Bioc 463a: 2013-2014

Experiment 1: Preparation and Analysis of Laboratory Buffers

Reading: Sections 2.3, 2.6, 2.7 in NB&B. Most of this material is assumed to be a review of Gen. Chem.

Objectives`

1. Be able to understand and use the Henderson-Hasselbach equation.
2. Be able to understand and calculate the ionic strength of a buffer solution.
3. Become proficient at pipetting and volumetric methods.

Introduction

The concepts covered in this experiment are typically first introduced in general chemistry: weak acids, conjugate bases, and the Henderson-Hasselbach equation. Unfortunately students do not really understand why they are learning this material so the tremendous significance and appreciation of these concepts gets lost. In experimental biochemistry, buffers while extremely important, are often taken as a "given". Regrettably, we often find that the lessons learned in "Gen Chem" are quickly forgotten; therefore we must re-acquaint students to those topics and their mathematical formalisms.

Beyond buffer preparation, the relationship between pH, pK_a, and the ratio of A/HA described by the Henderson-Hasselbach equation is a very important concept in biochemistry and not merely a mathematical exercise carried over from general or physical chemistry. All proteins or enzymes are comprised of amino acids some of which have side chains that are weak acids themselves (as well as the N- and C-termini). Therefore, at a given pH these amino acids can exist in different states of protonation (or deprotonation) that can possibly result in the presence of positive or negative charges that will dictate the interaction of these proteins with other proteins or small molecules. Additionally, many enzymes have titratable side chains which are involved in the catalytic mechanism and thus their activities are often strictly dependent upon the pH of the buffer and the pK_a of the ionizing groups! Therefore, a thorough understanding of simple buffers lays a strong foundation for understanding the pH dependencies of biomolecules!

Virtually all biochemical experiments are carried out in aqueous solutions containing specific salts that help **minimize** changes in the pH of the solution upon addition of a **small** amount of acid (H^+) or base (OH^-). These salts, when dissolved in solution, are referred to as buffers. They consist of ***a weak acid species*** (the protonated form, HA, or WA) that is responsible for consumption of added base, and its ***conjugate base*** (the deprotonated form, A, or CB) that consumes added acid or H^+. An excellent description of buffer action in solution as well as the relationship between the ratio of conjugate base to weak acid (i.e. [A]/[HA]) and the pH of the solution is given in Section 2.7 of NB&B. A "short and sweet" version is given under **Buffer Primer** (see below).

In addition to affecting the pH of a solution, buffers also affect the ionic strength of a solution because buffers are often ionic species, which have electrostatic charge. The ionic strength of a solution containing ionic species takes into account two factors (see below for more detail): the **concentration** of all the ions in solution and the **charge squared** of each species. Consideration of ionic strength (*I*) is very important for a number of reasons. Water soluble proteins are charged species that interact with other proteins, small charged molecules free in solution, or small molecules fixed on a solid surface (such as with ion

exchange resins). These interactions, which are Coulombic or electrostatic in nature, are strongly influenced by the ionic strength of the solution.

The pH of a buffered solution can also be affected by the temperature of the solution. Depending on the type of buffer, the pH either increases, decreases, or does not change when the solution is taken from room temperature to 4ºC (i.e. the temperature of a cold room, refrigerator, or ice bath). This temperature dependent behavior is most critical for protein or enzyme purification schemes in which both the pH and temperature of the procedure are critical to the isolation of an active form of the protein. There are easy and practical ways of dealing with this problem that will be discussed in class.

Finally, these experiments will require the dilution of stock buffer solutions to give a range of concentrations. This will require use of volumetric methods (i.e. pipetting). In most biochemistry laboratories, concentrated solutions of reagents are first prepared. The researcher then makes the appropriate dilution of the stock solution to give a final working concentration. Therefore, it is extremely important to be able to accurately and reproducibly use a variety of volumetric devices such as graduated cylinders, serological pipettes, and variable volume pipettors to make these dilutions.

In this set of experiments, we will:

- Prepare phosphate and Tris buffers at different pH values.
- Measure the pH of those buffers.
- Determine the effect of temperature on the pH of a buffered solution.
- Relate conductance to concentration and ionic strength of phosphate and TRIS buffers.
- Make accurate dilutions of stock solutions.

Buffer Primer: The Role of Weak Acids and Conjugate Bases in Buffers.

