MICROBIAL PHYSIOLOGY AND BIOCHEMISTRY

Water, pH and buffers

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Water as a universal solvent

Water is indispensable for life. In fact, life arose in aqueous solution. Water is life's true and unique medium. Water content of different life forms varies from about 99.8% in jelly fish to about 40% in fatty animal. Likewise the water content in tissues of organisms also varies. In general the more active a tissue is the higher would be its water content. Water which is often regarded as an inert space filling liquid is actually a chemically reactive substance which has profound influence on the structure of biomolecules that define life. Without water biomolecules such as proteins and nucleic acids might no longer truly be biomolecules. In addition, to its functions as a major and integral part of living protoplasm, water serves as a medium for the transport of food in the form of sap in glands, lymph and blood in animals and nutrient solutions for microorganisms, and facilitates chemical reactions by being a reactant or a product in metabolic reactions. The polymerization reactions in the cell metabolism involved with biosynthesis of macromolecules are accompanied with the elimination of water molecule whereas a number of other metabolic reactions in the cell require presence of water as a reactant.

The water in an organism may exist as free water or bound water. Free water is the liquid water containing truly dissolved solutes and serving as a dispersion medium for the colloidal particles in the protoplasm. The bulk of water however, is bound to the colloidal particles in the protoplasm. The water in the bound condition exhibits entirely different properties from water in the bulk (free water). The water in the bound form forms colloids (a colloidal solution has particles which range in size from 1 to 10 nm).These particles present a huge surface area for a given volume upon which smaller molecules are adsorbed. For example proteins adsorb water and become hydrated. Even in dry state they bind one third or more of their weight of water. Proteins and polysaccharides bind water and are called hydrophilic colloids. The protoplasm is a colloidal complex due to the presence of proteins and polysaccharides. They are dispersed through out the ground substance of the protoplasm where they are involved in cell activities. The cell activities in different organisms are regulated to a large extent by the bound to free water equilibrium. Under stress conditions the organisms shift the equilibrium in one or the other direction in order to preserve the species.

Microorganisms multiply most efficiently in a medium containing about 95% water. They differ in the extent to which they can be dried and still remain alive. Many microorganisms can be quick-frozen, dried from the frozen state (lyophilized) and remain alive, but dormant for years. The survival of microorganisms, in the form of spores under dry conditions is an adaptation of this form of life to drought.

What qualities in water molecule make it ideal for life? Biological success of water stems up from its extraordinary cohesiveness compared to any other liquid. Given its molecular weight it should have been a gas. However, inter molecular interaction mediated via H-bonds are such that water behaves as if it were of higher molecular weight. Hence it is a liquid at room temperature. The cohesive nature of water molecules affects the interactions between molecules in aqueous solution. The other quality of water that makes it important for life is its polar nature. The molecule of water is bent and not linear so the distribution of charge is asymmetric. The oxygen atom draws electrons away from the hydrogen which leaves the latter with a partial positive charge. The water is, thus, a polar molecule. These two properties of water namely the cohesiveness due to H- bonding ability and polarity make it an excellent solvent for polar molecules. Life on earth depends on the capacity of water to dissolve a remarkable array of polar molecules that serve as building blocks, catalyst, fuels and information carriers. These molecules can co-exist in water and can also interact with each

other. It weakens the interactions between polar molecules which the biological systems circumvent by creating water free microenvironments. Water greatly weakens electrostatic forces and hydrogen bonding between polar molecules by competing for their attractions. For example folding of proteins becomes possible only when water is excluded. The hydrogen bonding between carbonyl group and the amide group can easily disrupt by the replacement of amide hydrogen by hydrogen atom of water as hydrogen bond donor whereas the oxygen of water can replace the carbonyl oxygen atom as the acceptor. Hence a strong hydrogen bond between CO and NH group can only form when water is excluded. This results in packing up the amino acids with hydrophobic R groups in the interior of the molecule and polar amino acids on the surface. This arrangement assures that these molecules remain in thermodynamically stable forms.

Many crystalline salts and ionic compounds dissolve in water but are insoluble in non polar liquids such as benzene or chloroform. This is possible because water has a very high dielectric constant (the tendency to oppose electrostatic attraction between positive and negative ions) of a magnitude of 81. In other words it reduces the strength of electrostatic attractions by a factor of 81 compared to that in vacuum. This high value of dielectric constant is because of two factors, one the polarity as explained earlier and two, the capacity to form oriented solvent shells around ions. These oriented solvent shells produce electric fields of their own which oppose the fields produced by ions. Consequently the presence of water weakens the electrostatic attractions between ions. To understand it better let us follow the reaction between water and crystalline sodium chloride. The crystal lattice of NaCl is held together by very strong electrostatic attractions between alternating positive (Na^+) and negative (Cl^-) charges. Large amount of energy is required to pull them apart. Water however, is able to dissolve these crystals because strong electrostatic attractions between water dipoles and the positively charged $Na^+$and negatively charged Cl^-ions lead to stable hydrated sodium and chloride ions. This property of water is very well exhibited in enzyme catalysis acts in the presence of counterions. Counterions play a very important role in catalysis in non aqueous solutions compared to that in water. In water it is the high dielectric which screens the electrostatic interactions allowing opposite charges to be far apart, the low dielectric of the organic media does not allow this to happen and counterions are bound to the proteins to neutralize its ionizable groups. It has been known that the curve for enzyme catalysis on hydration is bell shaped exhibiting maximum activity at 10 to 12 %. Increased hydration is associated with increased flexibility of the enzyme. Dry enzymes would be very rigid but become flexible upon binding of water making catalysis possible. The reduction of enzyme activity at higher levels of hydration is due to conformational changes at the active site. Proteins denature in organic solvents but this does not happen in dry conditions as they are kinetically trapped. Water plays a very important role in protein folding. It is responsible for the packing and three dimensional structure of proteins, which is indispensable for protein bioactivity. The aqueous solution (55%M concentration of water) dictates the hydrophobic force which is the driving force of protein folding and other biological processes (see hydrophobic substances). The conflict between the hydrophobic side chain R groups and the polar nature of water guides them to lie buried in the interior forming a tightly packed core that contains more than 80% of the non polar side chains of a typical protein. The hydrophobic interactions are driven by the unfavourable entropy decrease that is caused by forming a large surface area of non polar groups with water. Though water is a natural medium for protein folding it is not indispensable. Small organic solutes osmolytes are used in nature by a variety of organisms to increase protein stability upon osmotic or water stress, high hydrostatioc pressures and dehydration. In addition to protein folding water has a role to play in stabilization.of nucleic acid structure also. It screens the electrostatic repulsions between phosphate groups thus stabilizing the classical double helical structure of DNA.

