Experimental Biochemistry
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Lecture – 01
Introduction

Welcome to the course on Experimental Biochemistry, which will be given by myself Professor Swagata Dasgupta and Professor (Refer Time: 00:26) of the School of Bioscience of IIT Kharagpur. I will start off with an introduction to experimental biochemistry, where we will be looking at Specific topics; specific concepts that are required to understand biochemical concepts, biochemical techniques particularly in the laboratory.

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For example, what do we mean by accuracy and precision? Measurements that we take, if we ask the question are they accurate, or are they precise, do these 2 mean the same things. Another very important aspect is about acids and bases, about pH, measurements of pH, and of course about buffers. We will go gradually today, and then in the next lecture we will learn about the different types of buffers, how they can be prepared. And each of these concepts, or each of these specific topics that we speak about will be demonstrated in the laboratory.

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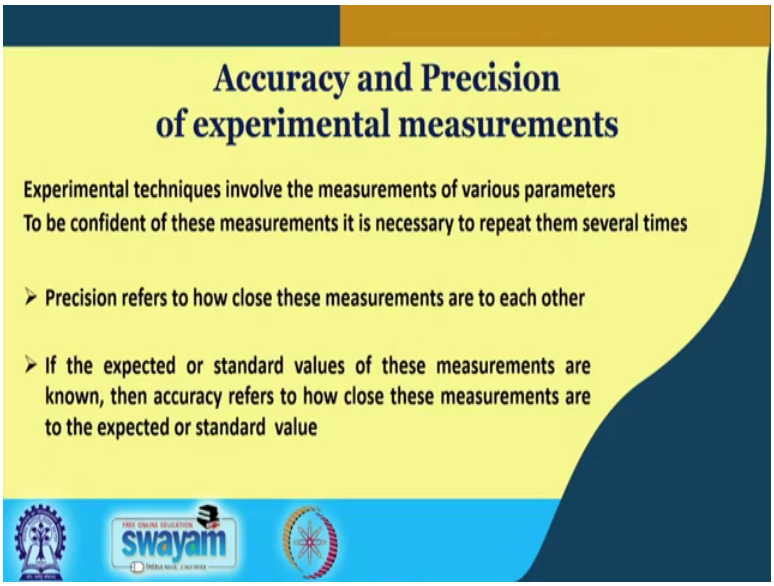

If we talk about accuracy and precision, we want to know that when we conduct a specific experiment, this experimental technique is going to involve the measurements of various parameters; whether it is just the volume of the sample we take, whether it is the temperature we are measuring or the pH that we are looking at. To be confident of these measurements, we have to repeat them several times so that they are reproducible. We call these reproducible results; they are obtained by repeating our results.

Precision refers to how close these measurements are to each other. If the expected or standard values of these measurements are known, then we say that the value is accurate. If I know that I have to measure a volume of 10 ml, and I get a volume of 10 ml when I measure this, then I know that I have had or I am getting an accurate measurement.

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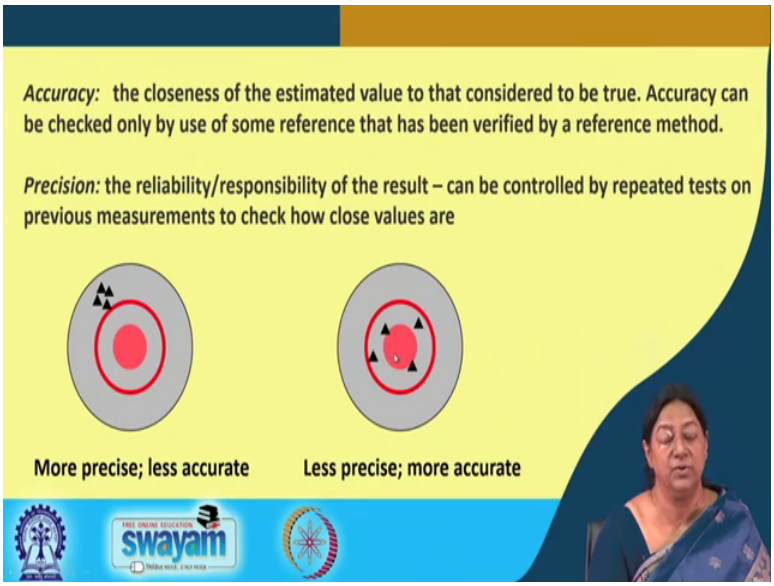

So, accuracy is the closeness of this estimated value which is considered to be true. Accuracy can be checked only if there is a certain reference value which it can be verified against. Precision is the reliability or the responsibility of the result that we get, and it can be controlled by repeated tests on previous measurements to check and to see how close these values actually are.

So, when we are talking about a target, if this red dot happens to be the target then here we see that the measurements that we have taken are more precise, but they are not close to the target. So, we call this a more precise value, but a less accurate one. On the other hand, these measurements of this set of measurements taken show that we have less precise values, but they happen to be more accurate.

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Now, we need a sense of accuracy a sense of precision in the biochemistry laboratory, because inaccuracy and imprecision can occur as a result of using standards or reagents that are not reliable.

In correct instrument calibration, poor techniques such as improper dilution, and a use of a method or technique that is not specific for the test that is being conducted. So, it is extremely important in the biochemistry laboratory apart from the safety measures that have to be taken into consideration, an understanding of what values what considerations and what parameters need to be specified or need to be measured in order to get a good experimental result.

