1. GENERAL INFORMATION AND RULES

For an efficient laboratory study, students must obey the rules below.

1.1 Laboratory Safety Instructions

(1) Wear laboratory coat to protect yourself against danger of various chemicals.

(2) Eating and drinking in the laboratory are strictly forbidden.

(3) Keep the working environment, tools and glass materials clean before and after using.

(4) Do not taste and smell chemical substances and do not touch them without wearing lab gloves.

(5) Keep closed the cap of vials containing any reagent or chemical.

(6) In case of any laboratory accident, inform the laboratory responsible immediately.

(7) Tools and devices used in the laboratory must be very clean.

(8) Keep the labels of bottles of reagents transferred to another tube or container upside down and do not let the label contaminated with reagent.

(9) Transfer the reagent from the bottle opening opposite to the label and close the right after the transfer.

(10) Use the reagents in exactly same amounts as indicated in the procedure.

(11) In the experiments which are performed by pipettes, use different pipette for each reagent to prevent contamination. Do not use the same pipette for different reagents.

(12) While heating the test tubes, hold the tube slightly bended and heat gently below the liquid surface. Meanwhile, shake the tube regularly and carefully to prevent splashing. The top of the heated tube is also turned to the side where anybody present.

(13) When a liquid has to be boiled for a long time, place the beaker on either the metal screen on the Bunsen burner or on the heated sheet. Perform the heating under the fume hood to avoid unpleasant smell.

(14) Never pour water and any aqueous solution onto concentrated acid (i.e. conc. H_2SO_4) to make dilution. Since this process is too exothermic, dangerous splash can occur and this

can lead undesirable consequences. Instead, add the concentrated acid slowly onto the water and mix with a glass rod at the same time.

(15) Another chemical that should not contact with concentrated sulfuric acid is potassium ferrocyanide. As a result of reaction of sulfuric acid with ferrocyanide, poisonous hydrogen cyanide (HCN) is released.

(16) Do not work near the flame while you are dealing with flammable liquids than can flame easily (such as ether, alcohol, carbon sulfide). If the solutions containing such liquids are to be heated, use the hot water bath to heat the flammable liquid.

(17) Never heat the test tube at the bottom. In particular, keep in mind that probability of boiling and splashing of the basic solutions in the test tubes is higher. Smelling and tasting of chemicals is forbidden and dangerous. Wear safety glasses in case of splashing during heating process.

(18) When working with urine and blood samples, wear lab gloves. Also, wash and bleach the used tubes and pipettes after the test.

1.1.1 First aid in laboratory accident

In case of possible laboratory accidents such as ingestion of chemical substances or contact with the skin, keep calm and report the situation immediately to the laboratory responsible and the following emergency measures should be taken before going to the doctor.

a) **Acid burns:** In case of that any acid is poured onto hand or splashed, wash the acid splashes with plenty of water, then with bicarbonate solution and then with plenty of water again. If the acid splashes have come into the eyes, wash your eyes with plenty of water and then bathed with 1% sodium bicarbonate solution and consult an ophthalmologist.

b) **Base burns:** Burns caused by bases should be washed with plenty of water, then 1% acetic acid solution and then again with plenty of water.

c) **Poisoning with ingested substances:** If acid is swallowed, first drink plenty of water then sodium bicarbonate solution. If base is swallowed, first drink plenty of water, then vinegar (or 1% acetic acid solution) or lemon juice (or citric acid solution).

1.2 Biochemical Analysis

Biochemical analysis is performed in biochemistry and clinical biochemistry laboratories. To diagnose an illness, to measure the intensity of the illness, to monitor the state of event of cure, to detect the biochemical molecules and to analyze those molecules quantitatively and qualitatively biochemical analysis methods are used.

The techniques used in biochemistry laboratories are in the class of biochemical analysis.

These methods are divided into three as chemical, physical and immunochemical methods.

1) Chemical Methods
   - Titrimetry (Volumetric Analysis)
   - Oxymetry
   - Precipitation methods
   - Complexometry
2) Physical Methods
   - Gravimetry
   - Colorimetry-Photometry-Spectrophotometry
   - Nephelometry
   - Turbidimetry

- Refractometry
- Polarimetry
- Gasometry
- Fluorometry
- Electrophoresis
- Chromatography

3) Immunochemical Methods
   - Radioimmunoassay (RIA)
   - Enzyme immunoassay (EIA)

1.2.1 Techniques used in biochemistry laboratories

Titrimetry is quantitative analysis of analyte (that is unknown amount of the compound in a solution) based on the reaction of analyte with a standard solution also known as titration solution whose weight or concentration has known.

The determination of the concentration of a given component in solution (the analyte) by addition of a liquid reagent of known strength (the titrant) until an equivalence point is reached (when the reactants are present in stoichiometric proportions). Often an indicator is added to make the equivalence point visible (e.g., a change in color).

Spectrophotometry is a method to measure the intensity of the absorbed light beam passes through the sample solution placed between the light source and detector. Concentration of the sample is calculated with respect to obtained strong or weak absorbance spectrum.

The basic principle is that each compound absorbs or transmits light over a certain range of wavelength. This measurement can also be used to measure the amount of a known chemical substance. Spectrophotometry is one of the most useful methods of quantitative analysis in

various fields such as chemistry, physics, biochemistry, material and chemical engineering and clinical applications.

The instruments that separate and send part of the light beam sent through the analyzed sample using filters are called colorimeter or photometer. The tools that make this selectivity through prisms are called as spectrophotometer.

If the UV-visible region passes through a solution with organic molecules in it, the solution selectively absorbs some of these rays (absorption), while others absorb very little or pass them (transmission).

The light intensity (I) from a colored solution placed in a cuvette is smaller than the light intensity (I_o) that enters the solution.

