of proteins in your saliva.

Our exploration begins with a technique that is used to identify the amount of protein in saliva.

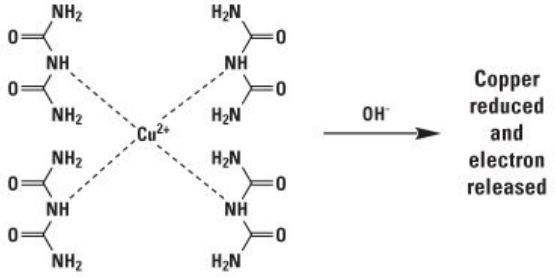
A protein is a very large molecule made of many connected pieces called amino acids. The order and frequency with which amino acids connect into a chain, determines how the protein folds and forms, and results in a specific function.



Proteins are so very small, as to be impossible to see when in water, even with a microscope. However, if we allow the protein to interact with something we can see, proteins become possible to identify and measure in solution.

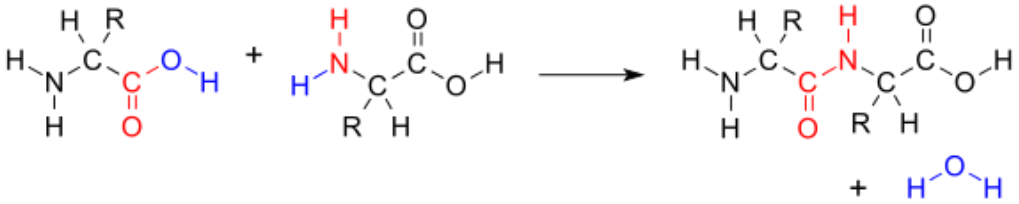
The atoms in proteins bond together by sharing or transfer of electrons. Electrons behave as waves and when they interact with light, energy can be absorbed from the light. The electron configuration in the atom or molecule determines how much, and what type of light can be absorbed. In one state, a molecule may only absorb energy from ultraviolet or infrared light. If the molecule bonds to a different molecule or changes its structure, it is able to absorb energy at a different wavelength or frequency.

Originally it was discovered that the biuret molecule, with a number of nitrogen atoms on it, would bond with copper ions changing its structure. The electronegative atoms of nitrogen in the molecule bond to the less electronegative copper ions. In doing so, the ability for the protein/copper complex to absorb light changes, and energy is absorbed at a different frequency of visible light. This absorbance by the complex makes a visible color change.

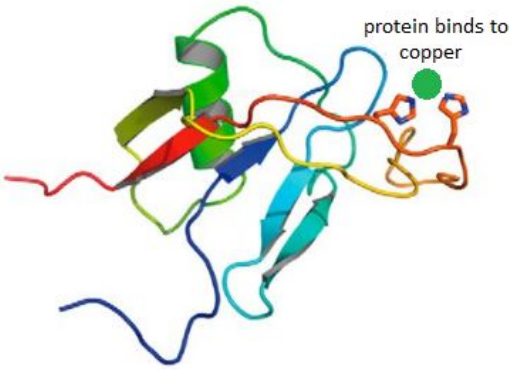


Biuret Reaction

The peptide bonding that occurs to build the long chain of amino acids that make up a protein has a very similar nitrogen-carbon bonding to what is seen in the biuret molecule. Below is a reaction of two amino acids forming the peptide bonds that are the foundation of the protein's structure.



In the same way that copper binds to biuret, copper will also bond to the nitrogen structures of proteins. Using the biuret reagent, we can observe the light blue color of the reagent change to a deep violet color in the presence of proteins. The more intense this change in color, the more protein is present.



Total Protein Assay:

An assay is a technique used in chemistry to make a measurement of something that is not directly measurable. The total protein assay involves creating six different solutions of known protein concentration. We are going to measure exactly how much protein goes into each solution. When the copper sulfate assay solution is added, each solution will have a unique color from violet to blue green. We will use these six solutions as a measuring stick to determine the amount of protein in other unknown solutions.

Once all six protein solutions are prepared, we'll add copper sulfate assay solution to each and observe the color change. The more protein present, the more violet the solution will appear. With the six solutions, the most concentrated closest to violet, and the least closest to bluish green, we'll then be able to use our known solutions to identify the amount of protein in an unknown solution by comparison.

