Medicinal Chemistry
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Tutorial-06
Enzyme Kinetics, Various Modes of Inhibition etc

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Welcome to the tutorials session. In today's session we will be looking at enzyme kinetics and associated concepts. So before we get into the enzyme kinetics let us first get an overview of what you know of some general concepts of kinetics. So we are all familiar with the concept of order. Order of a reaction is something that we have learnt extensively in the past, so let us just go over that quickly.

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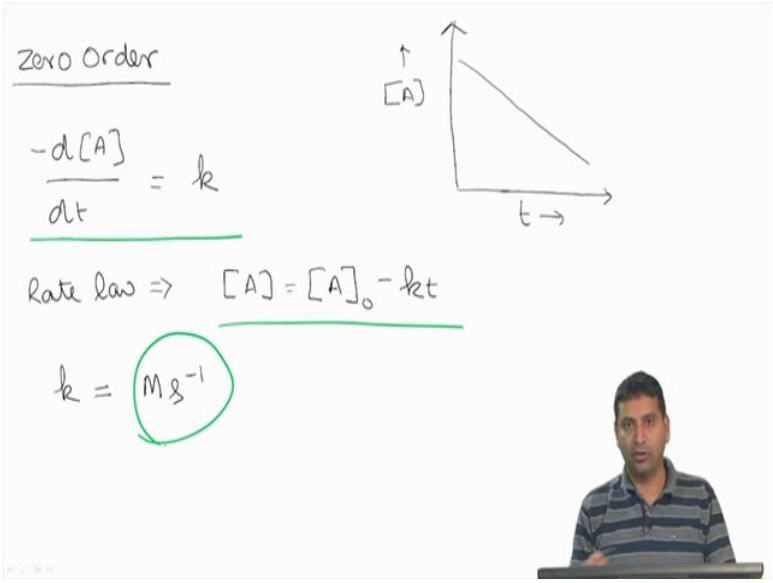

So the first major reaction that we should look at is a zero order reaction, rate of the reaction does not depend on the concentration of the starting material. So therefore dA by dt equals constant which is the rate constant k and the rate law is nothing but A into A not minus kt and the order of zero order reaction is molar per second and if you plot the concentration of the starting material versus time, you get a straight line so that is as shown here, zero order reactions are quite not very commonly encountered.

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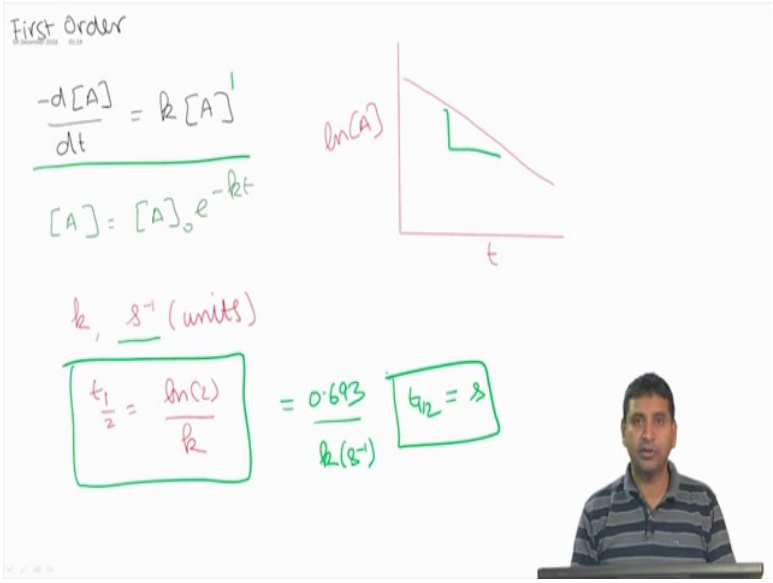

The more commonly encountered reactions are the first order reactions and keep in mind that if you have a second order reaction in which there is excess of starting material, then if there are two starting materials A and B and if B is in large excess then we approximate that to be a

first order reaction or a pseudo first order reaction. So the principles of that would be very similar.

So if you look at the rate of change of concentration of A with time you get it depends on the concentration of A and the superscript here or the coefficient is 1 and therefore this is called a first order reaction. So the rate law would be written as concentration of A equals the initial concentration that is time equal 0 times e power minus kt. So we will not go into all the details and all that but essentially if you plot ln of A versus time you will get a straight line and the slope gives us the rate constant.

So the units are per time that is here it is per second, it can also be per minute and so on and we are already familiar with the concept of half-life. Half-life is nothing but ln of 2 divided by rate constant which is basically 0.693 approximately divided by rate constant which is expressed in per second, so half-life the units are in seconds or it can be in minutes if your half-life is expressed in minutes and so on.

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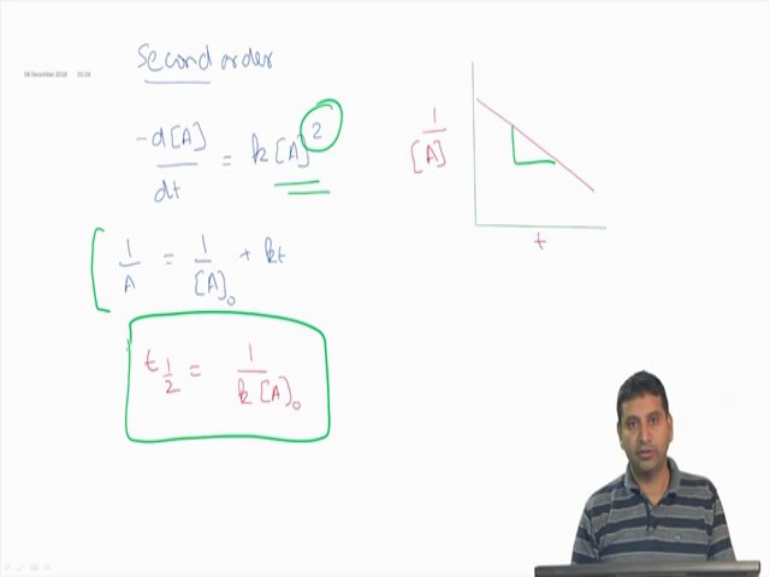

So the last order reaction we are not going to go into higher order reactions is the second order reaction, so here we have taken a very simplified example of a reaction of A plus A giving you products. So here the rate law is defined as d of A by dt equals k times A to the power of 2, so that is the order of the reaction. Now we are all familiar with what order and molecularity means, so molecularity is the number of molecules that are going to collide to give you the product, so here the order is equal to molecularity that is that it is a bimolecular reaction and it is also a second order reaction.

So if you do the math you get 1 by A equals 1 by A not plus kt and so if you plot 1 by A versus time you get a straight line the slope of this gives us the rate constant k and the half-life of a second order reaction is defined by the following term which is 1 by k times concentration of A not.

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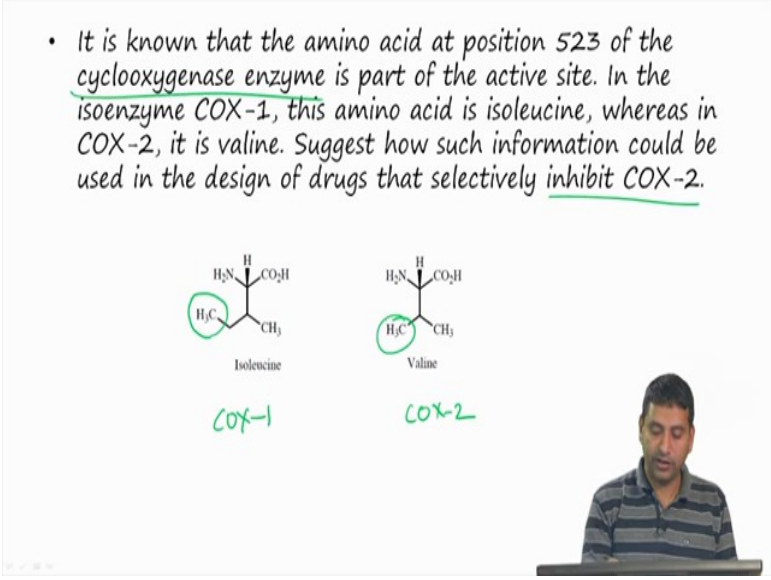

So now let us look at some questions, it is known that the amino acid position 523 of cyclooxygenase enzyme is a part of the active site. In the isoenzyme COX-1 which is a cyclooxygenase enzyme 1 this amino acid is isoleucine, whereas in COX-2 it is valine suggest how such information could be used in the design of drugs that selectively inhibit COX-2.

So in order to understand this first let us understand that isoleucine COX-1 is the amino acid at 523 and valine is COX-2, so now if you want something selectively inhibit the enzyme with valine but not with isoleucine then we need to look at the sterics for example. So here the major difference between isoleucine and valine is the extra methyl group.

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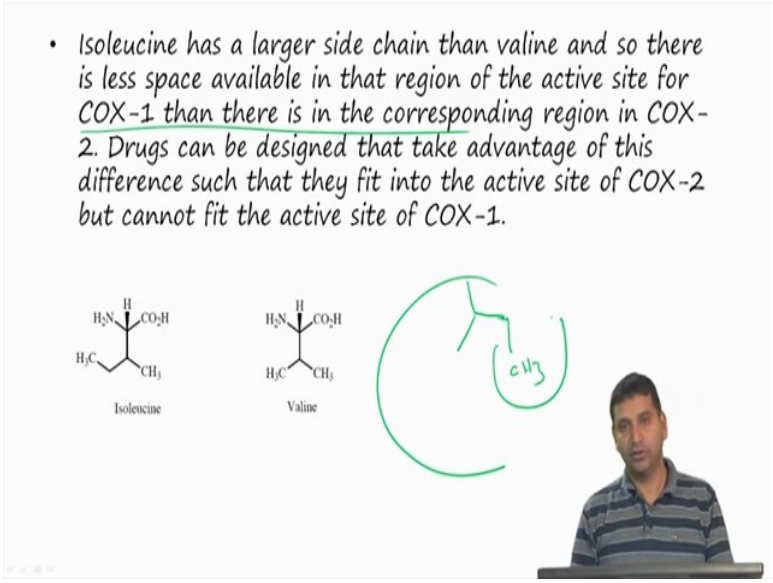

Since isoleucine has a larger side chain than valine, there is less space available in that region of the active site for COX-1 than there is for COX-2. So even the small difference can actually have a major impact and if we can design a drug that can take advantage of this then it is possible to design a compound that is selective to COX-2 over COX-1. So here in the active site you have isoleucine or you have another active site which has valine sorry it has isoleucine or it can have a less methyl group and so if we can figure out something that binds selectively to this then we would be able to design a selective compound.