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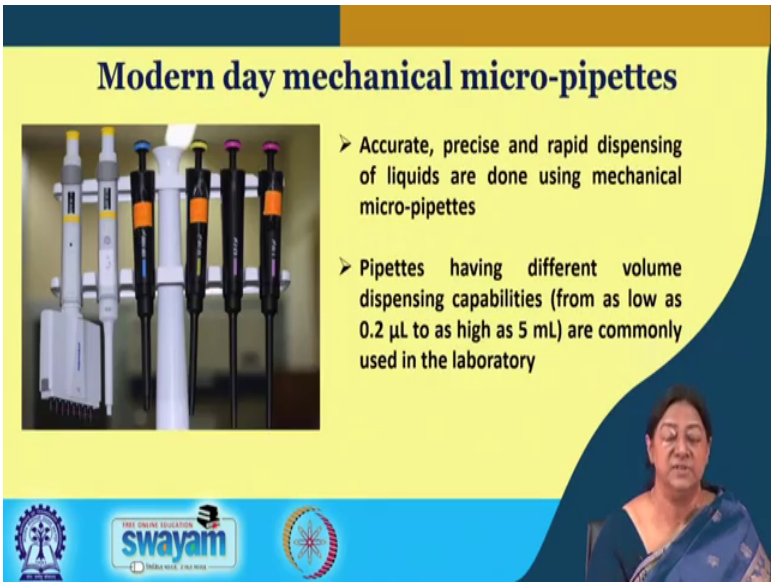

For example, we have modern day mechanical micropipettes. These will be shown to you in the laboratory. These are accurate, precise and rapid dispensing of liquids are possible using these mechanical micropipettes. They have different volume dispensing capabilities from as low as 0.2 micro liters to as high as 5 milliliters. These are the ones that are commonly used in the laboratory for dispensing biochemical samples which are usually less available in the sense that the volumes that we need are very minute, or they are very less volumes that we will we do require.

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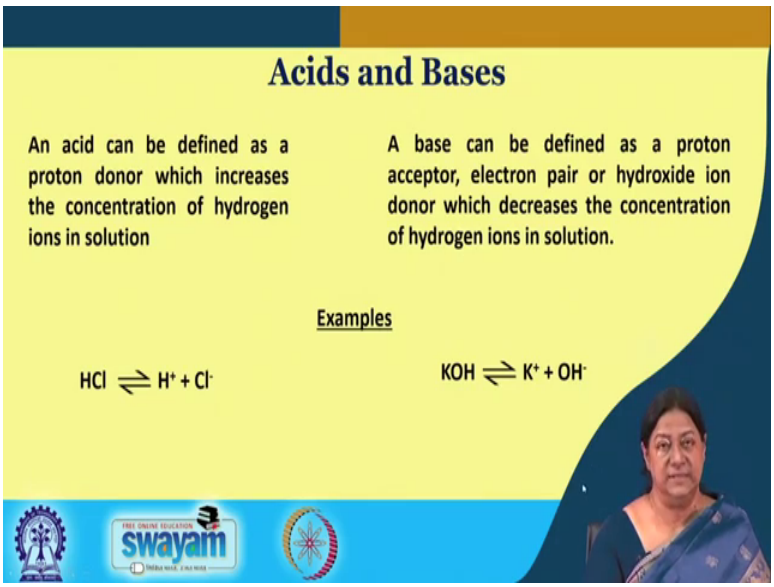

If we look at acids and bases; now, before we get into what we mean by buffers, before we get into how we can prepare the specific buffer solutions, we need to have a very basic idea of what we mean by acids and bases. This is something all of you have done in school, but we know that we can have an acid defined as a proton donor in the most basic sense, and we have a concentration of H plus in solution. If we look at a base it can be defined as a proton acceptor electron pair or hydroxide ion donor.

Now, what can we understand more using the acids and the pH?

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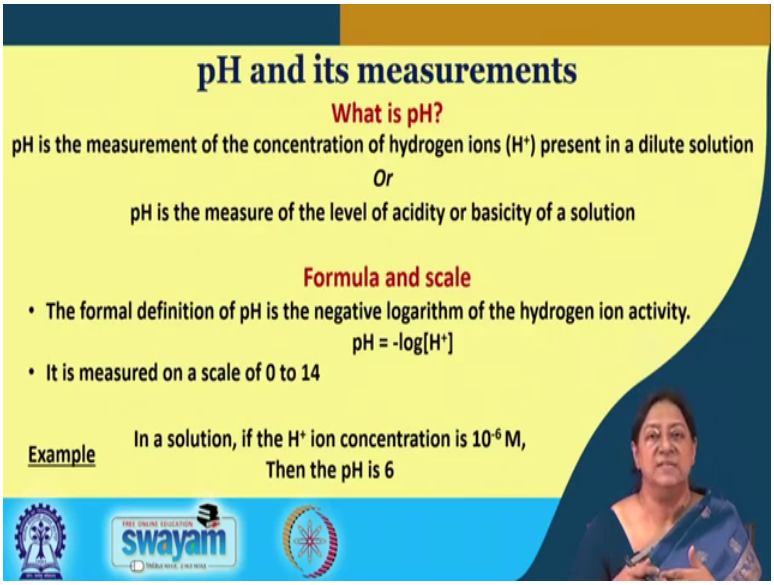

When we have acids we have a hydrogen ion concentration. This hydrogen ion concentration the measurement of this hydrogen ion concentration it is called the pH. This pH is actually the measure of the level of acidity or basicity of a solution, and there is a specific formula by which we can calculate what the pH of the solution is; and the formal definition of the pH is given here as the negative logarithm of the hydrogen ion activity. And when this solution is dilute we say that this is the negative logarithm of the hydrogen ion concentration.

It is measured on a scale from 0 to 14. For example, in a solution if the hydrogen ion concentration happens to be 10 to the minus 6 molar, then if we plug the value of 10 to the minus 6 molar for the hydrogen ion concentration, we get a value of pH that is equal to 6.