Apart from playing an important role in structure, stability, dynamics and function of biological macromolecules water is an active component in metabolic reactions. For example the synthesis of polymers such as polypeptides, takes place when amino acids combine together with the loss of a water molecule. Similarly water is added in hydrolytic reactions when substances are broken down. When starch or cellulose is broken down by enzymes water is a reactant. Under physiological conditions, a hydrolytic cleavage reaction where the concentration of metabolic precursors is very low (10^{-3} to 10^{-6} M) is thermodynamically irreversible. This plays an important role in metabolic pathways as many biological processes are driven by the hydrolytic cleavage of adenosine triphosphate.

Hydrogen bond

Hydrogen bonds play a very important role in maintaining the structures of biomolecules.They form between charged as well as uncharged molecules. In a hydrogen bond, a hydrogen atom is shared by two other atoms, a hydrogen donor to which the hydrogen is tightly bound and a hydrogen acceptor. The latter has a partial negative charge. In biological systems the donor atoms are generally oxygen or nitrogen atom with covalently bound hydrogen. The acceptor is also, either nitrogen or an oxygen atom. The strongest hydrogen bonds are those in which the donor, the hydrogen and the acceptor are collinear. The kinds of hydrogen bonds formed with respective bond lengths are given in table 1.The average bond energy associated with a hydrogen bond is 5 kcal/mol. They are weaker than covalent bonds and stronger than Van der Waals bonds. Their bond length is also intermediate between the two. The various structural motifs found in proteins such as alpha-helix, the beta pleated sheets are stabilized by hydrogen bonds between the acceptor carbonyl oxygen and the donor amide nitrogen of amino acids. The double helical structure of DNA is stabilized by hydrogen bonds between the bases in the two strands in addition to other forces of attraction.

Table 1: Hydrogen bonds and bond lengths

Bond	Bond Length (nm)
O-H..O	0.27
O-H..N	0.28
N-H..O	0.30
N-H..N	0.31

Hydrophilic Substances

Hydrophilic substances also known as polar substances are those which dissolve in water, for example, sodium chloride, glucose, paper etc. They transiently bond with water through hydrogen bonding. These substances are not just soluble in water but also in other polar solvents such as alcohol. They are of immense importance in biological systems as they enable different compounds to come together in a medium and interact. Many reactions required for obtaining energy for growth and multiplication in cells occur in water, hence the need for hydrophilic substances.

Hydrophobic Substances

These substances are repelled by water and therefore in aqueous solutions they tend to cluster together. Hydrophobes are not electrically polarized, and are unable to form H-bonds with

water. Water molecules are in a state of high energy if they are unable to form maximal number of hydrogen bonds possible either with one another or with solutes. When water molecules are adjacent to a hydrocarbon moiety they are in a state of higher energy compared to the molecules that are farther away from the moiety. Thus water at hydrophobic surface is in a high energy state because it makes fewer and weaker hydrogen bonds than water in the bulk of the solution. This is responsible for the hydrophobic effect which makes them water repelling. The interactions which hold these substances together are therefore appropriately called as hydrophobic interactions which result due to mutual exclusion of polar environment. This makes possible the self assembly of lipid bilayers without which the cells could not function as they do. Compared to hydrogen bonds hydrophobic interactions have little directionality but owing to the large numbers in which they are generally present they tend to produce systems that are highly stable. They are, therefore, a major driving force in the folding of macromolecules, the binding of substrates to enzymes, and the formation of membranes that define the boundaries of cells and organelles.

The living systems are so fabricated that they partition an aqueous hydrophilic cellular milieu from the aqueous environment through the imposition of a hydrophobic barrier in the form of cell membrane. This design insulates the cell's interior from the outside environment and any link or connection that should establish for the sustenance of the cell must cross the hydrophobic boundary. The cell needs to take up a large number of substances both hydrophilic and hydrophobic for its growth and multiplication. It is simple to understand that the substances which are hydrophobic (also known as lipophilic) are able to cross the membrane with greater ease and a faster rate than hydrophilic substances. The latter class of substances is therefore dependent upon the carrier molecules which act as receptors and transport them into and out of the cell. Guided by this golden principle of nature,"like dissolves like" many biological chemicals are deliberately made hydrophobic to increase their rate of uptake by the cells,e.g; drugs, pesticides ,etc.

Amphiphilic Substances

Many biomolecules contain both strongly non-polar and polar groups. Such molecules are called amphiphilic substances as they posess both hydrophilic and hydrophobic properties.Examples are phospholipids, glycolipids,cholesterol. A major proportion of the cell membrane lipids are formed by such substances (Table 2).

