ENZYME SCIENCE AND ENGINEERING

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LECTURE – 9

ROLE OF EFFECTOR MOLECULES IN ENZYME KINETICS

Today we will be talking about the role of effector molecules in the kinetics of enzyme catalyzed reactions.

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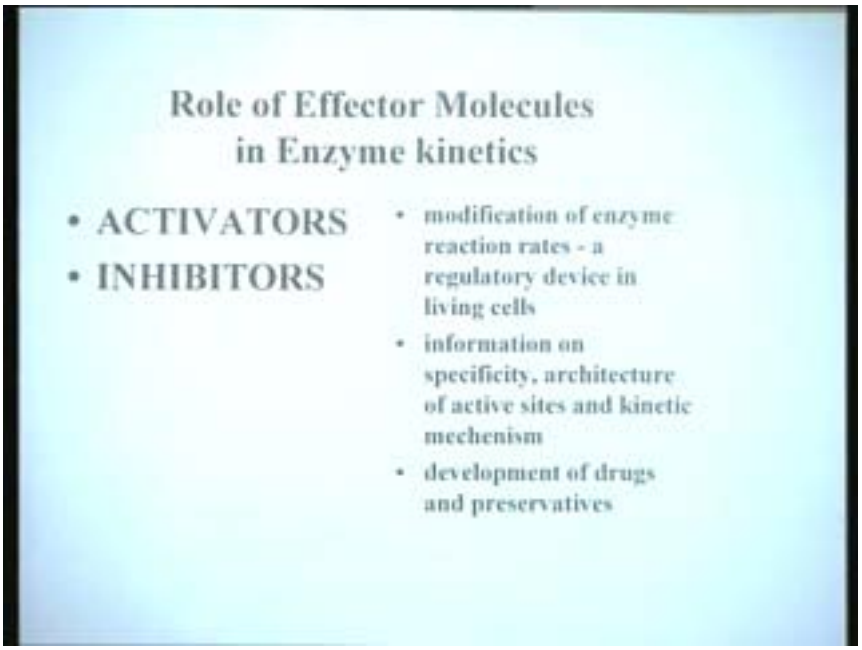

You will recall that so far we have seen the role substrate molecules play to influence the rate of an enzymatic reaction. They bind with the enzyme and have dynamic complexes that ultimately break down to form the product. Now this complex, enzyme substrate complex formation can be effected by a number of other external molecules which are not really directly the players in the enzyme reactions. That means neither they are substrate may or may not be products and such molecules are called effector molecules. Those molecules whose presence in a reaction mixture influences the rate of reaction, if it

is in increasing the rate of reaction we call them as activators if it is decreasing the reaction rate, we call them inhibitors.

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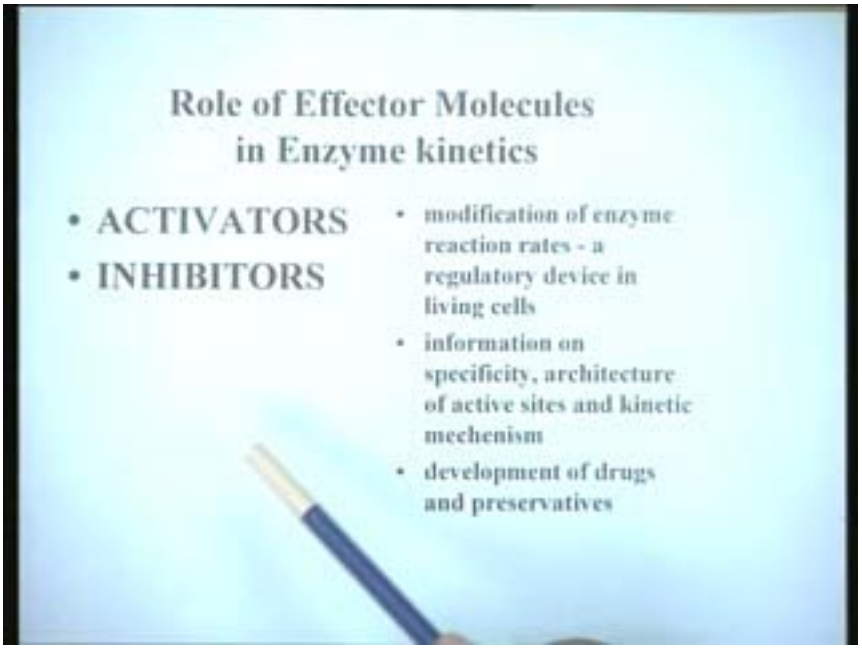

The role of these molecules is very important in the kinetics of enzyme catalyzed reactions for certain reasons. One is that they give you a picture on the reaction rate in the presence of those molecules. You can regulate the reaction rate to an enhanced value or to a decreased value in the presence of those molecules. In fact in the living cells also these molecules provide a regulatory device to control the rate of reaction. We will see when we go on subsequently how many of the molecules control the rate of reaction by inhibiting or activating a particular enzyme in the beginning of a metabolic pathway.

In addition to that, the understanding of the enzyme inhibitors or activators gives us very precise information on the specificity. Most of the information on the architecture of the active sites, which are the functional groups that are present on the active sites or the mechanism of the specificity, has resulted from the understanding of the interaction of inhibitor molecules or activator molecules in the enzyme catalyzed reactions.

Particularly in the case of multisubstrate reactions which we will be dealing subsequently where more than one substrate molecule takes part in the enzyme catalyzed reaction their kinetic mechanism is also understood by the role of certain external effector molecules. That is another major advantage. So these are the issues that provide us a very important understanding towards the role of effector molecules.

