ENZYME SCIENCE AND ENGINEERING

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LECTURE-18

EFFECT OF IMMOBILIZATION ON FUNCTIONAL PROPERTIES OF ENZYMES

Today we shall discuss about the effect of immobilization on functional properties of the enzyme molecules.

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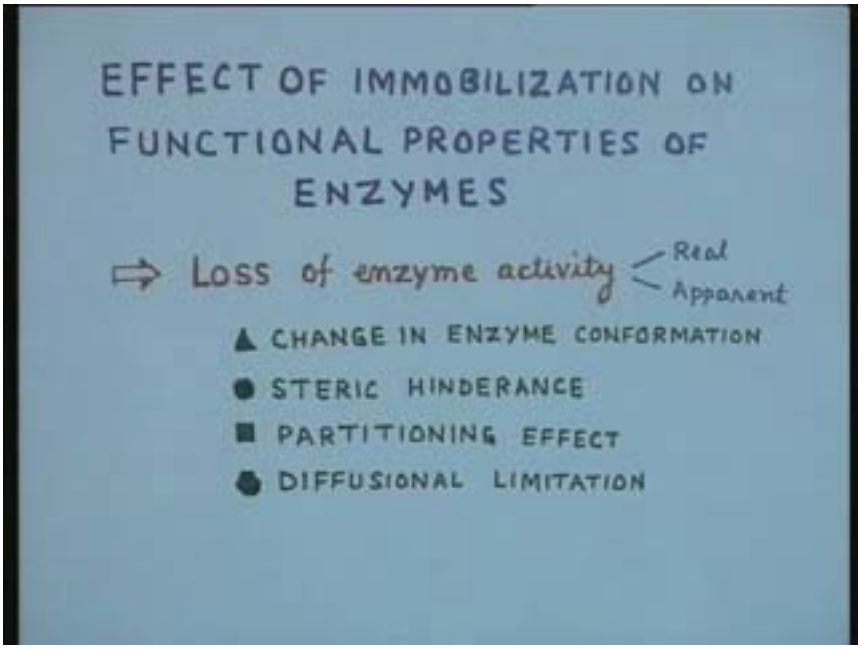

We have seen a number of immobilization methods that we have discussed in previous classes. We see that in most cases the enzyme undergoes loss of activity during immobilization. In fact no method can ensure that you can recover the enzyme in hundred percent yield. The yield can vary depending on the method of immobilization, depending on the carrier and the stability of the enzyme itself with respect to the conditions of immobilization. This loss of enzyme activity could be real or could be apparent. By real I mean that you cannot recover, after the immobilization, the original enzyme activity by physical interventions. On the other hand the apparent loss refers to the reduced enzyme activity that you are measuring but by certain physical interventions the original activity can be restored. It is not a permanent loss of activity but due to certain physical forces which make a interplay with the catalytic function, the enzyme activity seems apparently to have been lost. The reasons for the two types of losses, the real and apparent, could be attributed to one or more of the following four factors that are listed here.

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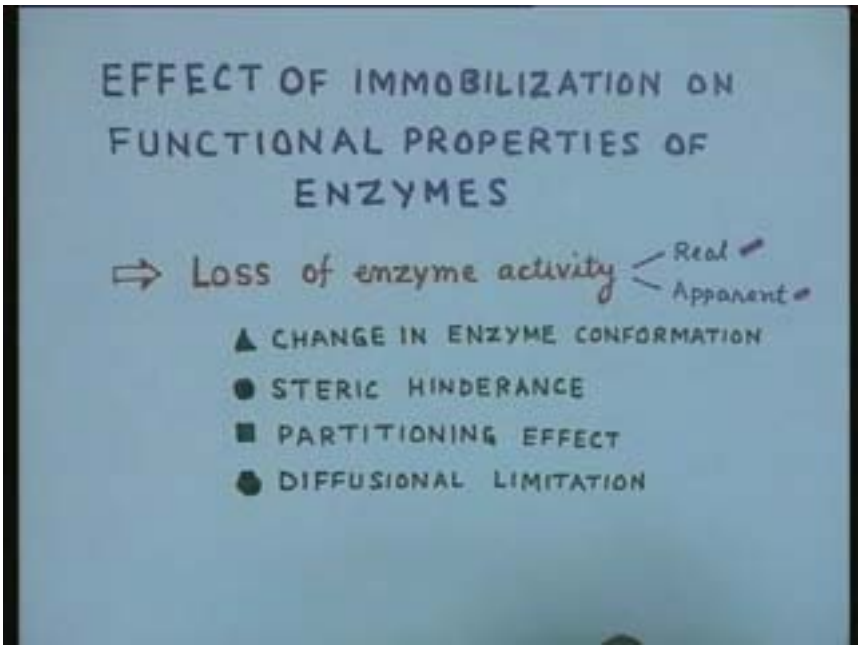

The first and foremost factor is the change in the enzyme conformation itself. When the soluble enzyme is immobilized on to a matrix the interaction between the matrix and the enzyme amino acids might lead to certain conformational changes possibly due to the result of involvement of some of the amino acids residues in the immobilization process. So therefore all the intermolecular forces that attribute to the enzyme conformation are altered. Very often it has been noticed that a very common feature is that upon immobilization an enzyme may undergo some kind of unfolding; some kind of a stretching.

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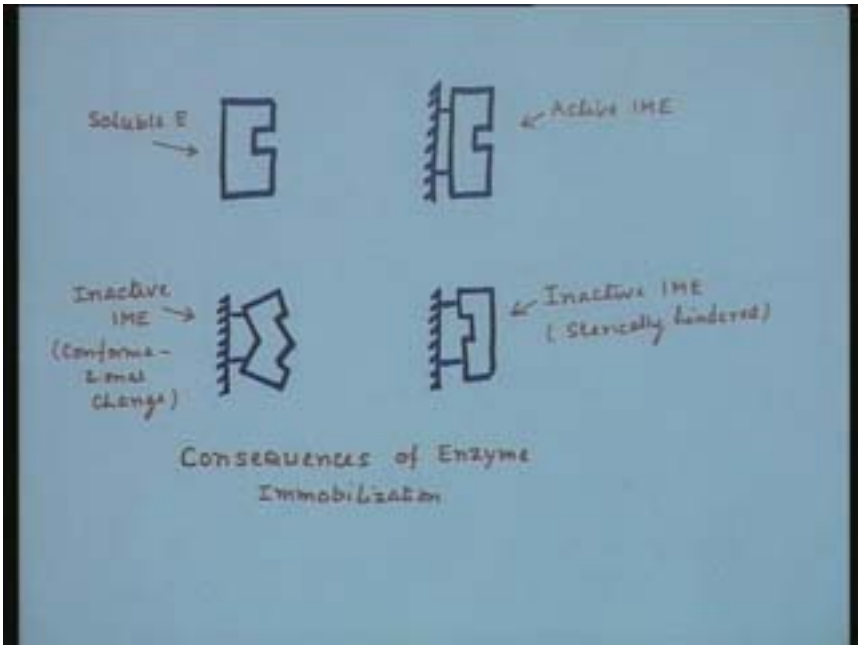

There are no parameters which can be simply measured. A detailed analysis or detailed study on the conformation, just like what we do as for identification of the globular structure the tertiary structure of the enzyme, one can study. But other wise a quick quantitative measure of the conformational loss is only through measurement of enzyme activity. For example here you see that this is a typical soluble enzyme shown here. It has been immobilized on to a solid matrix by some bonds; it could be an electrostatic bond; it could be a metal salt linkage; it could be covalent bond or it could also be as an entrapment within a gel. Entrapment within a gel fortunately does not involve the amino acids of the enzyme protein and therefore the chances of any conformational change during gel entrapment are minimum. Then during immobilization, the enzyme may under go some conformational changes. Just a hypothetical change I have shown here. It could be in any shape. It could be simply unfolding; it could be some kind of stretching and it may lead to a totally inactive enzyme preparation or a reduced activity of an enzyme preparation. Reduced activity means that the protein content will be reduced. The specific activity of the enzyme that means the enzyme activity in terms of international units per milligram of protein might get reduced. There have been certain exceptions. I am saying exceptions which are rare examples where the enzyme activity after the immobilization has also been analyzed. If the conformational changes are more favorable to the factors that are responsible for reduction in activation energy either by strain or any other mechanism that we discussed earlier by entropic or enthalpic changes which reduce the energy of activation for the enzyme catalyzed reaction, it might also lead to favorable conformational change. But those examples are indeed rare and not very common examples.

Then the second factor which is responsible for loss of enzyme activity is steric hindrance.

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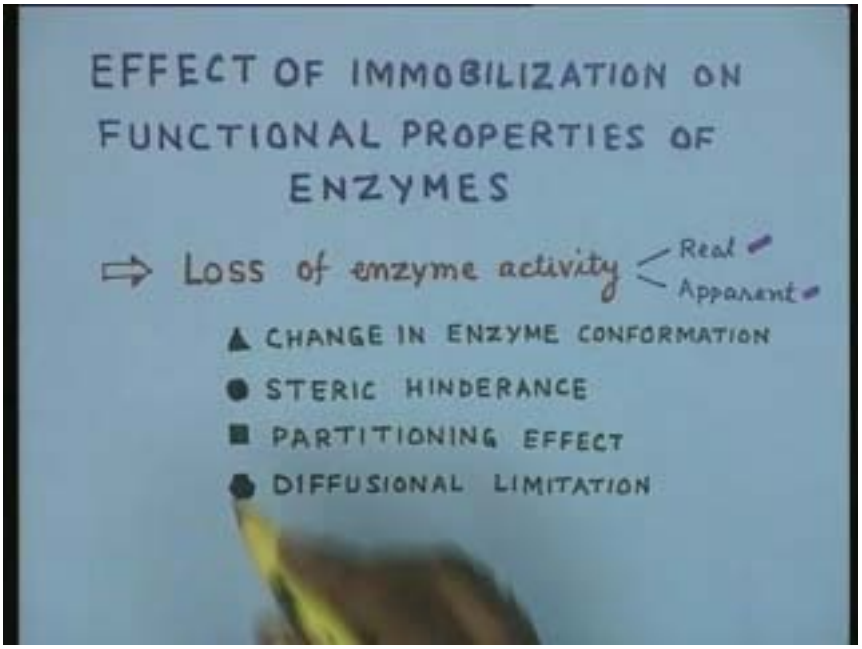

In many cases the immobilization process might involve the reduction in the access of the substrate molecule to the enzyme active site. You have achieved a good binding but the enzyme active sites are hindered from access to the substrate molecule. A typical case is shown here. In this case, the enzyme has been immobilized on sites which are in very great proximity to the active site and therefore the participation or the involvement of the substrate with the active sites is reduced and that also results in reduced enzyme activity.

