ENZYME SCIENCE AND ENGINEERING

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LECTURE-16

IMMOBILIZED ENZYMES – III

So we were talking about methods for immobilization of enzymes and in our earlier meeting discussed the first method called adsorption. This method includes various combination of methods including physical adsorption, ionic binding and metal salt linkage.

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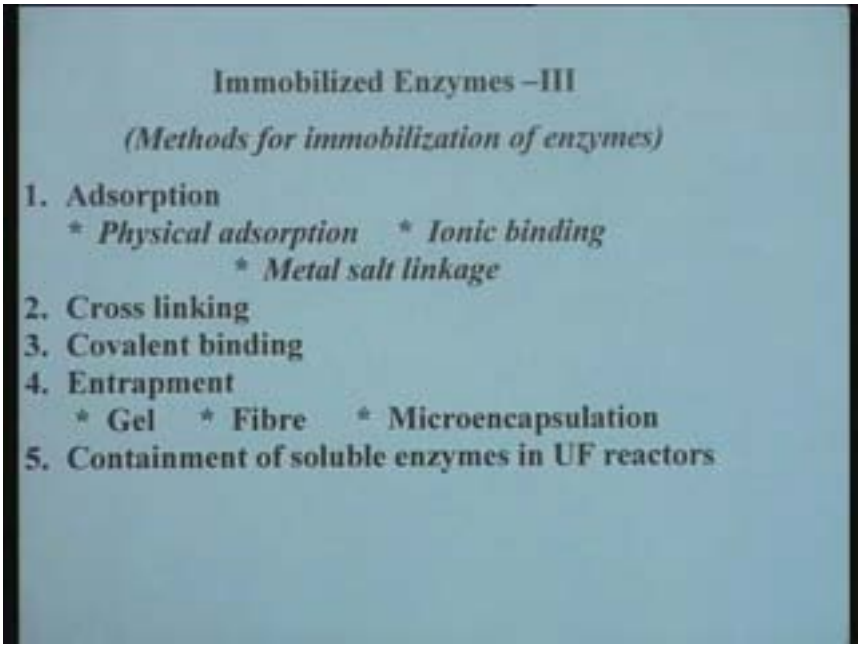

It also involves involvement of a carrier matrix and sometimes these are known as carrier binding methods. The other methods that are known and available for immobilization of an enzyme are cross linking, covalent binding, entrapment within a matrix-it could be a gel, fibre or a microcapsule and finally the containment of soluble enzymes in the ultra filtration reactors and they are the whole variety of immobilization methods out of which we will be talking in a short while about the cross linking methods.

The cross linking methods involve intermolecular cross linking of enzyme molecules in the presence or absence of a solid support. I would like to make it very clear the phrase, “presence or absence of solid support.”

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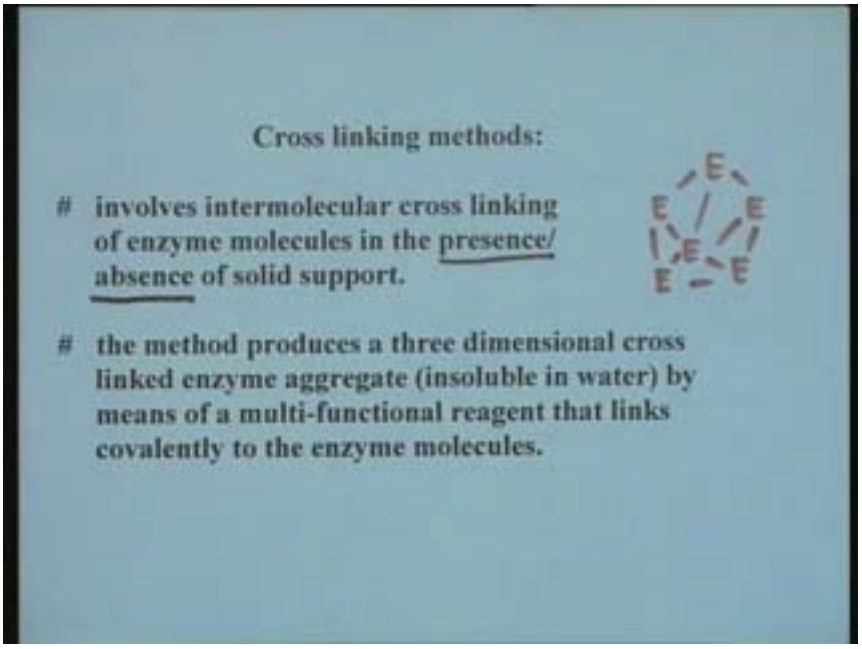