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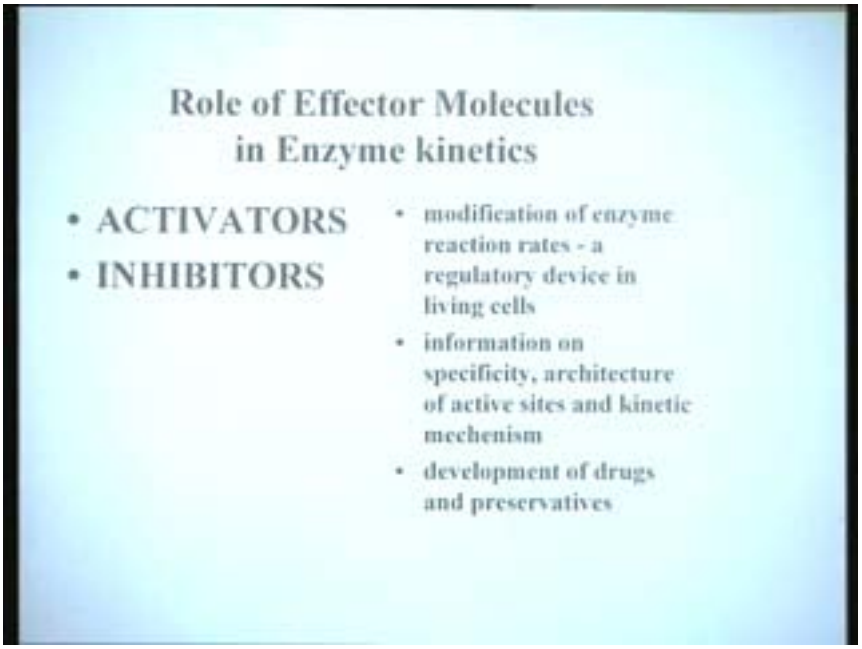

But even on the practical aspect probably from the industrial aspect also effector molecules are very important because in large number of drugs and the preservatives that are used commercially they basically work on the principle of modulating the enzyme reaction rate. We are aware of many of the sulphur drugs. In fact all the sulphur drugs are basically inhibitors of one or the other enzyme operating in the living system.

The only care that is taken in designing a drug is that the particular molecule which acts as an inhibitor for a particular toxic substance or let us say for an infection should not be toxic to the human beings. Otherwise it cannot be used as a drug. Most of the materials that we use, whether they are sulphur drugs or antibiotics, they are inhibitors or activators of certain enzymes. So from that point of view, understanding their interaction is quite important.

Activators are molecules, as name suggest, that enhance the rate of enzyme catalyzed reactions.

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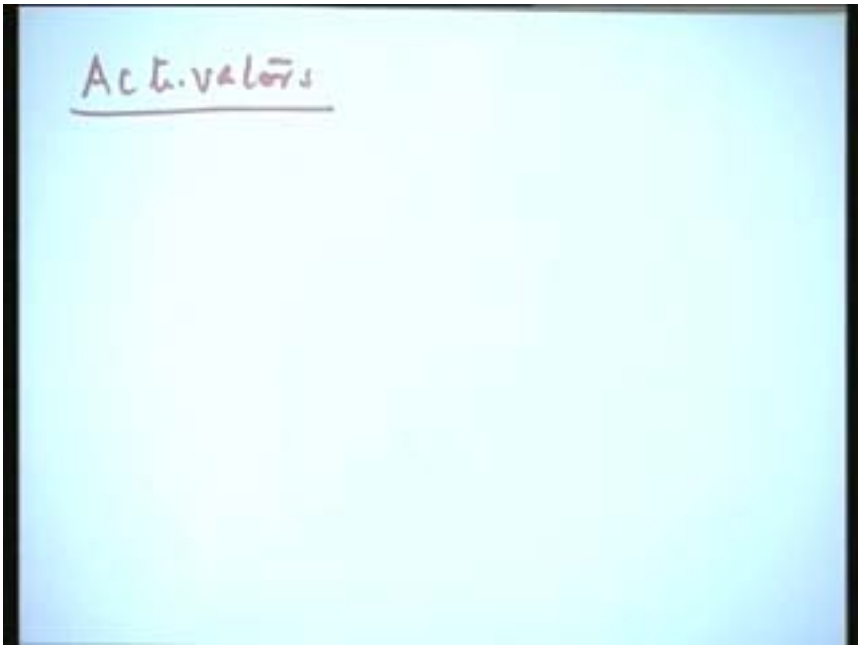

Some of the activator molecules may be absolutely essential for a given enzyme reaction. Some may not be so. They may only provide stability to enzyme substrate complex. Therefore the nature of activator molecules might differ from case to case. Typical examples of some of the activators are for example in the case of alpha amylase, calcium ions act as activators which is known to stabilize the enzyme substrate complex. In the absence of calcium ions, the enzyme substrate complex is not very stable or the dissociation constant of enzyme substrate complex is relatively high and therefore the rate of reaction will be very, very slow.

Similarly in the case of most of the Kinases, hexokinase, a typical example of the very first reaction in glycolysis, magnesium ions are absolutely required. That is another example of an absolute requirement of an activator. In the absence of magnesium ions the enzyme will not be functionally catalytically active and therefore these activators function by participating or facilitating in either enzyme substrate binding or in stabilizing the enzyme substrate complex.

The difference between cofactors and activators is a cofactor will have to participate in the catalytic mechanism. Take for example a cofactor like a NAD. It is used in the oxidation reduction reactions and in the absence of any such residue in the active site which can participate in the oxidation reduction reaction, the NAD provides a molecule which undergoes redox reaction and therefore catalyzes the reaction. It also plays a role in substrate binding and the role of cofactor is absolutely essential. The reaction cannot take place in the absence of cofactor whereas in the case of activator we are only enhancing the rate. The rate may not come to zero. It might have a very small insignificant rate in the absence of an activator and in the presence, the rate might enhance.

Mg^{2+} is an absolute requirement. So it falls in the category of both actually. It also participates in the catalytic mechanism. True example of activators will be calcium ions which do not take part in the catalytic function but provides stability to enzyme substrate complex. That means binding process is facilitated. So magnesium is something on interface.

Looking at activators from the mechanistic point of view, the role of activators can be considered as binding to the enzyme molecule to give you an activated enzyme. That means the free enzyme molecule will be inactive or has a very low activity. When the activator molecule binds to it, the EA complex becomes an active enzyme complex. This EA complex will bind with the substrate which is the active enzyme substrate complex. This enzyme substrate complex can break down to the active enzyme plus product with k_1, k_{-1} and k_2. If we consider that the dissociation constant of activator binding to the enzyme is K_a, then in that case the rate of such reaction can be expressed as

