ENZYME SCIENCE AND ENGINEERING

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LECTURE – 11

EFFECT OF pH AND TEMPERATURE ON ENZYME CATALYSED REACTIONS

So far we have seen the influence of substrate concentration, inhibitor concentration or activator concentration on the rate of enzyme catalyzed reaction and they very strongly influence the catalytic behavior. Besides the substrates, inhibitors or activators two major environmental parameters which form or which constitute a bulk environment for the enzyme catalyzed reactions are pH and temperature. Today we will be looking at these environmental parameters and how they influence the whole enzyme catalyzed reactions.

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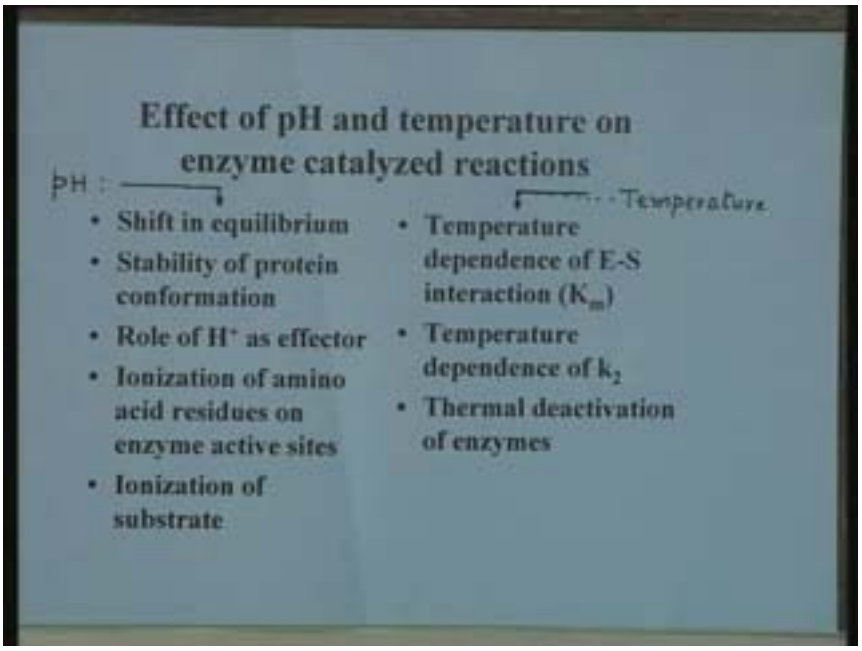

As you know enzymes are primarily very complex molecules and we had also looked into the complexity of the situation as regards to their catalyses, as regards to their specificity. The whole effect of pH and temperature therefore is not based on a single interaction like

in the case of substrate or an inhibitor which is usually their effect on the reaction rate and the formation of dynamic complexes with the enzyme.

In the case of pH and temperature the effects are multidimensional. If you look at the pH effects you will notice that the pH influences enzyme reaction rates or enzyme reaction in general in a number of ways.

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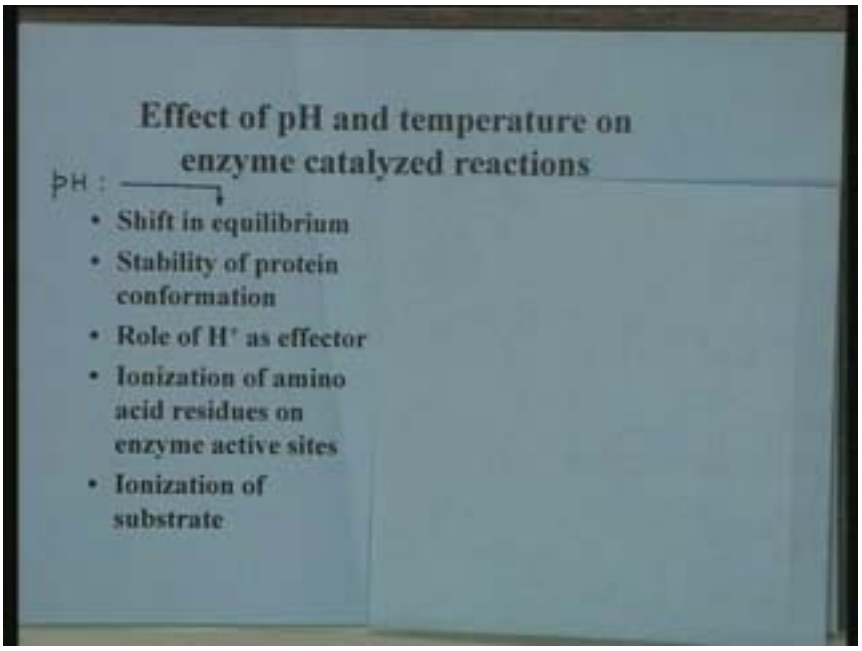

The first and probably the most common feature could be the shift in equilibrium. A number of enzyme catalyzed reactions that are reversible in nature primarily can be affected strongly by the pH, particularly the reaction like oxidoreductases. If you take for example ethanol oxidization by alcohol dehydrogenase, forming acetaldehyde plus NADH plus H. If you consider such a reaction you will notice that the concentration of hydrogen ions at any given pH can be regulated by the pH of the environment. By taking a buffered reaction mixture at a higher pH the concentration of hydrogen ions in the reaction mixture can be maintained at a very low level and the reaction can be driven in the forward direction. Similarly if you reduce the concentration or the pH of the buffer in which the reaction takes place you can also bring down the equilibrium constant of the reaction.

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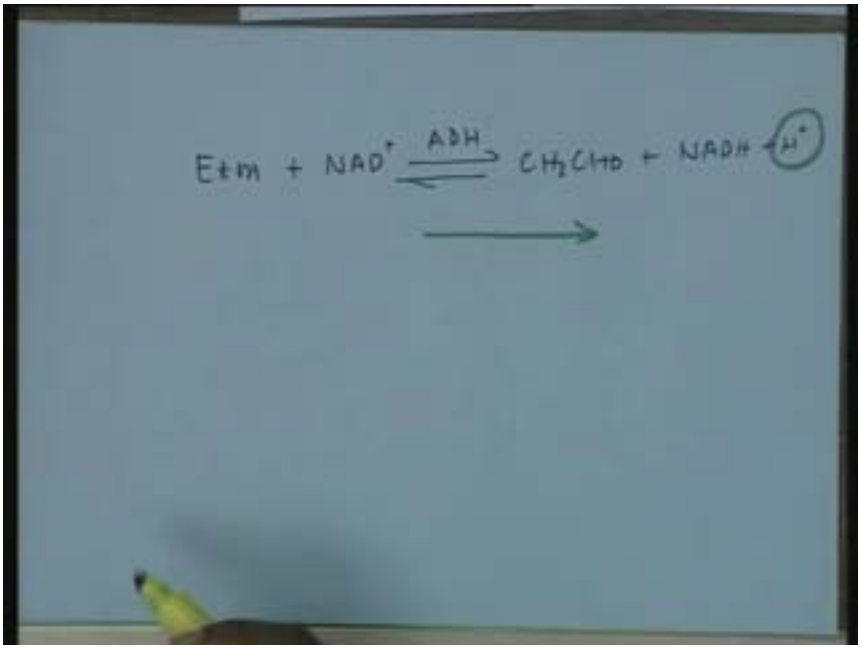

This shift in equilibrium although not directly related in any way must be considered in conjunction with the rate of the reaction. If you consider this reaction although this reaction may have an optimum pH of, say for example, seven that means the rate of reaction is maximum at pH seven. But at pH seven, the reaction may not be driven very largely to the forward direction. The reaction may stop as soon as some concentration of the product and hydrogen ions are built up. To be able to drive the reaction in the forward direction you need to adjust the pH so that the concentration of hydrogen ions can be reduced and therefore the reaction can be driven. But the reaction might proceed at a very slow pace. For example if you consider let us say pH nine. Just alter the pH value. At pH nine the concentration of hydrogen ions will be hundred fold less, one hundredth of at pH seven and therefore you can drive the reaction as far as the equilibrium constant is concerned but at a very slow rate.

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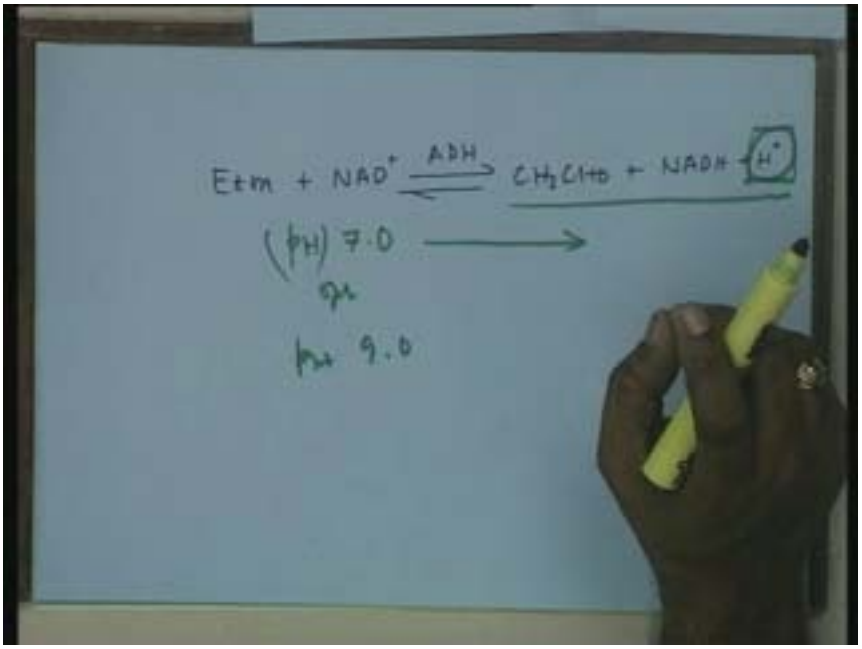

The shift in the equilibrium must be considered in terms of the rate. So pH optimum or the equilibrium constant they are two issues which are to be considered independently.

