ENZYME SCIENCE AND ENGINEERING

PROF. SUBHASH CHAND

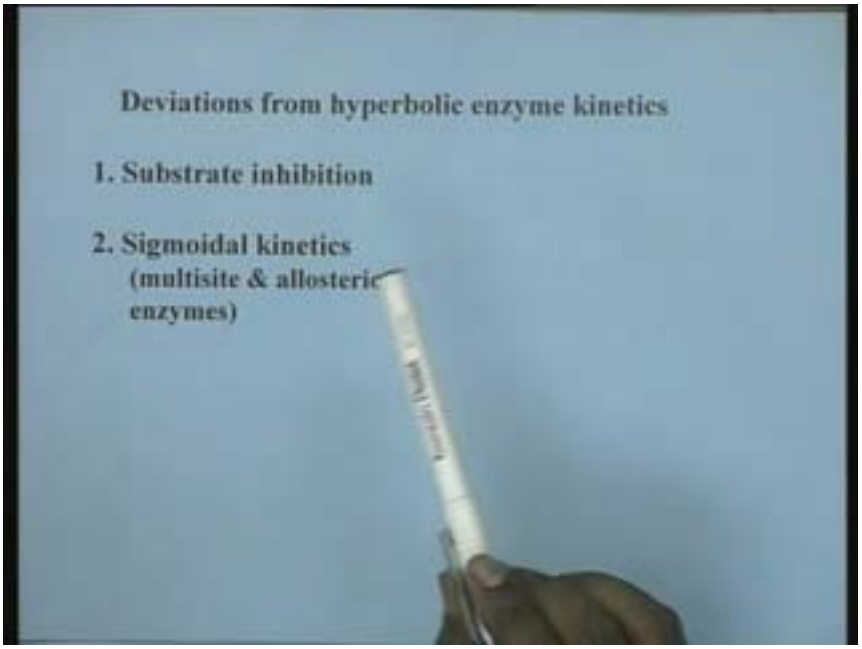
DEPARTMENT OF BIOCHEMICAL ENGINEERING
AND BIOTECHNOLOGY
IIT DELHI

LECTURE – 8

DEVIATION FROM HYPERBOLIC ENZYME KINETICS

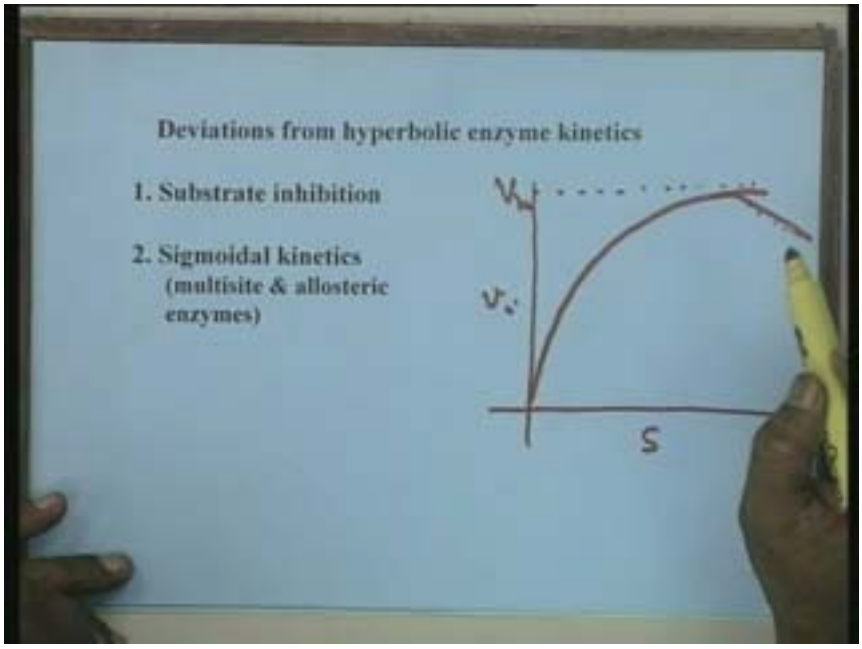
Today we will discuss two of those cases that do not represent the reaction velocity profile in terms of hyperbolic kinetics.

[Refer Slide Time: 1:15]



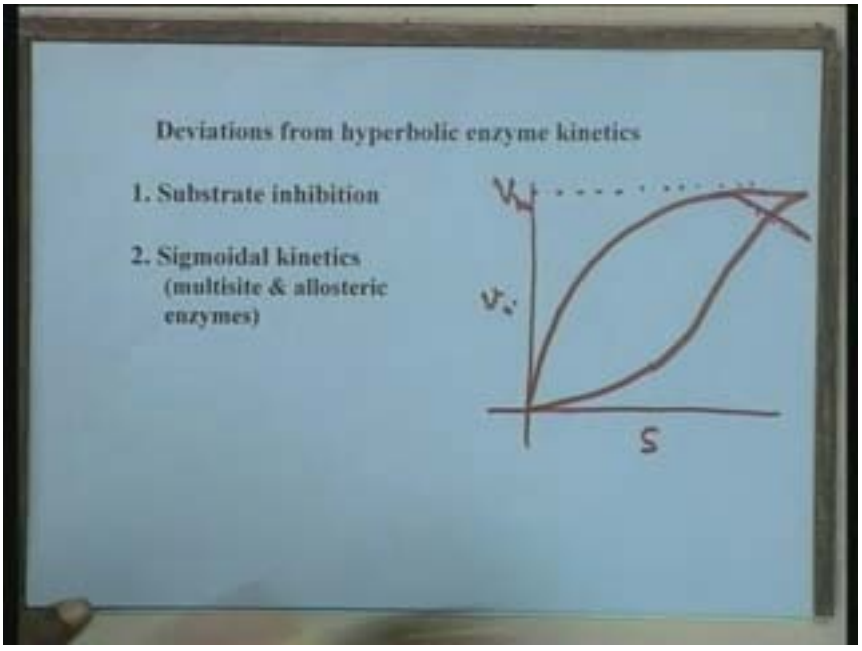
One of them is substrate inhibition the other is sigmoidal kinetics particularly as it applies to multisite and allosteric enzymes. If you look into the reaction profile, a typical substrate verses initial reaction velocity profile, for a hyperbolic kinetics goes like this with an asymptotic reaction velocity V_m. In the case of substrate inhibition the reaction velocity when you increase substrate concentration does not reach to a saturation level rather even earlier than that it deviates and passes through a maximum. When substrate concentration goes high the reaction velocity starts dropping and does not retain saturation level.

[Refer Slide Time: 2:10]



On the other hand in the sigmoidal kinetics instead of a reaction profile being represented by rectangular hyperbola it is in the form of a sigma. Ultimately it can reach the same kind of saturation behavior but the initial profile will be different in the case of a sigmoidal profile.

[Refer Slide Time: 2:32]

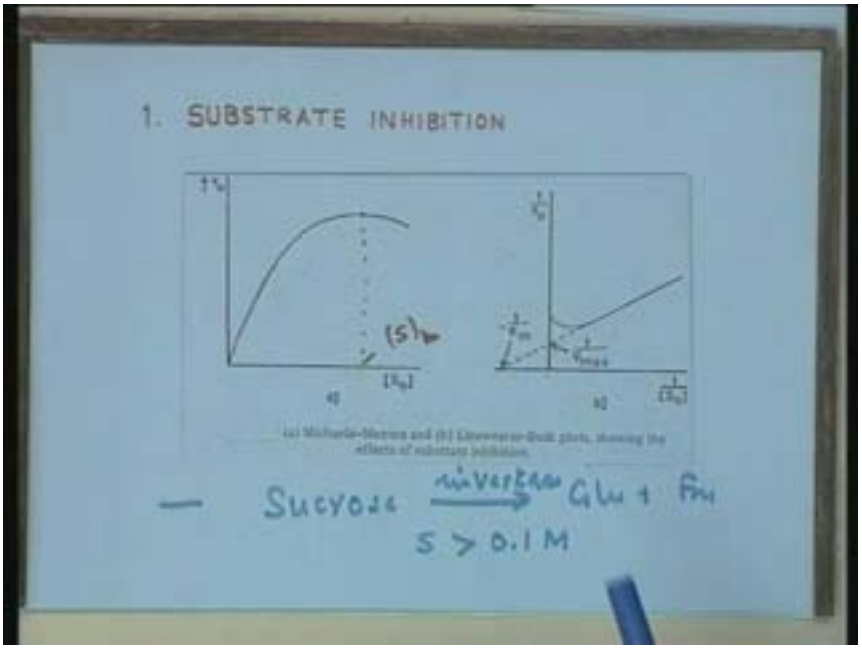


These two different situations will give you an idea, that in some cases and these are very important cases and have lot of physiological importance for the cell to have design such a enzyme which deviate from hyperbolic kinetics as we will see further.

Coming to the first case of substrate inhibition, the reaction profile passes through a maximum and at a particular substrate concentration it reaches a maximum value and then it starts dropping. This substrate concentration corresponds to maximum reaction velocity. This substrate concentration corresponding to maximum reaction velocity is not really something which is corresponding to maximum reaction velocity of enzyme catalyzed reaction. It is as a result of certain enzyme substrate interactions such that the rate gets tapered even before the saturation level reaches. This effect can also be visualized when you look for the classical Lineweaver plot. Instead of giving you a straight line it becomes convexed to the x axis and it can meet. That can give an indication that the enzyme catalyzed reaction is having some kind of a substrate.

There are number of examples in practice for such kind of a behavior in the reaction. One of the most conventional and simplest examples could be hydrolysis of sucrose to glucose and fructose by invertase. A very classical example, where at a substrate concentration of greater than 0.1M, 0.1M is not a very high substrate concentration, is about say 3.4 or 3.5%. Even at that level the effect of substrate concentration inhibition is noticed and the reaction velocity starts dropping.