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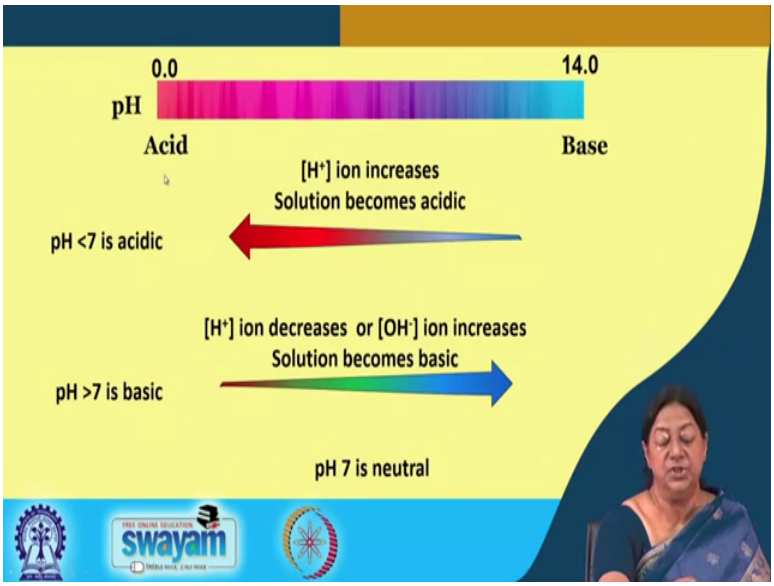

This pH range can go from 0 to 14, and as the pH becomes less than 7, pH 7 being neutral, we have increasing hydrogen ion concentration which means that the solution becomes acidic. On the other hand, when we go from this direction toward the basic direction we have the solution becoming basic meaning that the hydroxide ion concentration is increasing and the H plus ion concentration is decreasing and pH 7 corresponds to a neutral value.

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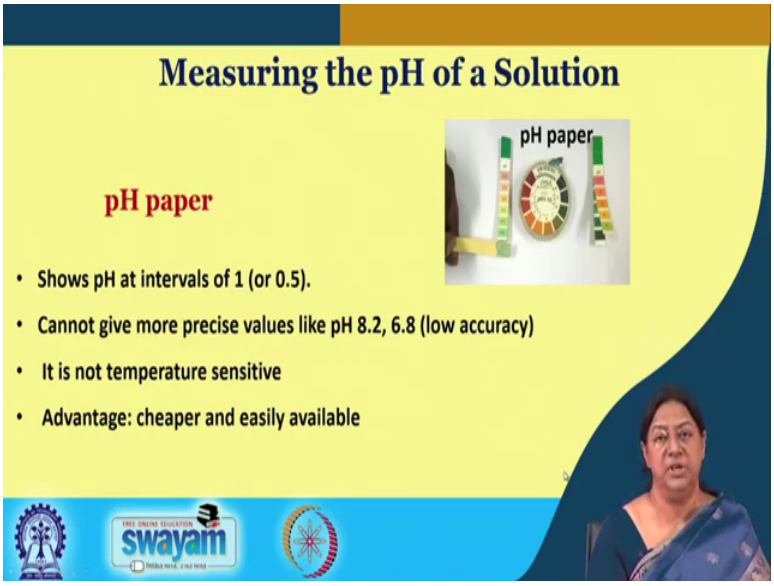

If we want to measure the pH of the solution, a crude way to measure the pH of the solution is the use of pH paper. The use of pH paper we can have a pH roll like this, where which shows pH of intervals of 1 or 0.5 or pH paper. But, it does not give us precise values that are required for biochemical experiments. And it is not temperature sensitive, but the advantage of that it is cheaper it is easily available and it is gives us a rough estimate of what the pH of the solution is.

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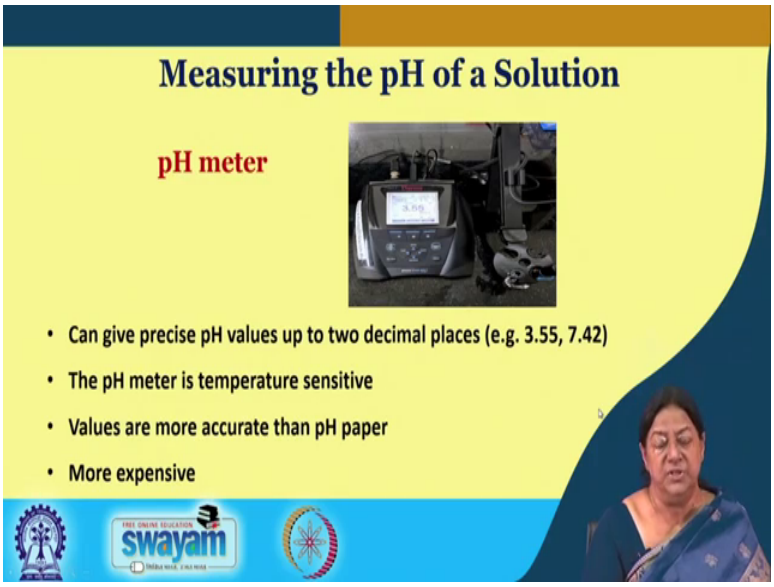

We use a pH meter the use of a pH meter will be demonstrated to you. This can give precise pH values for even up to 2 decimal places, which is sometimes required for biochemical reactions. The pH meter is temperature sensitive and we get values much more accurate than the pH paper, but it is more expensive.

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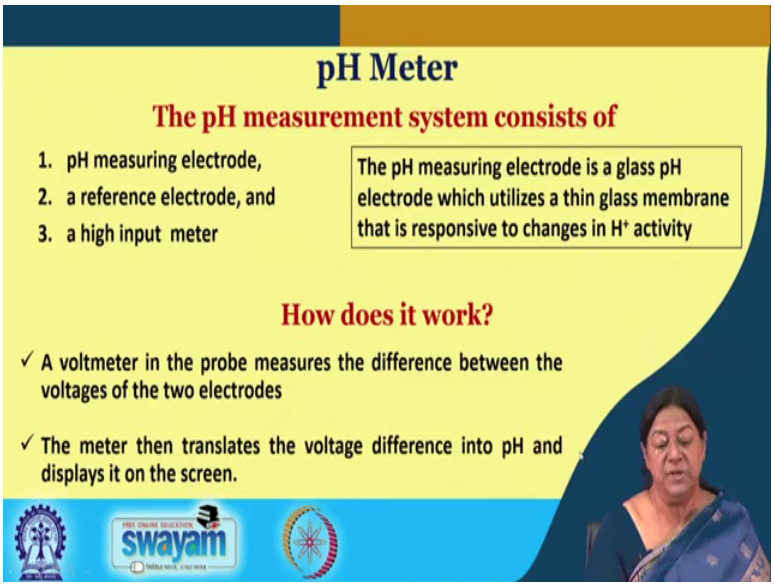

If we look at the pH meter, the pH measurement system in this case consists of a pH measuring electrode a reference electrode and an input meter.