The second issue which is important in the case of pH effects is the stability of the protein confirmation.

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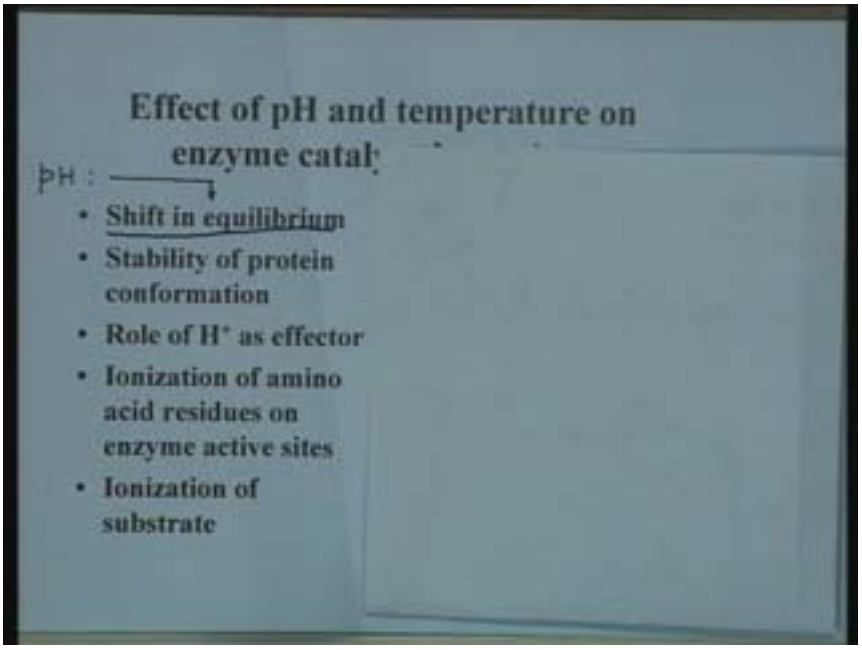

As we all know that proteins retain their native confirmation within a limited pH range, because electrostatic interactions, which are a major force, non-covalent force are

involved in the confirmation of the enzyme molecule. So within a limited pH range at which the right kind of amino acid residues are ionized the native confirmation of the protein is maintained. If you shift that pH very often gross alterations occur often called as denaturation. That means if there is an irreversible change in the confirmation then the enzyme is considered to have been deactivated.

We assumed the stability behaviour of enzymes. In earlier stabilization studies we saw that a two stage model is the most accepted model for enzyme which consists of two steps, native protein going into an unfolded form and then irreversible inactivation to inactive enzyme. While the first step is reversible, the second step is irreversible and always the approach to stabilize and a similar effect is noted even for the pH.

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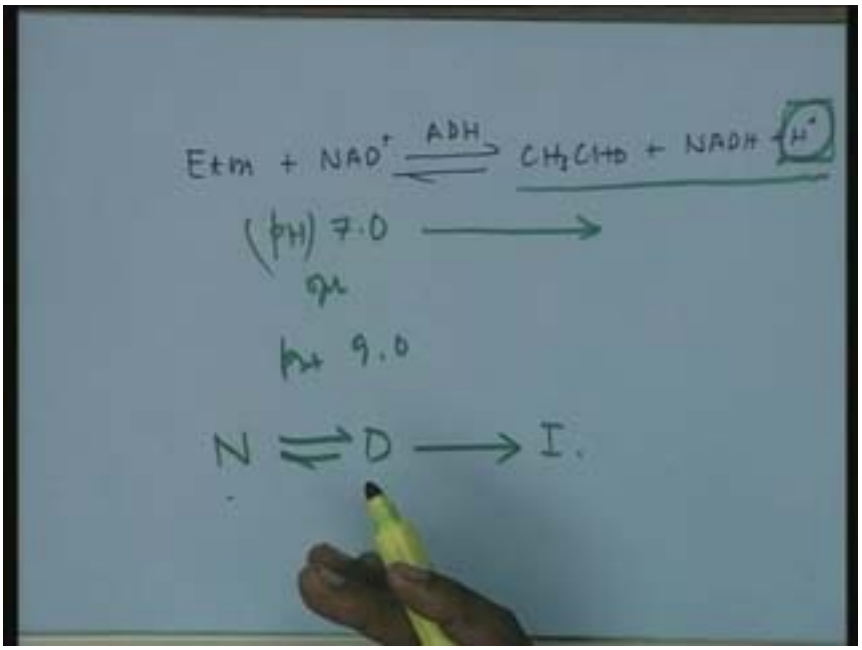

Initially if the pH alteration is mild if you go beyond a certain level of pH change you might observe only reversible change and therefore the enzyme can be brought back to a native confirmation but if you further change the pH it might undergo to a irreversible inactivation and which may not be possible to bring back.

The third role is in which the hydrogen ions play a role for either inhibitor or an activator. Wherever we are considering the involvement of ionisable substrates or the products that are formed which are ionisable, the hydrogen ions will play the role of an inhibitor or an activator. The fourth role of pH and probably one of the most important role which pH plays and which directly influences the rate of reaction is the ionization of the amino acid residues on enzyme active sites.

You know that on the enzyme active sites a number of amino acid residues are present which are charged and these charged residues will ionize as a function of pH of the bulk

environment and therefore it is required that they are at a particular ionization state in which the enzyme offers the maximum reaction velocity. The effect of pH is linked to the ionization state of the enzyme active site. That means if there is a particular state of ionization of the active site of the amino acid involved, whatever fraction of the enzymes present in that fraction will constitute the catalytically functional enzyme. Rest of the enzyme concentration will not be useful. This ionization state will affect the maximum reaction velocity of the enzyme because ultimately you are affecting the total effective enzyme concentration which is in the true ionisable state of the system.

The last effect is due to ionization of substrate.

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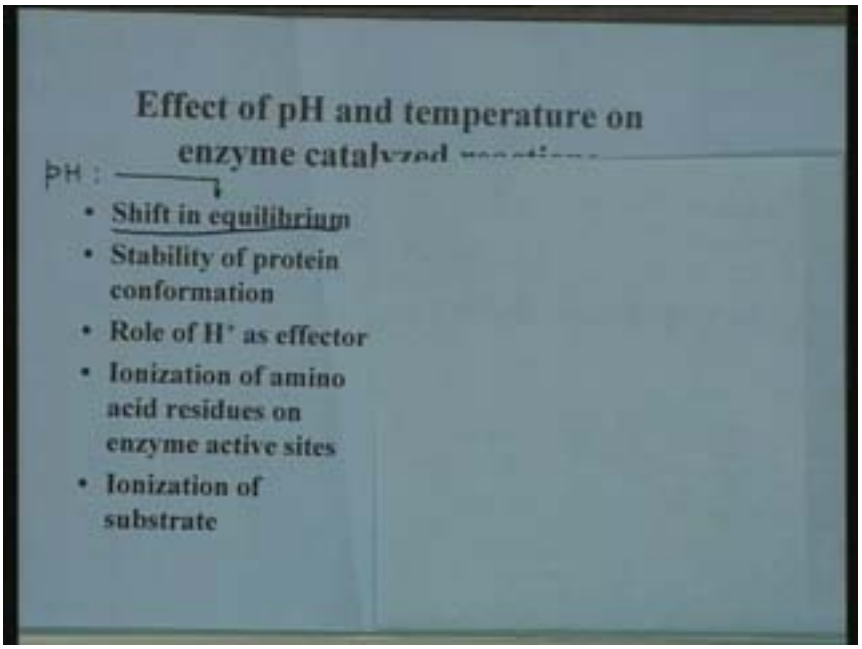

Although many of the substrates which are used in the enzyme reactions may be neutral, like ethanol or any other non-ionisable substrate, many of them also contain carboxylic group or hydroxyl group or amino group. The substrate will also ionize and depending upon the true ionization state of the substrate which is accepted by the enzyme at the binding site, the fraction of the substrate which will get bound will determine the pH effects. The ionization of substrate will influence the K_m value of the enzyme. The K_m value will be altered because the apparent concentration of substrate which we are providing is not the true substrate concentration which is interacting with the enzyme and it may be lesser than that because the ionized state which is catalytically acceptable to enzyme is less than that. Therefore the pH effects must be looked into all these parameters or all these effects in a comprehensive manner.

Practically it may be difficult to consider all these effects in one go because sometimes they may be counteracting and so the net effect of what we notice is a very complex phenomenon, all over. To understand the behavior and to derive certain meaningful

conclusions one may create or design experiments in which only one of the pH effect is considered prominent and in fact the pH effect studies have contributed a lot to understand the identification of the amino acid residues in the active site in conjunction with the chemical modification studies. Common technique to study the amino acid residues or identify the amino acid residues at the enzyme active site is by chemical modification of the different function of the groups that are present. In addition to that their effect on the ionization behavior and ultimately the rate effect and putting both the things together can give you a confirmatory result or conclusion on the identification of the amino acid residues on the reaction rate.

We will consider today mainly the ionization of amino acid residues on the active site which is primarily a major factor which influences the typical reaction profile that you are familiar with of the effect of pH on the enzyme.