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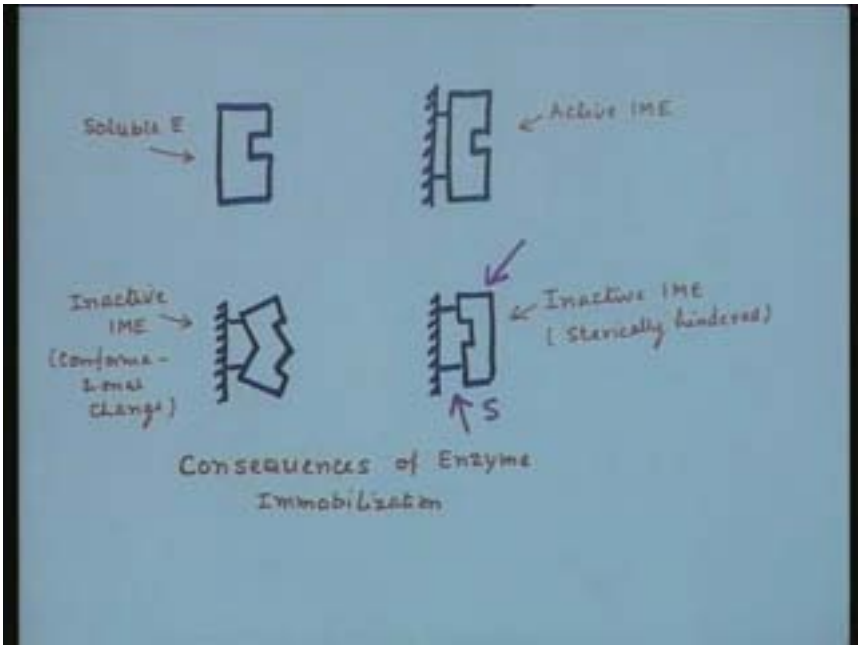

However such a enzyme activity can be recovered or this phenomena can be reduced if you can use a proper choice of the method for immobilization. In some of the cases the steric hindrances can be minimized by introduction of the spacer arm. For example if you are going to immobilize this enzyme you can introduce a kind of a hydrocarbon chain before it is really coupled to the enzyme molecule. That means between the binding sites introduction of a spacer arm which is usually a hydrocarbon chain and which can make the binding more flexible and there by the steric hindrances can be reduced.

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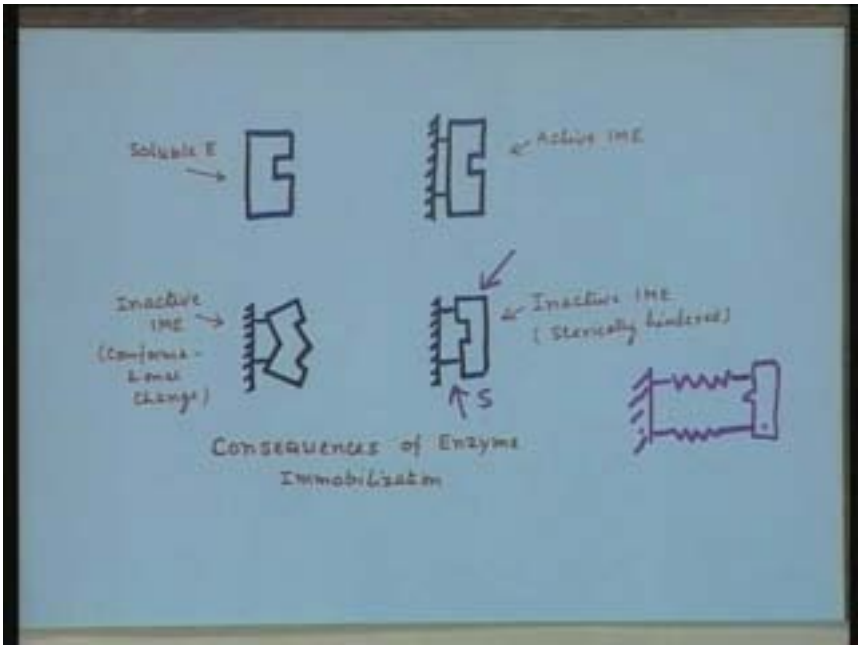

But in a particular method of immobilization the steric hindrance results in a definite loss of enzyme activity which is a real loss and cannot be recovered by physical methods.

By physical methods. For example if you have covalently bound an enzyme i.e., by covalent bond depending on the method of binding if you have not involved a spacer arm the loss will remain there. But you cannot recover that. Unless you modify enzyme immobilization procedure whereby a spacer arm is introduced then you can reduce the steric hindrance. The method can be modified to result in reduced loss as a result of steric hindrance. But once a preparation has been made with a definite amount of steric hindrance, the loss is real.

The third factor which contributes to loss of enzyme activity is partitioning effect.

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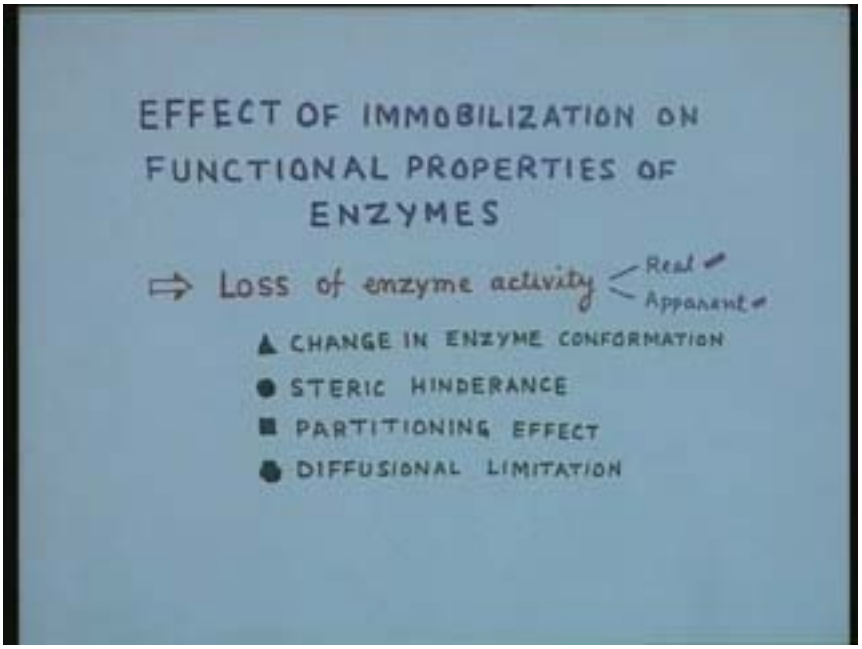

We must consider immobilized enzymes in the light of a shift from homogenous catalysis to heterogeneous catalysis. Instead of looking at the catalytic reaction in a homogenous phase where the catalyst is dissolved in the same medium as the reactant here the catalyst has a different phase-solid phase and the substrate is in the liquid phase. You lead into a heterogeneous catalysis and the various species that are involved in the reaction, it could be a substrate; it could be inhibitor; it could be simple hydrogen ions which contribute to the pH; they might get partitioned in the two phases depending on the chemical nature of the bulk and the carrier particles and this partition effect can also lead to an apparent loss of enzyme activity in a particular situation.

The fourth is the diffusional limitations. A result of incorporation of a solid particle in a reaction mixture and particularly we consider a porous solid particle where most of the enzyme has been immobilized on the interior of the particle. When a solid particle is immersed into a liquid phase there is also bound to be a stationary film of liquid around the particle. This film thickness can vary depending on the physical properties of the substrate solution; for example viscosity or density. They may control the film thickness and these might call also a diffusional constraint particularly with respect to transport of the substrate from the bulk to the site of enzyme catalyzed reaction. The two effects the partitioning effect and the mass transfer of the diffusional effect can be visualized here

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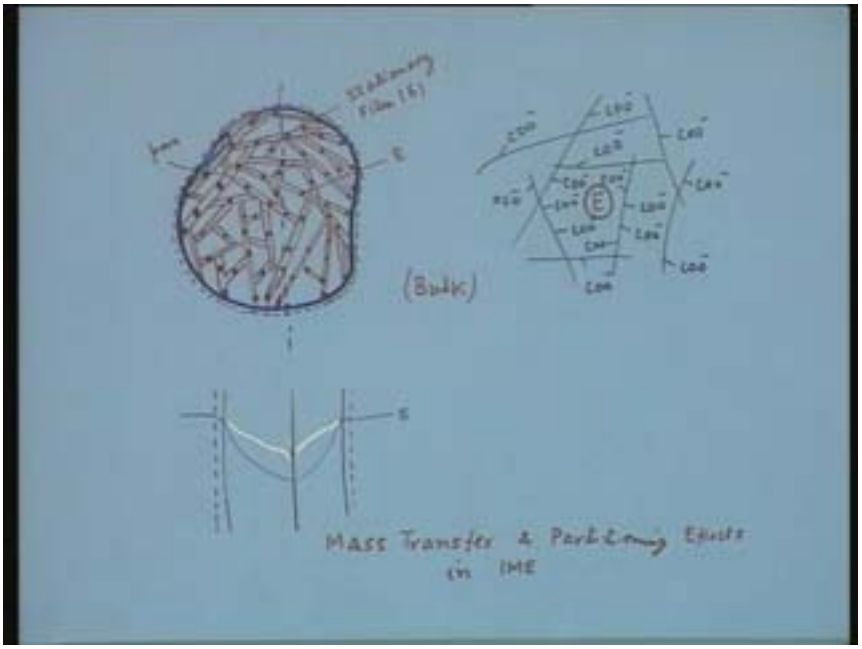

If you notice here for example in the right hand side diagram we have assumed a hypothetical carrier preparation which is poly anionic in nature. That means it is highly negatively charged and an enzyme molecule is entrapped or located in the midst of negative charges. Consider the situation in the bulk. The total charge inside the carrier particle or around enzyme where the catalysis is going to take place will be different than in the bulk. If in case the substrate also happens to be a charged molecule, so there will be some partitioning effects of the substrate between the bulk and so the real substrate concentration what we are considering in the bulk phase will not be same at the site of the enzyme reactions. I will come to that. I am just describing the right hand side one. There could be very clear cut partitioning effects with respect to hydrogen ions because they are positively charged and a poly ionic carrier will definitely have preference for hydrogen ions. Therefore the hydrogen ion concentration inside the carrier will be greater than the bulk. Similarly if the substrate is also positively charged you are going to have higher substrate concentration in the carrier or at the site of enzyme rather than the bulk or it could be the other way. If suppose the substrate is also negatively charged the reverse might happen. That means the bulk concentration vary more than at the site of the reaction. That might lead to the apparent effect on the enzyme activity.

