University of San Diego

Biochemistry Lab

Lab Math, Dilutions, Solutions…

CALCULATIONS: Preparation and handling solutions is an essential part of experimental biochemistry. One of the key tools any new science graduate can have is to be competent in preparing reagents, buffers, and accuracy in pipetting. The three most common concentration expressions biochemists use are molarity, percent (vol/vol, wt/vol and wt/wt) and normality. Say it with us, lab math is fun, lab math is fun, lab math is fun …..

SOLUTIONS: Solutions are a big part of biochemistry, biological and chemical based work. Making solutions can also be the most frustrating part of an experiment for a beginner. Making reagents correctly are often a make or break event for experiments. Stock solutions (or concentrated solutions) are often defined as percent, Molar or the informal "X" concentration. If a solution is made of one component, then the molarity is usually shown. When a number of chemicals are used to make a concentrated solution the concentration factor X is used. The purpose of this section and the required readings is to help you determine what calculations to use and how to prepare solutions for your experiments.

Preparation of Solutions:

Concentrations in percent:

a. Percent by weight (w/w)
   (X g of solute/100 g of solvent)*100 = X%

   Ex. a 10% (wt/wt) solution would be 10 g of solute and 90 g of solvent.

b. Percent by volume (v/v)
   (X ml/100 ml of total solution)*100 = X%

   Ex. A 10% (v/v) solution would be made by adding 10 ml of concentrated solution to 90 ml of dilutent.

Note: measure volumes separately, then add them together. Some liquids are more dense when mixed. I.e. simple alcohols and water when combined have a smaller volume than before being mixed!

c. Percent by weight per volume (w/v)
   (Xg of solute/100 ml total volume)*100 = X%

Ex. A 30% NaCl solution would be prepared by adding 30 g of salt to a vessel containing 50 ml of water then QS (*fill to **Quantity Sufficient***) to measure 100 total ml. Do not add 30 g to 100 ml of water, the resulting solution would be more dilute than planned.

d. Percent by Mg per volume (Mg %)
   mg of solution/100 ml of total volume

Milligram % is often used in clinical laboratories. For example, a clinical blood sugar value of 225 means 225 mg of glucose per 100 ml of blood serum.

Helpful Hint: Many times, you will not need to make 100 ml of a solution. If that is the case, simply multiply the percent of your desired solution (in decimal form) by the total volume of solution you wish to prepare. The resulting calculation is the mass of solid you need to add to solution. Don't forget to QS to the desired volume.

Concentration in molarity

Molarity is the most common concentration unit in biochemistry. Make certain that the abbreviation is a capital M. *Brackets [ ] indicate molar concentration, usually in M.*

Molarity (M) = Moles/ liter of solution
 = (grams of solute/molecular weight)/liter

Dilute solutions are often expressed in terms of a smaller unit therefore,

1 mM = 10^{-3} M = 1 mmole/liter = 1 μmole/ml

1 μM = 10^{-6} M = 1 μmole/liter = 1 nmole/ml

1 nM = 10^{-9} M = 1 nmole/liter = 1 pmole/ml

Concentration in normality

Normality (N) = the number of equivalents of solute and its equivalent weight (EW). The EW is the mass of one mole of ion (either H^+ or OH^-)

$$\frac{WT_g}{EW} = \frac{MW}{n}$$

Where n is the number of replaceable H^+ or OH^- per molecule for acids and bases.

The molarity and normality are related by:

$$N = nM$$

Dilutions $C_iV_i = C_f V_f$

This calculation is used for diluting concentrated solutions, usually those that are in percent, molar, or normality units. It is NOT intended to be used when dealing with "X" or serial type of dilutions. Use this only when you want to make a dilute solution starting from a concentrated solution. Do not use this calculation for solids – instead use the molarity calculation.

This is a little different method of determining concentration than what most of you are used to. However, it is very easy to use, but can be a large source of error for if the calculations are not performed correctly. The biggest problem in using this method is when you don't keep the units the same. When using milliliters on one side of the equation then you need to use milliliters on the other. The same goes for concentration units. *See page 139 in At the Bench for additional information.*

Biochem Lab Law: *write down the units no matter how trivial it seems*. This way any mistakes are found before its too late (at the end of day when your experiment didn't work). AND RECORD THIS IN YOUR LAB BOOK!