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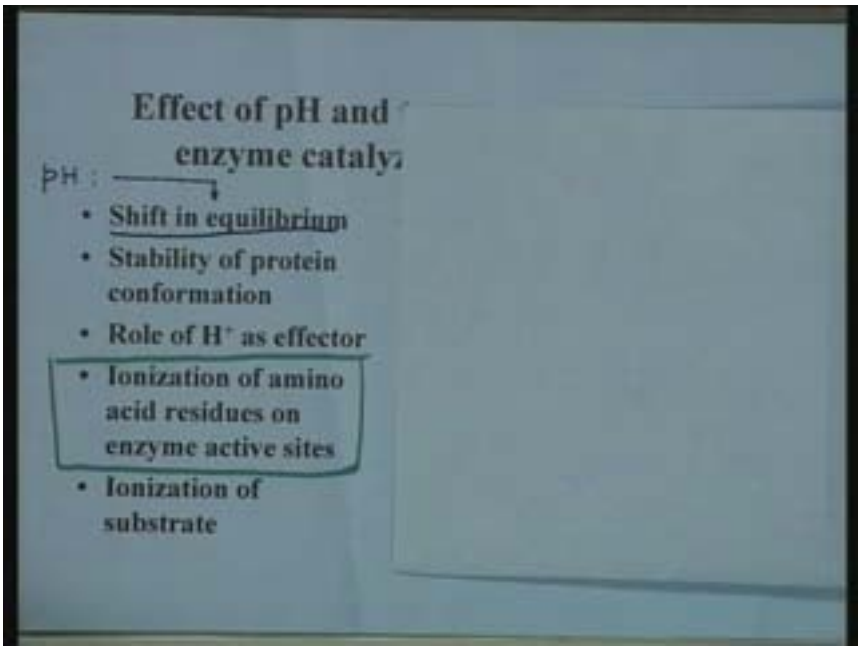

You often end up getting a variety of profiles when we study pH. When we study the enzyme reaction rate as the function of pH we get a variety of profiles.

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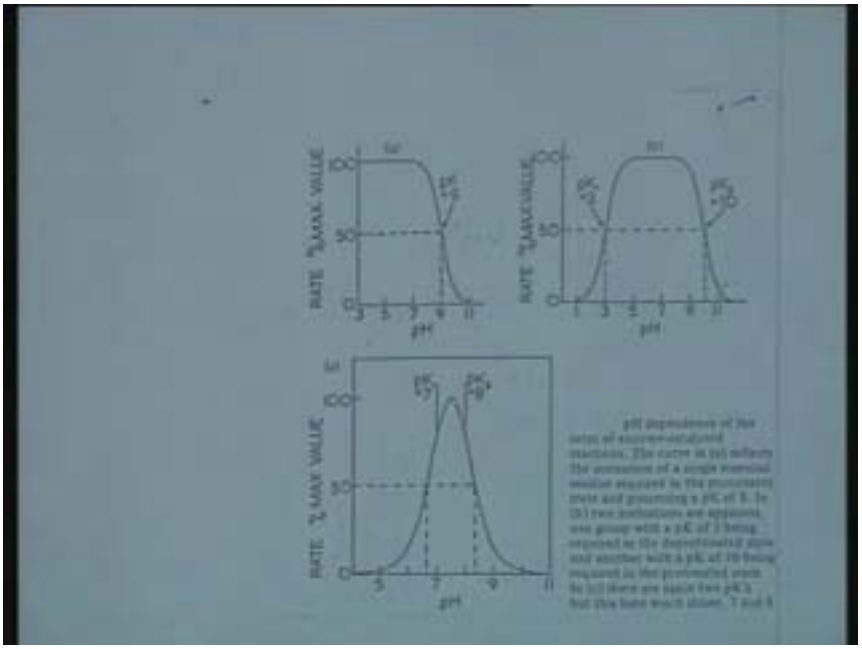

For example in the first case in the diagram, in part a you notice that, the effect of pH is shown against the percentage rate of the maximum value. Maximum value means the optimum pH, the optimum or the maximum at which it shows and with a single ionisable residue on the active site. When there is only one ionisable residue with the pK_a value of nine initially at a lower pH value the enzyme activity remains constant and when you go to higher pH value the rate falls and subsequently at an extreme value it comes down to zero. We will determine the pKa values and their role.

In the second case you notice that it may be a much broad based pH profile. A profile like this shows maximum value over a pH range, not a very broad range but at least in one or two units. This reflects probably the presence of two ionisable amino acid residues, one acidic and one basic on the enzyme active site and the profile is like this. Of the two amino acid residues one has pK_a three and one has pK_a ten, just a typical profile which you get. Most commonly you may end up getting a profile of a very sharp maximum and we are familiar with the typical terminology called bell shaped curve. You have very sharp pH optima which is considered at the peak of this.

Such a situation is a special case of this broad profile in the sense that here the pK_a value of the two ionisable groups are distinct apart, one at three and another at ten.

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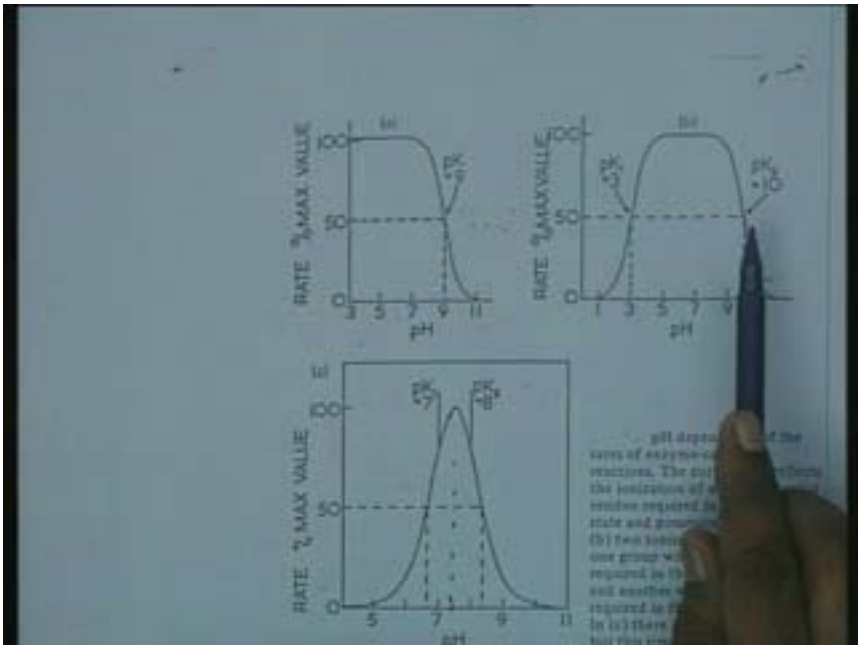

So their ionization doesn't really influence each other so much. One of the amino acid is ionizing and the other is not in a very highly ionised state and therefore you got a broad pH. But in case the pK_a value of the two ionisable groups are close enough that means at a particular pH, the ionization of one amino acid residue does also ionize, to different extent, the other amino acid residue. In that case they will tend to look as if both the ionizations result are merged and you get a sharp profile because here the pK_a values of the two amino acid residues are seven and eight which are pretty close. So that is a kind of scenario you get. It is a comparatively simplistic situation.

In practice in many of the enzymes for practical applications, you might end up with a profile like this which consists of not ionization of one or two amino acid residues but more than two, may be five, may be seven and may be six.

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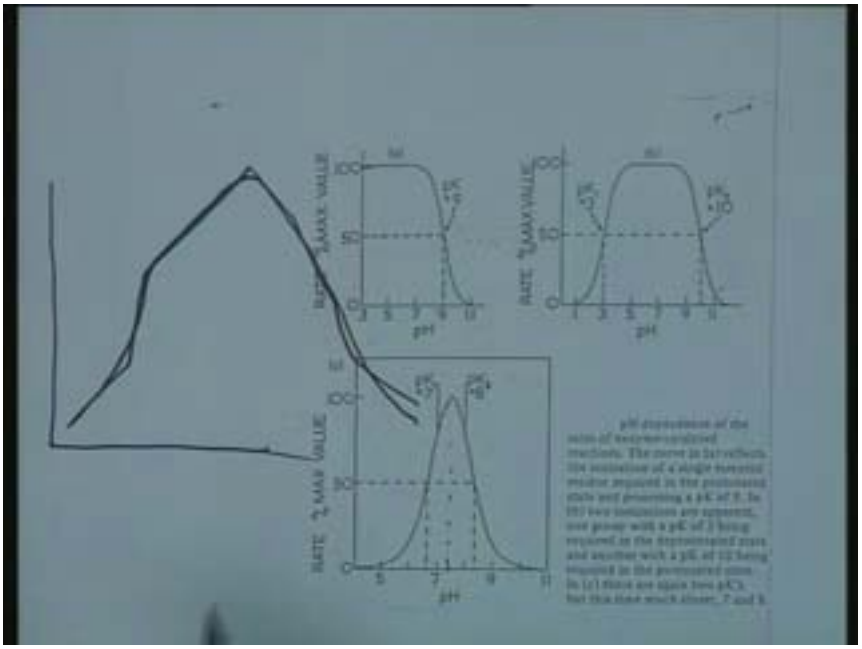

The net pH profile may be a sum total of all the effects and they can be broken down into linear segments and each linear segment will represent the ionization of one of the amino acid residues. The effect of pH on the reaction velocity must be considered as a parameter on the rate of reaction.