While initially the enzyme molecules are interlinked through a bifunctional reagent between some of the functional residues on the enzyme protein itself, the method produces a three dimensional cross linked enzyme aggregate. That means three or four or more enzyme molecules aggregate together through a covalent linkage mediated by a cross linking agent which is usually a multi-functional reagent and therefore the enzyme molecules form an aggregate that are insoluble in water. They retain the catalytic activity. That means the intermolecular cross linking between the enzyme molecules should not involve the catalytic functional group that is required on the enzyme molecule.

A characteristics feature of this method is that ultimately when you get this material you end up in a gelatinous mass. That means you don't get a particulate matter.

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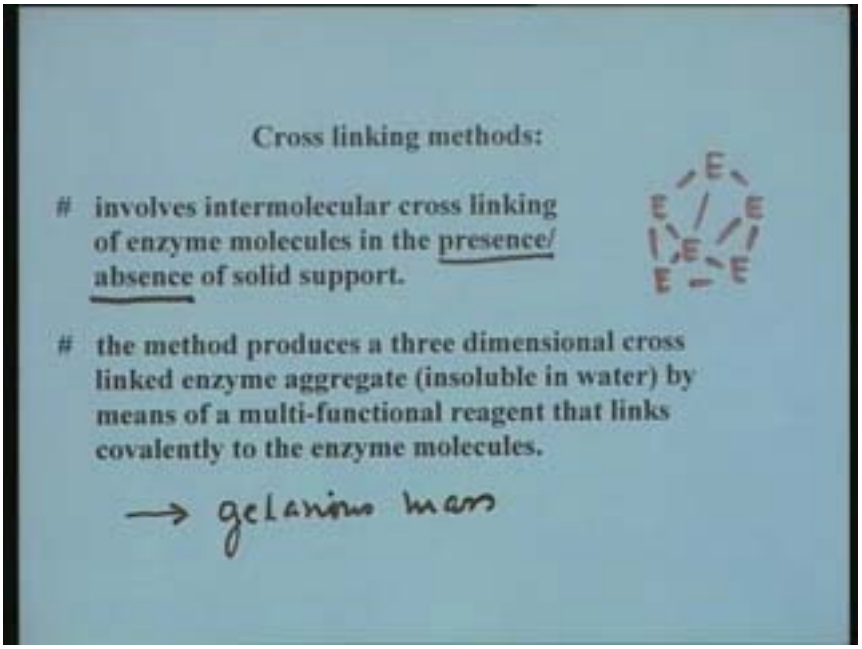

The enzyme molecule aggregates and comes out in the form of a precipitate, the gelatinous mass and when they are used in the enzyme reactors, they don't offer a good flow property in the reactor and therefore they are not a very desirable kind of a material which can be processed in the enzyme reactor. To overcome this very often they are used in the presence of a solid support. When we talked about adsorption we mentioned that adsorption being a reversible process, the immobilized enzyme preparations that are produced by adsorption usually do not have very high or long operational stability. To enhance the stability of the adsorbed material very often adsorption is combined with cross linking so that the cross linking is carried out in the presence of an adsorbate, a matrix on which adsorption can take place. Then the cross linking is carried out, so that you get a very stable immobilized enzyme preparation which also has the morphology of a particulate matter. If you recall the method of immobilizing enzymes by adsorption, one of the major drawbacks of the adsorbed enzymes is that they do not possess a very high operational stability.

To over come this problem if the adsorption process is coupled with cross linking of the enzymes so that ultimately what happens is that each of the enzyme molecule present here is supported on a matrix and also cross linked to each other so that it forms a multi variant force by which the enzyme will not be a desorbed because it is now covalently linked to each other.