Table 2: Amphiphilic lipids of membranes

Membrane lipid	**Hydrophilic unit**	**Hydrophobic unit**
Phosphoglycerides	Phosphorylated alcohol	Fatty acid chains
Glycolipids	Sugar residues	Fatty acid- and hydrocarbon chains
Sphingomyelins	Phosphoryl choline or phosphoryl ethanolamine	Fatty acid and hydrocarbon chains

A simple fatty acid such as palmitic acid has a single carboxyl group which is polar in nature and a long hydrocarbon tail which is non polar and intrinsically insoluble in water. In water it readily forms micelles, in which the negatively charged carboxylate groups are exposed and form H-bonds with water molecules and the non polar insoluble hydrocarbon tails are hidden within thus completely protected. Such micelles have a net negative charge on the surface and remain suspended because of mutual electrostatic repulsion. It must be emphasized that

there is no true bonding between the hydrocarbon tails in micelles. It is just an aggregation or clustering of hydrophobic portions of amphiphilic molecules out of contact with water that produces a system of high stability.

Acid -Base Reactions

An earlier classification of substances into acids and bases was based upon their characteristics such as conduction of electricity, reaction towards litmus paper, taste etc. Accordingly acids were defined as the substances which had sour taste, could conduct electricity, turn blue litmus paper red and bases were defined as the substance with bitter taste which could conduct electricity and turn red litmus paper blue. These are the operational definitions based upon certain tests. However, with time more appropriate conceptual definitions have emerged that go into the cause of the observed behaviour of the substances. The definitions put forth by Arrhenius, Bronsted-Lowry and Lewis are all conceptual definitions. The most useful one in Biochemistry is that introduced by J. N Bronsted and T. M. Lowry and must be thoroughly understood by students of biochemistry. According to Bronsted-Lowry Concept an acid is a proton donor and a base is a proton acceptor. An acid base reaction involves a conjugate acid base pair made up of a proton donor and the corresponding proton acceptor. The following substances for example are Bronsted acids.

$$HCl \longrightarrow H^+ + Cl^-$$

$$CH_3COOH \rightleftharpoons CH_3COO^- + H^+$$

$$NH_4^+ \longrightarrow NH_3 + H^+$$

And the generalized expression would be

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

In this generalized reaction HA is the Bronsted acid because it can furnish a proton. The anion A^- is the conjugate base because it can accept the proton to form the acid HA. In other words acetic acid is a proton donor hence a conjugate acid and the corresponding acetate anion is the proton acceptor the conjugate base. Together they constitute an acid base pair.

Since most of the reaction in a cell takes place in an aqueous solution it important to learn the dissociation or ionization of acids and bases in dilute solutions. An acid in dilute solutions transfers a proton to water which in its presence acts as a base i.e. proton acceptor to yield the acid H_3O^+. The reaction occurs as follows:

$$HA + H_2O \rightleftharpoons H_3O^+ + A^-$$

Each conjugate base has a characteristic affinity for a proton relative to the proton affinity of hydroxyl ion. Acids that have only a slight tendency to donate protons to water are weak acids and the acids with strong tendency to donate protons to water are strong acids. The tendency of an acid to dissociate is given by its dissociation constant at a given temperature, which is numerically represented as:

$$Ka = \frac{[H_3O^+][A^-]}{[HA][H_2O]}$$

Since the concentration of water in aqueous solution is itself a constant, 55.5 moles/liter, the equilibrium constant (dissociation constant) can be combined with concentration of water to obtain a new constant, Ka

$$Ka' = \frac{[H_3O^+][A^-]}{[HA]}$$

Because hydronium ion concentration is the same as hydrogen ion concentration,

$$Ka' = \frac{[H^+][A^-]}{[HA]}$$

where the brackets indicate the concentration in moles per litre.

It is convention in biochemistry to employ dissociation constants based on the analytically measured concentrations of reactants and products under a given set of experimental conditions, i.e. at a given total concentration and ionic strength, such a constant is called as apparent or concentration dissociation constant and is designated as **Ka'** to distinguish it from the true thermodynamic dissociation constant **K**, used by physical chemists which is corrected for deviation of the system for ideal behavior caused by such factors as dilution and ionic strength. In Bronsted-Lowry concept, acids and bases are treated alike, i.e. solely in terms of the tendency of the protons to dissociate from the donor species. Table 3 lists the apparent dissociation constants of some acids.

One of the most common types of weak bases encountered in biochemistry is the group of organic amines, e.g.; the amino groups of amino acids. Such compounds R-NH_2, do not contain hydroxyl groups and cannot dissociate as mentioned before. On the other hand such compounds can ionize in water to produce hydroxyl ions:

$$\underset{\text{(Conjugate base)}}{RNH_2} + \underset{\text{(Conjugate acid)}}{H_2O} \rightleftharpoons \underset{\text{(Conjugate acid)}}{RNH_3} + \underset{\text{(Conjugate base)}}{OH^-}$$