Let us take the simplest case. Assume like in the first case that the enzyme possesses only one ionisable amino acid residues. The ionization of the residue can be considered as

$$EH \underset{}{\overset{K}{\rightleftharpoons}} E^- + H^+$$

That is the neutral enzyme, which is a protonated enzyme can dissociate into the negatively charged enzyme and the protons with the ionization constant of K. Under these circumstances, the concentration of EH will be

$$EH = E^-.H^+/K$$

and the total enzyme concentration

$$E_0 = EH + E^-$$

$$E_0 = EH + K.EH/H^+ = EH\ (1+K/H^+)$$

$$EH = \frac{E0}{(1+K/H^+)} = E_0.H^+/\ H^+ + K$$

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If we assume that EH is the catalytically active form and not the negatively charged enzyme, then the substrate will bind only to EH.

$$EH + S \rightleftharpoons EHS \longrightarrow EH + P$$

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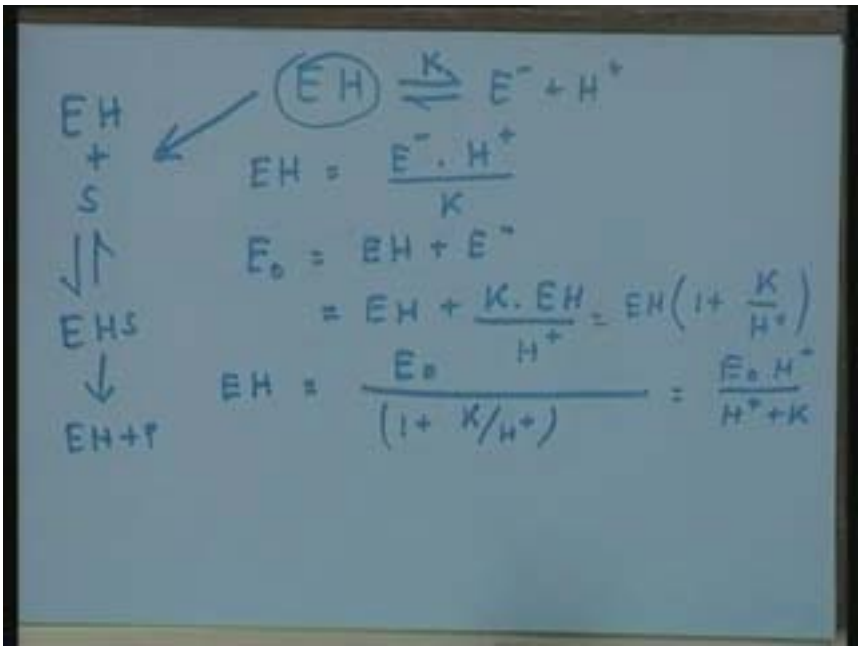

If you assume that EH is the catalytically active form that is the enzyme is present in the form of EH, in that case the V_m, the maximum reaction velocity will be

$$V_m = k.E_0.H^+/H^+ + k$$

The enzyme is present in the form of EH and therefore you multiply k, the rate constant k here, with the concentration of EH. If the total enzyme is present in the form of EH that is V_m^{true}. That is the maximum reaction velocity which one can achieve at some pH when predominantly all the enzyme E_0 is present in the form of EH. The concentration of E^- is negligible. That is a true V_m.

$$V_m^{true}/(1+k/H^+)$$

You arrive at a relation where by you see the V_m is the affected by a factor $1+k/H^+$

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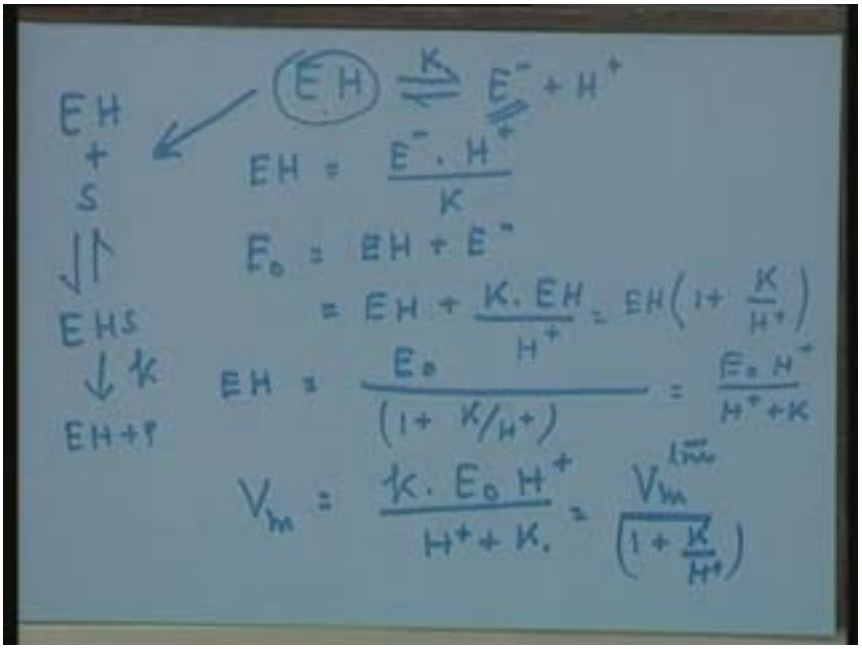

In such a case if you plot the profile of pH, which is negative logarithm of the hydrogen ion concentration, with log of V_m you end up with a profile like this. If you take logarithm on both sides

$$\log V_m = \log V_m^{true} - \log(1+k/H^+)$$

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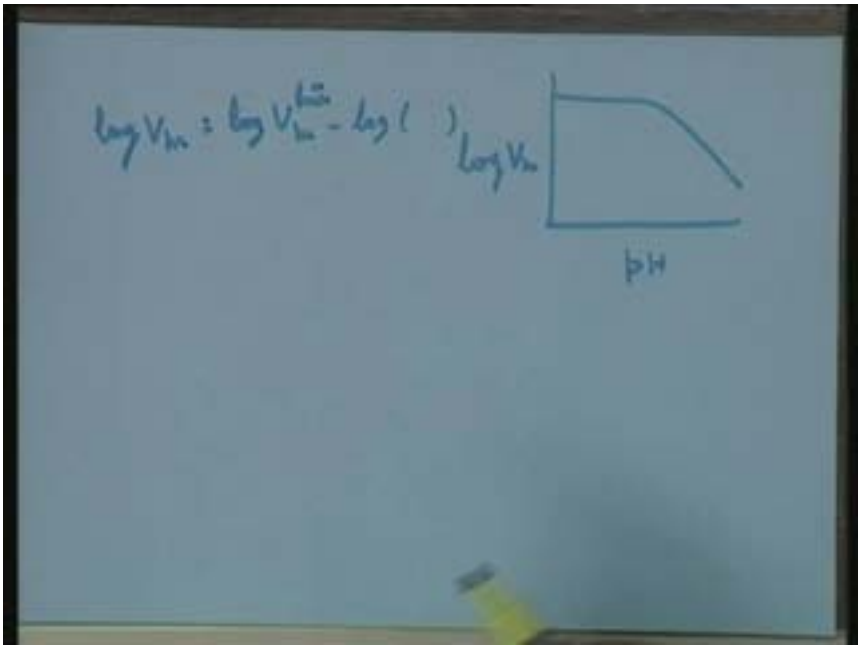

If you consider the extreme conditions that is when hydrogen ion concentration is much, much greater than the ionization constant or in the other words the pH is smaller than pK, then log V_m = log V_m^{true}. That means it remains constant. At a value of pH which is lesser than pK value of the ionisable residue, the V_m remains constant and at its original value. Only when the hydrogen ion concentration becomes smaller than k or pH becomes greater than pk,

$$\log V_m = \log V_m^{true} - \log k + \log H^+$$

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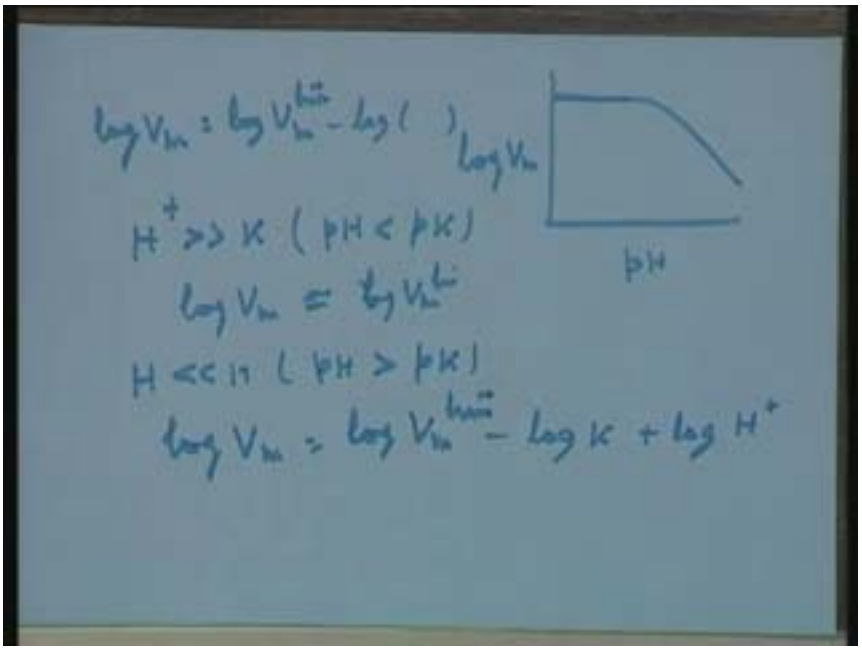

The hydrogen ion concentration will influence, V_m will be decreased and you get a downward fall. If you extrapolate these two lines, at the point where pH and pK are identical you will get a point of intersection at which the two lines will merge.

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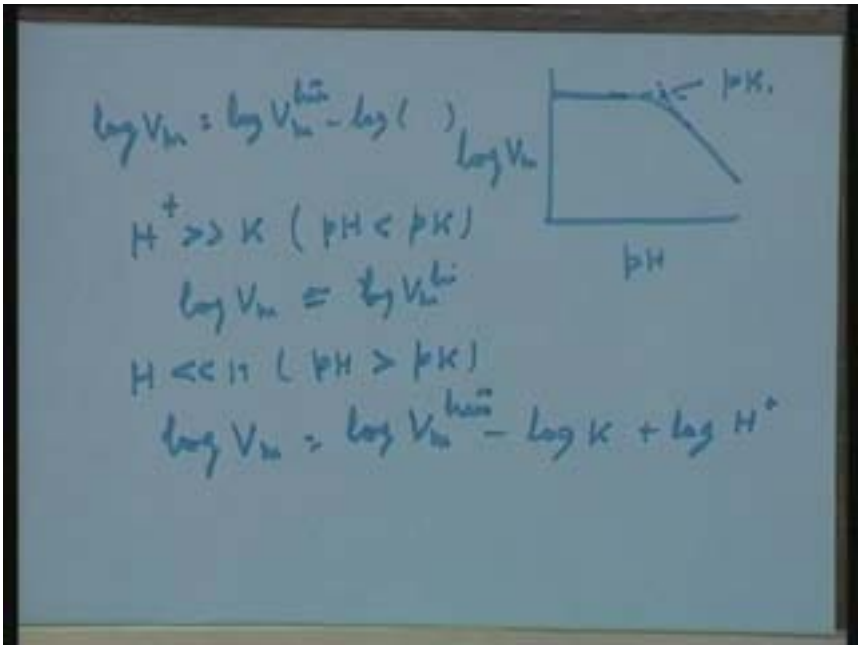

Another option available to us is instead of EH, E^- or the negatively charged enzyme is the active form.

