

Expt. 1: Biological Buffers

Goals:

1. Learn how to use the Henderson-Hasselbach (H-H) eqn.
2. Learn how to prepare buffers.
3. Learn something about physical properties of biological buffers which are salt solutions:
 - Temperature effects on pH.
 - Conductivity and Ionic Strength.

From Gen. Chem.:

1. *WHAT is an ACID?*
2. *WHAT is a BASE?*

Now:

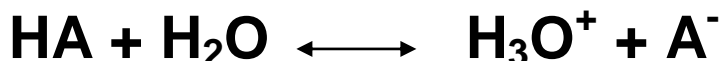
1. *What is a biological buffer?*
2. *What is its function?*
3. *How does it work?*

STRONG vs. WEAK acids

Strong acid dissociation (~100%):



Weak acid (i.e., dissociation $\ll 100\%$), HA, in H_2O :



This is a proton DISSOCIATIVE equilibrium, so:

$$K_{\text{eq}} = \frac{\text{products}}{\text{reactants}} = \frac{[\text{H}_3\text{O}^+][\text{A}^-]}{[\text{HA}][\text{H}_2\text{O}]}$$

Since $[\text{H}_2\text{O}] = 55 \text{ M}$, it can be considered a constant,

$$K_{\text{a}} = K_{\text{eq}}[\text{H}_2\text{O}] = \frac{[\text{H}_3\text{O}^+][\text{A}^-]}{[\text{HA}]} = \frac{[\text{H}^+][\text{A}^-]}{[\text{HA}]} \quad (\text{simplified})$$

By definition: K_{a} is the DISSOCIATION CONSTANT for a WEAK ACID in H_2O .

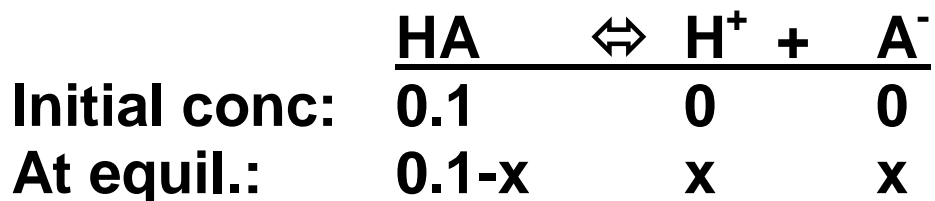
Now, the “p” term:

The “p” of any THING = $-\log(\text{THING})$

So: $\text{p}K_{\text{a}} = -\log K_{\text{a}}$

What does it mean to be a Weak Acid or how much dissociation occurs?

Suppose you prepare a 0.1 M weak acid solution:



$$K_a = \frac{[H^+][A^-]}{[HA]} = \frac{(x)(x)}{0.1 - x}$$

At this point you can solve for x (= [H⁺] or [A⁻]) using the quadratic equation or assume x << 0.1, then simplify eqn:

$$K_a = \frac{x^2}{0.1}$$

So: $x = \sqrt{\{(0.1)(K_a)\}}$

Now, if $K_a \ll 1$, then $\sqrt{\{(0.1)(K_a)\}}$ is going to be very small AND “x” is a measure of dissociation!

Total [Weak Acid] = 0.1 M

<u>K_a (M)</u>	<u>pK_a</u>	<u>[HA] (M)</u>	<u>[H⁺]</u>	<u>pH</u>
10^{-3}	3	0.09	0.01	2.0
10^{-4}	4	0.097	0.003	2.5
10^{-5}	5	0.099	0.001	3.0
10^{-6}	6	0.0997	0.0003	3.5
10^{-7}	7	0.0999	0.0001	4.0
10^{-8}	8	0.09997	0.00003	4.5

In all cases $[H^+] \ll [Weak\ Acid]_{total}$:

- As K_a decreases, $[H^+]$ decreases and $[HA]$ increases \rightarrow less H^+ dissociation
- As pK_a increases, pH increases

Stronger Weak Acids vs. Weaker Weak Acids

If Weak Acid "A" has $pK_a = 3.5 \times 10^{-3}$ M

And "B" has a $pK_a = 5 \times 10^{-8}$ M:

- Which dissociates to a greater extent?
- Which is the "stronger" weak acid?
- Which is more likely to be protonated at pH 7?