$$V_i = \frac{V_m . S}{K_m \{1 + K_a / A\} + S}$$

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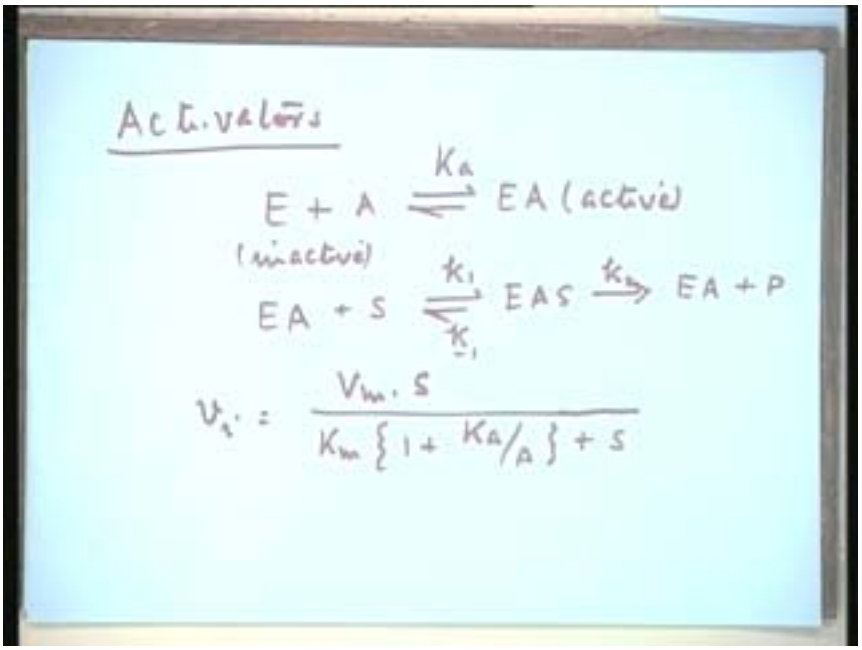

You ultimately end up in a rate expression in which the K_m term is modified. This can be written as

$$Vm . S / K'_m + S$$

The role of an activator works out to modify the intrinsic K_m value of an enzyme and the effect on the K_m value will influence the rate of the reaction. One can also see that the modified K_m value is

$$K_m' = K_m \{1 + K_a / A\}$$

That is when A becomes very, very large the activator concentration present in the reaction is much larger in comparison to K_a. That means when we have sufficient quantity of activator present as required, the K'_m approaches to that of intrinsic K_m or $K'_m/ K_m = 1$. Also when the activator is zero or when there is no activator present, the enzyme reaction will not take place because modified K_m value will be too large. There will be no effective binding taking place and that means the secondary step will be ruled out.

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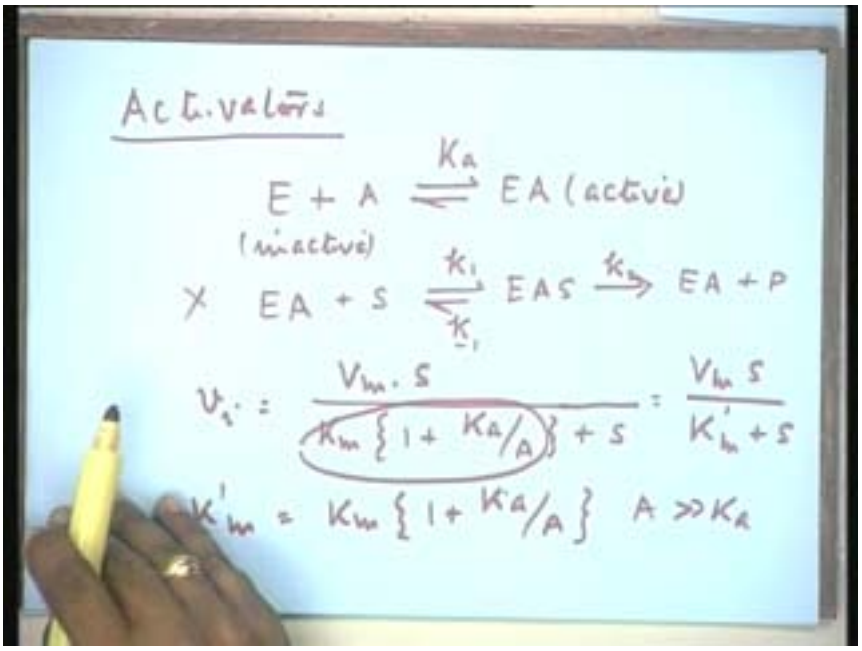

Therefore the activator molecules can influence the reaction rate in an enzyme catalyzed process by this simple way to modify the K_m value.

The second category of effector molecules that we noted was inhibitors.

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As the name suggests, these are those molecules which decrease the rate of an enzyme catalyzed reactions when present in the reaction mixture. They form complexes with enzyme molecule, enzyme substrate molecule, both or one of them. They form dynamic complexes with the enzyme and enzyme substrate complexes and thereby influence the rate of reaction. The inhibition patterns or inhibitors can be classified into two main classes. One is irreversible inhibitors, which means that the inhibitor molecule binds to the enzyme in an irreversible fashion and cannot be recovered or revoked by physical operations.

Very often when into a reaction mixture if you have added an inhibitor molecule, one can remove it by physical method say for example by dialysis or ultra filtration or any of those methods. But irreversible inhibitors cannot be removed once added to a reaction mixture and their effect is irreversible. That means ultimately they will tend to reduce the rate of reaction and provide a poisonous effect on the rate of reaction. In fact some times there are also called catalytic poisons because they irreversibly poison the catalyst.

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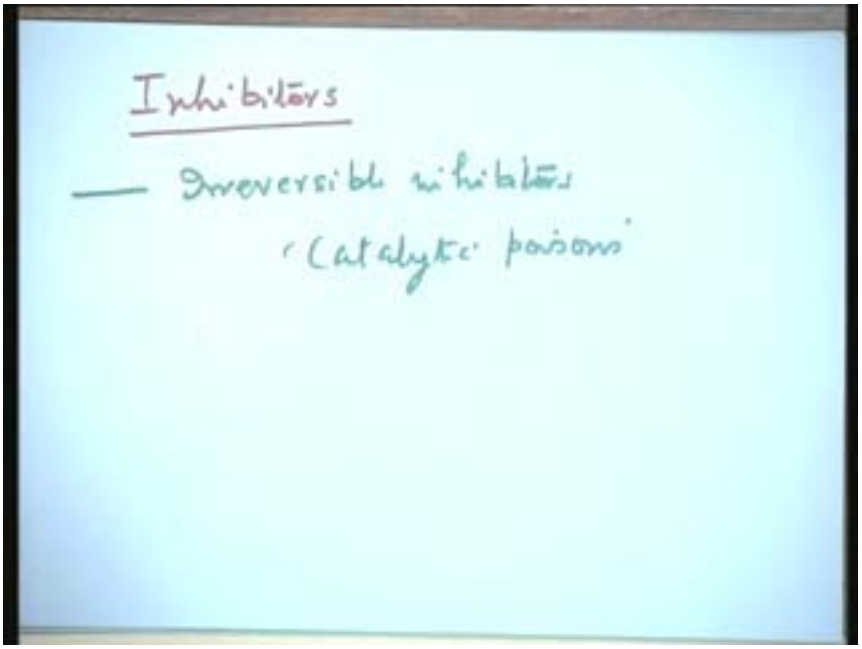

In most of the cases the effect is such that the reaction rate or catalytic function of the enzyme approaches to inactive form and thereby they cannot be used for catalytic functions. Reversible inhibition is caused by chemical modification of one of the amino acids residues on the active site. Most of the chemical modification reagents that are used for modification of the enzyme active sites do play a role in the irreversible inhibition. Most of the alkylating agents will alkylate some of the amino acids residues and thereby make the enzyme inactive. Many of the heavy metals like mercury or some of the poisonous gases also have the same action of irreversible inhibition whereby saving the person become difficult.