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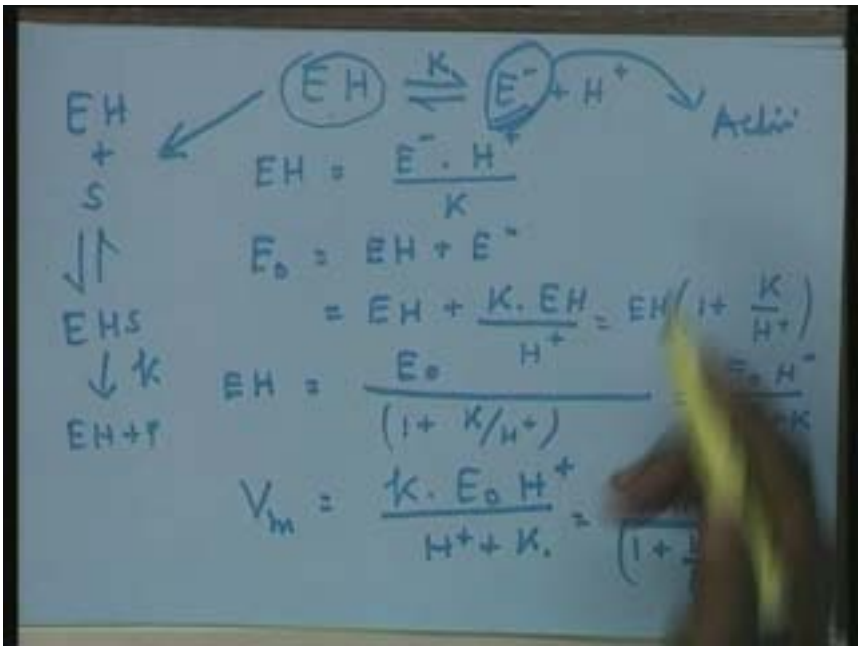

If the E^- is the active form, not the neutral enzyme in that case although …. (23.08) will be identical but the rate profile will be different. The rate profile will be

$$V_m = k\, E_0\, K/H^+ + K$$

You can again analyze the same way and the profile obtained will be reverse of this and you get pK value.

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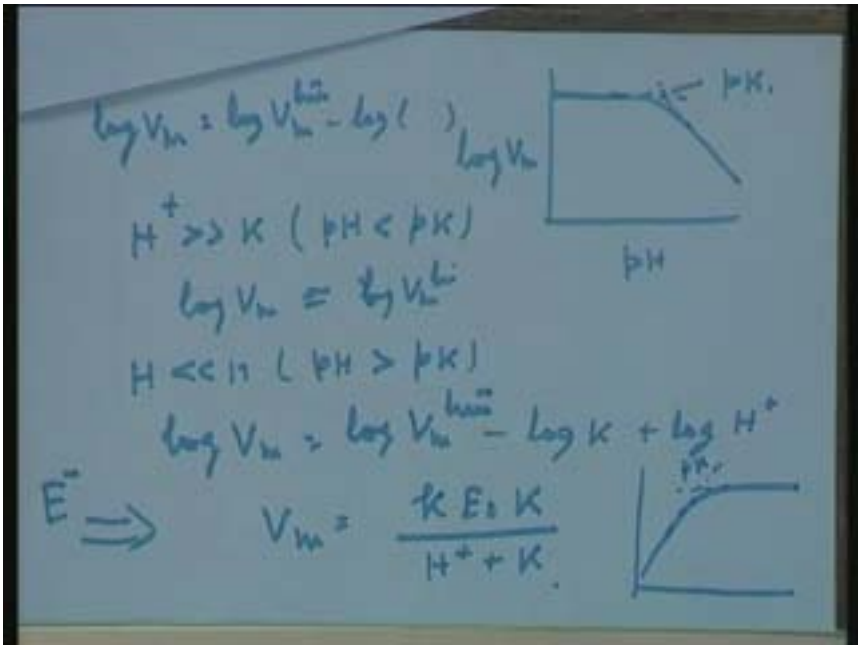

So only difference is the direction of the pH effect will be changed and the analyses will remain on the identical lines.

That is a very simplistic situation only to appreciate and understand the effect of ionization of the amino acid residue on the reaction rate. But if you take a little more realistic case that instead of having one amino acid residue, we have two amino acid residues or two ionisable residues there might be more than two other amino acid residues which are involved in the active site but they may be non-polar.

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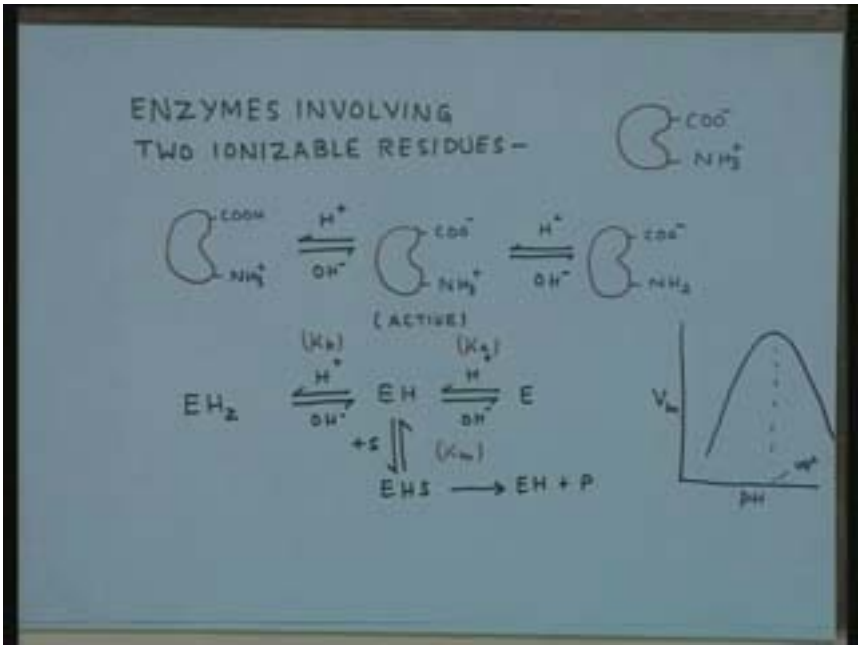

If there are two ionisable residues involved, one containing a carboxylic group and amino group and if you add acid or base or change the pH, the ionization state of the two residues will change. Both groups being in the ionized form, if it is the active form then you can see the effect of addition of acid or base in this equilibrium. If the middle thing is the ionisable form, then you can also predict that the left hand side form of the enzyme EH_2 is more reduced form than the EH and then EH is more reduced than E. If this EH is the form which is catalytically active, that means it binds to S the substrate and forms the product.

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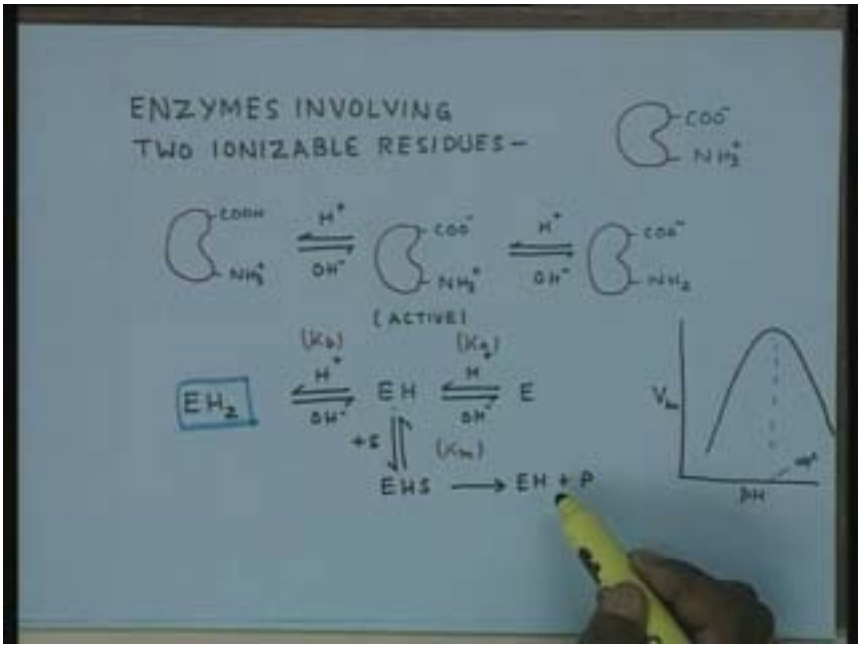

To analyze the system we have taken, we consider that there are three dissociation constants involved. One is the ionization of the two amino acid residues. If we represent K_a and K_b as the ionization constant of the two residues that are involved and the third we are taking as K_m which is the dissociation constant of the enzyme substrate complex. We define it as analogous to the dissociation constant, K_m. In such a case the typical profile that will be obtained of pH will be a bell shaped curve with the pH optimum at the middle of it which we consider as the typical profile and V_m.

If we look at the analyses of such a system you will notice that you can write the three different equilibrium and get the concentrations of individual enzyme species that are present in the three states. Let us consider the first one, the case of EHS that is the interaction of the EH with the substrate and we can write the concentration of EHS

$$EHS = EH.S/K_m$$

I am omitting the parenthesis to denote the concentration term. These terms represent the concentration terms.

