Fundamentals of Protein Chemistry
Prof. Swagata Dasgupta
Department of Chemistry
Indian Institute of Technology, Kharagpur

Module - 07
Enzyme kinetics and Enzyme inhibition
Lecture - 34
Enzyme Inhibition - II

In the previous lecture we looked at specific inhibition types. In this lecture we will continue our discussion on enzyme inhibition.

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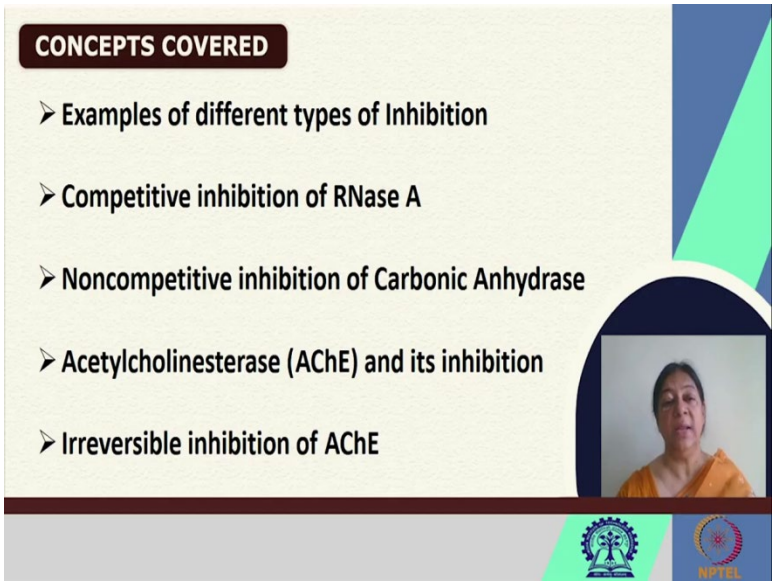

Inhibitor types in terms of how we can look at competitive, non-competitive and uncompetitive. In addition to irreversible type of inhibitions, in covalent connections with the enzyme.

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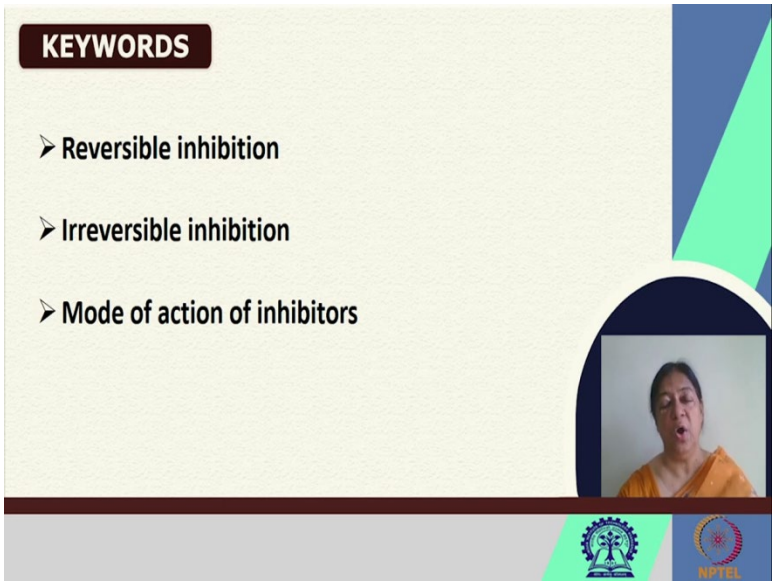

We are going to look at specific examples in this lecture, in terms of reversible inhibition, irreversible inhibition and mode of action of these inhibitors.

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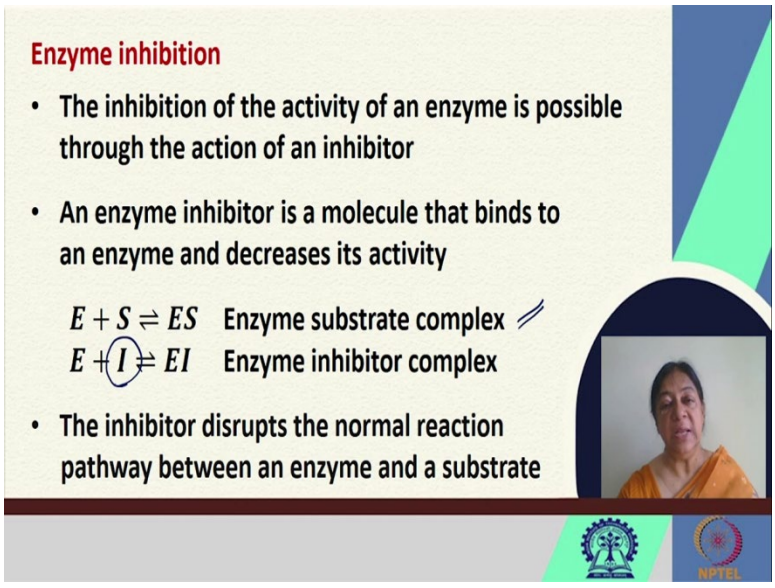

What we saw in the last lecture was, that the enzyme inhibition was a process that inhibited the activity of the enzyme by several methods. We have the formation of an enzyme substrate complex. The enzyme substrate complex would be interrupted by the presence of the inhibitor that would form an enzyme inhibitor complex. So, this would disrupt the normal reaction pathway not leading to the desired product.

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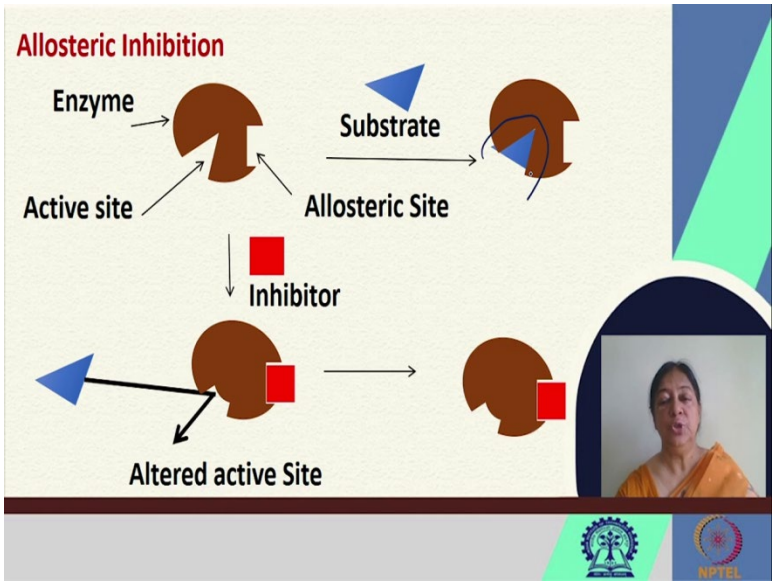

We also looked at allosteric inhibition, where we had the enzyme and the active site. When the substrate bound to the active site, we would have a specific formation of the enzyme substrate complex. However, if we had the inhibitor that bound to the allosteric site, this would modify the active site of the substrate in a manner, that the substrate will not be able to bind because of the altered active site.

We looked at several examples in the kinetic methodologies related to the competitive inhibition, non-competitive inhibition and uncompetitive inhibition.

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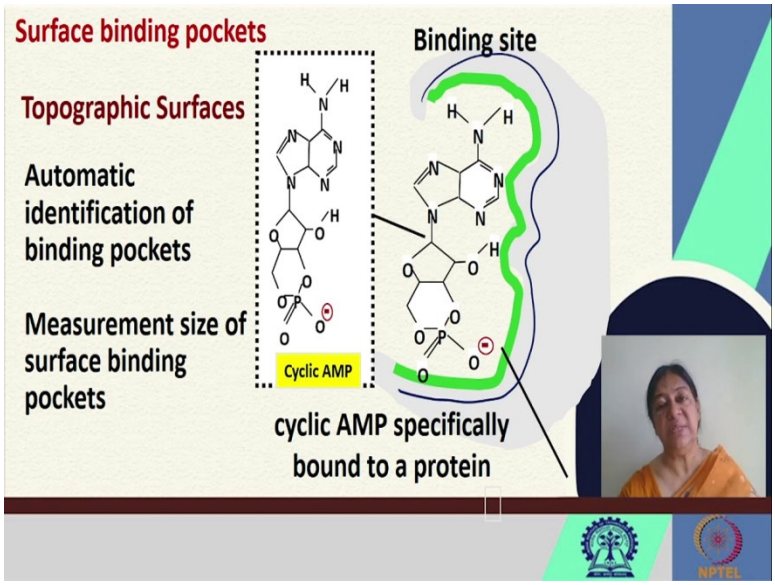

So we would have a surface binding pocket. When we look at the protein or an enzyme, we have a specific binding site. And if we look closely, we can look at specific binding pockets, as we

saw the geometric complementarity and a chemical complementarity in terms of the specific binding sites.

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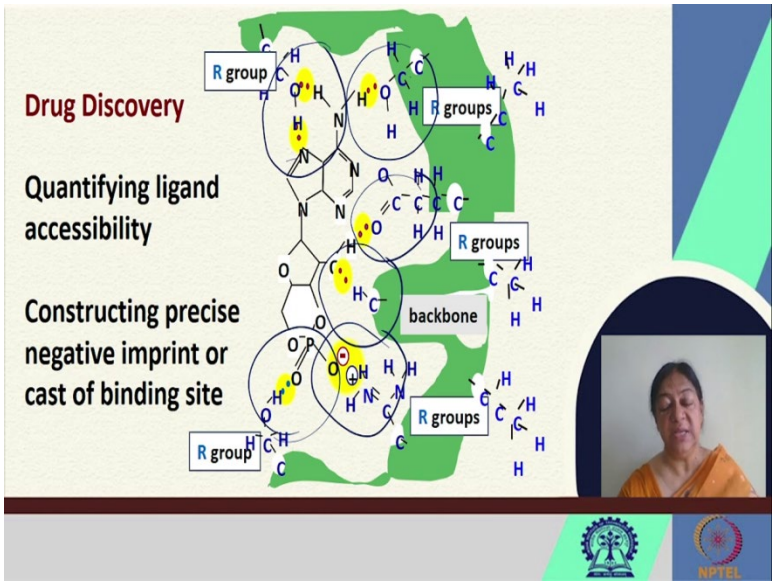

This amounts to a lot of ligand accessibility, ligand binding studies, drug discovery; where we are looking at the exact types of interactions, interactions that could be modified from the surface of the protein, with the knowledge of the active site. We can modify these interactions to make them tighter interactions, better interactions that would result in inhibition of the activity of the enzyme, in terms of the formation of an enzyme inhibitor complex that would not degrade into the products.

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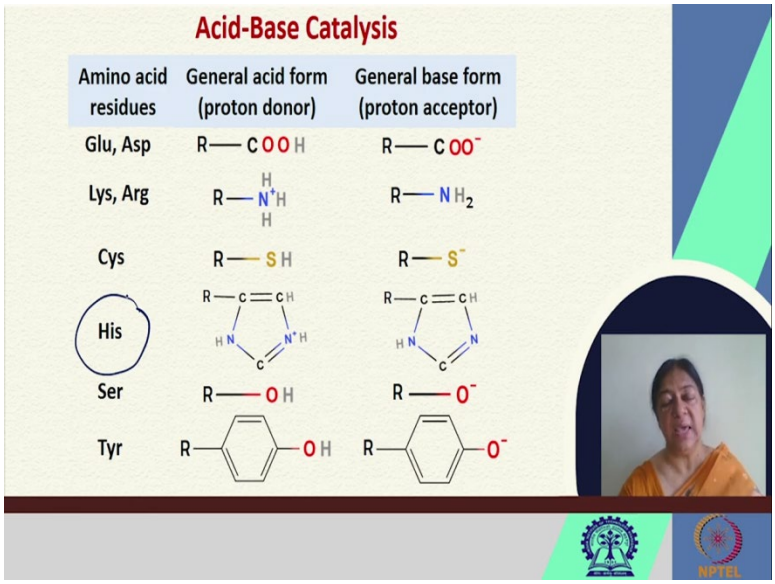

In an earlier lecture, we looked at the specific types of amino acids that were likely to be present in the active sites of proteins and we found out that many of these, particularly histidine, are most important because of the acid-base catalysis. The possibility of a proton donation and a proton acceptance in the pH range of the physiological pH. But we also saw the presence of other amino acids in terms of their roles, in say the catalytic triad reaction for chymotrypsin and other enzyme mechanisms as well, such as lysozyme, ribonuclease A and serine proteases.

