ENZYME SCIENCE AND ENGINEERING

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LECTURE – 10

REVERSIBLE INHIBITION

We were yesterday talking about reversible inhibition that is those inhibitor molecules which reduce the rate of an enzymatic reaction by reversible interaction with enzyme molecule and after the inhibition is over they can be physically separated and the enzyme can be recovered in its original catalytic functional form. These reversible inhibitors are often categorized into three major classes based on certain general features and some of the general features which are used to classify them are: One is pattern of binding of the inhibitor to either the enzyme molecule or the enzyme substrate complex or in another words it leads to specific mechanistic steps which are involved in the inhibitory effect.

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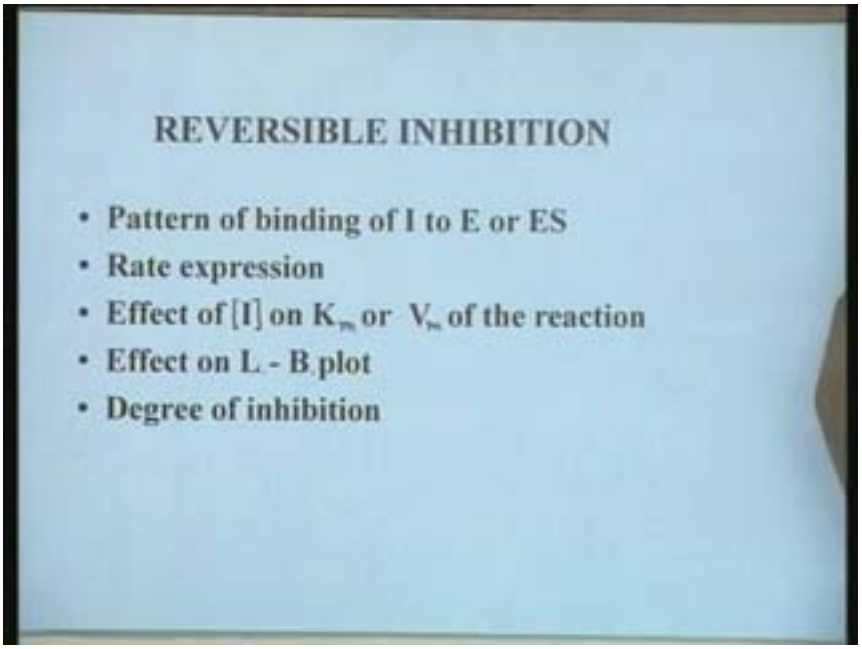

That means the enzyme reaction rate is modified or rather reduced to a new state in the presence of those inhibitor molecules. Based on that mechanism then the rate expression has evolved which also has characteristic features and for particular class of inhibitor. Then one looks at the effect of inhibitor on the Michaelis Menten constant and the

maximum reaction velocity of reaction. A particular class of inhibitor gives you a typical effect on the value of K_m or V_m. Then another feature is the effect on the double reciprocal plot, Lineweaver-Burk plot and the nature of Lineweaver-Burk plot which is influenced as a function of inhibitor concentration will also tell you about the class of a particular inhibitor.

Finally the type of inhibitor also influences the ultimate degree of inhibition. As I mentioned earlier the degree of inhibition is defined as

$$i = v_0\text{-}v_i/v_o$$

where i is the degree of inhibition and v_0 and v_i are the enzyme reaction rate at concentration of inhibitor at zero and at some particular concentration i for the enzyme reaction.

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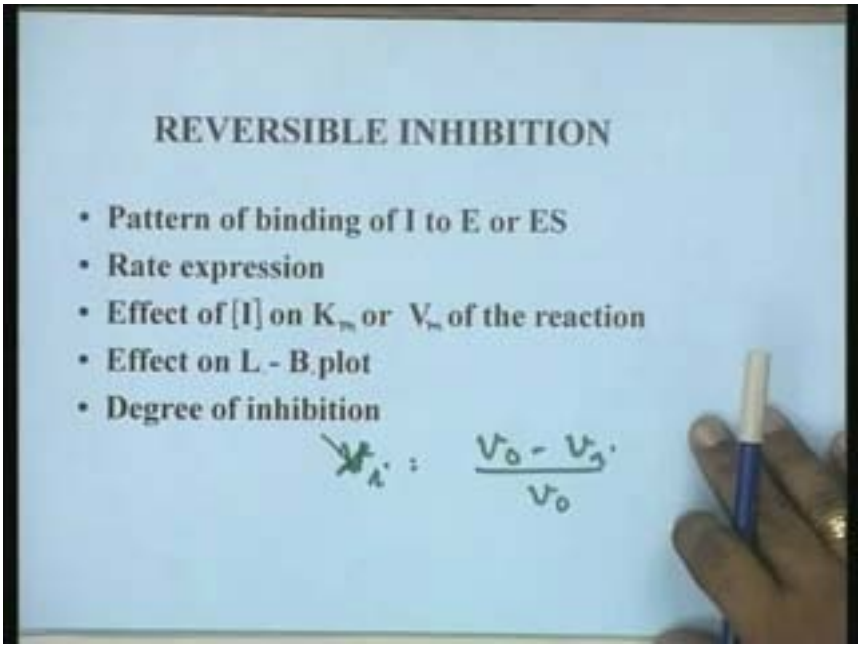

Any inhibition pattern is studied on these basic lines and yesterday we had looked at competitive inhibition and to summarize the nature of competitive inhibition the basic mechanistic step was that the inhibitor combines with the enzyme in a manner that prevents substrate binding. It may occupy the same binding site. It could also interfere with the binding due to steric hindrances in such a way that the binding of substrate is prevented fully or partially and substrate and the inhibitor are mutually exclusive. Their effect on the kinetic parameters of the enzyme reaction is just to increase the apparent Michaelis Menten constant. The maximum reaction velocity remains unchanged. The inhibition constant that is the reversible binding of enzyme with the inhibitor and the corresponding dissociation constant is equivalent to the concentration of inhibitor that

doubles the slope of initial reaction velocity that is inverse double reciprocal plot 1/v verses 1/S plot.

If you recall in the case of competitive inhibition we have seen that

$$1/vi = K_m/V_m\ (1+I/K_i)\ .1/S + 1/V_m$$

The Michaelis Menten constant is modified by the amount $1+I/K_i$ and therefore the value of K_i is equivalent to that concentration of the inhibitor at which the slope of the double reciprocal plot is double. That means from the initial slope, the value of slope doubles and that concentration of inhibitor gives you the magnitude of K_i.

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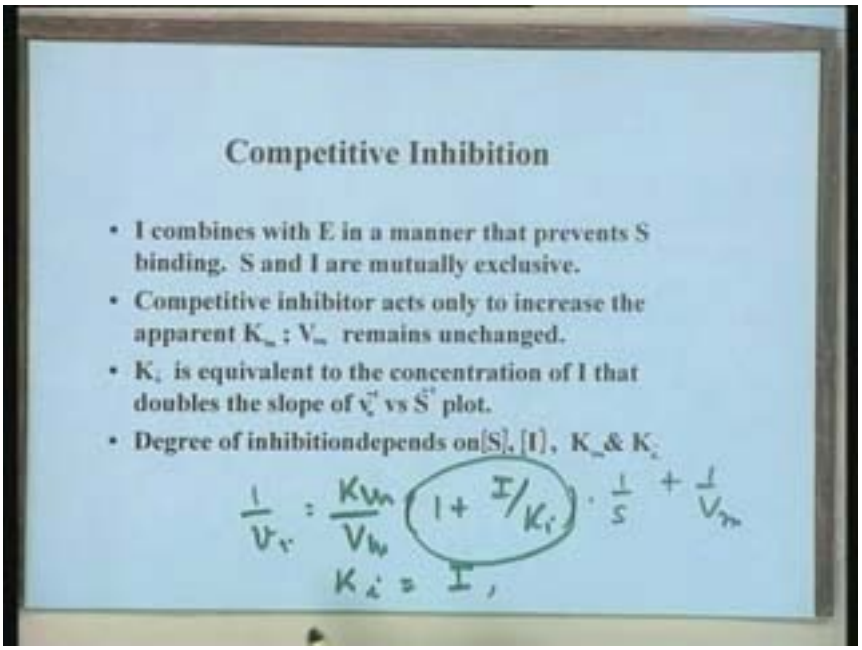

Then degree of inhibition for the competitive inhibition also depends upon all the parameters and the expression for degree of inhibition for competitive inhibitor can be written as

$$i = \frac{K_m.I/K_i}{\{K_m\,(1+I/K_i)\} + S}$$

The degree of inhibition depends on substrate concentration, inhibitor concentration and also on K_m and K_i. All the parameters influence the degree of inhibition at any given inhibitor concentration for the competitive inhibitor.

