Experimental Biochemistry
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Lecture – 35
Enzyme Kinetics II

We continue our discussion on enzyme kinetics and enzyme mechanisms, where we in the last class we understood how enzymes work, they are biological catalysts, they are proteins and they work in such a manner that they have a specific active site. And this active site has a geometric complementarity as well as a chemical complementarity with the substrate that in binds.

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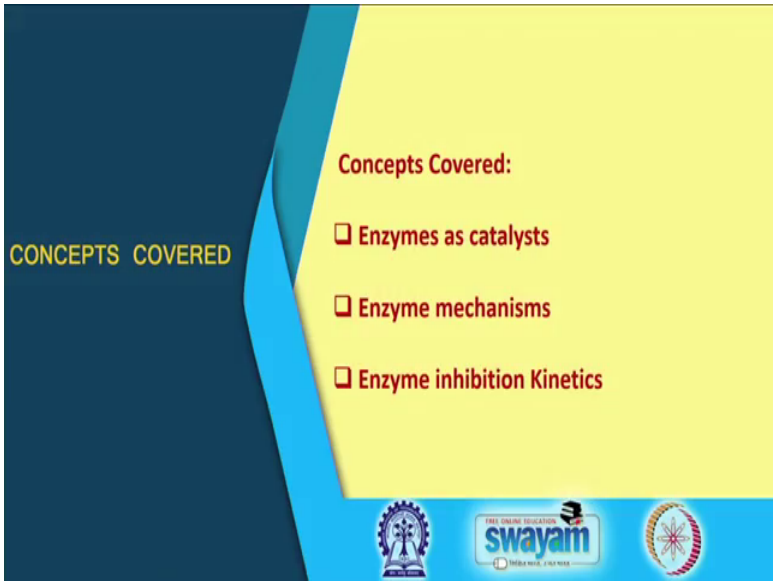

If we look at the concepts that are going to be covered in this class, we learned about enzymes as catalysts, their specific enzyme mechanisms that we are just going to look at to see how specific enzymes actually are in their way of action, and then we will look at enzyme inhibition kinetics. And I will briefly touch upon the experiment that will be demonstrated to you for this specific enzyme kinetics chapter.

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So, if we look at the active site of the enzyme as we looked at in the last class, we have this active site is lined with particular amino acid residues and it may contain a co-factor. The active site it has a specific charge to it, a hydrophobicity, some flexibility, and some reactivity, because it has to undergo a biochemical reaction. So, the substrate will sit in the active site, and it will form what is called we learned in the last class an enzyme substrate complex.

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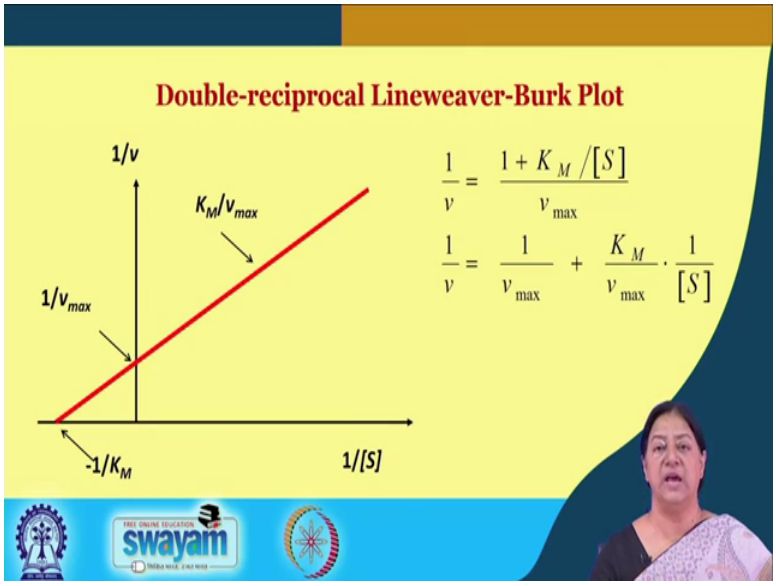

And what do we learn from this? We learn from this that we have a specific double-reciprocal plot. And what information can we get from this? The information that we can get from this is the maximum velocity that can be achieved and the Michaelis constant, Michaelis-Menten constant of the enzyme constant that will tell us something about the enzyme and something about the enzyme substrate complex, and something about the whole catalytic reaction.

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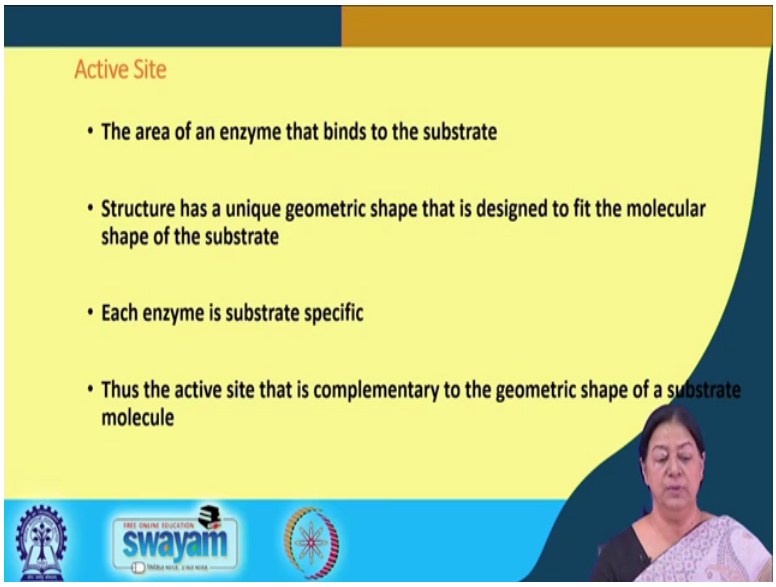

Now, as we proceed further, we will try and understand apart from what the active site is. Now, if we ask the question what is so important about the active site, it is important because this is the place, so this is the area the region on the protein that the enzyme will bind to the substrate. The structure has a unique geometric shape up as we have seen; and each enzyme is substrate specific. In addition to this, we will have specific geometric complemantarity like a shape complementarity.