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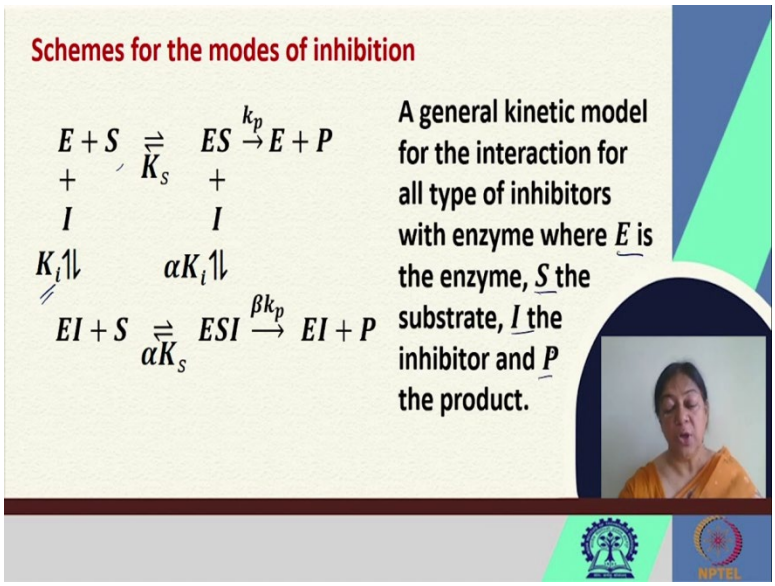

When we look at the schemes for the modes of inhibition, this scheme covers all the possibilities that might occur. Where we have a general kinetic model with the equilibrium conditions given here [refer to slide] and we have the enzyme, the substrate, the inhibitor and the product: all depicted in this diagram.

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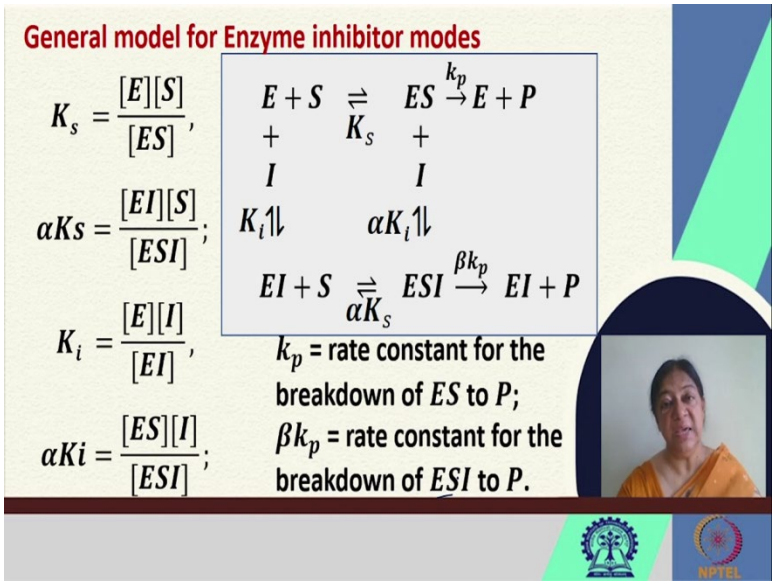

When we look at the specific equilibria involved, we have equilibria in terms of modifications with the presence of the enzyme, we have the specific rate constant associated with the formation of the product and also the rate constant for the formation of the product from the ESI complex, the ternary complex if this is possible.

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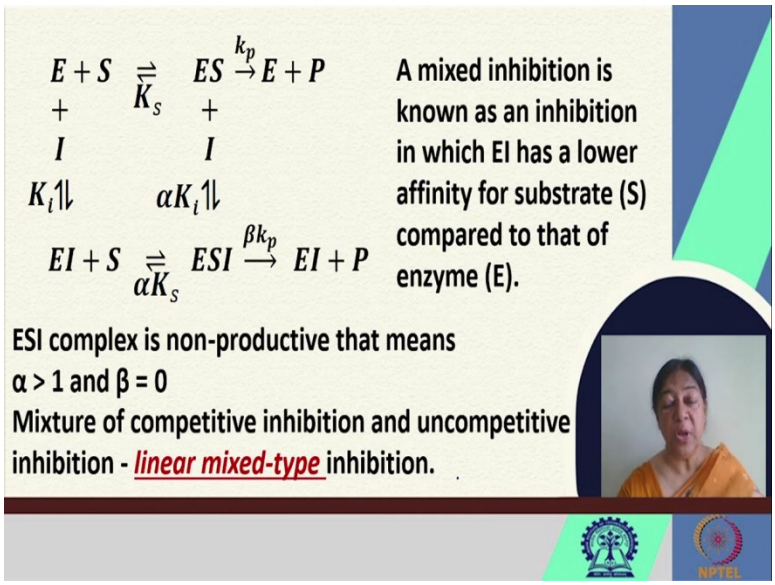

We have also looked at a possibility of mixed inhibition, where we could have a case where the enzyme inhibitor complex has a lower affinity for the substrate. It does form the ternary complex, but the substrate affinity for the enzyme is larger, but the enzyme inhibitor does have affinity.

The ESI complex could also be non-productive and sometimes we can have a mixture of competitive and uncompetitive inhibition in a linear mixed type of inhibition. The typical Lineweaver-Burk plot was shown in the previous lecture.

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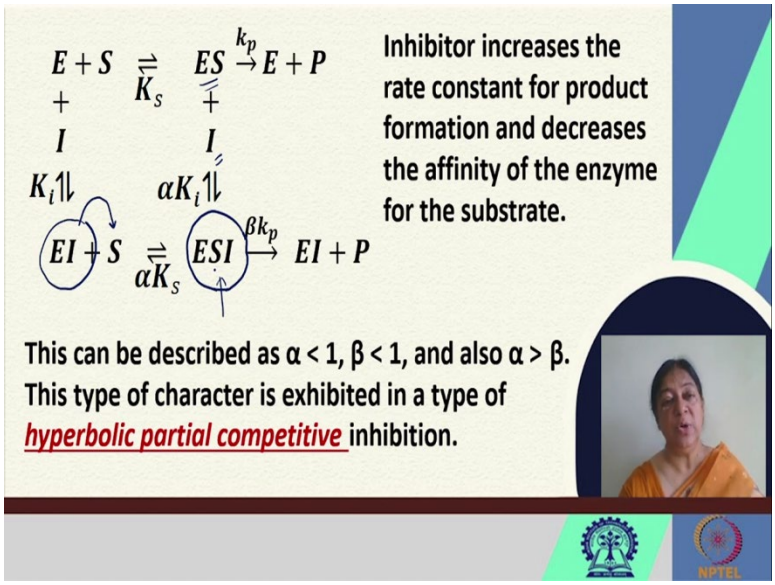

If now the inhibitor increases the rate constant for the product formation and decreases the affinity for the substrate, we can have a different type of character exhibited by this inhibitor, in what is called a hyperbolic partial competitive inhibition.

There are various possibilities of the inhibitor acting on the enzyme. It could act directly on the enzyme in the formation of the enzyme inhibitor complex. It could act on the enzyme substrate to bind to form the ternary complex or we could have the enzyme inhibitor bind the substrate to form the ternary complex, which may or may not result in the formation of the product. So, we could have this mixed type of inhibition.

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We will look at specific types of inhibition for a set of examples. When we look at competitive inhibition of this protein ribonuclease A, a very well studied protein, we need to understand what is the composition of the active site.

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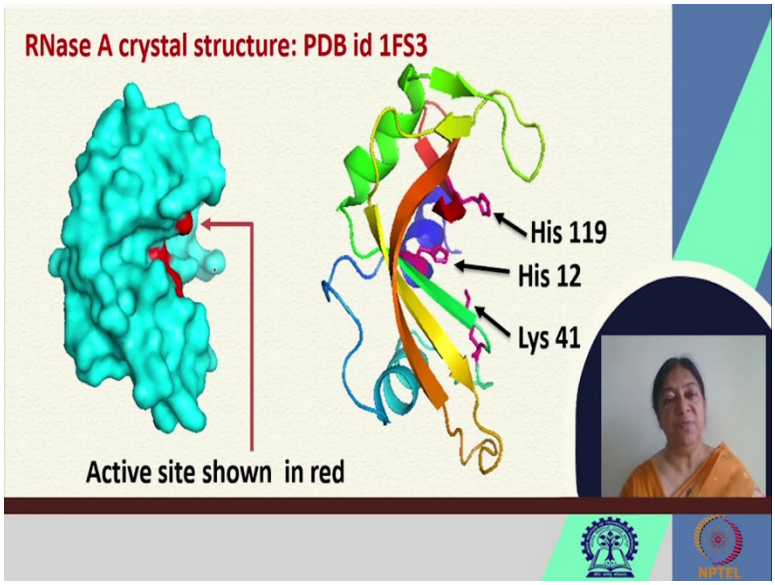

In the active site what we do have. We have histidine residues and we have lysine residues, that each of them have their specific roles to play and we saw briefly the enzyme mechanism involved in the formation of the product.

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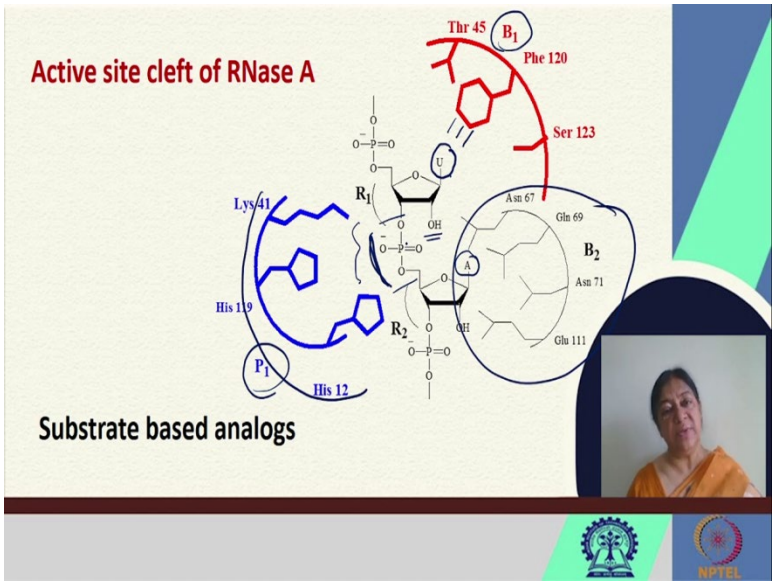

If we look at the active site cleft of RNase A in a bit more detail, we will see that there are specific sites; ribonuclease A cleaves ribonucleic acid. So, if we look at this [refer to slide], we have this as the main active site, where we have the catalytic activity associated with the histidines and the lysine. This is a phosphate recognition site, where we see that this is where we have the phosphate. Here we have the ribose sugar, the R_1 and the R_2, here we have a base recognition site.

We realize that if we want to inhibit the activity of this protein, we could prepare substrate based analogs. These could be prepared in a manner where we could modify the base, where we could affect this recognition; we could modify the phosphate connectivity where we could affect this recognition or we could work on the B_2 site. So, this [refer to slide] is the B_1 site that affects this base. We could have the B_2 site that would affect this base.