In solution, a buffer system consists of two species, a weak acid (HA) that serves as a proton donor and its conjugate base (A) that acts as a proton acceptor. These two species exist in equilibrium:

(1) $HA \Leftrightarrow A + H^+$

Note: In this discussion the charges of either HA or A have not been specified. The presence of a charge (or lack thereof) on a given species is dependent on what type of buffer is considered (i.e. compare the charge on the species in the $H_3PO_4 \Leftrightarrow H_2PO_4^{-1}$ equilibrium with $TrisH^+ \Leftrightarrow Tris^0$ equilibrium).

Thus, an equilibrium constant for the **dissociation** of the **weak acid** can be defined:

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

*Caution: The subscripted "**a**" means "dissociation of a weak **a**cid", NOT "**a**ssociation".*

The role of HA is to consume (or neutralize) a small amount of base by donating an H^+ while the role of the conjugate base, A, is to consume (neutralize) a small amount of acid by accepting the added protons. In either case the ΔpH of the solution is minimized. In order

for a buffer system to work efficiently, it must be able to serve **both** roles (meaning you have to have enough of both in solution).

The Henderson-Hasselbach (H-H) equation allows us to interchangeably relate three properties of a buffer system: (a) the **pH** of the solution, (b) the **pK_a** of the buffer itself, and (c) the **ratio of conjugate base (A) to weak acid (HA) = [A]/[HA]** (you are referred to your text or the lecture notes for a derivation of the H-H equation).

(2) $pH = pK_a + \log [A]/[HA]$

Note: The "p" of something is, by definition, "-log of" something. Thus, pH = -log $[H^+]$ and pK_a = -log K_a. This formalism is used in order to convert very small numbers (i.e. 1 x 10^{-7}) to positive numbers that can be easily added or substracted (i.e. 7).

Usually, pK_a values are obtained from a table, such as Table 2-2 (p. 60) in NB&B. Assuming the pH of the solution is known, we can calculate the ratio of A to HA by:

(3) $pH - pK_a = \log [A]/[HA]$

and

(4) $[A]/[HA] = \mathbf{n/1}$, where *n = the anti* $\log_{10}$ *of* $(pH - pK_a) = 10^{pH - pKa}$!!!!!
This is the point where math problems usually arise! In solving for [A]/[HA], the number, n, is obtained from your calculator: [A]/[HA] = n. While this is numerically correct, conceptually it is not. The ratio [A]/[HA] represents two distinct chemical species, so the numerical ratio should also include two numbers: [A]/[HA] = n/1. Putting the "1" in the denominator reminds us that HA is also present quantitatively in solution and the [total buffer] = [HA] + [A].

Equation (4) allows us to make some **simple predictions** about the relative amounts of A and HA that we have at a given pH. When $pH < pK_a$ then [A]/[HA] < 1/1; when $pH = pK_a$ then [A]/[HA] = 1/1; when $pH > pK_a$ then [A]/[HA] >1/1.

The **primary importance** of the ratio, [A]/[HA] = n /1 comes into focus when you must calculate the mole fraction of either A (F_A) or HA (F_{HA}) at a given pH:

(5) $F_A = [A]/([A] + [HA]) = n/n+1$ and $F_{HA} = [HA]/([A] + [HA]) = 1/n+1 = 1 - F_A$
Hint: You will be expected to thoroughly understand this relationship between [A]/[HA] and F_{HA} and F_A !!!!!

While the ratio, [A]/[HA], gives a clear mental picture of the relative amounts of the species present, the mole fractions of A and HA are necessary for the calculation of the actual molar concentrations of A and HA in solution:

(6) $[A] = (F_A)\,[Buffer]_{total}$ and $[HA] = (F_{HA})\,[Buffer]_{total} = (1-F_A)\,[Buffer]_{total}$

The ratio of [A]/[HA] is also very critical in determining whether or not you have an efficient buffering system at a given pH. ***To reiterate, the function of a buffer is to minimize the change of pH of a solution when an incremental amount of acid or base is added. In***

order to do this, there must be enough weak acid (HA) and conjugate base (A) present at the working pH. The rule of thumb for choosing a buffer is that the pK_a is within 1 pH unit of the working or desired pH (i.e., pH = $pK_a \pm 1$). Within this pH range, the ratio of [A]/[HA] varies from 1/10 to 10/1. *(Note: using simple algebra, you should be able to prove this to yourself)*. **Outside this pH range there simply is not enough conjugate base (when pH < pK_a –1) or weak acid (when pH > pK_a + 1) present in the buffer system to enable the ΔpH to be minimized upon addition of a small amount of acid or base.** If an experiment is to be carried out over a wide pH range (greater than $pK_a \pm 1$), then typically a two (or greater) buffer system, with individual pK_a values two pH units apart, is chosen. Using a two buffer system allows you to cover a 3 pH unit range and still be well buffered.