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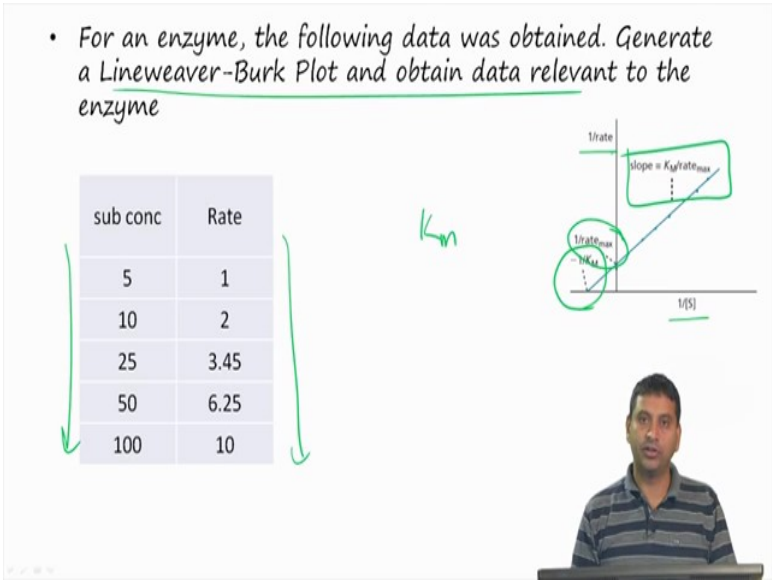

Now let us look do some plotting, so this plotting we will go through its step by step and we should be able to figure out lot of things in the Lineweaver-Burk Plot. So again recall

Lineweaver-Burk Plot is a plot of 1 by rate versus 1 by substrate concentration, so you have to repeat the experiment of enzyme kinetics a number of times with various substrate concentrations and then you measure the initial rate and then 1 by rate versus 1 by substrate concentration is the Lineweaver-Burk Plot.

So the question here is for an enzyme the following data was obtained, generate a Lineweaver-Burk Plot and obtain the relevant data for the enzyme. So the substrate concentration is given here that is we have changed it from 5 to 100 and the rate is given here and as you can see the rating increases with the substrate concentration. So what are the major parameters that we need to look at? The first one is the Michaelis–Menten constant or K_M, the second one is 1 by rate max which is the intercept over here and the slope is K_M by rate max.

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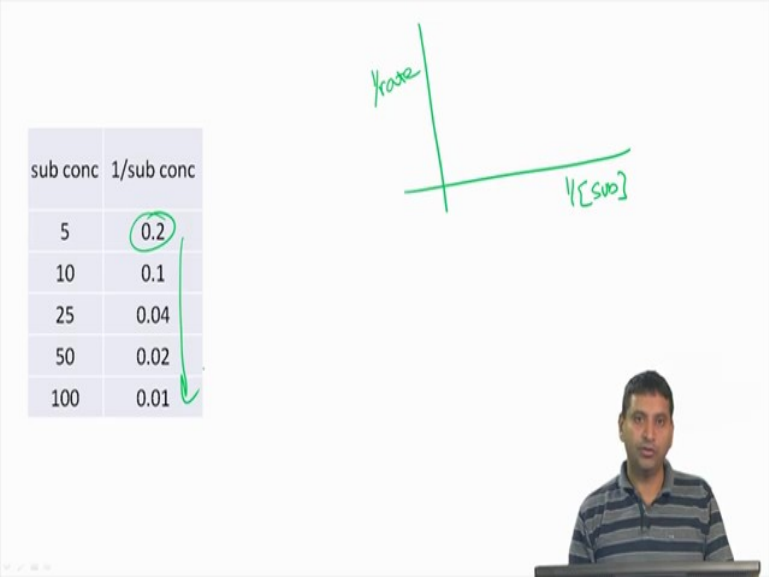

Now let us look at how to obtain all of these parameters in a very systematic manner. So first thing that we do is to take the inverse, so we need to take the (substrate) inverse of the substrate concentration because we plot 1 by substrate concentration on the X axis, 1 by rate on the Y axis. So let us first do an inverse, so if you take an inverse of 5 you get 0.2 and 25, 50, 100 and so on you get these numbers. So this can be done either manually or if you just put it in spreadsheet such as excel or other software's you can get this done very easily.

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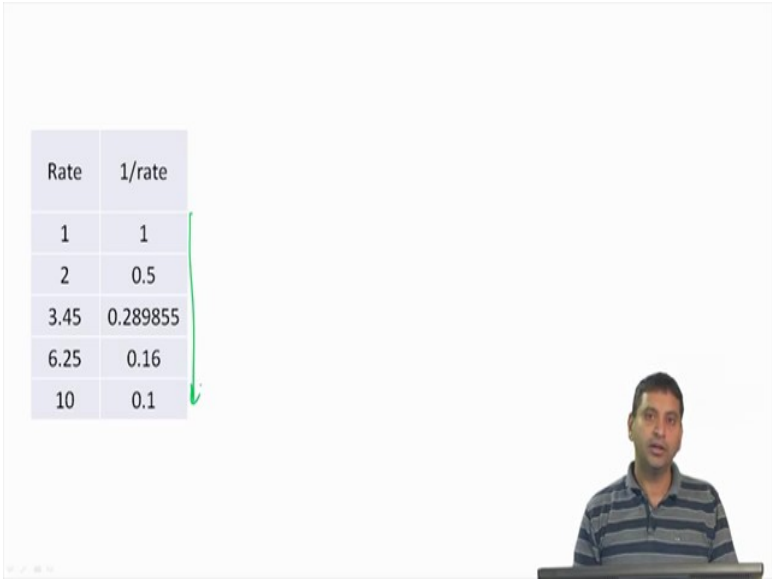

Similarly we will have to do inverse of the rate, so if you do the same inverse that is 1 by rate you get these numbers. Now you have to use you can either plot it again manually using a graph sheet or you can plot it using excel.

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So what we are looking for is basically this is the overall table that we get, so here is 1 by substrate concentration for that substrate concentration this is the rate and for this is 1 by substrate concentration you get this as the 1 by rate.

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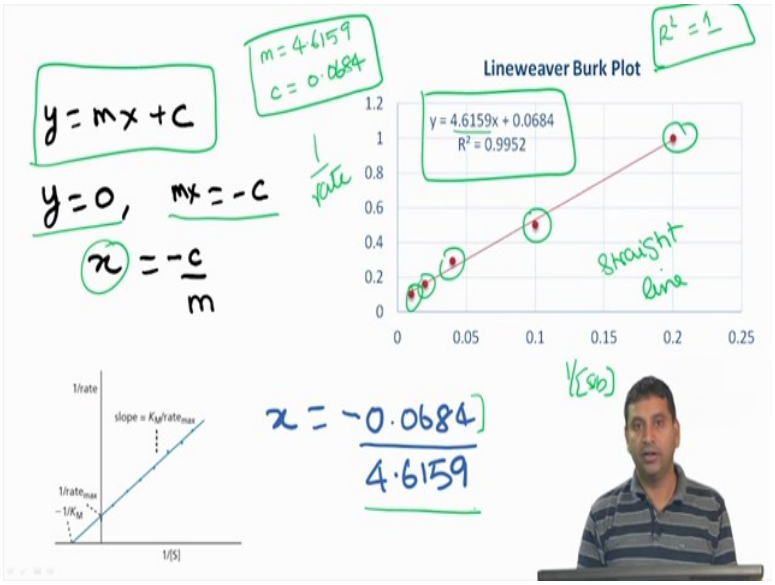

So if it make the plot you get on the Y axis is 1 by rate and on the X axis is 1 by concentration of substrate. So if you plot you should do this yourself, these numbers are available so you can just plot it, you get a straight line, you get a first you get this plot where you have lot of these points so each of these represents a data point and then you can do what is known as a curve fitting, you do a curve fitting to a straight line and that equation of the straight line is basically y equals m x plus c and you get that corresponding thing here which is y equals mx plus c. So we have looked at all of these stepwise.

So R square that is shown here is a measure of how well the line fits. So the higher the R square value, the closer it is to a line fit. So the maximum value of R square is theoretical value is 1 and so any number that is close to 1 is actually a good fit. So when we look at this plot we get an R square of 0.9952 which is very close to the theoretical limit and therefore this fit is excellent. So this data that we are going to obtain is very reliable and the experiment is quite reproducible as we know from other data and this is going to be a good you can make good meaningful interpretations from this data.

So now let us look at y equals mx plus c. So here m is 4.6159, let me write that down m equals 4.6159 and c which is the intercept is 0.0684. So these are the two numbers that we get after curve fitting. So when y equals 0 we can take y equals 0 then mx becomes minus c, so then x turns out to be minus c divided by m and so as we can see here if you take the c as the number from here and divided that by 4.6159 which is m, then you get a value of x, when y equals 0 you can get the value of x.

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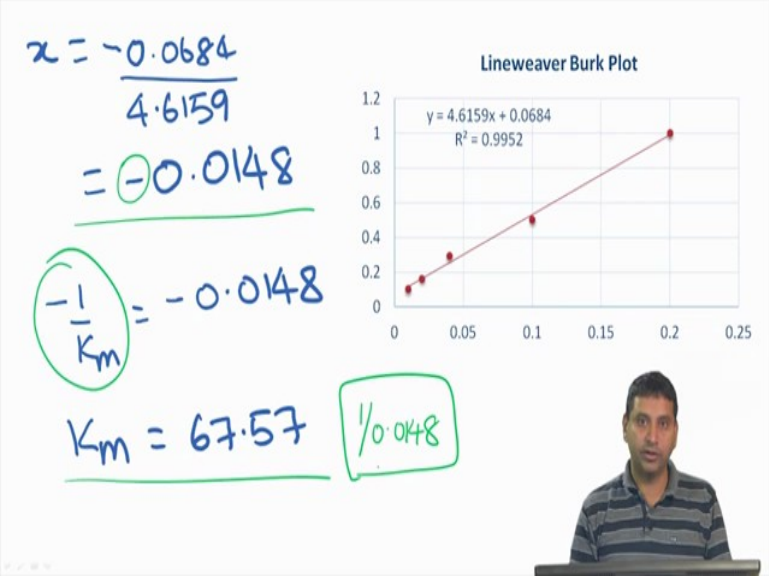

So this number turns out to be minus 0.01484 and this negative number comes because it is going to intercept at the negative part of the X axis. So from the Lineweaver-Burk Plot we know that the negative intercept or the intercept on the X axis is nothing but minus 1 by K_M. So then just I have to take the inverse of this number and then K_M becomes 67.57 which is nothing but 1 divided by 0.0148. So I would urge all of you to go back and do these calculations yourself, these numbers are available so please take some time and do these calculations and familiarize yourself with the technique.