Moving on to the H – H equation:

$$K_{\text{eq}}[\text{H}_2\text{O}] = K_a = \frac{[\text{H}^+][\text{A}^-]}{[\text{HA}]}$$

$$\frac{1}{[\text{H}^+]} = \frac{1}{K_a} \frac{[\text{A}^-]}{[\text{HA}]}$$

$$\log \frac{1}{[\text{H}^+]} = \log \frac{1}{K_a} + \log \frac{[\text{A}^-]}{[\text{HA}]}$$

$$-\log ([\text{H}^+]) = -\log (K_a) + \log ([\text{A}^-]/[\text{HA}])$$

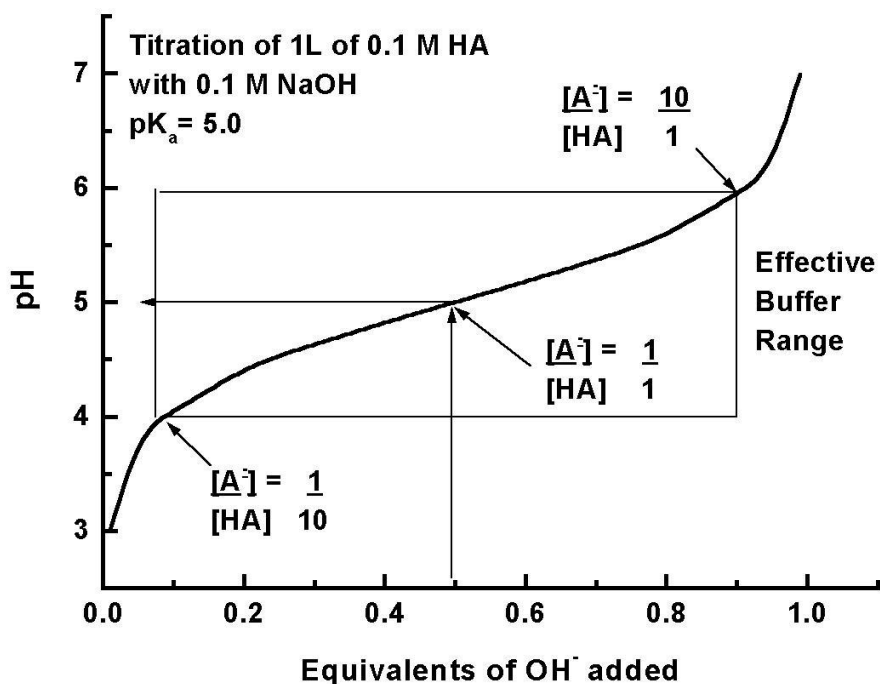
$$\text{pH} = \text{p}K_a + \log ([\text{A}^-]/[\text{HA}])$$

The H- H equation relates:

1. pH
2. $\text{p}K_a$
3. $[\text{A}^-]/[\text{HA}]$ (i.e. the concentrations of the two weak acid species present at any given time)

In other words: If you know 2 things, you can determine the 3rd.

A Titration Curve for a Generic Weak Acid



What is an EQUIVALENT?

Note the **RATIOS** of $[A^-]/[HA]$!! Why not just give a Decimal (what you get from calculator) value?
--A **RATIO** implies **TWO** species, so conceptually there should be **TWO** numbers. When $pH = pK_a - 1$, the $[A^-]/[HA] = 1/10$. There is 1 part A^- and 10 parts HA – not too hard to figure out which is in excess!
--A **DECIMAL** is **ONE** number, representing **TWO** species, conceptually not very gratifying, even though we all should realize that $0.1 = 1/10$.
--In this class, **RATIO = FRACTION**

What is PHYSICALLY occurring during this titration?

- 1. In aqueous soln, equilibrium produces:
 $HA \rightleftharpoons H^+ + A^-$**
- 2. As OH^- is added, it is neutralized by H^+ :
 $OH^- + H^+ \rightarrow H_2O$**
- 3. More HA ionizes to maintain equilibrium, producing more H^+ , which can react with more OH^- , until all of the HA is used. At that point, the pH increase is merely a function of OH^- added.**

Does all this Gen. Chem. stuff have some relevance to biochemistry?

In proteins, amino acid side chains are weak acids:

<u>Acidic (WA) Form</u>	<u>Basic (CB) Form</u>
RCOOH	RCOO ⁻
RNH ₃ ⁺	RNH ₂
RSH	RS ⁻
ROH	RO ⁻

The behavior of many proteins and enzymes show distinct pH dependencies, i.e. their weak acid nature controls the behavior!

Buffer Range vs. Buffer Capacity

Buffer range: the range of pH over which there is enough WA and CB for the buffer to be effective (i.e. maintain pH with the incremental addition of OH^- or H^+).

- $pK_a - 1 \leq pH \leq pK_a + 1$
- $[A^-]/[HA]$ varies from 1/10 to 10/1.

Buffer capacity: related to the amount of H^+ or OH^- that a buffer can neutralize.

- Capacity is related to total buffer concentration.