Water in this reaction serves as an acid to contribute a proton to the base RNH_2. Using Bronsted definition of a base as a substance that can accept a proton we can write the equation as:

$$\underset{\text{(Conjugate base)}}{A^-} + \underset{\text{(Conjugate acid)}}{H_2O} \rightleftharpoons \underset{\text{(Conjugate acid)}}{HA} + \underset{\text{(Conjugate base)}}{OH^-}$$

$$\text{Hence, } K_{ion} = \frac{[HA][OH^-]}{[H_2O][A^-]}$$

Or,

$$K_b = \frac{[HA]\ [OH^-]}{[A^-]}$$

The above equation can be used for calculating the hydroxide ion concentration of a solution of weak base. The chemical handbooks list the values of K_a of such substances (Table 3). An examination of the Table 3 clearly reveals, the Ka values are cumbersome to handle and therefore, pKa values are generally considered. Also note that strong acids have low pKa values and strong bases have high pKa values. Water itself may be considered a very weak acid of pKa of about 14; its conjugate base hydroxide ion is very strongly basic with a high affinity for a proton. The value of pKa of dissociable groups is an important qualitative characteristic of; biomolecules. We are aware that biological compounds such as organic acids, amino acids, proteins, purines, pyrimidines and phosphate esters, ionize to varying degrees in biological systems with characteristic pKa values. The determination of pKa value therefore is an important procedure in describing a substance. In the laboratory it can be determined by plotting a titration curve (Figure 1) which is done by adding known amount of alkali to an acid, the pH resulting after each increment of alkali is plotted against the equivalents of hydroxyl ions added. The pH intercept at the midpoint of the curve is numerically equal to the pKa of the acid titrated. It denotes equimolar concentrations of the proton donor (HA) and proton acceptor (A^-) species of the acid present. In fact the pKa of the acid can be calculated at any point on the titration curve if the concentrations of proton donor and proton acceptor species at that point are known.

The curve in Figure1shows the change in pH of acetic acid as a function of ml. of added NaOH when equimolar acetic acid is titrated with NaOH, the equivalence point is reached with equal volume of base. At the equivalence point a sharp increase in pH is observed as there is surplus base present in the solution. The flat portion of the titration curve before the equivalence point is called the buffer region. In this part of the pH scale, the acid and conjugate base are both present in significant concentrations and the solution resists changes in pH. As base is added to a solution in this buffer region, acetic acid reacts with it to form acetate ion, without a large change in pH. If additional acid were added to a solution in the buffer region, it would react with the conjugate base, acetate ion, and, again, the pH would not change appreciably. In the middle of the buffer region lies the half-equivalence point. Here the volume of base added is half that required to reach the equivalence point and half the acetic acid has been converted to the conjugate base, acetate ion. This means that the concentrations of acetic acid and acetate ion are equal.

Table 3: Dissociation constants and pKa values of some acids and bases useful in biological systems

Free acid or base	Ka (M)	pKa
Acetic acid	$1.74x10^{-5}$	4.76
Lactic acid	$1.38x10^{-4}$	3.86
Phosphoric acid	7.18×10^{-3}	2.12
Carbonic acid	1.70×10^{-4}	6.35
Succinic acid	$6.16x10^{-5}$	4.21
N-TRIS(hydroxymethyl)-2-aminoethanesulfonic acid"TES"	$3.6x10^{-8}$	8.04
Ethanolamine	$2.75x10^{-10}$	9.44
Ascorbic acid	$6.16x10^{-12}$	11.79
Histidine	$1.47x10^{-10}$	9.17