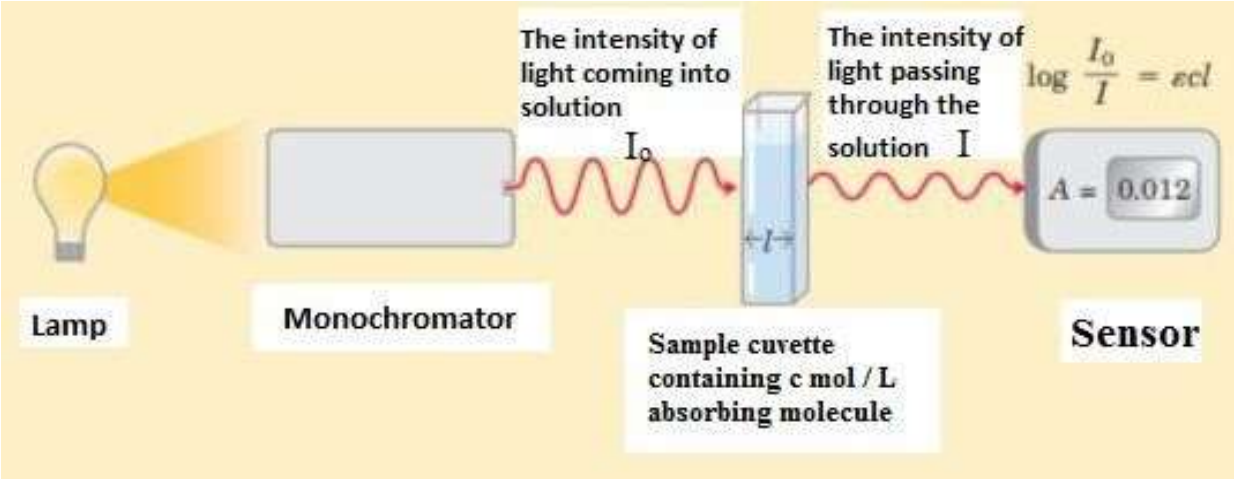


Figure 1.1 Working principle of spectrophotometer

The ratio of the light intensity passed through the solution to the light intensity entering the solution (I / I_0) is defined as transmittance (T). Transmittance is usually expressed as T%.

The logarithm of the inverse of the transmittance is defined as "Absorbance" (Optical density, A). This is the expression of how much of the light passing through the solution is absorbed.

$$A = \log\frac{1}{T} = -\log T = -\log\frac{I}{I_0}$$

There is a linear relationship between the amount or concentration of the substance dissolved in a solution and the absorbance (A).

In the spectrophotometric measurements, various tubes are prepared as blank , standard and sample:

Calibration standard is the solution used to make the optical settings of the device (setting 0 and 100). The standard solution sample generally includes all components of the measurement mixture, except for the substance to be determined. Volume of the standard solution is completed by addition of equal volume of buffer, water or reagent.

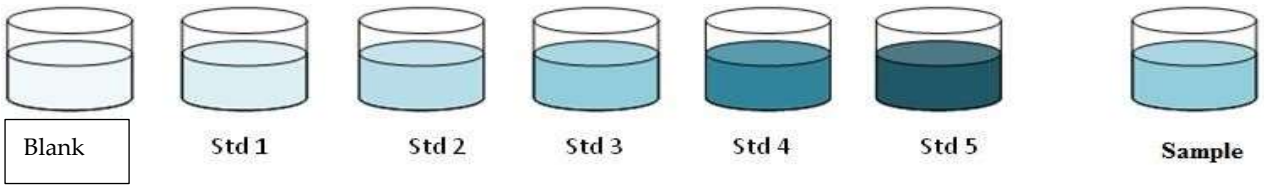
Blank solution is the most commonly used one; it is prepared by placing solvent in the reading cuvette. It is always used to reset the absorbance value.

Standard is the solution of the substance in the known concentration.

Sample includes the solution whose concentration to be determined.

The absorbance values of the standard solutions at different concentrations at a certain wavelength are measured after absorbance of blank solution was measured. Calibration curve is constructed by plotting the absorbance values of blank and standard solutions to y- axis versus concentration to x-axis of graph . Linear equation of the calibration curve is found in the form of y=mx+n.

After the calibration curve is plotted, absorbance of the sample solution is measured. The corresponding concentration of the sample (x value in the calibration curve) can be found from the calibration curve by inserting the absorbance (y value in the calibration curve equation).



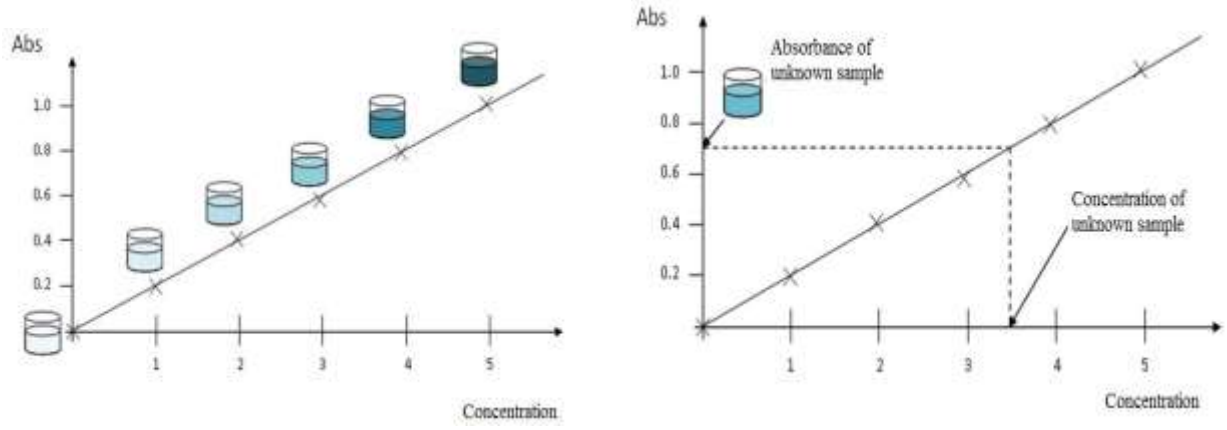


Figure 1.2. Plotting absorbance values of the standard solutions versus concentration at a given wavelength versus concentration graph and obtaining concentration of sample from this graph