Similarly if you look at EH_2

$$EH_2 = EH.\ H^+/K_b$$

$$E = K_a.EH/H^+$$

This is based on the equilibrium dissociation of the three species. Our total enzyme concentration E_0 will be equal to

$$E_0 = E + EH + EH_2 + EHS$$

The total enzyme is present in four different species: the free enzyme, the partially reduced, fully reduced and the substrate bound which is involved in binding only with the EH. Therefore we can substitute the value EH

$$E0 = EH\ \{1 + K_a/H^+ + H/K_b + S/K_m\}$$

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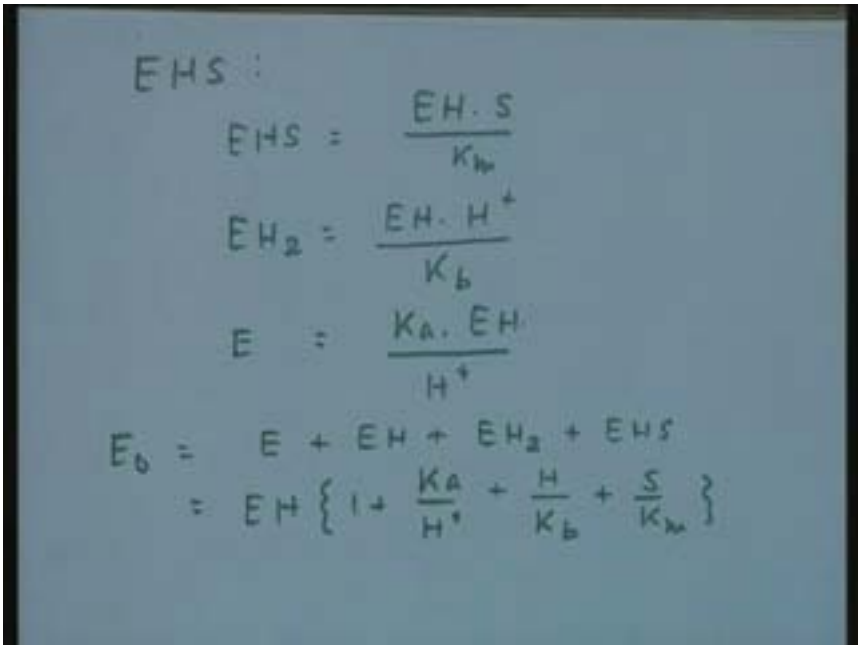

From this if we put the expression in the term for EHS

$$EHS = \frac{E_0.S/K_m}{\{1 + K_a/H^+ + H/K_b + S/K_m\}}$$

or reaction velocity will be

$$v = k[EHS]$$

That is the concentration of the enzyme substrate complex which has bound the doubly ionized enzyme complex.

$$V = k[EHS] = \frac{k.E_0.S}{K_m (1 + K_a/H^+ + H/K_b) + S}$$

The whole effect of the change of pH that is the hydrogen ion concentration is influenced by this parameter in the parenthesis. The parameter in the parenthesis influences or depicts the effect of pH on the reaction velocity and this parameter will pass through minima as hydrogen ion concentration is varied and you can get the minimum value by simply differentiating it with respect to hydrogen ion concentration. If you denote this function as F

$$dF/dH^+ = 1/k_b - k_a/(H^+)^2 = 0$$

If you equate it to zero you get

$$1/kb = k_a/(H^+)^2$$

Hydrogen ion concentration gives you this function F to be a minimum. When this function is minimum your reaction velocity will be maximum and that will be given by

$$(H^+)_m = \sqrt{k_a.k_b}$$

You get the minimum value of this function. This is not the minimum hydrogen ion concentration but is the value of this function F which is minimum.

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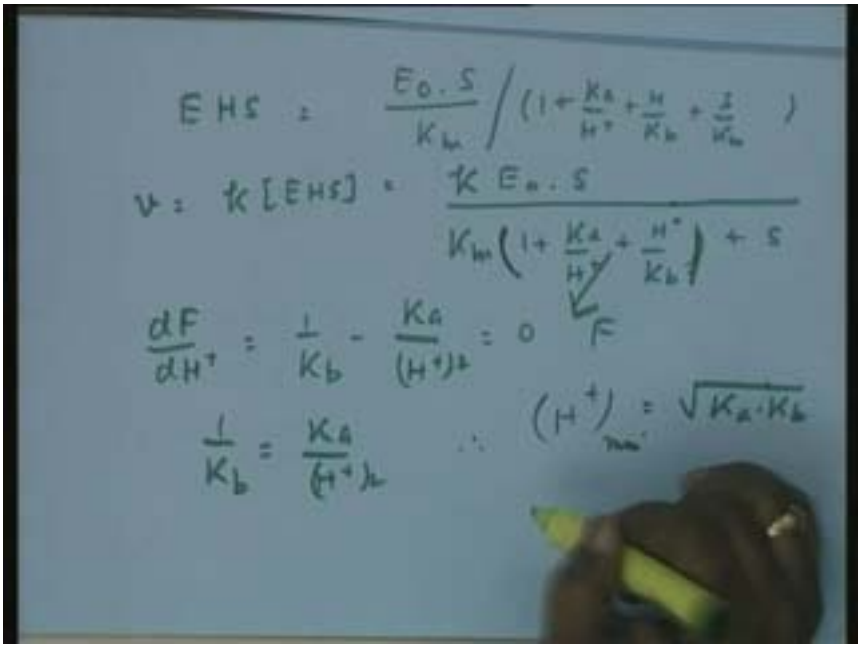

The rate will pass through a maximum when the function is minimum and at this concentration of hydrogen ion the reaction velocity will pass through a maximum. You can use this expression to denote to the effect of k, the effect of ionization constant of two amino acid residues on the reaction velocity of the enzyme catalyzed reaction.

You can increase on the complexity by taking more and more number of ionization. It is not necessary that there will be always one negatively charged and one positively charged amino acid residue. There might be more than one of each of the type. There might be two negatively charged, there might be one positively charged or there might be two acidic and one basic or more than that in the case of particular enzyme. The only difference you will notice in that case is that the pH optimum profile will not be a very sharp bell shape but can be broken down into different stages or segments, each of the segment representing the ionization state of that particular segment. It will be important to notice that the sharpness of the profile of the pH versus reaction rate will also depend on the proximity at which the pK value of the different amino acid residues lie. Very often just by a pH profile if the number of amino acid residues involved are not very

large, one can guess the likely number of amino acid residues involved and knowing the pK value of different amino acids that are known, that are likely to be present one can make judgment whether a particular ionisable amino acid residues present or not. That is the effect of pH as far as the ionization of enzyme active sites are involved.

The next stage which is again very important is to look at the ionization of substrates because it is not only important that the enzyme may ionize it may also be very commonly feasible that when you are handling the charged substrate molecules and very often the protein itself is a substrate which is a poly electrolyte and it will have many functional groups which will be ionisable.

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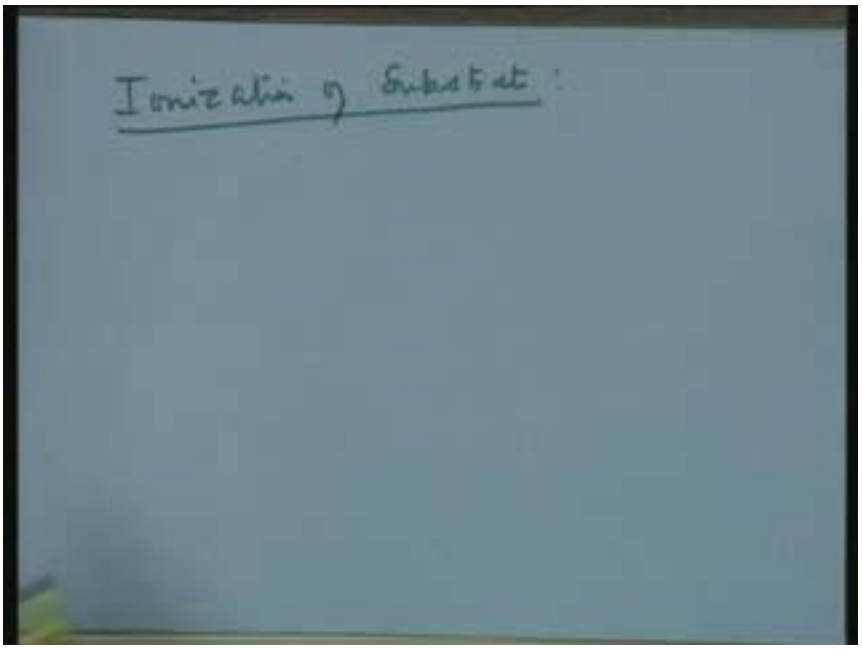

In those cases the binding of the substrate to the enzyme will depend upon the ionization state of the substrate. Depending on the number of charged functional groups on the substrate the situation might be more and more complex. But just to consider two simple model cases let us consider a substrate which in its unionized form is not catalytically active but is able to bind to the enzyme for catalytic function. The substrate itself is not catalytically active, it is only enzyme which is a catalytically active component and if suppose we assume a simplistic case wherein the substrate can ionize and give you a protonated form of substrate with the ionization constant of K.