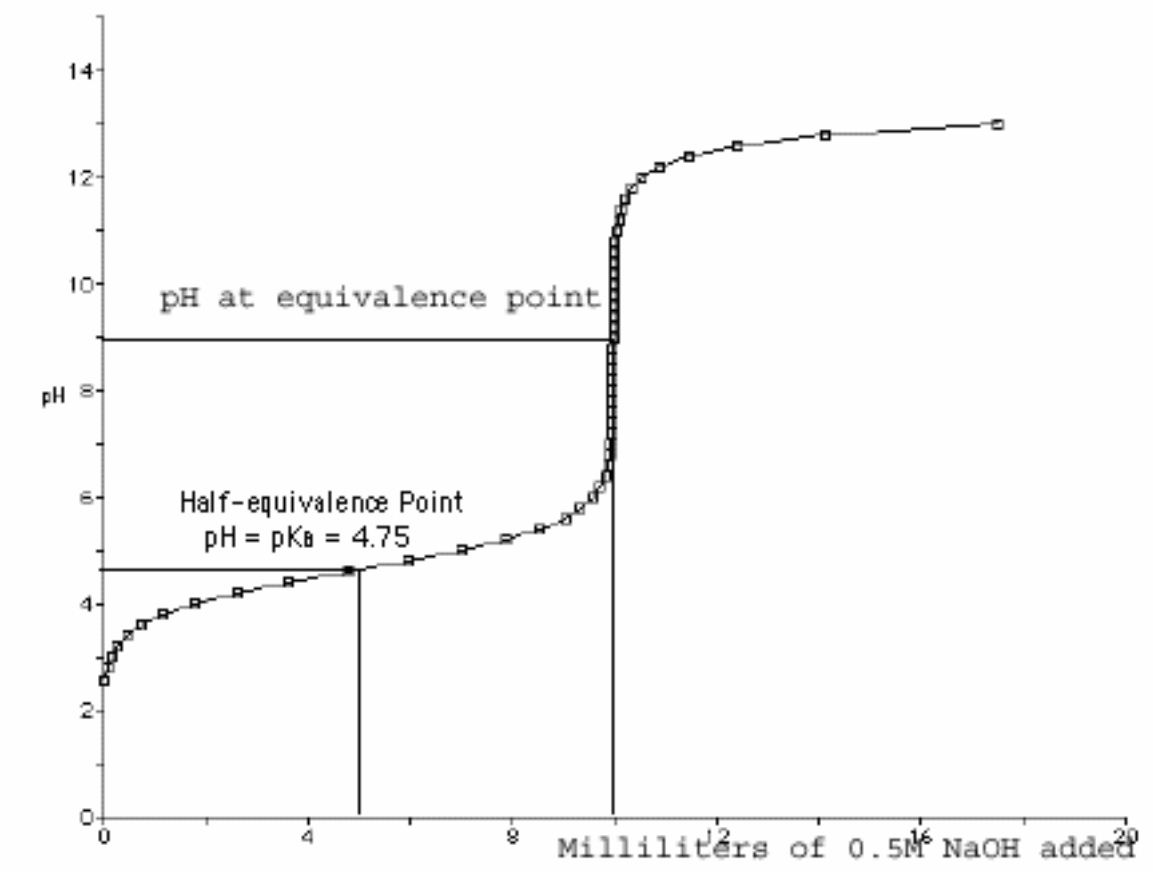

Figure1

If we examine the equilibrium expression at the half-equivalence point, $[CH_3COOH] = [CH_3COO]$ or $K_a = [H_3O^+]$. Taking log and multiplying both the sides by -1, pK_a=pH. So at the half-equivalence point, half the acetic acid has been converted to the conjugate base, acetate ion, and the pH will be equal to the pK_a of the acid.Since at its pKa any weak acid will be half ionized , this is one of the most useful ways of distinguishing between individual weak acids . The pKa is also a characteristic property of acids because the ionization constant, i.e; Ka is an inherent property of an acid. The curve defines an expression known as

Henderson-Hasselbalch equation. The equation as applicable to the ionization of weak acids is obtained by rearranging the law of mass action

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

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

Solving for H^+

$$[H^+] = Ka \times [HA] / [A^-]$$

Taking logarithms on both the sides

$$-\log[H^+] = -\log Ka - \log [HA] / [A^-]$$

Substituting pH for $-\log[H^+]$ and pK' for $-\log K'$

$$pH = pKa + [\log A^-] / [HA]$$

Or

$$pH = pKa + [\text{Proton acceptor}] / [\text{proton donor}]$$

Or

$$pH = pKa + \log [\text{Conjugate base}] / [\text{conjugate acid}]$$

Thus if the molar ratios of the proton donor and the proton acceptor species are known at a given pH the pKa can be calculated. Also, if the pKa is known with the molar ratios of the conjugate acid and the base, the pH can be calculated. A corollary derives from the fore account, when the concentrations of the conjugate acid and its base are equal, pH is numerically equal to pK'.

Applying Handerson Hasselbalch equation to a weak acid such as acetic acid ,we get

$$pH = pKa + \log [\text{Acetate}] / [\text{Acetic acid}]$$

However Handerson Hasselbalch equation is of no use in calculating the pH of strong acid like HCl as the equation applies to only weak electrolytes. Thus when the pH in a titration curve of HCl vs NaOH has to be calculated the milliequivalents of HCl remainig after the addition of alkali are to be calculated. For example when 100 ml of HCl are titrated with equimolar NaOH solution after addition of 30 ml of alkali pH can be calculated by determining the meq. Of HCl remaining in total volume of solution i.e; 7/130=0.054 M. The concentration of H^+, therefore in this solution would be 0.054 M and pH=-log (0.054) =1.27. Acetic acid, HCl are monobasic acids. However many of the common acids encountered in intermediary metabolism are polyprotic,e.g; succinate which ionizes as follows:

$$COOH\text{-}CH_2\_CH_2\text{-}COOH \rightleftharpoons COO\text{-—}CH_2\text{-}CH_2\text{-}COOH + H^+ \rightleftharpoons COO^-\text{—}CH_2\text{-}CH_2\text{-}COO^-$$

pKa=4.2 pKa=5.6

At pH 7.0 in the cell, succinic acid will predominantly exist as as the dianion and the titration curve will be biphasic.with two pKa values of 4.2 for anion and 5.6 for dianion.

Similarly the titration curve of a monocarboxylic monoamino acid is a biphasic curve owing to the presence of two ionizable groups,viz; an amino group and a carboxylic group. The presence of two types of charges make it a zwitterion. When such a substance is dissolved in water, the pH of the resultant solution is neutral and there is no migration of the aminoacid in the electric field. For a monocarboxylic amino acid, two pKa values corresponding to those of carboxylic groups of and simple amines are obtained. The carboxylic groups of organic acids dissociate in the pH range of 3 to 5 while protonated alkyl amines such as NH_4^+ are weak acids with pKa s in the range of 9 to 11.Those amino acids having more than one carboxyl or amino group will have corresponding pKa values for them. Thus, the pKa for the α carboxyl group of aspartic acid is 2.1 while that for β carboxyl group is 3.9 and the pKa for the amino group is 9.8. The pKa values for histidine are 1.8 due to α carboxylic group, 6.0 due to side chain R group and 9.2 due to α amino group. None of the monoamino monocarboxylicaminoacids have buffering capacity at physiological pH. They show buffering capacity in the zone of their pKa values, i.e; pH 1.3 to 3.3 and 8.6 to 10.6. The only amino acid with buffering capacity at pH 6 to 8 is histidine.That is why histidine acts as a physiological buffer.