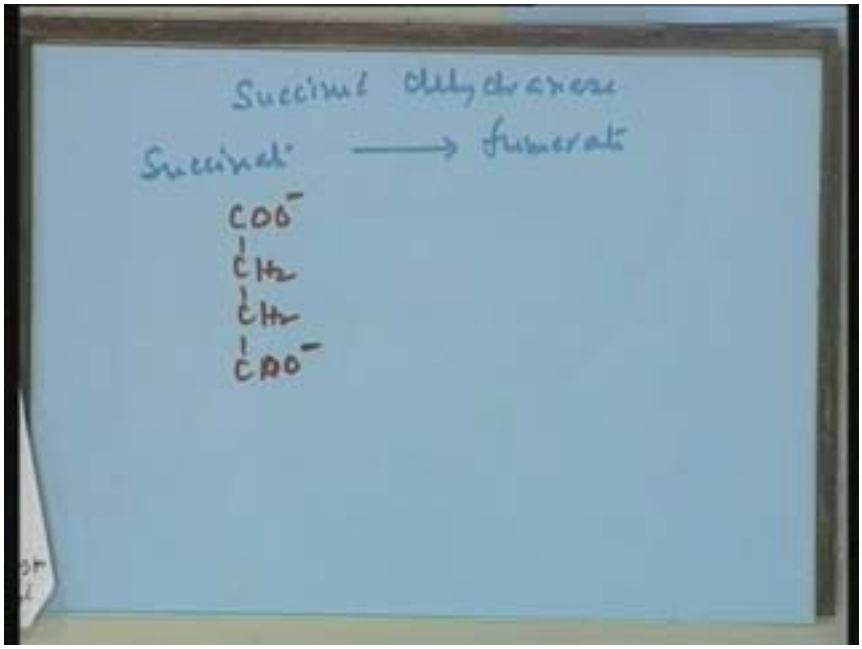
[Refer Slide Time: 4:40]



Another example of substrate inhibition is in the form of succinic dehydrogenase which catalyzes the interconversion of succinate to fumerate, a reaction which catalyzes the formation of a double bond. Succinate as a substrate has two carboxylic groups. As per the normal function of the enzyme the two carboxylic groups of the substrate are required

to be bound to the enzyme molecule. When substrate concentration becomes very high, the possibility or the statistical feasibility of one carboxylic group from each of the two substrate molecule binding to the same enzyme molecule becomes much higher. In that process when the two substrate molecules gets bound to one enzyme molecule and one carboxylic group of each of the substrate molecule then the reaction is not catalyzed and then ultimate enzyme substrate complex is a dead end complex. The structure of succinic acid is $^{-}OOC\text{-}CH_2\text{-}CH_2\text{-}COO^{-}$.

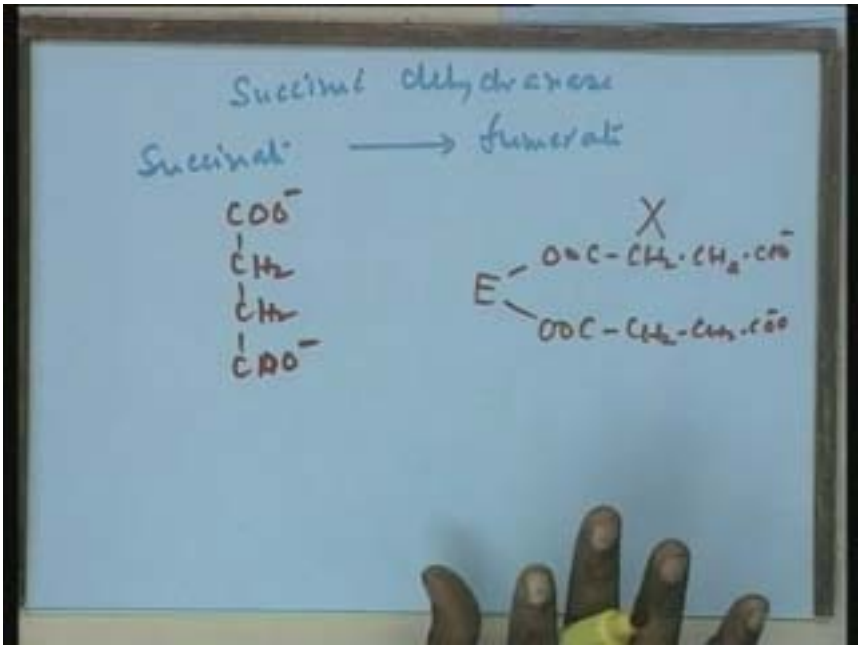
[Refer Slide Time: 6:16]



As per the normal reaction of succinic dehydrogenase two carboxylic group of substrate molecule are required to be bound to the enzyme active sites and at low concentration of substrate this can very well happen. When substrate concentration becomes very high the enzyme molecule might bind to two substrate molecules, one of the carboxylic groups from each of the succinic acids. So therefore the active sites are not functional yet because instead of one molecule there are two molecules attached and the enzyme substrate complex so results in the form of dead end complex which doesn't go into the final product forming stage.

For such a reaction there is a feasibility that you may end up in an enzyme molecule having such kind of a confirmation, instead of having the two carboxylic group of the same substrate molecule bound to the enzyme, there are two substrate molecules bound to the same enzyme and one carboxylic group of each of the substrate molecule and such a complex does not form the product.

[Refer Slide Time: 7:45]



Similarly in the case of hydrolysis of sucrose by invertase also by one of the possible mechanistic models when one substrate molecule binds, the enzyme substrate complex leads to a product formation. If the enzyme is bound to two substrate molecule a dead end complex is formed.

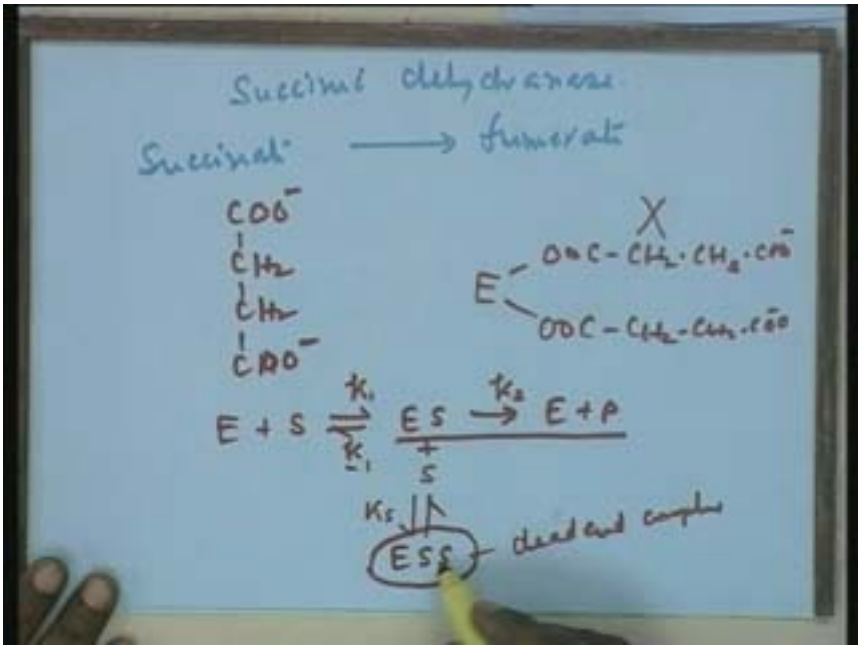
A simple reaction mechanism can be expressed like

$$E + S \rightleftharpoons ES \longrightarrow E + P$$

$$ES + S \rightleftharpoons ESS$$

We can write exactly in our nomenclature of k_1, k_{-1}, k_2 and this equilibrium of the second substrate binding can be expressed by dissociation constant of K_s, individual rate constant being k_s and k_{-s}. This ESS is a dead end complex. That means the product formation will take place from the substrate complex.

[Refer Slide Time: 8:50]



This will not lead to any product formation and whatever fraction of the total enzyme is bound in the form of ESS will not participate in the reaction with the result that reaction velocity will drop. That means at the saturation level you are assuming that the entire enzyme is bound to the substrate. The entire enzyme is not bound to the substrate here. Increasing the substrate concentration leads to more of ESS formation.

On the basis of equilibrium kinetics one can write the rate expression of such a system as

$$V_i/V_m = \frac{S}{K_m + S\,(1 + S/K_s)}$$

The V_m is the true maximum reaction velocity if there was no such substrate inhibition to take place and K_m is, the classical $(k_{-1} + k_2 / k_1)$, the Michaelis Menten constant and k_s is the dissociation constant of the second substrate binding.

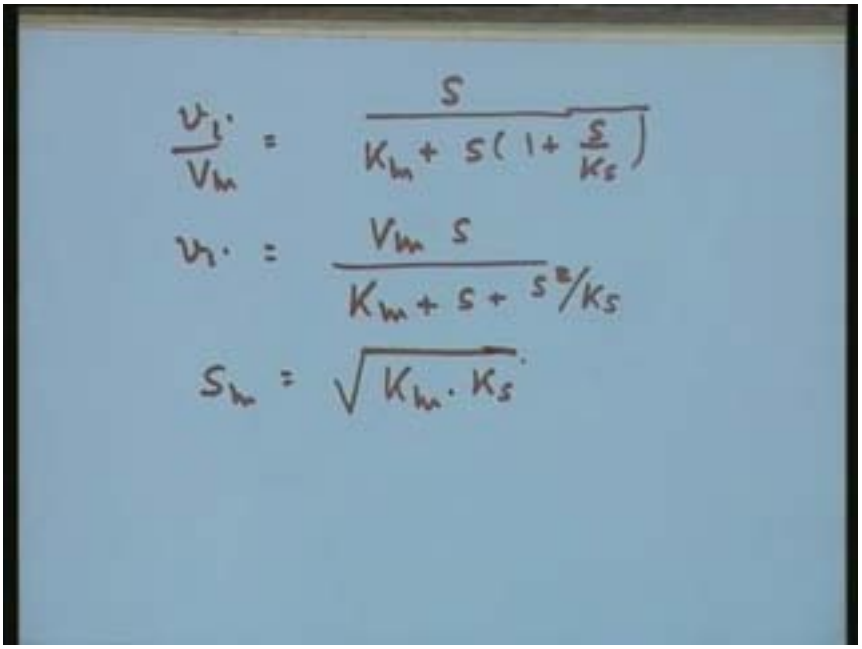
This can also be written as

$$V_i = V_m\, S / K_m + S + S^2/K_s$$

If you plot such profile the rate expression will pass through maxima and if you differentiate that dv over ds and equate it to zero you will end up in maxima and the maximum reaction velocity will be obtained at substrate concentration which is

