MEASUREMENTS OF GLUCOSE, CHOLESTEROL, AND PROTEIN IN BLOOD SERA

MATERIALS:
1.   Pyrex test tubes, mechanical pipettors for 40µL, 50µL and 5.0 ml. volumes; corresponding pipettes (0.10 ml. and 5.0 mL)
2.   Constant-temperature water bath, set at 37°C.
3.   Spectrophotometer and cuvettes
4.   Glucose standard
5.   Cholesterol standard
6.   Total Protein Standards in following concentrations: 2,4,6,8 g/dL
7.   Biuret reagent.
8.   Serum

The concentrations of glucose, protein, and cholesterol in plasma (or serum) can be measured using various techniques in the laboratory. Abnormal concentrations of these molecules are associated with specific disease states.

SAFETY:
The human sera we use is purchased and screened for pathogens. Despite this, we will use standard safety protocols; students will wear gloves at all times and unknowns (containing the sera) will be disposed as biohazards in appropriate containers.

OBJECTIVES:
1.   Describe how Beer's law can be used to determine the concentration of molecules in solution.
2.   Use a standard curve to determine the concentration of molecules in serum samples.
3.   Explain the physiological roles of glucose, protein, and cholesterol in the blood.
4.   Explain why abnormal measurements of plasma, glucose, protein, and cholesterol are clinically significant.

INTRODUCTION:
Organic molecules found in the body contain the atoms carbon, hydrogen, and oxygen in various ratios, and some of these molecules also contain the atoms nitrogen, phosphorus, and sulfur. Many organic molecules are very large and consist of smaller repeating subunits that are chemically linked via synthesis reactions. The term *monomer* refers to the individual subunits; the term *polymer* refers to the long chain formed from these repeating subunits.

When two monomers are bonded together, a molecule of water is released. This reaction is called **dehydration synthesis.**

When the chemical bond between monomers is broken, a molecule of water is consumed. This **hydrolysis reaction** releases monomers for use by cells.

Ingested foods are usually polymers – mainly proteins, carbohydrates, and triglycerides. In the stomach and small intestine, these polymers are hydrolyzed (in the process of *digestion*) into their respective monomers: amino acids, monosaccharide, fatty acids, and glycerol. These monomers are then moved across the wall of the small intestine into the blood of the

capillaries (a process called *absorption*). The vascular system transports them to the liver and then to all the other tissues of the body.

In a healthy person, the concentrations of the different classes of monomers and polymers in the blood plasma are held remarkably constant and vary only within narrow limits. When the concentration of one of these molecules in the blood deviates from the normal range, specific compensatory mechanisms are activated that bring the concentration back to normal (negative feed-back). Homeostasis is thus maintained.

When the concentration of any of the monomers or polymers in the blood remains consistently above or below normal, the health of the person may be threatened. Abnormal concentrations of different molecules in the blood are characteristic of different diseases and aid in their diagnosis. The disease *diabetes mellitus,* for example, is characterized by a high blood glucose concentration. Therefore, accurate measurement of the concentrations of different molecules in the blood is extremely important in physiology and clinical laboratories.

THE SPECTROPHOTOMETER

The Spectrophotometer is a device used in physiology and clinical laboratories to measure the concentration of a substance in a solution. This is accomplished by the application of **Beer's law,** which states that the concentration of a substance in a solution is directly proportional to the amount of light absorbed (*Absorbance, A)* by the solution and inversely proportional to the logarithm of the amount of light transmitted (*Percent Transmittance, %T)* by the solution

Beer's law will apply only if the incident light (the light entering the solution) is monochromatic – that is, light composed of a single wavelength. White light is a mixture of many different wavelengths between 380 and 750 nanometers (nm). The rods and cones within the eyes respond to the light waves, and the brain interprets these different wavelengths as different colors.

Violet	380--- 435 nanometers
Blue	436—480 nanometers
Green	481—580 nanometers
Yellow	581—595 nanometers
Orange	596—610 nanometers
Red	611—750 nanometers

By means of a prism or diffraction grating, the spectrophotometer can separate white light into its component wavelengths. The operator of this device can select incident light of any wavelength by simply entering the desired wavelength. This light inters a tube, the *cuvette,* which contains the test solution. A given fraction of the incident light is absorbed by the solution and the remainder of the light passes through the cuvette, allowing us to measure either the amount of light absorbed by the sample (Abs) or the percent of the light that is transmitted through the sample (%T).