1.3. Solutions and Preparations

A solution is a homogeneous mixture of at least two compounds. It is homogeneous because its composition and properties are uniform. A solution is composed of a solvent and one or more solutes. The solvent is the component that is present in the excess quantity or that determines the state of matter in which the solution exists. A solute is said to be dissolved in the solvent. If the solvent is not indicated, water is the solvent. A concentrated solution has a relatively large quantity of dissolved solute(s), and a dilute solution has only a small quantity.

When preparing the solution, the solids should be weighed on the surfaces that will not adhere to the surface during the weighing. If a solid is to be dissolved in the liquid, a little solvent is added to the beaker and then the solid is added and dissolved. Then this solution is transferred to the volumetric flask and completed to the final volume.

Various solutions are required for qualitative and quantitative analysis in laboratory studies. The purpose of preparing the solution is to provide a certain amount of solvent and solute in a certain amount of solution. For this purpose, some standard definitions are used.

Percent by weight (%w/w): The amount of substance in 100 grams of a solution. For example, when %37 HCl solution is used, it is understood that 100 grams of this solution contains 37 grams of HCl.

$$\textbf{Weight \%} = \frac{\textbf{Dissolved matter (g)}}{\textbf{Mass of solution (g)}}$$

Percent by volume (%v/v): It is defined as the volume of substance in 100 mL of a solution. For example, when a 5% ethanol solution is mentioned, it is understood that 100 mL of the solution contains 5 mL of ethanol.

$$\text{Volume \%} = \frac{\text{Amount of solute (mL)}}{\text{Solution volume (mL)}}$$

Percent by weight/volume (%w/v): It's a hybrid definition. For example, a 5% NaOH solution means that 5 g NaOH is prepared up to 100 mL with water.

$$\text{Weight - Volume (\%)} = \frac{\text{Amount of solute (g)}}{\text{Solution volume (mL)}}$$

Example 1: Calculate g/L% and w/v% of 500 ml solution in which 0.9 g NaOH is dissolved. (molecular weight of NaOH=40g/mol)

The solution contains 0.9 g / 500 mL so 1.8 g / L,

% w / v ; 1.8 g / L = 0.18 g / 100 mL = 0.16%

Example 2: How much NaCl is required to prepare 20% 100 mL NaCl solution?

% (w/v) = g of solid / 100 mL solution

%20 = 20 g NaCl/ 100 mL

Molarity (M) is defined as the number of moles of solute per liter of solution.

$$\text{Number of moles} = \frac{Weight\ of\ the\ compound}{Molecular\ weight\ of\ the\ compound}$$

$$\text{Molarity} = \frac{Moles\ of\ solute}{Liters\ of\ solvent}$$

1 mmol= 10^{3} mol	1 nmol= 10^{9} mol	1 mM = 10^{-3} M = 1 mmol/L = 1μmol/mL
1μmol= 10^{6} mol	1 pmol= 10^{12} mol	1 μM = 10^{-6} M = 1 μmol/L = 1 nmol/mL
		1 nM = 10^{-9} M = 1 nmol/L = 1 pmol/mL

Example 3: How much NaOH is required to prepare 0.01 μ M 250 mL NaOH solution?

$0.01 \times 10^{-6} \frac{mol\ NaOH}{L\ of\ solution} \times 250$ mL solution $\times \frac{1\ L}{1000\ mL} \times \frac{40\ g\ NaOH}{1 mol\ NaOH} = 0.1 \times 10^{-6}$ NaOH

0.1×10^{-6} g NaOH is weighed and diluted to 250 mL with water.

Example 4: Describe how you would prepare 100 mL of 6 M HCl solution? (Mwt of HCl = 36.5 g/mol, 37% and d = 1.18 g/cm^3)

$$6.0 \frac{\text{mol HCl}}{\text{L solution}} \times 100 \text{ mL solution} \times \frac{1 \text{ L}}{10^3 \text{ mL}} \times \frac{36.5 \text{ g HCl}}{1 \text{ mol HCl}} \times \frac{100 \text{ g reagent}}{37 \text{ g HCl}} \times \frac{1 \text{ L reagent}}{1.18 \times 10^3 \text{ g reagent}} = 0.05 \text{ L HCl} = 50 \text{ mL}$$

50 mL of HCl is diluted to 100 mL with water.

Note: When preparing the acid solutions, the concentrated acid is slowly added onto a certain amount of water.

Normality (N) is defined as the number of equivalent weights per unit volume. Normality is ratio of the amount of solute to the total volume of solution like molarity, but it is used for acids and bases.

$$Normality = \frac{Number\ of\ equivalent\ weights}{1\ L\ of\ solution}$$

$$Number\ of\ equivalent\ weights = \frac{Formula\ weight}{Number\ of\ equivalents\ (n)}$$

$Normality\ (N) = n \times M$ where n is number of equivalents and M is molarity.

Number of equivalents (n) is the number of H^+ ions released to the solution by the acids, number of OH^- ions released by the bases to the solution and the charge of the anion and cation of a salt.