On the left hand side I have shown the diffusional effects. You see the particles shown here; let us say a kind of a spherical particle not perfectly sphere but something which can be assumed to be spherical and with a network of pores within the particle and the enzyme is located while on the surface as well as on the bulk of the enzyme. In fact a very large percentage, more than ninety percent of the enzyme will be distributed on the porous network within the particle. Only very small fraction will be on the surface and for the reaction to take place the substrate has to diffuse from the bulk to the site of the enzyme reaction. Here you notice that in the case of the immobilize enzyme unlike a soluble enzyme what we are conventionally used to where the enzyme is in direct proximity with the substrate and can interact and reaction can take place without any

resistance other than the chemical reaction, the biochemical reaction. Here in addition to biochemical reaction you have another limitation. That means the bulk substrate has to diffuse through the stationary film which is on the surface of the particle. This surface, they have a thickness delta on the basis of the physical properties of the substrate molecule and the carrier particle. The thickness of the stationary film can be reduced by changing the flow properties in the reactor stream and as we will see further that the effect of thickness of the stationary film can be reduced if you increase the linear field velocity in a continuous reactor or increase agitation speed in a stirred vessel in a stirred reactor. So you can reduce it. But any way it has to transfer from the bulk phase to the stationary phase and their might be some concentration gradient in the substrate. From the surface, the substrate has to move to the enzyme active site and the enzyme is distributed all over the particle. Depending on the particle thickness, let us say diameter of the particle is d_p the substrate might be able to reach only up to a part of the carrier. Let us say it reaches up to here. The substrate value comes down to zero.

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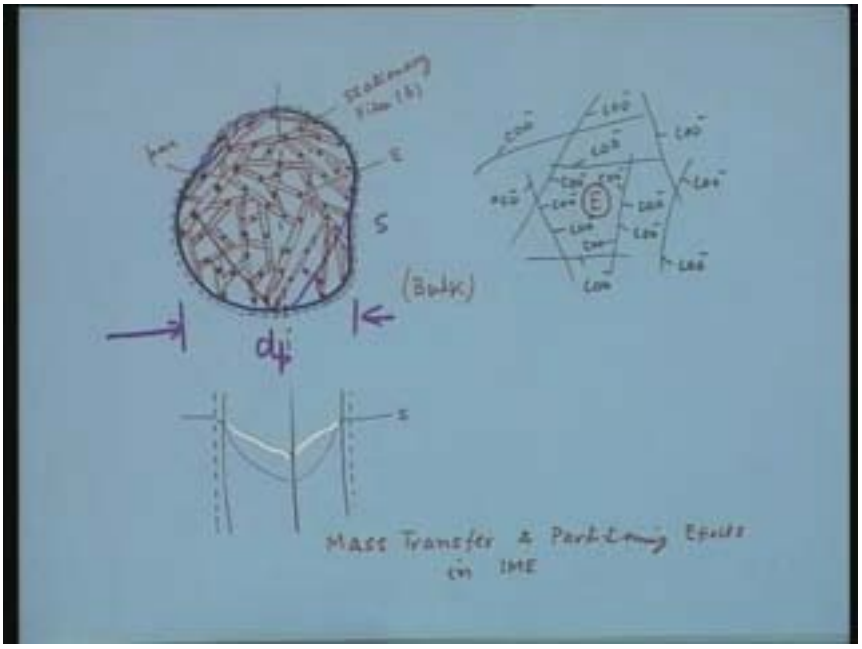

This means that a fraction of the total enzyme available in the immobilize preparation is not able to interact with the substrate because of diffusion limitations. This will depend on the relative rate of diffusion vis-à-vis, the rate of biochemical catalysis. The slower rate will dominate the effect. If suppose the biochemical reaction rate is very fast compared to rate of diffusion of the substrate into the carrier particle, it might happen that soon after the surface of the catalyst particle the substrate concentration might drop to a very small level or even to zero depending on the rate of diffusion which is smaller than that. Or on the other hand if the biochemical reaction rate is very, very slow compared to diffusion or diffusion is very fast you might have a reasonable concentration of substrate even at the centre of the particle and therefore the apparent loss of enzyme activity may not be serious. So in case of enzyme reactions where enzymes have a very high activity

or in other words a few are able to make a preparation which has a very high enzyme loading that means the amount of enzymes in terms of international units per gram of the carrier particle if that can be increased to a significant level, then the system can be operated which is under diffusion limitations. If you are able to prepare an immobilize enzyme preparation with a very high enzyme loading that means the catalytic rate is very high. Enzyme loading high means the catalytic rate will be very high because the enzyme activity in terms of international units, the reaction rate per unit weight of the carrier, if that is very high compared to the rate of diffusion then the whole system can be conceived to be totally diffusion limited. The biochemical reaction kinetics will not have a major role to play and the whole thing will be controlled by the physical diffusion. I have told you two kinds of effect of diffusion. One is the external film diffusion. The other is the diffusional limitations caused by the boundary layer film, inert film on the boundary as external film diffusion. On the other hand, the diffusional process within the pores of the particle or internal pore diffusion. The two types of diffusion, we will subsequently see their quantitative analysis in relation to the biochemical reaction but we shall understand that these two processes will put you four additional resistances over and above the biochemical reaction.

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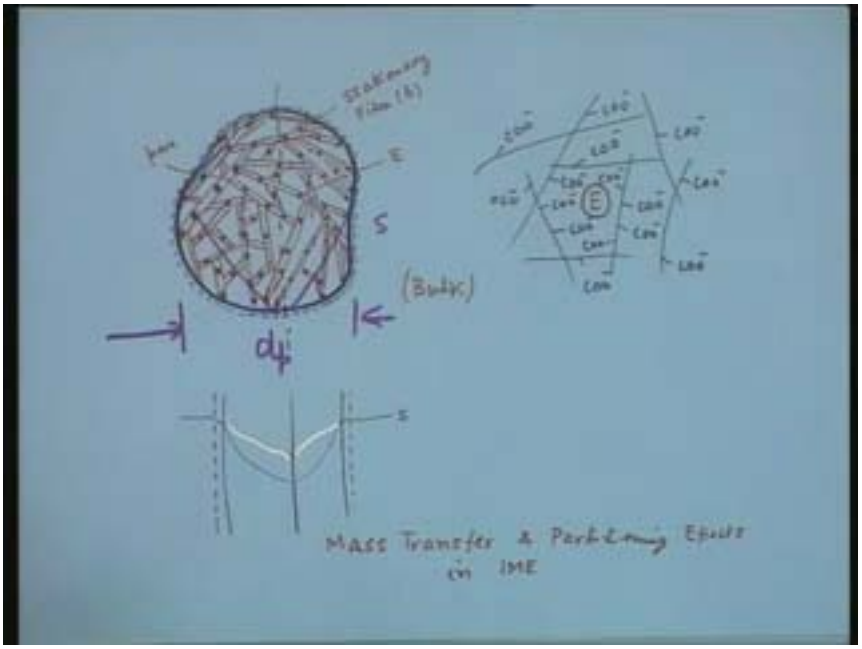

One is the transport of substrate from the surface to the enzyme active site and then reverse will be transport of product. Ultimately for all practical purposes our measurements or monitoring will be in the bulk. So the product has to also transport and it might also undergo a kind of a gradient. Just to illustrate a little more detail on the transport behavior consider a typical spherical particle with an external film shown here.

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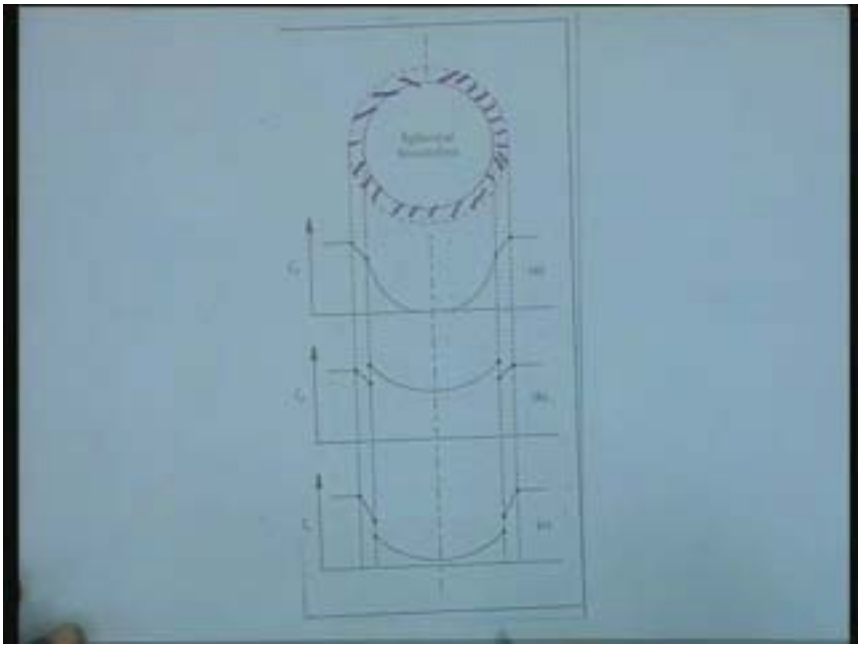

This is substrate concentration. “a” is shown as substrate concentration at the bulk phase, that means at the entry of the boundary layer. There will be a gradient of substrate in the boundary layer. This gradient will depend on the thickness of the boundary layer and you can minimize this by increasing a very high agitation speed in the stirred reactor or increase a very high linear flow velocity in a continuous reactor. There is a reduction but in the internal porous structure the substrate concentration may undergo also a gradient. This gradient it might so happen that it comes down to zero. The substrate concentration comes down to zero at this point and therefore all the enzyme present in this phase is not available and apparently it will look as if there is an enzyme activity loss.

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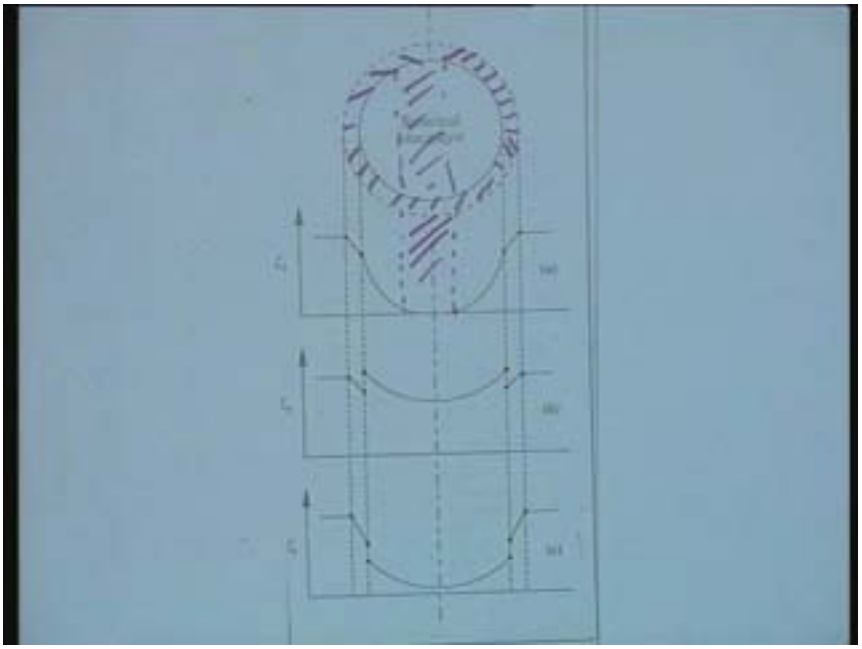

So the enzyme activity loss as a result of diffusional limitations is a totally apparent loss. If you reduce the particle size to the extent that the rate of diffusion is enough to counter or enough to take the substrate up to the interior of the particle. That means if you can reduce the particle let us say if this particle was only of this much size and this is the center, then it could happen that the enzyme might reach up to the center of the particle. The substrate might reach at the centre of the particle and all the particle is interacting with the substrate.