Ionic Strength of Salt Solutions

$$I = \frac{1}{2} \sum c_i Z_i^2$$

where c_i = molar concentration of the each ion
and Z_i = the charge on the ion.

Which term (c or Z^2) takes on greater significance when $c = 0.01 M$ AND $Z = \underline{\pm 1}$ or $\underline{\pm 2}$ or $\underline{\pm 3}$?

- For salt solutions, ionic strength is one of the most important physical properties.
- I takes into account BOTH concentration and CHARGE, which can have a profound influence on the behavior of the ions!
- Physical biochemists speak in terms of ionic strength rather than absolute salt concentrations.
- Almost every equation dealing with salt solutions involves a term for ionic strength.

How to calculate ionic strength for weak acid buffers is discussed in greater detail in Expt. 1 protocol, where you will calculate the ionic strength for phosphate and TRIS buffers.

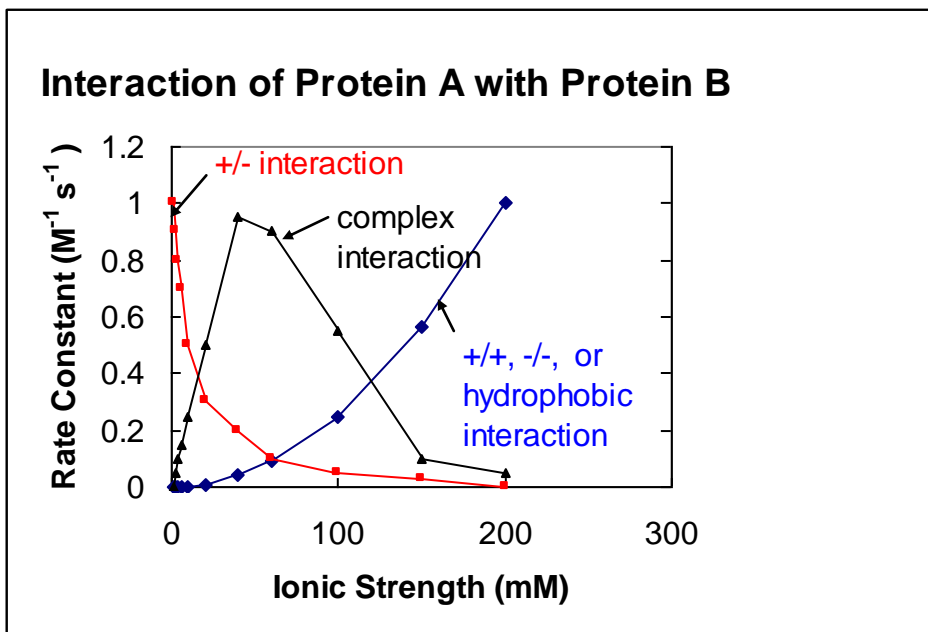
For solutions containing buffer salts and other salts (MgCl_2 , MgSO_4 , NaCl , EDTA, etc.), ALL ionic species MUST be accounted for in calculating the TOTAL IONIC STRENGTH of the solution: $I_{\text{total}} = I_{\text{Buffer}} + I_{\text{MgCl}_2} + I_{\text{NaCl}} + \dots$

Which salt would have a higher I at 100 mM concentration, NaCl or MgSO_4 ?

Why is Ionic Strength Important?

Many bimolecular biochemical reactions are controlled by electrostatic (Coulombic) interactions.

- **Proteins have charged surfaces.**
- **Electrostatics control either attraction or repulsion of macromolecules including other proteins.**
- **Electrostatic interactions are dramatically altered with increasing ionic strength.**



- **The nature of the change (increase or decrease) tells you something about the nature of the interaction.**

How is Ionic Strength Experimentally Varied?

- Chose a relatively low buffer concentration (~ 5 – 10 mM), then calculate I_{buffer} .
- Increase I_{total} by adding NaCl or KCl, because $I_{\text{NaCl}} = I_{\text{KCl}} = [\text{NaCl}]$.
- Calculate $I_{\text{total}} = I_{\text{buffer}} + I_{\text{NaCl}}$
- Alternatively you can make buffers at varying concentrations, taking into account H-H equation, pH, etc. (more cumbersome).

Conductivity of Buffer Solutions (or how can you determine the buffer concentration and/or ionic strength without tasting it to see how salty it is?)

Conductivity is the measurement of the how well a solution conducts electricity.

Ohm's Law states:

Voltage = Current x Resistance

$$E (V) = I(A) \times R (\Omega)$$

Conductance (C) = $1/R$ expressed as Ω^{-1} (expressed as Mho's or Siemens).