Example 5: Describe how you would prepare 500 ml of 0.4 N H_2SO_4 solution? (Mwt (H_2SO_4)= 98 g/mol, %96, d =1.841

g/cm^3)

$$\text{Equivalent grams } H_2SO_4 = \frac{98}{2} = 49 \text{ g/mol}$$

$$0.4 \frac{\text{mol } H_2SO_4}{\text{L solution}} \times 500 \text{ mL solution} \times \frac{1 \text{ L}}{10^3 \text{ mL}} \times \frac{49 \text{ g } H_2SO_4}{1 \text{ mol } H_2SO_4} \times \frac{100 \text{ g reagent}}{96 \text{ g } H_2SO_4} \times \frac{1 \text{ L reagent}}{1.841 \times 10^3 \text{ g reagent}}$$

$$= 0.0055 \text{ L } H_2SO_4 = 5.5 \text{ mL}$$

5.5 mL H_2SO_4 is diluted with water to 500 mL.

Dilution is the process of reducing the concentration of a solute in solution, usually simply by mixing with more solvent. Following equation is used for the calculations;

$M_1 \times V_1 = M_2 \times V_2$

M_1 : Initial concentration

V_1 : Initial volume

M_2 : Final concentration

V_2 : Final diluted volume

Example 6: Describe how you would prepare 400 mL of 25% alcohol solution from 95% ethyl alcohol?

$M_1 \times V_1 = M_2 \times V_2$

95 × V1 = 25 × 400 V1 = 105 mL

Take 105 mL of 95% ethyl alcohol and dilute it to 400 mL with pure water.

1.3.1 Solutions used in biochemistry

Osmosis: Movement of a solvent (such as water) through a semipermeable membrane (as of a living cell) into a solution of higher solute concentration that tends to equalize the concentrations of solute on the two sides of the membrane.

Osmotic pressure: When two different aqueous solutions are separated by a semipermeable membrane (one that allows the passage of water but not solute molecules), water molecules diffusing from the region of higher water concentration to that of lower water concentration produce osmotic pressure.

Osmolarity: The number of particles dissolved in 1 L solution.

Osmolality: The number of particles dissolved in 1 kg of water.

Isotonic solution: Solutions of equal osmolarity are said to be isotonic. Surrounded by an isotonic solution, a cell neither gains nor loses water. % 0.9 NaCl solution is isotonic to body fluid. This is called physiological solution (saline).

Hypotonic solution: A solution that has a lower concentration than the inside of the cell. Swelling is observed in the cell placed in hypotonic solution. If the cell is put into a hypotonic environment; cell absorbs water from its environment, and it swells. This situation is called as deplasmolysis.

Hypertonic solution: A solution that has a higher concentration than the inside of the cell. In this solution, water moves out of the cell and the cell shrinks.

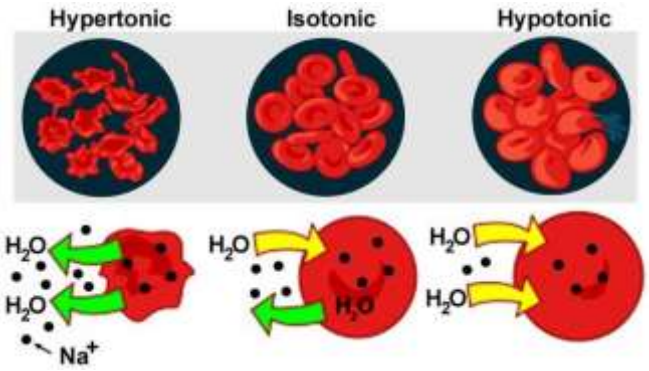


Figure 1.3. The behavior of cells according to the different solutions

1.4. Acid-Base Balance

Acids are proton donors and bases are proton acceptor substances. In other words, the acids give the hydronium ion and the bases give the hydroxide ion. The substances that are both the proton donor (donor) and the proton receptor (acceptor) are called the amphoteric. The ionization of acids and bases is different when dissolved in water. Accordingly, they can be separated as weak acid, strong acid, weak base and strong base. A strong acid fully dissociates into its ions in water. (Ka values are large, pKa values are small). A weak acid is an acid that partially dissociates into its ions in an aqueous solution or water (Ka values are small, pKa values are large). The negative logarithm of the concentration of H^+ ions in a solution is expressed as the pH of the solution. A neutral solution has a pH of 7 at 25° C. If a solution is pH <7 (higher than the H^+ ion concentration), the solution is acidic. If a solution has a pH>7 (H^+ ion concentration is lower), the solution is basic.

1.4.1 Buffers

A buffer solution (more precisely, pH buffer or hydrogen ion buffer) is an aqueous solution consisting of a mixture of a weak acid and its conjugate base, or vice versa. Its pH changes very little when a small amount of strong acid or base is added to it. The pH of the buffer solutions containing the weak acid and salt can be calculated by the Henderson-Hasselbach equation. If weak acid is shown as HA and its conjugated base is represented as BA;

$$HA \leftrightarrow H^+ + A^-$$

$$BA \leftrightarrow B^+ + A^-$$

$$K_a = \frac{[H^+][A^-]}{[HA]} \qquad [H^+] = K_a \frac{[HA]}{[A^-]}$$

When there is no salt in the environment, HA, which is a weak acid, has very little ionization. When BA is added to the acid solution, all A^- ions come from the more ionized BA. In dilute buffer solutions, non-ionized acid concentration [HA] is equal to the total acid concentration. At the same time, acid ion concentration [A^-] is equal to [BA], which is equal the total salt concentration.

