

# **Introduction, Water and Aqueous solution; pH and Buffers**

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<https://archive.nptel.ac.in>

## **Introduction**

**introduction to the good laboratory practices, and introduction to water and buffers**

This lecture briefly introduces the general guidelines and precautions one needs to adhere to before carrying out the experiments in a laboratory. The lecture also introduces the good laboratory practice, an internationally accepted regulation of Organisation for Economic Co-operation and Development (OECD) that ensures quality assurance of the scientific and industrial research. The lecture also briefly reviews the chemical and structural properties of proteins and nucleic acids, the classes of biomacromolecules studied in most of the experiments discussed in this course.

## **Guidelines/precautions for a laboratory course**

Let us see what all things are important for a laboratory course, especially a course utilizing biological specimens, chemicals, and possibly harmful radiation. Let us outline the dos and don'ts for a wet-lab course:

### **Do's**

1. Always wear the appropriate equipment of personal protection. They include an apron, a pair of gloves, safety glasses, and shoes.
2. Read thoroughly the detailed procedure before carrying out the experiment.
3. Identify the potentially hazardous materials required for performing the experiments. It is recommended to go through the Material Safety Data Sheet (please see the next section) supplied with the chemicals to understand their potential hazards and the safe usage.
4. Before starting the experiment, label your materials (tubes, vials, specimens, etc.).
5. Check the label on the container before taking out any substance.
6. Use cleaned spatula for taking out the solid chemicals.
7. Be well aware of the locations and the operating procedures of the safety equipments such as safety shower, first aid kit, fire extinguisher, eyewash, etc.
8. Use a chemical fume hood for strong acids and other fuming chemicals and solvents.
9. Use the recommended biosafety level containment while handling microorganisms.
10. Before using a biosafety cabinet, always ensure that the UV lamp is turned off.
11. Decontaminate your work bench and the equipments handled after use.
12. Decontaminate all the infectious materials before disposing them off.
13. Maintain a laboratory book and write down all the methods, procedures, and observations in detail.
14. Any spillage, accident, or injury should immediately be reported to your instructor.

## **Don'ts**

1. Do not sit on the laboratory benches.
2. Never eat or drink anything inside the laboratory.
3. Do not handle your contact lenses or apply cosmetics in the lab.
4. Never touch any chemical or reagent with bare hands.
5. Never try to taste or smell any chemical or reagent.
6. Do not pipette any of the chemicals through your mouth.
7. If the chemicals are taken from the original container, and you happen to take out more than the amount required, never transfer back the excess amount to the original container.
8. If concentrated acids are to be diluted, never add water to the concentrated acid; concentrated acid should be added to the water drop-wise with continuous stirring.
9. Never leave your experiments unattended.
10. Never point the open end of any glassware or vial towards yourself or any other person.

## **Material Safety Data Sheet (MSDS)**

A Material Safety Data Sheet is a document provided by the manufacturer/supplier that contains information about the potential hazard of the material. An MSDS comprises of sections on product information (product name, names and addresses of manufacturer and supplier, emergency numbers); ingredients of the material; physical properties of the material; fire or explosion hazard data; toxicological data; first aid measures in case of an accident; and information on safe handling, usage, storage, and disposal. You might have observed certain hazard warning symbols, also known as pictograms, on the chemical containers. These warning symbols allow immediate identification of the material as a hazard.

## **Globally Harmonized System (GHS) of Classification and Labelling of Chemicals**

Globally Harmonized System of Classification and Labelling of Chemicals, introduced by United nations in 2000, is an internationally accepted system for classifying and labeling the chemical. The chemicals are classified as physical

hazards, health hazards, and environmental hazards. Some of the routinely observed symbols are shown in figure 1.1.