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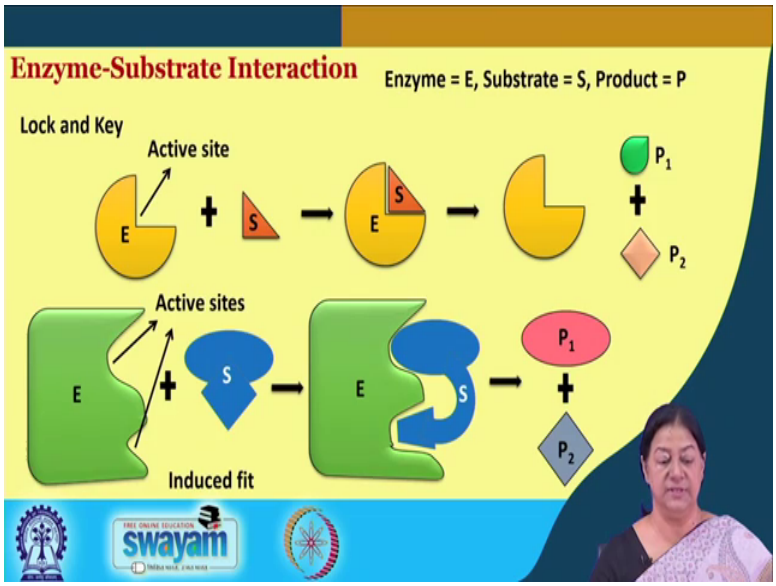

There are two different methods that we learned in the last class or two different models. One is the lock and key model, and one is the induced fit model. In the lock and key model, the active site will take in the substrate in a specific manner and release the product, and then the enzyme will be back to its original form to take in another substrate molecule. In the active site for the induced fit model, there is a certain flexibility that has been given to the enzyme as well as the substrate for an adjustment, for an accommodation purpose for the substrate to the enzyme active site.

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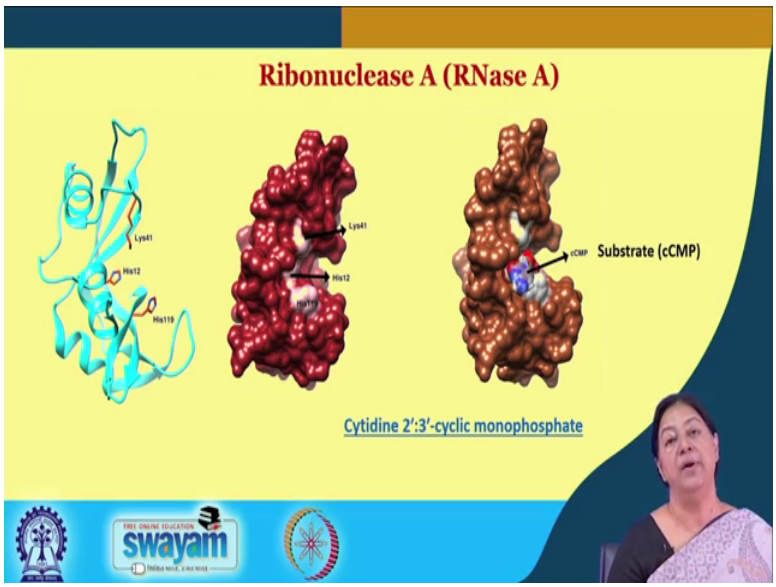

In the specific example that we looked at in the last class this is ribonuclease A. Ribonuclease A is a protein that is involved in our RNA degradation. Now, it is an extremely important protein in the body. And if you can this is the active site, these are the most important residues in the active site, the histidine 12, the histidine 119 and the lysine.

Now, what do we see about these amino acid residues? We see that they are basic in nature. And what does that mean? If they are basic in nature, then what is going to likely bind there something that is going to be negative in charge, because there has to be a complementarity in the charge, in the p k, in the shape and so on and so forth. So, if we look at the basic pocket, where we have the substrate that is going to bind, this is a specific substrate of RNase A; we call it cCMP. Here you can see this is ribonuclease A bound to the substrate. And what do we see? We see a perfect fit in the active site of the protein.

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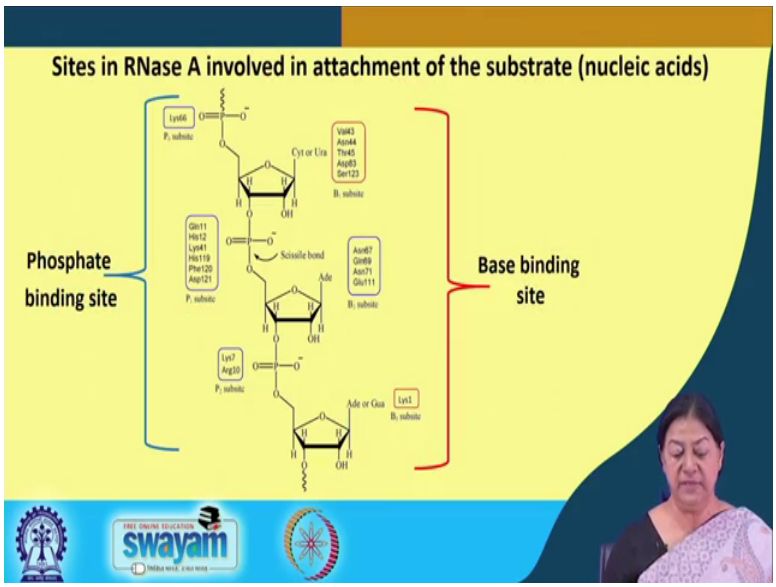

If we go a step further a bit of chemistry, if we look at the sites in ribonuclease A that are involved in the attachment of the substrate which is a nucleic acid, this is RNA. If we look at the RNA we see that the histidine 12, the histidine 119, and the lysine 41 that we talked about is in what is called the phosphate binding site. This we see is negatively charged. What does it mean? It means that is a complementarity, and this is extremely important in its mechanism.