Buffer Primer: Ionic Strength Calculations

The ionic strength (I) of a salt solution is a very important parameter that one encounters often when talking about these solutions physical behavior. Ionic strength takes into consideration two things: the **concentration** and the **squared charge** for each ionic species, according to the simple equation:

(7) $I = \frac{1}{2} \Sigma(c_i)(Z_i^2)$

Where c_i is the concentration of each ionic species and Z_i^2 is the charge squared for **each** ionic species. For a simple 0.1 M NaCl solution (assuming 100% dissociation yielding 0.1 M Na^+ and 0.1 M Cl^-) the value for I is easy to solve:

$$I = \frac{1}{2} \{(0.1\ M)(+1^2) + (0.1\ M)(-1^2)\} = 0.1\ M$$

For buffers the math is a little more complicated, but easy to determine if one is systematic in their calculations. In order to simplify the following discussion we assume that the pH = pK_a for a buffer so [A]/[HA] = 1/1 and $F_{WA} = F_{CB} = 0.5$.

In order to calculate for phosphate buffer: *Remember, buffers are prepared typically from salts of the species, therefore the salt contains cationic and anionic species, **ALL** of which contribute to ionic strength even though they might not have an effect on the pH of the solution!*

Let WA = KH_2PO_4 (that dissociates in solution to give $K^+ + H_2PO_4^{-1}$)
and CB = K_2HPO_4 (that dissociates to give $2K^+ + HPO_4^{-2}$).

Now:

(8) $I = \frac{1}{2} \{ \underline{[K^+](+1)^2 + [H_2PO_4^{-1}](-1)^2} + \underline{(2)[K^+](+1)^2 + [HPO4^{-2}](-2)^2} \}$

The first underlined sum in the expression is derived from KH_2PO_4 (also referred to as mono-basic phosphate) while the latter term is from K_2HPO_4 (also referred to as di-basic phosphate).

Recalling that [total phosphate] = 0.1 M and $F_{WA} = F_{CB} = 0.5$, then the concentrations of KH_2PO_4 and K_2HPO_4 are both equal to 0.05 M.

(9) $I = \frac{1}{2}\{(F_{WA})(0.1\ M)(1)^2 + (F_{WA})(0.1\ M)(-1)^2 + (2)(F_{CB})(0.1\ M)(1)^2 + (F_{CB})(0.1\ M)(-2)^2\}$
$= \frac{1}{2}\{(0.5)(0.1\ M)(1) + (0.5)(0.1\ M)(1) + (2)(0.5)(0.1\ M)(1) + (0.5)(0.1\ M)(4)\}$
$= \frac{1}{2}\{(0.05\ M) + (0.05\ M) + (0.1M) + (0.2\ M)\}$
$= \frac{1}{2}\{0.4\ M\} = 0.2\ M$

For a Tris buffer, a similar calculation can be made taking into consideration that:
WA = TrisHCl (that dissociates into $TrisH^+ + Cl^-$)
CB = Tris base which electrostatically neutral (Z = 0)

*Note that it is **absolutely necessary to include the concentrations of all the ionic species**, since they contribute to the total number of charged species present in solution, that is why the counter ions have been taken into account in the above calculations!*

The $(Z_i)^2$ *term* **contributes significantly** to *I*, especially when IZI > 1. For buffers containing EDTA, a buffering agent itself, which exists as $EDTA^{-2}$ and $EDTA^{-3}$ at pH 7 (pK_a ~ 6.3), the charge terms make the most significant contribution to the net ionic strength of the solution. The magnitude of the effect of charge squared for divalent, trivalent, and tetravalent ions is further amplified by considering the fact that typically concentrations of these ions are << 1 M!