The pH measuring electrode itself is a glass pH electrode which utilizes a thin glass membrane that is responsive to changes in hydrogen ion activity which is why it can measure the pH changes. It works by a precision system where we have the voltmeter in the probe that measures the difference between the voltage of these 2 electrodes, and the meter then translates this into a pH value that is demonstrated and given on this displayed on the screen.

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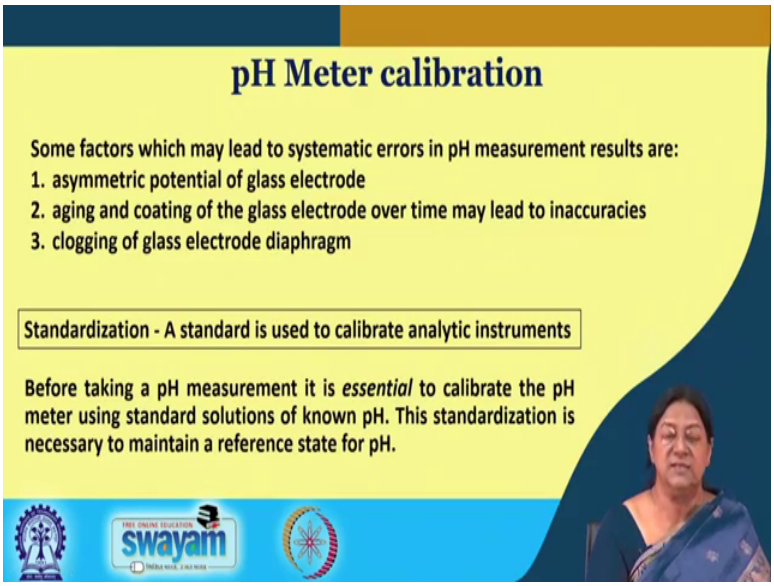

The factors that sometimes can lead to improper pH measurements is if the glass electrode is not stored properly, if the glass electrode is aged has is old or there is coating on the glass electrode and clogging of the glass electrode diaphragm. So, it does not measure the change in the hydrogen ion concentration, and because of this it does not measure a change in pH.

Now before we take a pH measurement using a pH meter, it is necessary to standardize the pH meter. Now what do we mean by standardization. This is a term that we will be constantly referring to as standardizing an instrument, when we standardize an instrument it means that we use a specific standard to calibrate an analytical instrument. So, how do we calibrate a pH meter? To understand this, we need a reference pH solution.

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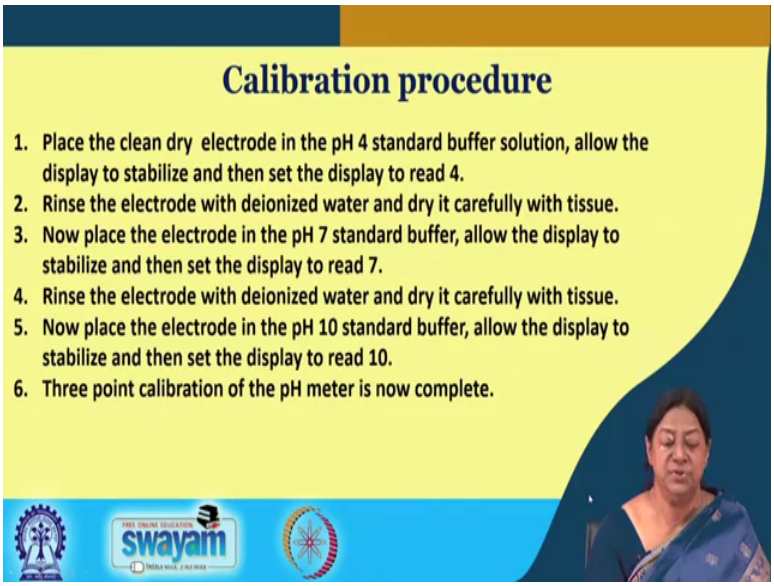

In the calibration procedure, we place the clean dry electrode in a pH 4 standard buffer solution that is provided by the vendor or it is provided by the company that gives you the machine where you get pH 4 7 and 10 tablets, that have very precise instructions as to how they are to be dissolved in water. And you measure the pH of this and calibrate your machine so that it reads the pH 4. So, you rinse the electrode with de ionized water and dip it in the solution of pH 4 and calibrate this means you rotates the dial so that it reads pH 4.

Then we rinse the pH electrode again will de ionized water and this time dip it into a solution of pH 7 a standard solution of pH 7; the dial is then again rotated so that the display reads pH 7 again it is rinsed with de ionized water and dried again with the tissue and placed in pH 10 standard buffer that allows the display to stabilize to reach the value of pH 10; which means that once this procedure is completed we have a 3-point calibration of the pH meter now complete. And it is now ready for use to determine the pH of any solution that we might want to do so.

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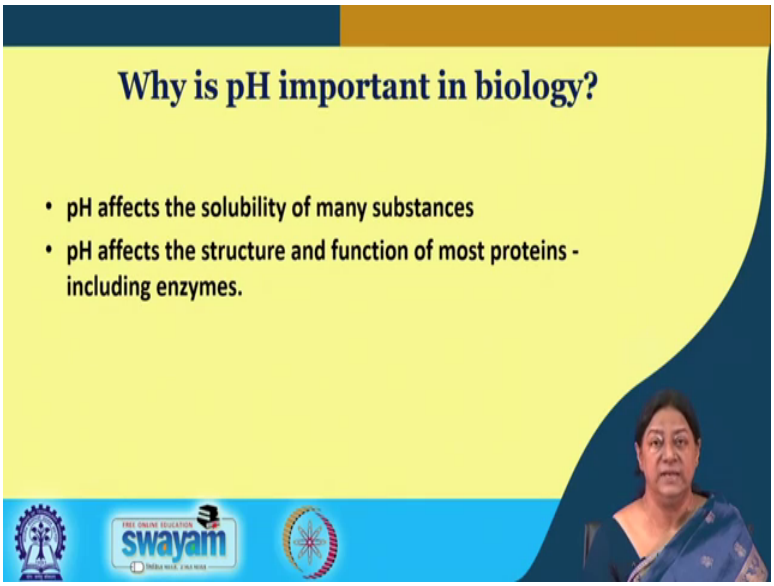

Now, why is pH important to biology? If we talk about biological systems, we know we are talking about proteins we know we are talking about DNA nucleic acids all these are biological macromolecules. Now biological macromolecules are very sensitive to pH, because they have in them moieties or they have in them amino acids nucleic acids that have the bases in them the nitrogen base nitrogenous bases, the hydrogen bonds the sugar phosphate backbone, each of these are very sensitive to changes in the environment in terms of the acidity and the basicity, and the pH affects the solubility of many of these biological substances.