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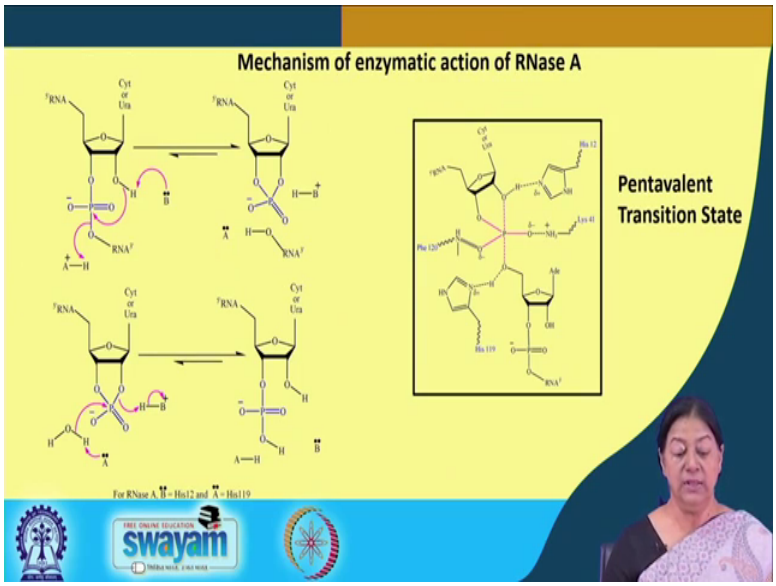

When we look at the mechanism of the protein without going into much detail we have the histidine and we have the two histidines that are involved. For example, here B is histidine 12, A is histidine 119. So, this is where the bases; this is where the acid is. So, in the first step of the catalytic reaction histidine 12 behaves as a base. In the second step of the reaction, it reverses its role. Why does it do that, because if it has now contributed as a base it has to get back to the original form of the histidine to be able to take in another substrate molecule as we learned in the last lecture.

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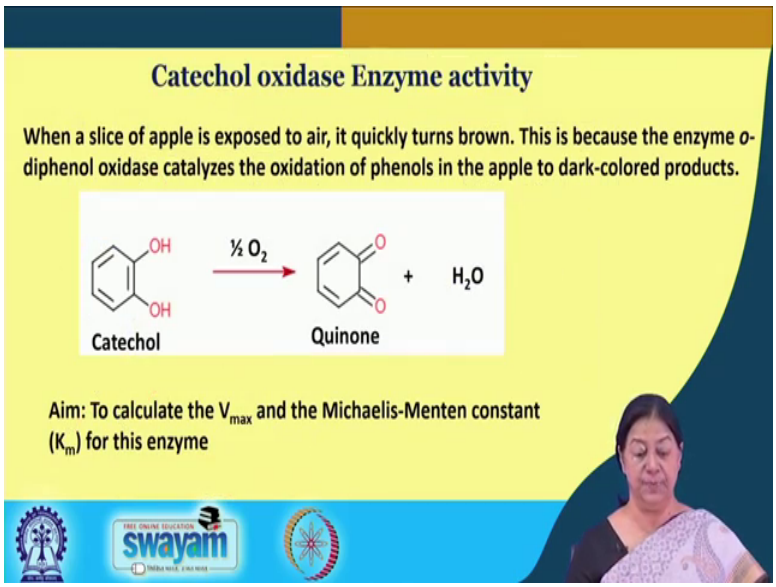

In the catechol oxidase enzyme activity, the reaction or the experiment that is going to be demonstrated to you. If you look at the slice of apple, all of you have eaten an apple, if you keep the apple exposed to air; what happens? It turns brown. Now, why does it turn brown there are enzymes that catalyze a reaction in the apple, there are polyphenols. So, we have poly phenyl oxidases. What do they do? They oxidize the polyphenol to dark-colored products. And what we what we can do is, then we can take an apple, we can look at the enzyme present in the apple, and what is the substrate without going to the details of the structure, but just an understanding of what is happening. So, if I say that it is a polyphenol oxidase, this catechol is a polyphenol.

What do we mean by a phenol, when we have these OH groups? So, I can take catechol as a substrate and then monitor the formation of the dark-colored product. And how will I do that? I will do because there is a color formation, I can do this in a spectroscopic manner. So, we aim here would be to find out the V max by the Michaelis-Menten and the Michaelis-Menten constant K m for the specific enzyme.

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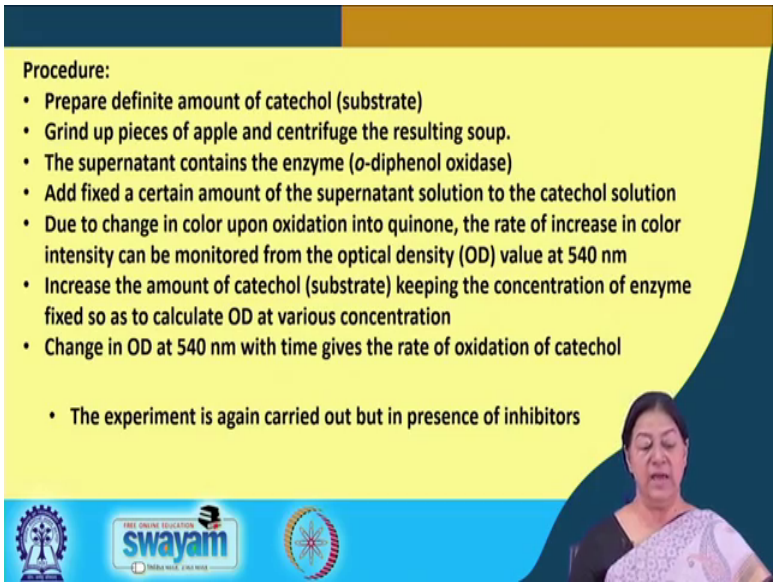

So, the way the reaction or the way the experiment is going to be done we have to prepare a definite amount or definite concentration of the catechol solution. Grind up pieces of apple and the resulting soup that you get is going to be basically your enzyme the solution. Now, the enzyme has an enzyme called ortho-diphenyl oxidase. Then what is going to happen is we are going to take the enzyme, add the substrate to it. And as the

dark-colored product is formed, we are going to monitor, that is means we are going to measure a parameter that is going to tell us that the dark-colored product is being formed. And this will be monitored by the optical density value that is the absorbance value at 540 nanometer.