The pH at which an amino acid carries no net charge is called the isoelectric point.,pI which is the arithmetic mean of pKa valuesi.e; 1/2 x (pKa_1+pKa_2). The predominant form is called the isoelectric species. The isoelectric pH of proteins influences their solubility. The proteins are least soluble at this pH. Under these conditions there are no electrostatic repulsions between the neighbouring protein molecules and they tend to coalesce and precipitate. However at pH values above or below the isoelectric point the protein molecules have a net charge of the same sign. They therefore repel each other and remain in solution. Since different proteins have different aminoacid composition with R groups that ionize at different pH they can often be separated from each other by isoelectric precipitation. When the pH of a protein mixture is adjusted to the isoelectric pH of one of its components much or all of that component will precipitate, leaving behind in solution protein with isoelectric pH values above or below that pH. The precipitated protein remains in its native conformation and can be dissolved in a medium having an appropriate pH and salt concentration. Isoelectric focussing another technique that makes use of isoelectric point of proteins for their separation by electrophoresis. Proteins are subjected to electrophoresis on a pH gradient . each protein moves until it reaches a pH equal to its individual isoelectric point. At that moment migration in the electric field stops because the net charge of the protein is zero.

pH Theory of buffer solutions

A careful examination of the titration curve in Figure 1 reveals it has a flat zone extending about 1 pH unit on either side of its midpoint. In this zone the pH of the system changes relatively little when small increments of hydrogen ions or hydroxyl ions are added. This is the zone in which conjugate acid base pair acts as a buffer. Buffer is defined as a substance which tends to resist changes in the pH when increments of hydrogen ions or hydroxide ions is added. At pH values outside this range there is little capacity to resist change in pH. The buffering action is maximum at the pH of the exact mid point of titration curve. This is the point at which the proton donor species equals the proton acceptor species in concentration and pH=pKa. Buffering power reduces as the pH is raised or lowered from this point. This is due to a change in the ratio of the conjugate base to conjugate acid. Each conjugate acid base pair has a characteristic pH at which its buffering action is maximum, the point at which pH=pKa.

The buffer solutions contain a mixture of weak Bronsted acid and its conjugate base;for example; a solution of acetic acid and sodium acetate. Intracellular and extracellular fluids of living organisms contain acid base pairs which act as buffers. The acid base pair $H_2PO_4^-$ - HPO_4 with a pK' = 7.2 is an intracellular buffer, ATP, the cell's energy currency otherwise, glucose –6-phosphate, a metabolic intermediate all contribute to the buffering power of the cell. The blood and the interstitial fluid of the vertebrates have bicarbonate buffer system to maintain the pH. The buffering power of the blood plasma is such that addition of 1 ml. of 10 M HCl to 1 L blood plasma lowers its pH from 7.4 to 7.2 whereas the same amount of acid when added to 1L of neutral physiological saline which is 0.15 M NaCl ,the pH declines to 2.0. Common buffers are mixtures of a conjugate acid and a conjugate base. Depending upon the range in which a buffer resists changes in pH upon addition of acid or base it is accordingly called acidic or basic buffer. An acidic buffer contains a weak acid and a salt of the weak acid (conjugate base). An alkaline buffer is a weak base and a salt of the weak base (conjugate acid). Together the two species resist large changes in pH by absorbing additions of hydrogen- or hydroxyl ions. When hydrogen ions are added to the buffered solution, they react partially with the conjugate acid present to form water and the conjugate base. Thus, some hydroxyl ions are taken out of the circulation. Buffered solutions do change in pH upon addition of hydrogen or hydroxyl ions. However the change is much less than that which would occur if no buffer was present. The amount of change depends upon the strength of the buffer defined as the amount of hydrogen ions or hydroxyl ions in moles per liter required to cause a given change in pH (for example 1 unit).

To understand the working of a buffer let us take the example of an acetate buffer. This buffer has two components, unionized acetic acid as the conjugate acid and the acetate ion as the conjugate base. When an alkali, for instance NaOH is added to a mixture of acetic acid and acetate the reactions occur as under:

$$CH_3COOH \rightleftharpoons CH_3COO^- + H^+$$

$$NaOH \rightarrow Na^+ + OH^-$$

$$H^+ + OH^- \rightleftharpoons H_2O$$

On further addition of alkali there is further dissociation of the available acetic acid to furnish additional protons and thus to keep the pH unchanged. When acid is added to the acetate buffer, the hydrogen ions combine with the acetate ions to make more acetic acid. The following reaction occurs:

$$H^+ + CH_3COO^- \rightleftharpoons CH_3COOH$$

The protons added combine instantly with the acetate anion present in the buffer solution to form the undissociated weak acid. Consequently the resultant pH change would be much less than would occur if the conjugate base were absent.

The alkaline buffer such as a mixture of ammonia and ammonium chloride works somewhat differently. Ammonia being a weak base is capable of accepting a proton from water which in its presence acts as an acid .The reaction occurs as follows:

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^-$$

When an acid is added to this buffer the protons are absorbed by ammonia to form ammonium ions

$$NH_3 + H^+ \rightleftharpoons NH_4^+$$

and hydroxide ions which form from the reaction between ammonia and water as shown earlier and form water. When an alkali is added to this buffer the hydroxide ions are removed by ammonium ions to form ammonia by the following reaction

$$NH_4^+ + OH^- \rightleftharpoons NH_3 + H_2O$$

The effectiveness of a buffer is determined by its buffering capacity. It is directly proportional to the molar concentration of the buffer components. The higher the molar concentration the greater its buffering action. By convention the concentration of a buffer is the sum of the concentrations of the weak acid and its conjugate base. Thus a 0.2 M acetate buffer could contain 0.1 moles of acetic acid and 0.1 mole of sodium acetate, in 1 liter of water. It could also contain 0.12 moles acetic acid and 0.08 moles of sodium acetate in 1 liter of water. Another factor which is important in influencing the effectiveness of a buffer is the ratio of the concentrations of the conjugate acid to conjugate base. It is again emphasized that a buffer has its maximum effectiveness near its pKa value. The selection of a buffer for a biochemical exercise would thus require that the buffer should have its weak acid with pKa value same as the pH requirement of the work.