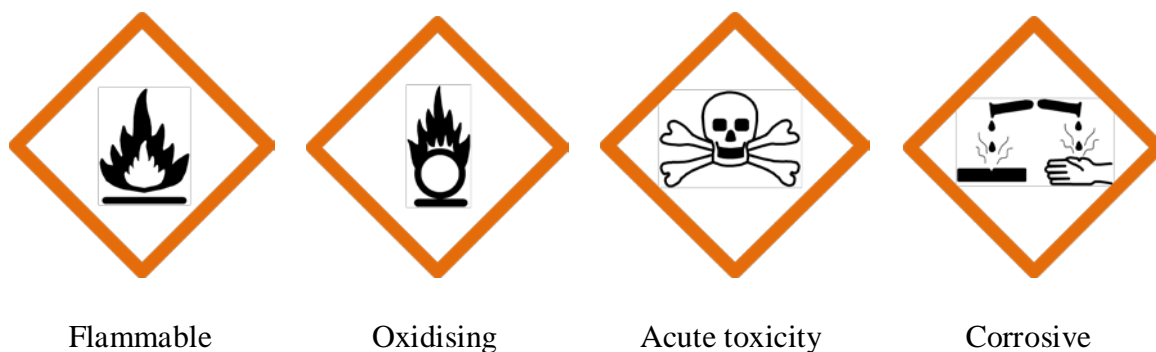


Figure 1.1 Some of the commonly observed hazard warning symbols (pictograms) on the chemicals.

### Good Laboratory Practice (GLP)

What exactly the GLP is? GLP is often confused with the standards of safe laboratory practices such as wearing aprons, gloves, and safety masks. GLP goes beyond the safe laboratory practices. An internationally recognized definition of GLP goes like this: *Good Laboratory Practice (GLP) embodies a set of principles that provides a framework within which laboratory studies are planned, performed, monitored, recorded, reported, and archived.* New Zealand and Denmark were the first to introduce GLP in 1972. United States was the next to introduce GLP in response to the poor scientific practices prevalent in US around that time. In early 1970s, US Food and Drug Administration realized the poor laboratory practices throughout United States. FDA also became aware of the fraudulent data produced by several toxicology laboratories; Industrial BioTest Labs being the most noted one. An international economic organization, the Organisation for Economic Co-operation and Development (OECD) published the principles of GLP in 1981 under the name 'OECD Guidelines for the Testing of Chemicals' that were internationally accepted. These guidelines are available on the OECD website: <http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm>.

GLP makes sure that the raw data generated during the study are traceable thereby ensuring the authenticity of the published data. GLP mandates a Quality Assurance unit that is required for monitoring and auditing the studies that are underway.



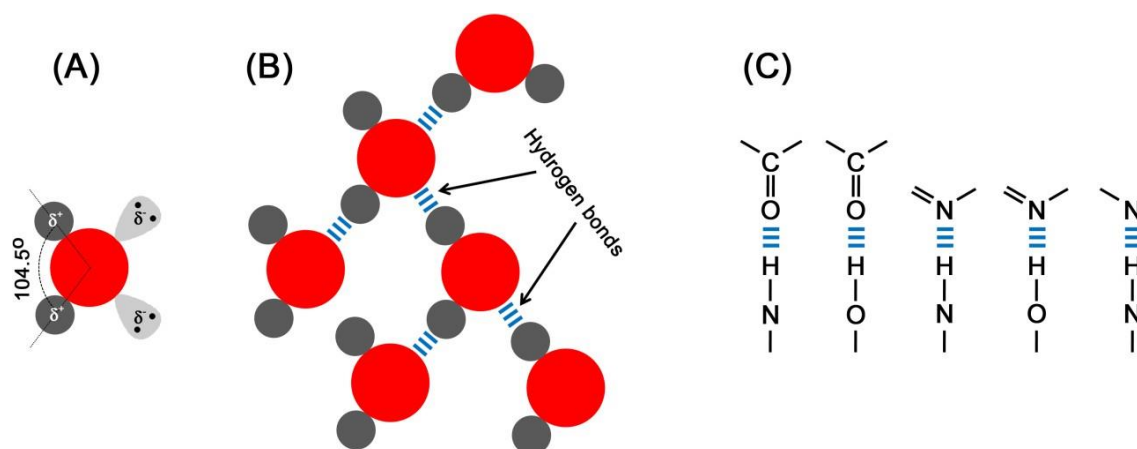
## Water and aqueous solutions

Water is the most abundant chemical component present in living systems. It constitutes around 60% or more of the total mass of most of the organisms. The first organisms are believed to have originated in the aqueous environment. Most of the biological reactions are carried out in water; many of them directly involving water as one of the reactants or products. It will not be incorrect if we call water as the biological solvent. To understand the chemistry of the biological processes and the behavior of biological molecules in water, we need to understand the physical and chemical properties of water. Biochemical experiments are mostly carried out in aqueous solutions having a defined chemical composition and pH. In this lecture, we shall be discussing the physical and chemical properties of water, concepts related to solutions, and pH.