As an experimentalist, if ionic strength is an important experimental parameter, it is very helpful to be able to do a fast mental ionic strength calculation, given a total buffer concentration and pH. ***You will be expected to know the simple algebraic relationship between I and the concentrations of phosphate and Tris buffers (the two most commonly used buffers) at their respective pKa's!!!***

High vs. Low Ionic Strength

It is not uncommon for researchers to say they are working at low, medium, or high ionic strength. Implied in this statement is an awareness of the relationship between ionic strength, the concentration of ions in solution, and their charges. Hence, high ionic strength can imply high total salt concentration (e.g., NaCl containing solutions) or ionic species with Z > 1. What does "low" or "high" ionic strength qualitatively mean? Frankly, these terms are relative, but as a rule of thumb they are related to the following ranges:

- Low: 0 mM < I <= 10 mM
- Medium: 10 mM <= I <= 100 mM
- High: 100 mM <= I

For example, the ionic strength of a typical eukaryotic cell is believed to be ~ 175 - 200 mM, which puts it in the low end of the high range. **To reiterate, remember that ionic strength is related to concentration of the salt in solution AND the charges on the ions squared.** The concentration of NaCl in a solution for which I = 175 mM is 175 mM, while the concentration of phosphate at the same ionic strength (and pH 6.8) is 87.5 mM! For a Tris buffer at its pK_a, the concentration would be 350 mM. *With respect to the ionic species involved, what single property of the ions influences this difference in concentrations of the different solutions, although they all have the same ionic strength?*

Buffer Primer: How to Make a Buffer

Method 1: Start with the weak acid (**WA**) species and titrate with a concentrated base such as 1 N NaOH. If you are working with high concentrations of buffer (i.e. > 0.1 M) you can weigh out the amount of base needed to bring you to the final pH. However, this latter method can result in considerable heating of your solution because the solvating of NaOH is a very exothermic reaction. *This method sometimes works well if you are dealing with dilute buffers, however, many people often "over-shoot" the desired pH when adding base, which then requires you to add more acid to bring the pH back down. This also necessitates having solutions of bases on hand.*

Method 2: Start with the conjugate base (**CB**) (usually as a Na^+ or K^+ salt) and add a concentrated (1 N) acid, such as HCl. This method is often used when working with Tris buffers. This method generally works well; however, sometimes you have to be concerned about the anionic species of the strong acid that you are adding. For instance, if Cl^- acts as an inhibitor of an enzyme, one would not want to use HCl, perhaps choosing acetic acid instead. *This has a similar drawback as Method 1: undershooting the desired pH by adding too much acid, in addition to the addition of various anionic species that may be deleterious.*

Method 3: Make up concentrated stock solutions (usually 1 M) of the weak acid and conjugate base forms. Determine the **dilution factor** needed to produce the final desired buffer concentration and determine the **CB/WA** ratio from the H-H equation (which will determine the pH of the final solution). Based on these two parameters, add the appropriate volume of each stock solution, add water to bring to the final volume, and check the pH with a meter. *This method works well when you are repeatedly making up the same buffer solution. It is also probably the most accurate method and usually the desired pH is obtained every time if you are careful with your volumetric work.*

Method 4: Use the H-H equation to determine the CB/WA ratio, then determine the weight of weak acid and conjugate base needed. Weigh out the salts, add H_2O to the final volume, then check the pH. *This method will work well if you are going to be using the buffer system only once. If you are going to be repeatedly using the same buffer,* ***Method 3*** *is preferred.*

For Complex Buffers: Sometimes buffers will contain a number of other species (Mg^{2+}, NaCl or KCl, SO_4^{2-}, EDTA, etc.). Addition of these species can have an effect on the pH of the buffer, so it is best to not make any final pH adjustments until all of the salts have **completely** dissolved.

Whatever method you choose: Always check the pH of your buffer using a carefully calibrated electrode at the working temperature!

Practical Buffer Considerations

As state above, virtually all biochemical studies are carried out in some type of buffer. The following is a list of considerations one should make when choosing a buffer:

1. Is the pK_a of the buffer and the pH at which the experiment will be done compatible? Remember the $pK_a \pm 1$ rule.

2. Is there sufficient buffering capacity for the experiment? This is related to the concentration of the buffer? Is the ionic strength appropriate for the experiment?