In a sense this gives us an idea for the design of an inhibitor for this protein. We could also remove the phosphate altogether in forming a nucleoside.

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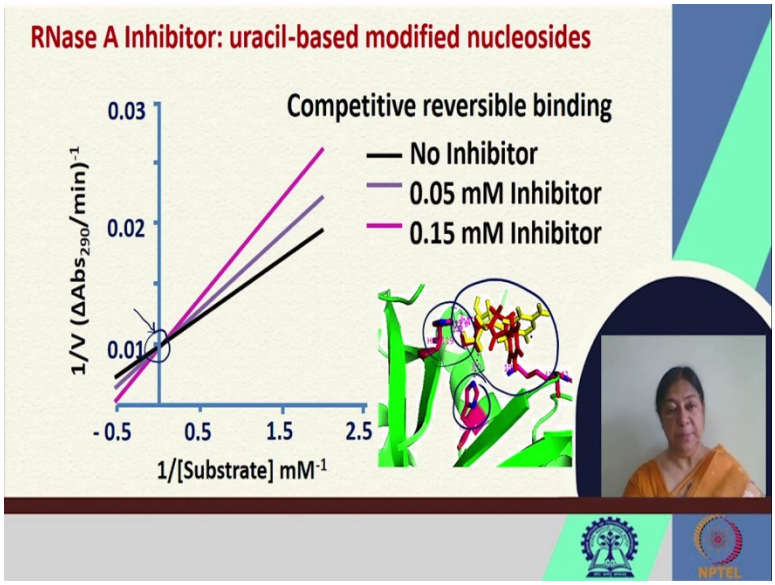

So, if we look at a uracil-based modified nucleoside and look for competitive reversible binding of this protein, we could then have a methodology to determine the inhibition, find out the kinetics and from the kinetics, the specific types of curves that are expected or the lines that are expected in the Lineweaver-Burk plot. We would get a line corresponding to the absence of the inhibitor that would give us, as we know from our previous enzyme kinetics lectures, that this would correspond to the $1/v_{max}$ value.

Now if we added our competitive inhibitor, we know that the point of intersection should be this[refer to slide]. We would get the same v_{max} because increase of the substrate concentration would lead to a formation of the product. So, we look at a specific example where we have the histidines and we have a set of competitive inhibitors that bind at this site, giving us competitive inhibition.

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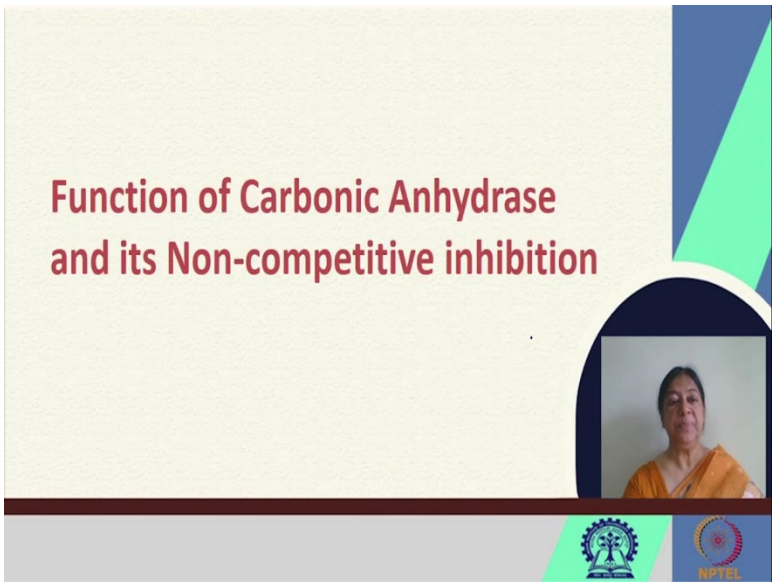

If we now look at an example for non-competitive inhibition in carbonic anhydrase.

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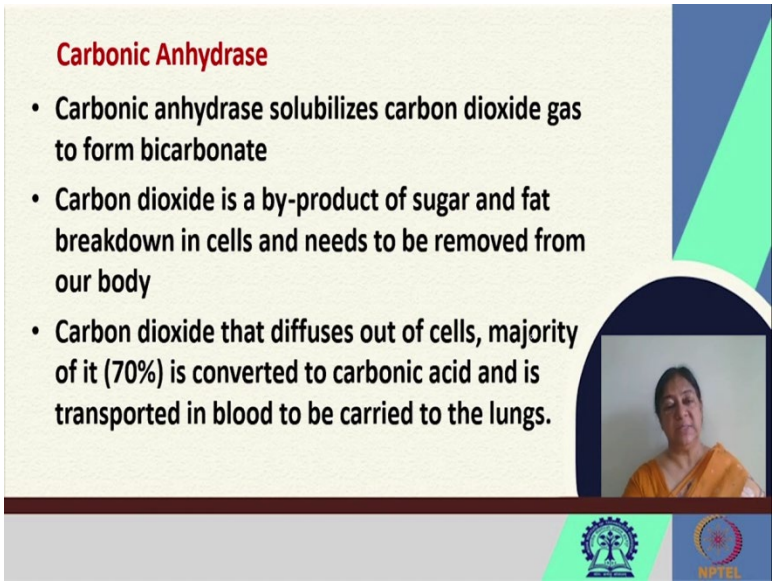

Carbonic anhydrase is a very important protein, as it solubilizes carbon dioxide gas to form the bicarbonate. The carbon dioxide we know is a by-product of all the sugar and fat breakdown in the cells and it has to be removed from the body, because it becomes toxic. So once it diffuses out of the cells, the majority of it is converted to carbonic acid and is transported by the blood to be carried to the lungs.

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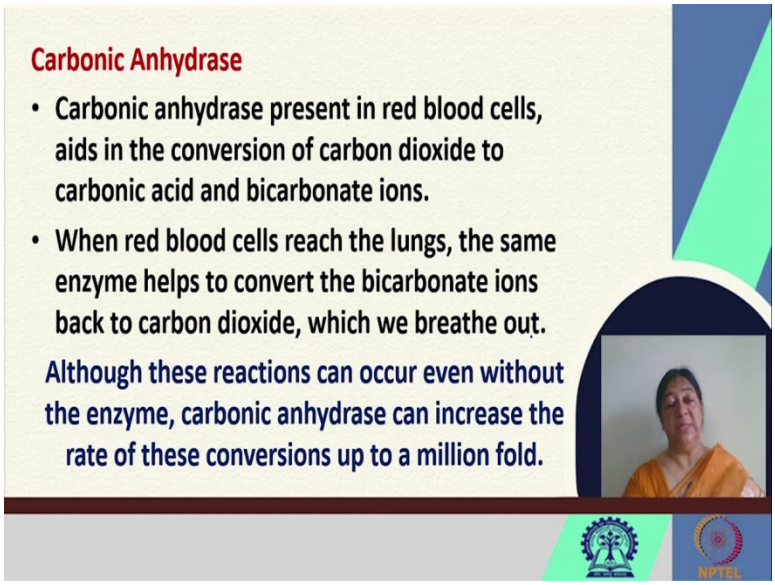

However, it is also present in red blood cells and this aids the conversion of the carbon dioxide to carbonic acid and the bicarbonate ions. These reactions sometimes can occur even without the enzyme, but carbonic anhydrase can increase the rate of these conversions manifold.

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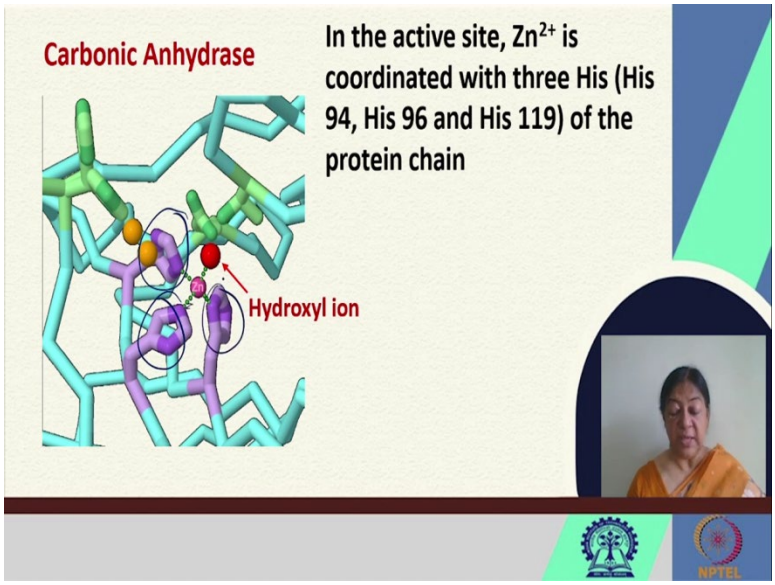

If we look [refer to slide] at the protein itself, there is a hydroxyl ion and we can see again the histidine residues that are connected to the zinc and we now have the carbon dioxide come into the picture.

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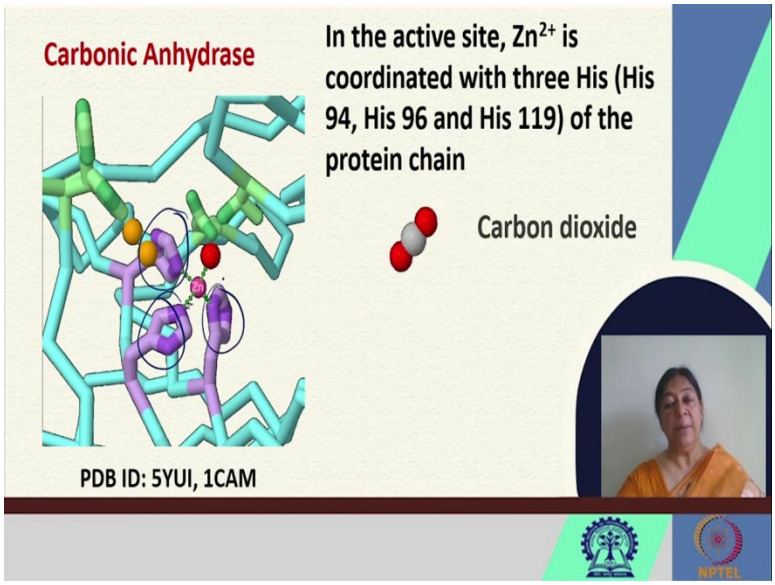

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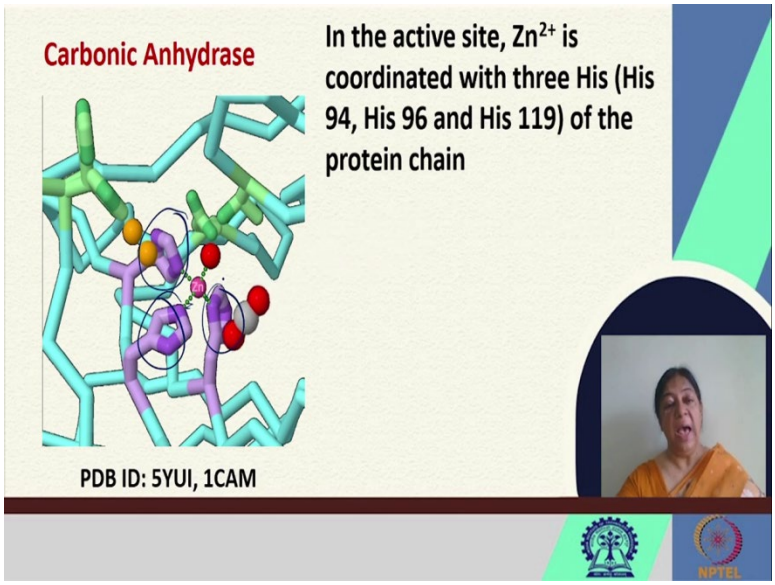