The "X" Factor

Vol of 1X needed/Concentration Factor = volume of stock solution to use.

Many laboratories use a number of solutions over and over again. Many times, it is easier to make a concentrated batch of a mixture of chemicals and simply dilute them out each time instead of starting from scratch each time you need the solution. When a solution is a single compound or buffer, the actual concentration is typically written on the bottle and use the CV=CV rule to calculate out how much you need. When the solution is a complex mixture (more than one chemical) in a solution, the X factor comes into play. When making a concentrated stock solution such as this you need to know the "working" concentration. That is the concentration of each of the chemicals when the solution is at the correct concentration to use. This is called 1X. All concentrated stock solutions that use the X factor use the working concentration as 1 X and then simply factor in how concentrated the stock solution is. To determine the amount of stock concentrate to use, determine how much 1X or working solution you need. Then divide the volume of working solution needed by the concentration factor. Confusing? It can be.

Ex. Almost every biochemistry laboratory runs some kind of gel, either for DNA or protein. The buffer needed to run this has three or four components. Instead of listing the molar concentration of each chemical in the solution, a stock concentrated solution might be labeled 10X SDS-PAGE Running Buffer. This tells the researcher that the stock solution is ten times more concentrated than it needs to be for use. Thus a dilution is in order. Let's say you need 50 ml of 1X running buffer to perform the electrophoresis. Then 50 ml/10X = 5 ml. That means you need 5 ml of 10X Buffer. Now you should be asking yourself, what do I add this to? Usually deionized or treated water.

Serial Dilutions

The nomenclature for dilutions can be especially confusing. There are several conventions for indicating the method of dilution. For example, a 1 to 10 dilution: there are two different meanings to this phrase and it is very important to understand which meaning is intended. For some people, a one to ten dilution means adding one part of an original solution to nine parts of diluent (usually some buffer or water) for a total of 10 parts. This will give a one tenth (1/10) dilution. Some people will call this a one in ten dilution. Another interpretation of a one to ten dilution is that one part of original solution is added to ten additional parts of a diluent. The final concentration of something like this will be different than the last meaning because now the original solution is diluted 1/11.

The former convention (one part / total parts) is frequently used because the dilution factory is easy to work with. In this case it is best to read "dilute 1:10" meaning "add one part of the sample plus nine parts of diluent for a total of 10 parts". Now it is easier to determine the calculations. If you wish determine the volumes of each part:

A dilution of 1:X means your concentrated solution should be diluted to $1X^{th}$ of its current concentration. Meaning that:

Total vol / total parts "X" = vol of each part

Add the concentrated solution to (X-1) volumes of diluent. ***Always add a small amount to a larger amount.***

> Ex. To prepare a 5 ml protein solution of concentrated BSA protein diluted 1:20 with Tris Buffer.
>
> First, determine the final volume of your solution. In this case it is five ml. Then divide by the total parts or X factor dilution.
>
> 5 ml / 20 = 0.25 ml
>
> The resulting answer tells you the volume for each part, and for this problem, how much concentrated BSA you need for this problem
>
> Then calculate the resulting volume you need to QS to achieve your final volume.
>
> - Add 0.25 ml of protein solution to 4.75 ml of Tris buffer.

Why the different nomenclatures and methods?

Biochemistry is truly a multidisciplinary field. Biochemists can come from chemistry departments, biology departments or biochemistry and molecular biology departments. Biochemistry is central for every one of the biomedical sciences such as pharmacology, physiology, cell biology, molecular biology and many others. Biochemists can be found working in nearly any kind of discipline. Because of all of these influences, several versions of what a dilution means remain in the field. We use the first meaning of dilution, a 1 to 5 means one part of original with four parts of diluent. Written 1:5. But always make certain that others you talk to are on the same page. The difference may seem small but the differences can be disastrous to the results of an experiment. For instance, you have a solution of glucose that is 3.5 M and your boss tells you to dilute the glucose one to two in water. What is the difference if you use the kind of dilution he didn't mean? Well if you use one part glucose plus one part water for a one to two as in the first method, the final concentration will be:

3.5 M x 1/2 = 1.75 M

If the second method is used, where one part glucose is added to three parts of water, the final concentration will be:

3.5 M x 1/3 = 1.16 M

Quite a difference isn't it? It is, especially if you will be injecting the sugar into a person!