So, it is a spectroscopic determination of seeing how much of the browning of the apple has occurred with time because we are following or we are doing an enzyme kinetic reaction. Then we increase the amount of the substrate that means, as we looked at a specific reaction where we have the velocity on the y-axis, and the substrate concentration on the x-axis. What does this tell us? This tells us that as we increase the substrate concentration, we are going to look at each of these specific reactions for an initial velocity.

Then what do we do we look at a Lineweaver-Burk plot. A Lineweaver-Burk plot is what it is a double-reciprocal plot, where we will plot 1 by v versus1 by s. And we will get a linear we will get a line. From that line, we will find out what the values of V max are and K m are. Now, this experiment will be carried out again with inhibitors.

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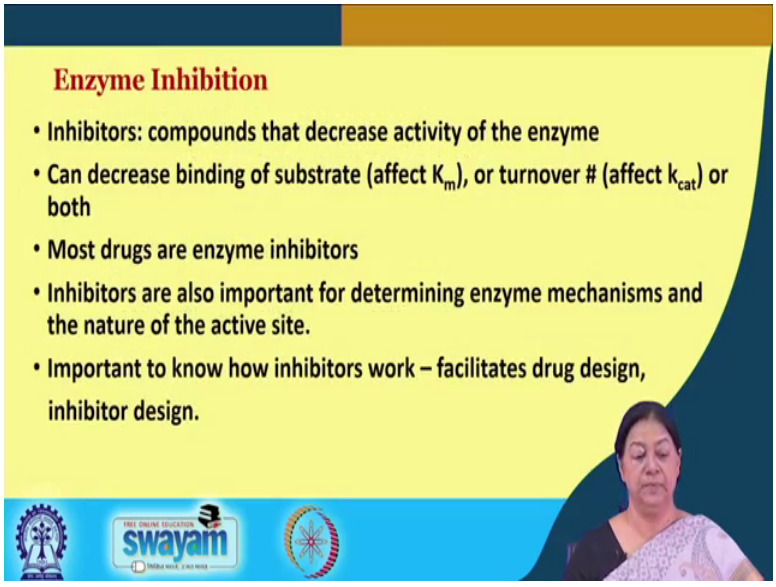

What do we mean by enzyme inhibition? Enzyme inhibition means that there are inhibitors that will decrease the activity of the enzyme; they inhibit the enzyme activity. So, what can they do or how can they do this? They can do this by decreasing the binding of the substrate because we know that if the enzyme has to have a catalytic

reaction go on, then the enzyme has to bind the substrate to its active site. If the enzyme is damaged in any way, it cannot bind the substrate. How do we damage an enzyme? An enzyme is a protein we can have we as we mentioned before, there has to be an optimum temperature and an optimum pH for a protein to remain in its folded state. And in that case, the active site will be intact.

If we have an inhibitor in this case, the inhibitor is a small molecule that may interact with the active site and prevent the catalytic reaction from occurring. If it does so, it is an inhibitor. It prevents the enzymatic reaction, the enzyme catalysis to proceed. So, what is going to happen? It is going to decrease the binding of the substrate that it is going to affect the K m value or the turnover number because it will affect product formation.

For example, most drugs are actually enzyme inhibitors. And the inhibitors are also important for determining enzyme mechanisms and the nature of the active site. And it is important to know how inhibitors work, because this facilitates what is called drug design or inhibited design. What do we mean by this? If we know the substrate, if we know the active site and if we want to inhibit the activity of the enzyme, then what we have to do is, we have to make an inhibitor, prepare an inhibitor that looks like the substrate.

So, what is going to happen? If it has the shape complementarity or it has some chemical complementarity with the substrate, it is going to bind to the active site, but the reaction may not proceed because it is not the actual substrate. So, we are making the protein or we are making the enzyme bind a molecule that is not the substrate, so the reaction will not proceed this is an inhibitor.

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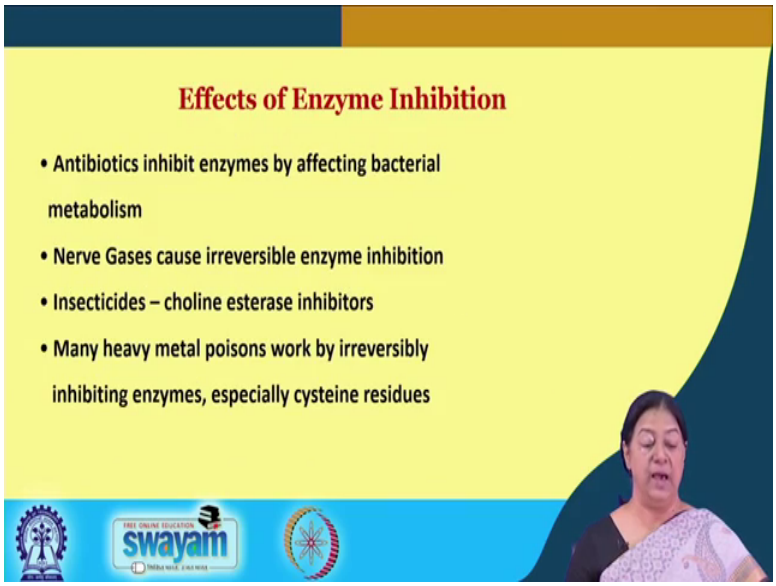

Now, what kinds of inhibitors can we have? For example, antibiotics inhibit enzymes by affecting bacterial metabolism, nerve gases called irreversible enzyme inhibition, insecticides, and heavy metal poisons work by irreversibly inhibiting enzymes. So, this inhibition of enzymes can have several effects on the enzyme, the inhibition of the products or the inhibition of the reaction, the decrease in the binding specific specificity can have effects on the enzyme structure or the enzyme way of working the enzyme mechanism.