$$S_m = \sqrt{K_m} \,.\, K_s$$

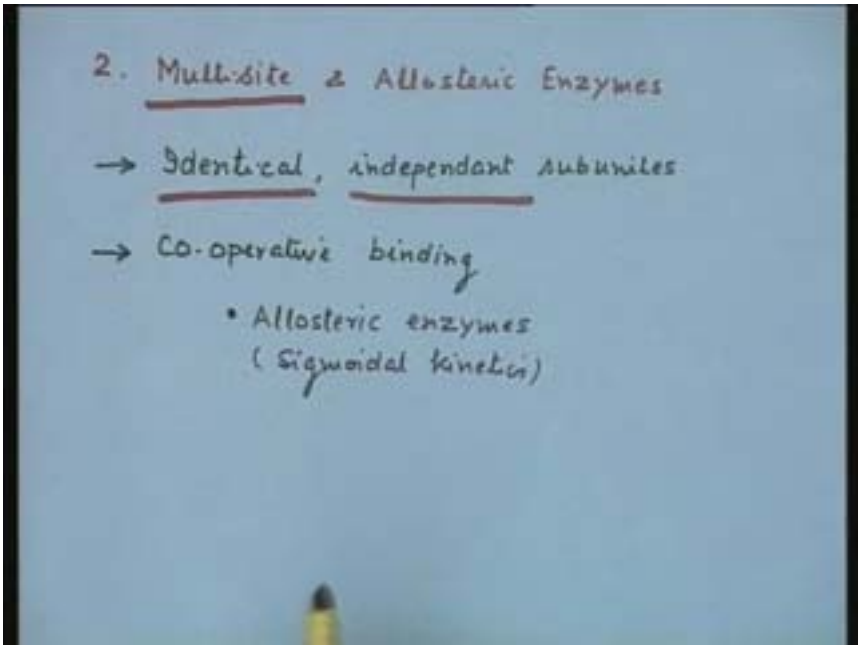
[Refer Slide Time: 10:54]



One can develop the expression to write the differential and equate it to zero and that will end up in the substrate concentration for the maximum reaction velocity that is the maxima. In such cases you notice that such enzymes do not really strictly follow the typical Michaelis Menten kinetics.

There are another group of enzymes which are multisite. The chemical nature of the enzyme provides for a quaternary structure in some cases and the quaternary structure is composed of multiple subunits arranged in a particular fashion so that their interaction brings in the confirmation thereby active sites are created. These multisite enzymes are alphabetic in nature which consists of more than one sub units and very often these subunits might be identical. Each subunit may have an active site for enzyme catalyzed reaction, multiple active sites per enzyme molecule. There are two kinds of situation one is that these active sites on these oligomeric enzymes might be identical and independent.

[Refer Slide Time: 12:36]



Notice the two terms which I have highlighted here. Identical means the catalytic sites have identical free energy change of binding. That means free energy of binding on either site is same and it does not matter to which site the substrate molecule binds first or second; it makes no different. They are identical in terms of confirmation. The independent subunits or the independent binding site implies that binding to one site does not bring in any conformational change so as to change affinity of other binding sites. Very often it might happen that initially the binding sites may be identical and they are free, free for the substrate to bind to any one of them. Once the substrate binds to one of the binding sites in some cases the binding site might induce a conformational change thereby making the affinity of the substrate molecule to the other binding sites more favorable or some times unfavorable. But when we talk of allosteric enzyme we are talking of a favorable interaction.

Multisite enzymes have identical, independent subunits. In such cases when all the catalytic sites on the enzyme molecule, even look at its different subunits, if they are identical and non-interacting or non-cooperative, they have no interaction among each other. The net result will be that you are handling something like if you consider that let us say one nano mole of an enzyme substrate concentration of n binding sites per mole will be equal to n pico mole of one binding site molecule. The rate profile will be hyperbolic in nature. The only difference is that the maximum reaction velocity term in that case will be $n.K_2.E_t$. That means maximum reaction velocity in the case of single binding site is

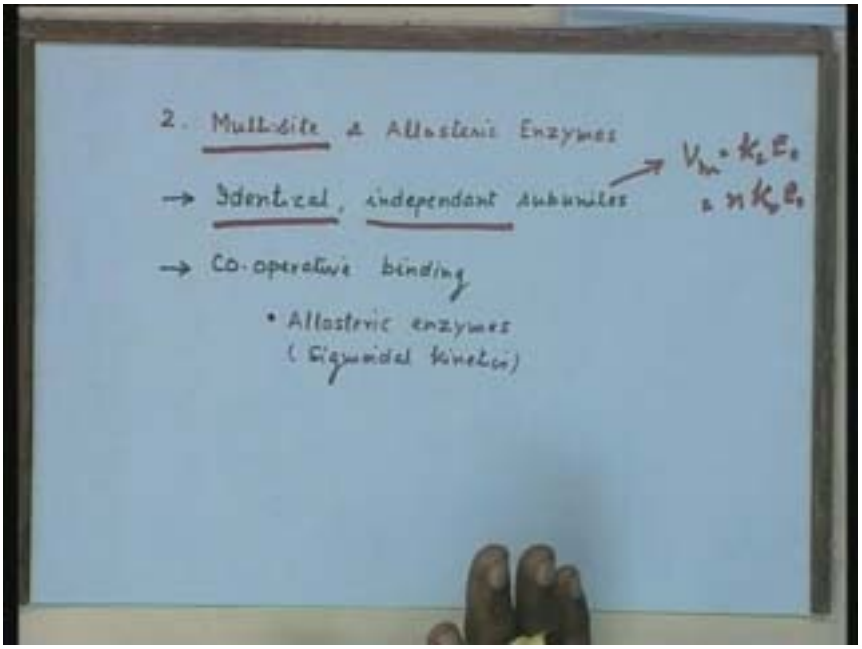
$$V_m = K_2 E_0$$

If there are n binding sites the only difference will be, it will become

$$V_m = n. K_2 E_0$$

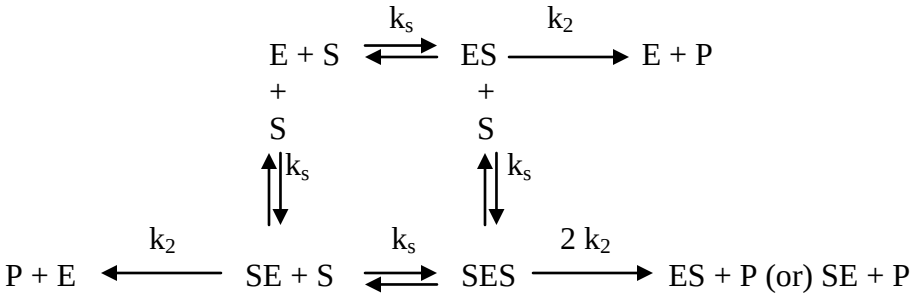
because all the n binding sites have to be saturated.

[Refer Slide Time: 15:12]



Except for that difference there will be no difference and the reaction profile will be hyperbolic in nature and one can examine such a situation in the case of a variety of identical independent subunits.

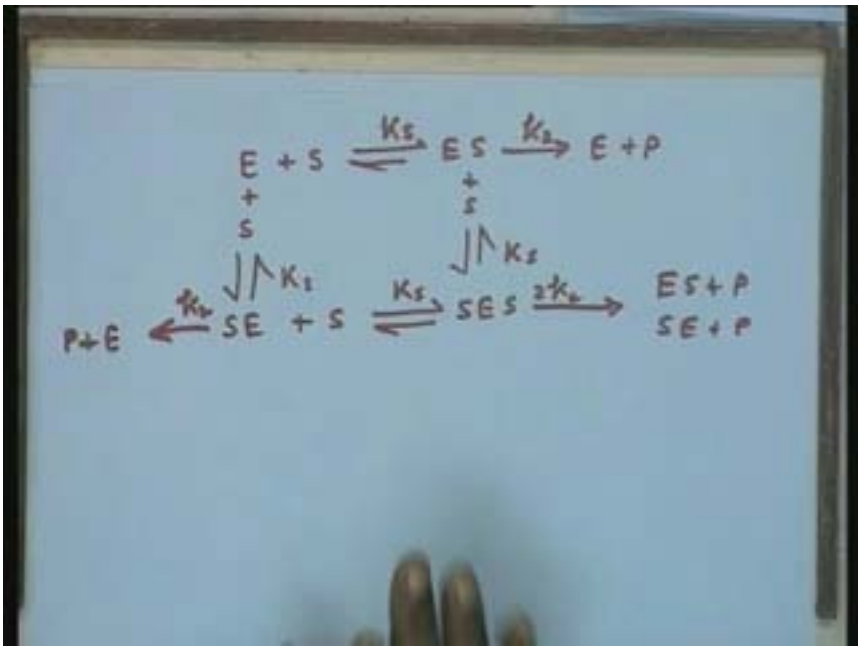
Just to illustrate my point with the simple example of a dimer having two identical binding sites, the reaction mechanism can be written as



In all the four cases of equilibrium, dissociation constant is k_s which are identical. That means all the binding sites are equal. They don't influence the binding to other site. The first binding site is occupied, the second binding site is free and depending on substrate concentration it can bind and form a binary complex where the two substrate molecules are bind that means both the binding sites. The only difference will be when SES gives you a product and the rate of product formation will be $2k_2$ because it will lead to ES + P

and SE + P. Similarly here also you can have a product formation to P + E. You can have from either direction the product formation and they are independent.