The role of irreversible inhibitors can be expressed as

$$E + I \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} EI \xrightarrow{k_{in}} E_{inac}$$

The first step shows a reversible process but the process doesn't stop here. Once EI is formed it goes into an inactive first order irreversible step which inactivates the enzyme and the concentration of EI at any given time can be expressed as

$$EI = (E_0\text{-}EI).I/\ k_i$$

where k_i is the dissociation constant for the inhibitor and then one can write the inactivation rate as

$$V_i = K_{in}\,[EI] = k_{in}\,E_0/1 + k_i/I$$

Therefore if I is in excess, if the magnitude of inhibitor is in excess, such that you can consider that there is a constant, then the whole expression is a first order rate constant that is $k_d.E_0$ where k_d a first order rate constant assuming that I is in excess and can be considered as constant.

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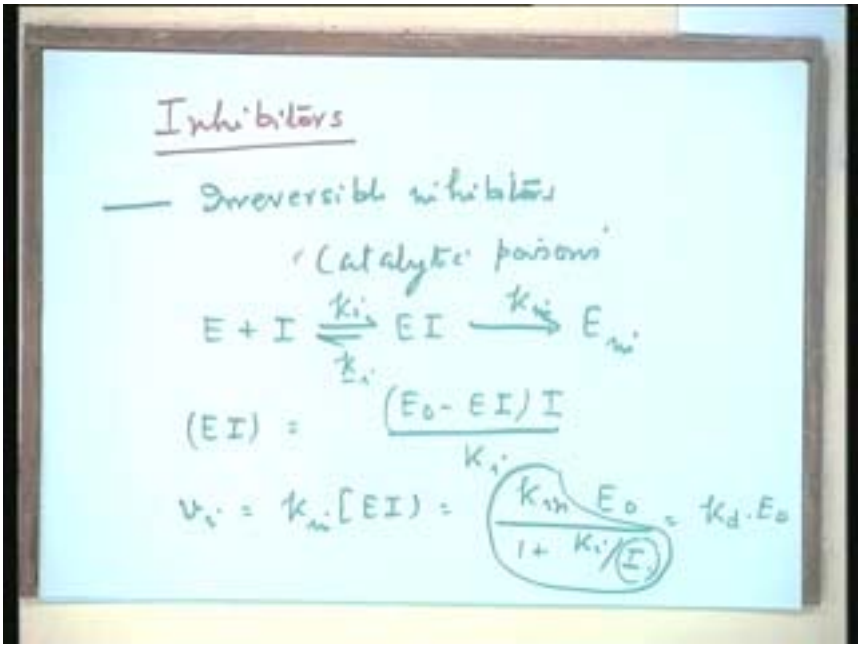

Such reactions are very often noted to be poisonous in nature. Most of the substances that are technically called poisons whether it is mercury or cyanide or nerve gas and things like that they all behave in the form of irreversible inhibitors. Except to understand their role to evolve methods to prevent the poisoning they don't have much of industrial value. It is mostly reversible inhibitors which play a key role in most of the industrial processes.

In the second category of reversible inhibitors, the inhibitor binds to the enzyme in a reversible manner.

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That means after the addition of the inhibitor to an enzyme substrate mixture one can physically remove it. The physical methods most commonly used are ultra filtration or dialysis such that the enzyme is fully restored to its initial activity. In the presence of inhibitor their activity will be decreased but once you remove it physically, the original activity of the enzyme is recovered. In a sense these molecules make dynamic complexes with the enzyme which have different catalytic properties to that of the native enzyme.

They bind in different ways. Different inhibitor molecules bind differently to the enzyme molecule or enzyme substrate complex such that the catalytic properties are altered and thereby the rate of reaction is altered. Our total orientation to study the inhibitors will be mostly directed towards the understanding the reversible inhibition. It is reversible inhibition which forms the major portion of the effector molecules that are of industrial, medical or biological value in the case of understanding the scientific basis of enzymatic catalysis.

There are certain general patterns which we follow in studying reversible inhibition and before we go to the individual cases I like to highlight that. The first thing that we must look at is the pattern of binding of inhibitor to enzyme or enzyme substrate complex.

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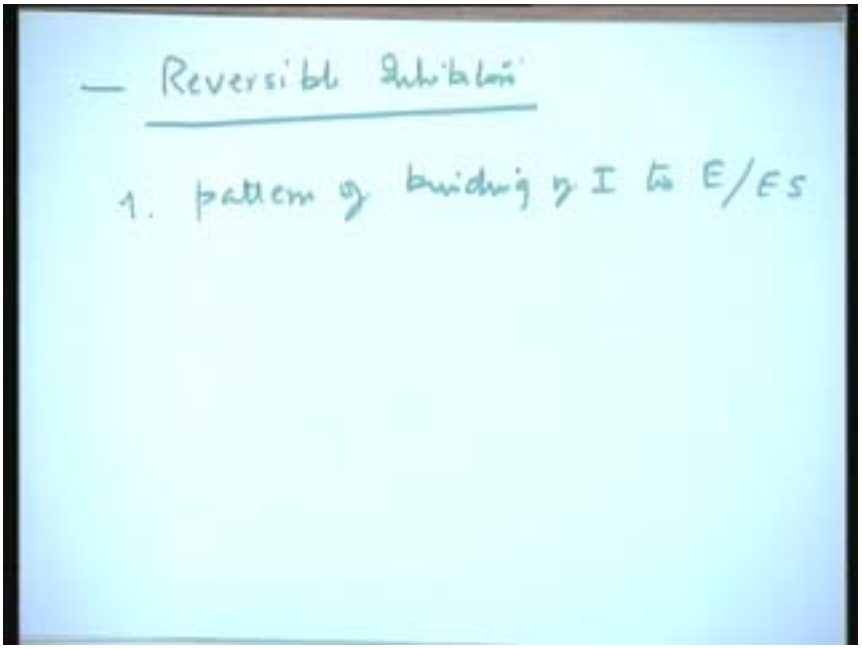

How does a particular group of inhibitors bind to the enzyme molecules? You are proposing an operational reaction mechanism which may be slightly different than that proposed by Michaelis Menten or others earlier as we discussed and that mechanism need to be understood very clearly and that will give you a physical picture of the enzyme substrate binding. The second thing is, on the basis of this binding process you develop a rate expression.