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Now, what kinds of, what types of enzyme inhibition can we have? We can have what is called reversible inhibition. In reversible inhibition, we can have competitive, non-competitive or uncompetitive. What does this mean? Reversible inhibition means that the it will the inhibitor will reversibly bind and dissociate from the enzyme, the activity of the enzyme is recovered when the inhibitor is removed, and it is usually non-covalent in nature. What does it mean? It means that there is no bond formation. If there is no bond formation, it means it is non-covalent in nature. So, there will be a loose binding of the inhibitor with the enzyme.

The other type is irreversible inhibition. What happens in irreversible inhibition? There will be inactivation by the irreversible association with the enzyme with the formation mostly of covalent bonds. So, if there is a molecule an inhibitor that comes and binds to the enzyme and forms a covalent bond with a active site residue, then it is too strong a bond to break, it is covalent in nature, and the enzyme has been destroyed; in the sense that, the enzyme has been irreversibly inhibited. How do these work?

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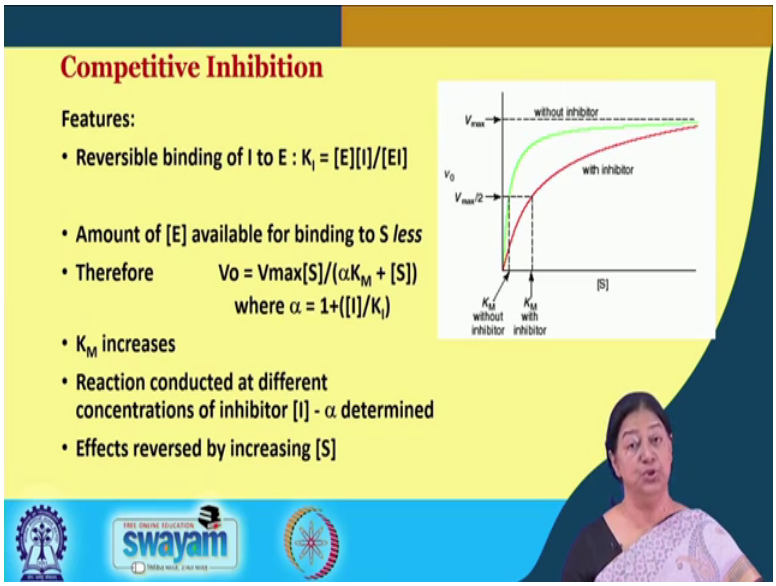

In competitive inhibition what we have is we have specific features associated with this. We will see what these features are. There is reversible binding of I to E. What is I? I is the inhibitor; E is the enzyme; S is the substrate. Now, the amount of E available for binding to S is less. Why is that, because some of the enzyme has been bound to the

inhibitor, some inhibitor has bound to the enzyme. So, the amount of enzyme left for the substrate to bind to it has decreased.

In a sense what happens, then there is going to be a specific factor that is going to be affected. What is going to be affected? The K M value is going to be affected. And what happens is we have a variation in the K M, the K M value is going to increase. And we can have the reaction or the way we proceed it can be conducted at different inhibitor concentrations as we will see to determine what is called the K I value and inhibition constant value that will tell us how effective our inhibitor is in inhibiting the enzyme activity.

If we look at how we can reverse the process, we remember that this is a non-covalent interaction which means that this is an enzyme inhibitor complex formation in a reversible fashion. So, it will form an enzyme inhibitor complex, but the inhibitor can also be moved, removed to get the free enzyme which is then available to the substrate. So, I can reverse the effect by increasing the amount of substrate molecules, as a result of which it will get to a specific value.

So, what happens is without inhibitor we have this green line with inhibitor there is a reduction in the initial velocity. Why? Because we do not have that many enzyme molecules. But if we keep on increasing the substrate concentration, there will be a time where the same V max will be achieved, because we will be able to get all the sub enzyme molecules for the reaction to proceed.

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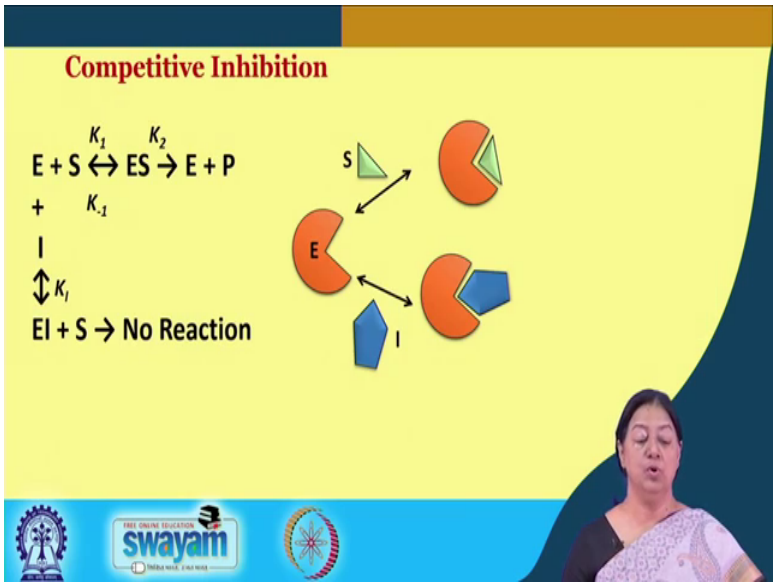

What do competitive inhibitors look like? So, this is our enzyme plus substrate forming the enzyme substrate complex that is then going to form the enzyme plus the product. So, this is our regular and Michaelis-Menten kinetics, but now we have an inhibitor. The inhibitor will also bind to the enzyme and form what is called an E I. Now, when this E I is formed, then it no longer can bind the substrate. So, if we look at this in this schematic, we have the enzyme molecule, the substrate comes and binds to the enzyme active site, this will form the product, this is the inhibitor.

When the inhibitor binds to the active site, it competes for the same active site. So, it is competitive inhibition. And when it competes for the same active site, once it binds to form the enzyme inhibitor complex, the substrate can no longer bind. But since this is a reversible reaction if I add enough substrate, I will be able to form the enzyme substrate complex and go onto form product.