If the above equation is arranged accordingly;

$$[H^+] = K_a \frac{[HA]}{[BA]} \quad \text{veya} \quad [H^+] = K_a \frac{[asit]}{[tuz]}$$

If the molecular ratio of the acid in the buffer to the salt and the K_a value are known, the negative value is calculated by taking the negative logarithm of both sides;

$$pH = -\log[H^+] = -\log[K_a \frac{[HA]}{[BA]}]$$

$$pH = -\log K_a - \log \frac{[HA]}{[BA]}$$

$$-\log K_a = pK_a \quad \text{ve} \quad -\log \frac{[HA]}{[BA]} = \log \frac{[BA]}{[HA]}$$

$$pH = pK_a + \log \frac{[BA]}{[HA]} \quad \text{veya} \quad pH = pK_a + \log \frac{tuz}{asit}$$

Example 1: Describe how you would prepare 500 ml of a solution 0.1 M of phosphate buffer (disodium monohydrogen phosphate heptahydrate and monosodium dihydrogen phosphate dehydrate) (pKa_2 value 7.2 and Na: 23, P: 31, O: 16, H: 1 g / mol))

at 7.4 pH phosphate buffer (disodium monohydrogen phosphate heptahydrate and monosodium dihydrogen phosphate dihydrate pKa_2 value 7.2 and Na: 23, P: 31, O: 16, H: 1 g / mol))

$7.4 = 7.2 + \log HPO_4^{2-}/H_2PO_4^-$ $\quad HPO_4^{2-} = a \quad H_2PO_4^- = b$

$a/b = 1.58 \quad a + b = 0.1 \quad a = 1.58b$

$2.58b = 0.1 \quad b = 0.039 \quad a = 0.061$

The amount of 0.061 M disodium monohydrogen phospate heptahydrate and 0.039 M monosodium dihydrogenphosphate dihydrate are calculated for 500 mL of buffer solution and pH is adjusted to 7.4 with 0.1 N H_3PO_4 and 0.1 M NaOH.

Example 2: Calculate the molarity of sodium acetate and acetic acid solutions to prepare 0.15 M acetate buffer at 5.06 pH (pKa value for acetic acid is 4.76).

$$5.06 = 4.76 + \log\frac{[CH_3COO^-]}{[CH_3COOH]} \quad 0.3 = \log\frac{[CH_3COO^-]}{[CH_3COOH]}$$

$$\frac{[CH_3COO^-]}{[CH_3COOH]} = 2$$

Since the total concentration is 0.15 M;

$$[CH_3COO^-] + [CH_3COOH] = 0.15\ M$$

0.05 M acetic acid and 0.1 M sodium acetate buffer should be prepared.

1.4.2. Biological importance of pH

For biochemists, the behavior of weak acids and bases that are not fully ionized when dissolved in water is important. Weak acids and bases play an important role in the regulation of metabolism. The pH should remain within certain limits to perform normal function of tissues and liquids in organism. For example, the pH of the blood serum is about 7.4 and the body can control the blood pH by chemical and physical mechanisms. Disorders of acid-base metabolism are common clinical problems observed of patients especially in intensive care units and emergency departments.

Normal pH is mandatory to maintain the activity of intracellular enzymes, for that reason pH changes can be fatal. Intracellular and extracellular pH is constantly in equilibrium. Some ion pumps and buffers inside the cell play a role in this equilibrium. Normally the blood H^+ concentration is 40 nmol/L. The pH of this concentration is founded as 7.4. Under physiological conditions the pH may show fluctuations around 0.04-0.05. The changes in the pH range of 0.1-0.2 indicate the presence of severe cardiovascular and neurological symptoms. The lowest possible H^+ concentration of life is 160 nmol/L (pH 7.8) and the highest concentration is 160 nmol / L (pH 6.8).

1.4.3. Buffer systems in body fluids and cells and regulation of acid-base balance

There are four major buffer systems that are providing acid-base balance in the body.

a) Carbonic acid / bicarbonate buffer system.
b) Primary phosphate / secondary phosphate buffer system.
c) Acid protein / proteinate buffer system.
d) Acid hemoglobin / hemoglobinate buffer system.

(a) **Carbonic acid / bicarbonate buffer system:** The most powerful buffer system providing the acid base balance in the extracellular system is the bicarbonate buffer system. Kidneys are controller most of the HCO^{-}_{3} concentration ,and lungs are controller most of the CO_2 concentration. Under normal conditions, in the blood pH changes only between 7.35-7.45, P_{CO2} changes between 37-42 mmHg and HCO_3^- concentration changes between 22-26 mEq/L. Decreasing in plasma HCO^{-}_{3} or increasing in CO_2 causes namely acidemia, increasing in HCO_3^- or decreasing in CO_2 causes clinical picture namely alkalemia.

The pH of the extracellular fluid is kept constant with the following reactions.

acidic environment, $H^+ + HCO_3^- \rightarrow H_2CO_3 \rightarrow CO_2 + H_2O$

basic environment, $OH^- + H_2CO_3 \rightarrow HCO_3^- + H_2O$

(b) **Primary phosphate / secondary phosphate buffer system:** The primary phosphate / secondary phosphate buffer system is more likely a buffer system of intracellular liquids. It is found in erythrocytes and kidney tubulus cells mostly. Primary phosphate / secondary phosphate buffer system plays an important role in the excretion of H^+ ions from the kidneys as $H_2PO_4^-$.

The pH of the medium is tried to keep constant with the following reactions:

acidic environment, $H^+ + HPO_4^{2-} \rightarrow H_2PO_4^-$

basic environment, $OH^- + H_2PO_4^- \rightarrow HPO_4^{2-} + H_2O$

(c) **Acid protein / proteinate buffer system:** It is one of the most commonly used buffer systems in tissue cells, and it also functions partially in plasma.

The pH of the medium is kept constant with the following reactions .

In excess of acid, $H^+ + \text{Proteinate} \rightarrow \text{Protein Acid}$

In excess of base, $OH^- + \text{Protein Acid} \rightarrow \text{Proteinate} + H_2O$

(d) **Acid hemoglobin / hemoglobinate buffer system:** It is a buffer system which is found in erythrocytes. It transports carbon dioxide in the form of HCO_3^-. 5% of CO_2 is free in plasma, 20% is in the form of carbo-hemoglobin in erythrocytes and 75% is transported as a HCO_3 in the blood.