Our stock protein solution has 5 grams of protein in every 100 mL of water, or 50 mg/mL, we will mix six solutions, each containing ½ the amount of the protein as the solution before it.

Experimental Overview:

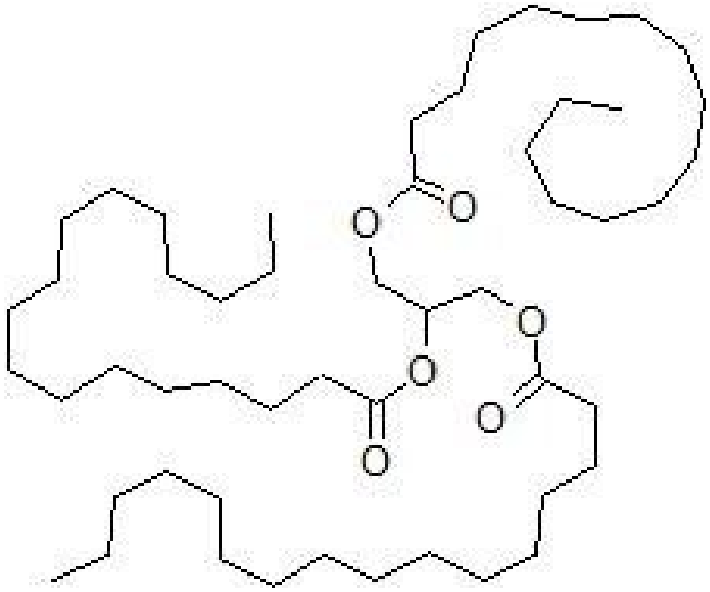
The purpose of this inquiry lab is to develop an assay using the Biuret reagent solution to determine the measurement of protein solutions in saliva. A solution of albumin protein at 5g/100mL will be diluted to form six standard solutions. With the addition of the Biuret reagent students will use the standard solutions to perform visual colorimetry in the determination of unknown protein concentrations.

Pre-Lab Questions:

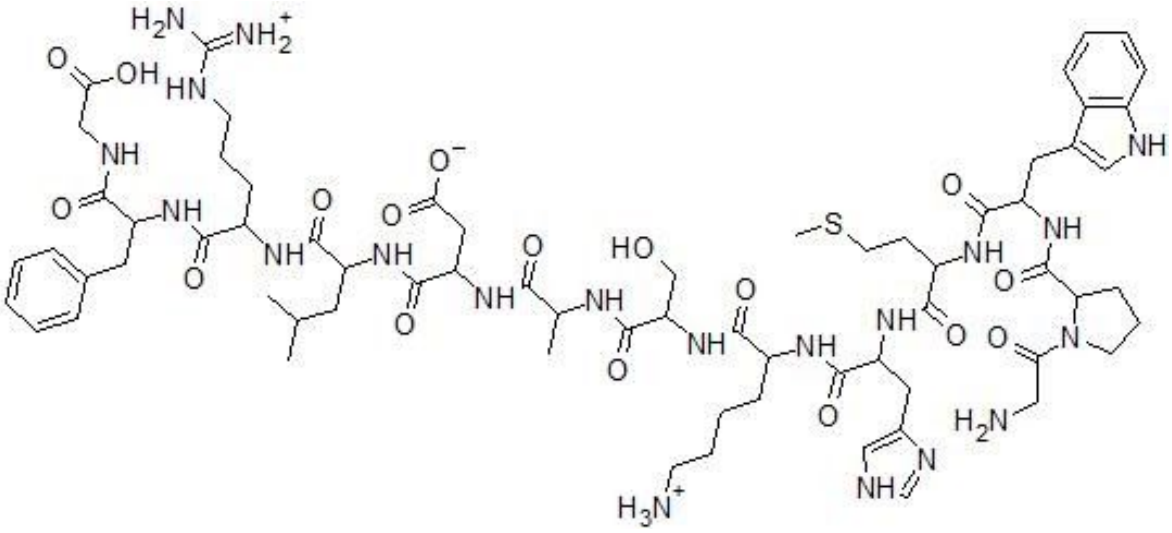
1. CHEM4.2 - Effect of different frequencies of light when absorbed by matter.
    a. Why is light absorbed differently when the copper sulfate solution is added to the saliva?
        i. What change is occurring between the copper ions and the proteins?
        ii. How does the information on the periodic table about the electronegativity of the atoms involved allow us to make accurate predictions as to how copper and proteins come together?

