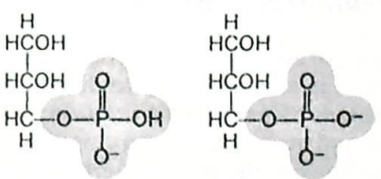
molecule of acid. Each step of the dissociation has associated with it a K_{ion} or K_a. In the case of phosphoric acid (H_3PO_4), three protons may be furnished on complete ionization of a mole of this acid.

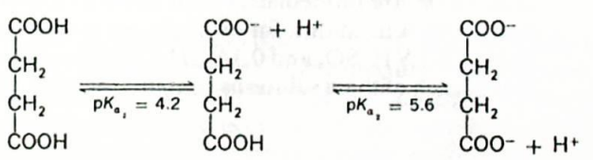
$H_3PO_4 \rightleftharpoons H^+ + H_2PO_4^-$	$K_{a1} = 7.5 \times 10^{-3}$	$pK_{a1} = 2.12$
$H_2PO_4^- \rightleftharpoons H^+ + HPO_4^{2-}$	$K_{a2} = 6.2 \times 10^{-8}$	$pK_{a2} = 7.21$
$HPO_4^{2-} \rightleftharpoons H^+ + PO_4^{3-}$	$K_{a3} = 4.8 \times 10^{-13}$	$pK_{a3} = 12.32$

This means that at the pH of 2.12, the first ionization of H_3PO_4 is half-complete; the pH must be 12.32, however, before the third and final ionization of H_3PO_4 is 50% complete. At the pH of 7.0, which is frequently encountered in the cell, the second proton of phosphoric acid ($pK_{a2} = 7.21$) will be about half-dissociated. At this pH, the monoanion $H_2PO_4^-$ and the dianion HPO_4^- of phosphoric acid will be present in approximately equal concentrations. A phosphoric acid ester, such as α-glycerol phosphate, has only two ionizations. These correspond, approximately, to the first two pK_a's of phosphoric acid, so that at pH 7, the most abundant species, indicated below, are present in approximately equal concentrations. The phosphate ester thus



appears to be "missing" the "last" pK_a. This is due in large measure to electrostatics. It is more difficult to remove a positively charged proton from a phosphate monoanion or phosphate ester monoanion than from unionized phosphoric acid or phosphoric acid ester. Thus, for both compounds, the first ionization corresponds to that of a similarly strong acid and the second to that of a similarly weak acid, since the same ionic charges are involved for phosphoric acid and its monoester.

Many of the common organic acids encountered in intermediary metabolism are polyprotic; for example, succinic acid ionizes according to the following scheme:



At pH 7.0 in the cell, succinic acid will exist predominantly as the dianion $^-OOC—CH_2—CH_2—COO^-$, commonly referred to as succinate. Most of the organic monoacids that serve as metabolites in the cell, for example, palmitic acid, lactic acid, and pyruvic acid, are acids of strength equal to or greater than that of acetic acid. Thus, like succinate, they will be present as their anions. This has led to the common use of the names of the anions (palmitate, lactate, and pyruvate) when these compounds are discussed in biochemistry. In writing chemical reactions, however, it will be the practice in this text to use the formulas for the undissociated acid. Table 1.2 lists the pK_a's for several of the organic acids commonly encountered in intermediary metabolism.

TABLE 1.2 The pK_a of Some Organic Acids

	pK_{a1}	pK_{a2}	pK_{a3}
Acetic acid (CH_3COOH)	4.74		
Acetoacetic acid (CH_3COCH_2COOH)	3.58		
Citric acid ($HOOCCH_2C(OH)(COOH)CH_2COOH$)	3.09	4.75	5.41
Formic acid ($HCOOH$)	3.62		
Fumaric acid ($HOOCCH{=}CHCOOH$)	3.03	4.54	
DL-Glyceric acid ($CH_2OHCHOHCOOH$)	3.55		
DL-Lactic acid ($CH_3CHOHCOOH$)	3.86		
DL-Malic acid ($HOOCCH_2CHOHCOOH$)	3.40	5.26	
Pyruvic acid ($CH_3COCOOH$)	2.50		
Succinic acid ($HOOCCH_2CH_2COOH$)	4.18	5.56	

1.13 DETERMINING pK_a VALUES

The pK_a of any dissociable groups is a characteristic property of a molecule and one that is relatively easy to determine. The pK_a may be determined in the laboratory by preparing a titration curve experimentally, using a pH meter, a buret, and titrant. As known amounts of alkali or acid are added to a solution of the unknown, the pH is determined, and the titration curve can be plotted. From this curve, the inflection point (pK_a) may be determined. This is possible for molecules with single titratable groups, such as acetic acid, and for molecules such as phosphoric acid that undergo multiple ionizations. However, when the pK_a's are closely spaced, for example, for citric acid (Table 1.2), the pK_a values do not correspond exactly to inflection points, and a more complex mathematical analysis is required to determine them.

At this point, we urge the student to review chemical stoichiometry. The meanings of gram molecular weight (mole) and gram equivalent weight (equivalent) and the significance of molarity, molality, and normality must be thoroughly understood. Biochemistry is a quantitative science, and the student must recognize immediately such terms as millimole and micromole. In connection with titrations, for example, we must be aware that the H^+ concentrations of $0.1N$ H_2SO_4 and $0.1N$ CH_3COOH are by no means similar, although 1 liter of each of these solutions contains the same amount of total titratable acid.