### Hydrogen bonding and physical properties of water

Water ( $\text{H}_2\text{O}$ ) is a molecule wherein the oxygen atom is  $\text{sp}^3$  hybridized; two of these hybridized orbitals make  $\sigma$  bonds with hydrogen atoms while the other two are occupied by the unshared pairs of electrons. Water, therefore, is a bent molecule with an H–O–H angle of  $104.5^\circ$  (Figure 2.1A). The large difference in the electronegativities of oxygen and hydrogen atoms make O–H a highly polar bond. Owing to the angular geometry of the molecule, the dipole moments of the two O–H bonds do not cancel each other out, imparting it a high dipole moment of 1.85 Debye. The oxygen atom is partially negatively charged while the hydrogen atoms are partially positively charged. This leads to an electrostatic attraction between the oxygen atom of one water molecule and the hydrogen atoms of the other. This interaction is referred to as the hydrogen bond (Figure 1.2B). It is due to these hydrogen bonds, that water molecules are held together making it a liquid at room temperature. Hydrogen bonds (H-bonds) between the water molecules impart it high melting point, boiling point, and heat of vaporization. Low molecular weight and the ability to form H-bonds also impart water a very high specific heat; it takes 1 calorie to increase the temperature of 1 gram of water by  $1^\circ\text{C}$  at standard temperature and pressure. Water, therefore, is as an excellent temperature buffer. Hydrogen bonds are not restricted to water; they are readily formed between an electronegative atom (called a hydrogen acceptor; usually fluorine, nitrogen, and oxygen) and a hydrogen

attached to another electronegative atom (called a hydrogen donor). Hydrogen bonds are weak but play very crucial roles in the structure and function of the biomolecules. Common hydrogen bonds observed in biological systems are shown in figure 2.1C.

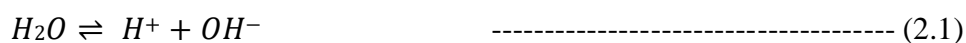


**Figure 2.1: Structure of water and hydrogen bonding.** Panel A shows the bent structure of water molecule; the big red sphere represents the oxygen atom while small spheres represent the hydrogen atoms. Panel B shows the hydrogen bonding between water molecules. Panel C shows the types of hydrogen bonds frequently found in biomolecules.

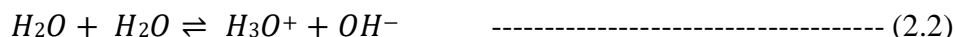
Polar solutes can form hydrogen bonds with the polar water molecules thereby dissolving in water. Non-polar solutes, on the other hand, do not make favorable interactions with the water molecules and clump together. Water can easily dissolve the charged solutes by hydrating and stabilizing the solute ions. Sodium chloride, for example, readily dissolves in water; the  $\text{Na}^+$  and  $\text{Cl}^-$  ions acquire greater freedom after leaving the salt crystal lattice. This increase in entropy makes the overall free energy change negative thereby favoring dissolution. The compounds that readily dissolve in water are termed hydrophilic while those that do not dissolve in water are termed hydrophobic. Certain molecules have spatially separated hydrophilic and hydrophobic regions; such molecules are termed as amphipathic compounds.

### Chemical properties of water

Pure water undergoes weak ionization to yield  $\text{H}^+$  and  $\text{OH}^-$  ions.



Free  $H^+$  ions do not exist in water; they exist as hydrated ions called hydronium ion ( $H_3O^+$ ). Equation 2.1 can therefore be written as:



The dissociation of water molecule can be expressed in terms of the equilibrium constant,  $K_{eq}$ .

$$K_{eq} = \frac{[H^+][OH^-]}{[H_2O]} \quad \text{-----} \quad (2.3)$$

Dissociation of water is so weak that the concentration of undissociated water can be assumed to be constant *i.e.* 55.5 M. Equation 2.3 can therefore be written as follows:

$$(55.5 M) K_{eq} = [H^+][OH^-] = K_w \quad \text{-----} \quad (2.4)$$

where,  $K_w$  is called the ion product of water. The concentrations of  $H^+$  and  $OH^-$  at 25 °C for pure water are  $1 \times 10^{-7}$  M each. Therefore, the ion product of water is  $1 \times 10^{-14}$  M<sup>2</sup>. When the concentration of  $H^+$  and  $OH^-$  are same, the water or any aqueous solution is said to be at neutral pH.