3. How can ionic strength be altered? Generally, there are two ways to adjust ionic strength. First, you can work at a relatively low buffer concentration (~10 mM) and adjust ionic strength by adding NaCl, or a similar type of salt. Second, you can work at different buffer concentrations. Sometimes, however, this choice can be problematic because of the next consideration.

4. Specific ion effects. Some buffers (phosphate is notorious for this) can cause specific ion effects. Specific ion effects are changes observed in a protein's properties or activity of an enzyme due to the binding of buffer species to the protein. Phosphate often has this type of effect because of its charged nature and the structure of the phosphate molecule. A second effect can be introduced by titrating your buffer to the desired pH with an acid that contains a counter ion (e.g., Cl^- is the counter ion of HCl) that acts as either an inhibitor or activator of an enzyme or protein.

5. How to choose the "optimal" buffer. Generally, when we begin to study a protein or enzyme, we will follow some other investigator's protocol, using the same buffer that is specified in the literature reference. While this is entirely suitable for initial investigations, one should eventually be bold enough to see what happens if you change the buffer (pH, ionic strength, type of buffer). Sometimes, you will find that the conditions chosen by some other investigator do not produce optimal results. Only by investigating the system thoroughly will you be able to say with certainty how that buffer (or reaction solution) affects the experiment. ***Never assume that just because someone else has done it a certain way, that this is the best way to do it!***

6. "Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems" is a very useful booklet covering the theory and application of buffers in biochemistry can be obtained from Calbiochem (consult a current catalog or go to their website: **www.calbiochem.com**).

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Experiment 1-1: Determination of pKa of a Laboratory Buffer.

Purpose: In this experiment you will prepare **either** phosphate buffers by mixing together different volumes of 0.1 M KH_2PO_4 (mono-basic phosphate) and 0.1 M K_2HPO_4 (di-basic phosphate) or Tris buffers by mixing together different volumes of 0.2 M TrisHCl (the acidic form) and 0.2 M Tris base. You will then measure the pH of the different buffer solutions. In order to minimize the time required for this experiment, you will as a group of four preparing the buffers, one pair of students making the acidic forms (KH_2PO_4 or TrisHCl) or the basic forms (K_2PO_4 or Tris base) at the given concentrations, then mixing them together to give a set of buffers covering the desired pH range.

Formula Weights:

KH_2PO_4 = 136
K_2HPO_4 = 174.2

TrisHCl = 157
Tris base = 121

Procedure:

1. Prepare **200 ml** of **EITHER 0.1 M** monobasic (KH_2PO_4) or dibasic (K_2HPO_4) phosphate or **0.2 M** TrisHCl or Tris base.
    - Using the formula weight, calculate the amount of salt you need to weigh out prior to coming to class. **Have TA sign-off on calculation.**

• Salt Species	• Amt. Needed	• Amt. Weighed Out
• KH_2PO_4	•	•
• K_2HPO_4	•	•
• TrisHCl	•	•
• Tris base	•	•

- After the salt has been weighed, add to a 250 ml beaker.
- Add a volume of H_2O < 200 ml and a magnetic stir bar to the beaker.
- Stir the solution until all of the salt is dissolved.
- Carefully transfer all of the liquid from the beaker to a graduated cylinder, **without** transferring the stir bar.
- Thoroughly rinse out the beaker, collecting the liquid in the graduated cylinder.
- Bring the volume to 200 ml by addition of H_2O. ***Do not over shoot! If you do overshoot 200 ml, you must calculate the precise concentration of buffer and adjust all subsequent calculations accordingly.***
- Final volume: _____.
- Final concentration: _____.

2. Prepare the buffers in the appropriate pH range using Tables 1.1 and 1.2 as guides. **Do these calculations prior to coming to class!** For these calculations, the pK_a values for phosphate and Tris buffer in the pH ranges we are studying can be obtained from Table 2-2 in NB&B (p. 60).

- Total volume for each buffer = 10 ml.
- From the H-H equation, calculate the mole fractions of the acidic and basic forms of each buffer (see Buffer Primer and Table 2-2 on p. 60 of NB&B) to give the desired pH.
- Using the mole fractions, determine the volume of each stock solution, which must be added using a final volume of 10 ml. **Have TA sign-off on calculations.**
- Using the 10 ml and 1 ml serological pipettes, mix the two buffer solutions in the correct proportion using the test tubes provided. Thoroughly mix the solution by gently tapping the test tube or use the vortex mixer.
- Using a pH electrode calibrated against two pH standards, measure the pH of all the solutions**. Make sure you thoroughly rinse the electrode with H_2O before you place it in the first solution to be measured.** To measure the pH of the buffer, pour the buffer from the test tube into a small beaker. After reading the pH, return the buffer to the test tube.