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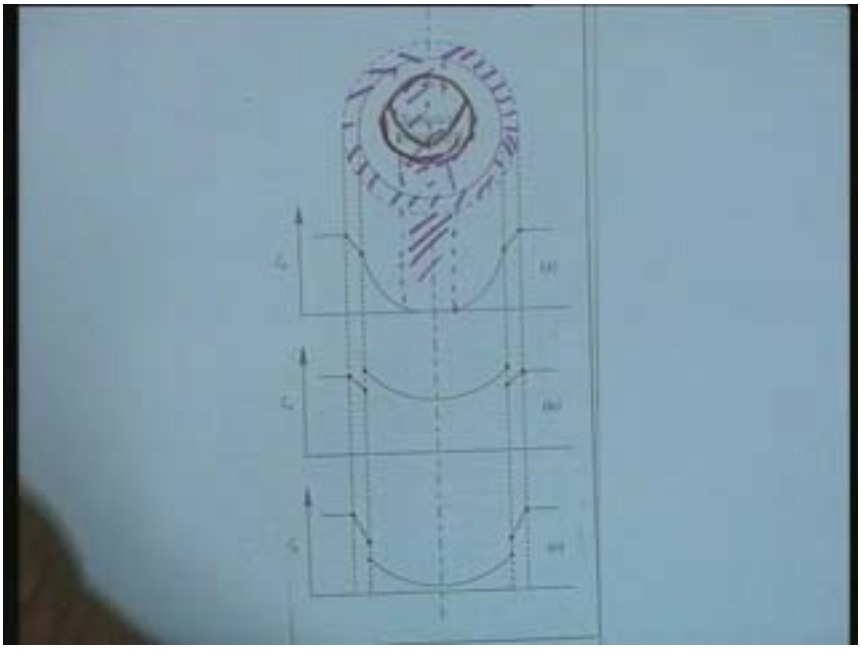

So there is no activity loss and that is what really I meant by the apparent loss of the enzyme activity that means by some physical interventions, by reducing the particle size one can overcome the internal pore diffusion. Similarly by increasing the agitation speed in the stirred vessel one can reduce or totally reduce the thickness of the film and thereby the gradient on the external film diffusion can be minimized.

In the part b and c, you notice the combined effect of partitioning and diffusion. You see here in the bulk phase, the part b shows that in the carrier particle the diffusional limitations are not very high. Although there is a very, very mild concentration gradient, the substrate is present all through the carrier particle. But the concentration of the substrate in the carrier is higher than the bulk. So there is a partitioning effect. That means the chemical nature of the carrier has more affinity for the substrate than the bulk. In the c part the reverse is there. The substrate has lower affinity that means suddenly from this boundary surface the concentration drops and there is a partitioning. So these pictures shows the first one is totally diffusion; the b and c part indicate the combined effect of diffusion and partitioning.

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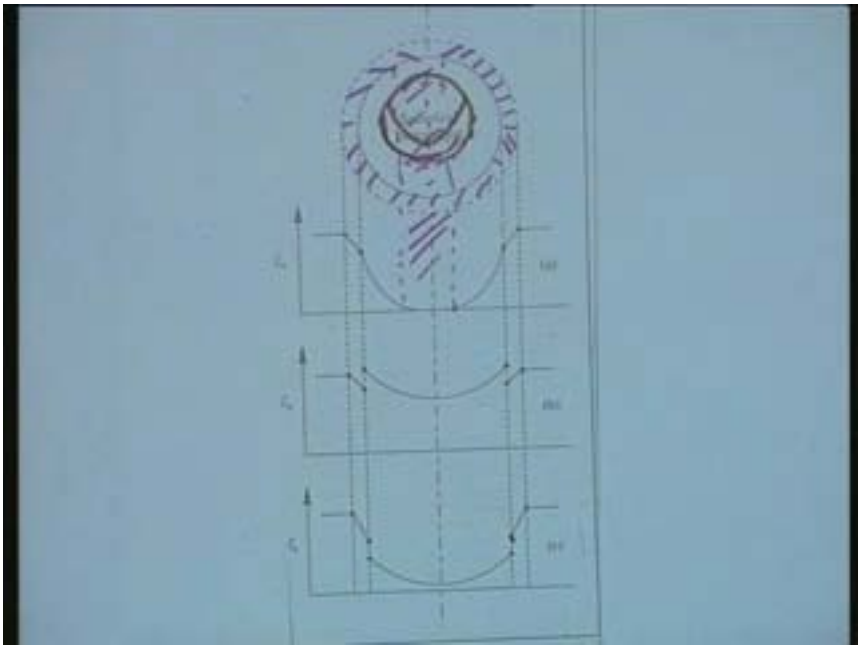

These are the four major parameters or the factors which cause the various effects on the functional properties of the enzymatic reaction.

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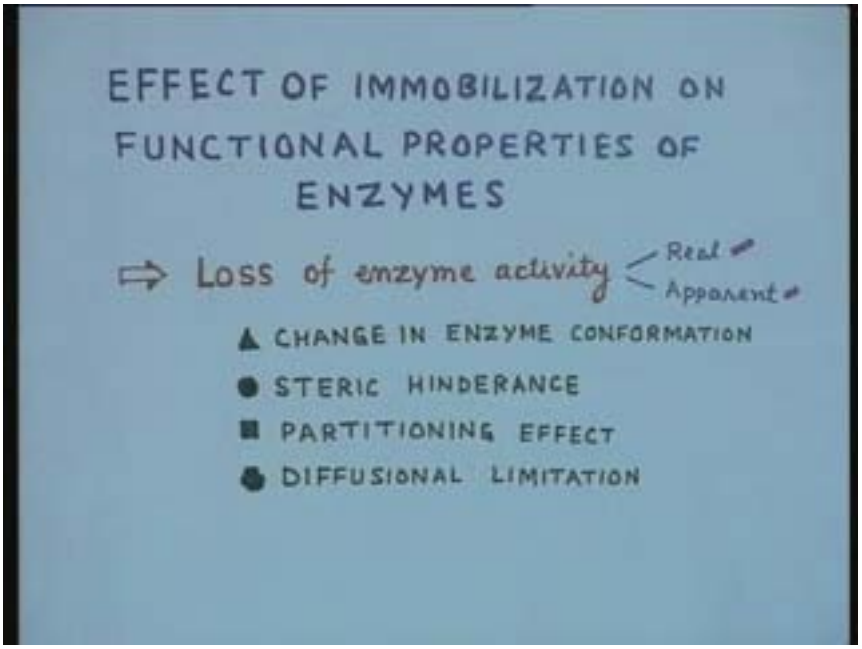

Whenever we have to look at any particular feature of an immobilized enzyme reaction, we have to look into these four effects and try to find an answer to the observed behavior. Let us look at some of them. The first is effect of pH.

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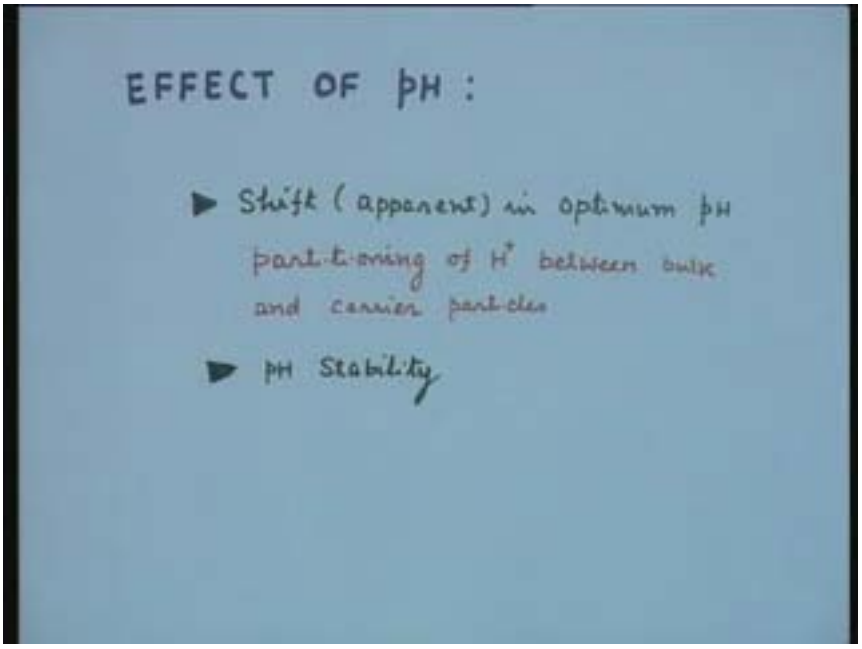

Very often we look at enzyme catalyzed reaction unknown to have an optimum pH and particularly in the case of immobilization by ionic binding it has been noted that there is an apparent shift in the optimum pH. When you take a soluble enzyme, a native enzyme and determine its optimum pH as against the optimum pH of an immobilized preparation

you find that the optimum pH has shifted and this shift is attributed mainly to the partitioning of hydrogen ions between bulk and the carrier particles. That means the partitioning effect of the hydrogen ions between the bulk and the carrier particle depends on the nature of charge. If suppose the carrier is negatively charged, there will be higher concentration of the hydrogen ions compared to the bulk and vice versa and therefore in the case of a negatively charged carrier, the apparent shift in pH will be on the alkaline side. Because for the higher pH in the bulk the actual pH in the carrier particle will be lower. Hydrogen ion concentration is more in the carrier. So there will be apparent shift in the pH optimum because what we are considering we are not varying the pH in the carrier particle; we are varying the bulk pH. So at even a higher bulk pH at the enzyme reactive site the pH will be lower and so we will consider it almost like an apparent shift in the optimum pH on the alkaline side and the reverse is true. If suppose you take a polycationic carrier, the shift will be on the acidic side.

The second effect of pH is on pH stability.