CO_2 molecules which are produced by cellular metabolism, pass into tissue spaces and blood plasma. If concentration of CO_2 has increased in plasma, it passes into erythrocytes.

In erythrocytes, CO_2 is converted to H_2CO_3 by the effect of carbonic anhydrase enzyme. H_2CO_3 is also separated into H^+ and HCO_3^-.

$$CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow H^+ + HCO_3^-$$

The composed H^+ ions are neutralized by hemoglobinate.

$$H^+ + Hb^- \xrightarrow{\text{Carbonic anhydrase}} HHb$$

1.5. Anticoagulants: Anticoagulants are the substances that prevent blood clotting. Some of the important anticoagulants are as follows.

Used in vitro (outside live): oxalate, citrate, fluoride, EDTA and heparin.

Used in vivo (inside live): heparin, dicumarol and its derivatives, human protein C (Vitamin K dependent glycoprotein)

Oxalates: It prevents haemophilia by binding calcium. A 20% of solution is used. 0.1 mL of solution is required for 10 mL of blood. Hemoglobin determination, erythrocyte, leukocyte counts, and hematocrit determination can be made. It is not suitable for the determination of certain enzymes like lactate dehydrogenase, examination of acid-base balance, analysis of electrolytes and calcium. It is not used for spreading apparatus.

Citrates: It prevents haemophilia by binding calcium. For 1 mL of blood, 5 mg of sodium citrate is required. 3.8% of solution is used.

Fluorides: It prevents haemophilia by binding calcium. For 1 mL of blood, 10 mg of sodium fluoride is required.

EDTA (ethylenediaminetetraacetic acid): It prevents haemophilia by making complex with calcium. For 1 mL of blood, 1 mg of Na-EDTA is required.

Heparin: Specifically, heparin binds to coagulation factors IX and XI and interacts antitrombin III. This interaction prevents haemophilia by increasing inactivation ability of thrombin. For 1 mL of blood, 75 IU of heparin is required.

Coumarine group drugs: Vitamin K antagonists inhibit the reduction of quinone derivatives to active hydroquinone derivatives. They inhibit carboxylation of Vitamin K-dependent of glutamic acid residues in amino-terminal regions of coagulation factors II, VII, IX and X.

Human protein C: It is activated by thrombin and inactivates the coagulation factors V and VIIIa.

1.6 Precipitation of Proteins in Biological Specimens:

Proteins retard biochemical analysis. Hence, proteins should be removed before the blood and body fluids analysis. For this purpose, proteins should be precipitated by acid, heavy metal or antibody and the precipitate should be discarded by filtration or centrifugation. The residual filtrate is referred to as the protein-free filtrate. Mostly heavy metals (zinc, cadmium, iron, mercury, copper, lead), concentrated acids and specific antibodies are used to obtain protein-free filtrates.

Acids (sulfosalicylic acid, nitric acid, trichloroacetic acid, etc.) or organic solvents (acetone, ethanol, methanol) are used for analytical precipitation.

Organic polymers such as salts (eg, ammonium sulfate), organic solvents (acetone, ethanol, methanol) or polyethylene glycol (PEG) are used for preparative purpose precipitation.

1.6.1. Precipitation with Acids:

Basic functional groups (such as free amino groups) of serum proteins react with the sulfosalicylic acid and the protein-sulfosalicylic acid compound is formed as a result of this reaction. This compound precipitates because it is not dissolved in water. Also, these proteins bind to the anions of trichloroacetic acid (TCA) to form salts which does not dissolve in water. Hence, they precipitate. Many proteins precipitate with the addition of 5% (v / v) TCA. For proteins which have a molecular weight less than 20.000 g/mol, it may be required to add 10% TCA. The liquid phase is removed by centrifuged and precipitate is washed a few times with buffer (or pure water if lyophilized) and finally, suspended in a small amount of buffer (or pure water).

1.6.2. Precipitation with salt (*Salting Out*):

Precipitation by adding of increasing amounts of neutral salts, is the most common method used. A neutral salt causes aggregation of proteins (forgathering) and precipitation by separating the proteins from the solution without causing denaturation.

The reason of precipitating effect of salts is that salt molecules absorb water molecules bound by the protein molecules. Salting-out depends on the hydrophobic structure of the protein surface. Hydrophobic functional groups (that do not like water) are usually found in the interior of the protein. When salts are added to the system, the water dissolves the salt ions and as the salt concentration increases, the water molecules around the hydrophobic groups in the interior part of the protein are removed by the salt ions, in this case the interaction of the hydrophobic groups with each other increases and the proteins precipitate. This method is called salting out.

Different proteins precipitate at different salt concentrations. Many proteins precipitate at 55% ammonium sulfate saturation. Ammonium sulphate [$(NH_4)_2SO_4$] is used as a salt which is cheap and effective, has a high efficiency and solubility, does not affect the pH mostly and does not cause overheating in the solution.

To reach the intended concentration (% saturation), required amount of salt can be found by calculation or by using a table which was prepared for this purpose.

1.7. Blood:

Blood is composed of cells and a fluid called plasma. Cells are erythrocytes (red blood cells), leukocytes (white blood cells) and thrombocytes. More than 99% of the cells are erythrocytes. Erythrocytes are the oxygen-carrying cells of blood. Leukocytes are cells that protect the body against infections and cancer. Thrombocytes are responsible for blood clotting.

Whole blood (total blood): Blood with all its components (as white and red blood cells, platelets, and plasma) intact that has been withdrawn from a donor into an anticoagulant solution. Determination of blood count (hemogram) and erythrocyte sedimentation rate (ESR) is necessary to obtain blood cells (erythrocytes, leukocytes, thrombocyte).