How do you calculate these dilutions if you are given a specific volume? How do you make 5.0 ml of 1:2 dilution of the 3.5 M glucose solution?

Using the first method: The final volume is 5.0 ml and consists of a one in two dilution. Each part is equal to the final volume divided by the total parts for that dilution.

In this case the total parts is 2 so
5 / 2 = 2.5 for each part.

Therefore you will need to add 2.5 ml glucose to 2.5 ml water.

Now try it using the second method: The final volume is 5.0 ml and now there are a total of three parts (1/3) in this dilution.

For this case you will divide the final volume by the parts one to two = three total parts.

5 / 3 = 1.66.

So you will need to add 1.66 ml of 3.5 M glucose to 3.33 ml water.

Note: if you want to determine the final concentration of any dilution, using the first method, simply divide the original concentration by the dilution factor. I.e. if you are starting with a 250 mM solution and dilute it 1:5 with water the solution is now 250 mM / 5 = 50 mM.

Real World Time

Biochemists often times use very low concentrations of reagents. Usually in the mM, µM range and sometimes in the nM range. It isn't always convenient to weigh out such small masses to make a µM solution of some chemical. Instead it's a better idea to start with a higher concentration so that the mass can be easily measured and then diluted from the solution.

Sometimes this can require several dilutions. For example, epinephrine (adrenaline) is added to lung cells in a research project. The total volume of media with the cells is 2.0 ml and the molecular weight of the epinephrine is 168.28. What mass of epinephrine do I add to the 2.0 ml to make a 0.10 μM solution of epinephrine?

0.1 μM = 1 x 10^{-7} M
= (moles)/volume (liters)
= (mass (grams)/MW)/volume (liters)
=1 x 10^{-7} M = grams / 168.28 / 0.002 l
= 3.36 x 10^{-8} grams

You can see there is no reasonable way to measure out that small amount of anything. So what do we do? Well let's start by weighing out a small amount of epinephrine and putting it in a small volume of buffer. 20.0 mg of solid and dissolve it in 5.0 ml of buffer. What is the final concentration now?

Answer: 23.8 mM

What volume of the 23.8 mM solution of epinephrine do I add to make a 2.00 ml solution with a final concentration of 0.10 μM?

Answer: 8.4 x 10-6 ml or 0.008 μl

This isn't reasonable is it? Generally a good rule of thumb is if a volume is less than 1.0 μl it is difficult to accurately measure it. Now what do we do? Well I could perform a serial dilution or make a couple of dilutions of the original solution.

Let's say you perform a 1:10 dilution of the original solution and then a 1:100 dilution of that solution. What would the final concentration be? Obviously there is a 10-fold decrease in the concentration for the first dilution. You start with 23.8 mM and end up with a 2.38 mM solution. The second dilution will result in a 0.0238 mM solution or 2.38 μM solution. Let's see if we can use this to add to the original 2.0 mls of cells, back to the $C_iV_i = C_fV_f$ calculation. Remember to keep the units straight!

$$C_iV_i = C_fV_f$$
2.38 μM x X μl = 0.1 μM x 2000 μl
X = 84 μl

This will work! With pipettors 84 μl can be easily measured. The final answer is to add 84 μl to 1.915 ml pH buffer and you are done!

Another way to do the same calculation is to know what volume of epinephrine to add to the cells. Ideally you want to add 10 μl of a more concentrated solution to the cells. Lets do some backwards planning to determine the concentration of the 10μl.

The initial concentration is the unknown, the initial volume is the 10 μl to add to the final volume of 2.0 ml and then achieve a final concentration of 0.1 μM.

$$C_iV_i = C_fV_f$$
X μM x 10 $μl_i$ = 0.1 μM x 2000 μl
X = 20 μM

So you need to make at least 10 μl solution of 20 μM! Are you going to then make a 10 μl solution? No, generally you will want to make slightly larger volume than you need without the final volume being excessive.

Let's figure out how to make a 1.0 ml solution of 20 μM epinephrine from the 23.8 mM original solution.

$$C_iV_i = C_fV_f$$
23.8 mM x X ml_i = .020 mM x 1.00 ml
X = 0.084 ml or 84 μl

This will work as well. Add 84 μl to 0.916 ml of buffer.

> Note: If you add less than 0.1% of the final volume in a dilution the changes in final volume are less than the error of your measurements! In other words don't sweat it. But only if the added volume is less than 0.1% of the final volume.