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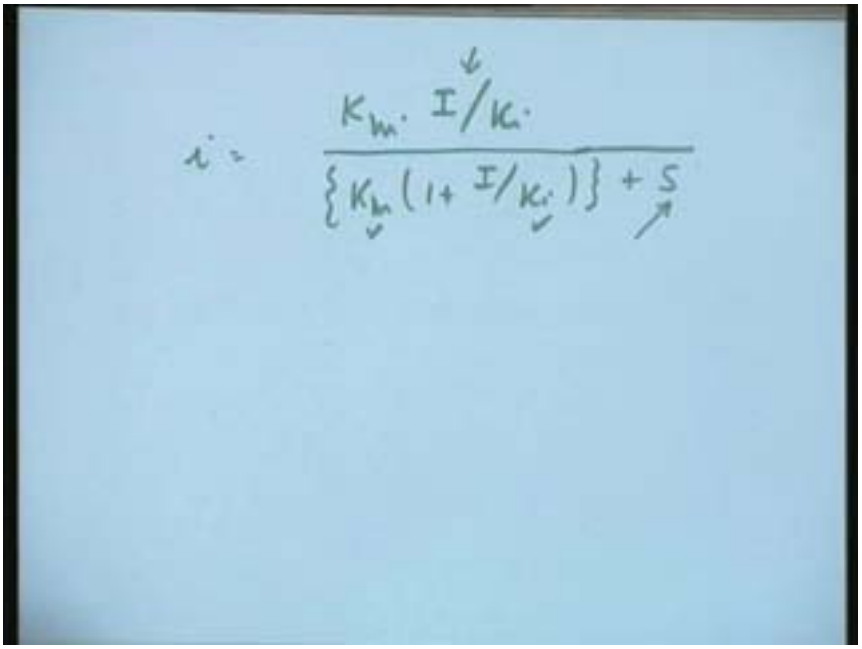

This is what we had discussed yesterday. Now if we come to the next major class of reversible inhibitor which is a non-competitive inhibitor, the classical feature of a non-competitive inhibitor in terms of binding with the enzyme or the enzyme substrate is that the inhibitor does not influence in any way the substrate binding.

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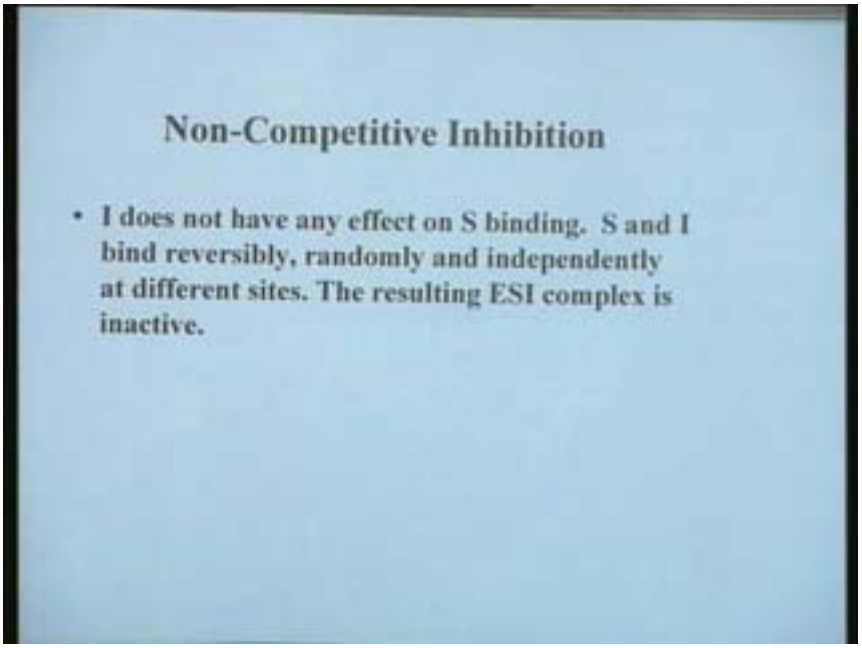
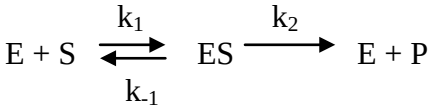
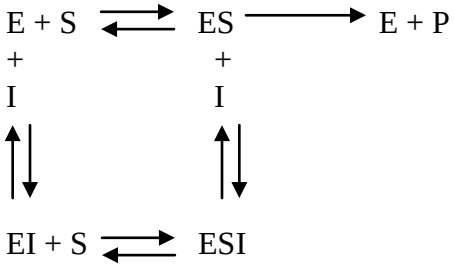


Substrate as well as the inhibitor binds reversibly, randomly and independently at different sites. That means the binding site on the enzyme molecule for the substrate is different from the binding site for the inhibitor molecule. The enzyme molecule binds to

both; substrate as well as inhibitor and the resulting ESI complex is functionally inactive. Therefore one can look at the reaction mechanism as



A classical route involving k_1, k_{-1} and k_2.



The enzyme can also bind to inhibitor giving EI and because the binding site of the substrate is different, EI can again bind to form ESI and the ES can also bind to I to give ESI. The enzyme substrate inhibitor complex, the triple complex, can be arrived at either from this route or from this route because the binding of substrate and inhibitor do not interfere with each other. They can bind reversibly, randomly and independently; there is no interaction within them. But only thing is that this ESI complex once formed is catalytically inactive and does not lead to product formation. It is only ES complex which will lead to product formation. In a simplistic mechanism we notice that we have a dissociation constant K_m for the enzyme substrate binding and dissociation constant K_i for the inhibitor binding. Also we assume that whether the enzyme substrate complex bind to inhibitor or the free enzyme binds to the inhibitor the dissociation constant is identical K_i. Similarly when substrate binds to EI complex, dissociation constant is K_m. There is no interference by binding of substrate or inhibitor onto the enzyme in their subsequent binding process.

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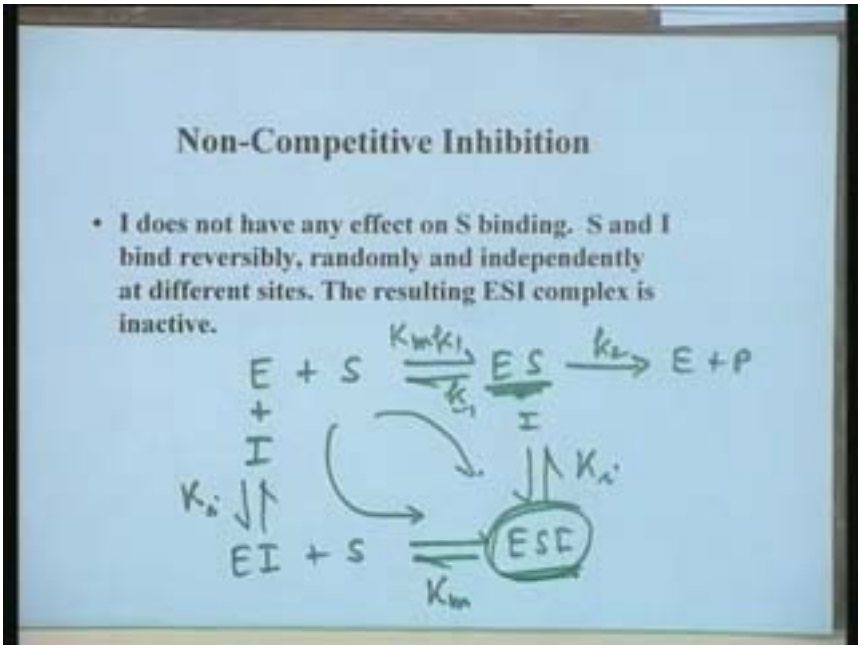