## The pH scale

The concept of pH originates from the ion product of water.

$$[H^+][OH^-] = 10^{-14} \quad \text{-----} \quad (2.5)$$

Taking logarithms:

$$\log[H^+] + \log[OH^-] = -14 \quad \text{-----} \quad (2.6)$$

Multiplying by -1:

$$-\log[H^+] - \log[OH^-] = 14 \quad \text{-----} \quad (2.7)$$

$-\log[H^+]$  and  $-\log[OH^-]$  are defined as pH and pOH, respectively where “p” denotes the negative logarithm. Equation 2.7 can therefore be written as:

$$pH + pOH = 14 \quad \text{-----} \quad (2.8)$$

The above discussed definition of pH does not pose any limits on the values it can take. It is however convenient to define a pH scale ranging from a concentration of 1



M H<sup>+</sup> to 1 M OH<sup>-</sup>. The pH scale thus obtained ranges from 0 – 14. It should be clear by now that pH is a scale for defining the acidity or basicity of aqueous solutions. A solution having a pH<7 is acidic while that having a pH>7 is basic.

pH is an important concept for living organisms. pH defines the ionization state and therefore the function of the biomolecules; most biomolecules function within a narrow pH range.

### **Expressing concentrations**

As most biological processes are carried out in the aqueous environment, it is important to know how the concentrations of the solutions are expressed:

**Concentrations based on volume:** concentrations represented as the amount of substance per unit volume of solution are the most widely used in a biochemical laboratory.

***Molarity (M):*** Molarity is the number of moles of solute per litre of solution. One mole is defined as the amount of substance that contains as many atoms, molecules, or other elementary units as the number of atoms present in 12 g of Carbon 12. One mole is the mass in grams numerically equal to the formula weight of the substance.

$$\text{Number of moles} = \frac{\text{mass of the substance in grams}}{\text{molecular weight of the substance}}$$

***Normality (N):*** Normality is the number of equivalents of solute per litre of solution.

One equivalent (or equivalent weight) of an acid or base is the mass that contains one mole of replaceable hydrogen or hydroxyl.

One equivalent (or equivalent weight) of a compound involved in oxidation-reduction reaction is the mass that donates or accepts one faraday of electrons (1 mole of electrons).

$$\text{Number of equivalents} = \frac{\text{mass of the substance in grams}}{\text{Equivalent weight}}$$

***Weight/volume percent (%w/v):*** The weight of solute in grams per 100 ml of solution.

***Osmolarity:*** Osmolarity is the molarity of the particles in a solution.

Osmolarity of 1 M solution of a dissociable molecule is  $n$  Osmolar, where  $n$  is the number of ions produced per molecule.

Concentrations based on weight:

*Weight/weight percent (%w/w):* The weight of the solute in grams per 100 grams of solution

***Molality (m):*** the number of moles of solute per 1000 grams of solvent

***Mole fraction ( $X_i$ ):*** Mole fraction of a compound in a solution is the fraction of the total number of moles represented by the compound in question. If a solution contains  $n_1$  moles of compound 1,  $n_2$  moles of compound 2,  $n_3$  moles of compound 3, and  $n_4$  moles of compound 4; the mole fraction of compound 3 is given by:

$$X_3 = \frac{n_3}{n_1 + n_2 + n_3 + n_4} \text{-----} (2.9)$$

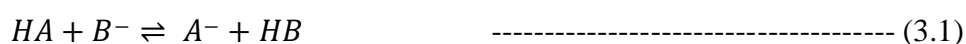
We shall be discussing about the buffers and preparation of buffers in the next lecture.

## Buffers

In this lecture, we shall be discussing about the ionization of weak acids and preparation of buffers using weak acids and bases. By the end of this lecture, you should be able to prepare a buffer of any strength (concentration) and any pH.

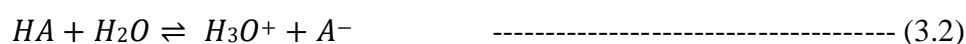
### Ionization of weak acids and weak bases

In biochemistry, it is convenient to use the Brønsted concept of acid and base; a substance that donates protons is an acid while a substance that accepts the protons is a base. When an acid loses a proton, a base is produced. The acid and the corresponding base are said to constitute a conjugate acid-base pair.



In the above ionization reaction,  $HA$  and  $A^-$  make one conjugate acid-base pair while  $B^-$  and  $HB$  make another conjugate acid-base pair.