A last bit of advice. Avoid adding a very small amount to a large amount. Such as adding10 μl to 10 ml. A very small error in measuring the small volume will translate into a very large error in the final calculation. It is better to make a more concentrated solution and perform a dilution. **Rule of thumb** *is the factor of 1000. Try not to add a sample that is less than 1000 times the final volume.*

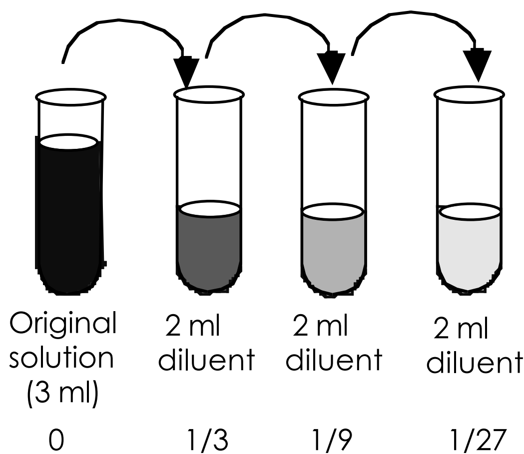
How do you know what numbers to use in these types of calculation? Trial and error and practice, practice, practice. Make certain that the numbers are reasonable. Right or wrong is not always the correct way to look at it.

Multiple Serial Dilutions

Serial dilutions involve the systematic dilutions of an original solution in fixed steps, such as 1:2, 1:4, 1:8 and so on. An example would be to start with 3 ml

of an original solution and carry out the following dilutions.

This type of dilution scheme is most often used in microbiology and immunology. Serial dilutions require careful attention and accuracy of measurement because errors made in an early part of the process will be carried out in all of the following dilutions.



Making solutions from solids

> It is important to keep the initial starting volume to about 70% of the final volume. This is important so that the addition of the buffer salts, solutions and pH adjustment do not increase the volume above the final amount.

Formula Weight vs. Molecular Weight vs. Daltons. The last two are easy, molecular weight (atomic mass units or gram/moles) and Daltons are the same thing. Daltons are used to describe the molecular weight of proteins, much larger than your ordinary chemicals. Thus a 10,000 dalton protein has a molecular weight of 10,000 g/mol. Formula weight and molecular weight are also the same thing IF no waters are associated with the compound of interest. You can tell when water is associated when the chemical, is written as such:

$MgCl_2 \cdot 4\,H_2O$. This means that each $MgCl_2$ has four tightly associated water molecules. Thus you must add the weight of each water when measuring out such hydrated compounds.

Buffers

A buffer system is a mixture of a weak acid and its conjugate base (or salt) that permits the solutions to resist large changes in pH with the addition of small amounts of H^+ or OH^-. In biochemistry, we use buffers in nearly all solutions that will be used with enzymes or where control of pH is important. The Henderson-Hasselbach equation is useful for calculating either the buffer composition or the pH of solutions. Just in case you've forgotten here it is again:

$$pH = pKa + \log \frac{[A^-]}{[HA]}$$

Helpful hint to remember the equation = drop the acid!

Two items are important to keep in mind when using a buffer in an experiment, the pKa of the buffer and the buffer capacity. The pKa is at the point that the solution has the highest buffering capacity; that is, the change in the pH of the solution with the addition of OH^- (or H^+) is least. For this reason, buffer solutions are made of the acids and its salt whose pKa values are close to the desired pH of the buffer. **As a rule of thumb, for the best buffering ability, the pH of buffers should be within 1 pH unit of the buffer's pKa.** The second point to remember when using buffers is the ability of a buffer to resist changes in pH (buffer capacity). Buffer capacity is different from the pKa-pH relationship just mentioned.

Buffer capacity is the number of moles per liter of H^+ or OH^- required to cause a given change in pH. Use this to determine if the concentration of buffer you are using is enough to resist changes in acid or base due to your system. In other words, is there enough of my buffer in solution to maintain pH as I add different chemicals or a reaction alters the proton concentration? Is a 10 mM solution enough to buffer a reaction that produces 100 mmoles of acid? In reality a 10 – 100 mM buffered solution is good for most biochemical needs.