Table 1.1 For Phosphate Buffers

pH Calc	Frac $H_2PO_4^{-1}$	Frac. HPO_4^{-2}	Vol. $H_2PO_4^{-1}$ (mL) *	Vol. HPO_4^{-2} (mL) *	pH Observed	ΔpH (Calc-Obs)
	1.0	0	10	0		
5.2						
5.8						
6.2						
6.6						
7.0						
7.4						
7.8						
8.2						
	0	1	0	10		

*** These are calculated volumes. In parenthesis, write down the actual volume added.**

Label and cover each tube with Parafilm. ***We will use these buffers next week in Expt. 2-3*****.**

Table 1.2 For Tris Buffers

pH Calc	Frac. TrisHCl	Frac. $Tris^0$	Vol. $TrisH^+$ (mL) *	Vol. $Tris^0$ (mL) *	pH Observed	ΔpH (Calc-Obs)
	1.0	0	10	0		
5.6						
6.0						
6.4						
6.8						
7.6						
8.0						
8.4						
8.9						
9.5						

*** These are calculated volumes. In parenthesis, write down the actual volume added. Label and cover each tube with Parafilm.** ***We will use these buffers next week in Expt. 2-3.***

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Experiment 1-2: Conductivity and Ionic Strength of Buffered Solutions.

As mentioned previously, a very important property of a solution used in biochemistry is the ionic strength which is directly related to salt concentration and the charge squared for each ionic species. While we can calculate the concentration of buffer salts in a solution, actually ***measuring*** the buffer concentration in a sample is done indirectly by determining the **conductance** of the solution using a **conductivity meter**. This reading is then compared to a previously obtained standard curve of conductance vs. buffer concentration.

Conductance occurs in salt containing solutions when an electrical current flows (or is conducted) between two electrodes of a DC electrolytic cell. Recalling Ohm's Law:

E (electrical potential in Volts) = **I** (current in Amps) x **R** (resistance in Ohms)

Since the current flowing through an electrochemical cell, at a given potential, is inversely proportional to the resistance, the conductance of the solution, **C**, is taken to be 1/**R**. Historically the unit of conductivity was referred to as a "**mho**" however the more modern unit is called a**Siemen**. Fortunately, **1 mho = 1 Siemen (S)**.

Operationally, the conductivity of a solution is related to: (1) the number of ions present, (2) the charge on the ions, (3) the ion mobility (i.e. the ability of each ion to conduct an electrical current), (4) the area of the electrode surface, (5) the distance between the electrodes, and (6) the temperature of the solution. Hence, a theoretical calculation of the conductance of a salt solution can be quite tedious. Typically, the conductivity of a buffer over a specific concentration (or ionic strength) range is empirically determined from which one constructs a standard curve. The conductivity of an unknown concentration of the same type buffer (at the same pH) is measured which allows one to graphically determine the actual concentration (or ionic strength) from the standard curve. This is often very useful for determining the buffer concentration (or ionic strength) at which a protein is eluted from an ion exchange column.

In this experiment, the conductivity of distilled water and various concentrations of phosphate and TRIS buffers, at pH values equal to their respective pKa's will be measured. (Because of the expense of the conductivity meter/electrode, an instructor will measure the conductivity for you). You will then determine the relationship between conductance, salt concentration, and the ionic strength of the buffer solutions.

Procedure:

One half of the class will measure the conductivity of phosphate buffers at different concentrations, while the other half will measure the conductivity of Tris buffers. At the end of the class, we will pool our results AND **everyone** is responsible for both data sets.

Calculate the ionic strengths of the phosphate (pH = 6.8) and Tris (pH = 8.1) buffers at the different concentrations. Enter these values into Tables 1.3 and 1.4. (Note: In order to calculate the ionic strength of the Tris buffer, it is necessary to know that it was prepared by the addition of equal volumes of 0.2 M Tris-HCl and 0.2 M Tris base AND Tris-HCl dissociates to give Tris-H^+ and Cl^-. Tris base is electro-neutral, thus has no charge, and is often written Tris^0 emphasizing its neutrality).