Conductance depends upon:

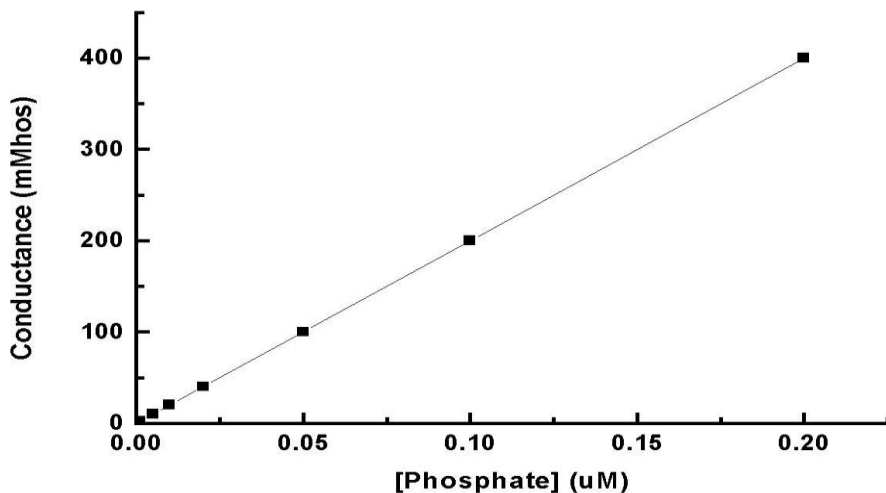
- 1. Number of ions.**
- 2. Ionic Strength.**
- 3. Ion mobility.**
- 4. Electrode surface area.**
- 5. Distance between electrodes.**
- 6. Temperature of the solution.**

The ability of solutions containing ions to conduct electricity is an important topic, especially with respect to understanding the ELECTROPHORETIC EFFECT. Several equations that can be used to determine the conductance of a given electrolytic solution, however, we will use the measurement of conductance to empirically determine buffer concentrations from standard curve of phosphate and TRIS buffers.

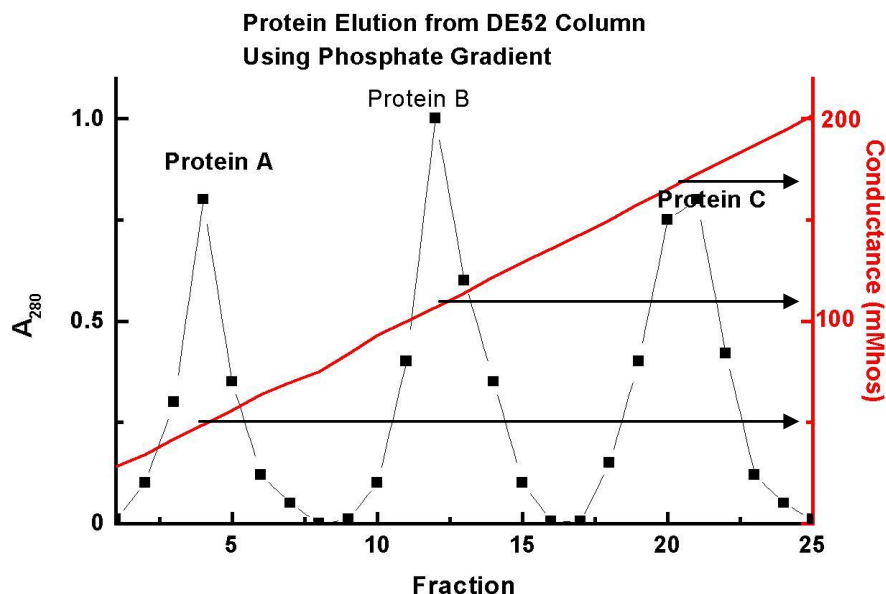
Procedure:

- 1. Measure the Conductance, C , of a buffer at increasing concentrations.**
- 2. Plot C as a function of concentration and/or ionic strength.**
- 3. Measure the Conductance of a buffer solution at an unknown concentration and determine concentration directly from your standard curve.**

Standard Curve for Phosphate Buffer



Elution Profile for a Mixture of Three Proteins



Using the Conductance values at which each protein elutes from the column, the exact [phosphate] needed to elute it can be determined from the standard curve.

Other Important Uses of Conductivity Measurements:

- **Determine if ion exchange column is properly equilibrated (with respect to I) before adding a protein solution that you want to “stick” or bind to the resin, then purify by increasing the ionic strength of the buffer going onto the column.**
- **Determine ionic strength of protein solution itself is sufficiently low before adding to ion exchange column.**
- **Determine “purity” of reagent grade H₂O. Most water purification systems use a conductivity meter to monitor the salt content of the effluent. (The little **Red / Green** light at the top of a water softening tank the signaling device of an ohm meter!).**