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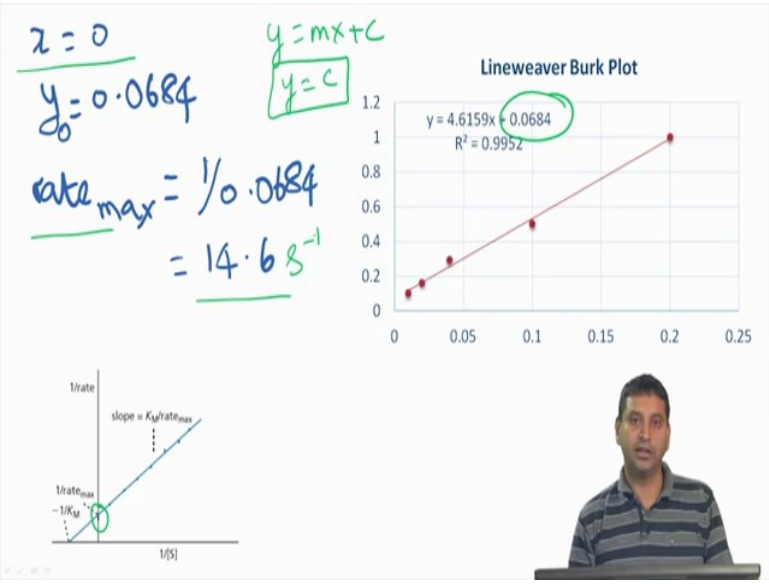

Now the next parameter is rate max and if you can see rate max is the intercept at the Y axis, so when intercept at Y axis, x becomes 0. So when I put y equals mx plus c if I put x equals 0,

so then y equals mx plus c, if I put x equals 0 then y becomes c, c as we know is this number 0.0684. So rate max is nothing but 1 divided by 0.0684 which is basically 14.6 and the units of this would be per second because we start with the time coordinate here is second.

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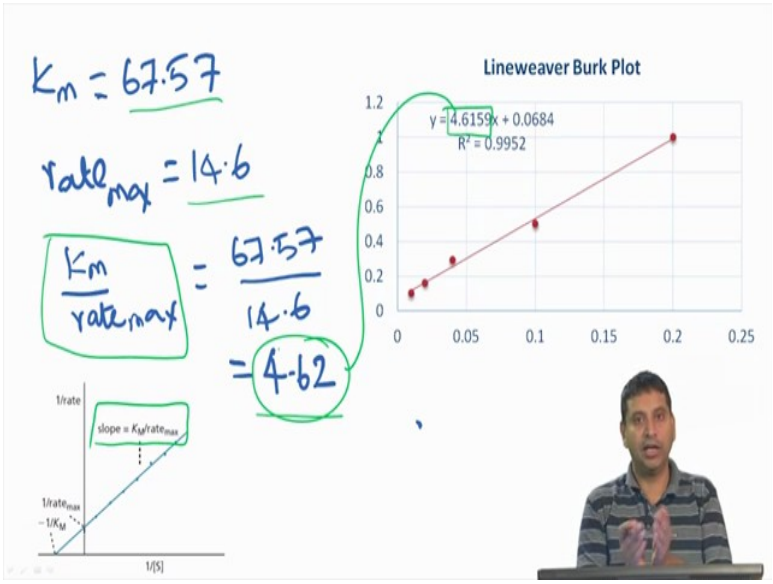

Now let us look and see if we are getting consistent data as far as the slope is concerned. So according to the Lineweaver-Burk Plot the slope should be K_M divided by rate max that is what shown here. Now we have independently derived what is K_M which is 67.57 and rate max is 14.6. So now if I divide these two numbers I get 4.62, so the slope that we obtain from curve fitting was 4.6159 these numbers are quite comparable in nature and therefore I think what we have done in this process is just to double check the calculations that we are doing everything correctly.

So when we start out this problem, what we need to do is to first take an inverse of both the numbers that is you take of the 1 by substrate concentration and then you take 1 by rate max, once you take these two you plot them as the X and Y axis and once you plot them you can then do a curve fitting to a straight line and when you do this curve fitting you get a slope which is you can fit it to a straight line equation y equals m x plus c, and then m is the slope and c is the intercept, so from these two numbers you can actually derive what is the Michaelis–Menten constant as well as rate max.

So these are going to be very useful when we are looking at problems where we want to understand the nature of inhibition. So the Lineweaver-Burk Plot on the whole gives us the good idea about the nature of inhibition. So once we do this exercise for a particular enzyme

then we have a very good way to understand how inhibition works and that is what we would like to do in terms of drug discovery.

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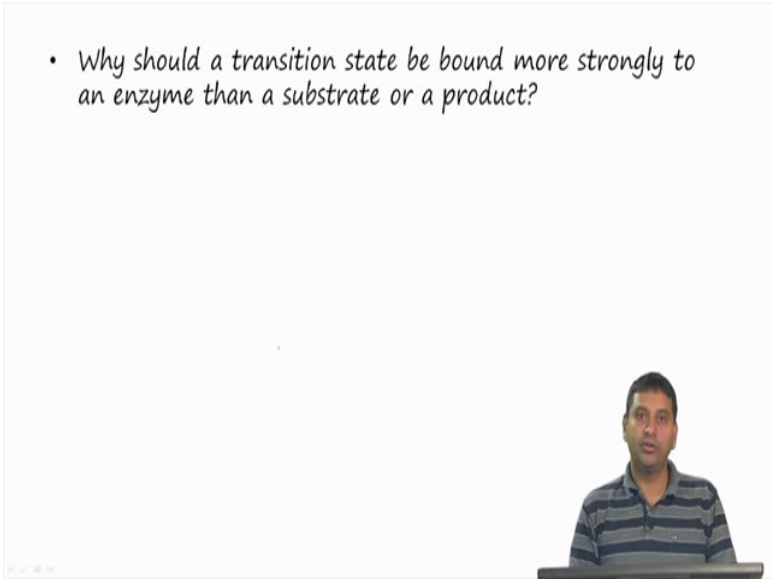

So the next question is why should a transition state be bound more strongly to an enzyme than a substrate or a product?

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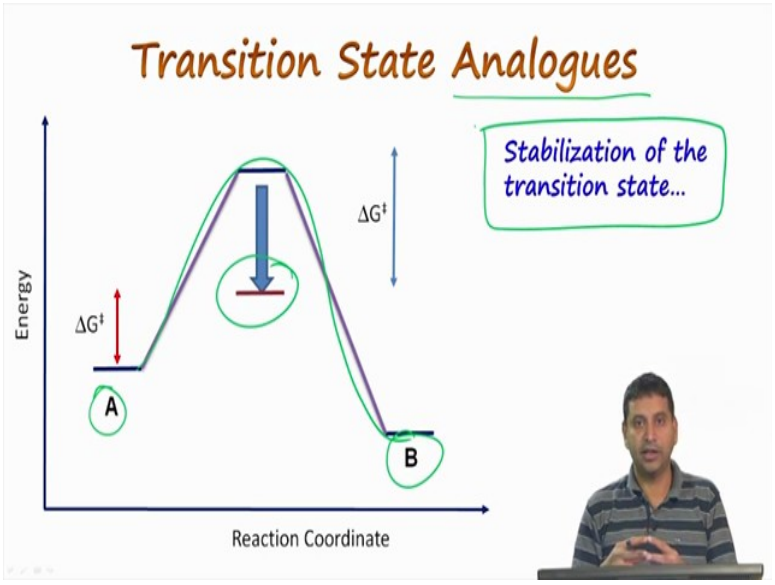

So let us go back and look at some of the concepts that we looked at which is basically transition state analogues. So in an energy profile that we have seen many times in this class of reaction A going to B, we have understood that there is a transition state in the process. So

one of the things that the enzyme does is to stabilize the transition state, so stabilization of the transition state is a key component of how an enzyme works?

(Now) Therefore we would hypothesize that the enzyme binds stronger to the transition state when compare to the starting material or the product and so this binding is going to afford some stability to the transition state when compared with the reactant or the product.

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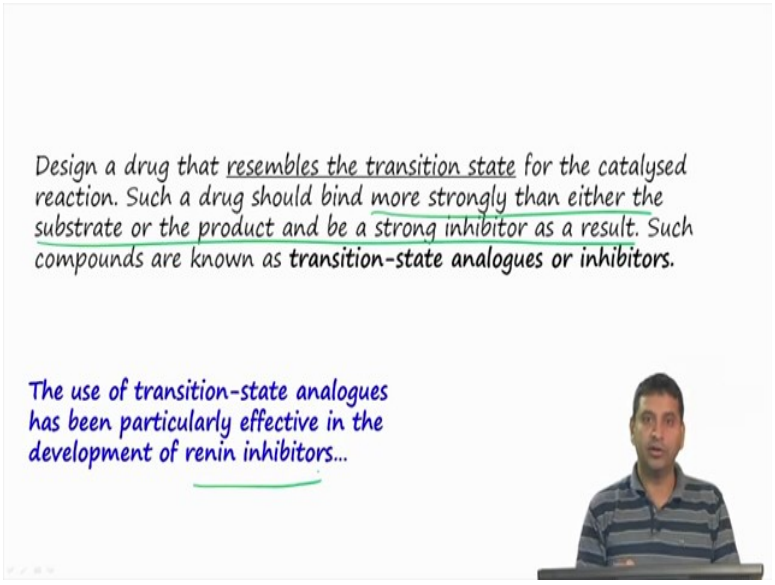

So let us just go back and look at this what we have seen in class that is if you design a drug that resembles the transition state then such a drug should bind more strongly than either the substrate or the product and so if it is going to bind more strongly than it is not going to dissociate if there is no turnover. So these compounds are known as transition state analogues or transition state inhibitors. The use of transition state has been particularly effective in the development of renin inhibitors which we have looked at in the class.

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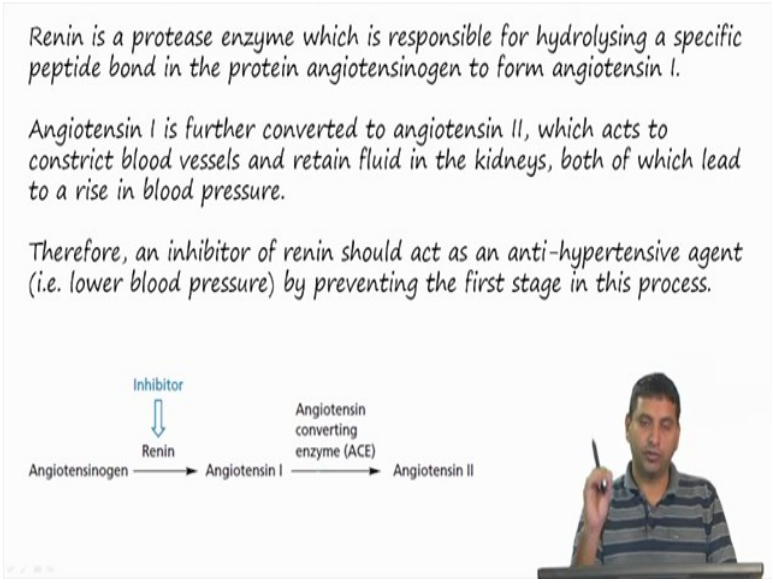

So let us just recap this once again, renin is a protease enzyme which is responsible for hydrolysing a specific peptide bond in the protein angiotensinogen. I will not go into too many details because we have already looked at in class, but essentially what we are looking at here is to design a compound that is going to prevent the hydrolysis for specific peptide bond.