2. CHEM2.1 - Mathematical model to represent the distribution and proportion of particles in solution.
    a. A calibration curve requires the use of several concentrations of the test solution. If you placed 2 mL of 25mg/mL solution in the first of the six wells, and then placed 1 mL of water in each of the following five wells,
        i. If you then took 1 mL of the 25mg/mL solution in the first well and added it to the second, after mixing what concentration of protein would you have? How many milliliters of the solution would you have? How many mL of solution would remain in the first well?
        ii. Describe how you might take the diluted solution in the second well and create a solution of 6.3 mg/mL in the next well?
        iii. How could you continue the serial dilution so you end up with six wells each containing half the concentration of the well ahead of it in the row?

3. CHEM1.6 - The function of macromolecules are related to their chemical structures
    a. The first compound shown is a triglyceride, with long hydrocarbon chains. The second is a polypeptide chain with amino acids, including oxygen and nitrogen, linking together. The molecular drawings below use the organic structural system. Each angle in the structure represents a carbon atom, and the number of bonds on carbon should always be four, so if any bonds are missing, hydrogen atoms are present making the missing bonds.
        i. In each structure, use a green crayon, marker or pencil to highlight all the carbon chains, and other regions where the structure will not have charge.
        ii. Use a red colored crayon, marker, or pencil to highlight the regions of the structure where negative charge would be present.
        iii. Use a blue crayon, marker, or pencil to highlight the regions of the structure where positive charge would be present.



Triglyceride



Polypeptide Chain

b. Which of the two structures had the most non-polar regions, green. Which had the most negative, and which had the most positive? What were the atoms that created the most charge on the structure?
c. Given the description in the background section of the Biuret Reaction, which of the two structures, do you believe might attach to the copper ion in the Biuret Reaction? Circle the place on the structure where you believe the copper ion will attach.

4. CHEM 4.2 - Explain the effects different frequencies of electromagnetic radiation have when absorbed by matter.Given the six solutions of decreasing concentration, and after adding Biuret Reagent, you will see that the most concentrated solution is purple, and each solution progressing in the row appears more blue and more dilute until the solution in the last well is a light pale blue color.
    a. How could you use these six solutions of decreasing color to measure a protein solution of unknown concentration?
    b. What techniques could you employ to make sure that your visual determination will be the most precise as possible?

Materials:

- Biuret Quantitative Assay Solution (Flinn B0237, 500 mL, $8.75)
- 24-well Reaction Plate (Flinn AP1447, 1 per lab group, $4.55)
- Graduated Beral-Type Pipet (Flinn AP1721, Pack of 20, $1.70)
- Albumin Powder, Protein (Flinn A0014, 100g, $14.60) dissolved in water at 5g/100mL
- Distilled water
- Waste beaker

Safety Precautions:

Biuret Quantitative Assay Solution causes skin and eye irritation. Albumin solutions are considered non-hazardous. Safety goggles, lab aprons, and gloves should be worn.

Laboratory Technique

Create the Calibration Curve for Protein Standard:

As you perform the procedure, describe the steps you take in your lab notebook. You'll need these notes to write up your lab report later.

1. Using a graduated pipette, place 1 mL of 5% protein solution in well 1, then add another 1 mL of distilled water.
2. Draw the 2 mL of solution into the pipette and dispense back into the same well two or three times to make sure the solution is very well mixed, then draw 1 mL of solution into the pipette and dispense it into the second well.
3. Now add another 1 mL of distilled water to the second well, mix the solution by drawing and dispensing two or three times and move 1 mL of the solution to the third well.
4. Continue the same procedure until you have mixed the last solution in the sixth well, then simply discard 1 mL from well six.