In the following exercises, the absorbance scale will be used rather than the percent transmittance scale because absorbance and concentration are directly proportional to each

other. This relationship can be described in a simple formula, where 1 and 2 represent different solutions.

$$\frac{\text{Concentration}_1}{\text{Absorbance}_1} = \frac{\text{Concentration}_2}{\text{Absorbance}_2}$$

One solution might be a sample of plasma whose concentration (e.g. of glucose) is unknown. The second solution might be a *standard*, which contains a known concentration of the test substance (such as glucose). When the absorbances of both solutions are recorded from the spectrophotometer, the concentration of the test substance in plasma (i.e., the unknown) can easily be calculated.

$$C_x = C_{std} \times \frac{A_x}{A_{std}}$$

Where
   C_x = the unknown concentration
   C_{std} = the standard concentration
   A_x = the absorbance of the unknown
   A_{std} = the absorbance of the standard

MAKING A STANDARD CURVE

If more standards are available, we can plot the concentration of known standards against their absorbances. A graph plotting the standard data points, including a straight line of "best fit" drawn closest to these points, is called a **standard curve.**

Standard	Concentration (mg/dL)	Absorbance
1	30 mg/100 mL	0.25
2	50 mg/100 mL	0.38
3	60 mg/100 mL	0.41
4	70 mg/100 mL	0.57
Unknown	??	0.35

In your lab notebook, practice the idea of using a standard curve by plotting the points above. Now suppose that a solution of unknown concentration has an absorbance of 0.35. The standard curve graph can be used to determine its concentration. What is the unknown's concentration using the standard curve? Ask your instructor to make sure you understand how to do this.

USE OF THE SPECTROPHOTOMETER

PROCEDURE:
1.   Set the wavelength dial to that of the correct wavelength in nanometers (provided in each exercise)

2.   Place the cuvette, which contains all the reagents *except* the test solution (e.g., glucose), into the cuvette holder. This tube is called the **blank** because it has a concentration of test substance equal to zero. It should therefore have an absorbance of zero.
PRESS ABS = O KEY

3.   Place the other cuvettes, which contain the standard solutions and the unknown, in the cuvette holder one at a time. Close the hatch and read the absorbance value of each solution.

Note: *Before placing each cuvette in the chamber, wipe it with a kimwipe. <u>Make sure no tape covers the light path</u>*

Ex. A. Carbohydrates: Measurement of Plasma Glucose Concentration

The monomers of the carbohydrates are the **monosaccharides,** or simple sugars. Two monosaccharides can join together by means of a dehydration synthesis reaction to form a **disaccharide.** *Sucrose* (common table sugar), for example, is a disaccharide of glucose and fructose, whereas *maltose* is a disaccharide of two glucose subunits.

The continued addition of glucose subunits to maltose will result in the production of a long, branched chain of repeating glucose submits, forming the **polysaccharide** *glycogen* (or animal starch). This polysaccharide is formed inside muscle and liver cells and serves as an efficient storage form of glucose. When the blood glucose level drops below normal, the liver cells can hydrolyze stored glycogen and release glucose into the blood. Conversely, when the blood sugar level rises above normal, the liver cells can take glucose from the blood and store it as glycogen for later use. In this way, the equilibrium between blood glucose and liver glycogen helps to maintain homeostasis of the blood sugar level. This process is regulated by hormones that include epinephrine, insulin, hydrocortisone, and glucagon.

PROCEDURE

Measurement of Plasma Glucose Concentration
1.   Obtain three test tubes, and label them
      U (<u>unknown</u>), *S* <u>standard</u>), *B* (<u>blank</u>).

2.   Using a mechanical pipettor, pipette 5.0 mL of the glucose reagent into *each* tube.

3.   Use a microliter pipettor to add 40 µL (0.04 mL) of the following solutions into each of the indicated test tubes to avoid contamination. Use different pipette tips for adding solution.

Note: *All tubes must contain equal volumes (5.04 mL) of solution: 1 decileter (dL) = 100 milliliters (mL).*

4.   Cover each cuvette with Para film and invert to mix. Repeat this step just prior to reading in the spectrophotometer (dense material will settle to the bottom and interfere with readings).

5.   Set the wavelength at 500 nm and blank the spectrophotometer by placing the BLANK cuvette inside, closing the hatch and pressing the ABS=0 key. Wait for the ABS to read 0.000 and remove the blank.

6.   Invert each cuvette quickly, remove the Para film and take your reading for the Standard ad Unknown solutions. Record.