It also affects the structural function of most proteins including enzymes, which we will look at later on during the course.

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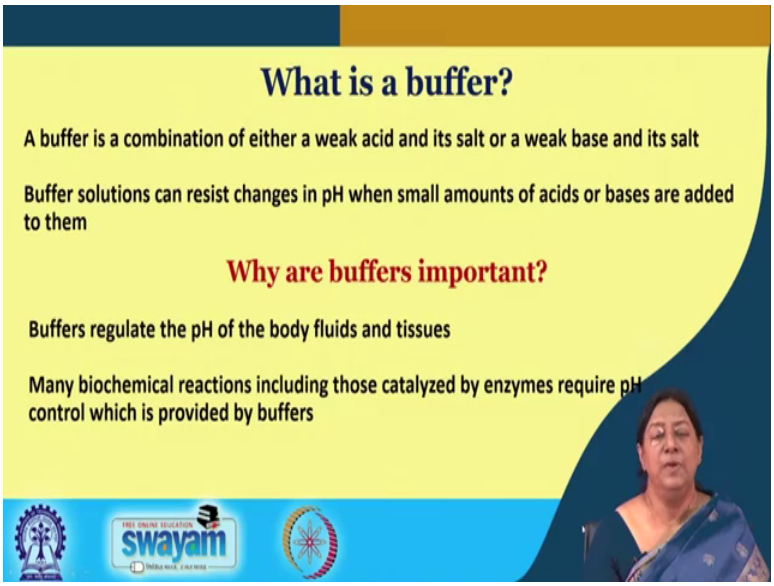

Now, when we come to a pH in terms of how we want our biological system to work, we need what is called a buffer solution. Now a buffer is a combination of a weak acid and it salt or a weak base and it salt. Buffer solutions actually can resist changes in pH when small amounts of acid or base are added to them.

Now, why are buffers important? Buffers actually regulate the pH of body fluids and tissues. The physiological pH or our blood pH is 7.4. Now addition of a small amount of acid, addition of a small amount of alkali does not change the pH to a very drastic amount or through a very drastic or very large amount, but it has what is called a buffering capacity that maintains the pH around 7.4. Now many biochemical reactions including those catalyzed by enzymes require pH controlled. Each enzyme has an optimum pH where it will work the best; an enzyme is a biological catalyst.

We will see how enzymes work later; we will do specific experiments with enzymes. And we will see how the pH dependence is extremely important much like the temperature dependence in the workings of a biochemical system.

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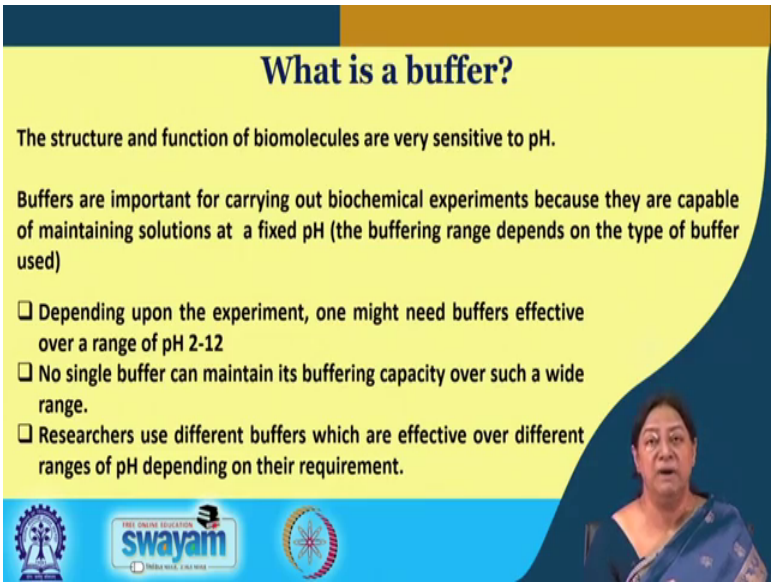

So, if you look at the buffer, it is the structure and function of bio molecules that is very sensitive to the pH, and the buffers are important for carrying out these biochemical experiments, because they are capable of maintaining solutions at the fixed pH. So, if we have a specific biochemical experiment that we are conducting.

We wanted to be at the specific pH of our experiment. For this we have to use these buffers. Specific buck buffers that are used for biochemical experiments, that will be demonstrated to you and will be explained to you as to how you can actually calculate the amount of salt required to determine or to prepare specific buffer solutions. Depending upon the experiment now, we might need buffers that are effective of our range of pH 2 to 12; there is no single buffer that can actually maintain it is buffering capacity to such a large extent.

So, depending on the type of experiment that you are conducting, depending upon the type of system you are studying, you will have to choose the buffer accordingly. And this buffer has to be effective over different pH ranges depending on the specific requirement of the experiment being conducted.