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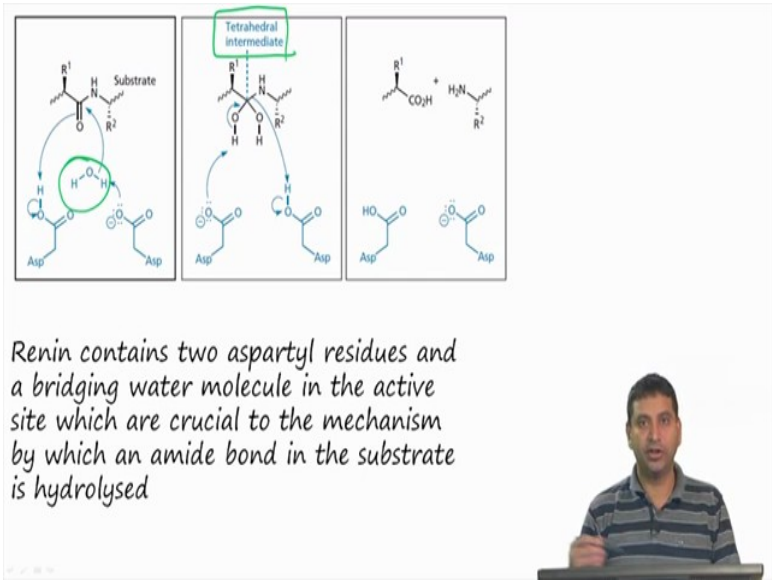

So if we look at how a peptide bond is hydrolysed, you have the mechanism that is proposed here is water attacks this and forms a tetrahedral intermediate and this tetrahedral intermediate then collapses and gives you the product.

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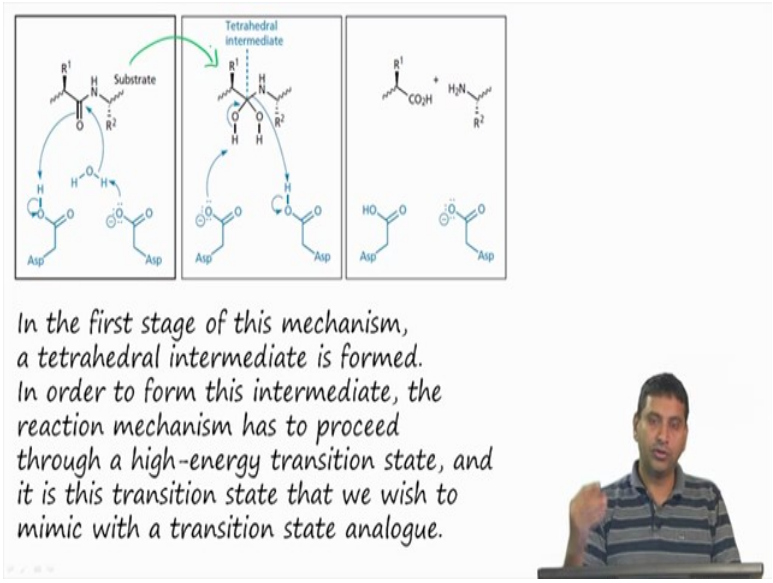

So in order for us to look at the transition state analogue we need to understand that there must be a transition state that is happening between the first step and the second step, so that transition state will have some tetrahedral character, it will also have some carbonyl character and this water will be bound partially start to make a bond with carbon and the other bond which is the double bond is going to slowly break.

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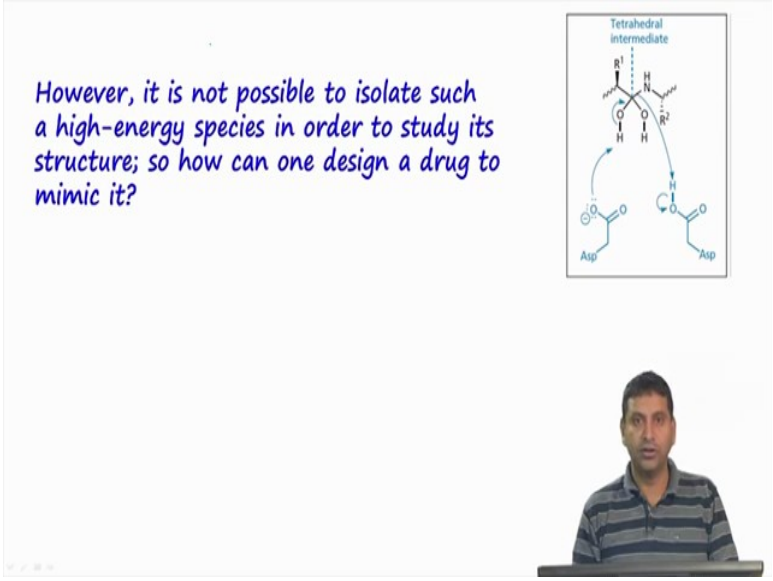

So as we know it is not possible to isolate such high energy species in study its structure, so we need to design a drug that mimics it.

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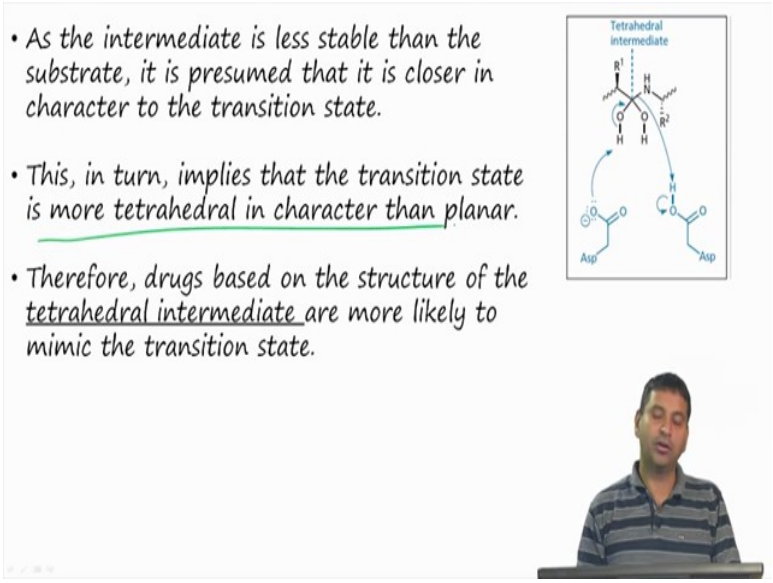

So as the intermediate is less stable than the substrate, now we can assume that this intermediate is what is going to be stabilized by the enzyme. So this in turn implies that the transition state is more tetrahedral in character than planar. So what we need to look at is we need to figure out how to develop a tetrahedral intermediate or a tetrahedral intermediate like molecule.

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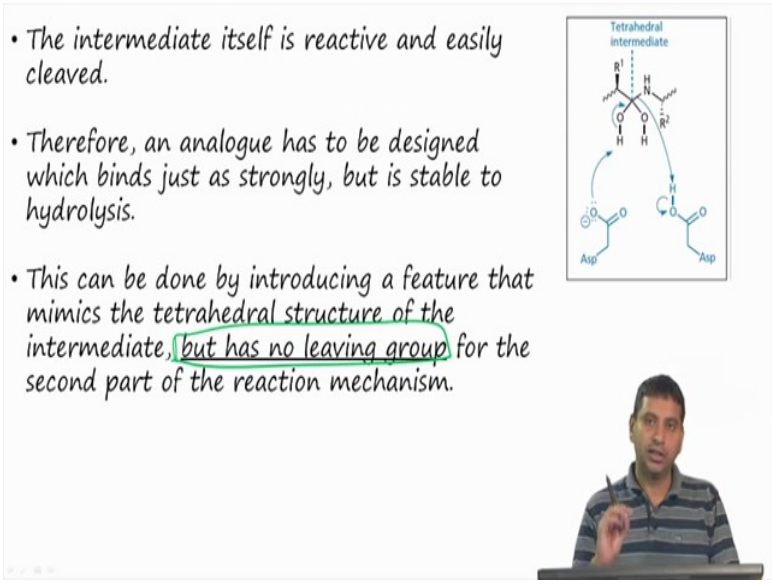

So the intermediate itself is reactive and so therefore it is not going to be useful in designing a compound, but if you have a compound that has no leaving group, so that it looks like a tetrahedral structure but it has no leaving group then we can make a transition state mimic.

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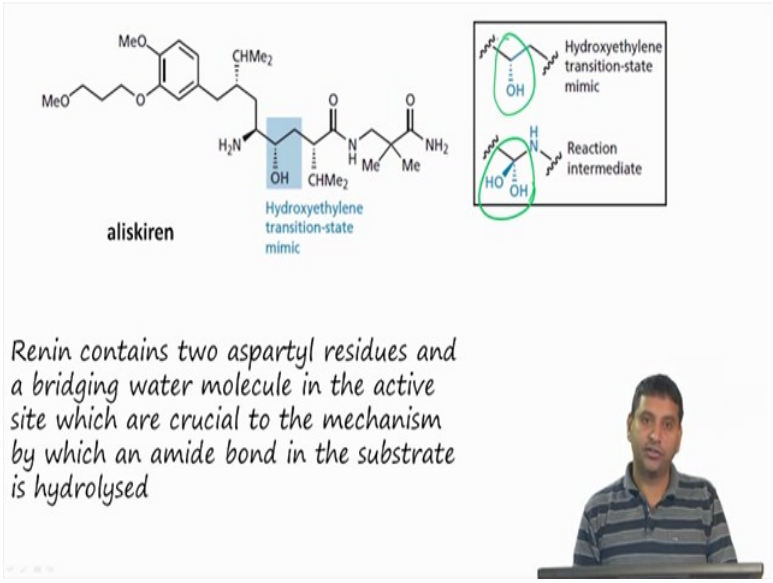

So the compound that we are looking at here is based on this reaction intermediate which has two hydroxyl groups and the compound that was designed has one hydroxyl group which has some character of the transition state and also has but also is not turned over and therefore it acts as an inhibitor.