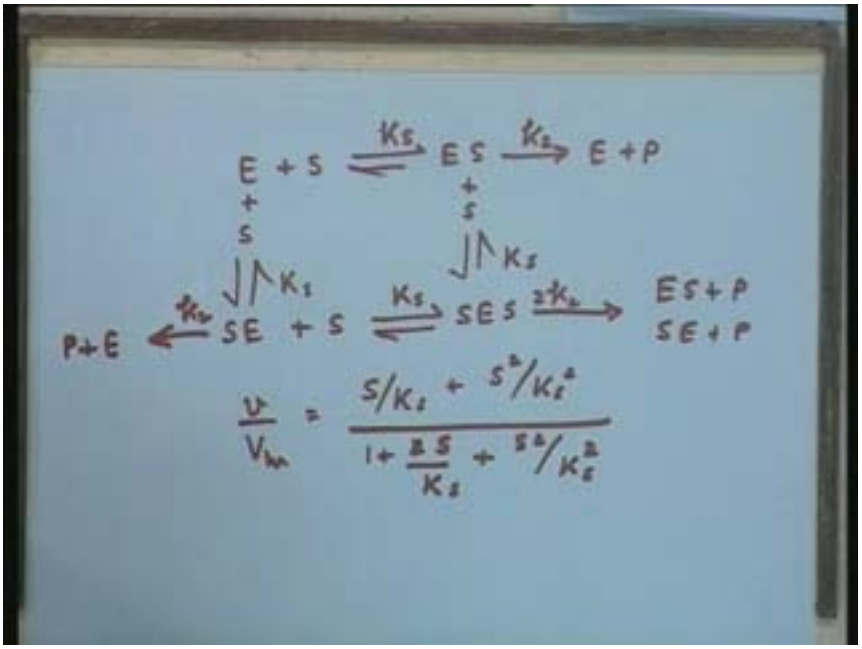
[Refer Slide Time: 17:06]



Similar situation might arise for a tetramer or a hexamer depending on the number of binding sites on the enzyme molecule. For such a simple case the rate expression can be written as

$$V/V_m = \frac{S/k_s + S^2/k_s^2}{1 + 2S/K_s + S^2/k_s^2}$$

[Refer Slide Time: 17:37]

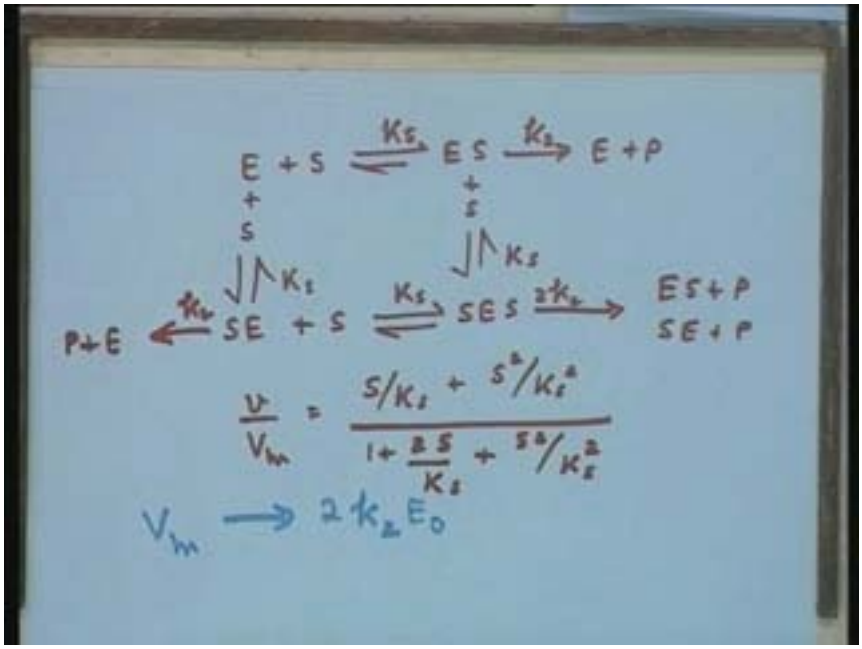


Consider that the enzyme molecule has binding sites on both the sides, S and ES. The only difference is S represents one active site and ES represents the other binding site that is occupied. That means that whether it binds on left hand site or right hand site the binding of the next substrate on to the vacant site has no effect. Both the binding sites can dissociate independently and lead to formation of a single enzyme substrate complex plus product. That means all the seats in this class are totally vacant. Anybody who gets into the room can occupy any of the seats and as many numbers of students can come in as long as they can occupy the number of seats available. Only difference you must notice is V_m term will correspond to

$$V_m = 2k_2.E_0$$

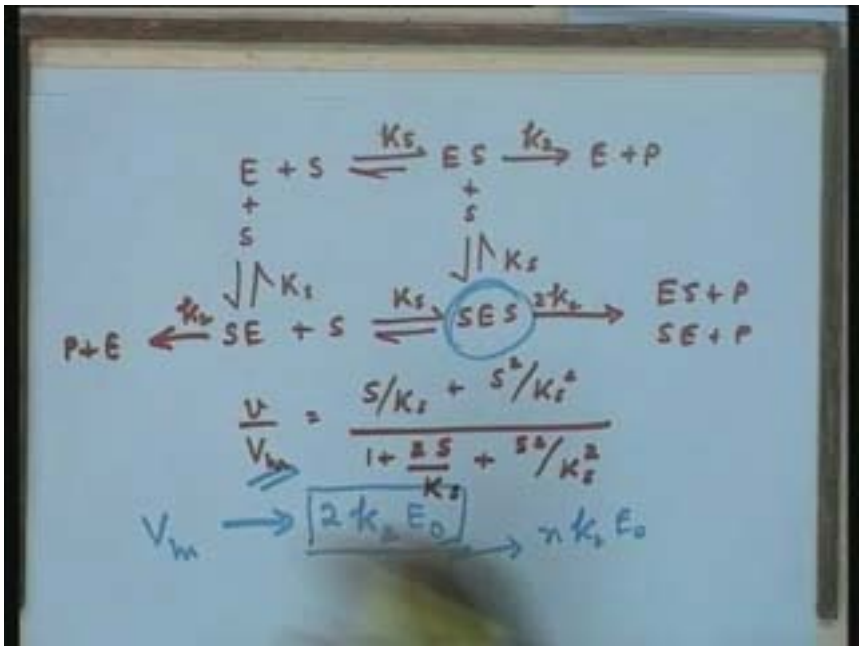
rather than E_s.

[Refer Slide Time: 19:01]



Eventually it will lead to that. If suppose the entire enzyme SES is in this form, both these sites are totally saturated because ES and SE also will lead to the product formation. The net effect will be that SES is giving you two products and the free enzyme. The only difference is here the definition of V_m must be taken as $2K_2.E_0$ or in another words if it is a multimeric protein or enzyme where n binding sites are there, it can become $n.K_2.E_0$

[Refer Slide Time: 19:46]



Otherwise the treatment can be identical as in the case of a hyperbolic profile. This in general you can write as

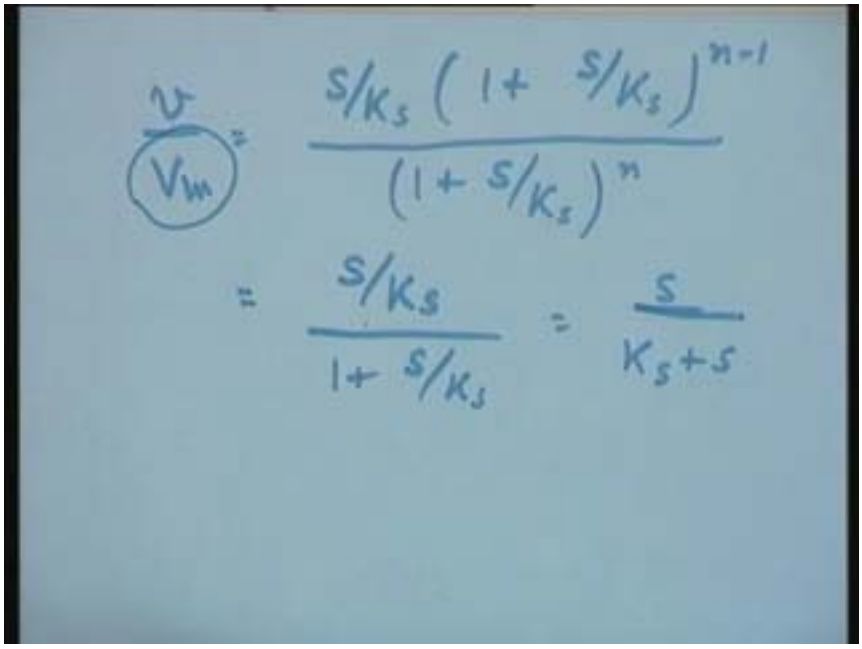
$$V/V_m = \frac{S/K_s(1+S/K_s)^{n-1}}{(1+S/K_s)^n}$$

If you just extrapolate the number of binding sites from two to four to six to n, these are the kind of rate expression you end up. This is equal to

$$V/V_m = \frac{S/K_s}{(1+S/K_s)} = S/K_s + S$$

which is same as hyperbolic rate expression. When there is n number of binding sites the only difference is that the definition of V_m will change.

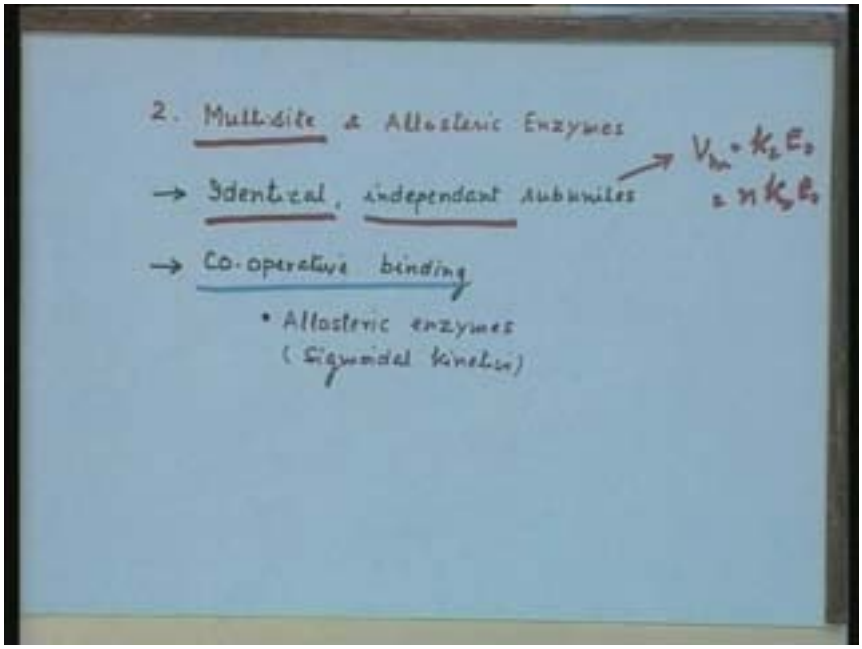
[Refer Slide Time: 20:51]



Rest of the rate profile will remain identical and there will be no difference.