Under such conditions one can write the rate expression for a non-competitive inhibitor as

$$v_0 = \frac{S.\,V_m}{K_m(1 + I/K_i) + S(1 + I/K_i)}$$

To simplify, to look at the effect on K_m and V_m values, if we divide both the numerator and denominator by $(1 + I/K_i)$

$$v_0 = \frac{S.\,V_m / (1 + I/K_i)}{K_m + S}$$

One can very clearly visualize that the effect of a non-competitive inhibitor is to influence the maximum reaction velocity and maximum reaction velocity is reduced by factor of $(1 + I/K_i)$ whereas the K_m value remains unchanged. There is no effect on the K_m value. In contrast to the competitive inhibitor where the K_m value was altered here the V_m value gets altered. The modified maximum reaction velocity is equal to

$$V_m' = V_m/(1 + I/K_i)$$

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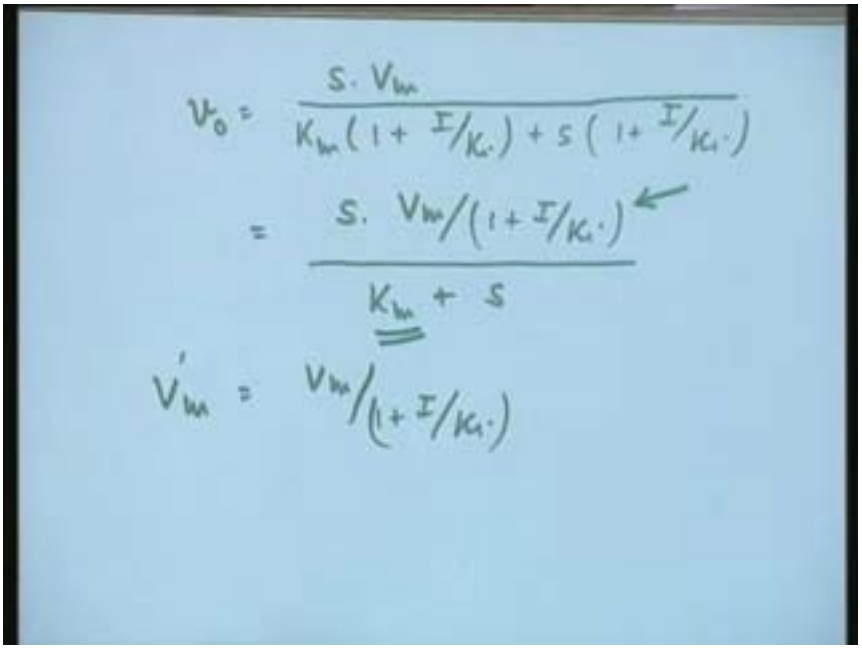

If you look at into the mechanic concept the total fraction of the enzyme molecule which was originally present for catalytic function has now got distributed into ES, EI and EIS and only a part of it which is in the form of ES is catalytically functional and therefore the maximum reaction velocity which is a function of enzyme concentration is reduced.

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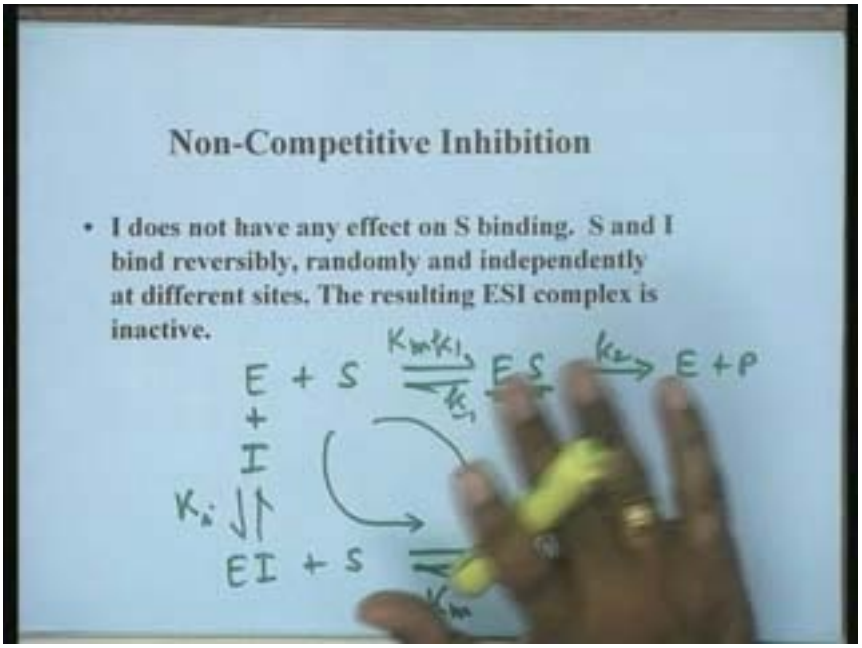

So the net effect on the maximum reaction rate is experienced. It does not influence the K_2 value, the turn over number, because as we know

$$V_m = K_2.E'_0$$

Here E_0, in the presence of non-competitive inhibitor, corresponds to that fraction of total enzyme which is in the form of ES. The ESI complex form is functionally inactive and therefore the K_2 is not altered. It is the E_0 which is modified or reduced by a factor of $1+I/K_i$. E_0 is a fixed quantity when there is no inhibitor in the reaction. Once the inhibitor is present the E_0 will be distributed into free enzyme, E, ES, EI and ESI. E_0 is not reduced but the effective total concentration of the enzyme, E'_0, which is catalytically functional is reduced and it is only the ES component which is catalytically functional.

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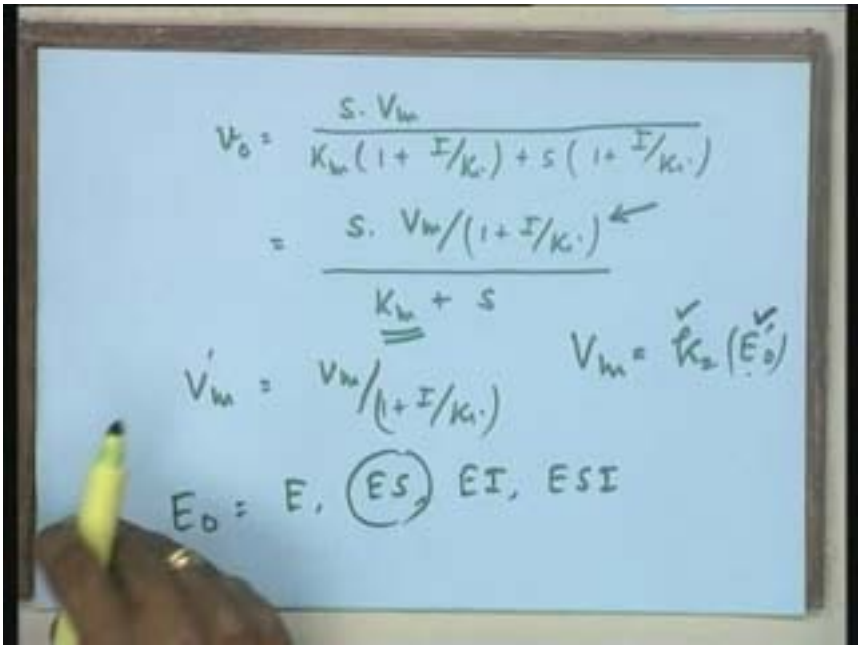

In many cases the binding of substrate can influence the catalytic function on the substrate binding. Binding wise it may be independent. There are two parts in the active site. One is the binding process which is not influenced which is elaborated by our dissociation constant. The binding part of the active site is demonstrated by the dissociation constant. The second part which is demonstrated is the catalytic function which is given by K_2, the turn over number. That can be influenced by binding of the inhibitor. Turn over number is unaffected here.

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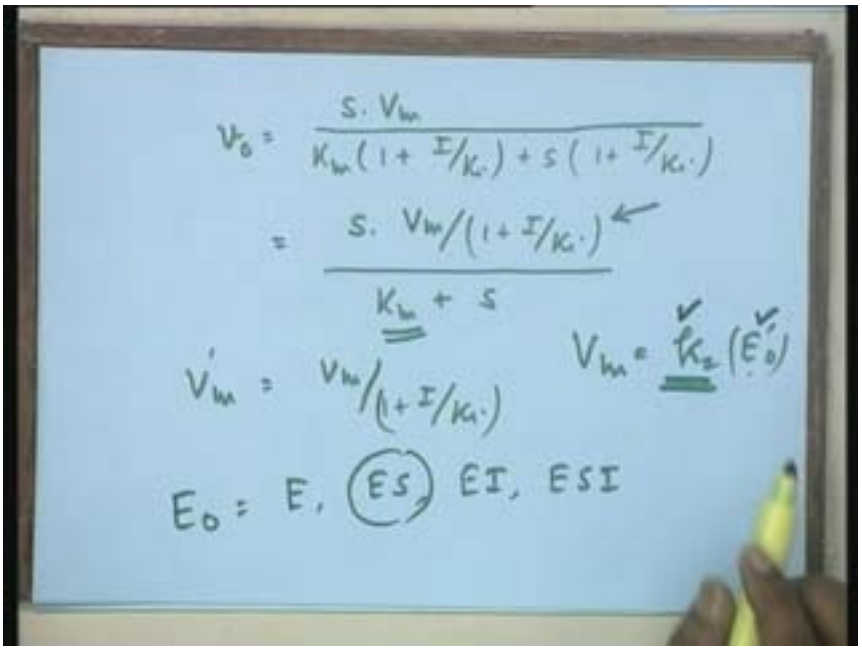

In the case of the non-competitive inhibition, the turn over number will be unaffected as far as the kinetic parameter is concerned. But it can influence the catalytic capacity of the enzyme molecule in total. This is where the question comes that when the substrate and the inhibitor both are bound it influences the whole molecule in such a way that catalytic function is lost. It is a dead end complex and then the binding site on the enzyme which is bound by the substrate is not able to give the product. But when I say K_2, I am referring K_2 in the sense of the original catalytic function which was present, which is retained and only in the presence of a bound inhibitor on the inhibitor binding site, the catalytic function is lost.