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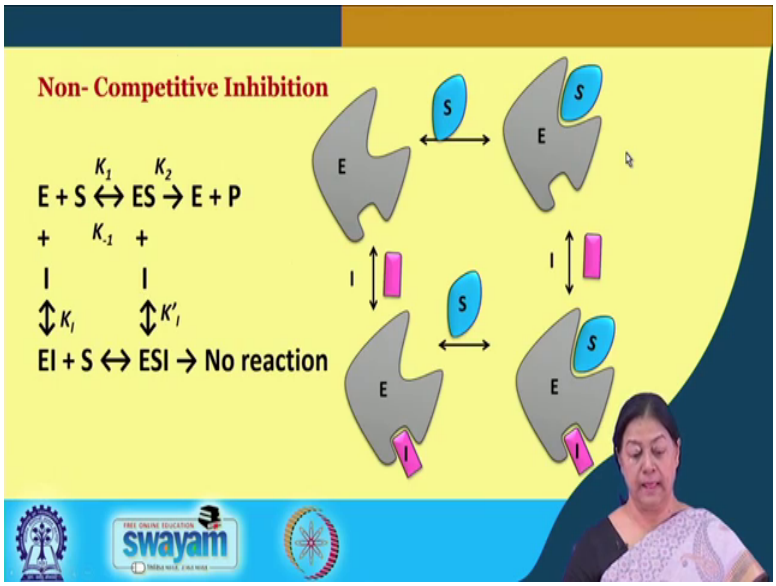

Non-competitive inhibition results in a fashion where we have the E S complex form that can also bind to the inhibitor, and the enzyme inhibitor complex also binds the substrate, but the complex that is formed what is called a ternary complex, where we have the enzyme, the substrate and the inhibitor, there is no reaction possible. So, this is what we call non-competitive inhibition, because the inhibitor is binding to a site that is not the active site for the substrate. What did we see for competitive inhibition, there was a competition between the inhibitor and the substrate, because they were going to the same active site. For non-competitive inhibition, this goes to a different site on the enzyme or the protein, and sometimes distorts the active site of the enzyme in such a manner that the substrate cannot bind anymore.

So, we have non-competitive inhibition. So, it will inhibit the activity, but it is not competitive in nature. So, if we were to design any molecules that would work as a competitive inhibitor say, what would we do? We would look for the substrate and they go for something that is called a substrate analog. What does that mean? That it would be a molecule that looks like the substrate, and then will go to the same active site as the substrate, but will not make the enzyme work to give any product.

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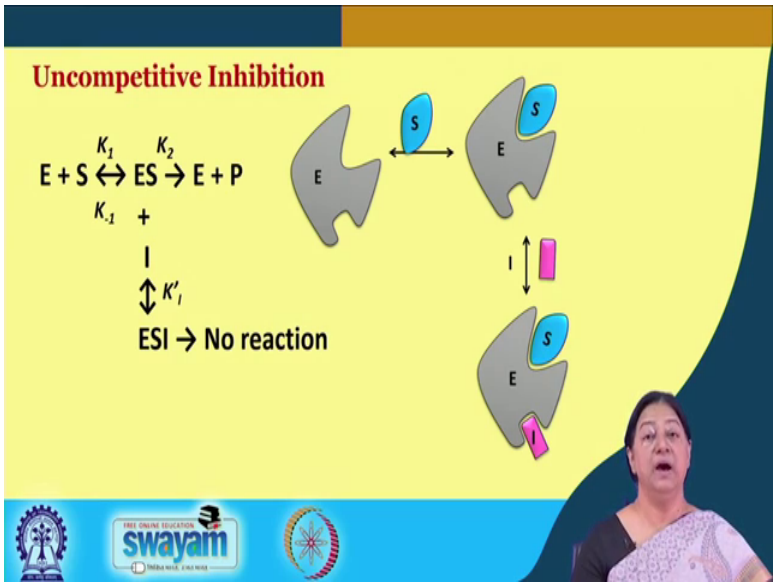

In case of uncompetitive inhibition, it is a bit different because the enzyme does not interact with the inhibitor directly the inhibitor interacts with the enzyme substrate complex. When it interacts with the enzyme substrate complex forming again the ternary complex there is no reaction possible. So, we have the enzyme substrate complex formed first, and then the inhibitor comes and sits at a different site, but if the reaction or the ternary complex is formed in such a manner that this product cannot be formed from the E S I ternary complex.

Now, how does this all work out in terms of the specific plot that we looked at the Lineweaver-Burk plot? We have to understand how this is going to affect the V max value, how it is going to affect the K m value, because that is going to tell us how effective the inhibitor actually is.

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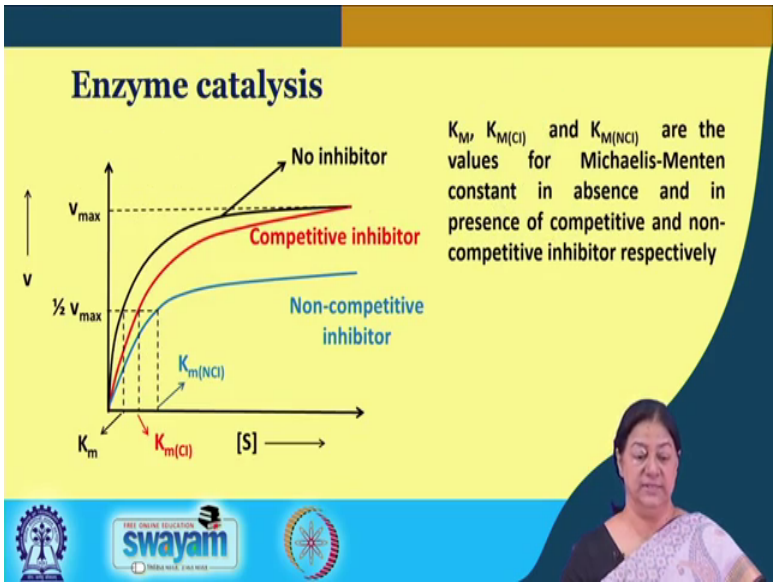

So, if we look at the enzyme catalysis now, the black line tells us there is no inhibitor. When we have a competitive inhibition, it tells us that the same V max will be reached, because we know that the enzyme will be available if we add sufficient substrate. But in non-competitive inhibition when even if we have the substrate, the enzyme is not available for the reaction because the non-competitive inhibitor has bound to such a site that it is destroyed the active site rendering the enzyme unavailable for product formation and substrate binding, and subsequent product formation.