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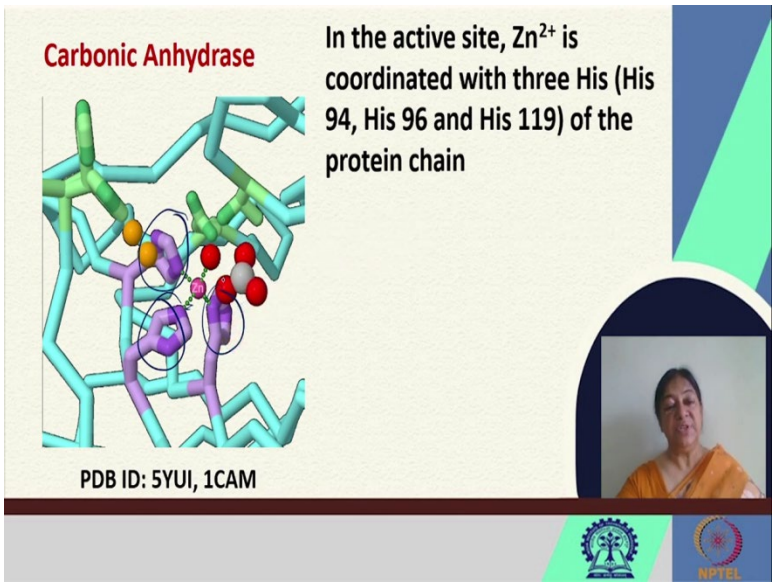

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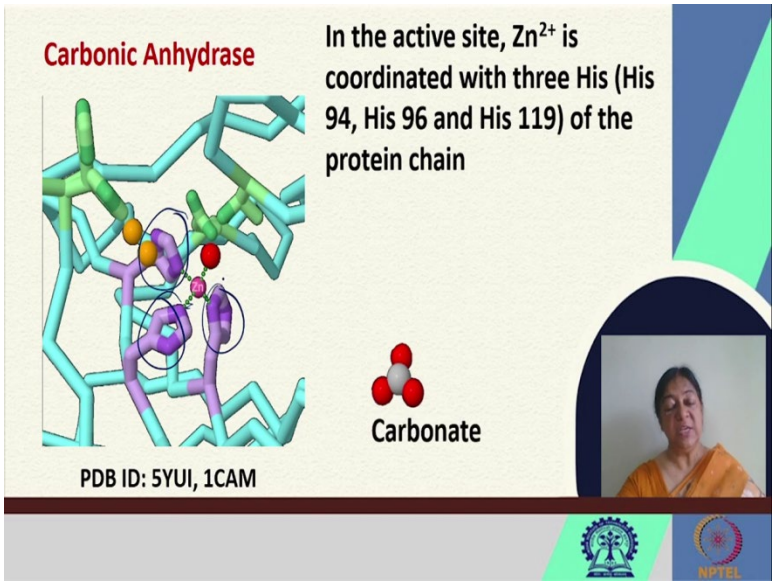

After a certain mechanistic process, we have the carbonate that is removed [refer to slide].

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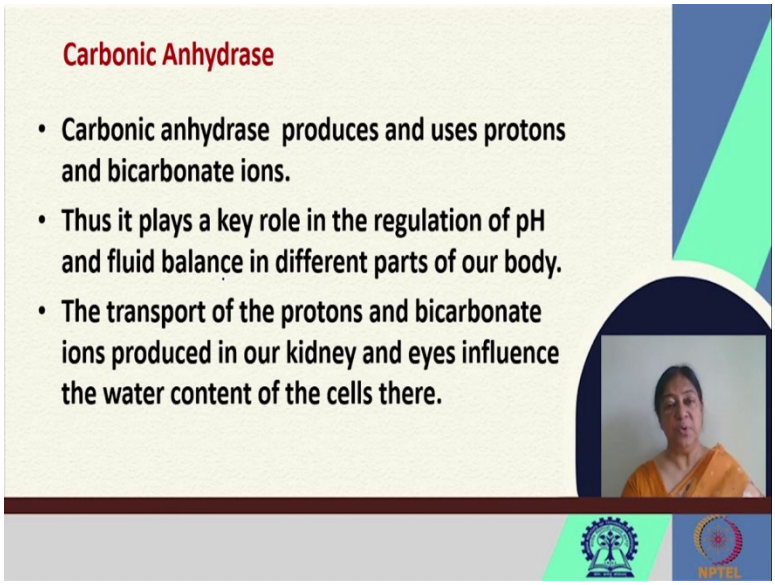

A further process of an understanding of this protein, leads us to show that carbonic anhydrase produces and uses protons and bicarbonate ions and as a result of this, it plays a very important role in the regulation of the physiological pH. So, the transport of this is extremely important.

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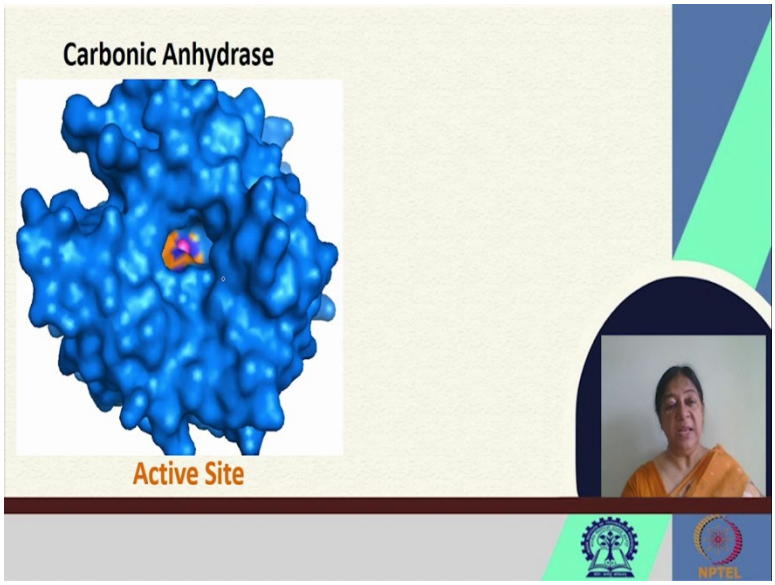

Similarly it has effect in other maintenance of the fluid content in the body.

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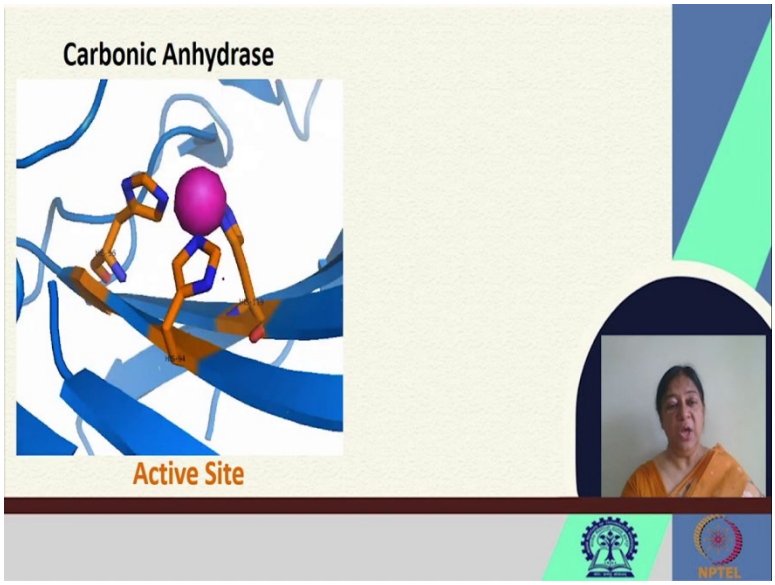

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So this [refer to slide] is the active site of the protein, where we can see the zinc ions connected to the several histidine moieties that hold the zinc in place. We have the carbon dioxide come into the picture.

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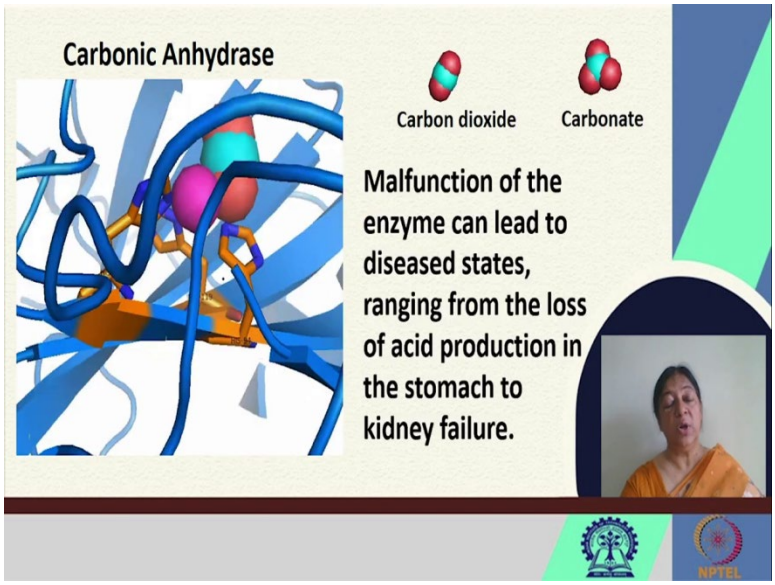

And what can happen with malfunction of the enzyme, this can lead to diseased states that could have an effect on the proton content. Loss of acid production in the stomach could also be a method and this could lead to several diseases.

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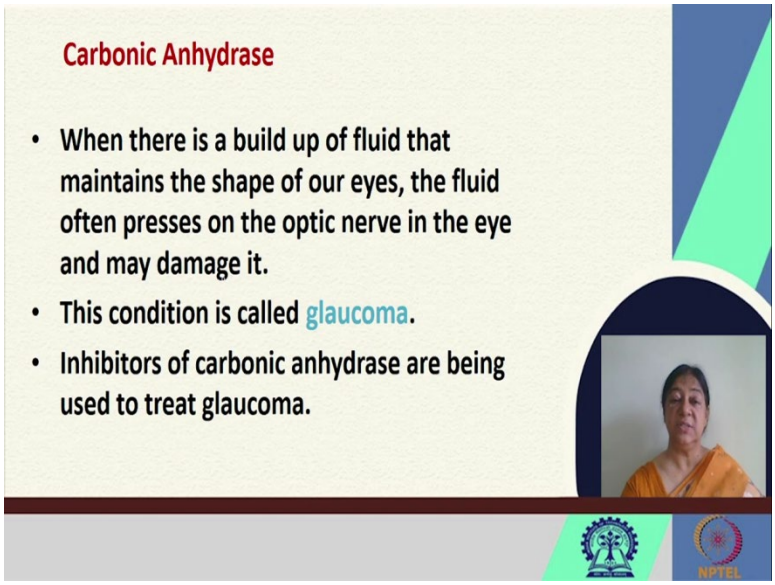

There is also a buildup of fluid that maintains the shape of our eyes. An additional fluid could have an effect on the pressure of the optic nerve and could damage it. This condition is known as glaucoma. Inhibitors of carbonic anhydrase are actually being used to treat glaucoma.