1.14 BUFFERS

Control of pH is an essential property of biological systems. The pH of human blood plasma is maintained within 0.2 pH unit of 7.2 to 7.3; values outside this range are not compatible with life. The enzymes (Chapter 4) that are responsible for the catalysis of reactions both within and outside of cells frequently exhibit their maximum catalytic action at some definite pH, and some have significant catalytic activity only within a narrow pH range. Metabolic reactions constantly produce Brönsted acids and bases, often not in balanced amounts. Any imbalance that tends to shift the pH outside the cell's pH range

for optimum activity must be compensated. The control of pH in natural biochemical systems is accomplished both by buffers, the subject of this section, and by energy-requiring, active mechanisms.

Biological systems contain dissolved proteins, organic substrates, and inorganic salts, many of which can act as buffers. In the laboratory, the biochemist wishes to examine reactions *in vitro* under conditions where the change in pH is minimal. He or she most often obtains these conditions by using efficient buffers, preferably inert ones, in the reactions under investigation. The buffers may include weak acids such as phosphoric and acetic, or maleic acids or weak bases such as ammonia, pyridine, and tris-(hydroxymethyl)amino methane.

With a thorough understanding of the ionization of weak electrolytes, it is possible to discuss buffered solutions. A *buffered solution is one that resists a change in pH on the addition of acid or alkali.* Most commonly, the buffer solution consists of a mixture of a weak Brönsted acid and its conjugate base; for example, mixtures of acetic acid and sodium acetate or of ammonium hydroxide and ammonium chloride are buffer solutions.

Let us consider the mechanism by which a buffer solution exerts control over large pH changes. When alkali (e.g., NaOH) is added to a mixture of acetic acid (CH_3COOH) and potassium acetate (CH_3COOK), the following reaction occurs:

$$OH^- + CH_3COOH \longrightarrow CH_3COO^- + H_2O$$

This reaction states that OH^- ion reacted with protons furnished by the dissociation of the weak acid and formed H_2O.

$$CH_3COOH \rightleftharpoons CH_3COO^- + H^+ \xrightarrow{OH^-} H_2O$$

On the addition of alkali, there is a further dissociation of the available CH_3COOH to furnish additional protons and thus to keep the H^+ concentration or pH unchanged.

When acid is added to an acetate buffer, the following reaction occurs:

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

The protons added (in the form of HCl, e.g.) combine exceedingly rapidly with the CH_3COO^- anion present in the buffer mixture (as potassium acetate) to form the undissociated weak acid CH_3COOH. Consequently the resulting pH change is much less than would occur if the conjugate base were absent.

In discussing the quantitative aspects of buffer action, we should point out that two factors determine the effectiveness or **capacity** of a buffer solution. Clearly, the molar concentration of the buffer components is one of them. The buffer capacity is directly proportional to the concentration of the buffer components. Here, we encounter the convention used in referring to the concentration of buffers. The concentration of a buffer may be defined as the sum of the concentration of the weak acid and its conjugate base. Thus, a 0.1*M* acetate buffer could contain 0.05 mole of acetic acid and a 0.05 mole of sodium acetate in 1 liter of H_2O. It could also contain 0.065 mole of acetic acid and 0.035 mole of sodium acetate in 1 liter of H_2O.

The second factor influencing the effectiveness of a buffer solution is the

ratio of the concentration of the conjugate base to the concentration of the weak acid. Quantitatively, it should seem evident that the most effective buffer would be one with **equal concentrations** of basic and acidic components, since such a mixture could furnish **equal quantities** of basic or acid components to react, respectively, with acid or alkali. An inspection of the titration curve for acetic acid (Figure 1.1) similarly shows that the minimum change in pH resulting from the addition of a unit of alkali (or acid) occurs at the pK_a for acetic acid. That this is so can be proved mathematically. At this pH, we have already seen that the ratio of CH_3COO^- to CH_3COOH is 1. On the other hand, at values of pH far removed from the pK_a (and therefore, at ratios of conjugate base to acid greatly differing from unity), the change in pH for each added unit of acid or alkali is much larger, that is, the buffer capacity is much less.

Having stated the two factors that influence the buffer capacity, we may consider the decisions involved in selecting a buffer to be effective at the desired pH value, for example, pH = 5. Clearly, it would be most desirable to select a weak acid having a pK_a of 5.0. If this cannot be done, the weak acid whose pK_a is closest to 5.0 is the first choice. In addition, it is evident that we would want to use as high a concentration as is compatible with other features of the system. Too high a concentration of salt frequently inhibits the activity of enzymes or

TABLE 1.3 Buffers

COMPOUND	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}
N-(2-acetamido-)iminodiacetic acid (ADA)	6.6			
Acetic acid	4.7			
Ammonium chloride	9.3			
Carbonic acid	6.1	10.3		
Citric acid	3.1	4.7	5.4	
Diethanolamine	8.9			
Ethanolamine	9.5			
Fumaric acid	3.0	4.5		
Glycine	2.3	9.6		
Glycylglycine	3.1	8.1		
Histidine	1.8	6.0	9.2	
N-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)	7.6			
Maleic acid	2.0	6.3		
2-(*N*-morpholino)-ethanesulfonic acid (MES)	6.2			
Phosphoric acid	2.1	7.2	12.3	
Pyrophosphoric acid	0.9	2.0	6.7	9.4
Triethanolamine	7.8			
Tris-(hydroxymethyl)aminomethane (Tris)	8.0			
N-Tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid (TES)	7.5			
Sodium diethylbarbiturate	8.0			
Ethylenediaminetetraacetic acid	2.0	2.7	6.2	10.3