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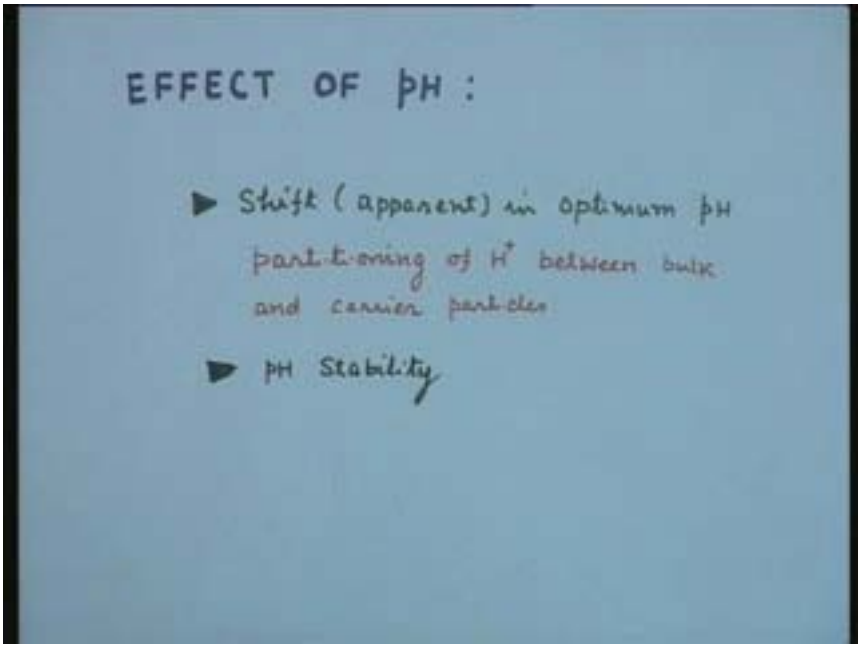

The same thing, the same partitioning effect. Particularly in some cases of the enzymes which are known to be more stable under alkaline side or acidic side, an immobilization can lead to increased or decreased stability of the enzyme depending on their behavior, although the pH stability as well as optimum pH in the real sense at the molecular level does not change. The behavior remains the same. It's only an apparent change in the monitoring system because we are measuring in the bulk and therefore the two effects are noted.

If you consider the stability fact of the enzyme with respect to pH certain enzymes are more stable under alkaline conditions. So the immobilization to an anionic polymer will

be able to give you a more stable preparation with respect to pH. I am talking of stability with respect to pH and not thermal. There will be no effect on thermal as a partitioning effect. But there will be some effect on the pH stability because the pH what you are offering to the enzyme particle in the bulk is not the same at the enzyme active site. It is reduced and if it is less stable at the alkaline pH, then even at the higher pH the enzyme particle may show higher pH stability.

Right. They are the same. The physical reason which attributes to both the effects is identical. That is partitioning of the hydrogen ions between the bulk and the carrier particle.

As an example if you look at some of the optimum pH of the immobilized preparations, which are reported in literature you notice that in most cases here the effects are mostly on ionic binding. But the pH effects are noted on a minor site in cases of gel entrapment and non-specific binding and particularly crosslinking effects also.

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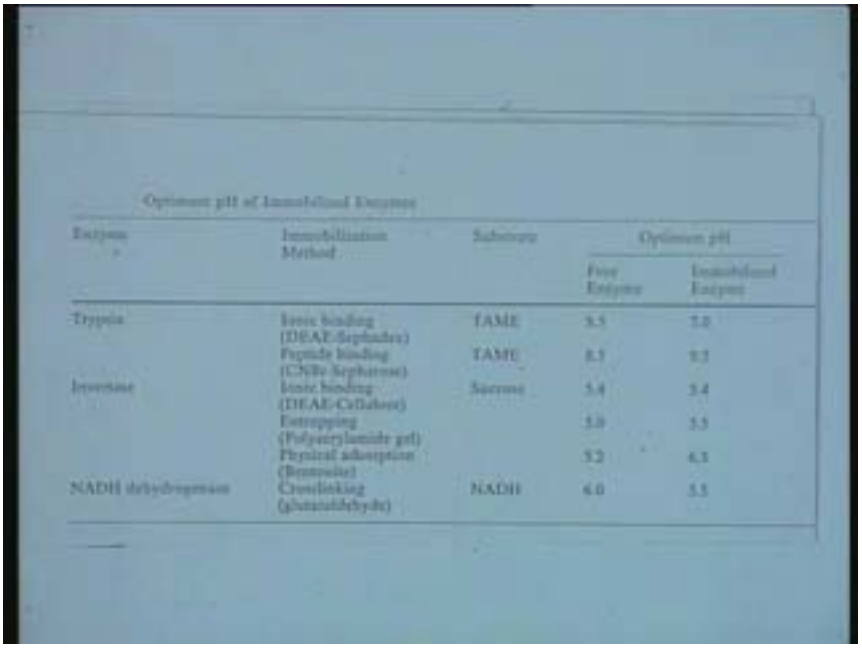

But they may be a result of a very complex system which cannot be defined very accurately but ionic binding can be defined very accurately. In trypsin by binding on to DEAE Sephadex the free enzyme pH which was 8.5 was reduced to 7.0. Similarly on invertase DEAE cellulose, 5.4 to 3.3, the immobilized enzyme optimum pH is reduced.

Yes. Diethylaminoethylether has lot of amino groups with positively charged matrix and therefore the enzyme activity is reversed. On the other hand look at an example here on physical absorption on bentonite. Bentonite is clay with high negative charge and this attributes to an increase in the optimum pH. Some optimum pH changes have also been reported in the case of crosslinking but their effect of change in the optimum pH is a

result of some other conformational interactions and not due to partitioning. Particularly the last example of the immobilization of NADH hydrogenase by crosslinking also indicates the shift of the optimum pH but here the optimum pH shift will not be attributed to partitioning effect.

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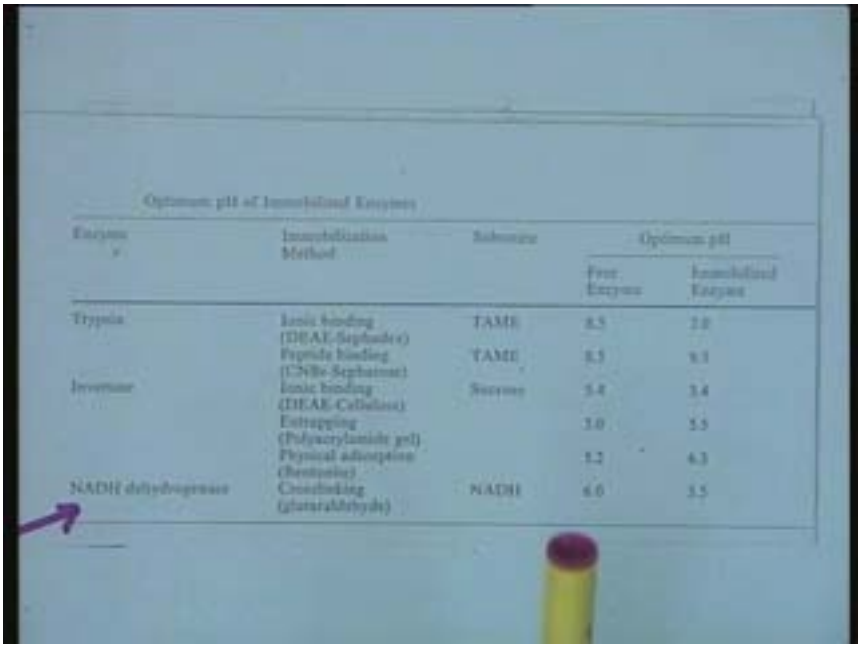

It could be due to some of the conformational changes as a result of crosslinking because conformational changes also can bring in some change in the optimum pH.

A quantitative analysis of the partitioning effect can be given in terms of a factor what we can call the partitioning coefficient, the partition factor which is the ratio of concentration of any specie on the enzyme active site divided by the concentration in the bulk

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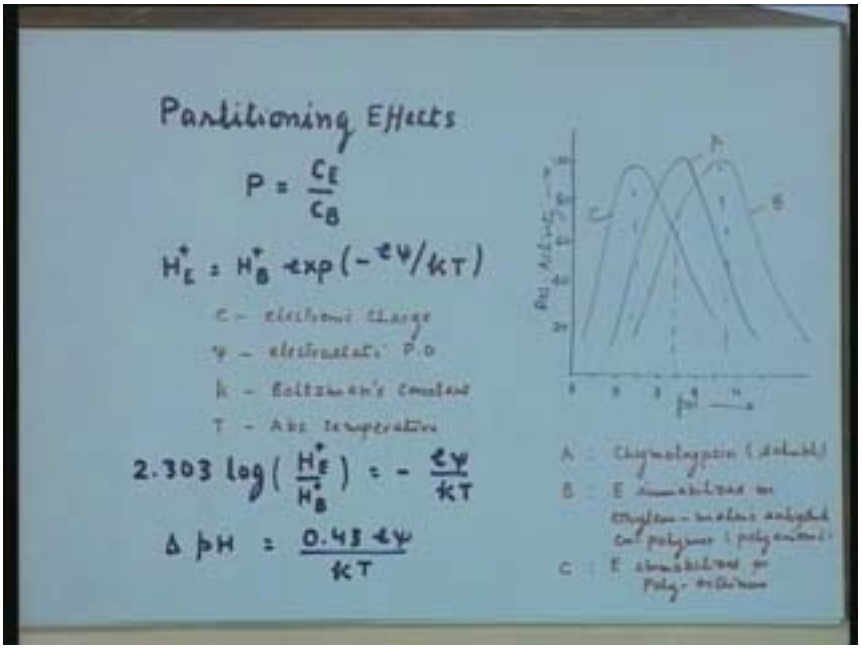

If you consider the pH effects or hydrogen ion concentration it can be described in the two phases. That means at the solid phase in the enzyme active site and in the bulk phase by Boltzmann type of distribution where you can give the concentration in the enzyme phase that is in the carrier matrix given by

$$H^+_E = H^+_B \exp(^{-e\psi/kt})$$

where E is the electronic charge, ψ is the electrostatic potential difference between the two phases, k is the Boltzmann constant and t is the absolute temperature. Simplifying this you can get ΔpH as equal to

$$\Delta pH = 0.43e\psi/kt$$

ψ is the electrostatic potential difference between the two phases. That means the potential as a result of the charge in the two phases. If you notice the shift up to what I have already told you see pH effect change. A is the native enzyme, B is the same enzyme chymotrypsin immobilized on ethylene maleicanhydride copolymer which is polyanionic and the phase shift is towards the alkaline side.

H is the hydrogen ion concentration which you can convert into pH. On the left hand side the profile c indicates a pH shift to the acidic side when the enzyme is immobilized on polyorthanine (31:29) which is a positively charged matrix. This has been one of the attributes in the case of immobilized enzyme preparation where many times we require a particular operational pH for certain other conditions. I must give you an example of glucose isomerase. Glucose isomerase, some of the native enzymes have a pH optimum of about 7.5-8, alkaline. An alkaline pH for isomerisation of glucose is not a desirable feature because it will undergo a lot of non-specific, non-catalytic, non-enzymatic reactions on glucose isomerisation producing products other than fructose. Other than

fructose, it will produce cyclose (32:21) and galactose and all other sugars which are not desirable in the product. A very significant property of apparent change in the enzyme optimum pH has bee used by immobilizing those enzymes to a positively charged matrix. You can use the enzyme in the bulk lower pH although the actual pH of the active site will be same 7.5 or 8 whatever is the optimum pH of the native enzyme. But if the reaction can be carried out at a pH in the bulk which is lower, the side reactions can be totally overcome. So that gives you another major advantage for using an immobilized preparation. The example of the glucose isomerase is a typical example which has used such a phenomenon.

The other effect of this immobilization is on the kinetic parameters.