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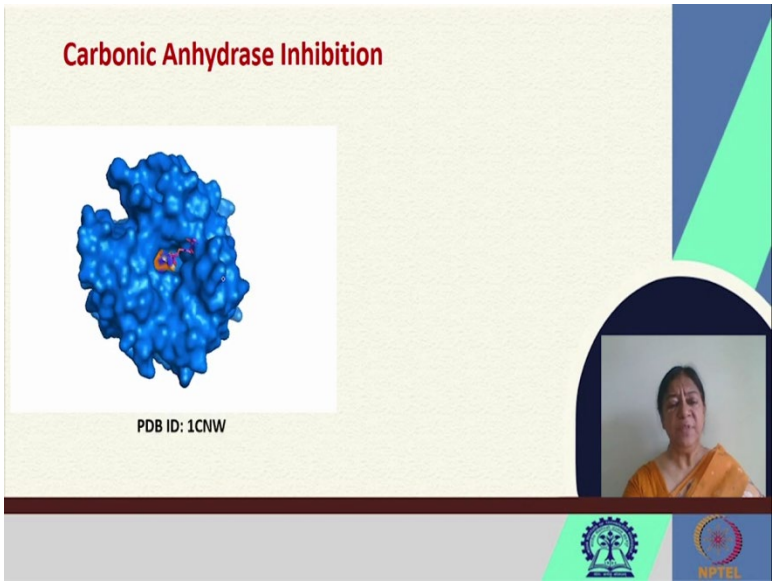

So if we look at carbonic anhydrase inhibition, we have the protein, we have the active site of the protein and we are aware that the active site has the histidine residues, which binds to the zinc ion.

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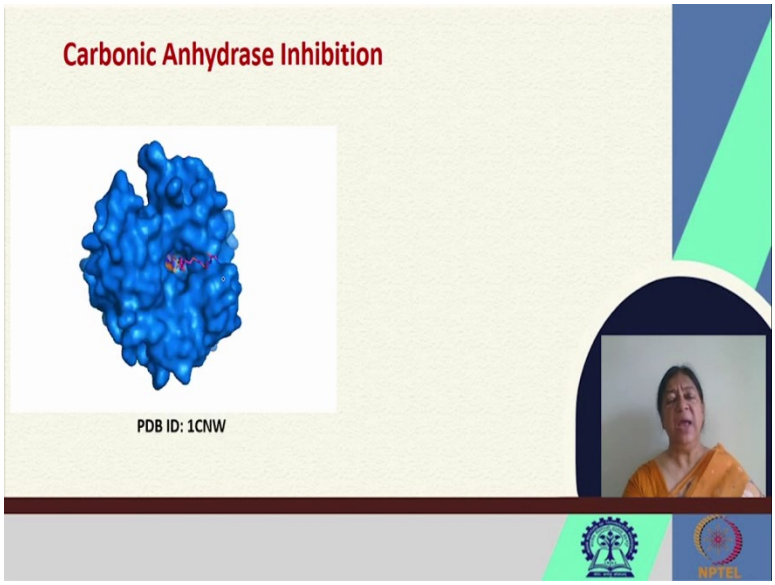

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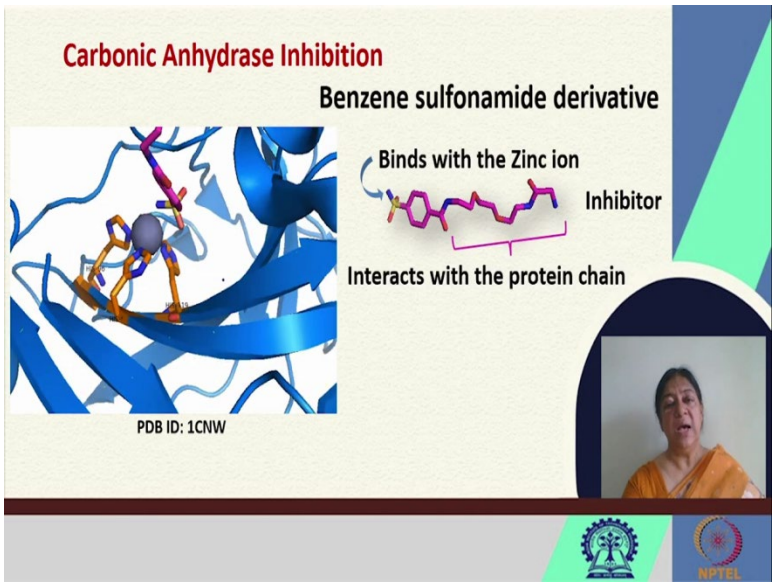

So, we would like a methodology by which we can look at an inhibitor that binds with the zinc ion.

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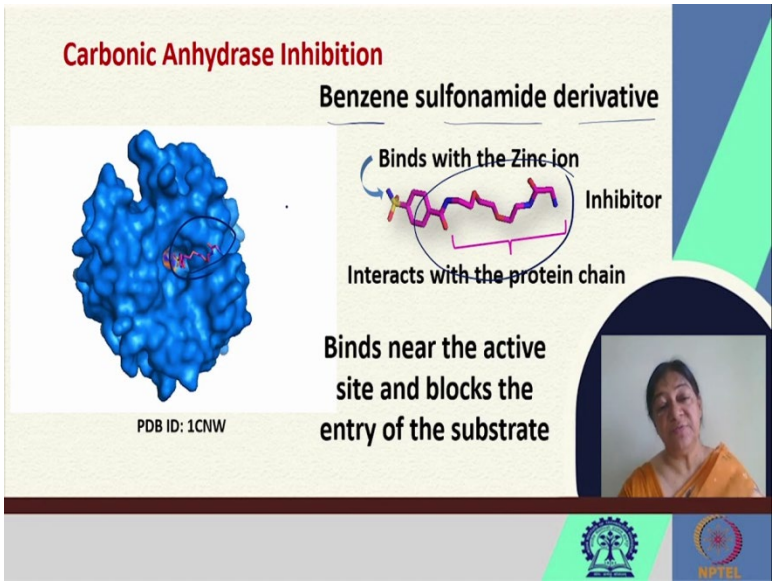

Another portion of this inhibitor, which is a benzene sulfonamide derivative, interacts with the protein chain in a manner that holds the position of the inhibitor in the active site. It binds near the active site, but it blocks the entry of the substrate in a manner that it acts in a non-competitive mode.

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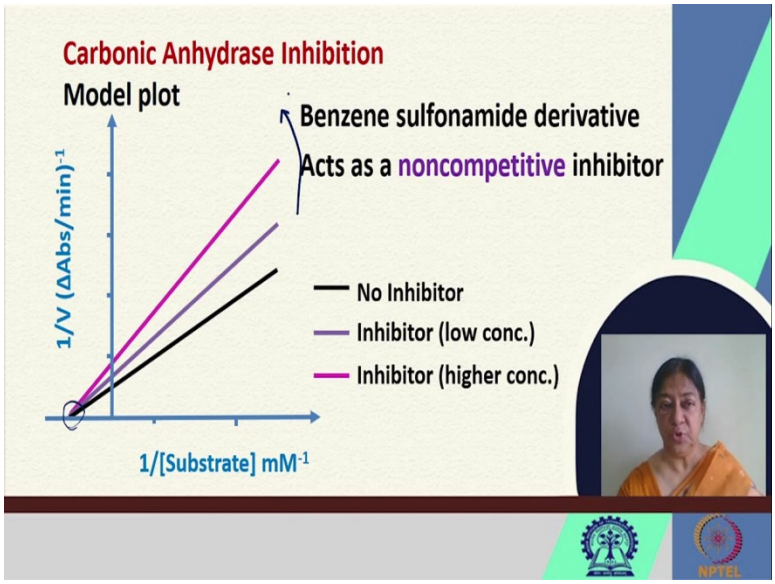

If we look [refer to slide] at a model plot of this protein, we will have in the Lineweaver-Burk plot, a methodology where we have no inhibitor bound. With inhibitor in this case, since we have some of the enzyme forming an enzyme substrate inhibitor complex, we would have a methodology that would not reach the same maximum velocity value that we would have observed in a competitive inhibition.

So, in this model plot we will see that the point of intersection of the two lines of the Lineweaver-Burk plot, should be on the x axis and not on the y axis as we saw before. This is the case where we have a low inhibitor concentration, followed by a high inhibitor concentration which will go in this direction.

We have this acting as a non-competitive inhibitor as is evident from this model plot.

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If we now look at the function of acetylcholine esterase. This has or shows non-competitive inhibition and also irreversible inhibition.

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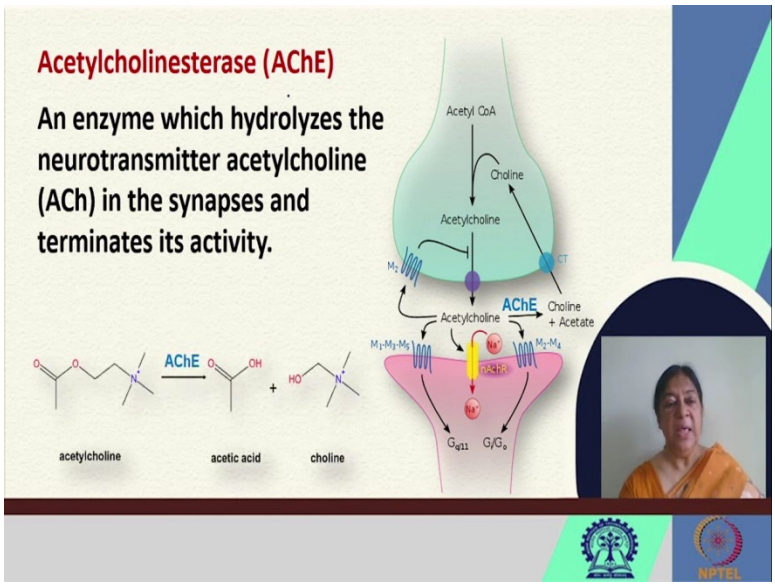

In its mode of action, it is an enzyme that hydrolyzes the neurotransmitter acetylcholine in the synapses and terminates its activity. There is a cascade of reactions that occur in a beautiful manner, that maintains cell signalling.

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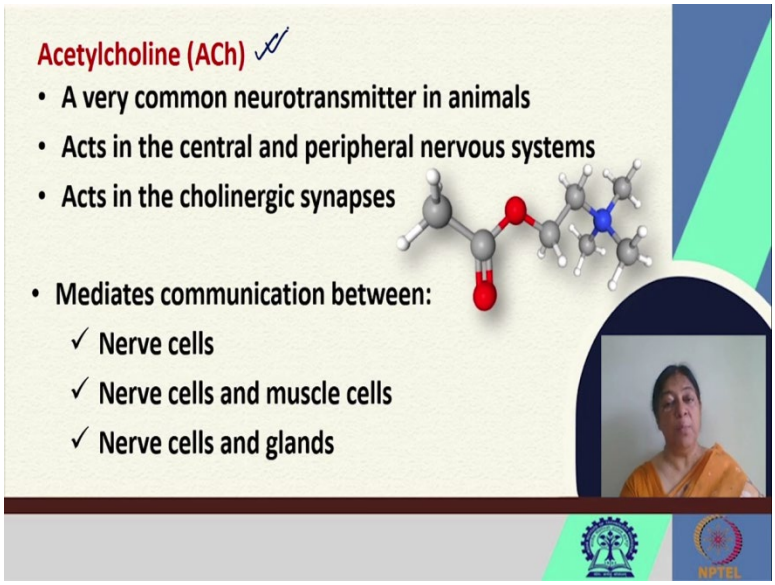

This enzyme is also very important as a neurotransmitter. The acetylcholine which binds to this acetylcholinesterase is a common neurotransmitter in animals and it acts in the central and peripheral nervous systems. And it mediates communication between nerve cells, nerve cells and muscle cells, nerve cells and glands. So we realize the importance of this molecule in our system.