Strong acids like  $HCl$ ,  $H_2SO_4$ , and  $HNO_3$  and strong bases like  $NaOH$  and  $KOH$  completely dissociate in dilute aqueous solutions. The weak acids and bases are those that do not dissociate completely when dissolved in water. The dissociation of a weak acid in water can be written as a reversible reaction:



The ionization can be represented by the equilibrium constant,  $K_{eq}$ :

$$K_{eq} = \frac{[H_3O^+][A^-]}{[H_2O][HA]} \quad \text{-----} \quad (3.3)$$

Again, the concentration of the undissociated water can be assumed to be constant (55.5  $M$ ). Equation 3.3 can therefore be written as

$$[55.5 \text{ M}]K_{eq} = \frac{[H_3O^+][A^-]}{[HA]} \quad \text{-----} \quad (3.4)$$

[55.5 M]  $K_{eq}$  is another constant; represented as  $K_a$  for the dissociation of acids. The equation 3.4 can therefore be rewritten as:

$$K_a = \frac{[H_3O^+][A^-]}{[HA]} \text{ or simply } \frac{[H^+][A^-]}{[HA]} \quad \text{-----} \quad (3.5)$$

Taking logarithm of equation 3.5

$$\log K_a = \log[H_3O^+] + \log[A^-] - \log[HA] \quad \text{-----} \quad (3.6)$$

Rearranging equation 3.6:

$$-\log[H_3O^+] = -\log K_a + \log[A^-] - \log[HA] \quad \text{-----} \quad (3.7)$$

$$pH = pK_a + \log \frac{A^-}{HA} \quad \text{-----} \quad (3.8)$$

where,  $p$  is a shorthand for negative logarithm ( $-\log$ ). Equation 3.8 is known as the Henderson-Hasselbalch equation. From Henderson-Hasselbalch equation,  $pK_a$  can be defined as the pH at which an acid is 50% ionized.

### Titration of a weak acid

Titration of a weak acid (acetic acid) with a strong base (NaOH) is shown in figure 3.1. Addition of NaOH neutralizes the  $H^+$  ions present in the solution; thereby increasing its pH. Consumption of  $H^+$  ions drives the dissociation of the  $CH_3COOH$  into  $CH_3COO^-$  and  $H^+$  (Le Chatlier's principle). As more and more of NaOH is added, the pH of the solution increases. This increase in pH is small when the solution reaches the pH close to the  $pK_a$  of acetic acid. A weak acid, therefore, provides a resistance to change in pH near its  $pK_a$  value. This resistance to change in pH by an acid near its  $pK_a$  is the concept of a buffer.

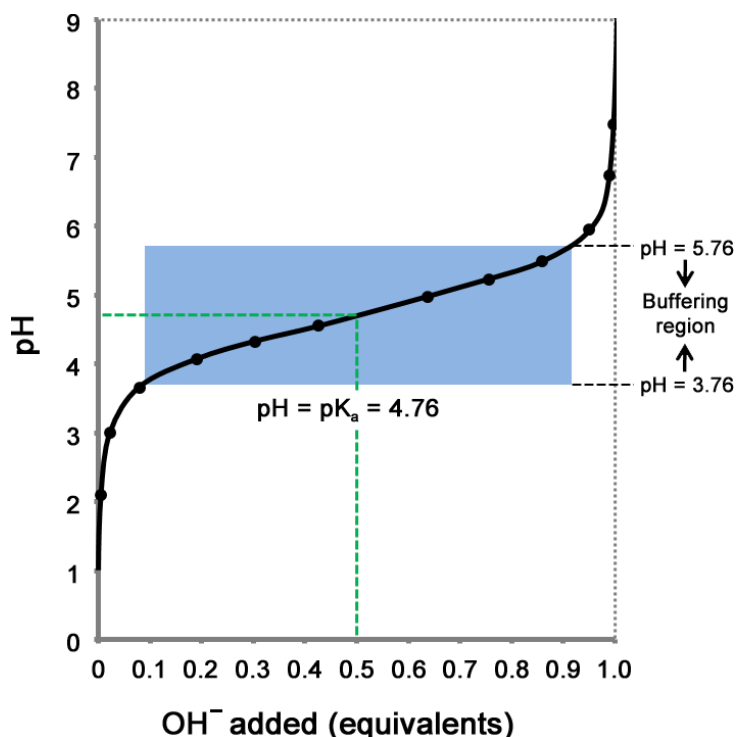


Figure 3.1: Titration curve of acetic acid. Acetic acid is titrated with NaOH and pH is measured after every addition. The pH is plotted against the amount of NaOH expressed as a fraction of the total amount of NaOH required to ionize all the acetic acid. Addition of NaOH drives the dissociation of  $\text{CH}_3\text{COOH}$  into  $\text{CH}_3\text{COO}^-$  and  $\text{H}^+$ . Addition of NaOH causes increase in pH; notice the buffering region (blue) wherein addition of NaOH causes little increase in pH.