The double reciprocal plot in the presence of non-competitive inhibitor is given as

$$1/v = K_m/V_m(1+I/K_i).1/S + 1/V_m(1+I/K_i)$$

In the case of a non-competitive inhibitor both the slope as well as intercept will undergo a change and both this slope and intercept has increased by a magnitude $1+I/K_i$ and therefore if you look at the pattern of the Lineweaver Burk plot you get an increasing concentration of I. Let us assume that this is I = 0 and some values of I as I_1 and I_2. The slope and the intercept are both increasing by a magnitude of $1+I/K_i$.

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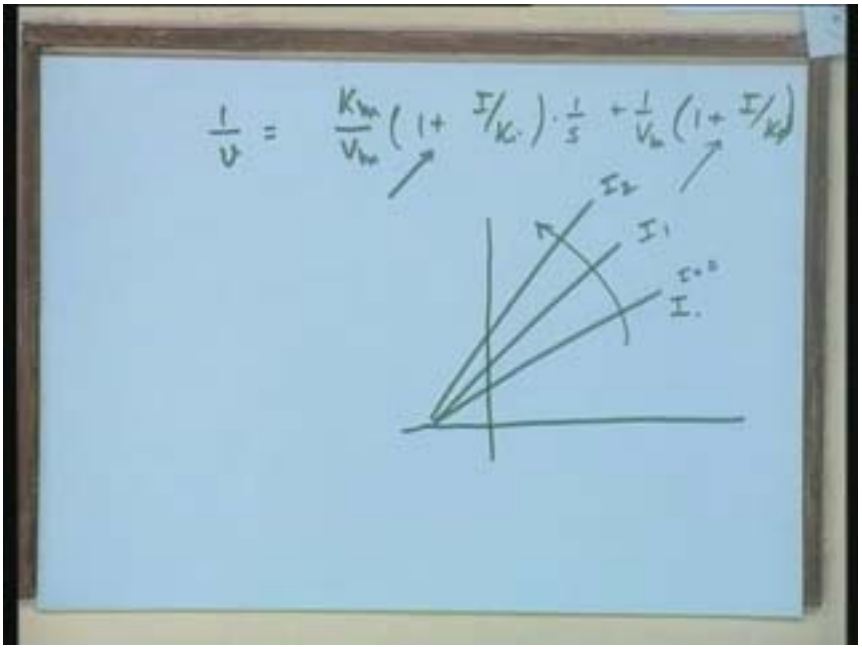

Therefore one can determine the value of K_i as we saw in the case of competitive inhibitor by a secondary plot. That means either you determine slope or intercept at different inhibitor concentration and make a plot between I and slope or intercept. You will get a straight line because slope will be equal to

$$\text{Slope} = K_m/V_m(1+I/K_i)$$

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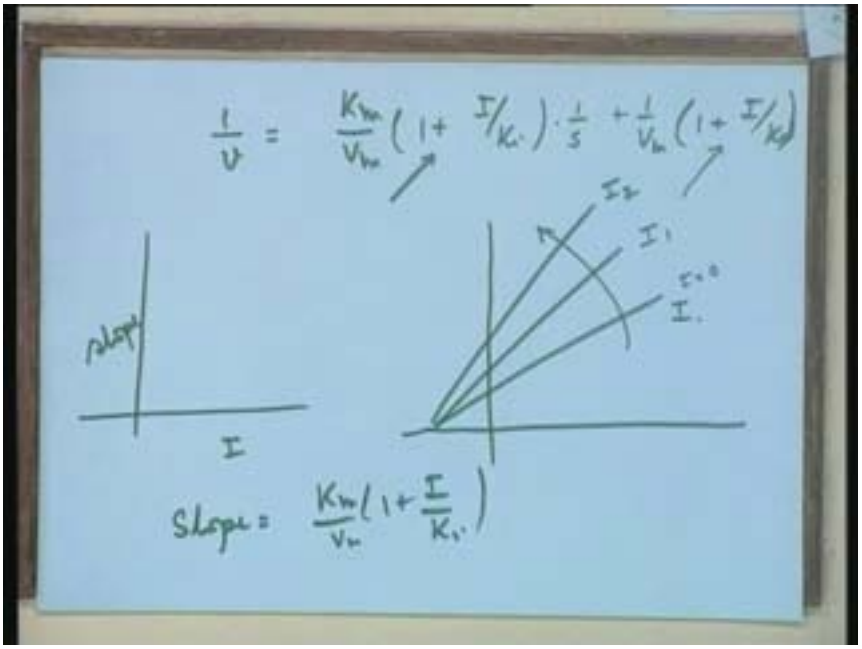

Therefore one can determine the value or magnitude of Ki either by plotting the slope verses the inhibitor concentration or by intercept. Either way one can determine and that can give you the magnitude of inhibition constant.

The third category of the reversible inhibitor is un-competitive inhibition. In this category of reversible inhibitors the inhibitor binds reversibly to the enzyme substrate complex. It doesn't have any mechanism to recognize the free enzyme.

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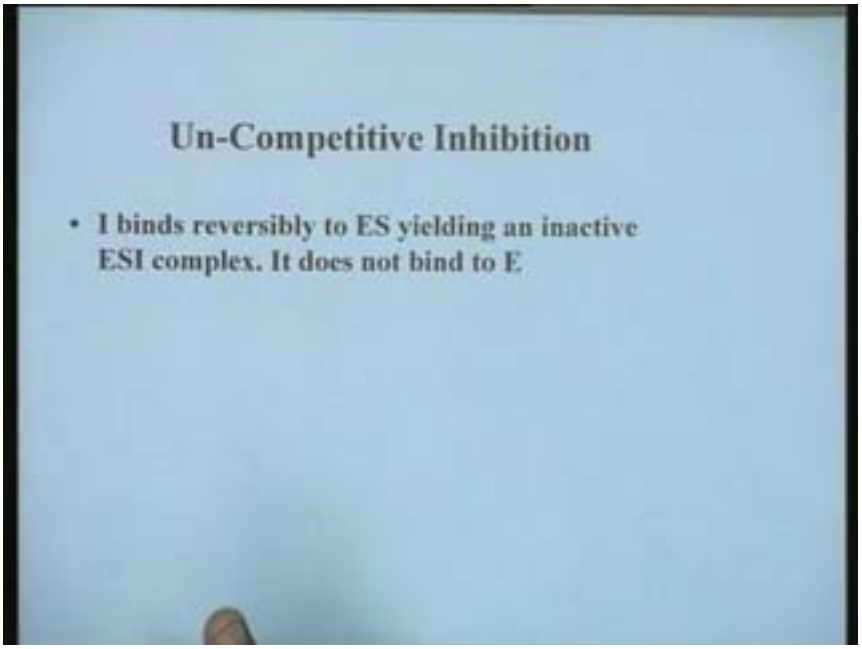
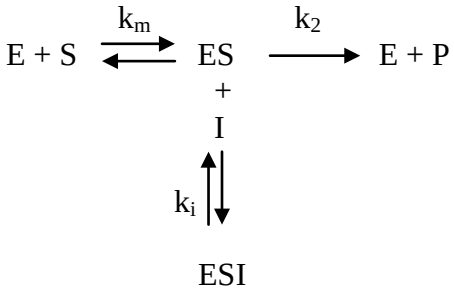


Only when the substrate is bound to the enzyme it can bind to the enzyme substrate complex and after binding of the inhibitor the ES complex becomes an inactive complex and the inhibitor does not bind to the free enzyme.



ES can bind to inhibitor giving you ESI. This is the dissociation constant K_i, classical K_m and K_2. The inhibitor can bind to only to the ES complex and does not bind to the free enzyme.