After your serial dilution your well plate should have six solutions, in six different wells, all consisting of 1 mL of protein solution with the following concentrations:

25mg/mL	12.5mg/mL	6.3mg/mL	3.1mg/mL	1.5mg/mL	.8mg/mL

Let's see how well you performed your dilution:

1. After rinsing your graduated pipette, add .5 mL of the copper assay solution to each well, mix, and observe the color formed.

If all the colors appear the same, it is likely the precision with which you prepared the wells was poor. If done correctly, you should see a violet color in the first well followed by violet-blue the second, with each well progressing less and less violet until the last is a bluish green.

Saliva Collection Protocol

1. Take a square of parafilm, and chew it like gum. The parafilm will stimulate saliva flow.
2. Allow saliva to pool in your mouth, then with your head tilted forward, gently guide the saliva into the graduated test tube.
3. Fill to the desired volume.

Guided Inquiry Design and Procedure

Form a working group with other students and discuss the following:

1. You will be given three solutions of unknown protein concentration. How can you use the Biuret reagent and your calibration curve to measure the concentration of these solutions?
2. After making your measurements, go to your teacher and get the actual concentrations of the three unknowns. How can you determine the precision of your calibration curve? Calculate the percent error in your measurements.
3. Now that you've analyzed the precision of the visual colorimetry method. Collect a small sample of saliva from each of the members in your group. Saliva contains some mucus and large particles.
    a. In what ways can you prepare the saliva to ensure these larger particles do not hinder your ability to use the calibration curve to measure the protein content in the saliva?
    b. If the saliva is too murky to get a good reading, how could diluting the saliva allow you to get a more precise reading?
4. Use the calibration curve to measure the protein levels in each saliva sample, and use the percent error from your initial unknown protein measurements to determine the precision of your saliva measurement. How big is the span of possible error for your measurement in +/- mg/mL?
5. Describe what you observed in the amount of protein found in the saliva of each member of your group.
    a. Were the protein levels consistently the same?
    b. If not, why do you believe the protein levels were different?

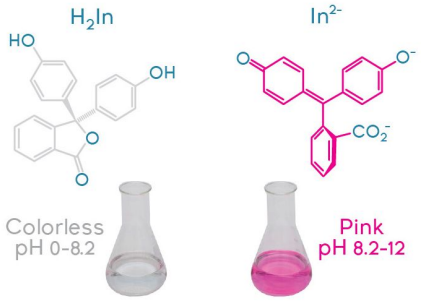
Opportunities for Inquiry

In your lab group, discuss the following:

1. The synthesis of proteins in your saliva is dynamic and often changes in reaction to environmental factors. What are some of the environmental factors that may have an effect on how much protein is generated by your body in your saliva?
2. Choose one of the factors you discussed, and with your group provide an explanation on how and why this factor would change protein levels in your saliva. Write a hypothesis in the form of "If I experience _____, then the levels of protein in my saliva will _____."
3. Discuss with your lab group how you could test your hypothesis. Design an experiment in which you could determine more precisely how this specific factor affects the protein level in the saliva.

Review Questions

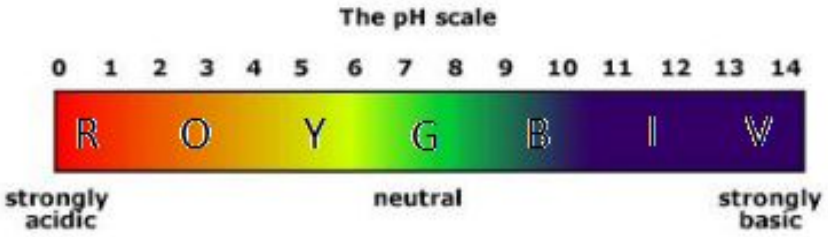
1. In a manner similar to how we used absorbance of light to determine protein concentrations in saliva, molecular changes can be used to measure the levels of acid, $H^{=}$ ions, in water. Phenolphthalein is a molecule that interacts with acid and results in a color change.



    a. The image above shows the chemical structure for phenolphthalein when in the presence of acid, H^+ ions, and when in the presence of a base, OH^- ions. What changes occurred that resulted in the color change?
        i. What do you note about the electronegativities of the atoms involved in the structural changes in the phenolphthalein?

ii. Why is the oxygen atom more likely to bond with H^+ ions than the carbon atoms in the structure?