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

$$K \uparrow\downarrow \quad SH^{+}$$

You can arrive at the rate expression for such a system as

$$V_0 = \frac{V_m \cdot S}{K_m(1+ H^+/K) + S}$$

You will notice that in such a case the net effect of the ionization of substrate is an increase in the K_m value. The K_m value is modified to the true K_m

$$K'm = K_m(1+H^+/K)$$

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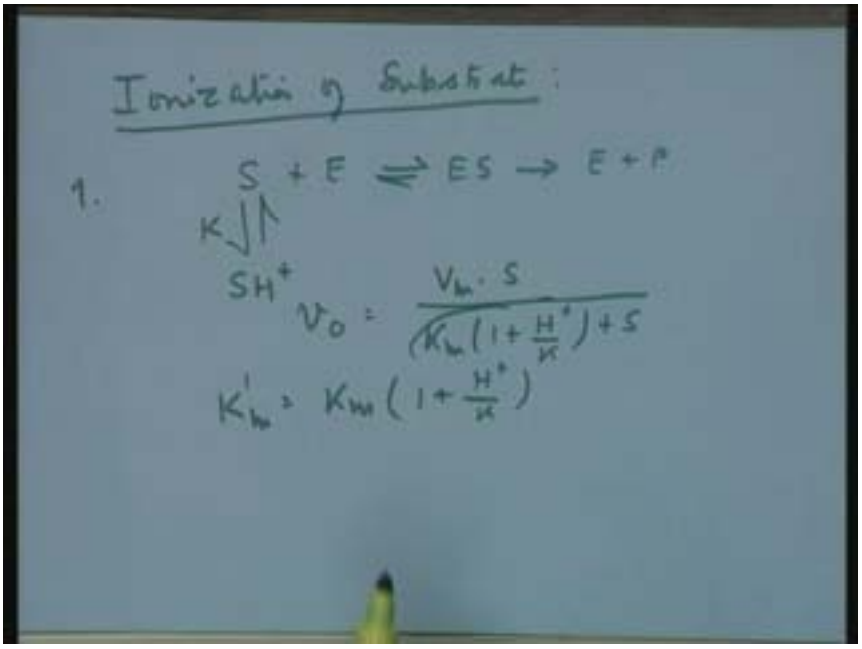
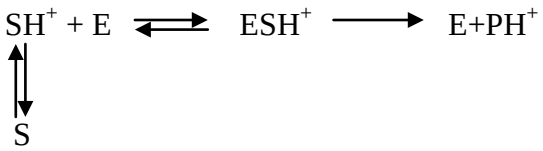


The expression is analogous to that of competitive inhibition. That means in such a case the hydrogen ion acts as a competitive inhibitor for the substrate. The neutral substrate and protonated substrate is the reactive form which is acceptable to the enzyme. Part of the substrate which got protonated by binding with the hydrogen ions becomes a dead end complex as happened in the case of a competitive inhibitor and therefore you get a similar profile which gives you a K_m value. The K_m apparent, if you consider K'_m as K_m apparent varies with the hydrogen ion concentration. So the effect of pH on the K_m value can be noted to look at the pattern of inhibition.

In the second case you assume that it is the protonated form which is catalytically acceptable to the enzyme function.



In such a case although the analysis will remain the same only the form of the reaction velocity will change. Again there will be a difference in the K_m value you will again get an apparent K_m. But the magnitude of apparent K_m will be slightly different and will be given by

$$V_0 = V_m.S/ K_m (1+K/H^+)S$$

If you analyze the two apparent K_m terms, in this case this one and in this case K_m. $(1+K/H^+)S$. You will notice that only at extreme values of hydrogen ion concentration there will be difference in the behavior of the enzyme.

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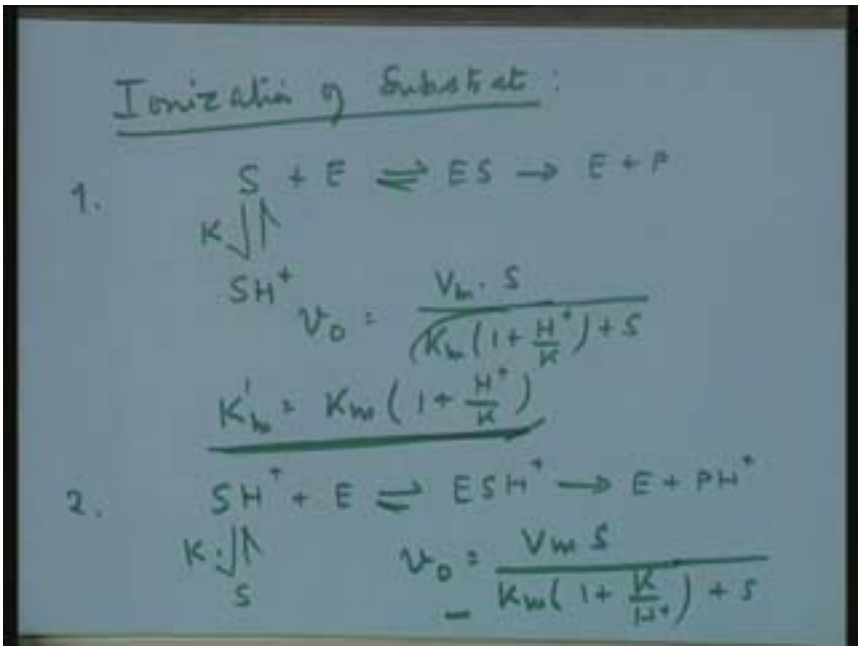

Otherwise they will be almost similar kind of behavior. In both the cases you will notice that the ionization of substrate influences the K_m value of the enzyme that has increased and this increase is in magnitude dependent upon the fraction of the substrate which is present in the form of the substrate molecule which is acceptable to the enzyme and ionization substrate. Similarly if there are more than one ionisable groups in the case of a substrate a similar analyses can be arrived at.

We should consider SH^+ because that is the form of substrate concentration which is the total substrate concentration for all practical purposes.

$$V_0 = V_m.S/ K_m (1+K/H^+)S$$

We have seen so for that this pH effects are quite complex and all the parameter that are influenced by pH cannot be considered in one go. They need to be considered in isolation

one by one and depending upon the information we want to collect. Sometime the information required is only to understand the optimum pH value of the reaction. So life is very simple. You conduct the enzyme catalyzed reaction in a buffered medium with different pH values and get the reaction rate and use the one which has the maximum value.

Sometimes you want to identify the amino acid residue at the active site. You need to make analysis of the enzyme reaction at the pH enzyme rate profile, detailed analysis to get the pK values of the amino acid residues involved and thereby make deduction. This needs to be confirmed again by chemical modification studies. Therefore such an analysis can give you an indication but may not be able to give you confirmed data.

The second environmental parameter which is very important is temperature. Like pH, temperature is also a multidimensional parameter which influences different aspects of the enzyme catalyzed reaction. Probably the very first step at which the temperature influences is the interaction of enzyme substrate complex, an equilibrium process, you know is a reversible equilibrium process. The key parameter which represents the ES interaction, in our case we have noted at least for typical Michaelis Menten kinetics, is the K_m Michaelis Menten constant.

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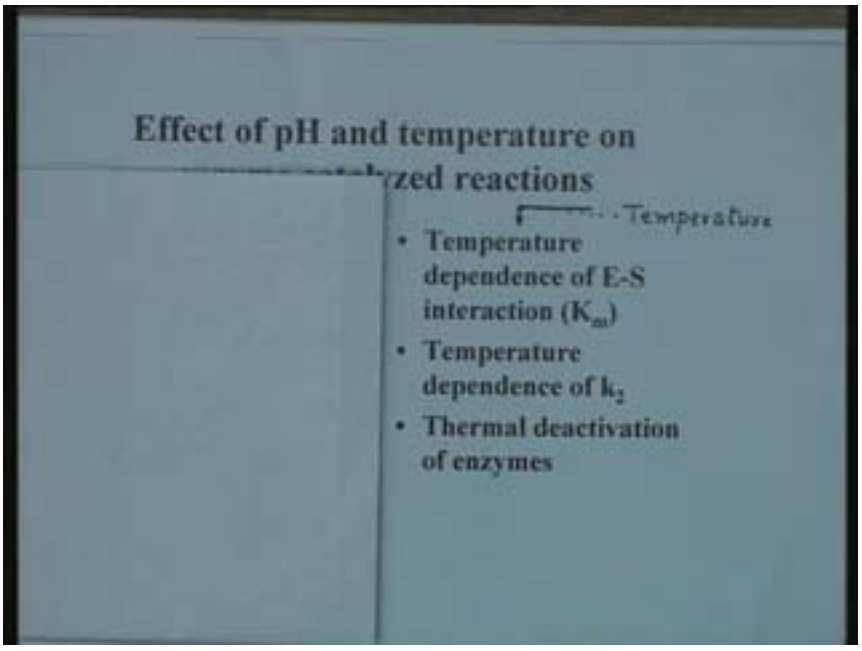

The temperature will influence the effect on K and the study of effect of temperature on the K_m is a very interesting issue because it gives you lot of insight into the thermodynamic aspects of the enzyme substrate binding like the enthalpic and entropic parameters involved.

If you look at the classical Vant hoff equation for an equilibrium process, the equilibrium process here is

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

with the dissociation constant of K_m.

The free energy change will be equal to

$$\Delta G^0 = RT \ln K_m$$

Note that I have purposely removed the minus term from here as K_m is not the equilibrium constant, is dissociation constant almost like inverse of that and which again also can be equated to $\Delta H - T\Delta S$. I think all of you should be familiar that conventionally in biochemical systems we are used to use these terms with prime and I am sure that you must have understood the meaning of prime for the thermodynamic parameters in biochemical system.

$$\Delta G' = RT \ln K_m = \Delta H' - T\Delta S'$$

That is only for the sake of convention but the laws of thermodynamics do apply and the Vant hoff equation is also applicable, the second law of thermodynamics is also applicable and therefore you can write

$$\ln K_m = \Delta H/RT - \Delta S/R$$

So a very simple situation arrives that if you determine the K_m values for an enzyme catalyzed reaction experimentally at a number of temperatures and then make a plot between $\ln K_m$ and $1/T$ you end up in a straight line with a slope of $\Delta H/R$. So one can easily get the magnitude of ΔH, the enthalpy change during the enzyme substrate binding. Also the sign of slope I have shown only the positive slope it could end up in a negative slope also. The sign also indicates whether the enzyme substrate binding is exothermic or endothermic. You can get a thermodynamic picture of the enzyme substrate binding and also you will notice that the interaction on the X-axis will give you $\Delta S/\Delta H$. If you put this equal to zero you get $\Delta H/\Delta S$ as the interaction point and thereby you can calculate both ΔH and ΔS for a given enzyme catalyzed reaction by studying the effect of temperature on the K_m value of a reaction.