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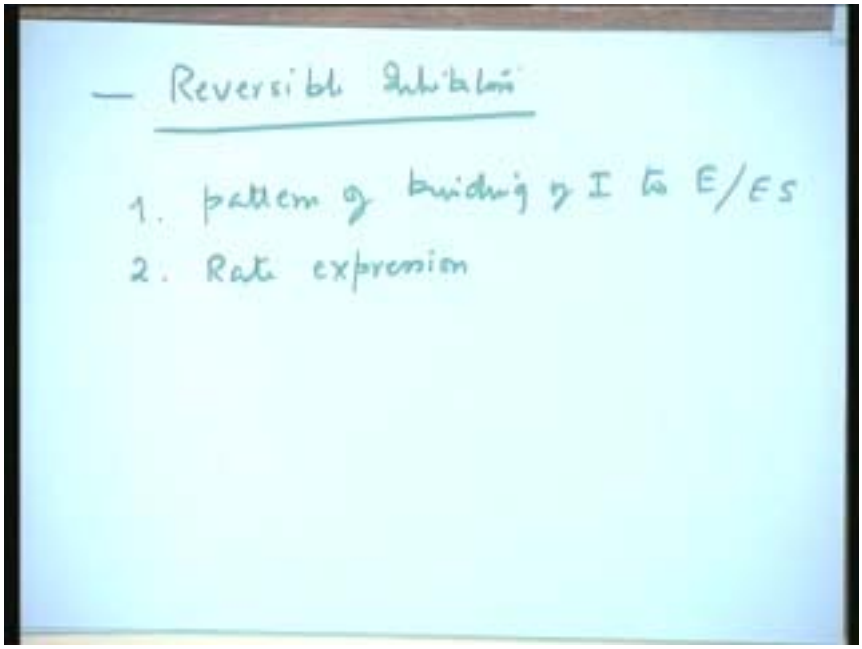

We always tend to compare a rate of expression with that of Michaelis Menten rate expression. Then look at the effect of the inhibitor concentration on K_m or V_m or both of the reaction.

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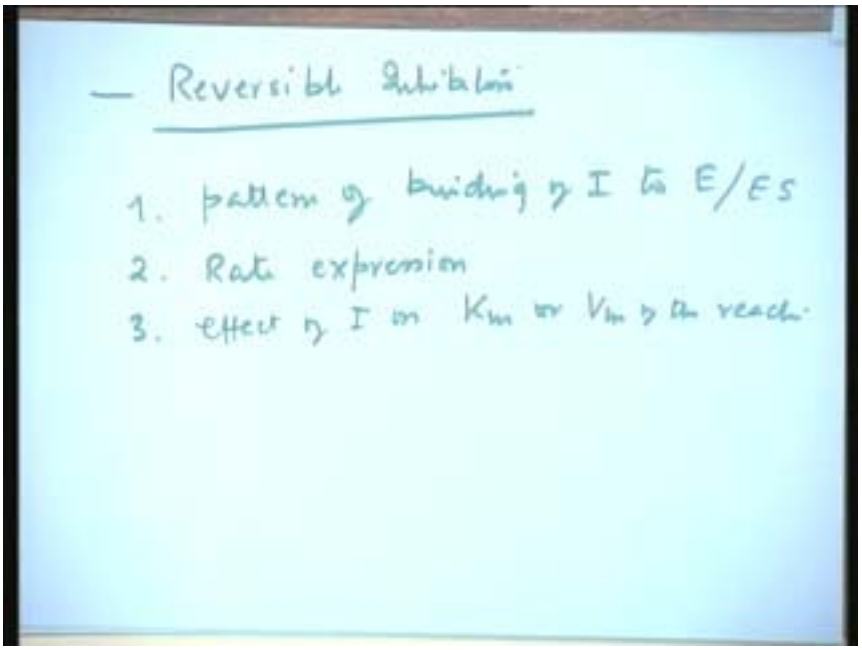

First we have looked at the binding pattern. Based on the binding pattern, we develop a rate expression compare it to the Michaelis Menten equation and see how the K_m and V_m of the native enzyme are influenced by the presence of inhibitors. Then we see the effect on Lineweaver Burk plot. That is another basic expression of kinetic behavior in the case of enzyme catalyzed reaction.

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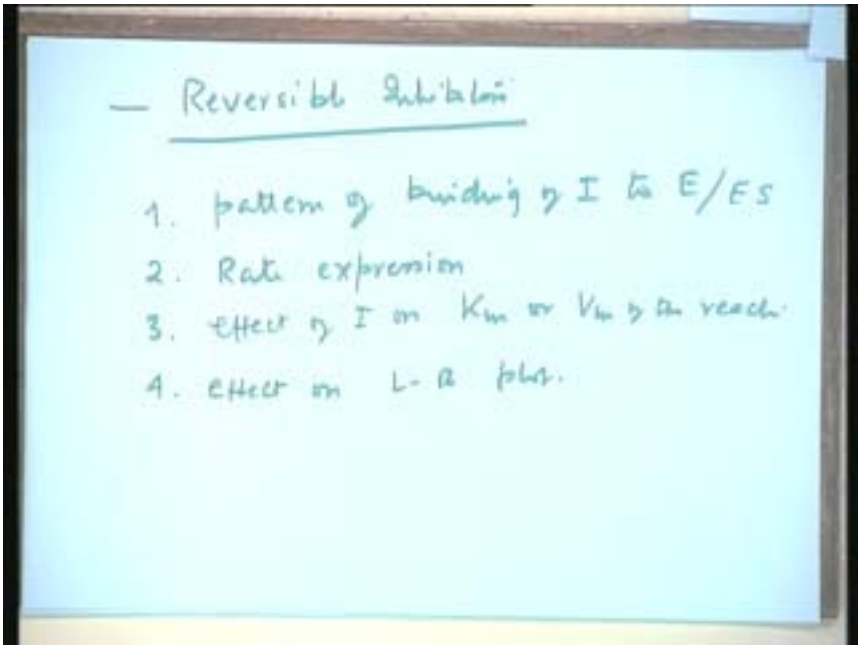

If a double reciprocal plot is plotted between 1/S and 1/V as a function of inhibitor concentration how does this represent? Fifthly another important pattern is for each class of inhibition, there is degree of inhibition. That means we define a term degree of inhibition called i which is nothing but v_0-v_i/v_0.