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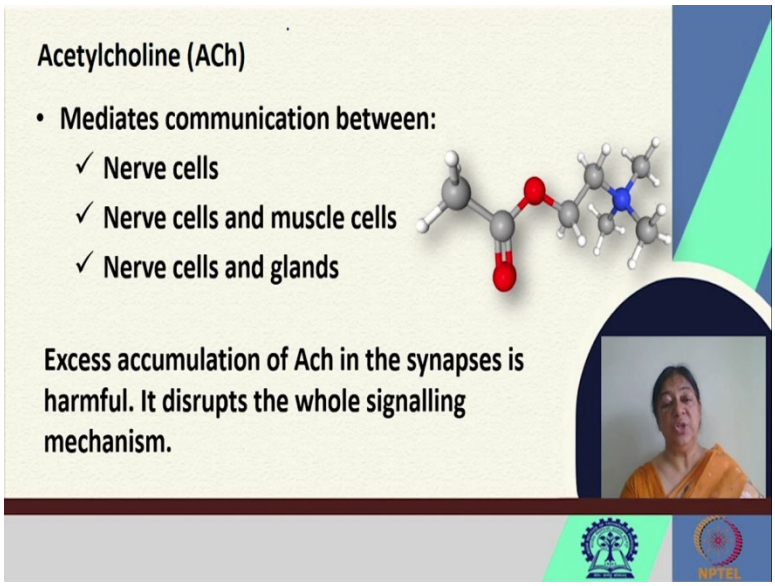

This mediation is therefore required and the excess accumulation in the synapses is harmful because it disrupts the signal mechanism.

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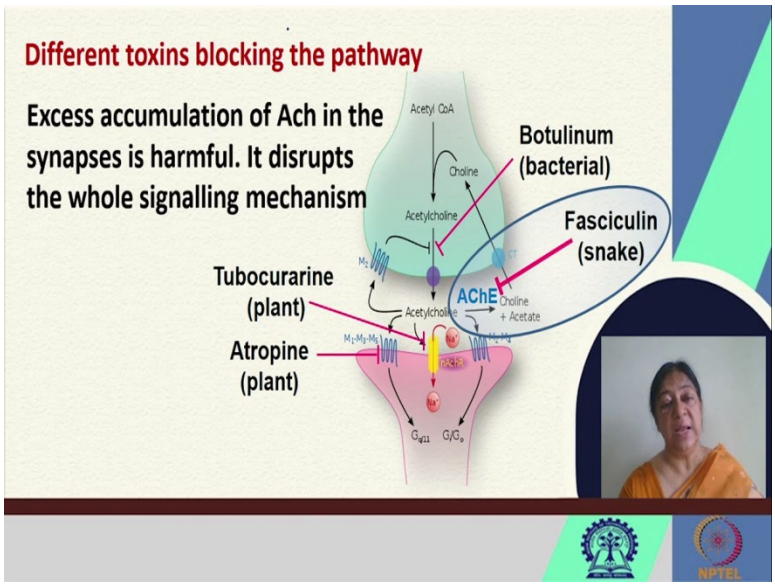

So, we can have several toxins that actually can bind or block the pathway of this protein. In a knowledge that an excess of the acetylcholine in the synapses will disrupt the whole signalling mechanism, we look at how it can be targeted by these natural toxins.

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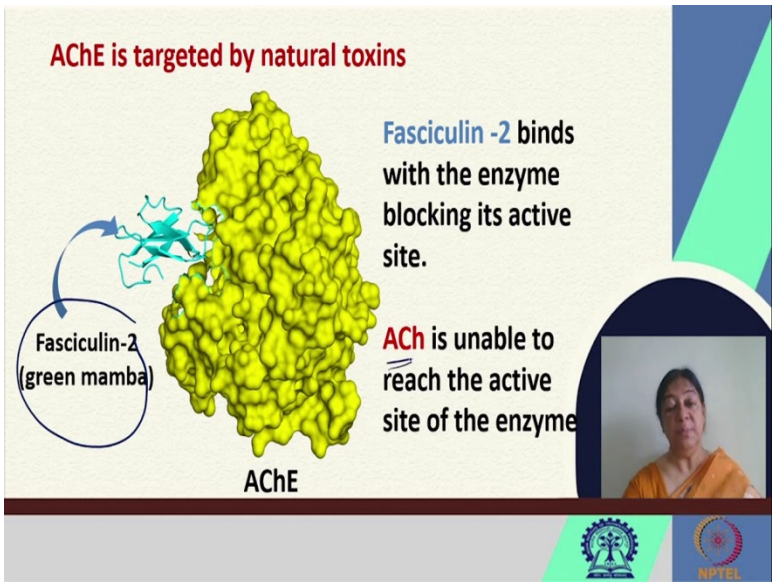

This [refer to slide] is acetylcholinesterase enzyme. Fasciculin-2 (green mamba), is snake toxin that binds to a specific point on this in the enzyme, blocking its active site. And the

acetylcholinesterase is unable to reach the active site of the enzyme, thus affecting the signalling process.

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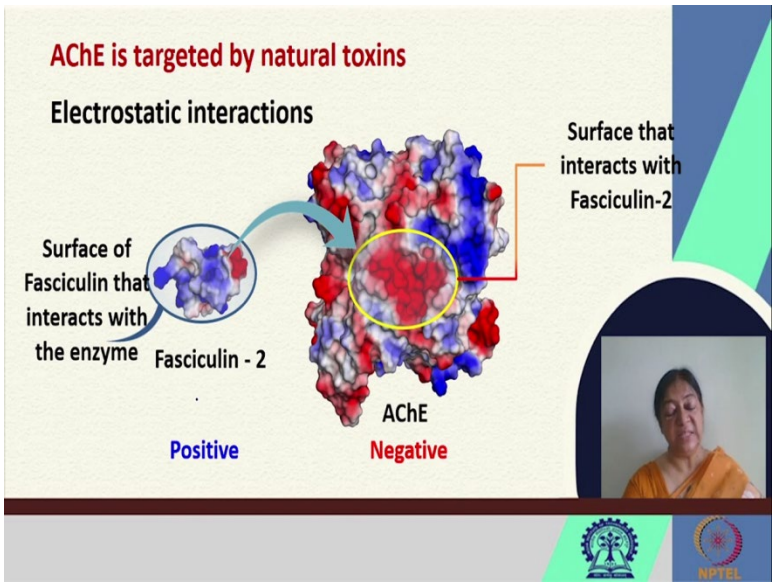

If we look at how we have the complementarity in terms of its connectivity with the enzyme, we can have a look at a surface charge representation. The surface charge representation shows us that this [refer to slide] surface is the part which acts with or interacts with the enzyme. We have the active site of the protein that is marked in red here, which is mostly acidic in nature and any inhibitor therefore even designed to act on this particular enzyme, would be basic in nature; as we can see from the active surface that interacts with this inhibitor.

So we have a positive surface here, we have a negative surface here, from the acidic amino acid residues. This interaction would give us a good inhibitor that would able to affect the activity of the enzyme.

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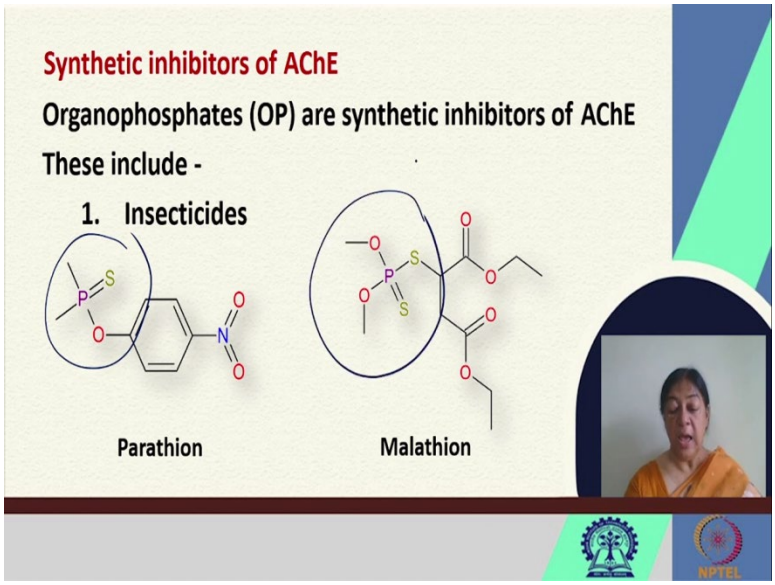

Synthetic inhibitors of this protein have also been designed. For example, organophosphates are synthetic inhibitors of acetylcholinesterase. These include insecticides that have this organophosphate group.

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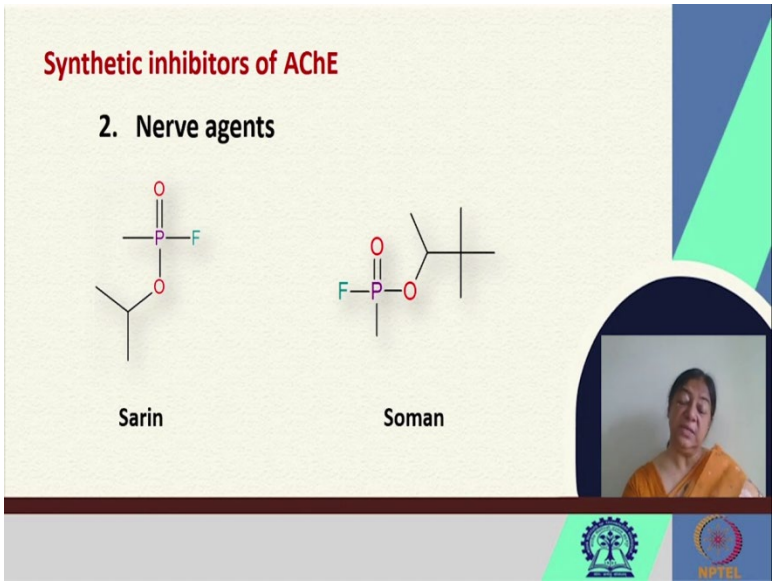

We have nerve agents such as Sarin and Soman. These are particularly affected in acting on acetylcholinesterase and affecting its binding.

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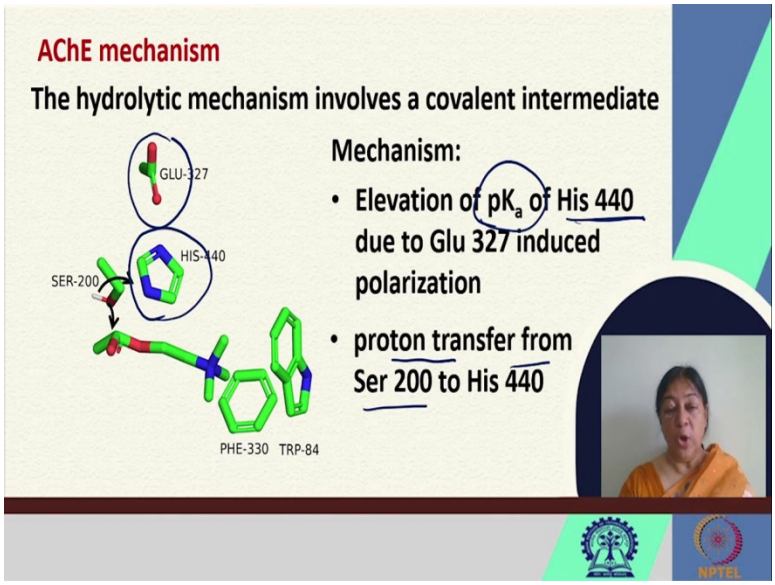

Let us now look [refer to slide] at the acetylcholinesterase mechanism in a bit more detail to understand the irreversible inhibition. We understand that irreversible inhibition is due to the formation of a covalent bond. In the hydrolytic mechanism, that involves a covalent intermediate in the normal mechanistic pathway of this enzyme, there is an elevation of the pK_a of histidine 440 that is located here in the enzymatic active site and this occurs due to a polarization that is induced by the glutamic acid 327. This results in a proton transfer from serine 200 to histidine 440.