So, what we have is we have specific K m values associated with conditions, where there is no inhibitor, K m values associated with a CI that is a competitive inhibitor, and k m values associated with NCI, because in this case we will have no competition from the inhibitor.

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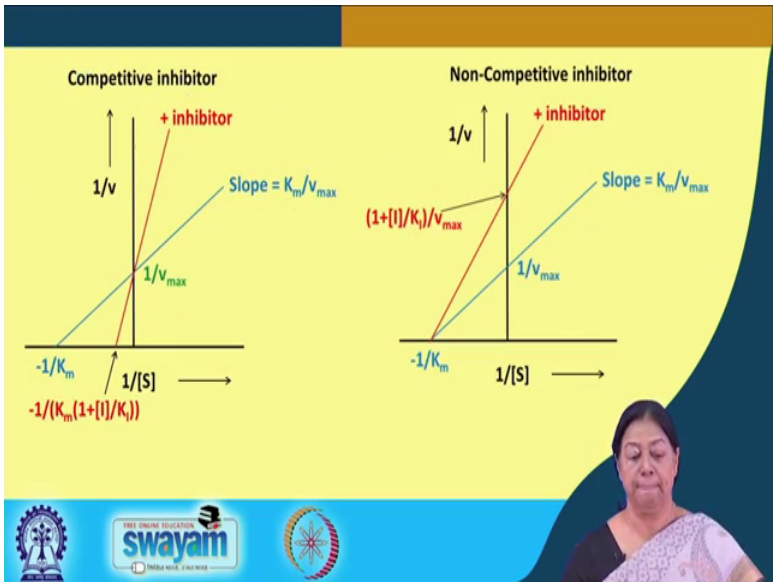

If we look at the plots now, the 1 by v versus 1 by s is called the Lineweaver-Burk plot. In the Lineweaver-Burk plot, we look at the specific case where we have the v max. So, this is the case where we have no inhibitor. We have no inhibitor as we add inhibitor, we will know that to reach the same v max, we have to have a larger substrate concentration. And the addition of inhibitor will create such a line that intersects at the y-axis, because the same v max is attained and we get a different K m value. For the case of the non-competitive inhibitor, the v max reached is not the same the K m value is the same, because whatever enzyme reactions can actually occur will give you the same K m value.

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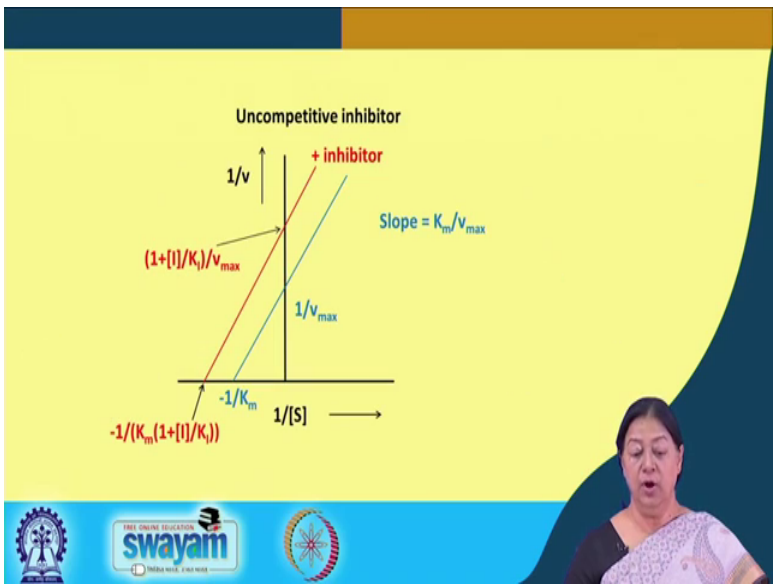

For the uncompetitive one we will get parallel lines. What does the uncompetitive inhibitor do? It interacts with the E S complex.

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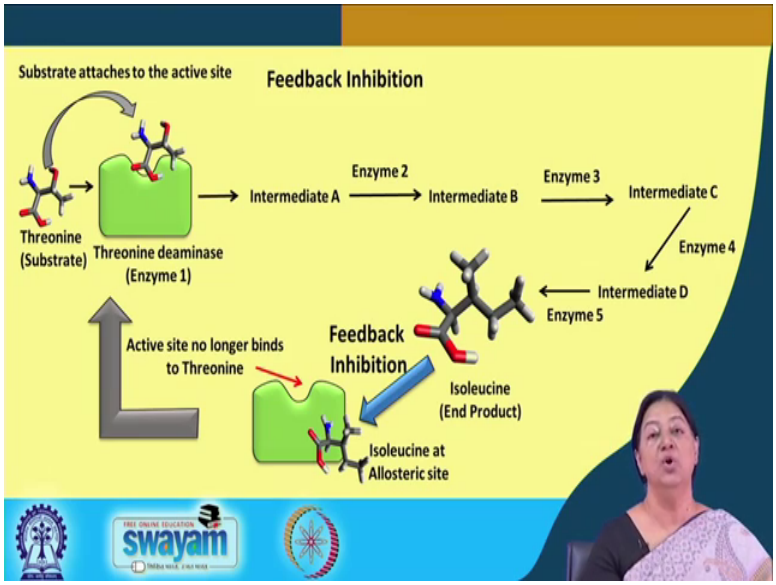

Another very interesting feature that occurs naturally in our body is something called feedback inhibition. It is just a simple thing in the sense the way nature has designed a specific reaction. This is a reaction say this is enzyme 1. It goes through a series of steps to form. So, this is threonine that is one amino acid. This threonine goes through a series of steps to form isoleucine which is the end product. Now, in this step, so we have threonine deaminase go through intermediate A to B to C to D, and finally, to isoleucine.