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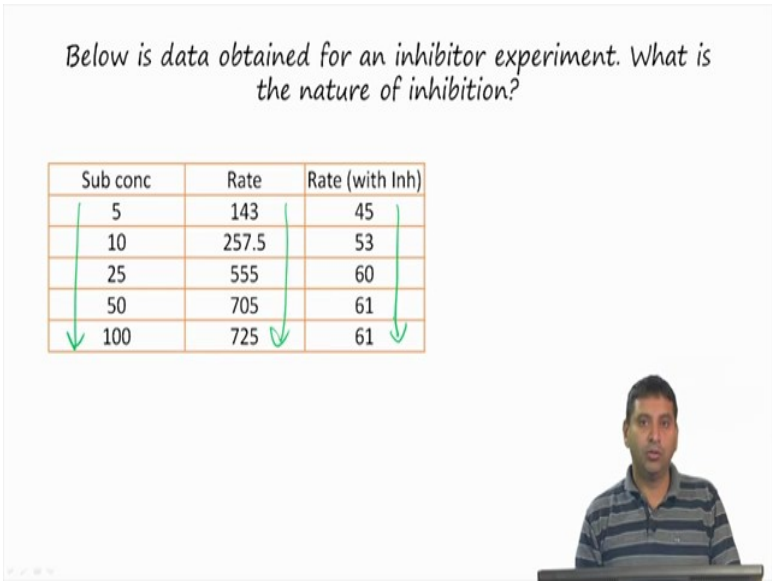

Now let us look at the next enzyme kinetics experiment, so here we are going to look at an inhibitor and the question is what is the nature of inhibition? So again the data that we have been given is a substrate concentration which varies from 5 to 100 and the rate is given here, rate can be measured in per second or per minute depending on what units we are putting in here and this is the rate with the inhibitor. So we know that the inhibitor works because if you

just look at this table you see that the rate without the inhibitor is much higher than the rate with the inhibitor, but the question is what is the nature of inhibition?

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Sub conc	Rate	Rate (with Inh)	1/sub	1/rate	1/rate(inh)
5	143	45	0.20	0.006993	0.021993
10	257.5	53	0.10	0.003883	0.018883
25	555	60	0.04	0.001802	0.016802
50	705	61	0.02	0.001418	0.016418
100	725	61	0.01	0.001379	0.016379

So in order to understand this we need to derive the Lineweaver–Burk Plot, so for doing the Lineweaver–Burk Plot, we have already looked at systematically what the steps are, so the first step is to take an inverse so you take 1 by substrate concentration which is from the substrate and then you do 1 by rate which is basically this 1 divided by this and the last one is the 1 by rate of the reaction in the presence of the inhibitor, so these are the numbers that we get they are all very long numbers I am not going to read them out but now you can start by plotting 1 by substrate concentration versus 1 by rate for the uninhibited reaction or the parent reaction.

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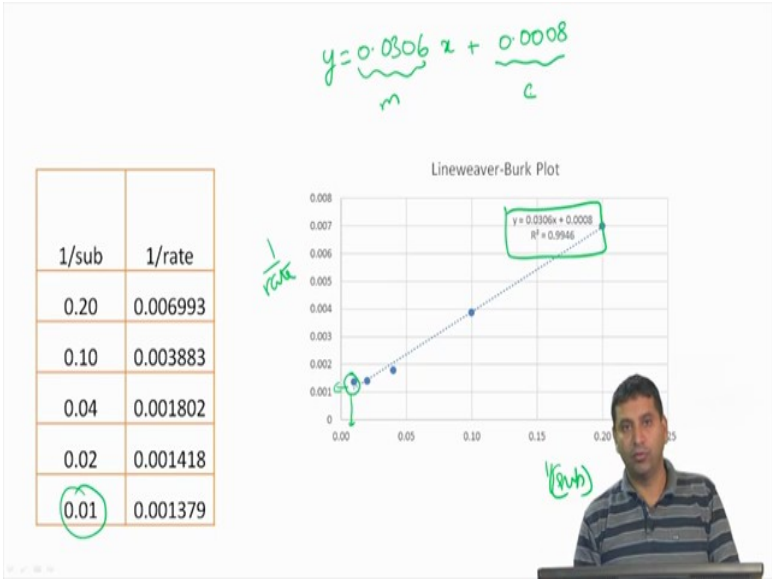

So when you do this you get the following plot, again this is 1 by rate versus 1 by substrate concentration. So as you can see here for example at 1 by substrate concentration of 0.01 the rate of the reaction is 0.001379 so here is the X axis and here is the Y axis. So again we will go through the same process of fitting it to a line and we get this curve fitting data y equals 0.0306 which is nothing but m and then x plus the intercept is 0.0008 so this is c, so again this is repetition of what we did in the previous case.

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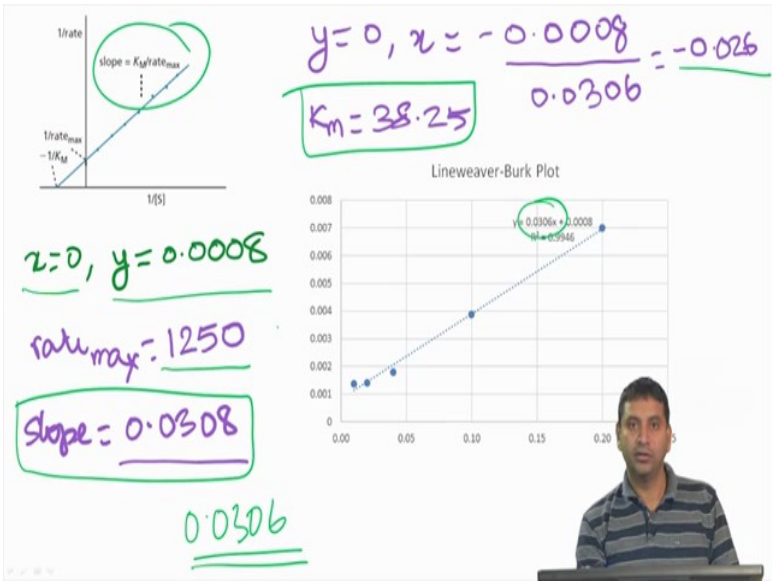

Now if we were to derive the Michaelis–Menten constant y equals 0, x then becomes 0.008 which is the c divided by m and you get this value of 0.026. So we know that we can take the inverse of this negative inverse of this number that is 1 by that number and then making it

into a positive value will give us 38.25. So the K_M here is 38.25 and the rate max again, if you go through the steps you get x equals 0, y equals 0.0008 and then you take the inverse of that then you get the rate max is 1250.

Now if we look at the third criterion which is basically the slope which is K_M divided by rate max then we get a value of 0.0308 and if you recall the previous value that we have of the slope 0.0306 so these two numbers are extremely close and they are comparable so there is no error in our calculation. So together from this Lineweaver–Burk Plot we have obtained K_M which is 38.25, rate max is 1250 and from this we can now look at the kinetics of the inhibitor.

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So when we do the same process when we apply the same process for the inhibitor when y equals 0, I mean you get a straight line as shown here, the slope of the straight line is 0.0306 and the intercept is 0.0158 so these are the values that we get, this is c and this is m and from this when y equals 0, x becomes 0.0158 divided by 0.0306 and this turns out to become this number and if you take the inverse of this you get 1.94, so the K_M is 1.94 in the presence of the inhibitor. So when x equals 0, you get y equals 0.0158 and so the rate max is 63.29.

So as you can see here the Michaelis–Menten constant is 1.94 and the rate max is 63.29, so both these numbers indicate you know that inhibition is indeed occurring. Now just by looking at the plot we can see that the slopes are similar if not identical because they look like parallel lines so indeed the slope is identical which is 0.0306 and so now let us compare the two situations.

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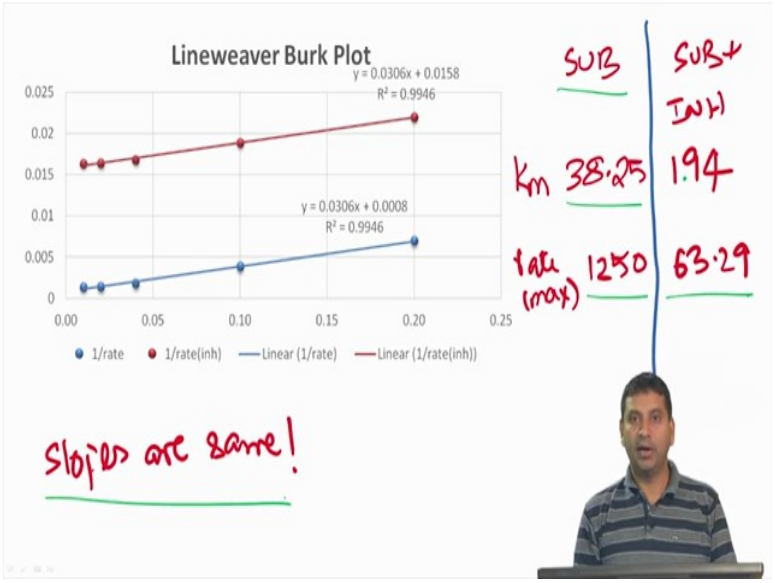

So in the presence of the substrate alone the Michaelis–Menten constant is 38.25 and in the presence of the inhibitor it is 1.94, the rate max is 1250 which is a fairly large number in the presence of the inhibitor it goes down to 63.29, but as we can see from the plot the slopes are identical and so let us go back and look at what kind of inhibition this is.

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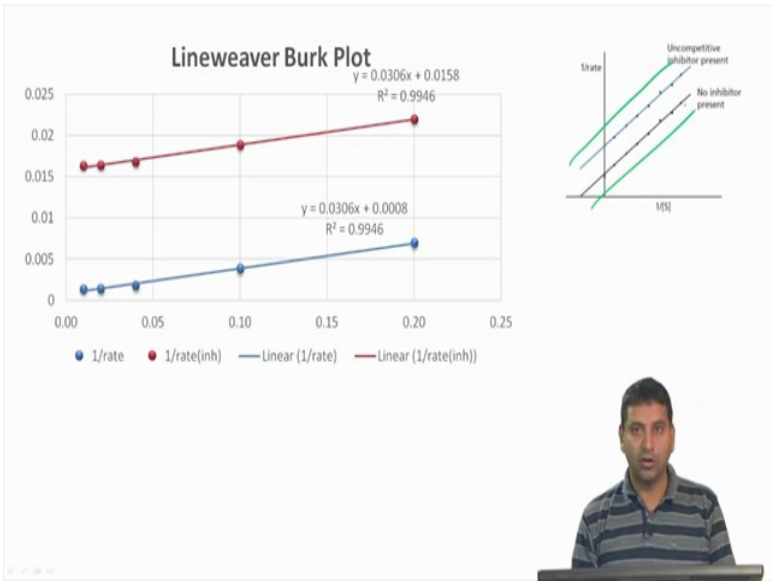

So if you have an uncompetitive inhibitor present then you would get this kind of a situation where the two slopes are the same and that is how this uncompetitive inhibition is understood. So based on the Lineweaver–Burk Plot we are able to figure out that this mechanism of inhibition is an uncompetitive mode of inhibition.