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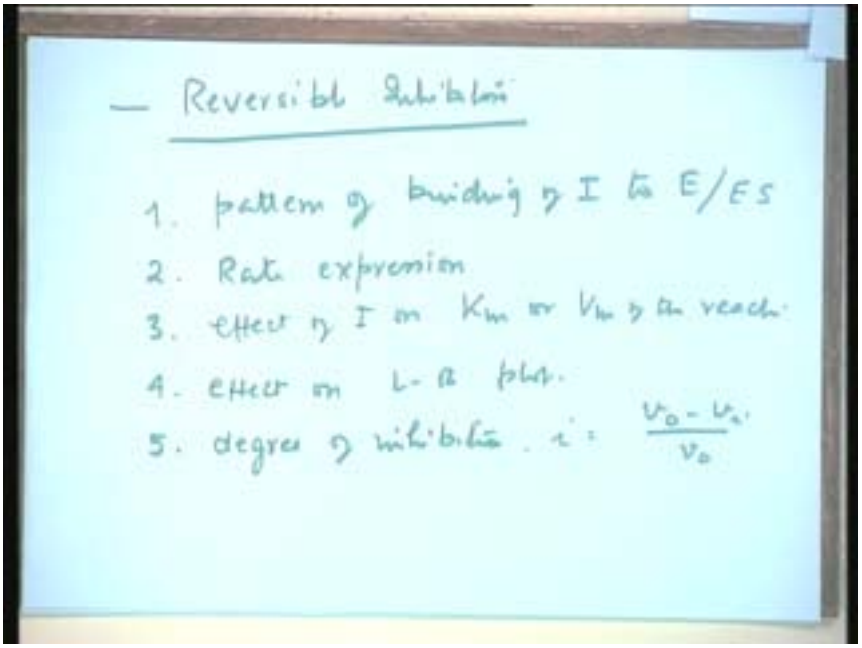

If v_0 is the rate of uninhibited reaction, classically Michaelis Menten kinetics, and v_i is the rate expression for inhibited reaction, the degree of inhibition that is produced by the presence of inhibitor is obtained even from a theoretical rate expression. You can work out an expression for degree of inhibition and that will have a characteristic pattern which can distinguish a particular type of a reversible inhibition. All other reversible inhibition studies will be based on these five approaches in general.

Probably one of the simplest and the most commonly encountered pattern of reversible inhibition is what we know as competitive inhibition.

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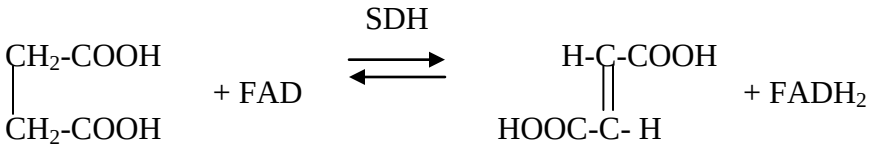
As the name suggests, competitive inhibitor combines with the free enzyme on the same site which is the substrate binding site and therefore the inhibitor competes for the binding site with the substrate. Usually both inhibitor and substrate molecules are mutually exclusive and compete with each other. The inhibitor binding site on the enzyme molecule and substrate binding site on the enzyme molecule are identical. This should be taken in a very broad sense. They could be on the same site where the substrate binds or even they could be on a site in proximity of that or in such a way that the binding of the substrate is influenced. It is not always necessary that the binding site has to be exactly the same as that of substrate. The purpose is that the presence of inhibitor makes the binding of the substrate difficult in a competitive mode. That means the proximity may be such that the inhibitor molecule has such a confirmation that the original binding site is disturbed and substrate binding is not feasible or alternatively after binding of the inhibitor molecule the substrate binding site might be modified in such a way that substrate binding is not feasible.

All those cases, variety of cases will come under the category of competitive inhibition and the purpose is that the presence of inhibitor must reduce because reversible inhibition depends on the concentrations of the two species, inhibitor and substrate and they will affect the binding mechanism.

A very typical example and probably the most easily understood example is of oxidation of succinic acid to fumaric acid.



This enzyme is succinic dehydrogenase, SDH. This is inhibited by a molecule closely resembling the substrate, malonic acid which has two carboxyl groups but only one methyl group, unlike succinic acid. It is very similar in confirmation and very often enzyme active site mistakes it for succinic acid and therefore it acts as a competitive inhibitor in the same reaction.

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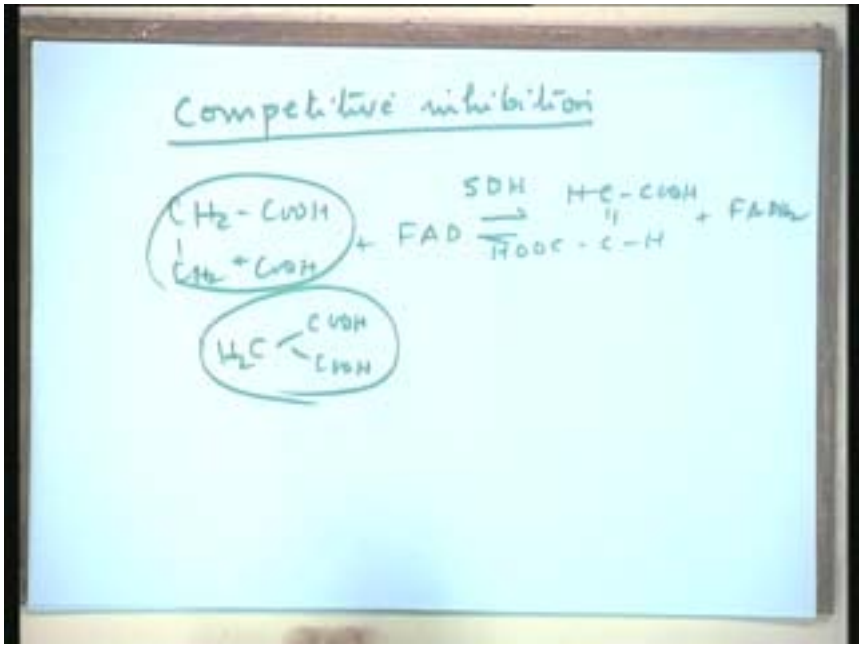

Another example of a competitive inhibitor is sulfanilamide which is a sulpha drug. It acts as a competitive inhibitor for folic acid biosynthesis from para amino benzoic acid. The amino group on the aromatic ring is identical in both the molecules. This is sulfanilamide and this is para amino benzoic acid.