7.   Using Beer's law formula, calculate the concentration of glucose in the unknown plasma sample.

Ex. B. Lipids: Measurement of Plasma Cholesterol Concentration

Lipids are an extremely diverse family of molecules that share the common property of being soluble (dissolvable) in organic solvents such as benzene, ether, chloroform, and carbon tetrachloride, but *are not soluble in water or plasma*. The lipids found in blood can be classified as **free fatty acids (FFA),** also known as nonesterified fatty acids (NEFA), **triglycerides** (or neutral fats), **phospholipids** and **steroids.**

The triglycerides consist of three fatty acids bonded to a molecule of the alcohol glycerol*.* Triglycerides with few sites of unsaturation and that are solid at room temperature are called *fats*, whereas those with many sites of unsaturation and that are liquid at room temperature are called *oils.*

Measurement of Plasma Cholesterol Concentration

1.   Obtain three test tubes, and label them *U (*unknown), *S* (standard), *B* (blank).
2.   Using a mechanical pipettor, pipette 5.0 mL of the cholesterol reagent into each tube.
3.   Use a microliter pipettor to add 50 μL (0.05 mL) of the following solutions into each of the indicated test tubes. Use different pipette tips for adding each solution to avoid contamination.

Tube	Serum	Standard	Water	Reagent
Unknown (U)	50 μL	----	-----	5.0 mL
Standard (S) (200 mg/dL)	---	50 μL	-----	5.0 mL
Blank (B)	-----	-----	50 μL	5.0 mL

Note: *All tubes must contain equal volumes (5.05 mL) of solution: 1 decileter (dL) = 100 milliliters (mL)*

4. Cover each cuvette with Para film and invert to mix. Repeat this step just prior to reading in the spectrophotometer (dense material will settle to the bottom and interfere with readings).

5. Transfer the solutions to three cuvettes. Standardize the spectrophotometer at 500 nm, using solution *B* as the blank.

6. Record the absorbance values of Standard and Unknown solutions.

7. Using Beer's law formula, calculate the concentration of cholesterol in the unknown plasma sample.

C. Proteins: Measurement of Plasma Protein Concentration Using a Standard Curve

Proteins are long chains of amino acids bonded to one another by condensation reactions. Each amino acid has an amino (--NH$_2$) end and a carboxyl (--COOH) end, as shown by the general formula:

When amino acids bond (through a **peptide bond)** to form a protein, one end of the protein will have a free amino group and the other end will have a free carboxyl group.

There are more than twenty-two different amino acids in nature, each differing from the others with respect to the combination of atoms in the R (functional group)*.* The amino acid **glycine**, for example, has a hydrogen atom (H) in the *R* position, whereas the amino acid **alanine** has a methyl group (CH3) in the *R* position.

Proteins in the plasma serve a variety of functions. Some proteins may be active as enzymes, hormones, or carrier molecules (transporting lipids, iron, or steroid hormones in the blood), while others have an immune function (antibodies). The **plasma proteins** are classified according to their behavior during biochemical separation procedures. These classes include the *albumins,* the *alpha* and *beta globulins* (synthesized mainly in the liver from amino acids absorbed by the intestine), and *gamma globulins* antibodies produced by the lymphoid tissue).

In addition to the separate functions of the different plasma proteins, the total concentration of proteins in the plasma is physiologically important. The plasma proteins exert an osmotic pressure, the **colloid osmotic pressure,** which pulls fluid from the tissue spaces into the capillary blood. This force compensates for the continuous filtration of fluid from the capillaries into the tissue spaces produced by the *hydrostatic pressure* of the blood

PROCEDURE

Measurement of Total Plasma Protein Concentration

1.   Obtain seven test tubes, and label them 1 – 7.

2.   Using a mechanical pipettor, pipette 5.0 mL of Biuret reagent into each tube.

3.   Use a microliter pipettor to add 50 µL (0.05 mL) of the following solutions into each of the indicated test tubes. Use different pipette tips for adding each solution to avoid contamination.

Note: *All tubes must contain equal volumes (5.05 mL) of solution.*
4..   Transfer the solutions to seven cuvettes. Standardize the spectrophotometer at 550 nm, using solution *B* as the blank.

5.   Mix each cuvette as you did in previous exercises, then record the absorbance values of the unknown (*U)* and the five standard solutions.

6.   In your lab notebook, draw a graph of absorbance versus total protein concentration (g/dL). Plot the standards and draw a standard curve (best-fit LINE). Then use the graph to determine the unknown concentration.

Clean Up
Please dispose of all materials as instructed. Pour the unknowns into appropriately marked Biohazard beakers and place unknown cuvettes in bleach tubs. All other vials are not biohazardous (they did not contain sera).   Wipe down your bench.

Post-Lab
In addition to your pre-lab, data, graphs and observations, answer the following questions in your lab notebook.

1. Why are blanks needed for spectrophotometer analysis?
2. Which technique is more accurate at determining the concentration of an unknown – Beer's Law or a Standard Curve? Why?
3. Describe the function of plasma proteins. Where do your plasma proteins really come from?
4. Does the glycogen in your liver come from glycogen in your diet? Explain.