Phosphate buffer groups: Prepare at least 30 ml of a stock 0.1 M phosphate buffer at **pH 6.8** using the mono- and di-basic phosphate solutions you have already prepared. Refer to

Table 1.3 to calculate the volumes of buffer and H_2O needed to prepare 10 ml of phosphate buffer at the given concentrations.

Note: in order to calculate the volume of 0.1 M phosphate to be diluted to 10 mls, use the equation:

$$(volume_{initial})(concentration_{initial}) = (volume_{final})(concentration_{final})$$

Tris buffer groups: Prepare at least 30 mL of a 0.2 M Tris stock solution at pH 8.1 from your TrisHCl and Tris base solutions you have already prepared. Refer to Table 1.4 to calculate the volumes of buffer and H_2O needed to prepare 10 mL of Tris buffer at the given concentrations.

Enter the calculated value for the ionic strength of the solution in Table 1.3 and Table 1.4 **PRIOR** to coming to class. Record the conductivity of the solutions in class.

Table 1.3

$[P_i]$(M)	**$V(P_i)$**	**$V(H_2O)$**	**Conductance (Mho's)**	**I (M)**
0	_____	_____	_____	xxxxx
0.01	_____	_____	_____	_____
0.035	_____	_____	_____	_____
0.05	_____	_____	_____	_____
0.075	_____	_____	_____	_____
0.1	_____	_____	_____	_____

Table 1.4

[Tris](M)	**V(Tris)**	**$V(H_2O)$**	**Conductance (Mho's)**	**I (M)**
0	_____	_____	_____	xxxxx
0.05	_____	_____	_____	_____
0.075	_____	_____	_____	_____
0.1	_____	_____	_____	_____
0.15	_____	_____	_____	_____
0.2	_____	_____	_____	_____

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Experiment 1-3: Effect of Temperature on the pH of Biological Buffers.

1. Using the buffers from Expt. 1-2, prepare 10 mL of 0.1 M phosphate or 0.2 M Tris buffer at their respective pKa's, then:
   - Measure the room temperature and pH values of the buffers.
   - Place the buffer solutions in an ice bath.
   - Allow the solution to come to thermal equilibrium (this should take no more than 10 minutes) then record the temperature and pH of the solution. (Do not remove the solution from the ice bath).
   - Record data in Table 1.3.

Table 1.5

	Phosphate	**Tris**
Room temp. of buffer:	_____	_____
pH of room temp. buffer:	_____	_____
Ice bath temp.:	_____	_____
pH of ice bath buffer:	_____	_____
Calculate ΔpH/ºC:	_____	_____

Note: $\Delta pH/^{o}C = \frac{pH(\text{room temp}) - pH(\text{ice bath})}{\text{Room temp} - \text{ice bath temp}}$

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Lab Report for Expt. 1 (20 pts):

Proper presentation of your data AND the ability to verbally describe and draw meaningful conclusions from the data is a very important trait every scientist must cultivate. For the first lab report, you will compose the figures outlined below. For each figure you are expected to:

1. Write one or two sentences stating **what the data represents**.
2. Differentiate between the actual observed data and a theoretical curve.
3. What equation, if any, was used to derive the theoretical curve drawn through your data.
4. What significant biochemical constants, parameters, or conclusions can be drawn from the data.

Format for Report:

1. Title page with you name.
2. Results and Discussion section.
3. Figures clearly labeled with figure number. Make sure you have included axis labels for each figure with the appropriate units.

Alternatively, if you want to be creative and embed your figures in your text, you may do so in this lab report. (In subsequent lab reports you will be given a specific format that will be rigorously adhered to). Regardless of how you create your report, it will be judged on how well you can follow the instructions given below and whether you address the questions and concepts!

Report Organization:

A. Create the following Figures using Tables 1.1, 1.2, 1.3, and 1.4.

Figure 1: from the data in Table 1.1 or Table 1.2, plot pH(calculated) vs. Frac. Conjugate Base (either K_2HPO_4 or Tris base) **as a solid line** (do not use symbols, however connect data points using a smooth curve). (To eliminate confusion you are plotting Y vs. X). This curve represents a theoretical curve for your data based on a specific equation and assuming a specific value for pK_a . On the same graph, plot pH(observed) vs. Frac. conjugate base as **individual data points** (do not connect the data points, we want to see how far they differ from the theoretical curve).