### Common ion effect

Common ion effect is the change in the ionization equilibrium due to the addition of a solute that provides an ion taking part in the equilibrium. Let us take an example with acetic acid.

*Question: For 0.1 M solution of acetic acid.*

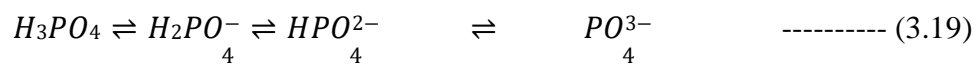
- Calculate the concentrations of all the chemical species present at equilibrium (Given  $K_a$  of acetic acid is  $1.7 \times 10^{-5} \text{ M}$ ).
- Calculate the concentrations of all the species when 0.05 M of sodium acetate is added to the solutions.

### Preparation of buffers

Let us now see how we make buffers. Suppose you are asked to prepare a buffer of pH 7.4, the pH of human blood. The first thing you need to do is to select appropriate conjugate acid-base pair. The selection is based on the  $pK_a$  of the acid; an acid can be used to prepare a buffer within a pH range of  $pK_a \pm 1$ . This means that you cannot prepare an acetate buffer of pH 7.4 because acetic acid-acetate conjugate base pair is a

good buffer only for the pH range of 3.76 – 5.7. Let us see the dissociation of phosphoric acid:

$$K_{a_1} = 7.6 \times 10^{-3} \quad K_{a_2} = 6.16 \times 10^{-8} \quad K_{a_3} = 4.79 \times 10^{-13}$$



For phosphoric acid,  $pK_{a_1} = 2.12$ ,  $pK_{a_2} = 7.21$ , and  $pK_{a_3} = 12.32$

We can therefore see that the  $pK_{a_2}$  of phosphoric acid is suitable for preparing the buffer of pH 7.4. Let us see how to prepare a phosphate buffer of pH 7.4.

*Calibration of pH meter:* pH meters need to be calibrated every time a buffer is to be prepared. A pH meter is calibrated with standard buffers of pH 4.0, 7.0, and 9.2. The pH meter is calibrated with pH 7.0 buffer; the second standard buffer to be used is that of pH 4.0 if the buffer that needs to be prepared is acidic and that of pH 9.2 if the buffer to be prepared is basic.

1. Switch "ON" the pH meter and allow it to stabilize for 20 *min*.

2. Take out your standard buffers and allow them to come to room temperature (standard buffers are usually stored at 4 °C).
3. The electrode of the pH meter is kept in 3 M HCl.
4. Take out the electrode and wash it thoroughly with distilled water.
5. Measure the pH of "pH 7.0 standard buffer", set this as pH 7.0 in the pH meter.
6. Take the electrode out of the standard buffer and wash it thoroughly with water.
7. Measure the pH of "pH 9.2 standard buffer", set this as pH 9.2 in the pH meter.

*Method 1: Preparing buffer with H<sub>3</sub>PO<sub>4</sub>*

1. Prepare 100 ml of 1 N NaOH solution
2. Take 400 ml distilled water in a 1 litre conical flask.
3. Add 11.53 ml of H<sub>3</sub>PO<sub>4</sub> dropwise in the 400 ml distilled water

a. The amount of H<sub>3</sub>PO<sub>4</sub> required

$$= \frac{\text{Mol.wt.} \times \text{Molarity} \times \text{Volume in ml}}{1000} \text{ grams}$$

$$= \frac{98 \frac{\text{g}}{\text{mol}} \times (0.2 \text{ M}) \times (500 \text{ ml})}{1000} =$$

9.8 grams

b. Concentrated H<sub>3</sub>PO<sub>4</sub> is 85 % (wt/v) with a density of 1.685 g/ml

c. Therefore, the volume of concentrated H<sub>3</sub>PO<sub>4</sub> required:

$$= \frac{\text{amount required in grams}}{\text{Concentration (\% wt/v)}} \times 100 \text{ ml}$$

$$= \frac{9.8}{85} \times 100 \text{ grams} = 11.53 \text{ ml}$$

4. Wash the pH electrode thoroughly with distilled water and place it in the H<sub>3</sub>PO<sub>4</sub> solution.
5. Add 1 N NaOH dropwise; shake well and measure the pH.
6. When the pH of the solution nears 7.4, be careful in adding NaOH so that pH does not overshoot.
7. Transfer the contents of the flask in a 500 ml volumetric flask.