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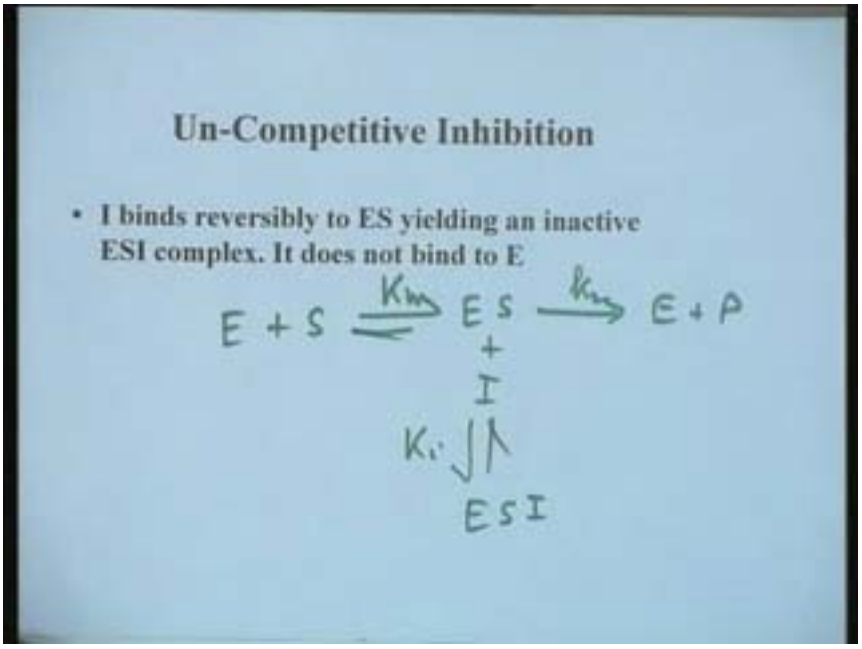

Such situations are very rare as far as the unisubstrate reactions are concerned. Very few cases, almost rare cases are there where one notices this kind of un-competitive inhibition. Most of these cases are usually related to bisubstrate reactions particularly because you notice here that the inhibitor binds after the substrate is bound. So there is a sequence of binding. In cases only where there is ordered sequence of binding of the ligands, the un-competitive inhibition is feasible. In cases where the binding is random, that means the inhibitor and substrate can bind randomly without any sequence, the mechanism of un-competitive inhibition is very rare. The idea of un-competitive inhibition has been very, very useful in understanding the reaction mechanism for bi substrate reactions. When we look at bisubstrate reactions we notice that this inhibition pattern gives you lot of information about the sequence of binding of the substrate in the case of a bisubstrate reaction.

In the case of an un-competitive inhibition the reaction velocity can be written as

$$v = \frac{S.\,V_m}{K_m + S\,(1 + I / K_i)}$$

To bring it to the same format of Michaelis Menten equation, if you can divide both numerator and denominator by $(1 + I/K_i)$

$$v = \frac{S.\,V_m\,/(1 + I / K_i)}{K_m/(1 + I / K_i) + S}$$

$$K_m' = K_m / (1 + I / K_i) \; ; \; V_m' = V_m/(1 + I / K_i)$$

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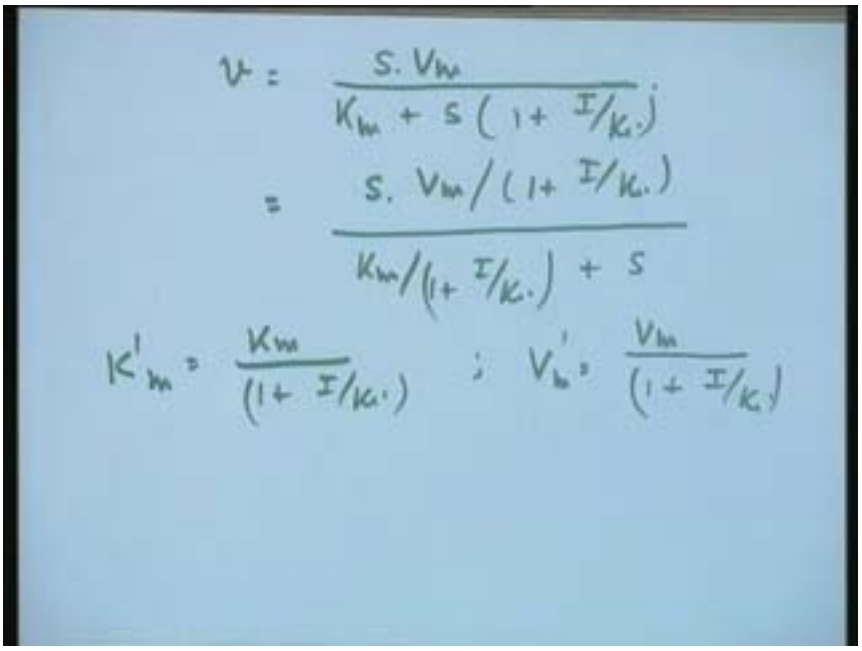

Both the parameters are reduced by a factor of $(1 + I / K_i)$ and the double reciprocal plot in the presence of un-competitive inhibitor will be

$$1/v = K_m/V_m.1/S + 1/V_m(1 + I / K_i)$$

Therefore you will notice that the un-competitive inhibitor does not change the slope. The slope remains unaltered but the intercept is increased. This is your intercept and the intercept $1/ V_m$ on the y axis is increased by a factor of $(1 + I / K_i)$.

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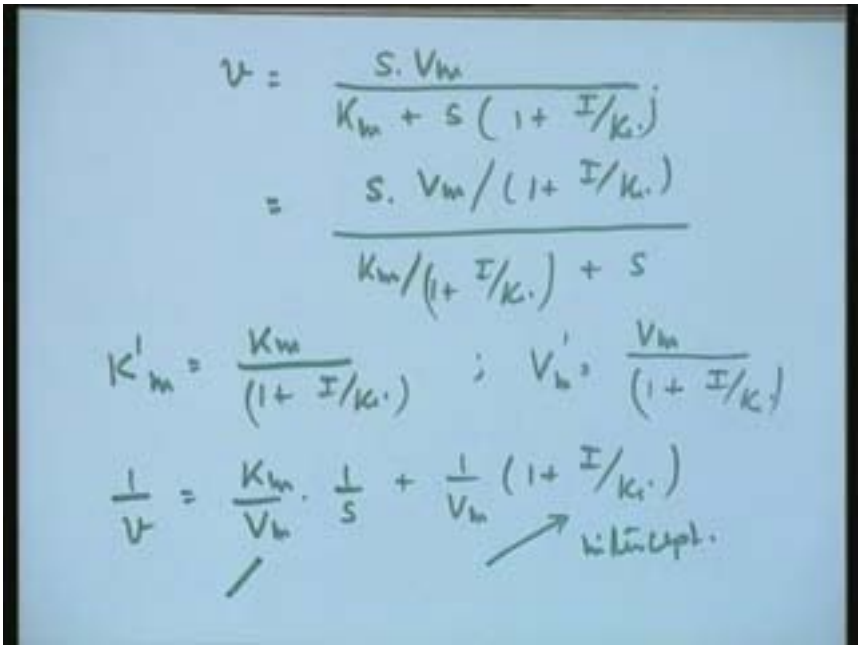

The typical pattern of an un-competitive inhibitor looks like parallel lines where the slope doesn't change but the intercept value $1/V_m$ is changed. It is increased by a factor $1+I/K_i$. If you make a secondary plot of $1/K_m^{'}$ verses I,

$$1/K_m^{'} = 1/K_m + I/K_m.K_i$$

you will get the value of K_i.

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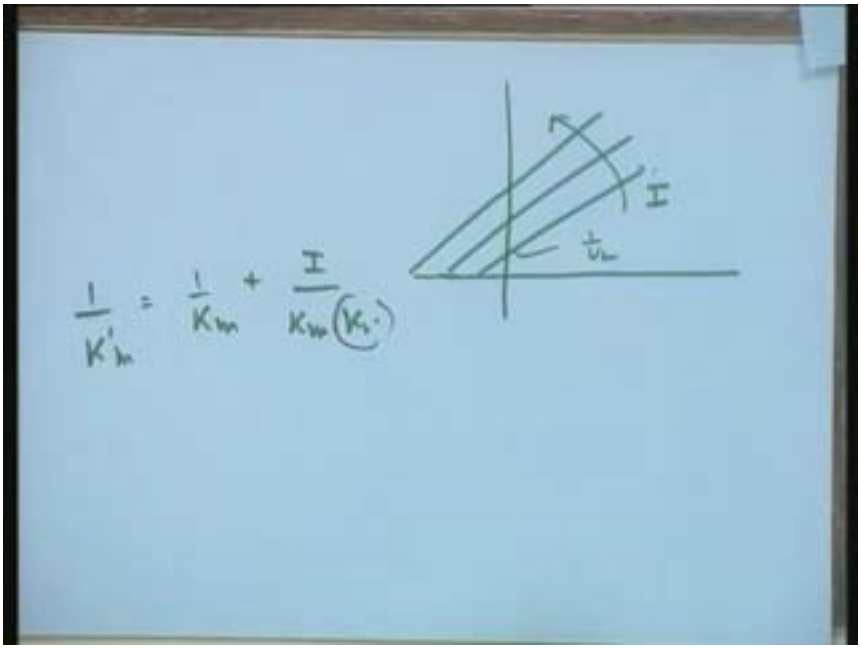

In all these cases by following a typical pattern that is by understanding the reaction mechanism, the binding of the inhibitor and substrate to the enzyme and their interaction between each other one can really arrive at a rate expression. One can also look at the double reciprocal equation, Lineweaver Burk equation, make a double reciprocal plot, see the nature of double reciprocal plot and identify the nature of inhibitor which we are talking about by understanding these.