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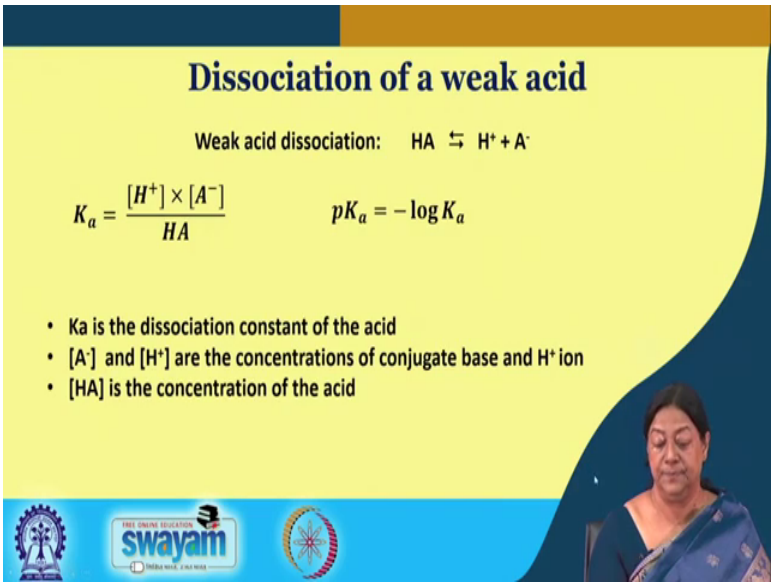

So, let us see how we can actually determined. First of all, what we mean by a buffer solution? And how I should determine which buffer solution I might want to use.

If we look at the dissociation of a weak acid, we understand that we have a weak acid designated by H A that has an equilibrium H plus and A minus being a weak acid, it does not dissociate or ionize completely. There is an equilibrium constant associated with disassociation given by the concentration of the H plus, the concentration of the conjugate base A minus and the concentration of H A which is the acid.

Now, if we look at the pK a value this is a specific constant for a specific type of acid, because this dissociation measures an equilibrium. And this is the K a is the dissociation constant of the acid, and A minus H plus are the concentrations of the conjugate base and the H plus ion, and H A is the concentration of the acid.

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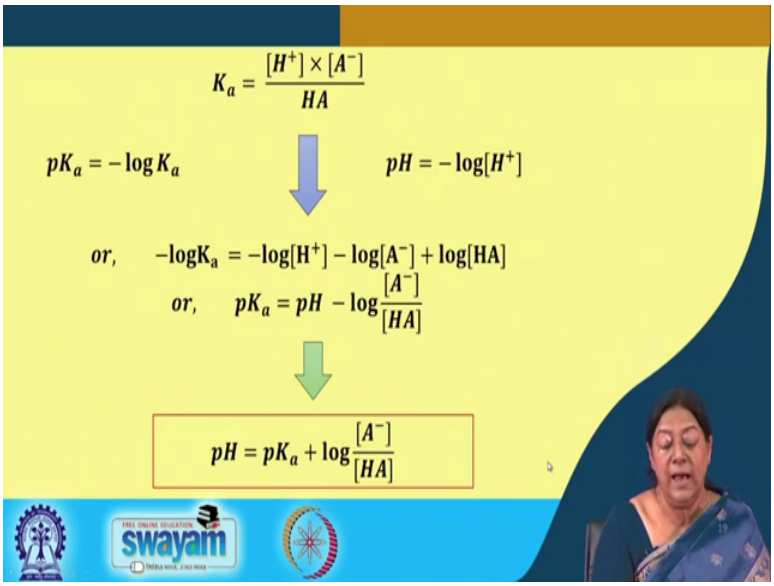

So, when we look at a value, considering the K a the H plus concentration we know is defined by the negative logarithm of the H plus concentration is defined or we define it as the pH. So now, we can reorganize this equation into a system where we say that minus log K a is going to be equal to minus log H plus minus log A minus plus log H A which is nothing but a bit of algebra on this specific expression.

What this works out to be is pK a equal to pH minus log A minus H A. We rearrange this to give us an equation that tells us that the pH is equal to the pK a plus the log of the concentration of the ratio of the salt or the conjugate base concentration divided by the acid. Now this is an extremely important relationship in our studies.

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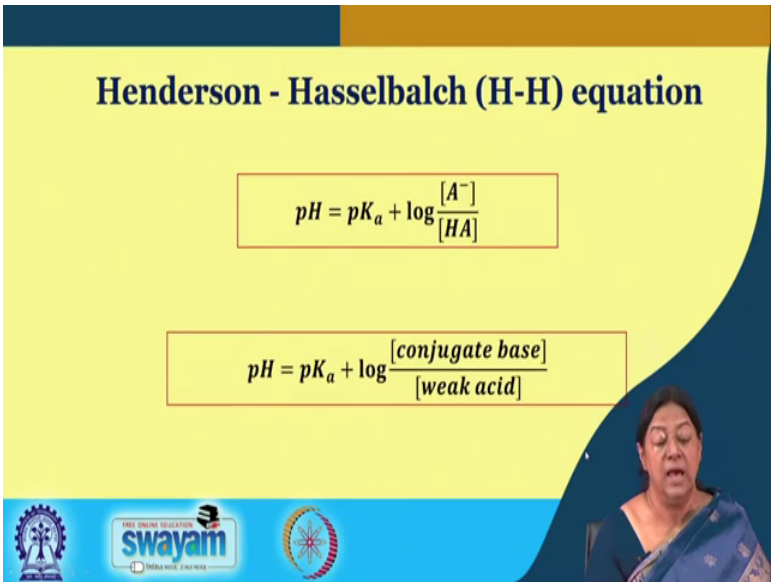

It is called the Henderson-Hasselbalch equation, and it is used extensively in determining the pH of buffers, and to determine the concentrations of salt or acid required to prepare specific buffers of specific pH with a knowledge of the pK a of the specific acid concerned.

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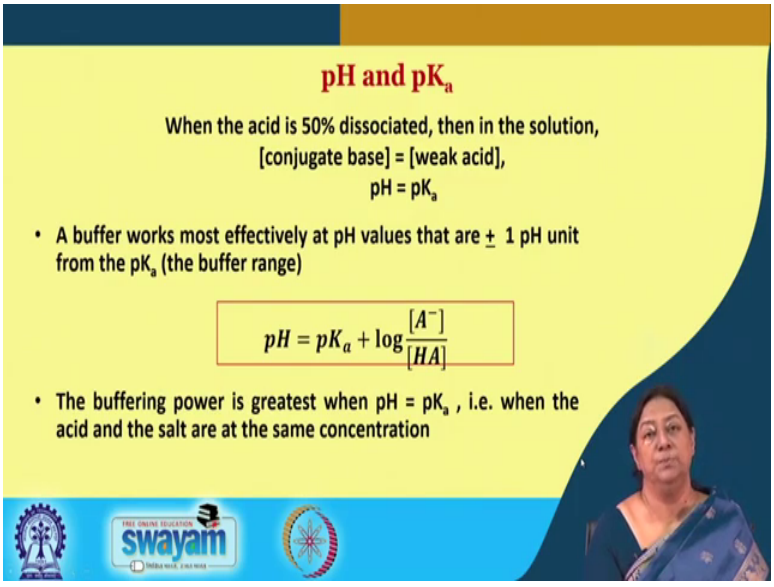