Now, what happens here is if there is sufficient number of isoleucine, sufficient amount of isoleucine present, what happens is that, this first reaction going on to intermediate A is not required. If it is not required, a very interesting feature we know that occurs in nature is called feedback inhibition. In feedback inhibition, what we see is the substrate attaches to the active site, and there are certain steps in the reaction that take through two isoleucine, which is the end product. Now, isoleucine except as what is called an allosteric site it is a non-competitive that will not give a reaction, but act on the enzyme in a manner that will not allow the substrate to bind.

Now, what happens here the active site no longer binds threonine, because isoleucine come and sits in a manner in the enzyme, because there is sufficient isoleucine present. It does not need to be found anymore. So, what it does is, it prevents its own formation by

not allowing, allowing threonine to bind to it. So, this is what is called feedback inhibition. And it is a very common biochemical reaction. But you see the beauty of it is that threonine no longer binds, because if threonine did bind to this, then what would happen is it more of isoleucine would be formed.

So, we have the uncompetitive, the competitive, the non competitive inhibitors that give us an idea of what is going on. Now, in a reaction that is going to be demonstrated in the specific experiment that is going to be demonstrated the browning of the apple slice. What happens there we have a polyphenol that has an oxidation catechol oxidase or of diphenyl oxidase that will act on it forming a dark-colored product. And we learned in our; we learned at the beginning how we can go through this that will tell us how we can monitor the color change that will tell us that there is browning occurring. But how is it going to occur in terms of inhibitors?

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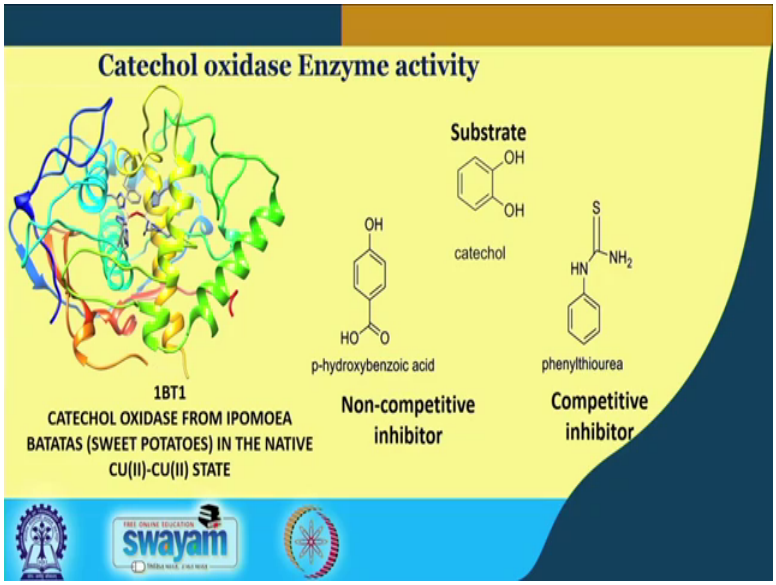

We will see that. So, this is the enzyme and this is the substrate. So, this is where you will do the initial reaction, we will have the catechol oxidase where we will get from the apple and the catechol which is going to be a substrate, and we are going to monitor it. In addition, there will be two inhibitors that you will be looking at one is a non-competitive inhibitor, one is a competitive inhibitor. And the two plots that we saw previously here, the two plots will be plotted and you will see that you will get a competitive inhibitor for

one of the products or inhibitors and one non-competitive inhibitor. So, this is what you are going to monitor in your specific experiment that you will do.

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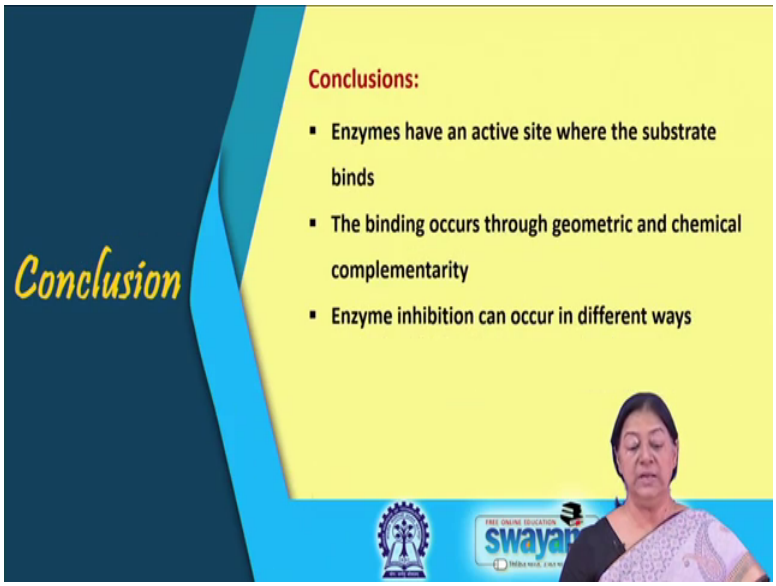

So, what we did learn here is that the enzymes have an active site, where the substrate binds. The binding occurs through the geometric and chemical complementarity. We looked at the specific reaction in terms of ribonuclease A, and we learned how the active site can bind the substrate. Then we looked at different types of inhibitions, reversible

inhibition, irreversible inhibition. In irreversible inhibition, what do we have? We have covalent bond formation. And reversible inhibition means that we have an enzyme inhibitor complex, and enzyme substrate complex, and enzyme substrate inhibitor complex in some cases where we have a ternary complex. In case of competitive inhibition, we see that the inhibitor competes for the same site as the substrate. So, it is the active site inhibitor, the competitive inhibitor is an active site inhibitor. Non-competitive and uncompetitive will bind to different parts of the enzyme which is the protein molecule and prevent the substrate from binding to the active site by a change in the enzymatic structure.

Thank you.