Figure 2: from the data in Tables 1.3 and 1.4, plot **Conductance vs. [Buffer]** for phosphate and Tris buffers **on the same plot**.

Figure 3: from the data in Tables 1.3 and 1.4, plot **Conductance vs. Ionic Strength** for phosphate and Tris buffers **on the same plot**.
{Figures 2 and 3 can be put on same page}

B. Referring to your data, use the following questions **as a guide** to compose your thoughts and to discuss the results. There should be a one or two paragraph description and discussion of each figure as well as the data in Table 1.5 based on the questions listed below.

1. For Figure 1, clearly state which equation was used to generate the theoretical curve and include that equation in your report. What value for pK_a was used to calculate F_{CB} in Table

1.1 or 1.2? This must be cited as a reference. From the data which you obtained, what important scientific constant can be determined? How well does the observed value for this constant agree with the literature value (i.e., the value you got from NB&B)? What does ΔpH represent? Is the variation the same at each ratio of [CB]/[WA] or Is there a pattern to the variance? Taking into consideration every thing you did (weighing out the salt, making the solutions, or mixing together the two solutions) and the equipment used (pipettes, pipettors, pH meter, etc.), what are the likely sources of error?

2. Using Tables 1.3 and 1.4, determine a simple algebraic relationship relating concentration to ionic strength for phosphate and Tris buffers at their respective pKa values. **You will be expected to know this simple relationship by heart!** Using Figures 2 and 3, discuss how conductance relates to buffer concentration versus the ionic strength of the buffer. Why are the physical properties of salt solutions (i.e., your buffers) often best described by ionic strength rather than simply the actual concentration of the species?

3. Refer to Table 1.5 addressing the following points. Are the temperature effects on the buffer pH values the same or do they vary with respect to the two buffers with respect to the sign of the change as well as the magnitude of the shift in pH? How well do these changes agree with the literature data handed out in class? Since the pH of a buffered solution is a function of the equilibrium between the weak acid and conjugate base forms, explain in you own words the temperature effects seen in Expt. 1.5 (i.e., how is the equilibrium between the WA and CB form shifted shifted by decreasing temperature?). *It is very important to remember that a shift in an equilibrium due to some change in experimental conditions means one species is stabilized and the other destabilized as a result of the change in the conditions!*

Supplimental Questions (2 pts each) To receive credit, ALL work must be shown:

1. Glycine (an amino acid) is a critical component of the running buffer in polyacrylamide gel electrophoresis experiments. Referring to Figure 3-5 in Lehninger Principles of Biochemistry and the pKa values given in Table 2-2 of NB&B (pKa_1 is for the -COOH group and pKa_2 is for the $–NH_3^+$ moiety), calculate the net charge on glycine at pH 6.8. How would the net charge change as the pH approached a value of 8.8?
2. An enzyme contains a histidine (cf. Table 3-1 and Fig. 3-5 in Lehninger Principles of Biochemistry) at its active site. Full enzyme activity is seen when the imidazole side chain is deprotonated. Assuming the pK_a for the side chain is 6.0, what percent of the maximal activity would you expect to see at pH 5, 6, and 7.0?
3. As part of a purification procedure for a protein you want to study, you load the protein on an ion exchange column using 10 mM phosphate buffer at pH 6.8. A linear buffer gradient from 10 mM to 0.2 M phosphate is begun in order to elute the protein from the column. The fraction at which the protein elutes from the column has a conductance of 380 uS/cm^2. Using your Figures 2 and 3, determine the concentration and ionic strength of the phosphate buffer at which the protein elutes. Indicate on the curves in Figures 2 and 3 using an (X) where you would expect the protein to elute.
4. Many protein purification protocols require that procedures be carried out at 4ºC. Often, researchers will calibrate their pH electrodes using pH standard solutions, which are buffers themselves, making the final adjustment of a buffer's pH in a cold room. Why do you think this is preferred over calibrating the electrode and meter at room temperature and then making the pH determination at 4^oC?

5. Suppose you are asked at the last minute to verify pKa values for 10 weak acids to be published in a biochemistry handbook that will be published very soon. Describe how, you could verify the "literature" pKa values in one easy experiment using only two solutions for each weak acid.