So, let us look at what pH and pK actually mean. So, if I have the expression as we just looked at the pH being equal to the pK a plus log A minus by H A. The question is what information can I get from this. First I see that if I have 50 percent association, this

means that the concentration of A minus and the concentration of H A are equal. This means that the pH is equal to pK a, so when the pH is equal to pK a. I say that the concentration of the conjugate acid and the concentration of the acid the conjugate base and the acid are equal. And the buffering capacity which will be just talk about in a moment is greatest when the pH is equal to the pK a.

We also mentioned that a buffer works most effectively at pH values that are plus minus 1 pH unit from the pK a we will see what this means in a moment.

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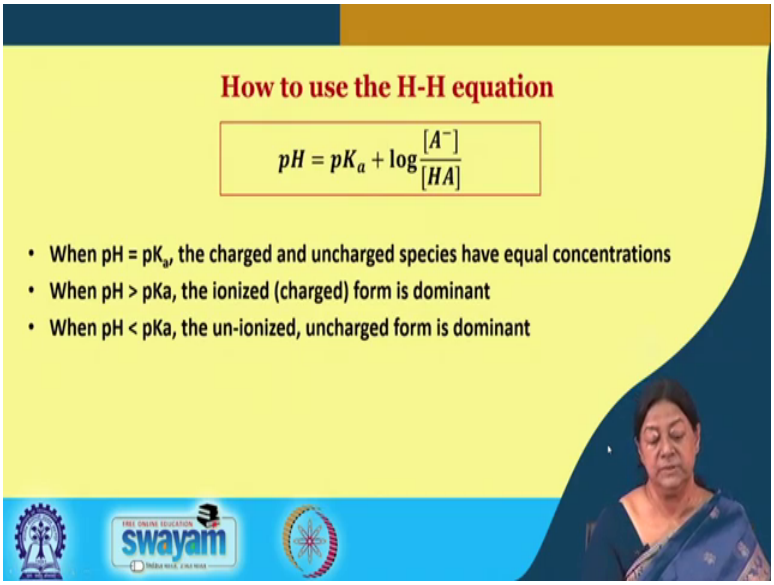

When we have this specific equation, we learnt that when we have the uncharged species that is H A and the charge species in equal concentrations then the pH is equal to the pK a. When the pH is greater than the pK a, the ionized form is dominant; that means, there is more of the ionized form then the un ionized or uncharged form making the pH greater than the pK a.

When the pH is less than the pK a, this means that, the uncharged or the denominator in this case H A is dominant. So, when we have pH is equal to pK a the charged and uncharged species have equal concentrations. When the pH is greater than the pK a, the ionized or the charged form is dominant, because this makes this concentration higher rendering this a positive quantity; which means, that the pH is greater than the pK a.

Again, when the H A concentration is higher than the A minus this becomes a negative quantity making the pH less than pK a.

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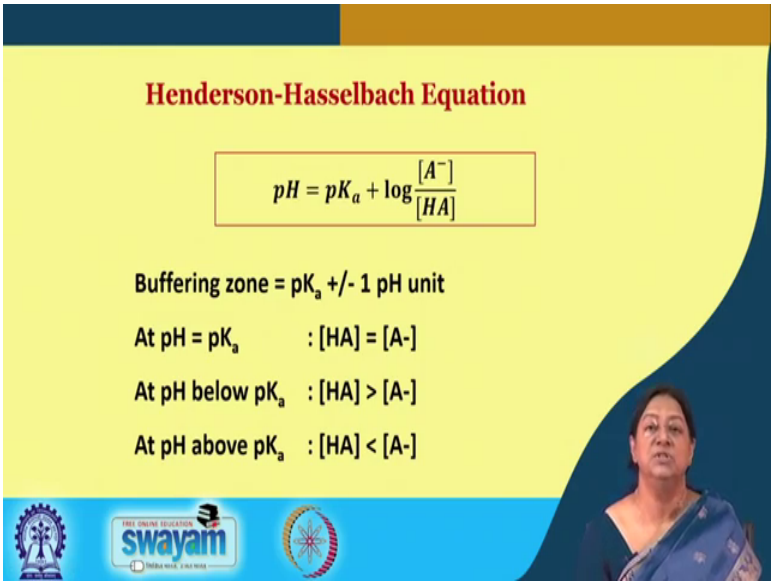

So, we have what is called a buffering zone. When there is 10 percent dissociation or 10 percent of A minus formed from HA if we say this is 10 percent and this is 90 percent, then this amounts to a quantity that is about pK a plus rather one we make a ratio of 10 by 90 so 1 by 9.

So, pK a plus 1 by 9 making this a negative quantity which makes it around pK a minus 1 approximately. If we consider that there is 90 percent dissociation meaning that A minus is 90 and HA is 10. So, we have a ratio of log 90 by 10, meaning around log 9, then this means this is around a pK a plus 1.

So, we say that the buffering zone in this specific quantity or in this specific situation is such that if the pH is equal to pK a we understand that H A is equal to A minus. If the pH is below the pK a then H A concentration of H A is greater than A minus, at pH above pK a H A is less than A minus. And the understanding that we have 10 percent dissociation on either side would mean that the buffering capacity would work from the pK a to plus minus 1 pH unit.