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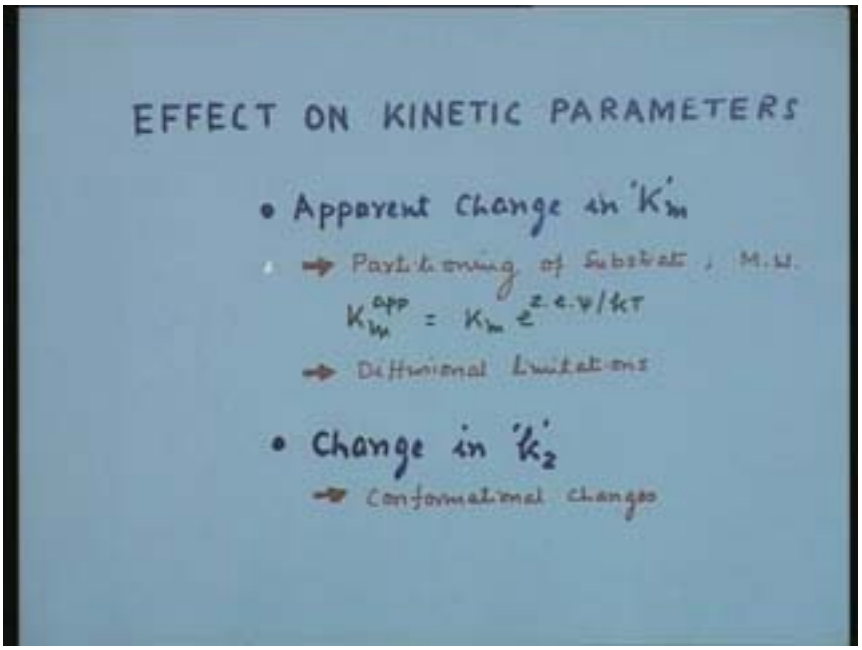

Can kinetic parameters also undergo some apparent change? Although the kinetic parameters of the enzymes does not change in the real sense but there are apparent changes in the kinetic parameters. First we consider the k_m, the Michaelis Menten constant. In most cases you will notice, if you look into literature, that the k_m value undergo a change. The changes can be of various nature. Different methods have yielded increase in k_m value, decrease in k_m value and sometimes no change in k_m also. The change in k_m value can be attributed to diffusional limitations as well as partitioning of substrate. Very often wherever the substrate is also a charged molecule similar partitioning effect will take place. There will be a difference in the substrate concentration between the bulk and the enzyme active site as we saw in the substrate profile of the immobilized enzyme particle that in the bulk phase and in the immobilized carrier particle, there will be a substrate gradient and depending on the chemical nature that will undergo and k^{app}_m can also be related to k_m of the native enzyme by

$$k^{app}_m = k_m e^{z.e.\psi/kt}$$

where z is the number of charges. It's an observed change. …… (35:10) does not change really; on the on the substrate if we consider the charges on the substrate.

The second reason for the apparent change in k_m would be attributed to diffusional limitations.

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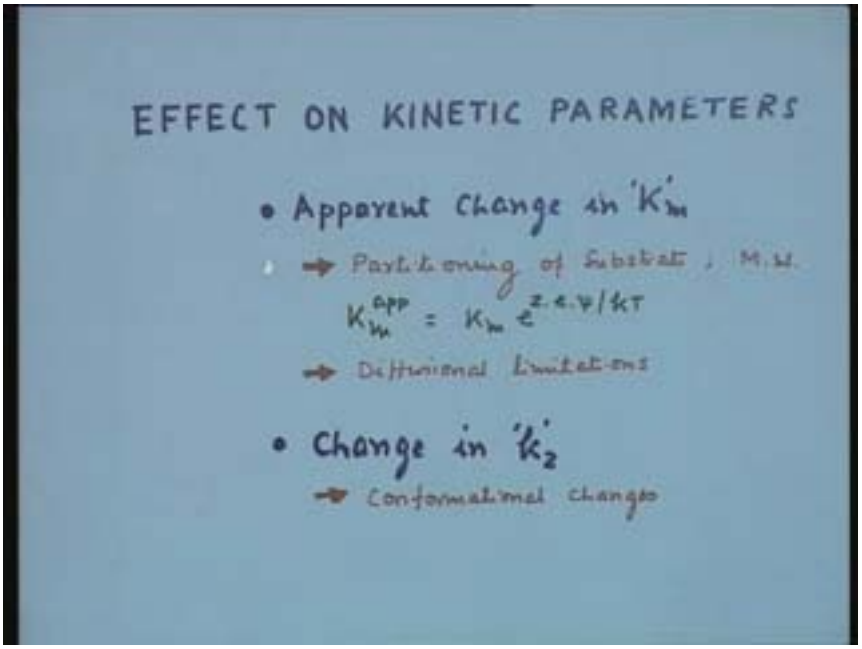

The effects due to diffusional limitations on k_m have been clearly demonstrated by experimental data because if you just use an immobilized enzyme preparation in a reactor and measure k_m by reducing the particle size you are able to come to a k_m value which is of the native enzyme which only implies that the diffusional limitations are playing a role. One of the result reports the k_m value of the immobilized glucoamylase on different substrates: soluble starch, amylopectin, amylose and maltose; four different substrates which can be acted upon by glucoamylase or amyloglucosidase.

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Michaelis Constant of Immobilized Glucoamylase (CABRAL, 1982)

Substrate	K_m (g/L)	
	Free Enzyme	Immobilized Enzyme
Soluble starch	1.0	15.1
Amylopectin	1.2	16.0
Amylose	3.0	16.5
Maltose	10.8	4.87

For the free enzyme the k_m values are given here on the left hand side. For the immobilized enzyme you see the k_m values have significantly increased except on maltose where it has decreased. This also gives you the feature that the molecular weight of the substrate can also influence the partitioning effect because the maltose being the smallest molecular weight molecule substrate here, it can easily and freely pass through a carrier particle and thereby one can achieve a much higher k_m value compared to the insoluble one.

k_m in the case of maltose has decreased. It has decreased means higher affinity; the concentration of the substrate in the enzyme active site is much higher. This concentration is in the bulk. So it affects particularly high molecular weights substrates and they behave in a reverse way. Therefore their concentration usually at the site is much smaller because of the diffusional barrier and therefore the k_m value is …. (37:48). In most cases they are usually considered to be an increased k_m value which is not a desirable feature. This has to be taken care while preparing the immobilized enzyme preparation so that the k_m value does not increase because otherwise it will lead to a less sufficient catalysis.

Another data is on the influence of particle size on the Michaelis constant of immobilized enzyme. If you look at soluble starch as substrate as a function of particle size, particle size being in micro meters the k_m value increases as the particle size increases.

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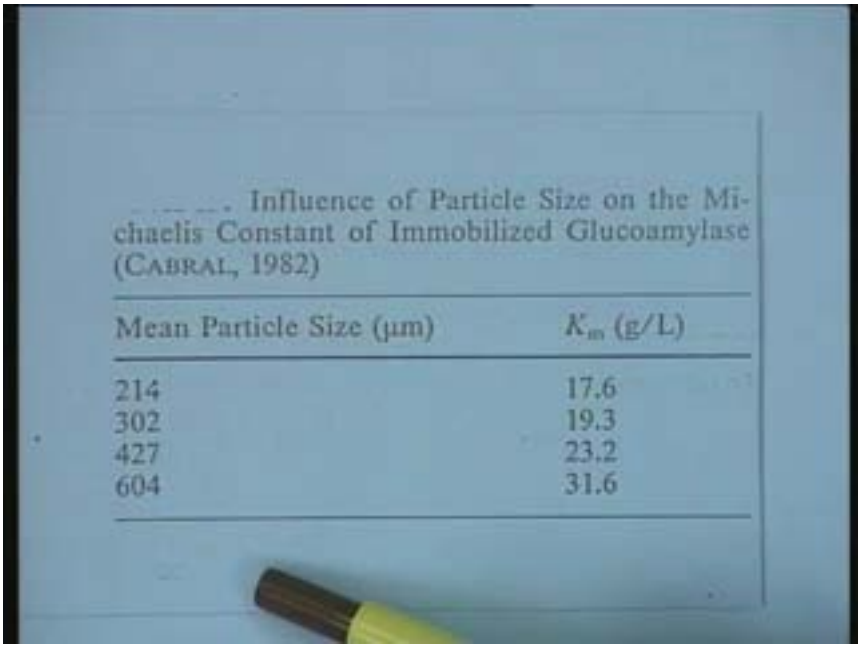

This clearly demonstrates the role of diffusional limitations on the effect of k_m and with the particle size increasing the system becomes more and more diffusion controlled for the particle is bigger. For example roughly about two fold increase causes 19.3 grams per liter to 31.6, a very significant increase in k_m value.

Similarly on the external film diffusion also the linear velocity of the immobilized glucoamylase using the 30% weight by weight 40DE corn syrup, the data here are important in the case of starch syrup because starch syrup when we use for hydrolysis we use a very thick syrup.

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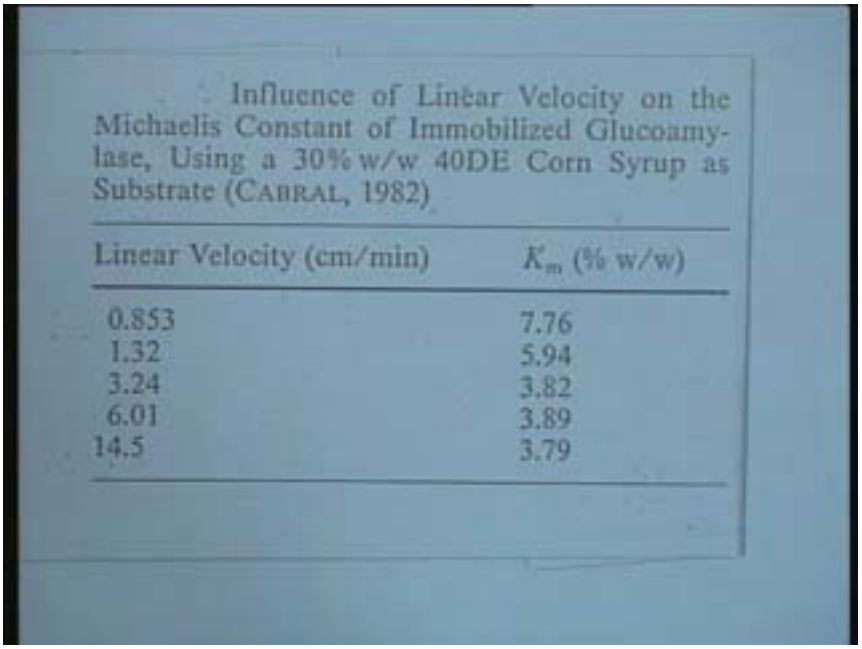

Influence of Linear Velocity on the Michaelis Constant of Immobilized Glucoamylase, Using a 30% w/w 40DE Corn Syrup as Substrate (CABRAL, 1982)

Linear Velocity (cm/min)	K_m (% w/w)
0.853	7.76
1.32	5.94
3.24	3.82
6.01	3.89
14.5	3.79

Even in the case of glucose isomerase when we carry out the industrial reactions, the substrate concentration which is used is about 2.5-3 molar glucose which means almost about 45-50% weight by volume. So it is very thick syrup and at such thick syrup, the boundary film can be very thick. The film thickness can be very high and external film diffusion in those cases where we use substrates as very thick syrup is usually very high and external film also becomes a major parameter and as shown here if you consider a packed bag reactor with a liner flow velocity from 0.85cm/min to almost as high as 14.5 almost twenty fold increase gives you a k_m value which decreases from 7.76 to 3.79 almost half the initial value of k_m. That means if you are able to overcome external film diffusion the increase in linear velocity means you are reducing the thickness of the boundary film and that's why when we talk of k_m we are talking only apparent change and not the real change.