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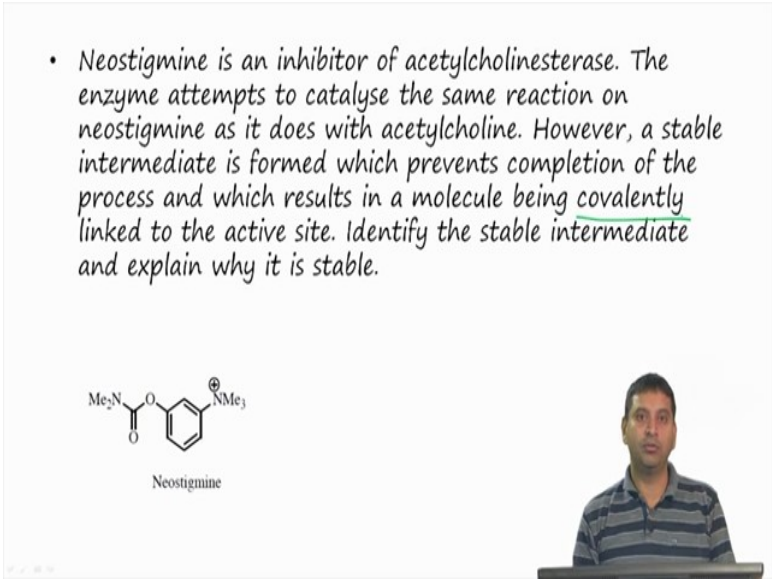

Next question is Neostigmine is an inhibitor of acetylcholinesterase. The enzyme attempts to catalyse the same reaction on neostigmine as it does with acetylcholine. However, a stable intermediate is formed which prevents the completion of the process and which results in a molecule being covalently linked to the active site. Identify the stable intermediate and explain why it is stable?

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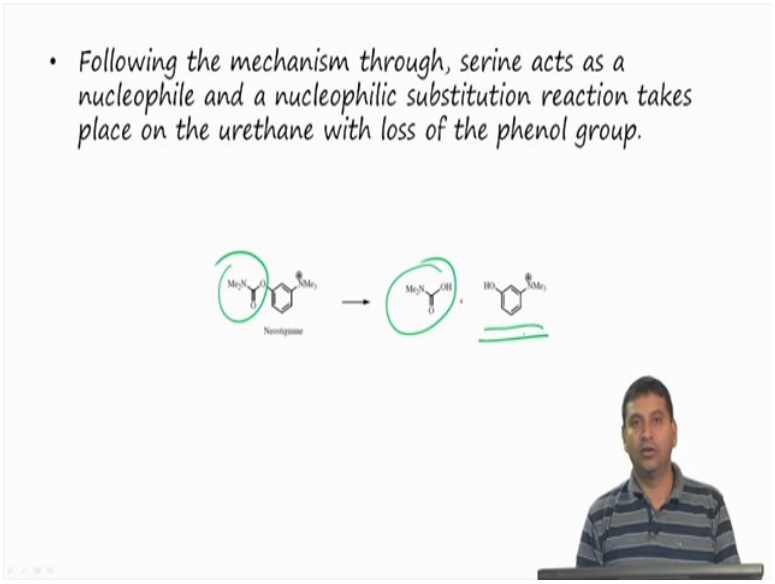

So this is the mechanism that is proposed for neostigmine, so you have carbamate over here and then the carbamate is undergoing hydrolysis to produce this product the loss of urethane with the phenol group.

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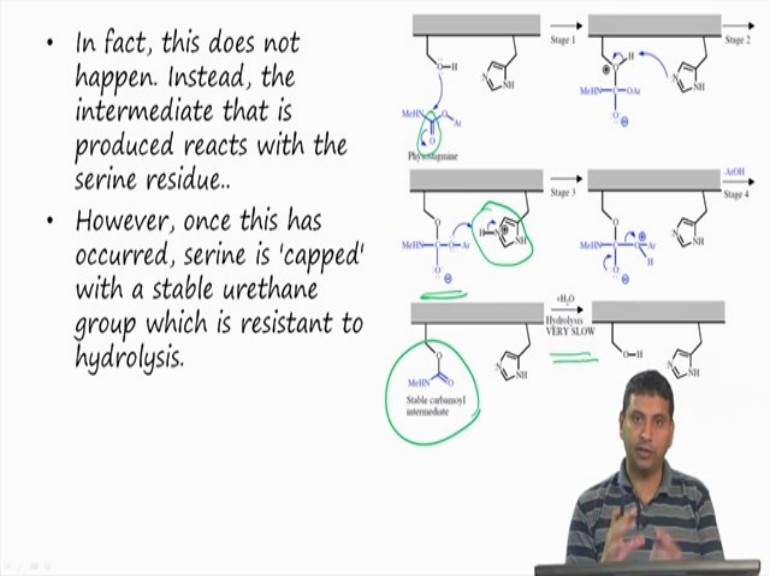

So in fact this does not happen and what indeed happen is actually a very different process, so when the serine residue reacts with the carbonyl over here, it produces the tetrahedral intermediate, the neighbouring residue picks up a proton and gives you a ionic species like this and subsequently, because of this acid based catalysis of the histidine ring over here you have a transfer of the phenol ring to the histidine and then once this happens you get a very stable carbamoyl intermediate which does not decompose further. So, therefore this hydrolysis is actually very slow and so this is how this molecule goes and caps the serine and forms a stable urethane.

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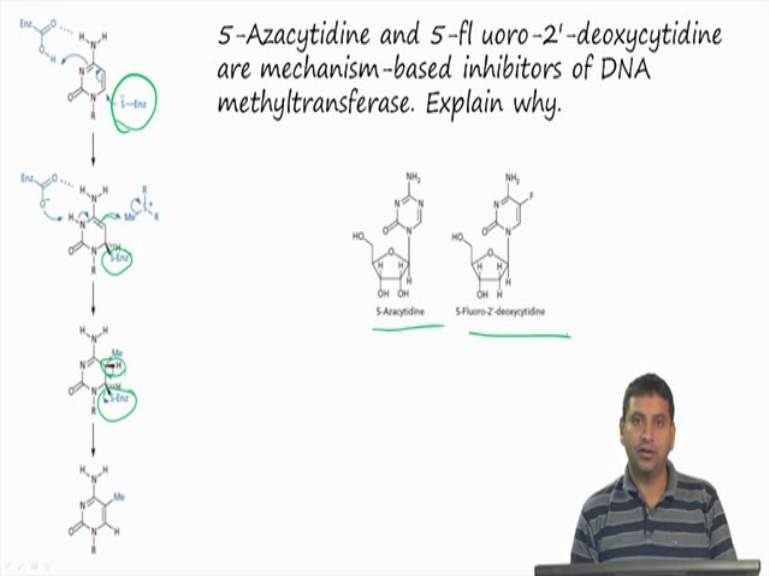

The next question is 5-Azacytidine and 5-fluoro-2 prime-deoxycytidine are mechanism based inhibitors of DNA methyltransferase. Explain why? So for this let us look at how mechanism of DNA methyltransferase happens. So this enzyme has a thiolate which attacks on this carbon over here and produces an intermediate such as this where you have an S enzyme over here, then arrows can be pushed and there is a loss of the proton before that here that reacts with S-Adenosyl methionine which is basically a methylating agent and produces this methylated compound which can then loose the enzyme, sulphur, as well as hydrogen to give you the product. So this is the mechanism by which DNA methyltransferase functions. So the question is these two molecules are they mechanism based inhibitors and we need to explain why?

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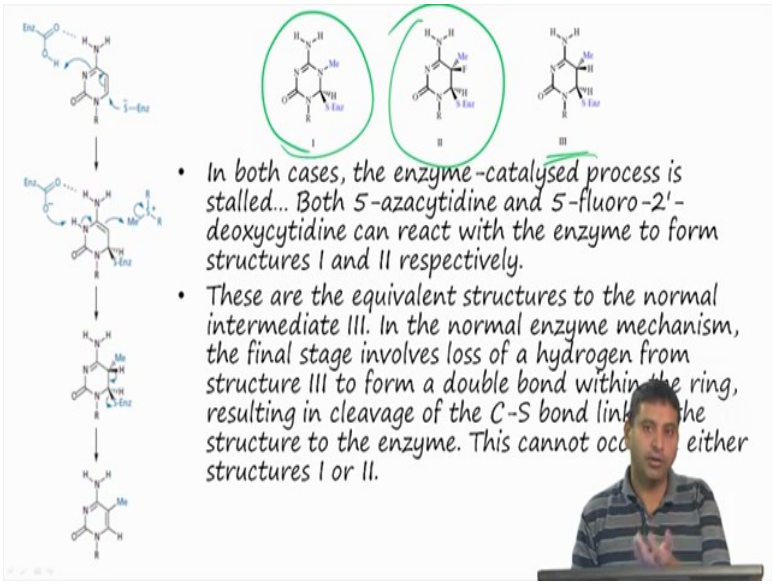

So if do the same or propose a similar mechanism to these molecules what would happen is you would get intermediate such as this, so here in the first case you get a intermediate where the sulphur attacks and forms the stable compound like this. So in both cases the enzyme-catalysed process is stalled. Both 5-azacytidine and the 5-fluoro-2 prime-deoxycytidine can react with the enzyme to form structures 1 and 2 but these do not degrade further.

These are the equivalent structures of the normal intermediate 3 as shown here, but then it does not subsequently result in a loss of a proton to give back the enzyme. So in the normal enzyme mechanism the final stage involves the loss of a hydrogen from the structure 3 to form a double bond, but this does not occur in these cases and therefore what happens is that it stalls at this point and it does not go further.

So therefore this is a mechanism based inhibitor because we know the mechanism of DNA methyltransferase, we are able to propose or design a compound which goes and does exactly what we wanted to do and except that it covalently modifies the enzyme and prevents it from acting further.

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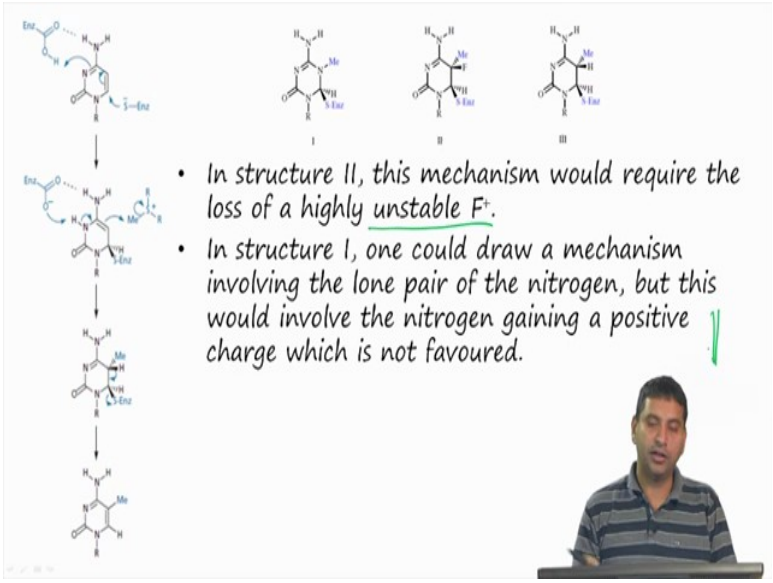

So in structure 2 this mechanism would require the loss of unstable F plus which does not happen and in structure 1 if you could draw a mechanism involving a lone pair of the nitrogen but this involve nitrogen gaining a positive charge which is not favoured. So knowledge of the mechanism of action is going to be very useful in designing mechanism based inhibitors.