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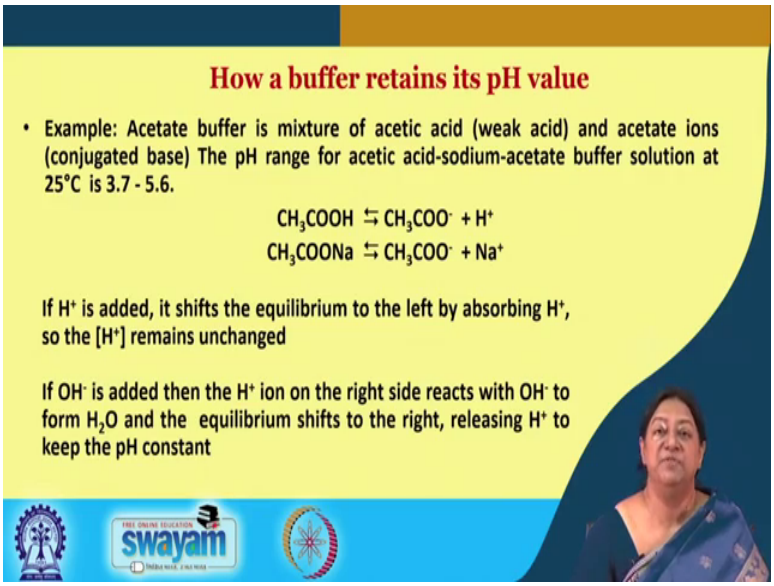

Now, how does a buffer retain it is pH value. For example, if we consider the acetate buffer. The acetate buffer is a mixture of acetic acid or weak acid and acetate ions that is the conjugate base. So, in this case, we can have an acetic acid sodium acetate buffer solution. So, the pair in this case is acetic acid and sodium acetate. The acetic acid dissociation is such that CH 3 COOH dissociates into CH 3 COO minus plus H plus CH 3 COONa that is sodium acetate again dissociates into CH 3 COO minus plus Na plus.

Now, if we look at what has been written here, the pH range for this particular buffer solution is between 3.5 to 5.6 at 25 degree centigrade; meaning that the pK a value of acetic acid is in between this. Because the range we know that the buffering range is going to be pK a plus minus 1. Now what happens if a small amount of acid is added? A buffer is supposed to withstand this small amount of acid addition or a small amount of alkali addition. So, if we add a small amount of H plus, all we add a small amount of oh minus, the buffering capacity of this pair of the weak acid and it is conjugate base or the weak base and it is conjugate acid should be able to counteract the addition of this small amount of acid or the small amount of oh minus.

For example, if H plus is added in this case, the shifts the equilibrium to the left by absorbing H plus. So, the H plus itself is going to remain unchanged. So, when I add H plus, the system is going to shift to the left, releasing or reducing the quantity of H plus in solution. When oh is added, there is OH minus that adds to the acetic acid oh minus

that reacts with the H plus to form water again maintaining the pH of the solution. It is important to realize that when we consider these specific solutions the specific understanding, that the buffer solution that we are going to consider the measurements of buffer solutions that we are going to make. The preparations of buffer solutions that we are going to show you depend upon this equation here.

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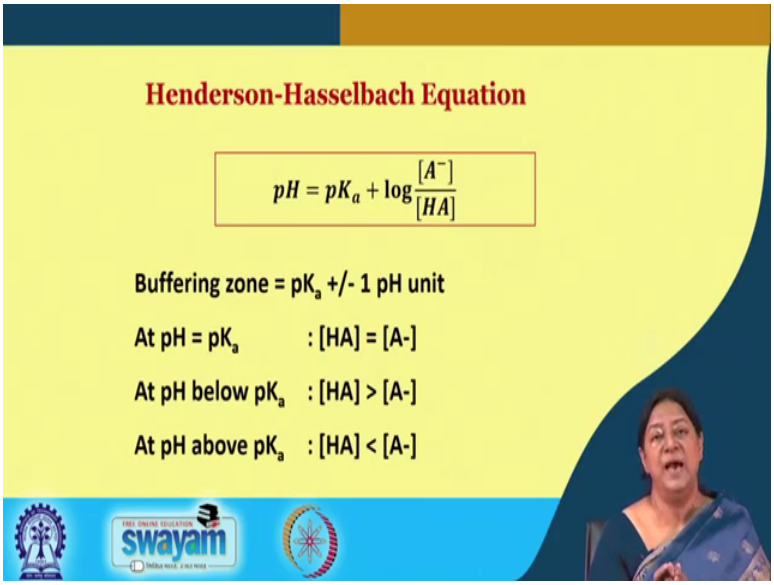

So, if we have a specific pK a of a solution, and we know the concentration of the A minus or the concentration of the H A the dissociation 10 percent, 20 percent, 30 percent; depending upon this dissociation, we can measure the pH of our solution. There are some acids that are going to have multiple H plus ions that are dissociating. For example, if we look at phosphoric acid.

In phosphoric acid we have H 3 PO 4. So, the salts that can be formed are going to be disodium hydrogen phosphate, we can have mono sodium dihydrogen phosphate. And we will see how this phosphate buffer can be prepared, and how this phosphate buffer is extremely important in the many biochemical systems or biochemical reactions that go on despite some disadvantages that it does have.

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So, we conclude by saying, that we have accuracy and precisions that are very much essential for experimental measurements. Especially, when we are looking or we are analyzing solutions that are of extremely small volume. We understand that when we look at biochemical reactions, when we look at biochemical systems, the volumes that we are working with are extremely small. So, the accuracy and the precision the use of the micropipettes, the use of specific instrumentation is extremely important.

pH measurements are another very important aspect of a biochemical systems biochemical reactions, and studies in the biochemical laboratory, in a research laboratory, in a teaching laboratory. Understanding or determining how the pH changes, how pH can be measured, and how we can prepare solutions, prepare buffers of specific pH values that are going to be conducive to the experiments that we are conducting with the knowledge that the bio systems that we are working on are extremely important in the biochemical aspects that we are studying.

Finally, the choice of the buffer, as I mentioned before understanding which buffer to use, which system we are studying; this is extremely important in our understanding of how we are going to look at our specific bio systems, specific proteins, specific enzyme catalysis reactions and so on.

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The references that we will be following for the course are; Modern Experimental Biochemistry by Boyer, and a specific paper related to the Hydrogen Ion Buffers for Biological Research that gives you an idea of how the different buffers are made, and how they can be prepared. We will be looking at some of these in our next lecture.

Thank you.