8. Add water to make the final volume of the solution to 500 ml.
9. The required buffer is ready.

Note: The same method can be used for preparing the buffer starting with  $\text{NaH}_2\text{PO}_4$ . The amount of  $\text{NaH}_2\text{PO}_4$  required:

$$= \frac{\text{Mol. wt of NaH}_2\text{PO}_4 \times \text{Molarity of buffer} \times \text{Volume in ml}}{1000}$$

$$= \frac{119.98 \frac{\text{grams}}{\text{mol}} \times 0.2 \frac{\text{moles}}{\text{litre}} \times 500 \text{ml}}{1000} \approx 12 \text{ grams}$$

12 g of  $\text{NaH}_2\text{PO}_4$  is dissolved in 400 ml water in a 1 liter conical flask and then steps 5-9 are followed.

*Method 2:* Preparing buffer with  $\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$

$\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  completely dissociate in water to provide conjugate acid ( $\text{H}_2\text{PO}_4^-$ ) and conjugate base ( $\text{HPO}_4^{2-}$ ). As is evident from the Henderson-Hasselbalch equation (equation 3.8), the pH of the solution is determined by the ratio of conjugate base to the conjugate acid. Let us calculate this ratio:

$$\text{pH} = \text{pK}_a + \log \frac{A^-}{AH} \quad \text{----- (3.8)}$$

For the given pair of conjugate acid and base,  $\text{pK}_{a_2}$  of phosphoric acid is to be used:

$$7.4 = 7.21 + \log \frac{\text{HPO}_4^{2-}}{\text{H}_2\text{PO}_4^-} \quad \text{----- (3.20)}$$

$$\log \frac{\text{HPO}_4^{2-}}{\text{H}_2\text{PO}_4^-} = 0.19 \quad \text{----- (3.21)}$$

$$\frac{\text{HPO}_4^{2-}}{\text{H}_2\text{PO}_4^-} = 10^{0.19} = \frac{1.5488}{1} \quad \text{----- (3.22)}$$

Therefore,  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}:\text{NaH}_2\text{PO}_4$  ratio for preparing the required buffer is 1.5488:1. For preparing one liter of buffer, the required number of moles of phosphate species = 0.2 i.e. 200 mmoles.

The required number of moles for a  $V$  ml of buffer is given by  $= \frac{\text{Molarity of buffer}}{1000} \times V$  (ml)

Therefore, the number of moles required for the 500 ml of 200 mM buffer =  $\frac{0.2}{1000} \times 500 = \mathbf{0.1}$

Therefore, moles of conjugate acid ( $\text{NaH}_2\text{PO}_4$ ) required  
 $= X_{\text{acid}} \times \text{total number of moles}$   
 $= \frac{1}{1.5488+1} \times 0.1 = \mathbf{0.039}$

Moles of conjugate base ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ) required =  
 $X_{\text{base}} \times \text{total number of moles}$   
 $= \frac{1.5488}{1.5488 + 1} \times 0.1 = \mathbf{0.061}$

Therefore, the amount of  $\text{NaH}_2\text{PO}_4$  required:

$= \text{number of moles of } \text{NaH}_2\text{PO}_4 \times \text{molecular weight}$   
 $= 0.039 \text{ moles} \times 119.98 \frac{\text{grams}}{\text{mole}} = 4.68 \text{ grams}$

The amount of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  required:

$= \text{number of moles of } \text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O} \times \text{molecular weight}$   
 $= 0.061 \text{ moles} \times 268.07 \frac{\text{grams}}{\text{mole}} = 16.35 \text{ grams}$

Let us now see how to prepare the buffer:

1. Weigh accurately 4.68 g of  $\text{NaH}_2\text{PO}_4$  and 16.35 g of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ .
2. Transfer the salts into a 1 liter conical flask and add 400 ml distilled water.
3. Shake the flask well to achieve complete dissolution of the salts
4. Wash the electrode of the pH meter thoroughly with distilled water.
5. Place the pH electrode into the phosphate solution and measure its pH.
  - a. Although the amounts of the conjugate acid and conjugate base are weighed so as to achieve a pH of 7.4, the pH of the solution is seldom 7.4.
6. If the pH of the solution is <7.4, add 1 N NaOH dropwise, shake the flask well and measure the pH. Keep doing this until a pH of 7.4 is achieved.