In the second and more complex case when we talk of co-operative binding if you consider two binding sites on enzyme molecule they may be identical.

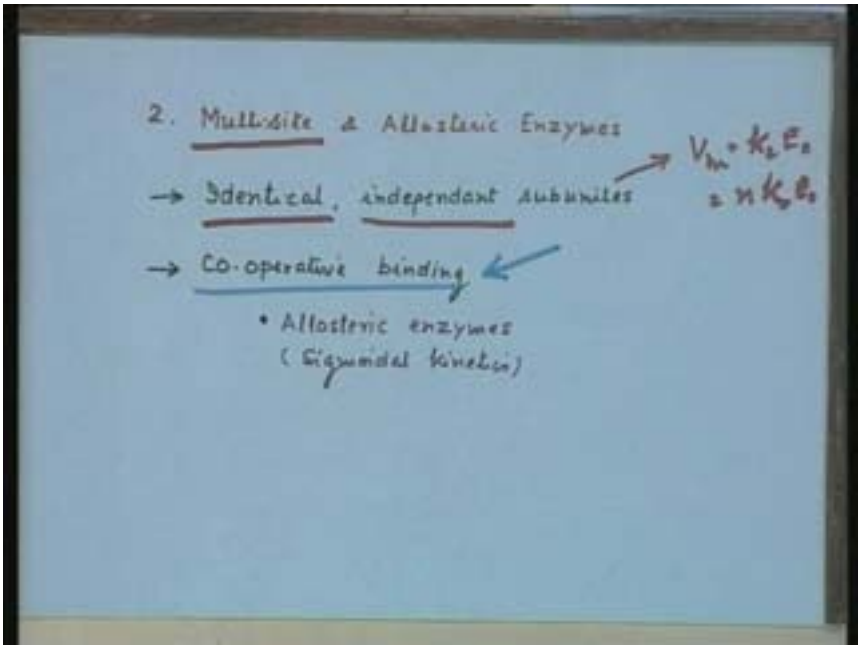
[Refer Slide Time: 21:14]



That means the free energy change for binding process may be the same. That is what we mean by identical binding sites. That means to start with the substrate molecule can hook up to any of them irrespective of the choice but after it gets bound it makes a conformational change on the enzyme molecule. That means it influences the confirmation of the other binding sites therefore altering its affinity.

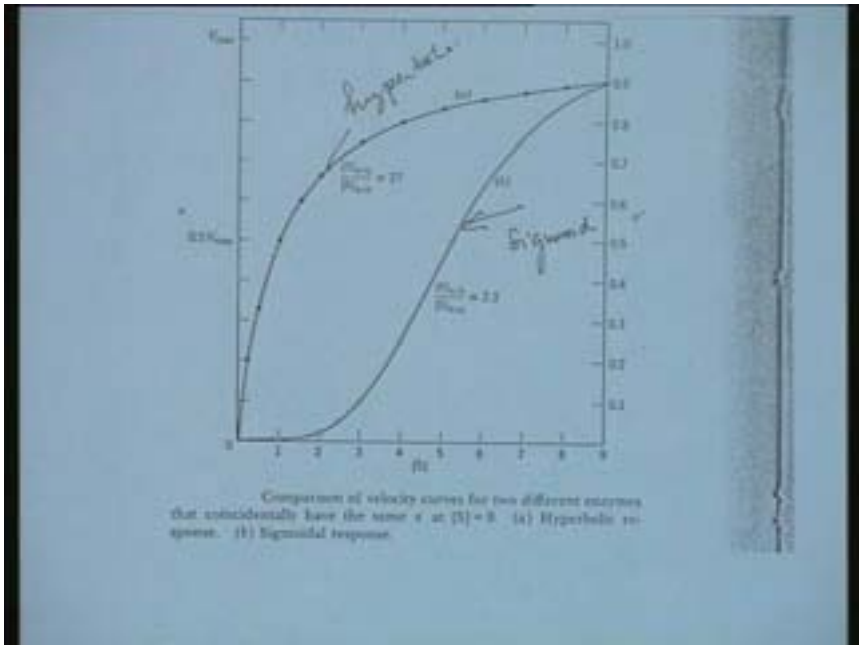
There are number of mechanistic hypothesis which have been proved. We will come to that. The first binding site is free for all the substrate molecule and once this is bound if it makes a change on the confirmation of the other binding sites such that the affinity of the substrate to the binding site gets altered, if you want a positive favorable effect, the affinity must increase or the K_m value must decrease, dissociation constant must decrease. If the k_m value of the subsequent binding sites decreases with the binding of the first substrate molecule we call it co-operative binding.

[Refer Slide Time: 22:49]



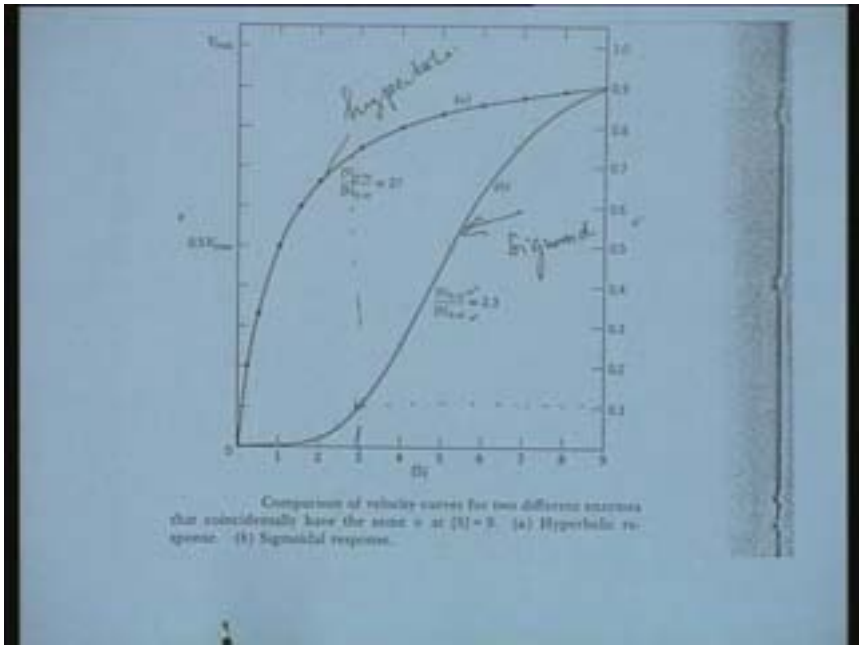
The significance of co- operative binding lies in actual practice in the case of allosteric enzymes. All of you know probably with your background in the biochemical metabolism that with each metabolic path way you have certain regulatory enzymes which behave in allosteric manner. By allosteric manner I mean that substrate binding process in case of those enzymes is co-operative in nature and they do not exhibit the typical hyperbolic kinetics rather they exhibit sigmoidal kinetics. If you just compare the two kinds of profiles, this is a sigmoidal profile and this is the hyperbolic profile.

[Refer Slide Time: 24:07]



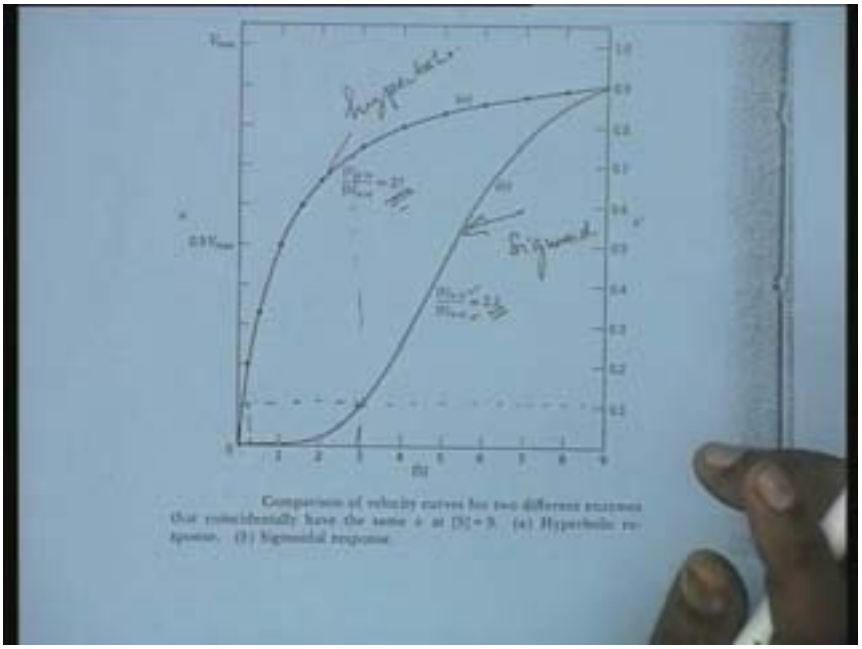
For two different enzymes which are coincidentally having the same velocity at substrate concentration let us say nine arbitrary units in both the cases whether it follows a hyperbolic kinetics or sigmoidal kinetics the enzyme velocity reaches the same value at a particular substrate concentration. Only initial phase changes and the first one is typical hyperbolic profile, the second one is the sigmoidal profile which is exhibited by allosteric enzymes. The allosteric behavior has in some way an advantageous situation for the regulatory enzymes to function. The role of regulatory enzymes in a metabolic pathway is the control of metabolic pathway. That means it should be able to switch on and off the path way with a very subtle change in substrate concentration. These two profiles have identical reaction velocity at high substrate concentration. For the two enzymes to act, in the early phases when substrate concentration increases from zero to three arbitrary units whatever are the units, in the case of sigmoidal kinetics the reaction velocity is only 0.1 in some arbitrary units. Whereas in the case of a saturation kinetics or hyperbolic kinetics the reaction velocity can reach to almost 0.75, 75% of the saturation value, a very high level. That means at a very small change of concentration, the reaction velocity can be very high. On the other side if you take let us say two different substrate concentrations required for two different levels of activity to reach for example say between 0.75 and 0.1, that is substrate concentration for reaction velocity to reach 0.75 and 0.1.