Serum: The clear yellowish fluid that remains from blood plasma after clotting factors (erythrocytes, leukocytes, thrombocyte) are removed. Serum is obtained from the tube that blood is injected to the anticoagulation-free tube. Bilirubin and carotenes cause serum to be yellow. It is preferred for many analyzes.

Plasma: Blood plasma is a yellowish liquid component of blood that normally holds the blood cells in whole blood in suspension. In other words, it is the liquid part of the blood that carries cells and proteins throughout the body. Plasma is obtained from the blood that has been withdrawn from a donor into an anticoagulant solution. It is required for some specific analysis. Plasma is the liquid part of the blood, it consists of many organic and inorganic substances dissolved in water. The most important of these substances are proteins. Proteins comprise approximately 7% of the total weight of the plasma. Plasma proteins are divided into 3 main groups. These are albumin, globulins and fibrinogen. The difference between plasma and serum is that the plasma contains fibrinogen.

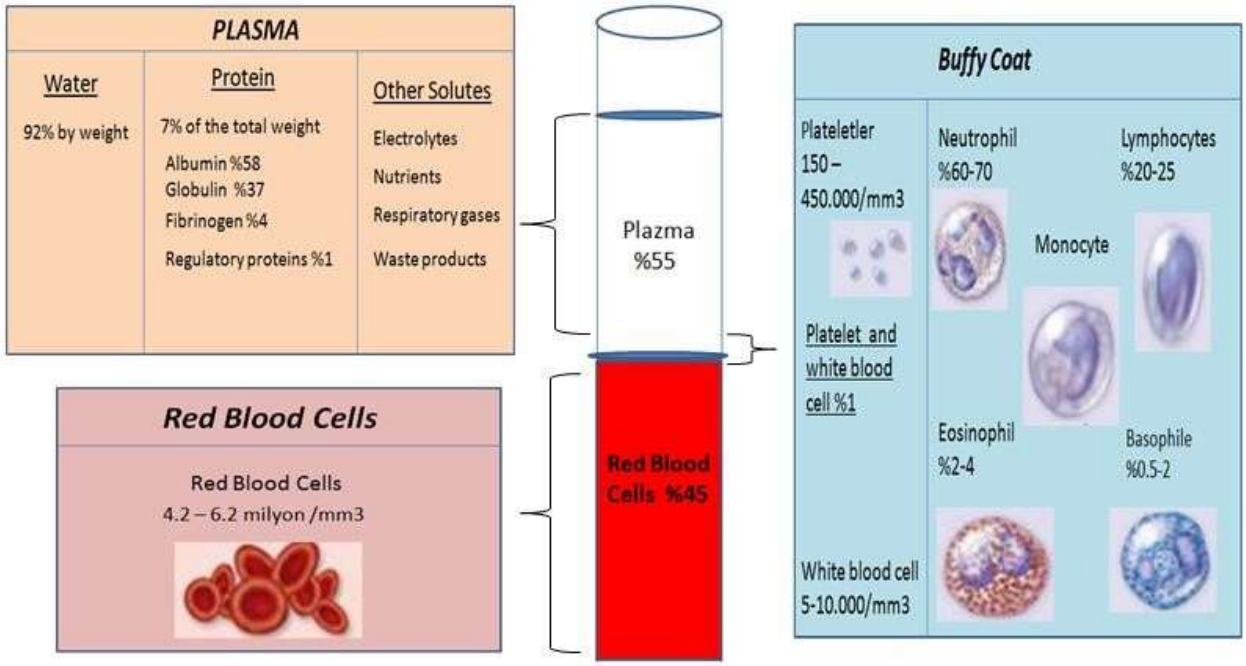


Figure 1.4. Serum and plasma contents

1.8. Experimental Studies

Experiment 1.8.1. Preparation of Protein-Free Filtrate with Trichloroacetic Acid

Principle of the experiment: It is based on the precipitation of insoluble salts of blood proteins with trichloroacetic acid.

Experimental Procedure: 1 mL of blood or serum is added to a test tube and 0.5 mL of 20% trichloroacetic acid (TCA) solution is added dropwise. The well shaken tube is incubated for 10 min and filtered through filter paper. The filtrate contains all substances other than protein.

Solutions:

%20 TCA: 20 mL of TCA is taken and completed to 100 mL with pure water.

Experiment 1.8.2. Separation of Globulins and Albumins in Serum by Ammonium Sulphate Precipitation

Principle of the experiment: Globulins are precipitated in a half-saturated ammonium sulfate solution and albumins are precipitated in a fully-saturated ammonium sulfate solution. Globulins and albumins in serum are dissolved in dilute solution. The globulins precipitate when the diluted solution is saturated with ammonium sulfate solution until it becomes half-saturated. While the globulins remain on the filter paper during filtration, the albumins are still dissolved in half saturated ammonium sulfate solution. Thereby, during the filtration albumins pass through the filter paper. This solubility difference in ammonium sulphate solution gives rise to the separation of the globulins and albumin from each other. Furthermore, albumins are precipitated when the filtrate is saturated with more ammonium sulfate and the solution turns into fully-saturated ammonium sulphate solution from half-saturated ammonium sulphate solution. The reason for the precipitation (dissolution) of dissolved proteins by the action of neutral salts such as ammonium sulfate is due to the withdrawal of water bound to the protein molecules called salting-out.

Experimental Procedure: 1 mL of serum is placed in a test tube. Add 1 mL of water to the serum in the test tube and mix the serum which dilutes the solution. Add 2 mL of saturated ammonium sulfate solution to the diluted serum in the test tube and mix. Thus, it is observed that a white turbidity occurs in the half-saturated ammonium sulfate solution. The turbid mixture in the test tube is filtered. The crystalline ammonium sulphate crystals are gradually added into the clear filtrate and the solution is mixed, and the resulting saturated ammonium sulfate solution is obtained. Re-turbidity is observed in saturated ammonium sulfate solution.