The other parameter which is important in the case of kinetic parameter is change in k_2 that is the turn over number or in other words you can say if you multiply with the enzyme concentration it becomes V_m.

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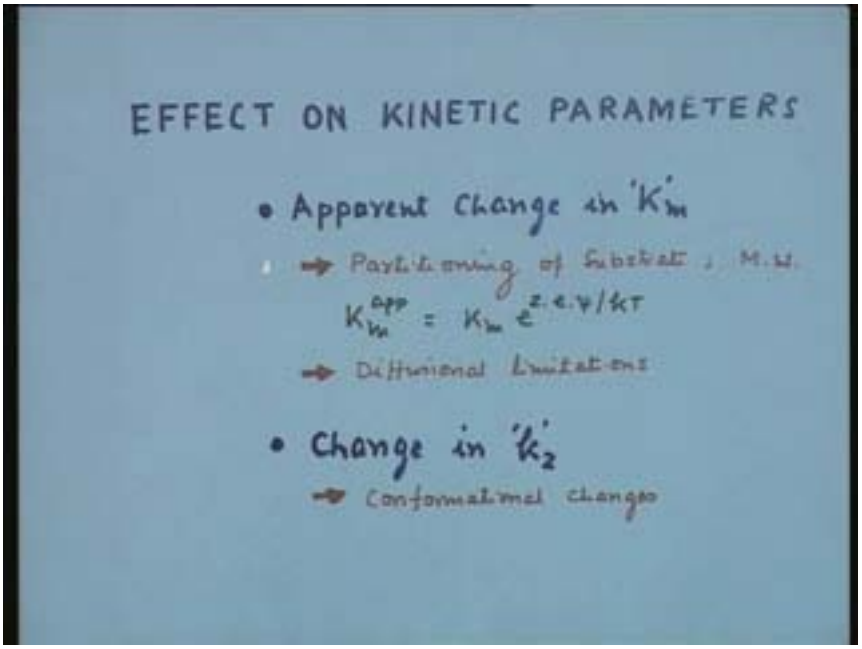

Enzyme k_2 change is solely attributed to conformational changes. That is if the enzyme undergoes a conformational change its specific activity will undergo a change. In some cases the change has been favorable. There have been reports, although rare not very common, that the enzyme activity increases as a consequence of immobilization. Another functional parameter is temperature which is important for all catalyzed reactions.

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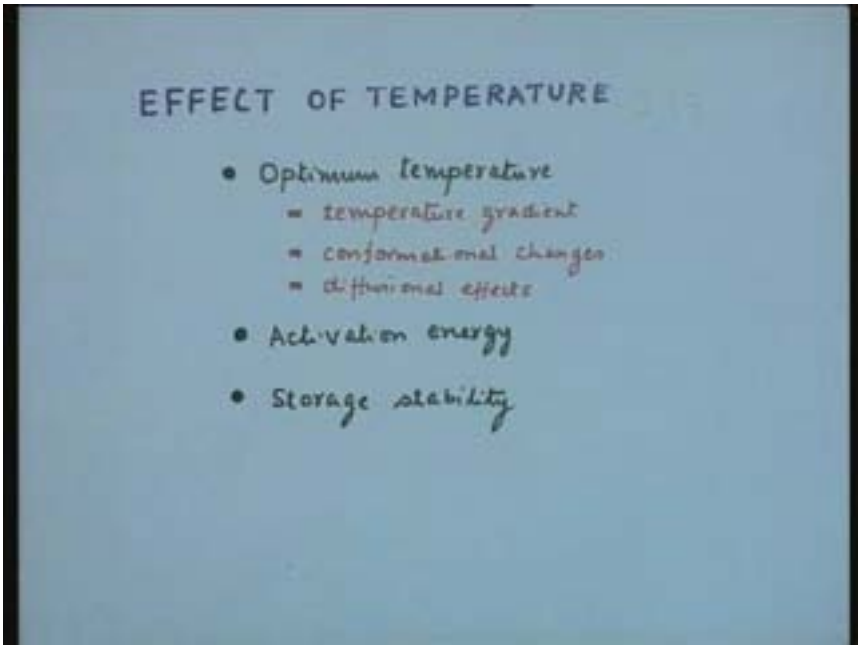

This temperature has mainly three consequences; one is the optimum temperature of the reaction. Just like optimum pH, we also need an optimum temperature for a reaction which is known for a native enzyme. In many cases there has been report in the change in the optimum temperature also. The change in the optimum temperature is a result of temperature gradient across the bulk and the enzyme particle and this is very prominent when you talk of gel entrapped enzyme preparation. The gel acts almost like a thermal insulating material and therefore the temperature at this site is relatively much less as compared to the bulk temperature and therefore apparently the apparent optimum temperature will be higher than what is required. At the active site it is the real optimum temperature but in the bulk it is slightly higher because there is a gradient of temperature between the bulk. Optimum temperature has also been attributed to conformational changes particularly in the case of adsorbed enzyme preparation or covalently coupled preparations where there is no likelihood of any temperature or you can even notice the temperature changes in the case of a non-porous matrix where all the enzyme is just immobilized on the surface of the matrix. There is no porous network and in those cases if there is optimum temperature change it is attributed to conformational change and diffusional effects are also very prominent with respect to temperature effects mainly because the diffusion itself is a process which is very significantly affected by temperature. In fact the diffusion process is much more sensitive to temperature effects than many of the catalytic reaction rates. The rate of diffusion can be enhanced significantly as a result of increase in temperature and particularly in the case where the system is diffusion controlled the temperature optimum might undergo significant change.

In the case of enzymes which are immobilized by carrier binding methods, for example, either by adsorption or by covalent binding where there are no there are no likelihood of much temperatures gradient in the bulk and the enzyme active site this can be very easily checked. In case if you consider a non-porous matrix where all the enzyme is supposed to be on the surface there is no porous network. In those cases the change in the optimum temperature will be attributed to conformational effects only because there is no chance of any diffusional effects. There is no chance of any temperature gradient. In different cases different kinds of changes in optimum temperature have been noticed.

Then the second parameter which is affected by the temperature is the energy of activation. The effect of temperature on reaction rate gives you a parameter activation energy and which is considered also to be a characteristic parameter for any chemical reaction and in the case of activation energy the major reason, the contributing factor is the diffusional limitations that is the rate of diffusion because as I mentioned that the diffusion process itself is highly sensitive to temperature and the activation energy in the case of immobilized enzyme preparation is a net resultant of the two temperature dependent processes the chemical reaction and the diffusion. So the net effect of the temperature on the overall reaction rate which is a combined parameter between the rate of diffusion and the rate of reaction can be different than the native enzyme because there is no diffusional limitation.

In the case of activation energy and optimum temperature I mentioned that different systems have shown different kinds of behavior. There is increase in activation energy there are reports on decrease in activation energy. Similarly there is increase in optimum temperature; there is decrease in optimum temperature and there are cases where there are no changes also.

But one parameter what is understood as storage stability which is attributed to the thermal deactivation of the enzyme with time is always favorable in the case of immobilized enzyme preparation mainly because of the additional intermolecular forces and the intermolecular forces that are involved which keep the process of deactivation to a minimum.

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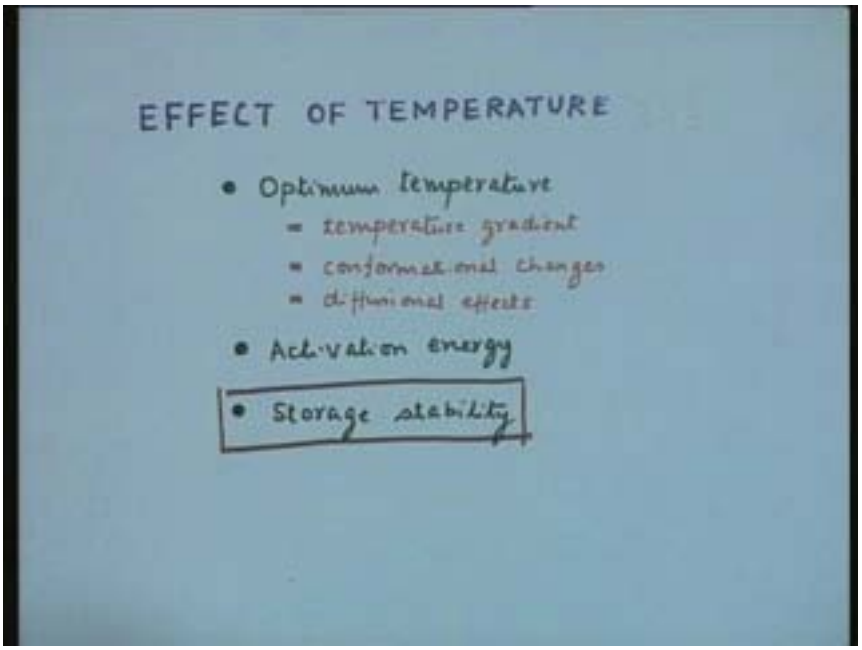

In almost all cases of the immobilized preparation the storage stability has noted to be higher in terms of its half life. So that is almost a proven fact and that is one advantage we have to take of immobilized enzyme for all operational purposes. Take an example of optimum temperature of various immobilized invertases. As you notice here the native invertase has the optimum temperature of 55^{o}C and by immobilized preparation by different methods or like physical adsorption on activated carbon is 40^{o}C, ionic binding to DEAE cellulose is 30^{o}C which is a very significant change.