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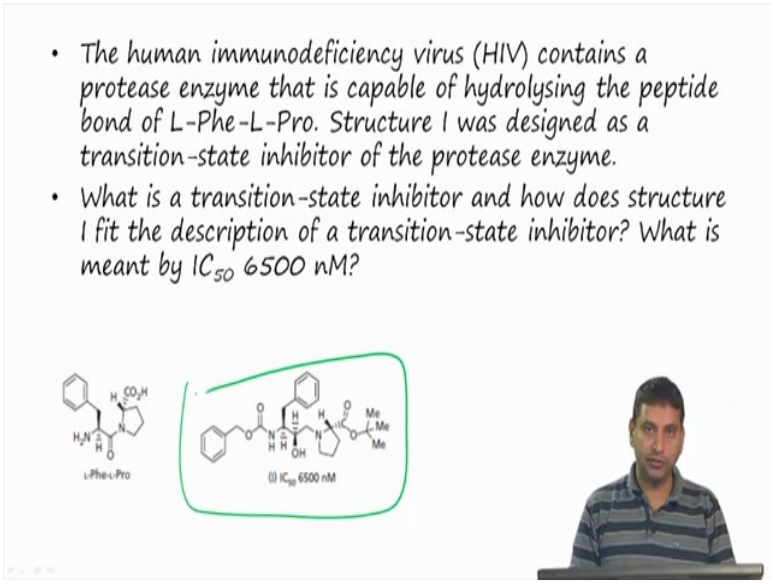

The next question the human immunodeficiency virus which is HIV contains a protease enzyme that is capable of hydrolysing the peptide L-phenylalanine Lproline. The structure 1 which is shown here was designed as a transition state inhibitor of this protease enzyme, what is the transition state inhibitor and how does structure 1 fit the description of the transition state inhibitor and what is meant by 6500 nano molar IC_{50}?

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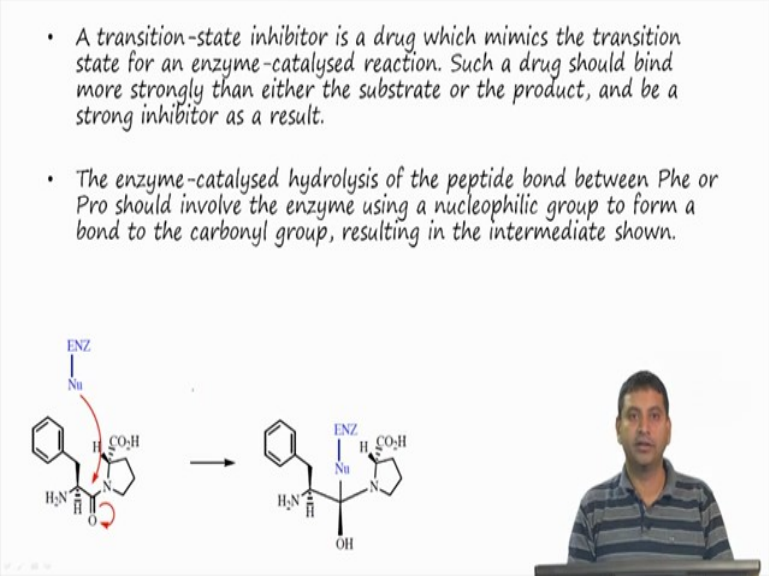

So we have again looked at this in detail just recently, but a transition state inhibitor is a drug which mimics the transition state of an enzyme. This molecule should bind more strongly to the enzyme because the enzyme is going to stabilize the transition state more than or bind better to the transition state more than the starting material or the product. So the enzyme catalyse hydrolysis involves again a tetrahedral intermediate as shown here.

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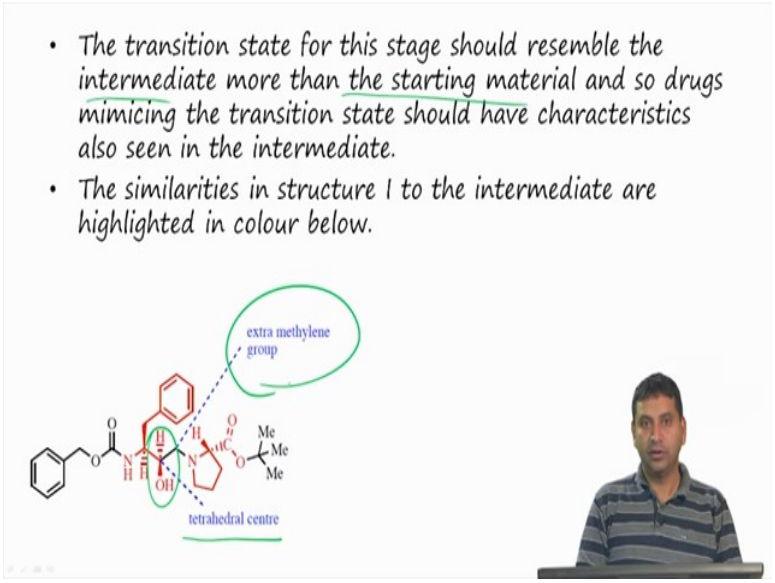

So the transition state for this stage would resemble the intermediate more than the starting material and so drugs mimicking the transition state should have the characteristics as seen in the intermediate. So what we have done here is we have used this again this concept of OH which looks like the transition state intermediate. So the similarities in structure 1 are highlighted here as shown here, there is a tetrahedral centre and then there is also an extra methylene group here which helps with the stabilizing the transition state.

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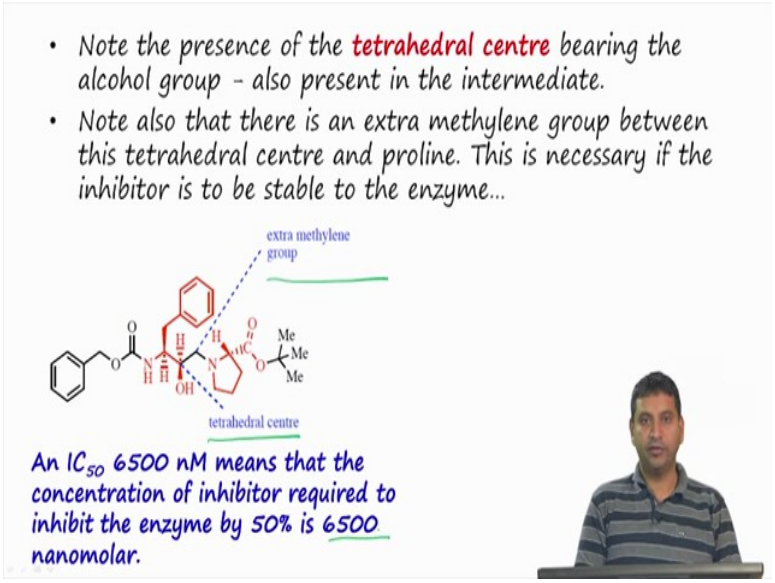

So this is shown here again, so you have a tetrahedral centre and you have this extra methylene group which is necessary if the inhibitor is to be stable to the enzyme that means there should be no turnover by the enzyme. So IC_{50} is inhibitory concentration, the

concentration required to inhibit 50% of the activity, so 6500 nano molar IC_{50} means that at 6500 nano molar you would expect to see 50% of enzyme inhibition.

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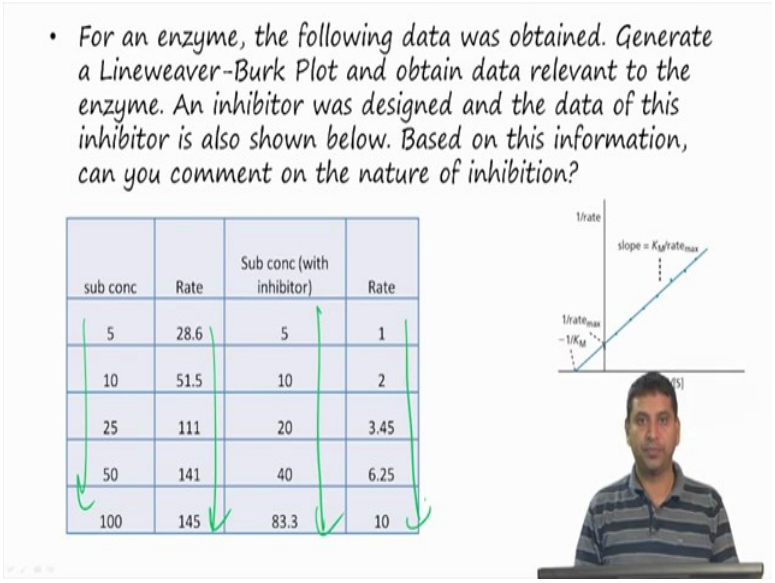

For an enzyme the following data was obtained. Generate a Lineweaver–Burk Plot and obtain the data relevant to the enzyme. Based on this information, can you comment on the nature of inhibition? So this is the data that is given, so here is a substrate concentration, here is the rate and this is with the inhibitor substrate concentration with the inhibitor and this is the rate.

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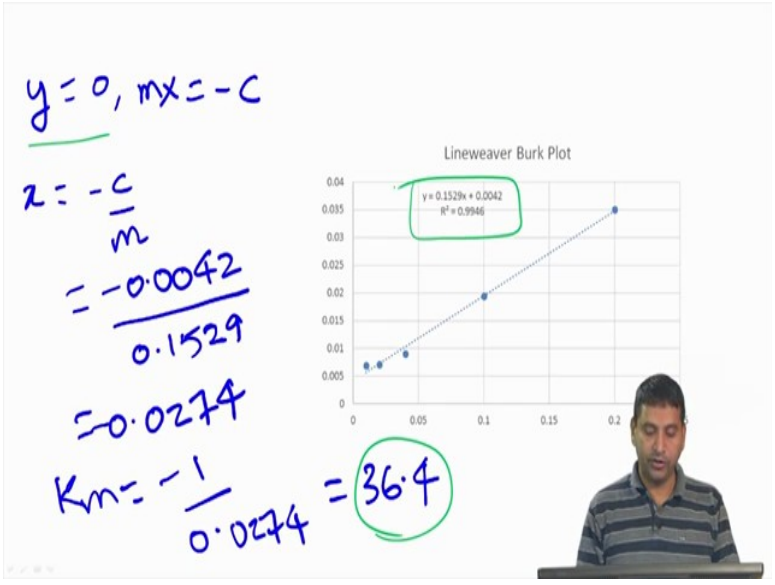

So again we will not repeat the steps involved here, you assign y equals 0 and then you get the K_M of 36.4 and you fit it to a straight line and you get the following parameters from that you obtain K_M which is 36.4.