How does one make up a buffer? There are two ways of looking at this problem. First you need to calculate the ratio of the acid and base that you

will need to have, and then determine the concentration of the acid and base you want in your buffer. This is commonly the way a polyprotic acid (an acid with more than one dissociable hydrogen ion) is used. (ie. This is how phosphate buffer is usually prepared.) Many of the buffers used in laboratories are zwitterions, molecules with both a negative and positive charge. These buffers act as both the acid and the base in a buffer system. These buffers are usually good to use because concentration and temperature have little effect on them. Examples of these types of buffers are HEPES, tricine, BES and others. These buffers are typically prepared by determining the concentration of buffer required and adjusting the pH to the desired endpoint.

For example if you are starting with Tris-HCl (the acid form of the molecule) there is no real buffering capacity because there is no base form to accept any protons. With the addition of a strong base such as NaOH the acid form is converted to the base form.

Finally when making buffers there is a practical procedure to use that is often overlooked. Once you have determined how much of a weak acid and its salt you need, fill 3/4 of the final volume in an appropriate sized beaker. Add the acid and base to the beaker and check the pH. If necessary add HCl or NaOH (or whatever strong acid and base required) to adjust the pH of the solution to the proper value. Pour the solution into a graduated cylinder or volumetric flask, rinse out the remaining buffer from the beaker into the cylinder and bring the volume to the final level. Finally check the pH again.

Additionally, some buffers, especially Tris, can change buffer when the temperature changes so it is important to adjust the pH at the temperature that the buffer will be used.

Make certain that you read the section in the book about making solutions, pH meters, determining pH and weighing chemicals. This includes very straight forward but helpful information that will make your life in the laboratory less frustrating.

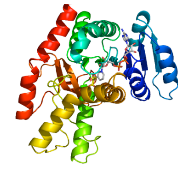
pH Meters: Just a quick word about using pH meters. Never trust that they are calibrated correctly. Many experiments have gone wrong or not been able to be repeated because the pH meter was incorrectly calibrated or had drifted by the time the buffer was made. It is good practice to place the probe in a pH standard buffer that is near your desired pH and check. Is it within 0.1 pH units? If so and that is enough accuracy for your needs then go with it, if not, recalibrate. Most pH meters use pH 7 as a center point, and a second calibration point at either 4 or 10. Choose the pH pair that will bracket your needs.

Be careful when handling the pH probe. It is made of very thin and porous glass and tapping or dropping the probe will result in irreparable damage to a 100$ probe. Do NOT wipe the probe, use plenty of water to rinse off the probe from a squirt bottle.

Statistics: How do you know when to throw out a bad data point? This is a difficult question. More often than not, the experiment should be repeated. If the particular experiment allows, you might be able to repeat just the point in question. Sometimes the differences are obvious. If the data points should lie on a particular line or curve and one point is "way off" then that point should be repeated or discarded in drawing the line (although you still show the point on the graph). Experience and judgment is often the guide to these kinds of problems. Not all "bad" points are actually bad. Many important discoveries have been made when the results do not follow expected outcome.

When making several measurements of a single sample, there are several ways in which to know whether or not to reject the point. When a single measurement deviates substantially from a set of three or more replicate measurements, the researcher must decide whether the data point should be rejected. There are several methods to examine the data one of the simpler methods is the Q test, which will be discussed here. The Q-test is best applied when the number of observations is low. The difference between the questioned point and its nearest neighbor ($|X_? - X_{nearest}|$) is divided by the difference between the highest value and lowest value $|X_{max} - X_{min}|$, one of which is $X_?$.

$$\frac{|X_? - X_{nearest}|}{|X_{max} - X_{min}|} = Q \text{ test}$$



As an example consider the following data set of five replicate values: 5.0, 5.2, 5.7, 5.8, 7.5. Intuition suggests throwing out the 7.5 value, but an objective criteria is preferred, so we will use the Q-test. Q-test is compared to the standard Q critical values (90% confidence) in the table listed below.

Number of points	Q critical value
3	0.94
4	0.76
5	0.64
6	0.56
7	0.51
8	0.47
9	0.44
10	0.41

If the Q test > Q critical then you may reject the questionable point. In the example, the data yield Q test = 1.7 / 2.5 = 0.68 which is greater than the Q critical value for n=5 (0.64). The value in question, 7.5 can then be rejected.