The Henderson-Hasselbalch relationship indicates that the pH of a buffer solution does not depend on the total concentration of the buffering acid and conjugate base but only on the pK_a and the ratio of the concentration of these two species. On the other hand, the buffering capacity of a solution quantifies the amount of H_3O^+ or OH^- the solution is capable of neutralizing before the acid or conjugate base form is saturated and the pH begins to fall or rise precipitously (Figure 2).This will depend on the total concentration of the acid and conjugate base buffer ions. For example, a solution 0.2 M in total acetate, distributed between acetic acid, CH_3COOH, and acetate ion, CH_3COO^-, will be able to neutralize more H_3O^+ or OH^- than a solution at the same pH that is 0.1 M in total acetate. Also, the buffering capacity may be different towards addition of acid than towards base.This will be true unless the pH of the buffer solution is identical to the pK_a of the buffering acid-base equilibrium. The following statements define the buffering capacity:

The acidic buffering capacity of a solution is the number of moles of H_3O^+ per liter of buffer required to lower the pH by 1 unit. The basic buffering capacity of a solution is the number of moles of OH^- per liter of buffer which will raise the pH by one unit.

Since pH influences all the activity in the cell its maintenance in physiological and biochemical exercises becomes inevitable. This is achieved by the use of buffer solutions. Thus, preparation of a buffer of desired pH is an important aspect of biochemical exercises. A simple way to make a buffer is to mix equimolar amounts of acid and its salt .If the buffer is an acetate buffer the resultant pH would be 4.73 (Table 3 and Henderson- Hasselbalch equation). When the ratio of the proton donor and proton acceptor species is the same the pH is equal to the pKa value. An increase in the concentration of acid (denominator) would lower the pH while that of conjugate base (numerator) would increase the pH of the buffer solution. Thus, fixing of the ratios is important.

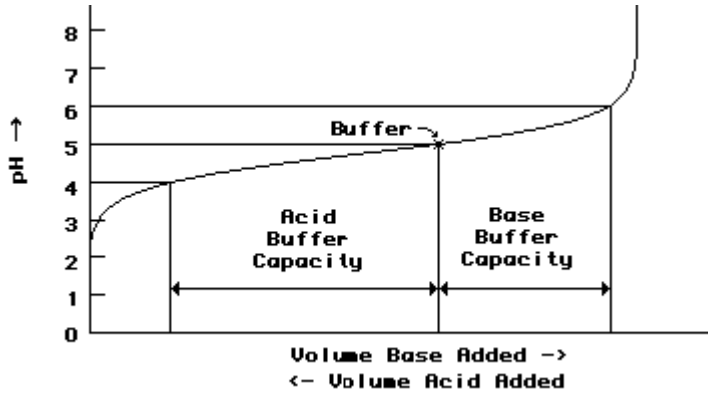

Figure 2: The acid and basic buffering capacities are the respective amounts of acid or base added to change the pH by one unit

A sample problem. How would you make an acetate buffer of pH 5.21and concentration of 0.25 M? Using Henderson –Hasselbalch equation,

$$pH = pKa + \log [A^-] / [HA]$$

substituting the known values

$$5.21 = 4.73 + \log [A^-] / [HA]$$

$$0.48 = \log [A^-] / [HA]$$

$$\text{Antilog } 0.48 = \log [A^{-1}] / [HA]$$

$$3 = \text{Acetate/Acetic acid}$$

Thus the ratio of concentration of the conjugate base to that of acid should be 3.In other words at pH 5.21, for every mole of acetic acid there would be three moles of acetate. If the buffer strength is 0.25 moles, for a litre of buffer one would require 0.25x0.75 = 0.18 moles of acetate and 0.25x0.25 = 0.0625 moles of acetic acid. It, therefore, follows that for the preparation of said buffer 14.76 grams of sodium acetate and 3.7 grams of acetic acid in 1L of solution would be required.

Although according to Henderson-Hasselbalch equation, the pH of a buffer depends only on ratio of conjugate base to conjugate acid yet the pH changes upon dilution. This is due to the fact that the degree of dissociation of weak acid increases as the solution is diluted. Also, as the buffer is diluted extensively, its contribution towards the H^+ and OH^- ion concentration of solution approaches that of water and the pH approaches 7.

Biological buffers

In all living organisms the fluids in the cells have a characteristic and nearly constant pH. Also, the fluids surrounding the cells have a characteristic and nearly constant pH. This is possible because of the presence of buffers in the systems. Many biological compounds capable of ionization are important biological buffers, e.g. TCA cycle intermediates such as citric acid, malic acid, and isocitric acid. They accumulate in the vacuole and play a major role in regulating the pH of the plant cell. Yeasts accumulate large amounts of phosphate esters which regulate the pH of the cell. In animals complex buffer systems are found in the

blood. Such buffers are appropriately called physiological buffers because the pH-level affects functioning of enzymes within the body, and the level is optimally kept around 7.4 in order to preserve efficient enzymatic function. "Physiological" generally just refers to the function of an organism, whether it be physical, biochemical, or otherwise. One such buffer comprises of carbonic acid –bicarbonate anion. Although the ratio of conjugate base to weak acid is 20:1 it is still a very efficient system since the weak acid is in rapid equilibrium with dissolved carbon dioxide in the blood plasma.

$$H_2CO_3(aq) \rightleftharpoons CO_2(aq) + H_2O(l)$$

Carbonic anhydrase enzyme which catalyzes this equilibrium is present in red blood cells.