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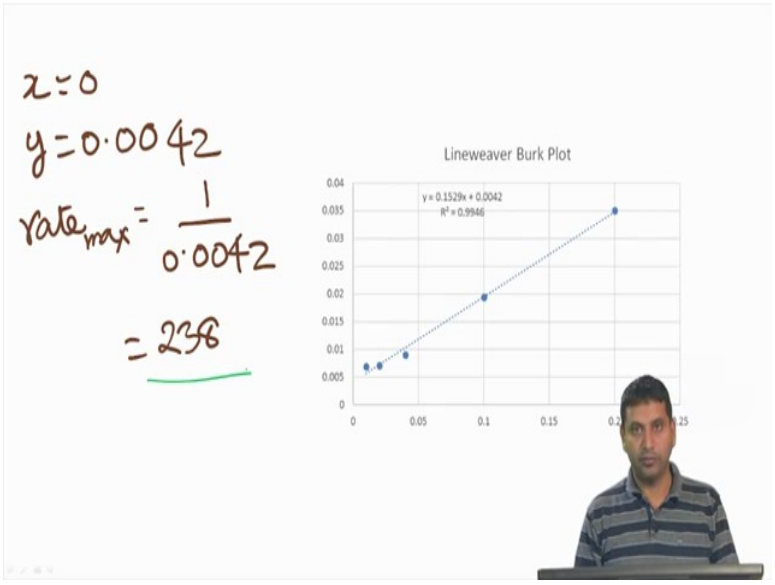

Similarly we get a rate max of 238 from this Lineweaver–Burk Plot.

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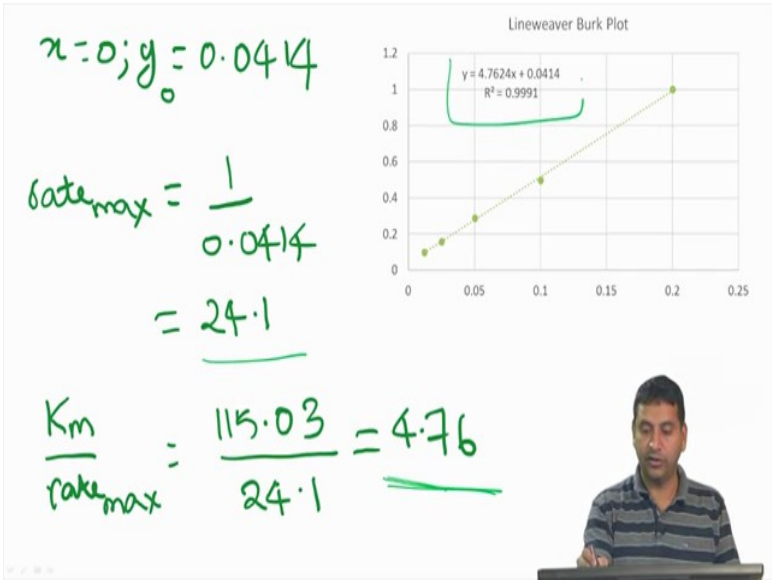

So now we do the same thing for the rate of the with the inhibitor, so in the presence of the inhibitor the K_M is 115.03 and the rate max is 24.1 and if you plot it you get K_M by rate max of 4.76 which is consistent with the slope of the reaction.

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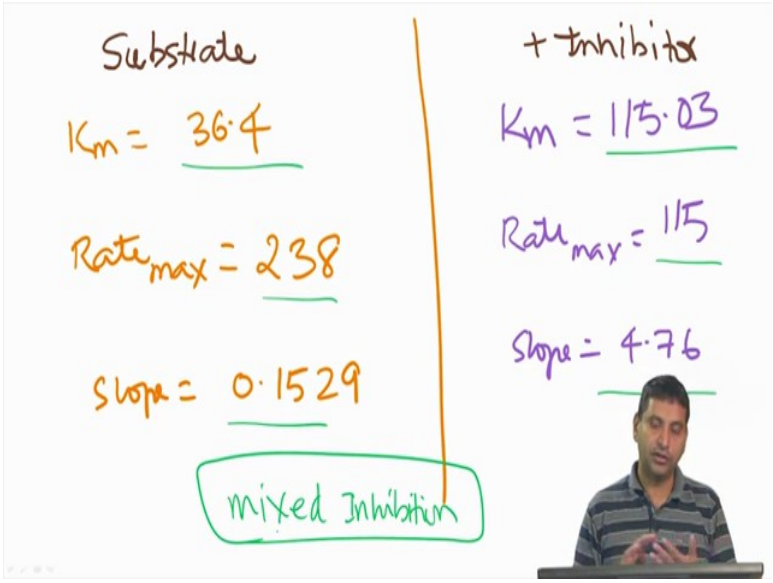

So now if you put these two plots next to each other you see that the Michaelis–Menten constant plus inhibitor does go up so you do see an increase and the rate max is also going down as you would expect, but the slope of these two situations are totally different. So this is a case of a mixed inhibition because we do not see parallel things, we do not see an intercept, we do not see the slope being the same so this would qualify as a mixed inhibitor.

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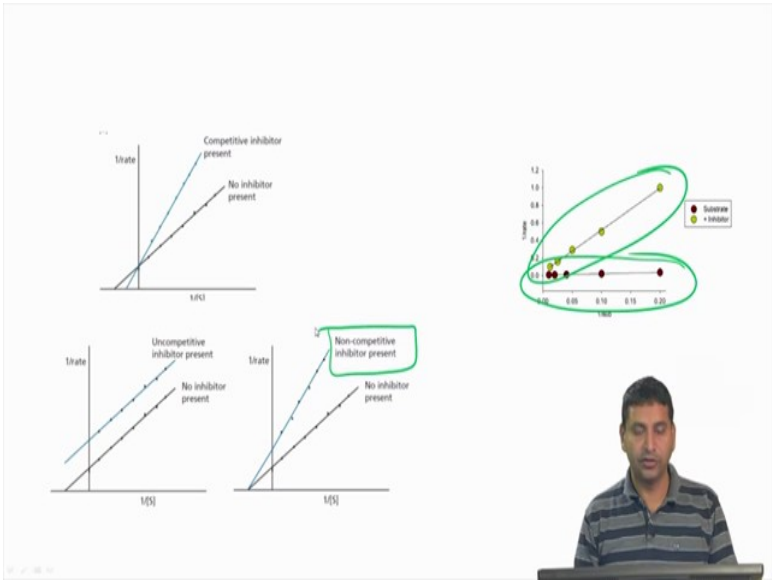

Because if you see here none of these characteristics are actually going to play a role so you have this is the slope with the inhibitor and this is the plot with the substrate plus inhibitor ad so this would qualify as a mixed inhibition, but it is actually closer to a non-competitive

inhibitor and therefore we are unable to conclude what exactly the mechanism of inhibition is but it would be a mixed inhibition.

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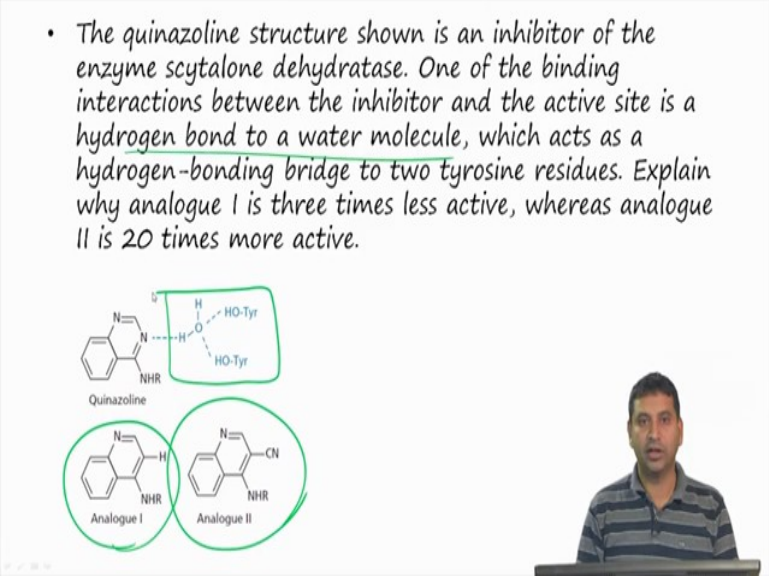

The quinazoline substrate shown is an inhibitor of the enzyme scytalone dehydratase. One of the binding interactions between the inhibitor and the active site is a hydrogen bond to a water molecule, which acts as a hydrogen bonding bridge to the two tyrosine residues. Explain why analogue 1 which is shown here is three times less active, whereas analogue 2 is 20 times more active. In order to address this question we have already been told that the two tyrosine residues are going to bind to the water.

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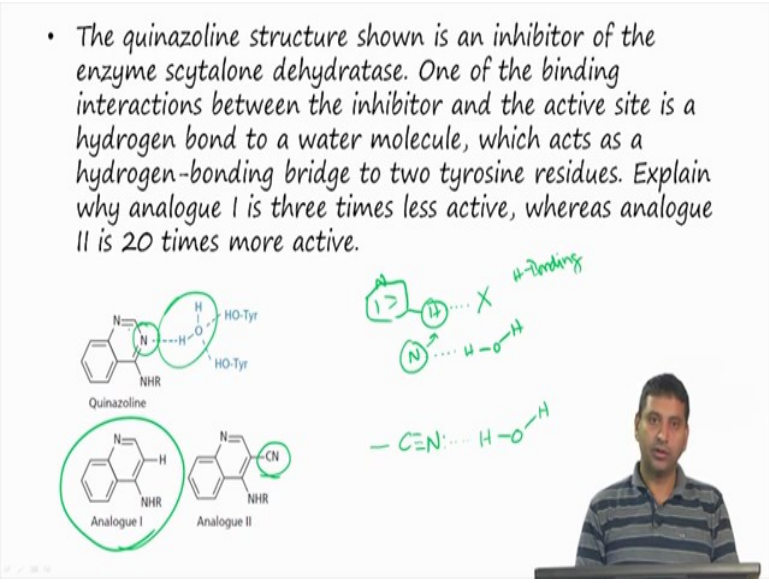

So in order to address this question let us look at the structure of analogue 1, so analogue 1 basically has a hydrogen over here (which) wherein earlier there was a nitrogen which was involved itself in a hydrogen bond, but now instead of the nitrogen you are replacing it with the CH. So this is incapable of hydrogen bonding, so one could expect that this would not bind as effectively as quinazoline to the water molecule and so therefore it is three times less active.

But now when I move to the cyano group, the cyano group here is very interesting because it is a C triple bond N and it has a lone pair which is available which can perhaps interact itself much better when compared with the parent molecule. So this helps us understand why this analogue is 20 times more active compared to the parent molecule.