2. Universal indicator for acids and bases also serves as a tool to determine acidity of solutions. The calibration of the standardized scale for universial indicator is as follows:

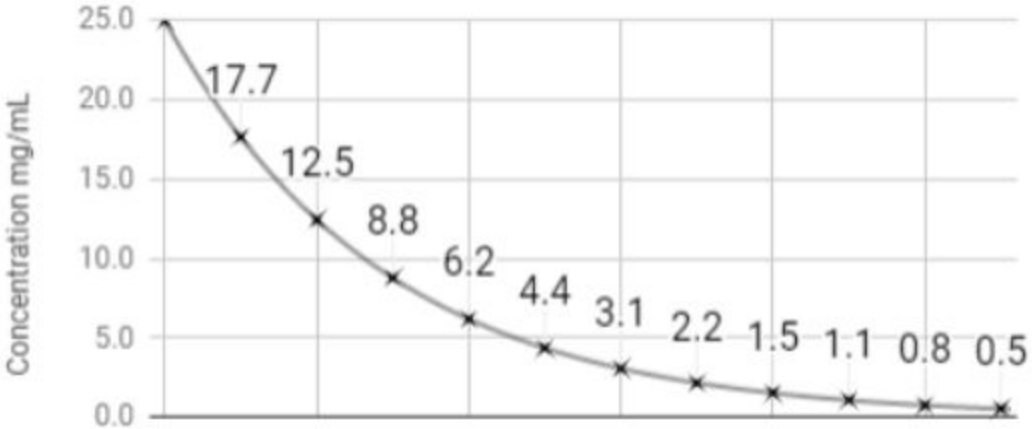


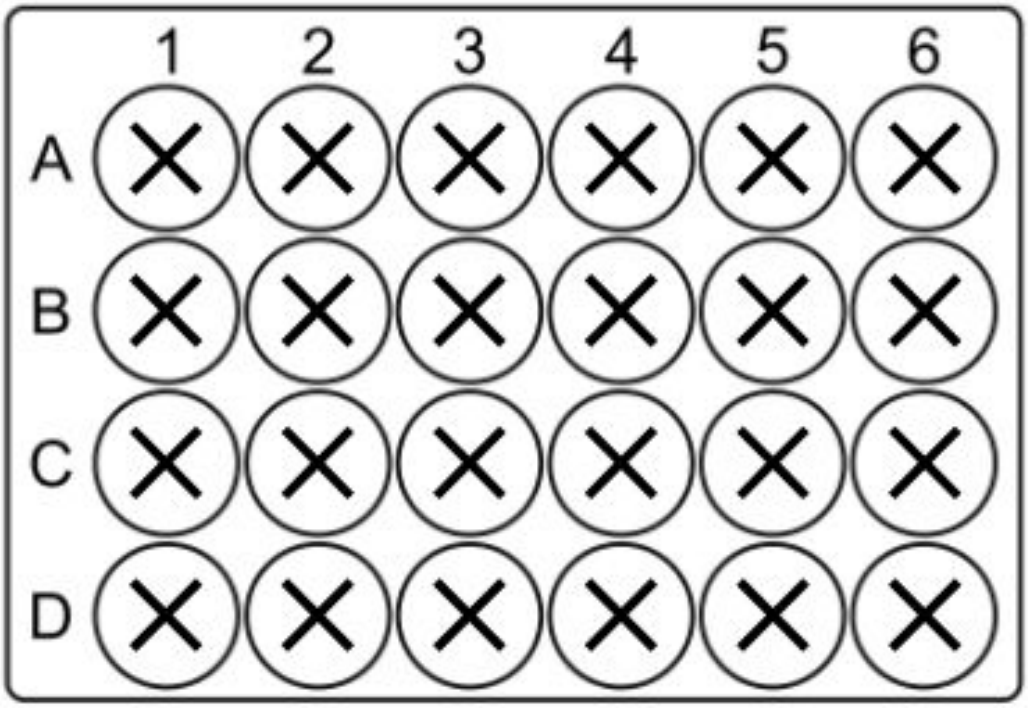
a. How is the manner in which acidity levels in water using universal indicator similar to the manner in which the biuret reagent allowed us to determine the protein levels in the saliva?