This para amino benzoic acid is the starting substrate for synthesis of folic acid. This synthesis is inhibited by this inhibitor.

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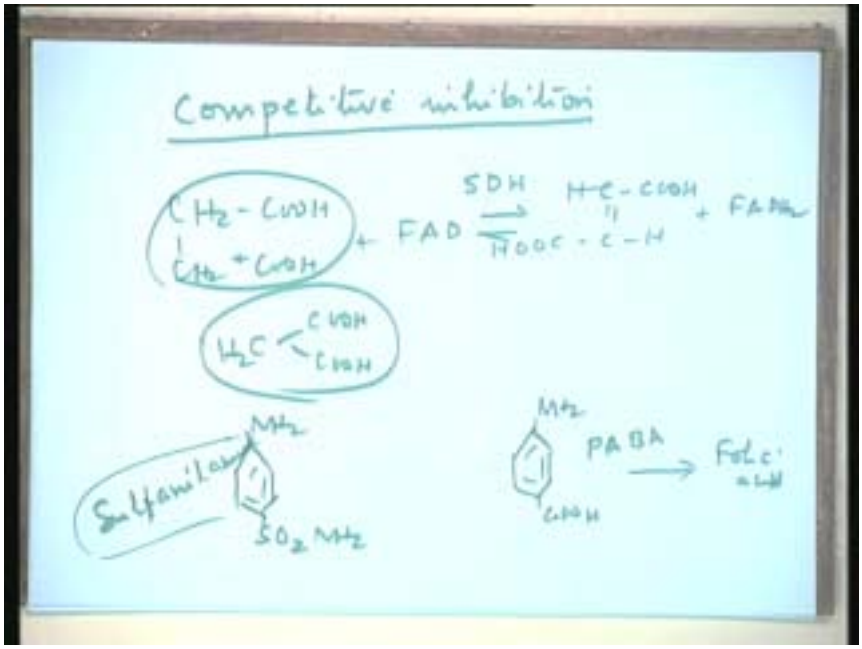

Such a synthetic reaction route although takes place in microbial systems does not take place in the human beings. Therefore this acts as a very potent sulpha drug. This reaction is not available in the human beings but since microbial systems are totally dependent on synthesis of folic acids it can easily inhibit the growth of microorganism and sulfanilamide is a very potent antibacterial agent in the case of human systems.

If you look for the reaction scheme or the binding pattern which is followed by competitive inhibitors we can write the first stage as enzyme substrate binding.

$$E + S \underset{}{\overset{k_m}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$+$$

$$I$$

$$k_i \uparrow\downarrow$$

$$EI$$

Then the same free enzyme molecule binds to the inhibitor and gives you EI. We can consider the dissociation constant of ES as k_m. When I say k_m I am referring to $k_{-1} + k_2/k_1$. It is analogous to dissociation constant. Just for simplicity I have written here and this is k_2. This is k_i, the dissociation constant for the inhibitor.

$$ki = E.I/EI$$

$$k_m = E.S/ES$$

You can write the rate expression for such a binding mechanism and the rate expression will come out to be

$$v_0/v_m = \frac{S}{k_m (1 + I/ k_i) + S} = \frac{S}{k'_m + S}$$

Now if you compare it to Michaelis Menten equation which is S/k'_m+S. Just like an activator molecule, here also the k_m gets modified to a new value $1+I/k_i$ and therefore as the concentration of I increases the value of k_m also increases and the affinity decreases for the substrate. The modified Michaelis Menten constant in the presence of inhibitor is

$$k'_m = k_m (1 + I/ k_i)$$

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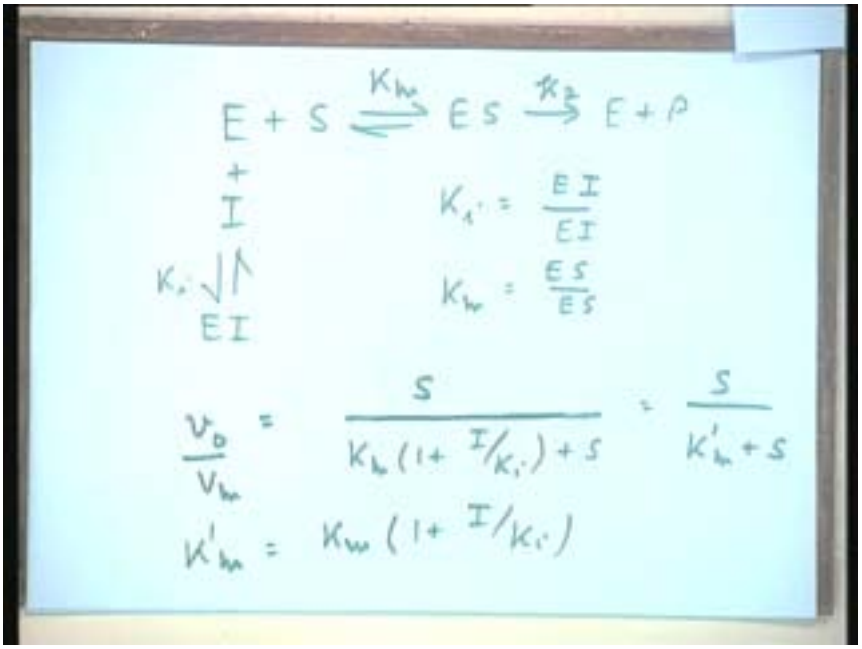

So you notice here that the K_m changes by a magnitude $1 + I/ K_i$ but the V_m remains unchanged, V_m.S on the numerator; there is no change in the magnitude of V_m. Maximum reaction velocity remains the same but the k_m changes. That is a very typical feature of identifying a competitive inhibitor.

If you make a double reciprocal Lineweaver-Burk plot between one upon substrate concentration verses one upon reaction velocity, 1/S Vs 1/V, at different inhibitor

concentrations, you will notice that with increasing inhibitor concentration the value of k_m will undergo change. k_m will tend to increase or the slope of the profile will increase without affecting the point of intersection on y- axis which is represented as $1/V_i$.