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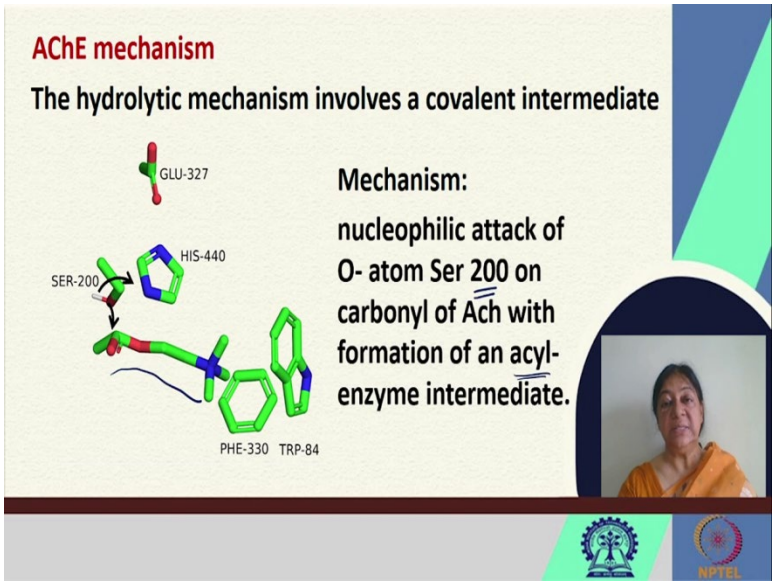

What now happens is a nucleophilic attack of the O- atom of serine 200 on the carbonyl of the acetylcholine, which results in the formation of an acyl-enzyme intermediate. We have seen the formation of a covalent intermediate in our mechanism discussion with serine proteases.

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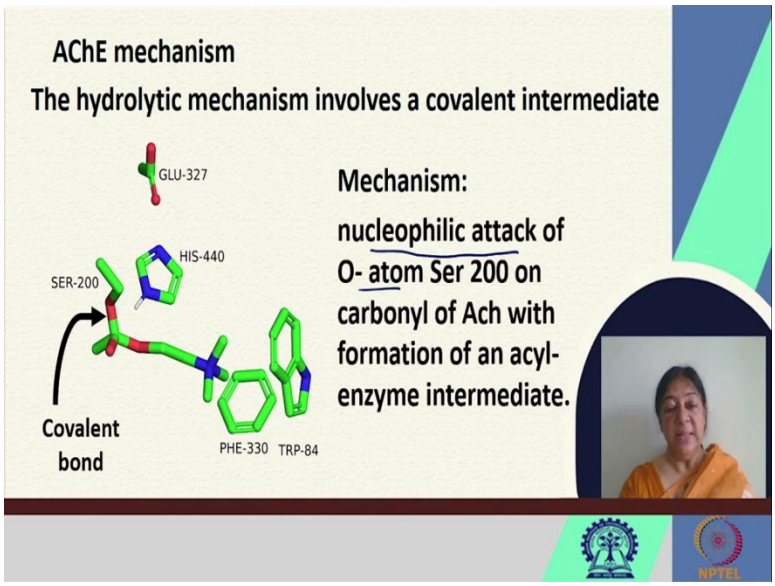

There is a covalent bond formation after the nucleophilic attack of the O- atom, which results in the formation of an acyl-enzyme intermediate, then there is subsequent reaction.

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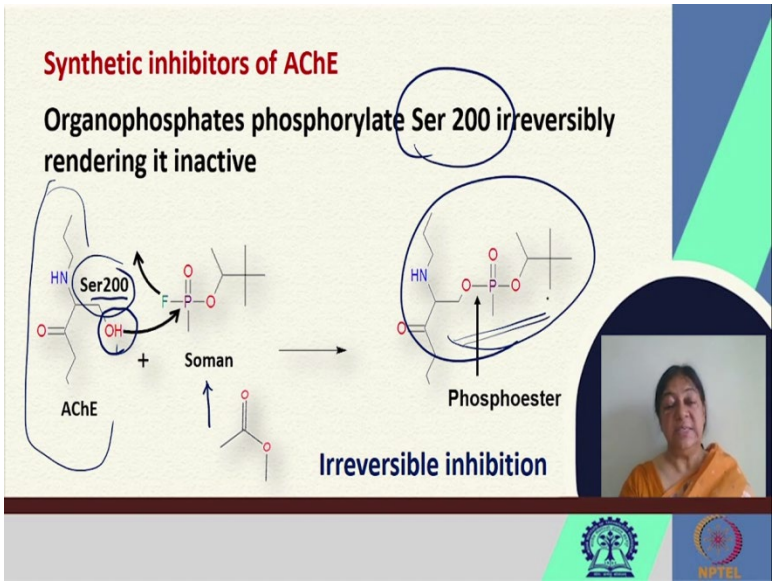

However, when there are synthetic inhibitors of acetylcholinesterase, their mode of action is such that they phosphorylate the serine 200. As a result what happens is this is no longer available for activity. Here is our serine 200, this O- would have been available after we have the proton abstraction, we have the O- ready for a nucleophilic attack.

In the presence of an organophosphate that acts as an irreversible inhibitor, what happens is, there is this [refer to slide] formation of phosphoester that renders. So this is the part of the

enzyme that we are talking about, that has the serine 200 to it and this has a permanent covalent interaction with serine 200 resulting in an irreversible inhibition.

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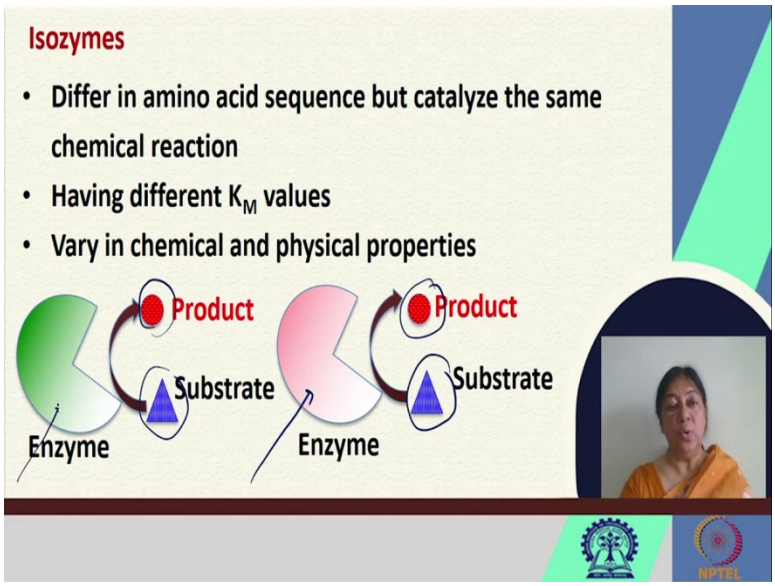

Our next discussion will be on isozymes. What are isozymes? Isozymes are enzyme molecules rather they differ in amino acid sequence, but they catalyze the same chemical reaction. So they have different Michaelis constants, they vary in their chemical and physical properties. We have a product and we have a substrate that are similar, we have the enzyme that is different. These are known as isozymes.

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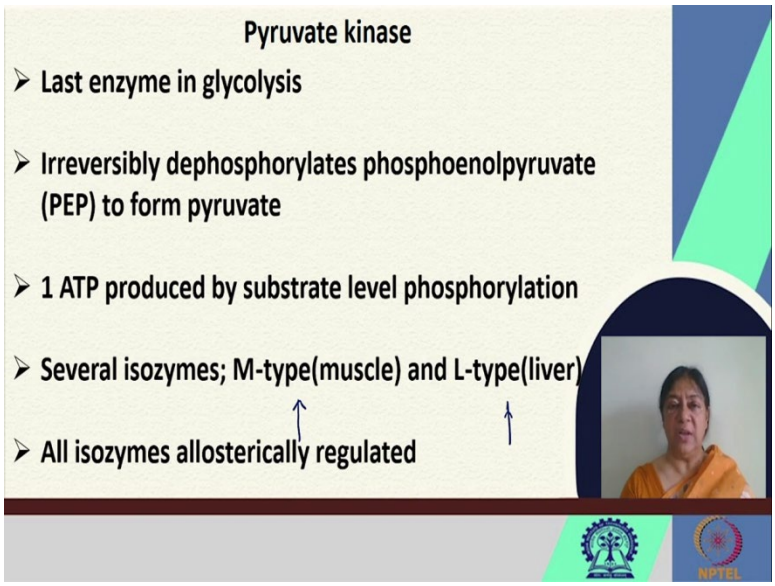

In pyruvate kinase, one such enzyme it is the last enzyme in glycolysis. And it irreversibly dephosphorylates phosphoenol pyruvate to form pyruvate and then we have 1 ATP produced by substrate level phosphorylation. This is important, in that it has several isozymes the M-type that is the muscle type and the liver type that is the L-type. Each of them have their own mode of action and we can look at these allosterically regulated isozymes.

So we have looked at competitive inhibitor types, non-competitive types, irreversible types; we will look at some allosteric regulation with the example of pyruvate kinase.

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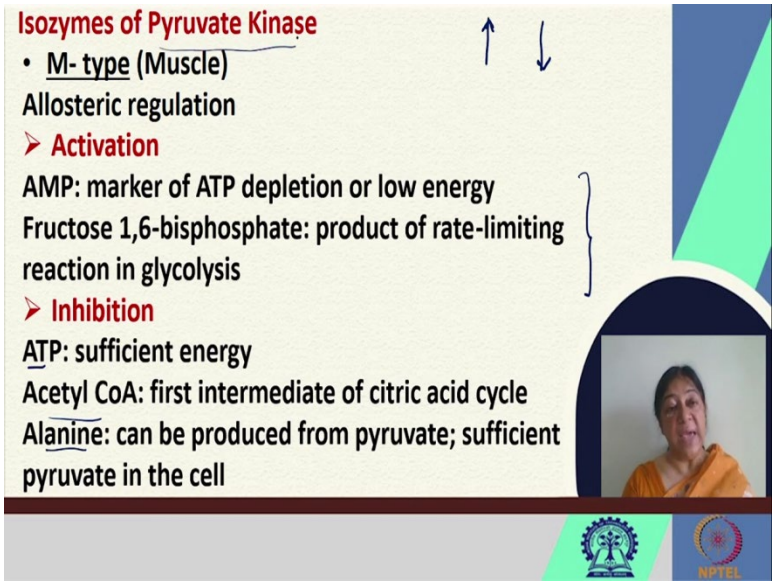

The isozymes of pyruvate kinase involve the M-type that has the allosteric regulation. The allosteric regulation would mean, there would be some molecules that would activate its enzymatic catalytic process and there would be some that would inhibit the process. So, in the activation process of pyruvate kinase for the M-type of the isozyme, there are these several activators that can activate the enzymatic activity.

There are also some inhibitions like ATP, Acetyl CoA, or Alanine that would be produced from pyruvate and what would happen is, this would inhibit the activity of the isozyme of pyruvate kinase of the M-type.

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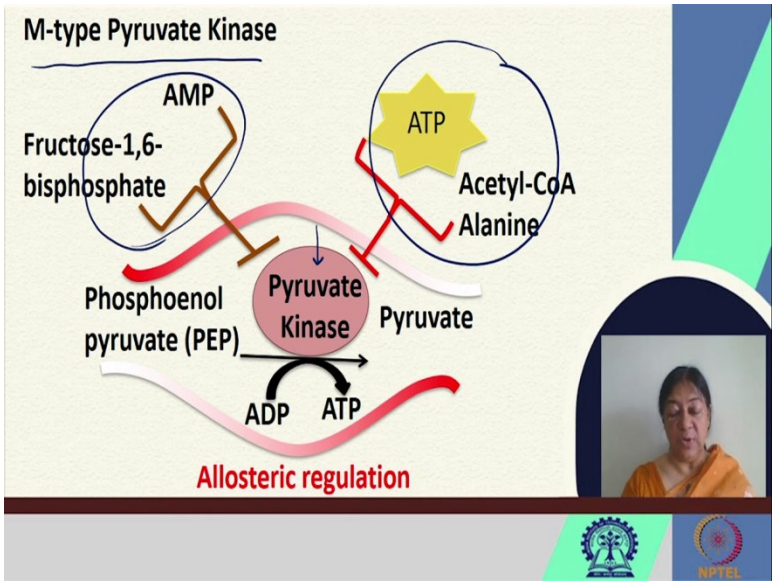

So, this would be an example of how the M-type would be activated. So if this [refer to slide] is our enzyme pyruvate kinase and we have these that are activating, they would act on a specific region of the protein that would activate the catalytic process. However, these would be inhibitors of the process and we would have what is called allosteric regulation.