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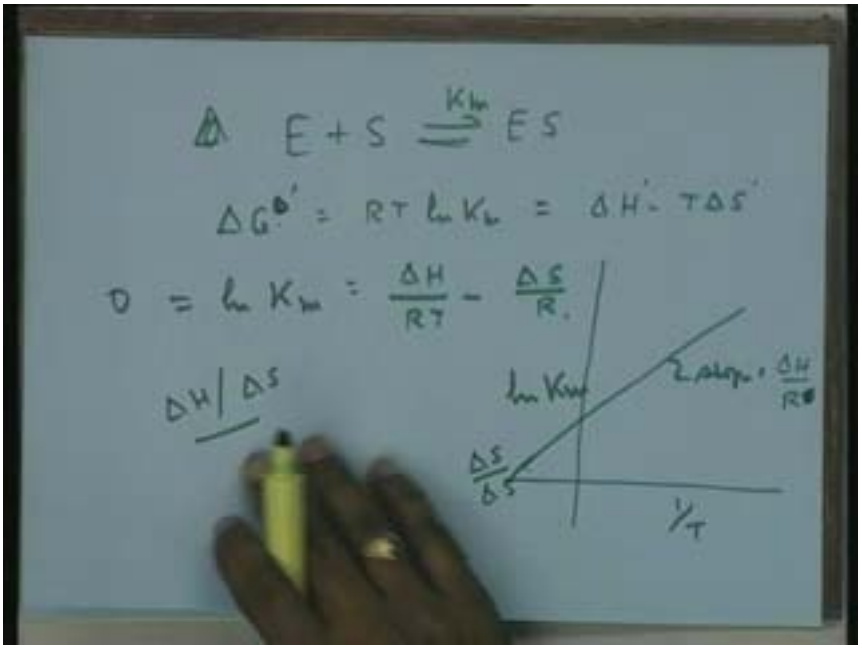

The second effect of temperature in the case of a enzyme catalyzed reaction is the dependence of K_2 that is the turn over number or the rate constant of the irreversible step of the conversion of ES into P and that classically follows a Arrhenius kind of a pattern that is

$$K_2 = A\, e^{-E^*/RT}$$

We have seen this analysis even in our initial lectures on catalysis that means the catalytic pattern of the enzyme as the catalyst and one can again analyze in the same manner

$$\ln K_2 = \ln A - E^*/RT$$

Very familiar profile 1/T versus ln K_2, you get a negative profile and this is a slope of E^*/R and that gives you a magnitude of energy of activation for the given enzyme catalyzed reaction.

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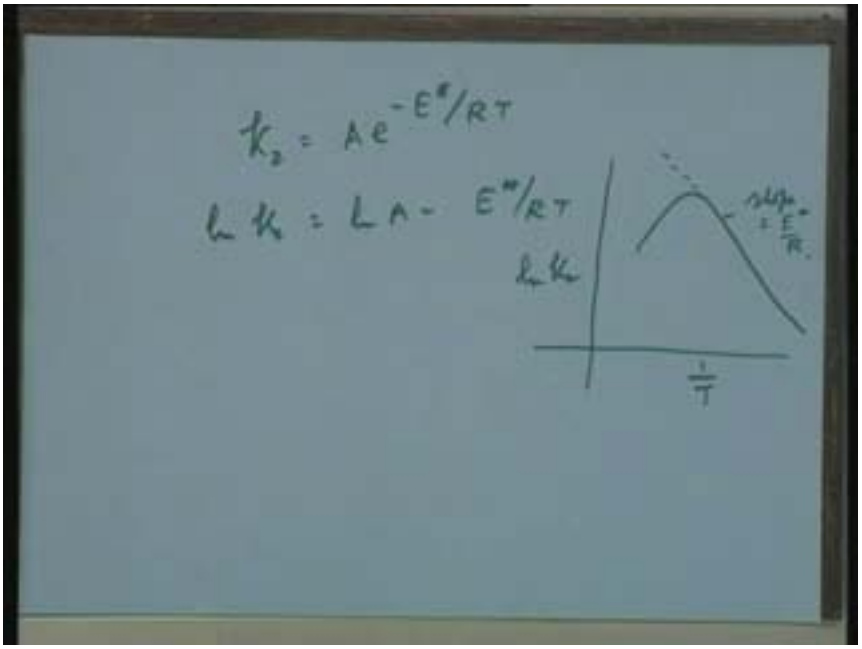

So the effect on K_2 that is the turn over number is primarily almost like any other chemical reaction.

The third effect on the enzyme catalyzed reaction of the temperature is the deactivation of enzymes.

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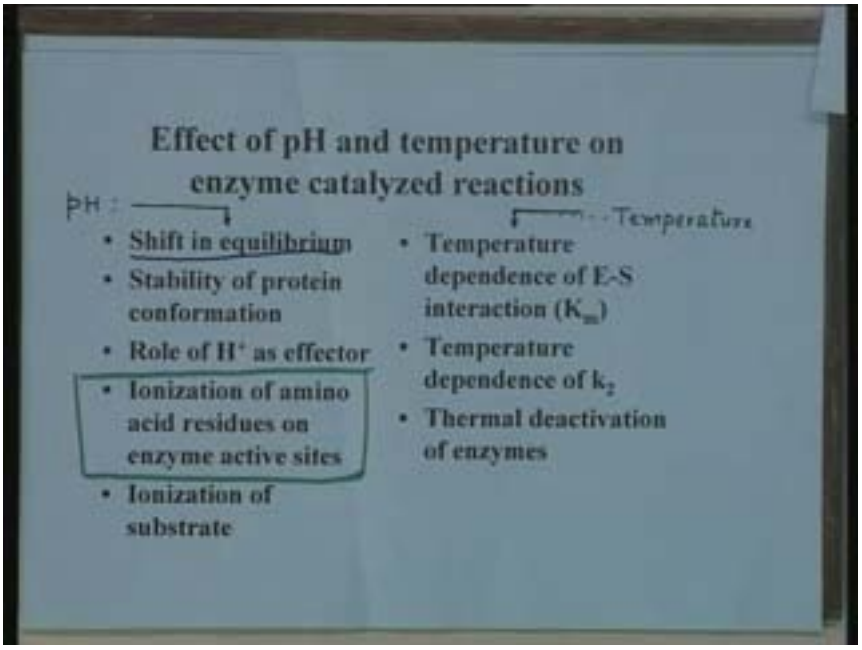

Just like in the case of pH effects on the deactivation of enzyme, temperature is also mediated by inactivation process. That means as long as you are keeping the temperature

range in the environment of the reaction within a close range the enzyme may be functional but if the temperature ranges are shifted drastically to the higher side the deactivation occurs and a very broad picture of deactivation has been discussed earlier which is represented by a first order deactivation kinetics where you can define as

$$E = E^0 e^{-Kd.t}$$

Here also you can make a first order kinetics analysis that is your ln E/E_0 versus time will give you a profile with a negative slope and slope will be equal to $-K_d$ and this will be a function of temperature. The magnitude of the deactivation constant will be a function of temperature.

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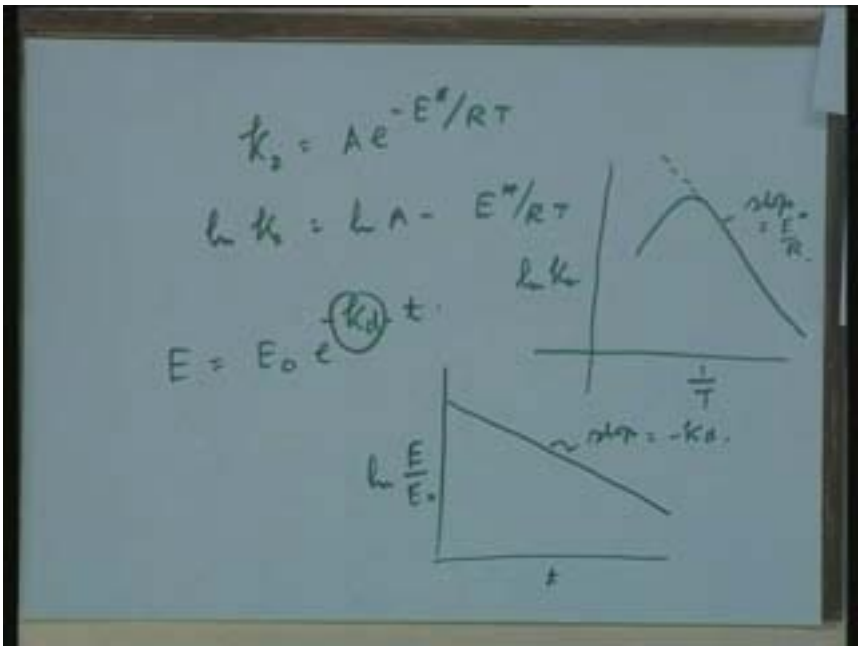

One must look at the deactivation effect, the third effect and the second effect that is on the K_2 as opposite forces. While with the increasing temperature on the one side the reaction rate increases, the deactivation constant also increases. The practical operational temperatures of enzyme reactions therefore are chosen on the basis of not just the optimum temperature that means the optimum temperature for the enzyme reaction rate but also taking into account the deactivation kinetics because the interest will be on the total productivity of the enzyme per unit enzyme rather than only initial reaction rates.

All these studies are based on initial reaction rate but if you look at the practical application that will be based on total productivity and both the effects the deactivation as well as increase of reaction rate with temperature should be considered.

So to summarize we have seen the effect of two major environmental parameters which are experienced by the enzyme catalyzed reaction the first is pH. The effect is quite

complex and the effect can be considered on the basis of the shift in the equilibrium or on the ionization of either substrate molecule or on the enzyme active sites. The other parameter which is temperature also influences in a multiple fashion. It influences to increase the reaction rate in a pattern of the Arrhenius plot. It also influences the deactivation of the enzyme molecule and finally a very important tool which it provides is to understand the thermodynamic status of the enzyme substrate interaction and one can by computing the value of K_m at different temperatures one can get a picture of the ΔH and ΔS for the given enzyme catalyzed reaction.