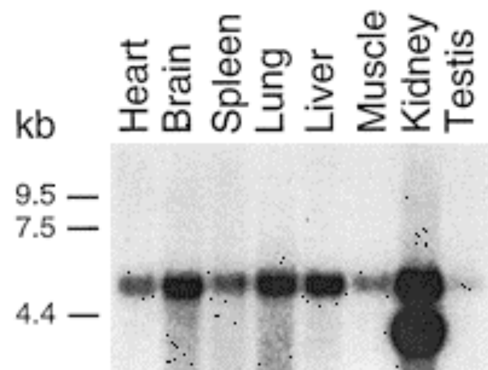
T tests, Anova and statistical analysis of trends and differences among and between groups will be discussed later in the semester.

Graphing: <u>Graphs are to be made on the computer</u>. It doesn't matter which program you use. Any graph should follow the following guidelines:

- Each graph should be from 1/8 to 1/4 of a page in size.
- The independent variable (that which you control) goes on the x-axis
- The dependent variable (the measurement you are taking) goes on the y-axis
- Each axis must be properly labeled. Do not include a legend within the graph
- Make the graph fit the data. Limit the axis to the values in the graph.
- Pictures of gels, blots or other figures must be completely labeled so the reader can understand the data you are presenting. What is in each lane should be indicated. The molecular weights should also indicated on the side and any points of interest highlighted by an arrow. See figure 1 below.
- *When you have multiple data points (n=>1) for a single reading, do NOT graph all points on one graph. Instead, average them. If the number of replicates is three or greater, then determine the standard deviation and include that information in your graph.*
- The title for each graph is in the figure legend
- A figure number and legend will be found underneath each graph. Each legend should be a mini statement on what you did to get the graph. It is kind of a specific methods section for each graph.
- The graph should be imbedded in the text.
- Do not include the table unless requested.

Examples of figures for the lab book:

Fig. 1. Northern Blot: Tissue distribution of rPLD transcript. mRNA from various rat tissues were analyzed using a preblotted poly(A)+ RNA from various rat tissues hybridized with a probe excised from rPLD in pBluescript SK by NotI in the multiple cloning site of the vector and Nsi1 in rPLD. It was labeled with [^{32}P]dCTP by random priming containing rPLDcoding sequence, corresponding to amino acid residues 231-590. Hybridization was performed at 42 °C for 16 h in the presence of 40% formamide and 10% dextran sulfate.



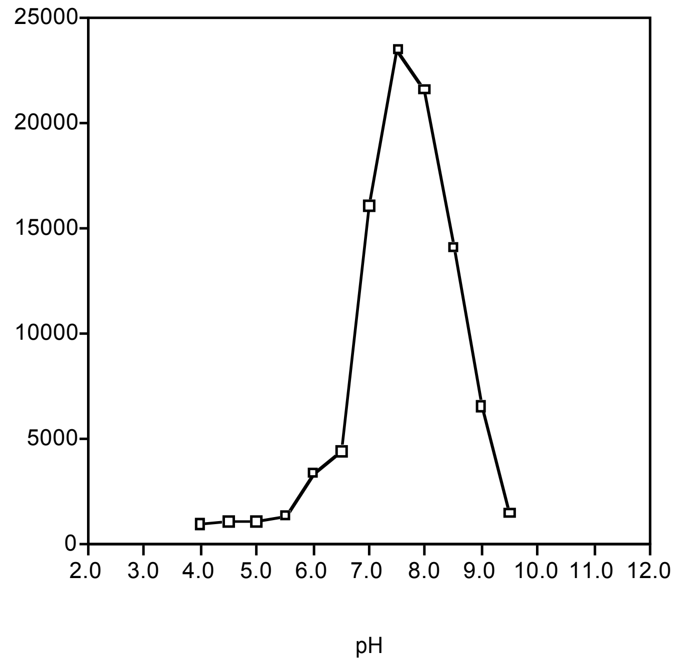


Fig. 2 Effect of pH on the activity of RhoA-activated PLD. PLD activity was assayed for 60 min at 37°C in the presence of 30 µM GTPγS and 50 nM RhoA. PLD activity was determined by the formation of radioactive phosphatidylbutanol as described under "methods" except that 50 mM succinate was used to buffer pH 4.0 through 6.0 and 50 mM Bis-tris propane was used to buffer pH 6.5 through 9.5. Results are representative of two experiments conducted in duplicate.