Experiment 1.8.3. Experiment of milk casein production and identification

Principle of the experiment: Casein in milk is a phosphoprotein; it is denatured with acetic acid and is characterized by protein identification methods.

Experimental Procedure: 5 mL of milk is put into a test tube and diluted with water with a dilution ratio 1:1. A solution of 2 N acetic acid is added dropwise onto the diluted milk in the tube and a precipitate is formed in the form of pellets. The precipitate formed is taken up on filter paper by filtering the mixture through filter paper; the filtrate is collected in another tube (the filtrate will be used in the next experiment). A biuret test is performed on the precipitate on the filter paper for the protein identification. Presence of casein protein is indicated by observing (+) the result. Reagent is added to the precipitate on the filter paper.

Solutions:

2N acetic acid: Take 11 mL of 99.9% acetic acid and dilute it to 100 mL with distilled water.

Biuret reagent: 6 g of Na-K tartrate, 1.5 g of crystallized copper sulfate, and 300 mL of 10% NaOH are mixed and dissolved with pure water and final volume is adjusted 1 L by addition of pure water.

Experiment 1.8.4. Lactoglobulin precipitation and identification assay with lactalbumin in milk

Principle of the experiment: Lactoglobulin with lactalbumin in the milk is denatured by heat and can be identified by protein determination methods.

Experimental Procedure: Place 3 mL of the filtrate obtained in Experiment 1.8.3 in a test tube. Add 2 drops of methyl red indicator to the filtrate in the tube and mix. Then, add 5% NaOH to the mixture in the tube until the color becomes yellow and solution becomes basic. The mixture in the tube is heated and boiled by a faint flame carefully. Consequently, a turbidity or precipitation is observed. The mixture in the tube is cooled and filtered through a filter paper after cooling; the precipitate is remained on filter paper is taken up and the filtrate is collected in another tube. Lead acetate assay ,which is one of the protein identification experiments, is applied

to the precipitate on the filter paper. In the light of the positive results, it can be concluded that lactalbumin and lactoglobulin are present.

Solutions:

Methyl red indicator

5% NaOH: Take 5 g of NaOH, dissolve in distilled water, and complete the final volume to 100 mL.

0.005 M $Pb(C_2H_3O_2)_2$: Take 0.16 g of Pb $(C_2H_3O_2)_2$, dissolve with distilled water and complete the final volume to 100 mL.

40% NaOH: Take 40 g, dissolve with distilled water and complete the final volume to 100 mL.

Experiment 1.8.5. Preparation of Buffer Solutions

Preparation of the Acetate buffer (pH= 4)

For the preparation of 0.1 M acetate buffer, 82 mL of 0.2 M acetic acid and 18 mL of 0.2 M sodium acetate solution are mixed and final volume is completed to 200 mL.
The pH is adjusted to 4 with a pH meter.

Preparation of Phosphate buffer (pH= 7)

For the preparation of 0.1 M phosphate buffer, 39 m of 0.2 M monobasic sodium phosphate and 61 ml of 0.2 M dibasic sodium phosphate solution are mixed and final volume is completed to 200 ml. The pH is adjusted to 7 with a meter.

Preparation of Carbonate buffer (pH= 10)

For the preparation of 0.1 M carbonate buffer, 27.5 ml of 0.2 M sodium carbonate and 22.5 ml of 0.2 M sodium bicarbonate solution are mixed and final volume is completed to 200 ml. The pH is adjusted to 10 by pH meter.

Experiment 1.8.6. Dilute Solution Preparation and Spectrophotometric Determination

1- Prepare 1 M $CuSO_4$ solution (M_w= 159.55 g/mol)

2- Prepare the following $CuSO_4$ solutions by diluting the total volume to 1 mL with 1 M $CuSO_4$ solution.

1:2, 1:5, 1:10, 1:50 and 1:100

3- Determine the absorbance value of each dilution at 700 nm.

4- Find the unknown concentration of $CuSO_4$ by using absorbance value of the unknown $CuSO_4$ solution and standard calibration graph obtained in step 2.

Experiment 1.8.7. Effect of Osmotic Pressure on Cells

Principle of the experiment: All cells are surrounded by a biological membrane that acts as a selective barrier. The passage of substances from this biological membrane takes place according to certain transport principles. The changes in the cell caused by concentration difference between the inside and outside of the cell are observed via microscope.

Experimental Procedure: The membrane of an onion is separated and placed flat on the slide and the lamella is closed. After examining the prepared microscope slide under a microscope, a drop of 2 M NaCl solution is added between the microscope slide and lamella and the cells are monitored. In the same way, a drop of pure water is added to the onion membrane and the changes in the cells are monitored. The cell behavior between two media is compared.

**The experiment can also be performed using green plant leaves.

REFERENCES

(1) Öztop, H.N. ve Candan, F. Biyokimya Laboratuvarı, Cumhuriyet Üniversitesi Yayınları, Sivas.

(2) Fahrünnisa P. 2000. Biyokimya, Ankara.

(3) www.ekimya.com/2/15039a.gif.

(4) Aktümsek, A. ve Nurullahoğlu, Z. Ü. Pratik Biyokimya, Nobel Yayınları, Ankara, Mart, 2007.

(5) http://www.rose-hulman.edu/~brandt/publications/422_Manual_3rd_Ed.pdf.

(6) Skoog, D.A, West, D.M, Holler, F.J, 2007, Analytical Chemistry, Bilim Yayıncılık, Ankara, 2007.

(7) http://www.hematoloji.org.tr/files/image/kan/kan1.jpg