$$CO_2(aq) \rightleftharpoons CO_2(g)$$

Additional H^+ is consumed by HCO_3^- and additional OH^- is consumed by H_2CO_3. The much higher concentration of hydrogen carbonate ion over that of carbonic acid in blood plasma allows the buffer to respond effectively to the most common materials that are released into the blood. Normal metabolism releases mainly acidic materials such as lactic acid .The acids react with hydrogen carbonate ion and form carbonic acid.

$$\text{Lactate} + HCO_3^-(aq) \rightleftharpoons \text{Lactic acid} + H_2CO_3(aq)$$

The carbonic acid is converted through the action of the enzyme carbonic anhydrase into aqueous carbon dioxide.

$$H_2CO_3(aq) \rightleftharpoons CO_2(aq) + H_2O(l)$$

An increase in $CO_2(aq)$ concentration stimulates increased breathing, and the excess carbon dioxide is released into the air in the lungs.The dissolved carbon dioxide is in turn in equilibrium with carbon dioxide in the atmosphere and depending upon the partial pressure of carbon dioxide in the gas phase it will either escape in the air or enter the blood. Thus, this buffer system works by maintaining the ratio at 20: 1 and increasing or decreasing the total amount of buffer components.

Another buffer found in the blood is present in the form of oxygenated hemoglobin and unoxygenated hemoglobin. The pKa value for oxygenated hemoglobin is 6.2 and that for unoxygenated species it is 7.7. Thus, in the lungs where the oxygenated form predominates the blood tends to become acidic whereas in the peripheral tissue where the partial pressure of oxygen is relatively low the unoxygenated form dominates and the pH tends to increase. These two effects are compensated by the low concentrations of the carbon dioxide in the lungs relative to that in the peripheral tissues, acoounting for the buffering action of the blood.

The internal fluid of cells have dihydrogen phosphate- hydrogen phosphate buffer. The former is the conjugate acid and the latter the conjugate base. The two are in equilibrium as per the following equation

$$H_2PO_4^-(aq) \rightleftharpoons H^+(aq) + HPO_4^{2-}(aq)$$

When, additional hydrogen ions enter the cellular fluid, they are consumed in the reaction with HPO_4^{2-}, and the equilibrium shifts to the left. If additional hydroxide ions enter the

cellular fluid, they react with $H_2PO_4^-$, producing HPO_4^{2-}, and shifting the equilibrium to the right. The equilibrium-constant expression for this equilibrium is as follows:

$$K_a = \frac{[H^+]\,[HPO_4^{2-}]}{[H_2PO_4^-]}$$

The value of K_a for this equilibrium is 6.23×10^{-8} at 25°C. From this equation, the relationship between the hydrogen-ion concentration and the concentrations of the acid and base can be derived.

$$[H^+] = K_a \frac{[H_2PO_4^-]}{[HPO_4^{2-}]}$$

Thus, when the concentrations of $H_2PO_4^-$ and HPO_4^{2-} are the same, the value of the molar concentration of hydrogen ions is equal to the value of the equilibrium constant, and the pH is equal to the pK_a (-log K_a), namely 7.21. Buffer solutions are most effective at maintaining a pH near the value of the pK_a. In mammals, cellular fluid has a pH in the range 6.9 to 7.4, and the phosphate buffer is effective in maintaining this pH range.

Buffers are routinely employed in biochemical and biological research to maintain the pH during the course of a reaction that produces or utilizes hydrogen ions. The criteria for the selection of the buffer revolve around the requirements of the experiment. For example most biological reactions take place at near-neutral pH between 6 and 8, so buffers with pK_a values in this regime would provide maximum buffering capacity. Also we have noticed that the ability to maintain a near constant pH increases as the concentration of the buffer increases. However, it is not always possible to use a relatively concentrated buffer. The tissue or cells under investigation may be sensitive to high ionic strength. There are certain circumstances where we want the pH to change appreciably, for example when the extent of a reaction is measured by the pH change. In this case we would use the lowest concentration of buffer possible without allowing the pH to move out of a range optional for the reaction under study. In addition the solubility in hydrophilic substances and high stability that is resistance to enzymatic and nonenzymatic degradation also needs to be considered. They should not absorb light when used in spectrophotometric assays. Table 4 lists some commonly employed buffers in biological research.

Table 4: Some common laboratory buffers

Compound	pKa
H_3PO_4/NaH_2PO_4	2.12
2-(-N-morpholino)-ethanesulfonic acid MES	6.15
$H_2CO_3/NaHCO_3$	6.37
N-(-2-acetamido-)iminodiacetic acid ADA	6.6
NaH_2PO_4/Na_2HPO_4	7.21
N-2hydroxy ethylpiperazine-N'-2ethanesulphonic acid HEPES	7.6
Tris-(hydroxymethyl)aminoethane (Tris)	8.0
$NaHCO_3^n/Na_2CO_3$	10.25
Piperazine-N,N'-bis- 2-ethanesulfonic acid PIPES	6.8

Suggested Readings

1. Biochemistry by J.M.Berg, J.L.Tymoczko, L.Stryer.2006. 6th ed.W.H.Freeman
2. W.H.FreemanStudents companion for Stryer's Biochemistry by L. Stryer & R.I. Gumport.1996. 4th ed. Freeman & Co.
3. Lehninger's Principles of Biochemistry by D.L. Nelson & M.M. Cox 2004 4th ed. Freeman & company
4. Biochemical Calculations by I.H. Segel. 1976. 2nd ed. John Wiley& Sons.
5. Biochemistry by D. Voet. .J. Voet, 1995. 2nd ed. John Wiley & Sons