3. Was the calibration curve we used precise? Is there a way you might increase the precision with which we use the calibration curve to make our measurements?

Data Collection Page

Protein Standard





Time(min) 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
(After taking a picture, cross off the time.)

Teacher Notes

Saliva Investigation - Total Protein Content

Materials Needed:

- Biuret Quantitative Assay Solution (Flinn B0237, 500 mL, $8.75)
- 24-well Reaction Plate (Flinn AP1447, 1 per lab group, $4.55)
- Graduated Beral-Type Pipet (Flinn AP1721, Pack of 20, $1.70)
- Albumin Powder, Protein (Flinn A0014, 100g, $14.60) dissolved in water at 5g/100mL
- Test Tubes with Screw Caps, Plastic, Pkg. of 30 (Flinn AP7116, $17.10)
- Parafilm (Flinn AP1501, 4", 125 ft, $25.50)

Additional Materials Needed:

- Distilled Water
- Beakers (3)
    - Waste
    - Distilled Water to Rinse Pipet
    - Distilled water to use in making solutions
- Stirring rods

Pre-Lab Preparation:

Biuret's Reagent

This reagent is most easily purchased already made, but if it is necessary to mix it yourself here is the procedure:

1. The reagent is composed of the following chemicals:
    a. .3% copper II sulfate pentahydrate
    b. .9% potassium sodium tartrate
    c. .5% potassium iodide
    d. .6% sodium hydroxide
    e. 97% water
2. To make 100mL, measure out the following:
    a. .12 g of copper II sulfate pentahydrate and add to a 100 mL volumetric flask filled halfway with water, swirl until mixed.
    b. .36 g potassium sodium tartrate, add next to the same flask and mix.
    c. .2 g potassium iodide, add and mix.
    d. .24 grams sodium hydroxide, add this last, (if you add it without already adding the potassium sodium tartrate, the hydroxide will precipitate with the copper forming a black precipitate.)
3. Fill the rest of the way with distilled water to make 100 mL.

5% Albumin Powder

1. Measure precisely 5 grams of albumin powder and add to 100 mL flask.
2. Mix well while heating gently until all the albumin has dissolved.

Unknown Protein Solutions

Use the 5% albumin solution and create three solutions of random concentration by diluting the stock solution to desired concentrations.

Saliva Preparation

Fresh saliva may be used in the experiment, however the presence of mucus and particulates often found in saliva may make the full strength sample hard to measure. It is recommended that the saliva is collected prior to the day of experimentation, frozen overnight, thawed, and either centrifuged at 1000g for 2 minutes and the supernatant collected, or filtered. This will ensure the saliva is clear when used in the experiment.

Safety Precautions:

Biuret test solution contains copper II sulfate and sodium hydroxide and is a corrosive liquid. It is moderately toxic by ingestion and is dangerous to eyes and skin. Excess biuret test solution should be neutralized with acid to achieve a neutral pH. The albumin solutions are harmless. Follow all general guidelines and specific procedures from federal, state and local regulations for proper disposal.

Connecting to Next Generation Science Standards

- HS-PS1-2 - Explain and predict simple chemical reactions using electron states, trends, and patterns.
    - Electronegativity of nitrogen and its bonding to copper ions
- HS-PS4-4 - Evaluate the validity and reliability of claims of the effects that different frequencies of electromagnetic radiation have when absorbed by matter
    - Visual colorimetric analysis of solutions involves the absorption of light at specific frequencies.

Lab Hints

- As students perform the serial dilution for the protein calibration curve, be sure they mix the solution well before moving 1 mL to the next well.
- Bubbles in the saliva and protein samples make the concentration determination difficult. If students use a pipet above the surface of the solution to touch the bubbles, the pipet will usually draw them up enough to remove them.
- Saliva is generally less transparent than the solutions used to make the standard. It is helpful to create a serial dilution of saliva as well as dilute saliva is more transparent. Students may then extrapolate the concentrations of dilute samples to determine the concentration of the full strength sample.
- It is generally helpful to have students collect and prepare saliva samples prior to the lab by freezing and thawing the saliva, and filtering or centrifuging the samples to remove the conglomerate of larger particles. The aliquot tends to be fairly clear.
- Using the Data Collection Page at the end of the student section of the lab provides x's beneath each well to facilitate in visual determination of the concentration of the unknowns.