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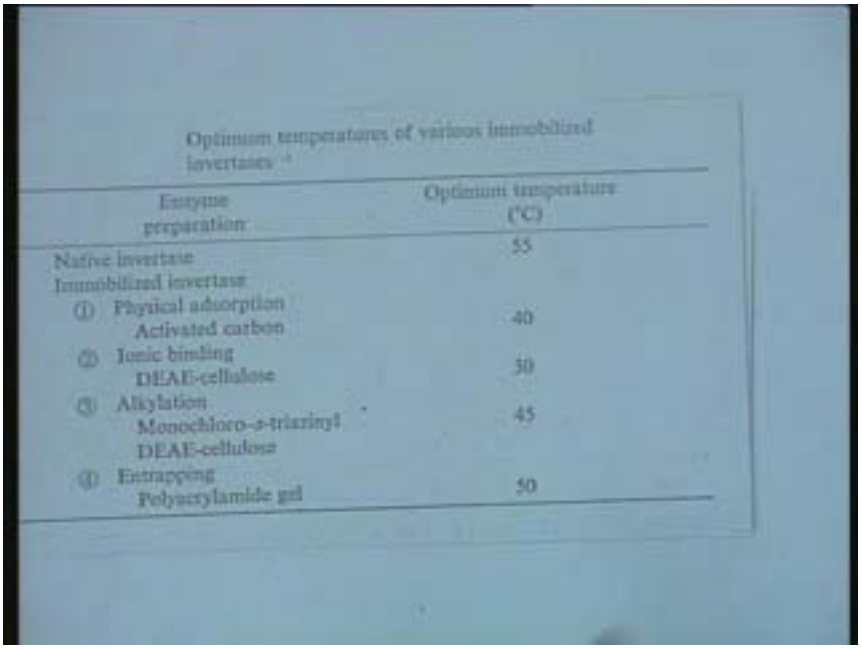

Optimum temperatures of various immobilized invertases

Enzyme preparation	Optimum temperature (°C)
Native invertase	55
Immobilized invertase	
① Physical adsorption	
Activated carbon	40
② Ionic binding	
DEAE-cellulose	30
③ Alkylation	
Monochloro-s-triazinyl DEAE-cellulose	45
④ Entrapping	
Polyacrylamide gel	50

As I mentioned some time earlier also that the adsorption process undergoes slight unfolding of the enzyme protein and unfolding of the enzyme protein is an endothermic process which results in reduction in the optimum temperature; alkylation by covalent binding is 45ºC, entrapment is 50ºC. Here entrapment is solely as a result of insulation effect, the temperature gradient.

In the case of gradient, but here that means there is some conformational change also occurring. There are some minor conformational changes but not as high as alkylation or ionic binding but there is some conformational change also occurring. In fact optimum temperature should be higher than entrapment if it is solely by temperature gradient. No. I am not talking about the stability. I am talking of the initial reaction rate as a function of temperature; not stability.

Look at the data on the activation energy of various immobilized aminoacylases. I will come to operational stability a little later. Activation energy for the immobilized aminoacylases immobilized by different methods also undergoes a change.

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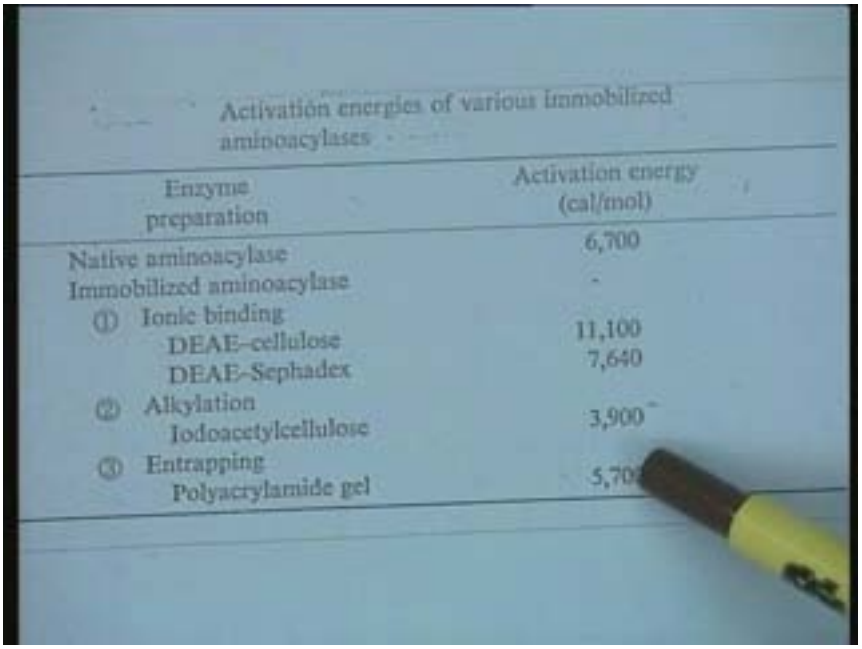

Activation energies of various immobilized aminoacylases

Enzyme preparation	Activation energy (cal/mol)
Native aminoacylase	6,700
Immobilized aminoacylase	-
① Ionic binding	
DEAE–cellulose	11,100
DEAE-Sephadex	7,640
② Alkylation	
Iodoacetylcellulose	3,900
③ Entrapping	
Polyacrylamide gel	5,70

As you will notice that in both the types of behavior there is a significant increase on DEAE-cellulose by ionic binding or DEAE-Sephadex slightly less increase but on covalent binding there is decrease in activation energy and these change in the activation energy is a resultant sum total of the diffusional effects and the biochemical reactions and the entrapment in polyacrylamide also results in decrease in the activation energy.

Why? It should decrease. The diffusion will entirely depend on the preparation. There are both kinds of effects because the preparation can be made to be both diffusion limited as well as if the enzyme loading is not very high it can be biochemical reaction limited also and the activation energy measurements will indicate that figure. The measurement of activation energy is also used as a kind of a experimental data to check whether the system is diffusion limited or not. So it will entirely depend on the loading on the preparation. Here the figures are given under certain experimental conditions in the literature and under which depending on the diffusion limitation or the loading of the enzyme on the carrier will dictate the final activation energy parameter.

Finally the most important parameter which we are concerned within the case of immobilized enzyme is their use in continuous reactors or long term reactions whether we can use them in a stirred vessel or in a packed bed or in a …… reactors or a variety of reactor configuration that are known to us from chemical reaction engineering and we can see that the parameter which is most important to look at the immobilized enzyme preparation property is operational stability that over a period of time when we use it, it should remain stable.

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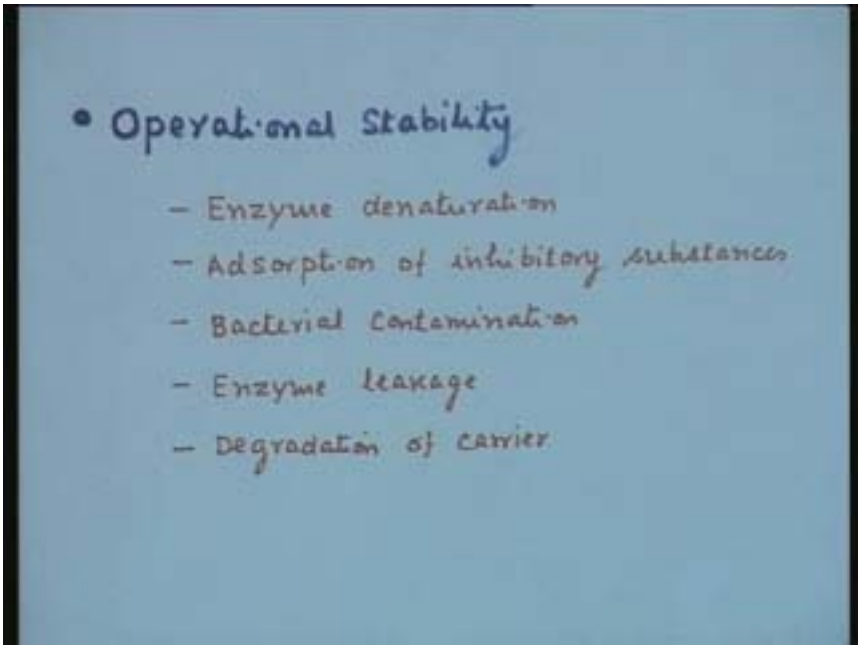

We measure it in terms of half life and the various reasons which cause the loss of stability during operation are enzyme denaturation, the thermal deactivation of the enzyme, adsorption of the inhibitory substances because the feed stream might include certain toxic substances which might inhibit the enzyme activity. Bacterial contamination is another very often faced problem in the case of enzyme reactor particularly if you are handling say for example a good nutritive substrate. Hydrolysis of proteins or even glucose syrup can undergo bacterial contamination. Then enzyme leakage which depends on the method of immobilization you are following. If you are using adsorb preparation there will be a continuous reversible desorption process and leakage might be there. Finally, degradation of carrier itself. During use, the whole matrix is under certain hydrodynamic condition and which might cause the degradation of the carrier.

You can get different kinds of profile as far as the operational stability of the immobilized enzyme is concerned. Y-axis is the activity factor, ln E/E_o

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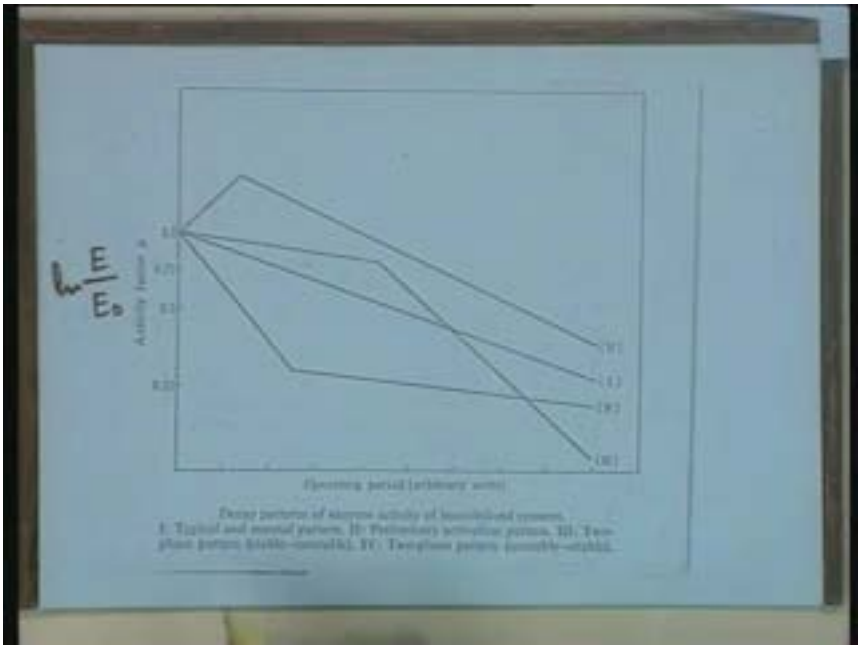

In the logarithmic scale you must get a first order decay as shown in the first case. These are the four different kinds of operational stability that have been noted and you will notice that they all …. (54:15) to certain discrepancies in the immobilization procedure. For example in the case of second profile, this one, there is an initial increase of the enzyme activity and then the normal decay. Very often such a profile is noted in the case of immobilized cells. In the case of immobilized cells initial autolysis of the cell releases some enzyme and then the enzyme remains entrapped and shows normal first order decay. But initial autolysis of the cell increases gives some …. (54:51) activity. In the third one, there are two steps in decay. Initially the stability is very high; suddenly there is a bacterial contamination action and the decay becomes very fast or some fault in the operation takes place. Contamination is a very usual problem in continuous reactors and therefore you have a very fast decay. In the case of fourth one initially you have very high decay and then it is continuous. This is some kind of incomplete immobilization. For example if you consider an adsorb preparation some of the enzyme is weakly bound which gets dissolved very fast and then the really adsorbed enzyme preparation shows you a normal decay. So different kinds of operation activity can be seen.