In many cases we will also notice that the actual inhibitor does not follow a very typical pattern and very often we end up with a profile that intersects neither on the y-axis nor on the x-axis and intersects somewhere in the middle point. Such a situation can be considered something away from the ideality, a kind of mixed type of inhibition. One of the most commonly observed mixed type of inhibition is what we call as linear mixed type inhibition.

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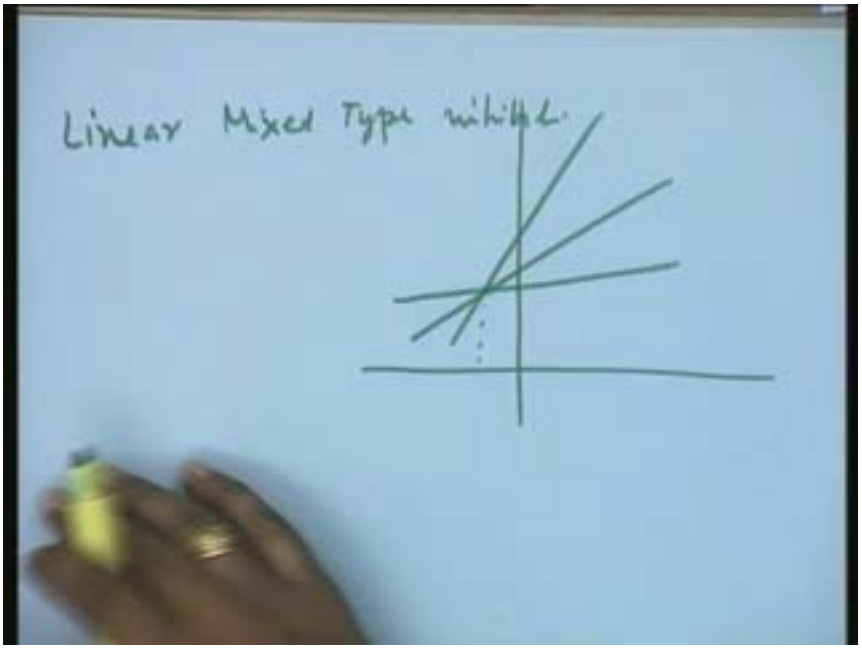

This is a typical case of a non-competitive inhibition. In non-competitive inhibition we considered the binding of the inhibitor to either the substrate or the free enzyme. In that case we assumed, one basic factor, that the dissociation constant for binding of the inhibitor to the enzyme and the binding of the inhibitor to the enzyme substrate complex is identical. But in many cases this may not be so.

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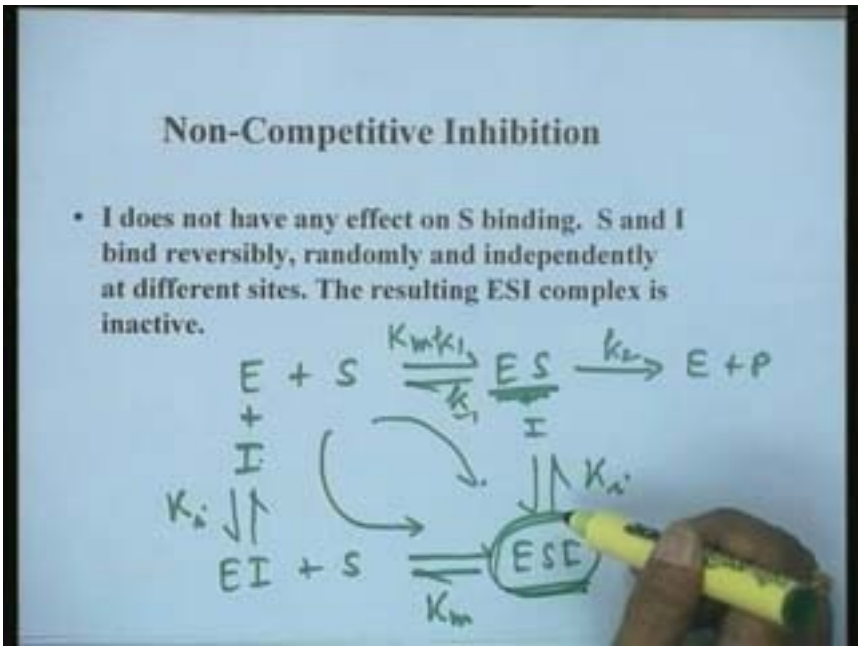
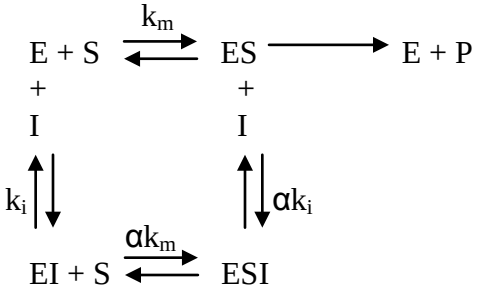


The dissociation constant for binding of inhibitor to enzyme may be different from the dissociation constant for inhibitor binding to ES complex and there might be an interaction factor of alpha.

In such a case ES going to E plus P. E binds to I with the dissociation factor of K_i to form EI. ES binds to the inhibitor with inhibition constant of αK_i to form ESI and substrate binds to EI. If the inhibition constant for this is K_m and if the system has to come to equilibrium, then the equilibrium constant for reaching ESI from either side must be identical and this inhibitor constant will also become αK_m.



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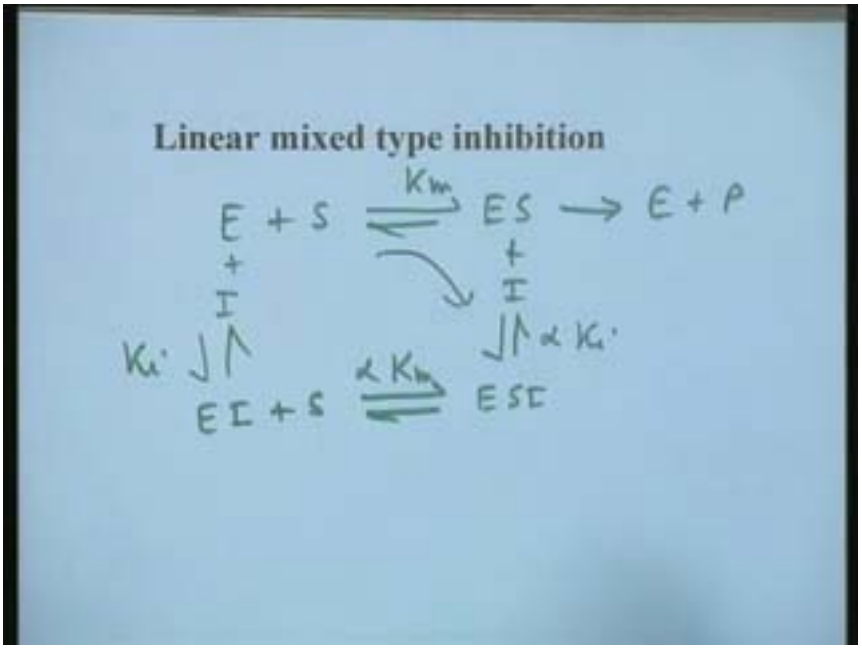

In most cases it so happens that the inhibition constant for binding of inhibitor to the free enzyme or binding of inhibitor to the enzyme substrate complex are not identical as assumed in the non-competitive inhibition and therefore the presence of inhibitor on the enzyme changes K_m also by the same factor αK_m and in such cases the rate expression becomes modified.

$$v_i = \frac{V_m \cdot S}{K_m/(1 + I / K_i) + S(1 + I / \alpha K_i)}$$

$$1/v_i = \frac{Km}{Vm} \cdot \frac{(1 + I / K_i)}{S} + \frac{(1 + I / \alpha K_i)}{V_m}$$

For cases where $\alpha > 1$ you will get a profile in which the double reciprocal plot of 1/S Vs 1/v at different inhibitor concentrations will intersect at a point on the left hand side of the y-axis. If you consider the slopes and the intercepts of this plot, a replot of the slope will give you K_i. If you replot the modified intercept as a function of inhibitor concentration it will give you αK_i and therefore value of α can be determined.