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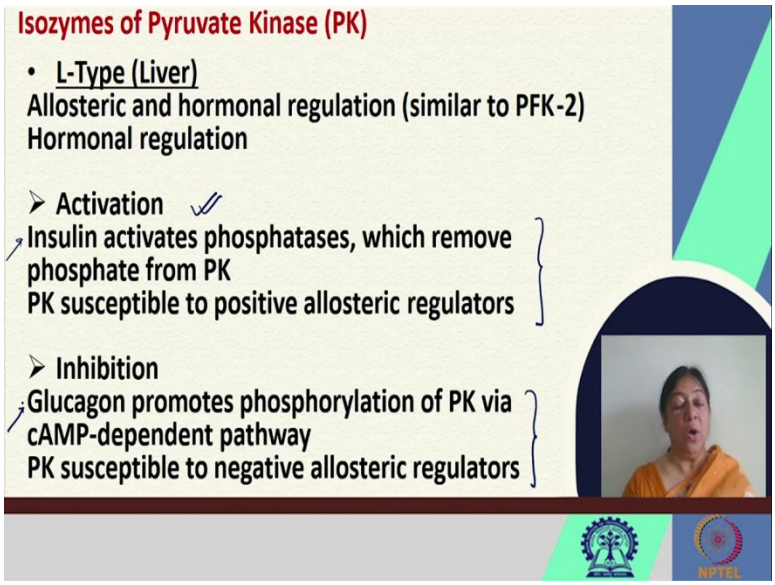

Similarly if we look at the isozymes of pyruvate kinase of the liver type, we would again have allosteric. In this case, there is also hormonal regulation where again we would have activation with some specific types of molecules present in the body and also inhibition that would result in the activity of the pyruvate kinase being affected in this particular L-type lysosome; the L-type pyruvate kinase.

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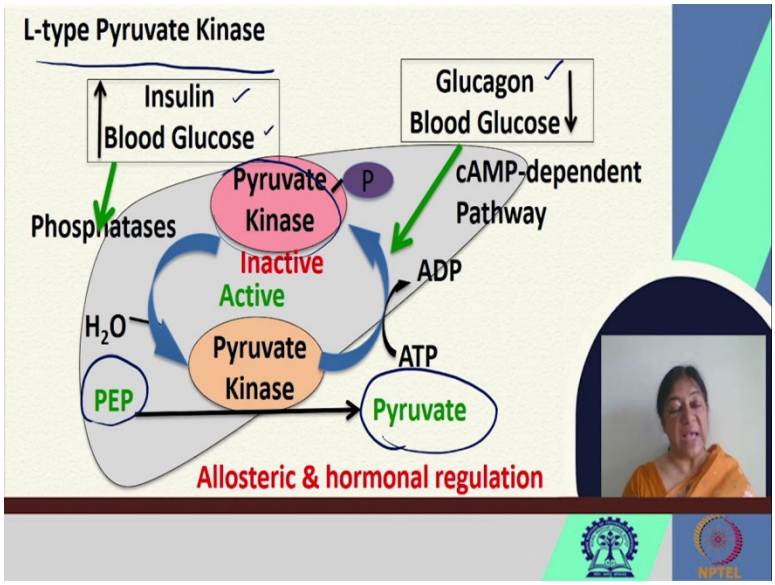

If there is say an increase in insulin blood glucose, then what happens, we have the action of the pyruvate in a manner that would result in an active form of the pyruvate kinase. However, there could be specific inhibitors of the protein. So, we have the phosphoenol pyruvate going to the pyruvate in this particular reaction. We would need the active form of the enzyme to perform this action.

This enzyme therefore would be activated, as we saw by the specific activations that are required. Insulin would be one such activator, on the other hand glucagon would be an inhibitor. We would have the activation of the enzyme pyruvate kinase to perform its activity in the presence of say insulin and we would have an inactivation in the presence of glucagon that would act as an inhibitor, preventing the pyruvate kinase from its action.

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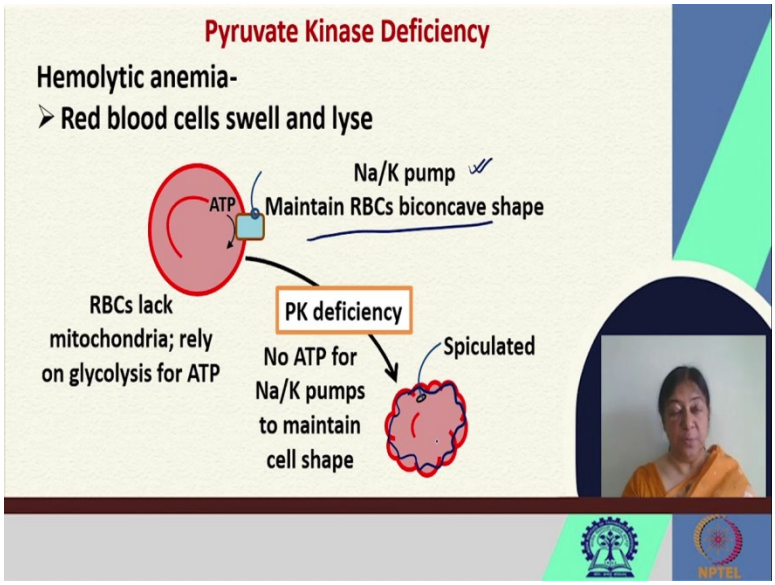

We look at the reasons for understanding the pyruvate kinase and how we can have specific inhibitors designed for this enzyme. It is involved in hemolytic anemia, where the red blood cells can swell and lyse. And what happens is usually to maintain the shape of the red blood cells, we have the sodium/potassium pump that; something that we will see in membrane transport.

So this maintains the RBCs by concave shape. However with the pyruvate kinase deficiency, the ATP pump does not work and what we have is, we have small spikes on rendering this non-spherical, which is called a speculated RBC.

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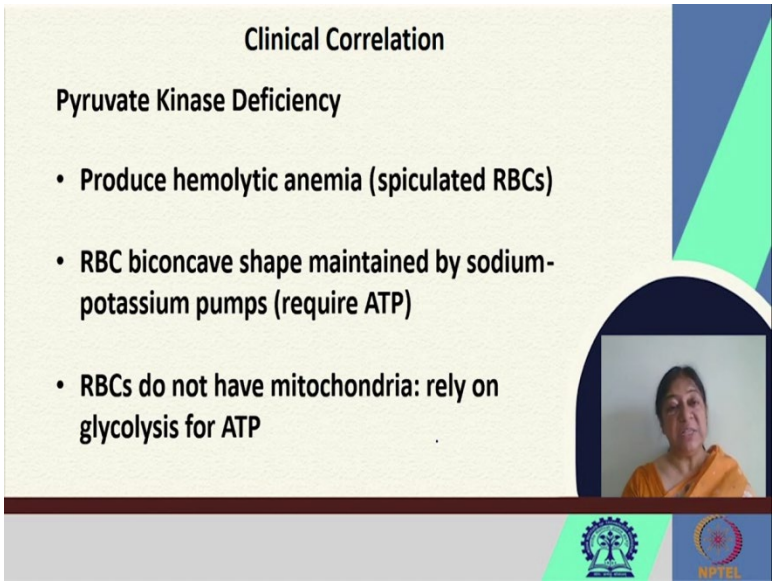

The clinical correlation is such that this pyruvate kinase deficiency could lead to speculated RBCs production of hemolytic anemia and the biconcave shape would not be possible in the presence or the inactivation of this protein, because it is very important for the process to go on in a manner because the glycolysis process is what is important for the RBCs, as they lack mitochondria.

The importance of looking at isozymes of the different type that would catalyze the same reaction in a sense, but the enzymes themselves would be different; like in this type we saw the muscle type and the liver type.

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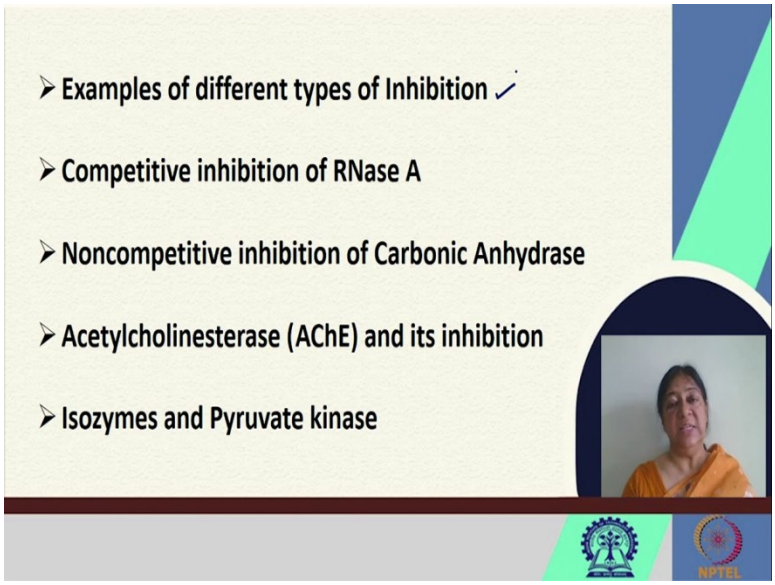

So what we have looked at in this lecture, is the examples of different kinds of inhibition in terms of specific examples, the kinetics associated with this in the Lineweaver-Burk plots and how with increasing concentration of the inhibitor, we would have varying values for the v_{max} in terms of non-competitive inhibition, varying values for the Michaelis-Menten constant for the competitive inhibition.

And we also looked at the example of acetylcholinesterase, where we have irreversible inhibition, due to covalent bond formation with a specific active site residue. In this case, serine 200 that is phosphorylated in a manner that would prevent the nucleophilic attack on the substrate. And we looked at isozymes with the specific example of pyruvate kinase in its mode of action.

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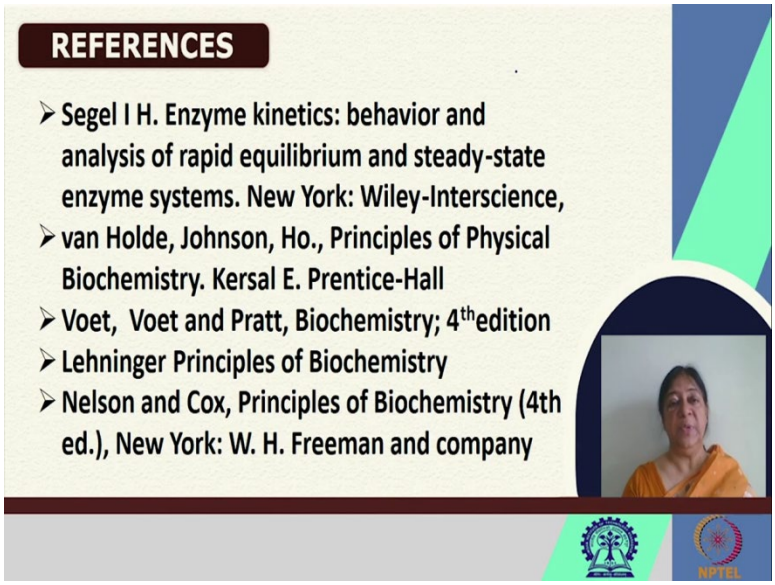

These [refer to slide] are the references.

Thank you.