[Refer Slide Time: 26:43]



In the case of saturation kinetics or hyperbolic kinetics the ratio of the concentration of substrates for the two levels to reach will be almost about twenty seven times. That means at 0.1, it can reach only at a very small substrate concentration here. The ratio is twenty seven fold. On the other hand in the case of sigmoidal kinetics this ratio is only 2.3.

[Refer Slide Time: 27:10]

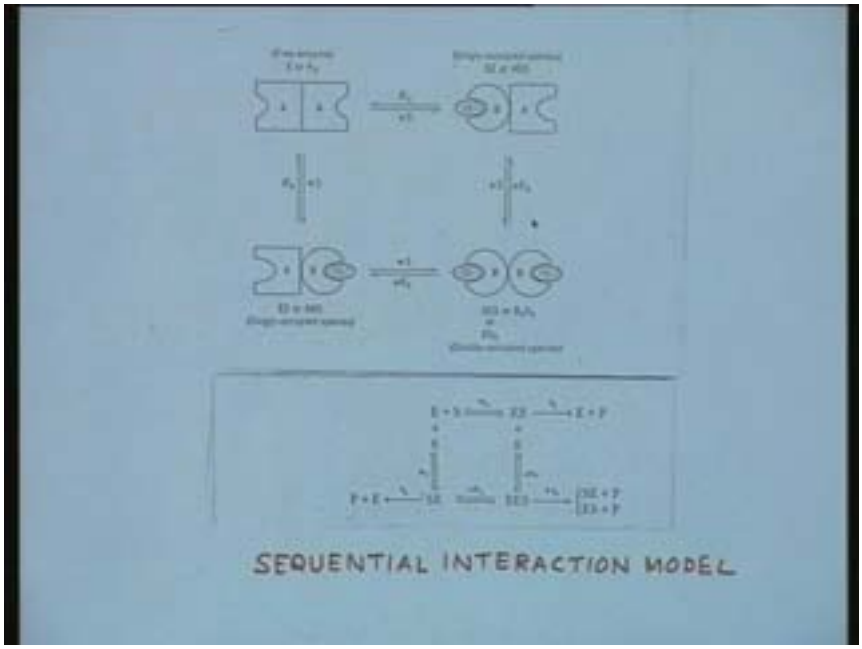


In the case of living cells the intercellular concentration of substrate does not change over a very large range. There will be hardly any situation where a substrate concentration can change twenty seven fold or such a high level of change. Whereas for control purposes it will require a very small concentration change by which the reaction velocity can be regulated and that is what can happen in the case of a sigmoidal kinetics. That means just by changing concentration ratio of two to three fold you will notice that the reaction velocity can be altered from 0.1 to 0.75 and that is the advantage with this kind of reaction behavior the regulatory enzymes obtained in the case of cellular metabolism

With this kind of a kinetic behavior the enzymes are able to act almost like an on and off switch. That means if the substrate concentration comes down reaction velocity is very slow; the metabolic pathway is slowed down; it is not made to zero. If the substrate concentration slightly increases the pathway is fully on and the product formation or whatever is corresponding intermediate in pathway are fully available and that advantage provides to the cellular metabolism.

Such a behavior of sigmoidal kinetics has been very appropriately explained at least by two mechanistic hypotheses. One and probably in its simplest form is on the basis of sequential interaction model. This is an extension of Koshland's induced fit hypothesis which we discussed when we were talking about the specificity and how the enzymes obtain their specific action.

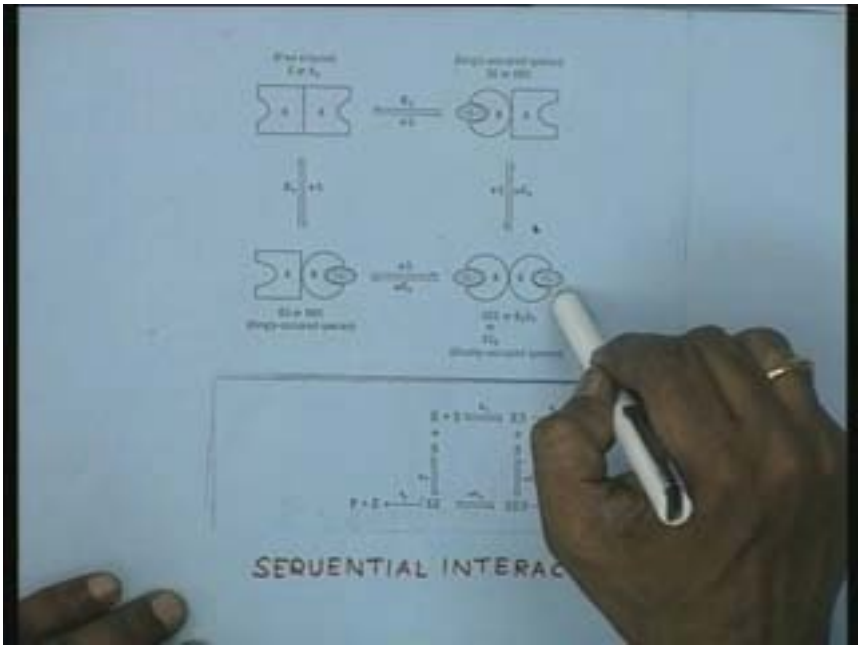
[Refer Slide Time: 29:33]



We talked about the Koshland's induced fit hypothesis whereby when the substrate molecule binds to one of the binding sites the enzyme undergo a conformational change so as to fit on to the substrate molecule. This is something like an extension to that and which means that when the substrate molecule binds on one of the binding sites of the enzyme it immediately triggers off a conformational change whereby the affinity for the next binding site becomes much higher and they expressed it with an interaction factor. For each binding of the substrate to a binding site the affinity reduces by a factor let us say α, as interaction factor.

That is what you see in a diagrammatic picture like here. If this is the free enzyme which has two binding sites, identical binding sites the substrate molecule binds first to let us say, A. It immediately brings in a conformational change on the second binding site whereby the dissociation constant reduces to αK_s. The conformational change leads to the dissociation constant or Michaelis Menten constant for the second binding site and then second binding site also gets bound.

[Refer Slide Time: 31:03]

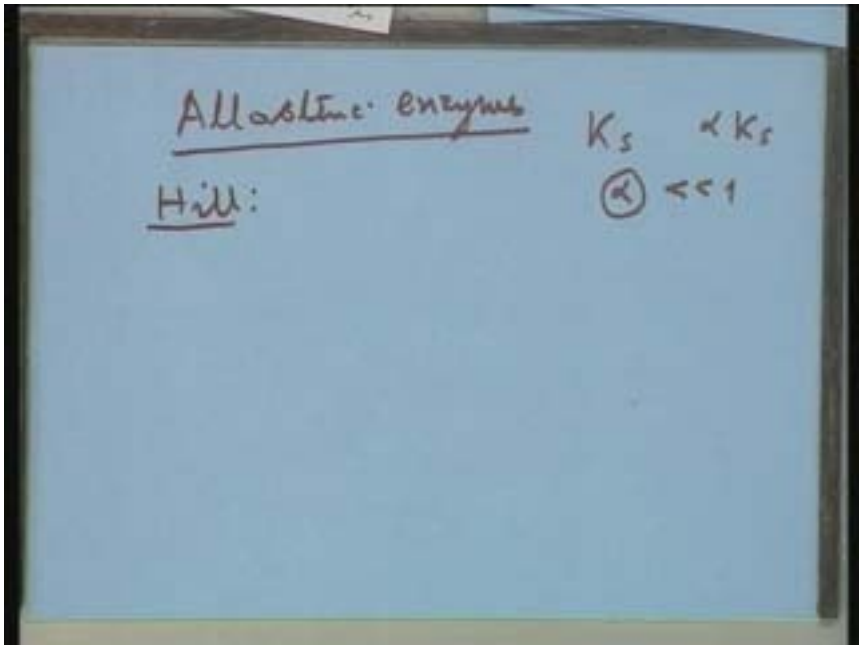


It is logical that if this dissociation constant is low the substrate will have much more affinity for the second binding site. Similarly if it initially binds with K_s on to other binding site it can influence the conformational change on the other binding site and it can meet. Both the binding sites are occupied and such a system can be represented by a mechanistic model whereby you have the first binding as K_s either here or here. The second binding process is represented by dissociation constant of αK_s either here or here. The product formation with ES will be E + P. Similarly when both the binding sites are occupied, the product formation rate will be twice the K_p because then it can go either to SE or ES, there is no difference.

In such a case you will ultimately end up with the kinetic equation or kinetic expression which will express the sigmoidal reaction velocity profile response. The situation which I have shown here is rather too simplistic. That means we have tried to explain with the two binding sites. In many cases particularly with the regulatory enzymes, the number of binding sites may be four or six or eight even tetramers are a very common example in the literature and therefore the rate expression will be quite complex and their analysis can become difficult.