If you write the double reciprocal equation for the same thing, what we wrote earlier

$$1/v_o = k_m/V_m(1+I/k_i).1/S + 1/V_m$$

If you plot 1/S verses $1/v_o$ you get a series of profiles with increasing I. The slope increases but the magnitude of $1/V_m$ remains unaltered. That is what we notice. Another fact is if one were interested to determine the value of k_i in comparison to k_m and V_m and the characteristic pattern of inhibition, you can use the double reciprocal plot as a basis for determining the value of k_i.

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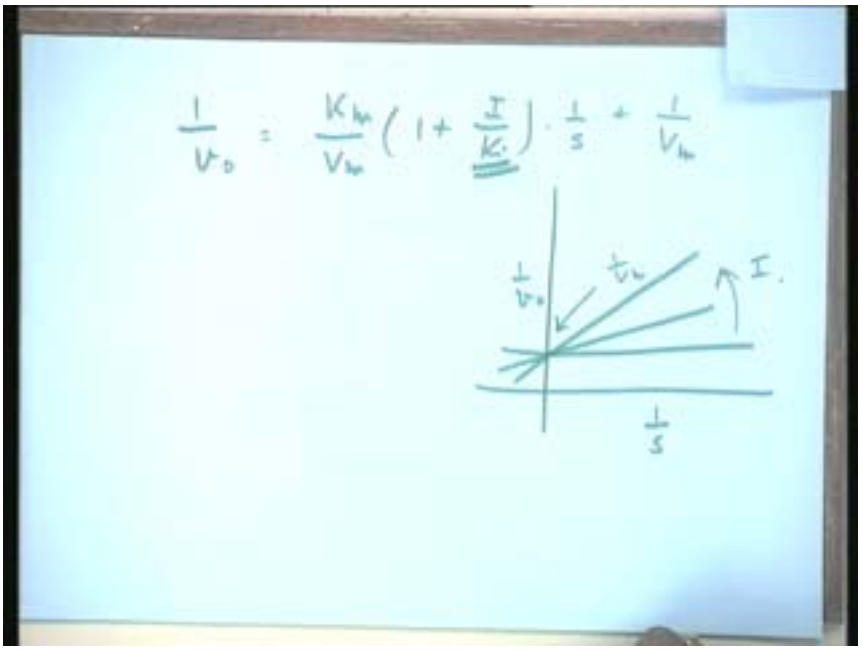

If you look at the slope of reciprocal plot slope is

$$\text{Slope} = k_m/V_m\,(1+I/k_i)$$
$$= k_m/V_m.k_i.I + k_m/V_m$$

If you get a secondary plot where you plot the value of slope, obtained from the primary double reciprocal plot, as a function of inhibitor concentration you will again end up in a straight line with the slope of $k_m/V_m.k_i$.

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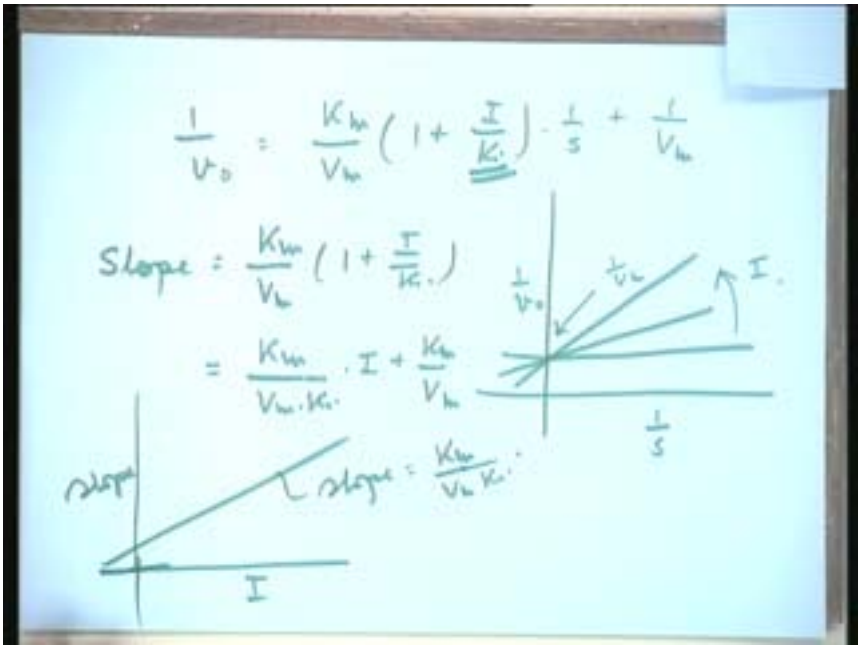

k_m and V_m is the slope at I=0 which is already known to us and therefore the magnitude of ki can be easily determined. Similarly you can also plot the apparent k_m. The slope also means the apparent k_m. So either you can plot apparent k_m or you can plot slope. It will be entirely the same thing. Therefore one can get the magnitude of k_i by plotting a secondary plot.

So to summarize the competitive inhibition, we must notice that the inhibitor molecule binds to an enzyme molecule at a site either on the same side which is ear marked for substrate molecule or at a site in proximity to it in such a way that it interferes or restricts the binding of the substrate molecule onto the enzyme site and therefore it competes for the binding process on the enzyme molecule. There is no binding of the inhibitor molecule with the enzyme substrate molecule once the substrate molecule has bound to the enzyme, the inhibitor is ineffective. Either inhibitor has to bind to the enzyme or the substrate has to bind to the enzyme. That means the total enzyme concentration is distributed between inhibitor and substrate and that is one. Second thing is the enzyme catalyzed reaction in the presence of a competitive inhibitor results in an increased k_m value and the V_m value is totally not affected. Third thing is that the effect of the competitive inhibitor can be successfully overcome to a significant extent if you increase the substrate concentration to a very large value as compared to inhibitor concentration. Because they are competing on the same side, if the concentration of substrate is much, much larger than inhibitor then the possibility of substrate molecule binding to enzyme is much more and once substrate molecule is bound the enzyme will no longer be available for inhibitor to act upon. Finally the double reciprocal plot of 1/S verses 1/V at different inhibitor concentrations followed by a secondary plot of slope verses inhibitor concentration can be used to determine the magnitude of k_m, V_m and k_i for a reaction which involves competitive inhibitor. We will stop at this point.