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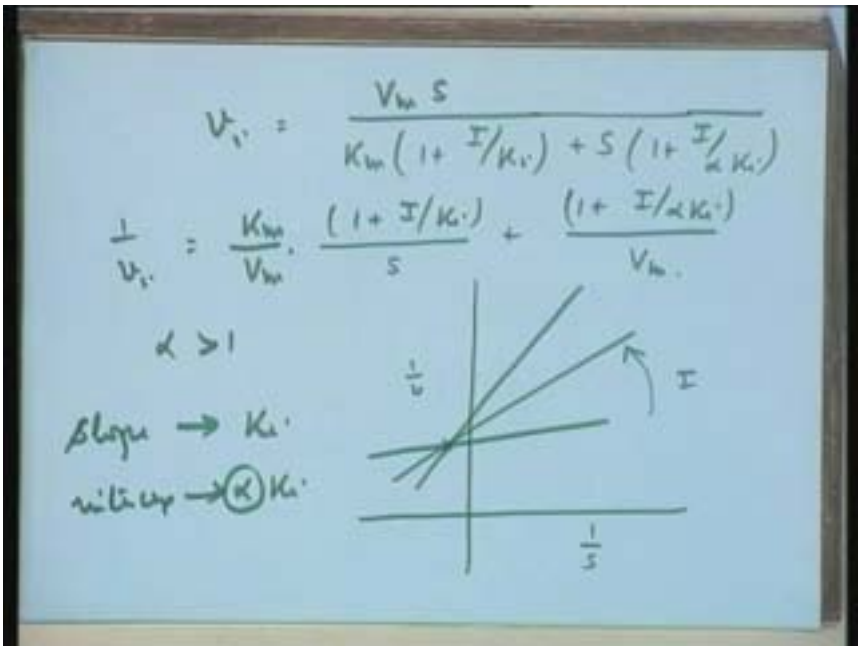

That means magnitude by which the dissociation constant of binding of inhibitor is modified can be determined. Such profiles are indicative of modification of dissociation constant as a function of substrate binding in the presence of non-competitive inhibitors. Such cases are very commonly found and in most of the practical cases they are not usually ideal type of inhibition patterns. They are mixed type of inhibition patterns and mixed inhibition pattern are attributed to modification in the dissociation constant as they are obtained.

Another general feature which gives you the properties or the characteristic of any type of inhibition pattern is degree of inhibition. If you recall from all the rate expressions that we wrote for the different types of inhibition pattern

$$i = v_0\text{-}v_i/v_0$$

For non-competitive inhibitors, if you substitute the rate expression for the inhibitor and uninhibited reaction for the non-competitive reactions the value of I will be I / K_i+I which indicates that the degree of inhibition is independent of substrate concentration, unlike in the case of competitive inhibition where increasing substrate inhibition can significantly decrease the degree of inhibition. That means the extent by which the rate of the enzyme catalyzed reaction is reduced by the inhibitor can be reduced by increasing the substrate concentration in relation to inhibitor concentration. In the case of non-competitive inhibition whatever be the concentration of substrate, it does not influence the degree of inhibition and it is totally dependent on the concentration of inhibitor and the Ki.

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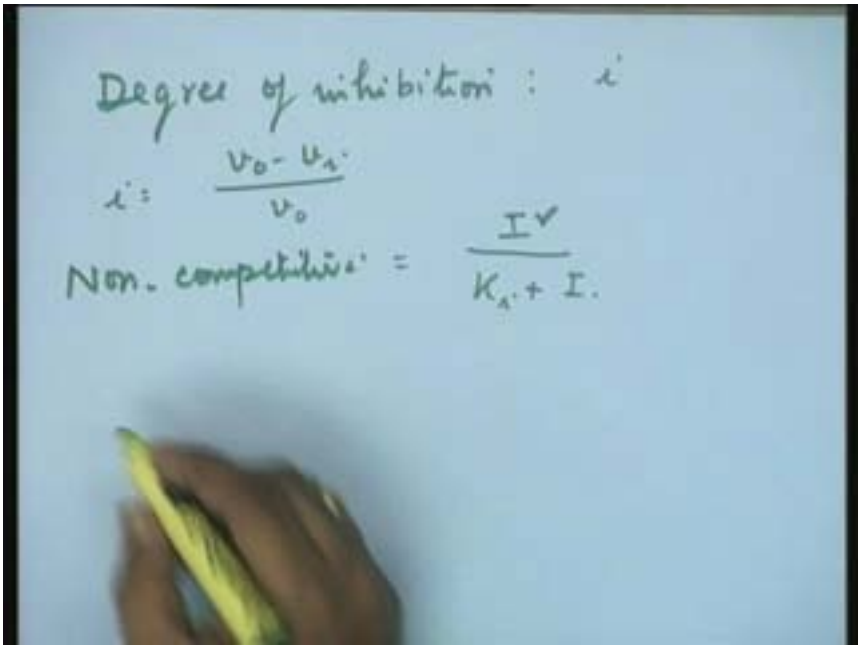

In the case of un-competitive inhibitor the degree of inhibition is

$$S.I / K_i(K_m+S).(1+I / K_i)$$

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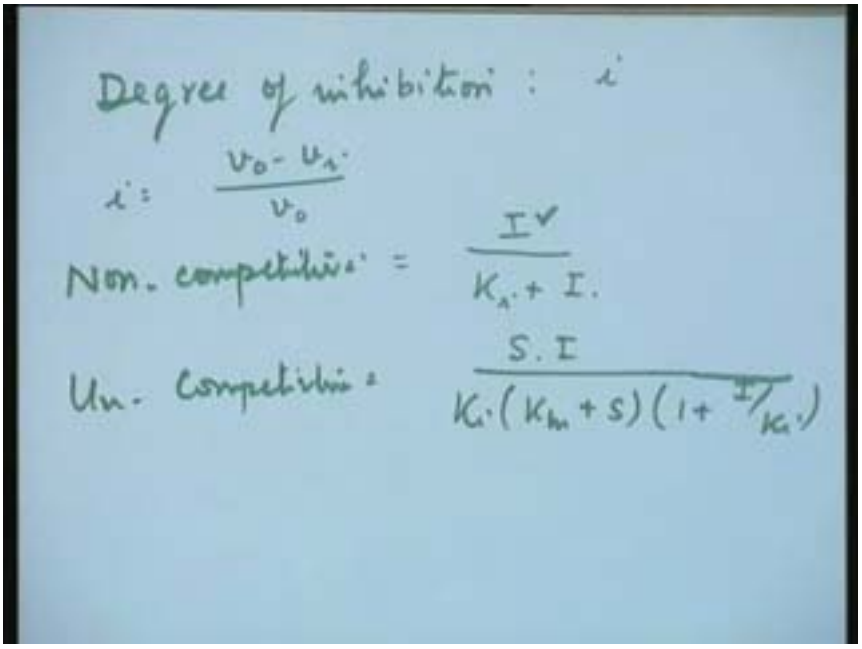

The degree of inhibition will rise from S = 0 when there is no reaction to a limiting value of $1+I/K_i$ at very high value of substrate concentration. When the substrate concentration becomes very high, the degree of inhibition will be $1+I/K_i$ but it will depend like

competitive inhibition on all the parameters, the substrate concentration, inhibitor concentration, K_i and K_m.

In the case of mixed inhibition, the degree of inhibition will be largely dependent upon the magnitude of α. The mixed inhibition is a special case of non-competitive inhibition where otherwise substrate concentration does not influence the degree of inhibition. Because the binding sides are different and they don't interfere with each other but in the case of mixed inhibition, the magnitude of inhibition will be dependent on the ratio of dissociation constant for free enzyme and enzyme substrate complex with the inhibitor. α will determine the magnitude of the degree of inhibition.

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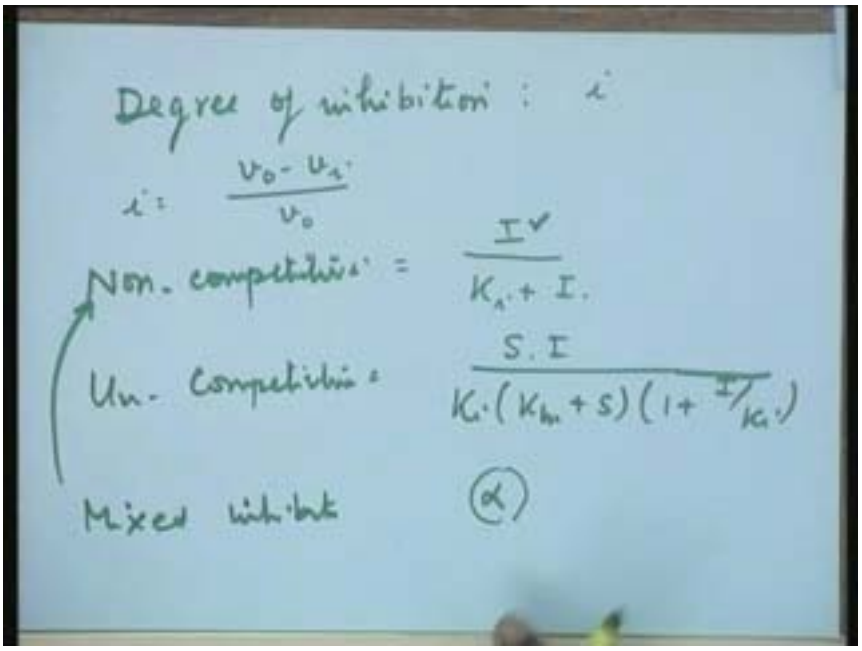

So these are some of the typical categories of inhibition pattern which are commonly noted and they have a key role to play in understanding the mechanism of variety of complex reactions particularly the bi-substrate reactions. The inhibition patterns obtained for bisubstrate reactions gives you lot of information about the binding pattern of different substrate or the sequence of binding of the different substrate in the case of a multisubstrate reaction.