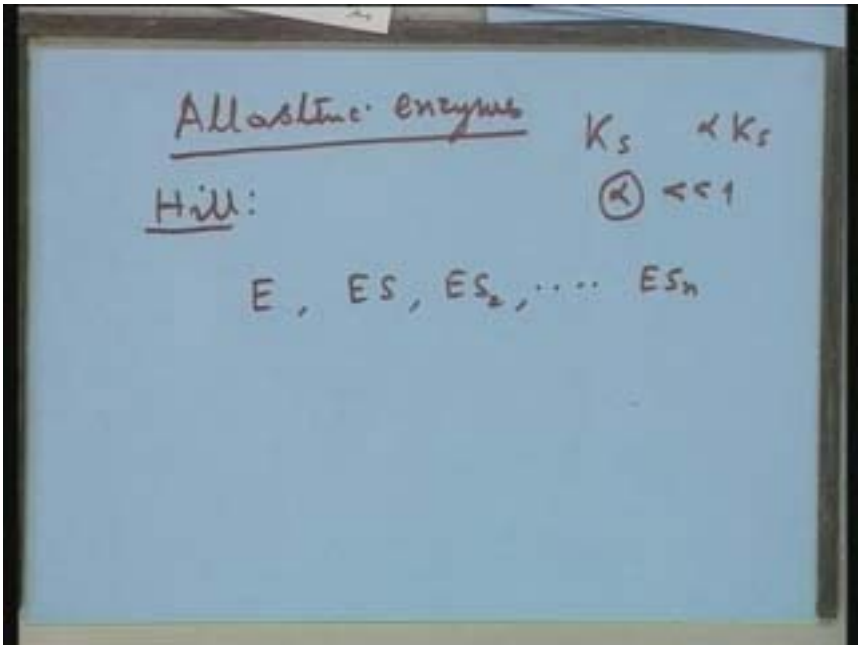
To simplify the situation there is an equation available corresponding to Michaelis Menten equation which can be applied to allosteric enzymes. In the case of allosteric enzymes we have a simplified rate equation which can express or which can describe the rate behavior of the allosteric enzymes. This was given by Hill. One of the basic assumptions here which we made is that the interaction factors, what I mentioned earlier as K_s and αK_s, the ratio alpha is very, very small than one. That means the co-operativity is very high.

[Refer Slide Time: 33:51]



Once a substrate molecule binds to one of the binding sites it makes a drastic conformational change so that the affinity is increased very significantly. If you consider n binding sites what will happen in such a situation is that with each binding site being occupied by the substrate, the next subsequent binding sites will have much more affinity. Ultimately at equilibrium or in steady state condition the net effect will be that the bulk of the enzyme will be in the form of ESS. Theoretically at equilibrium the enzyme must be available in the form of E, ES, ES_2, ……, ES_n.

[Refer Slide Time: 34:37]

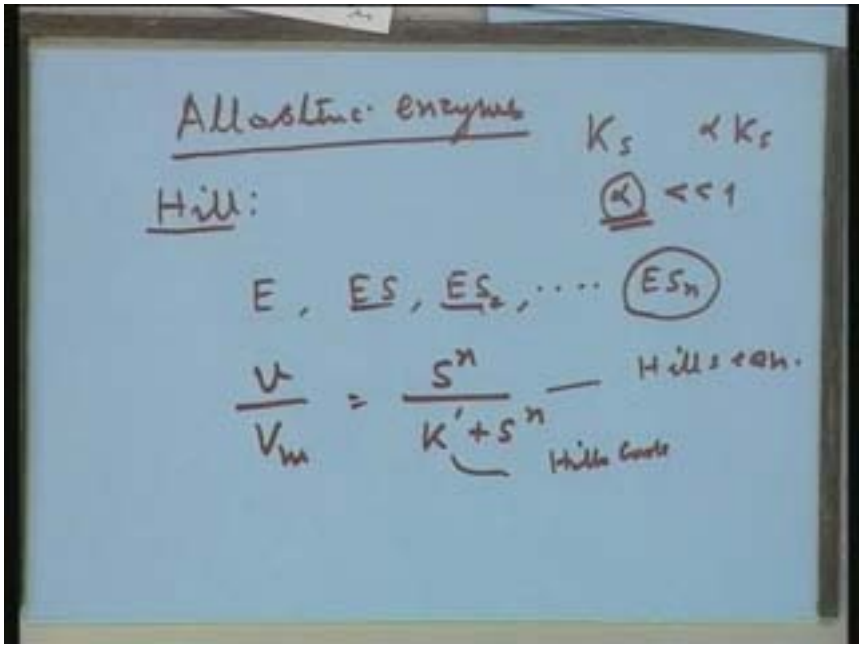


Normally if there are n binding sites and the binding process continues sequentially then the enzyme must be available in different species in difference forms as E, ES, ES_2, up to ES_n. But if the interaction factor alpha is very, very small, at each stage affinity will increase by a very significant level or what we understand as co-operativity is very high. In that case the assumption by Hill was that most of the enzyme will exist in the form of ES_n. There will be small concentrations but they can be neglected. Based on that assumption then he gave a rate expression which is

$$v/V_m = S^n/K' + S^n$$

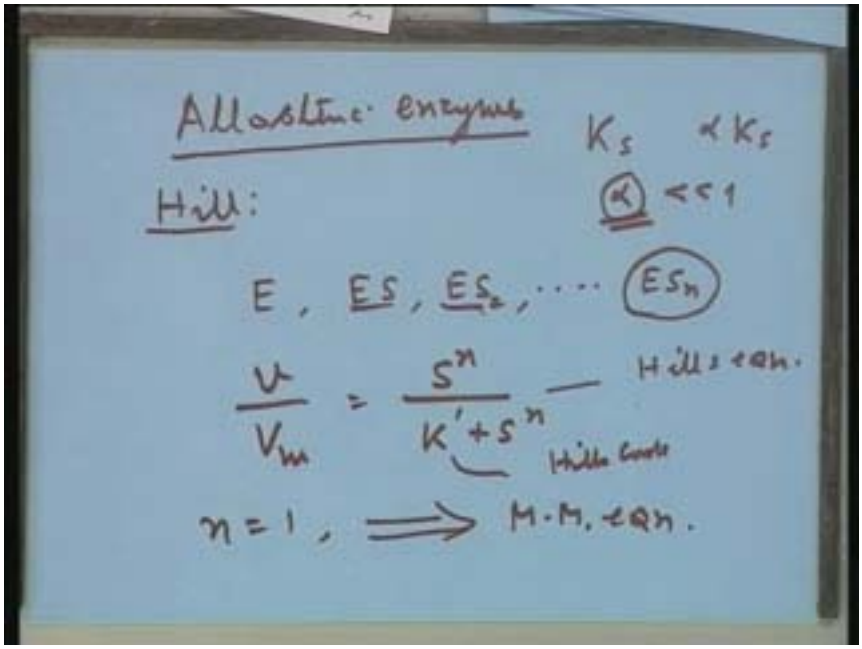
This is called Hills equation. Here n is the number of binding sites per enzyme molecule and K'is the Hill's constant. This K'is different than the Michaelis Menten constant.

[Refer Slide Time: 35:55]



Michaelis Menten constant is the substrate concentration at which reaction velocity is 50% of that of the maximum reaction velocity. That situation will not apply here, if you analyze. If you put n = 1, then the equation will get into Michaelis Menten kinetics.

[Refer Slide Time: 36:20]



V_m here is the maximum reaction velocity when all the binding sites are occupied by the substrate. The substrate concentration is high enough but again this is not identical to the V_m of the Michaelis Menten equation because V_m of the Michaelis Menten equation is an asymptotic value. You can never approach it practically. It is a theoretical value. Whereas in this case of V_m, you can approach to that value when all the enzyme binding sites are totally occupied by the substrate molecule. If you just take the same yardstick of explaining K' as we have taken in the case of Michaelis Menten equation,

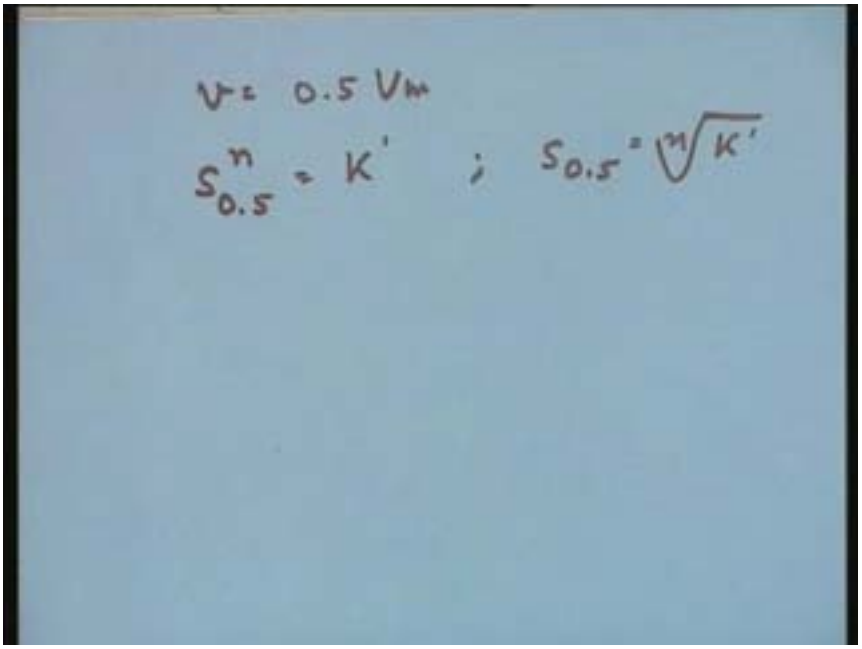
$$v = 0.5\ V_m$$

you will end up as

$$S^n_{0.5} = K'$$

in the case of Hill's equation. Or $S_{0.5}$ substrate concentration for 50% maximum reaction velocity is $n\sqrt{K'}$.