7. If the pH of the solution is  $>7.4$ , add 1 *N*  $\text{H}_3\text{PO}_4$  dropwise, shake the flask well and measure the pH. Keep doing this until a pH of 7.4 is achieved.
  - a. Addition of  $\text{H}_3\text{PO}_4$  increases the concentration of a phosphate species in the solution; you will therefore end up with a buffer that will have a pH of 7.4 but the concentration of the buffer will be slightly more than 200 *mM*.

### Which method of buffer preparation one should choose?

Which method do you think is better for preparing a buffer. Method 1 might appear better than method 2 because we are required to titrate with NaOH; this implies that we are not going to add any phosphate species that could change the strength of buffer. There is, however, a possibility of overshooting the required pH while adding NaOH. What do we do if we actually overshoot the required pH? We are left with two options:

- i. Discard all the contents and prepare fresh buffer.
- ii. Add  $\text{H}_3\text{PO}_4$  to bring the pH down to 7.4; this would, however, increase the strength of the buffer.

Increase in buffer strength, however, is not that big a problem. The prepared can be diluted with water so as to obtain the buffer of desired strength. But, is there a way out that we don't change the buffer strength and still achieve the correct pH no matter how casually we perform the titrations? The answer is "yes". This is how we go about it:

We know that  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{NaH}_2\text{PO}_4$  are required in 1.5488: 1 molar ratio. We can prepare the 200 *mM* solutions of both  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{NaH}_2\text{PO}_4$  in a 1.5488:1 volume ratio. As we need to prepare 500 *ml* buffer, we would perform the calculation for a slightly higher volume, say 550 *ml*.

1. Prepare  $\frac{1}{(1.5488+1)} \times 550 \text{ ml} = 215.8 \text{ ml}$  of  $\text{NaH}_2\text{PO}_4$  solution in water. Let us prepare 220 *ml* of the  $\text{NaH}_2\text{PO}_4$  solution (volume rounded off to the above 'multiple of 10' integer):

$$\text{a. Weigh } \frac{\text{Mol.wt} \times \text{Molarity} \times \text{Volume in ml}}{1000} = \frac{119.98 \times 0.2 \times 220}{1000} \text{ grams} =$$

5.28 *grams* of  $\text{NaH}_2\text{PO}_4$ , dissolve it in 200 *ml* distilled water and add distilled water to make a final volume of 220 *ml*.

$$2. \text{ Prepare } \frac{1.5488}{(1.5488+1)} \times 550 \text{ ml} = 334.2 \text{ ml of } \text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O} \text{ solution in water.}$$

Let us prepare 340 ml of the  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  solution (volume rounded off to the above 'multiple of 10' integer):

$$\text{a. Weigh } \frac{\text{Mol.wt} \times \text{Molarity} \times \text{Volume in ml}}{1000} = \frac{268.07 \times 0.2 \times 340}{1000} \text{ grams} =$$

18.23 *grams* of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , dissolve it in 300 *ml* distilled water and add distilled water to make a final volume of 340 *ml*.

$$3. \text{ Mix } \frac{1}{(1.5488+1)} \times 500 \text{ ml} = 196.17 \text{ ml of } \text{NaH}_2\text{PO}_4 \text{ with } \frac{1.5488}{(1.5488+1)} \times 500 \text{ ml} =$$

303.83 *ml* of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ . This gives you the 500 *ml* of 200 *mM* phosphate buffer.

4. Measure the pH of the solution.

5. If the pH of the solution is less than pH 7.4, titrate with 200 *mM*  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  until the pH reaches 7.4; if the pH is greater than 7.4, titrate it with 200 *mM*  $\text{NaH}_2\text{PO}_4$  until the pH reaches 7.4.

6. The 200 *mM* phosphate buffer with pH 7.4 is ready.

Although we have discussed the preparation of phosphate buffer, the concept and the protocol for making the buffer are general.