Teaching Tips

- Discussion of electronegativities, electron structures, and absorbance of light by electrons is recommended during the explorations in the lab.
- This lab investigation can be used to reinforce or introduce discussion of periodic trends, electron states, light absorbance, reactions between metals and nonmetals, and solution concentrations.
- It may be useful to model how to perform a serial dilution prior to experimentation by the students.
- Students may be encouraged to collect photographic data of reference solutions and unknowns for later analysis.
- If saliva samples are significantly cloudy, saliva may be centrifuged or filtered prior to analysis
- This lab will help students develop skills necessary to perform further investigations involving changing concentrations and kinetics.
- The activity may be modified to use the copper solution as a test to identify the presence of proteins and focus solely on the color change of the reaction.
- The activity may also be modified to extend the use of the assay to investigate factors that may change protein levels in saliva. Students can explore how other transition metals may interact with proteins to form color changes, iron, nickel, manganese.
- The activity may be focused on the exploration of redox changes in copper ions when in contact with electronegative portions of macromolecules.
- If colorimeters are available, visual colorimetric identification of concentration may be expanded to include absorbance measurements made with colorimeter and analyzed using Beer's law.

Answers to Pre-Lab Questions

1. Electron configurations and energy levels change when the copper ion attaches to the protein, resulting in a different frequency of absorbance.
    a. As the copper bonds to the nitrogen in the proteins electron configurations change to a different energy level.
    b. Copper as an ion has a positive charge, and the high electronegativity of nitrogen compared to carbon creates a partial negative charge on the nitrogen, which attracts and bonds to the positive copper ion.
2. A calibration curve requires the use of several concentrations of the test solution. By mixing 1 mL of 50 mg/mL solution with 1 mL of water in the first of six wells in a reaction plate, you would create 2 mL of a 25mg/mL solution.
    a. 1 mL would dilute the solution by ½.
    b. By adding 1 mL of the 12.5 mg/mL solution to another 1 mL of water, the solution would again dilute by ½.

c. After mixing each new solution, you will have 2 mL of the solution. Take 1 mL of the newly mixed solution and place it in the next well with another 1 mL of water. Continue the pattern until the last well, where you will remove and discard 1 mL of the solution.

3. Given the six solutions of decreasing concentration, and after adding Biuret Reagent, you will see that the most concentrated solution is purple, and each solution progressing in the row appears more blue and more dilute until the solution in the last well is a light pale blue color.
    a. By comparing the depth and color of an unknown solution to the standards and matching it closely to one of the standards, it is possible to conclude that both the unknown and its matching standard will have near the same concentration.
    b. By placing an x beneath the wells of the standards and the unknown, you can use the ease of visibility of the x as well as the colors to match the concentrations. If an unknown does not match any one of the standards exactly, but falls between the two, you could use a curve of the concentration and the standards to extrapolate a more precise measurement.

Sample Data Tables:

Standard Protein Solutions:

Well #	Protein Concentration mg/mL	Color and transparency	Observations
1			
2			
3			
4			
5			
6			

Calibration of Standard:

Unknown #	Measured Concentration mg/mL	Actual Concentration mg/mL	Percent Error	Notes
1				
2				
3				

Testing of Saliva

Dilution Percentage	Measured Protein Concentration mg/mL	Notes
100		
50		
25		
13		

7		
4		

Answers to Review Questions

1. Oxygen is very electronegative, with a partial negative charge. It is attracted to the positive charge of the H^+ ion.
   The electronegativity of the carbon atom is not different enough from the atoms around it to create a dipole and a partial charge. It is necessary to have a partial negative charge to attract the positive charge of the H^+ ions.
2. The universal indicator molecule is changed in how it absorbs light as it interacts with the hydronium ions in the water, just as the proteins changed in how they absorbed light when they interacted with the copper ions.
3. Our measurements involved making a judgement call on how close the color of the unknown solution was compared to our standard. The standard measurements were approximately 3 mg/mL apart, and any measurement between the marked wells would be an estimate. The measurement could be improved with the use of a colorimeter, which can measure the absorbance to a much greater precision

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