[Refer Slide Time: 37:59]

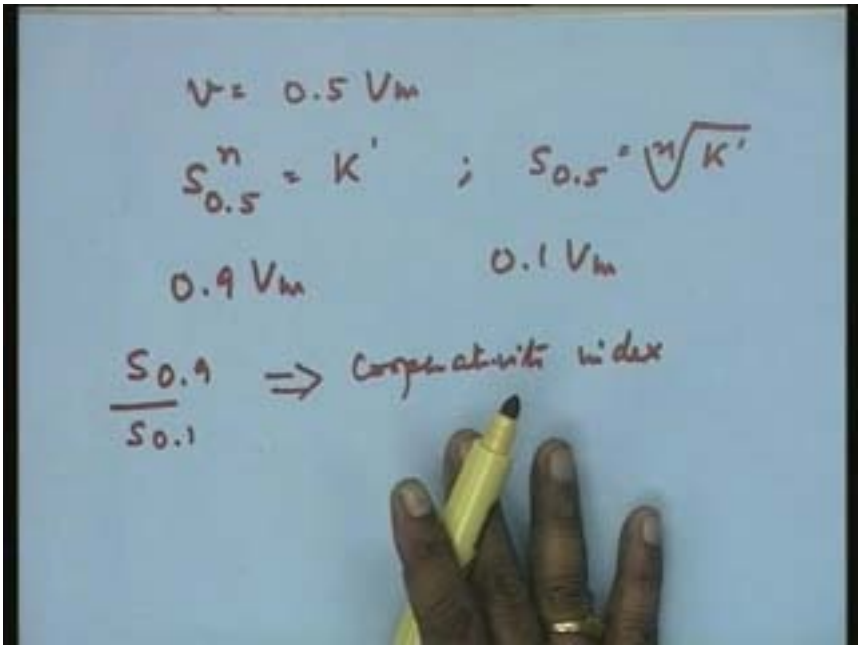


It was half of the maximum reaction velocity their. So that brings in a distinction between the two parameters that we are talking about.

The level of σ means the level of co-operativity for different enzymes as to how much it deviates from the hyperbolic performance or how much more efficient it is in terms of regulatory behavior. When we talked about the ratio of two substrate concentration for two levels of reaction velocity the smaller it is, it is a better regulatory system. Very sharp change in substrate concentration can bring in the regulation. Similarly one can express the co-operativity index of any given allosteric enzyme by expressing it in terms of the ratio of substrate concentration for achieving two different levels of reaction velocity.

As conventional practice we take the two reaction velocity as 0.9 V_m and 0.1 V_m. That means 10% and 90 % and the ratio of the two, $S_{0.9}/S_{0.1}$ is conventionally called as co-operativity index. This is just a conventional practice. You can define co-operativity index as long as you mention; if it is $S_{0.75}/S_{0.1}$ or $S_{0.9}/S_{0.1}$, or it could be any ratio. It can be used to express co-operativity index.

[Refer Slide Time: 40:00]



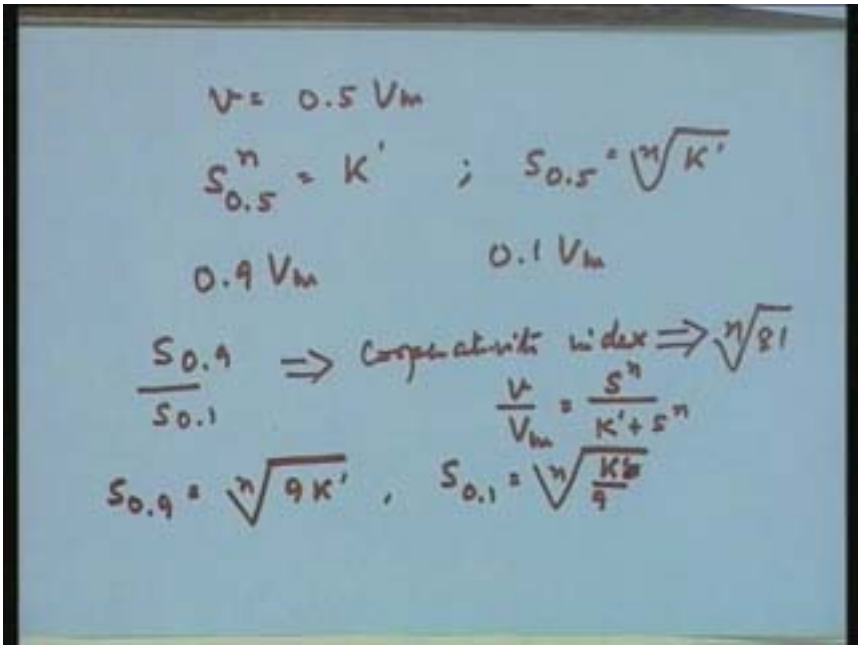
It is only a matter of convention that we are following the ratio of 90% and 10% values. If you just get the value of $S_{0.9}$ and $S_{0.1}$ you know original hills equation

$$v/V_m = S^n/K' + S^n$$

$$S_{0.9} = n\sqrt{9}\ K', \quad S_{0.1} = n\sqrt{K'/9}$$

and the co-operativity index will be $n\sqrt{81}$.

[Refer Slide Time: 41:05]

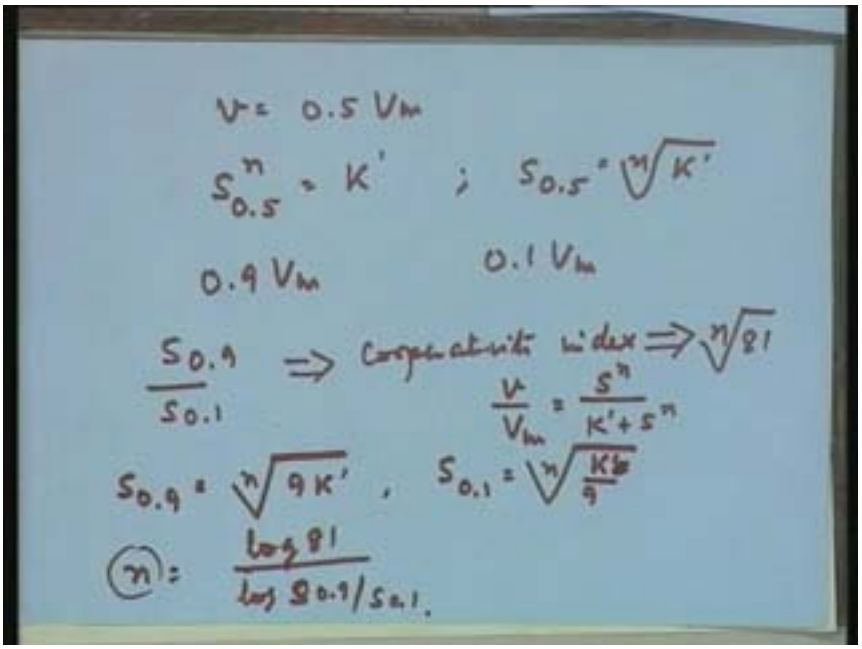


Or in another words n will be equal

$$n = \log 81 / \log S_{0.9}/S_{0.1}$$

Therefore n is the number of binding sites.

[Refer Slide Time: 41:24]



If one can determine the value of $S_{0.9}/S_{0.1}$ratio, one can determine the number of binding sites. Number of binding sites, n theoretically should be an integer. Number of binding sites will be one, four, two, three, five any integer. But practically if you notice you will never get an integer value. You will get a fraction say 3.4 or 3.6. The reason is the basic assumption in the hills equation is that the enzyme at equilibrium will be in the form of ES_n whereas there will be small concentration of all other enzyme species. That means with a partly substrate bound form and so therefore the final value of n will be in fraction. As a matter of conventional practice if we get let us say 3.6, we consider the next integer that is binding sites are four because the number of binding sites if we get less is primarily because of the other not completely saturated enzyme molecule.

If you want to plot this expression you get

$$v/V_m = S^n/K' + S^n$$

You can simplify this to get

$$n\log S + \log (V_m - v)/v = \log K'$$

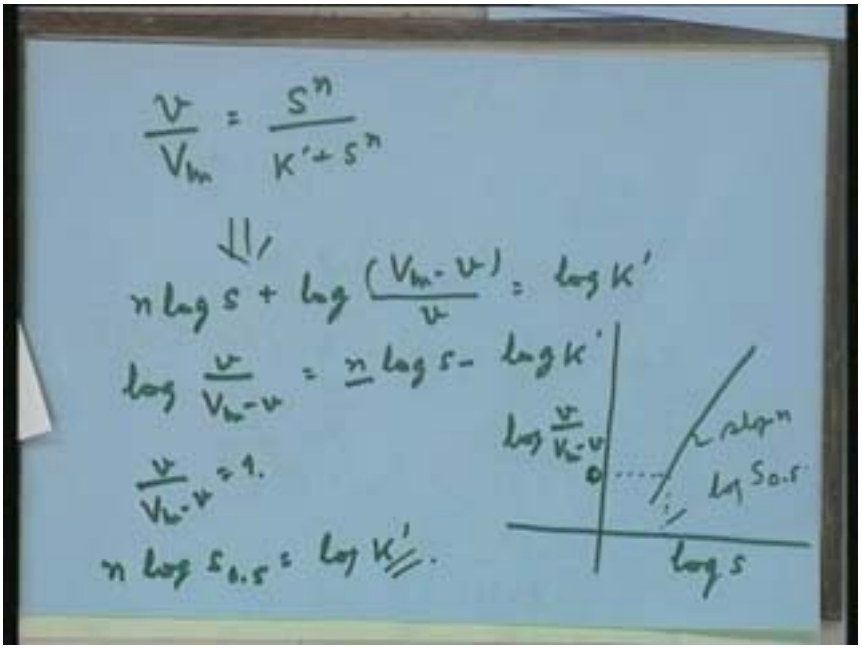
$$\log v/(V_m - v) = n\log S - \log K'$$

If you make a log log plot of log S verses log v/V_m-v, you must end up in a straight line with the slope of n and on log log plot you will straight get a slope and get the number of binding sites. At a value of log S if you put v/ V_m-v as one, the log format will be zero. I am putting v/V_m-v as one and at that value the corresponding value of log S will be log $S_{0.5}$. That means if you recall the log $S_{0.5}$ what we deduced earlier from our equation was

$$n\log S_{0.5} = \log K'$$

If you know the slope and determine the value of n and also determine log $S_{0.5}$ that means substrate concentration for 50% of maximum reaction velocity you can get the value of K' what is known commonly as Hill's constant.

[Refer Slide Time: 45:23]



So that gives you a neat analytical procedure to define your kinetic behavior of the allosteric enzymes that are available in the living systems mostly for regulatory purposes.