In the case of single substrate reaction, that is what we have been talking so far, the life is simple because there is only one substrate and we are concerned with the binding of the substrate. In the case of bisubstrate reactions we have much more possibilities, much more types of mechanistic approaches that are feasible and they are taken into account.

When we say very high substrate concentration, $S >> K_m$ and we are talking of zero order design and then the degree of inhibition at very high substrate concentration will be significantly very high because then it will reach the limiting value of $1+I/K_i$.

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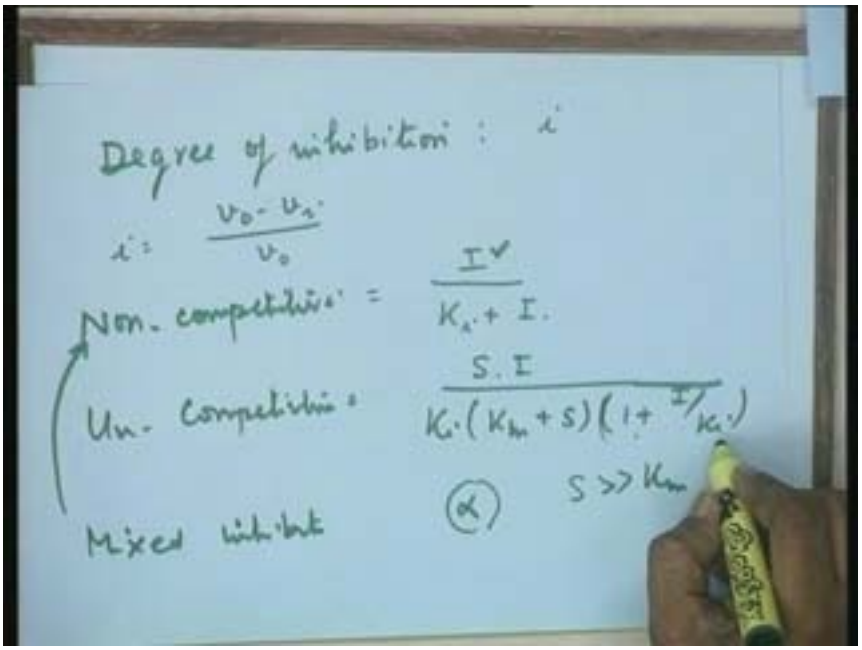
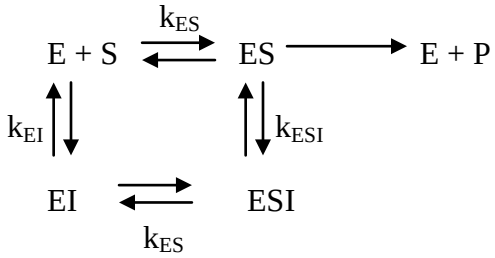


K_m becomes K and S gets cancelled and then degree of inhibition will be $I/ K_i.(1+I/K_i)$. It reaches a limiting value of $1+I/K_i$. But otherwise at low substrate concentration that will be some where in between zero and that value.

Another way is to look at the inhibition pattern in a generalized way rather than specific cases. If you look at a very generalized scheme of inhibition patterns



If you consider each of the reversible steps, we are not here really concerned with the irreversible step because that determines the rate of the reaction, the interaction of the reversible step the dissociation constant for this step is K_{ES}, this is the K_{EI}, this is K_{ESI} and this is also K_{ES}.

In the three different cases that we talked about for the type of inhibition, in the case of competitive inhibition magnitude of K_{ESI} will tend to infinity. K_{ES} and K_{EI} will have

definite magnitude. That means the enzyme can bind either to inhibitor or to a substrate and K_{ES} and K_{EI} will have definite magnitude but the K_{ESI} either will be infinity and that is the typical case of a competitive inhibitor.

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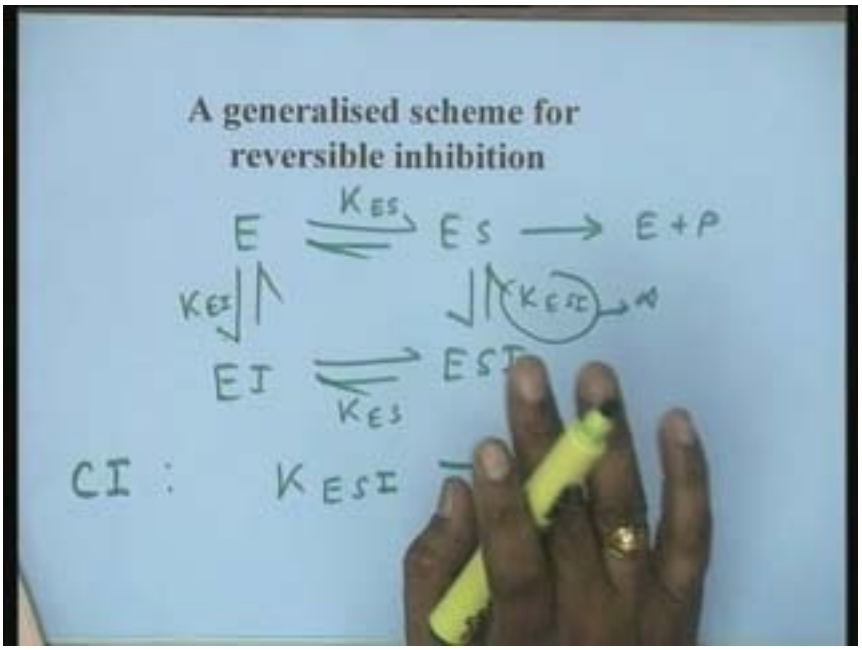

In the case of a non-competitive inhibitor K_{ESI} will be equal to K_{EI}. That means these two dissociation constants are identical. In the case of mixed inhibition they are different by a factor α and in the case of un-competitive inhibition the magnitude of K_{EI} is infinity or rather tends to infinity. That means the inhibitor cannot bind to the enzyme. The three cases are typical examples of three extreme situations of the inhibitor binding.

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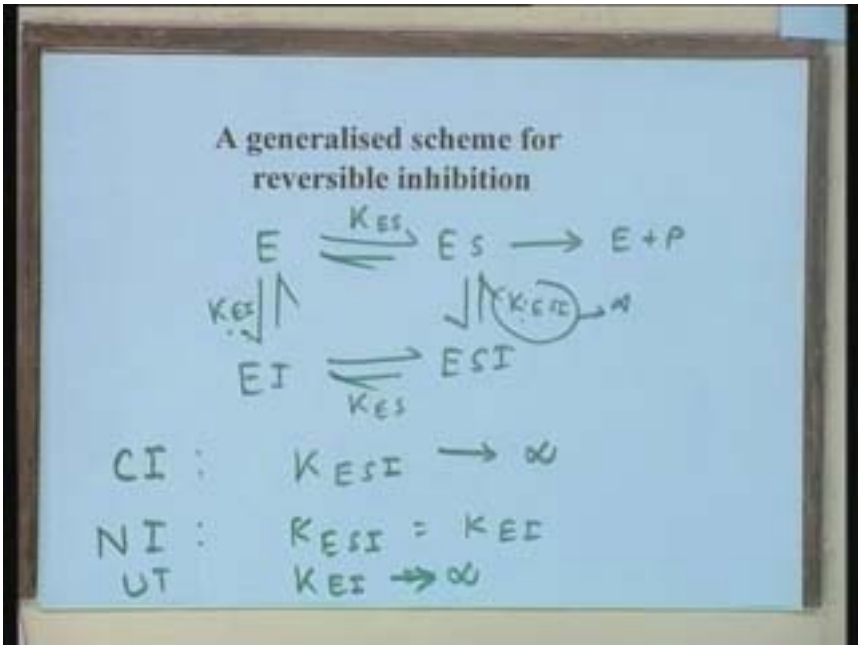

In the competitive inhibitor the binding of the inhibitor to ES complex is not feasible and the dissociation constant is infinity. In the case of non-competitive inhibition it can bind to either E or ES and the dissociation constant is identical. In the case of mixed inhibition they are not identical but they have a magnitude and both are related by ratio of α and which determines the degree of inhibition. In the case of un-competitive inhibition, the dissociation constant for the binding of inhibitor to the free enzyme approaches to infinity. That means binding to free inhibitor is not feasible. With that view I think we would like to look at the other bisubstrate reactions subsequently and we will stop at this point.