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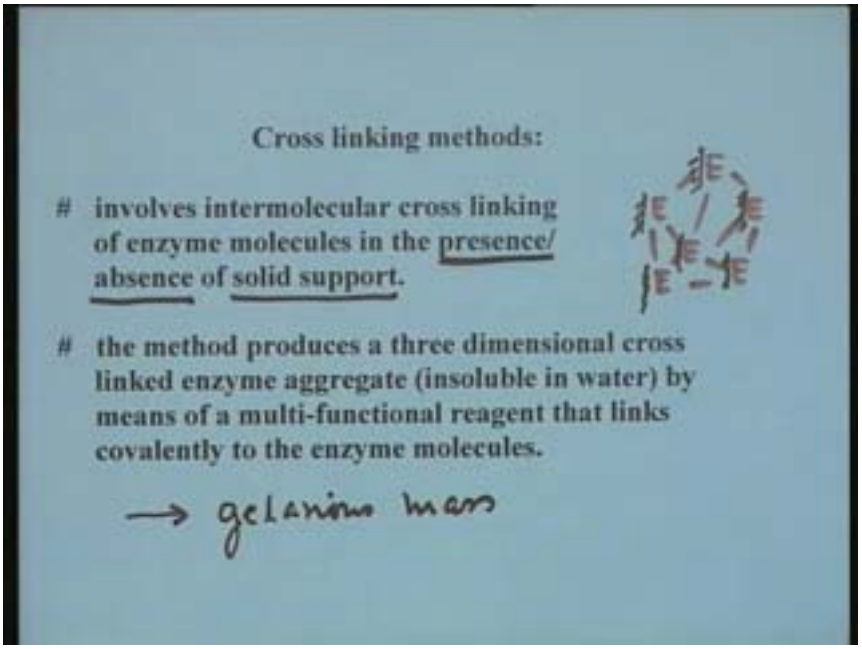

The other advantage is that the disadvantage of the cross linked enzymes, that is the gelatinous morphology will also be removed because the cross linked preparation in the presence of the adsorbent will have the morphology of the carrier particle. The adsorbent whatever we have used that morphology will be available and thereby one can get a much better preparation which can be used in reactor systems. One of the important things that we should recall is that these intermolecular cross linking requires certain very reactive reagents so that the functional groups of the enzyme can covalently bind under mild reaction conditions. Mild reaction conditions means, again I will I have to repeat, under those conditions in which the enzyme does not lose its confirmation or the functional reactivity. For those purpose we need certain reagents which are multifunctional and also very, very reactive. Some of the typical reagents, although it's not the complete list but some of the typical reagents which are commonly used are diazobenzidine, it has two functional groups and 1,5 – difluro – 2,4 – dinitrobenzene - two nitrate groups and fluro groups and trichloro-s-triazine and hexamethylene diisocyanate. The most commonly employed cross linking reagent over the years has been glutaraldehyde.

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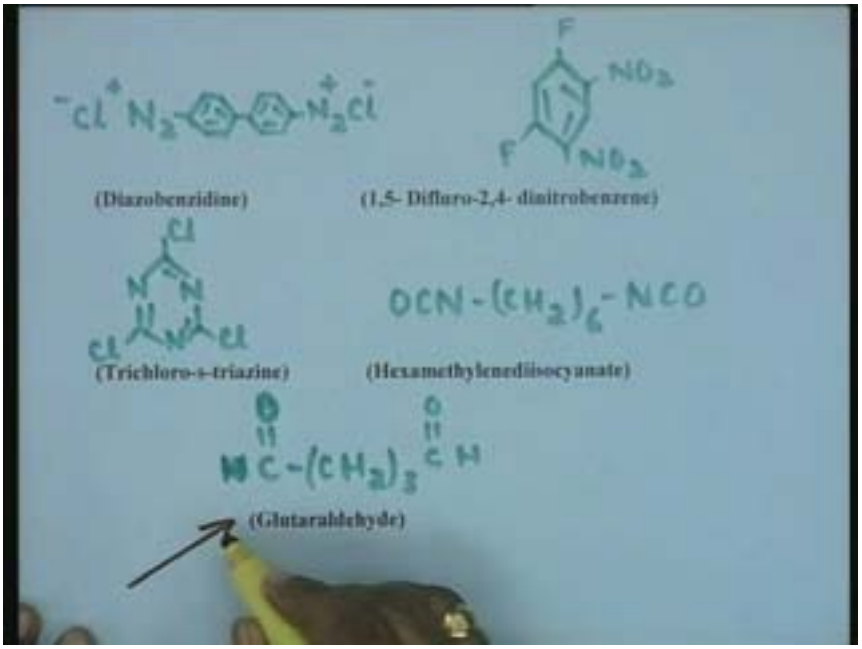

The most commonly used method of cross linking the enzyme molecules is by the use of glutaraldehyde which possesses two aldehyde groups separated by a hydrocarbon chain, the three $–CH_2$ groups. This reagent is very commonly used for all crosslinking experiments even routine as well as for practical. The glutaraldehyde is composed of two different reactions.

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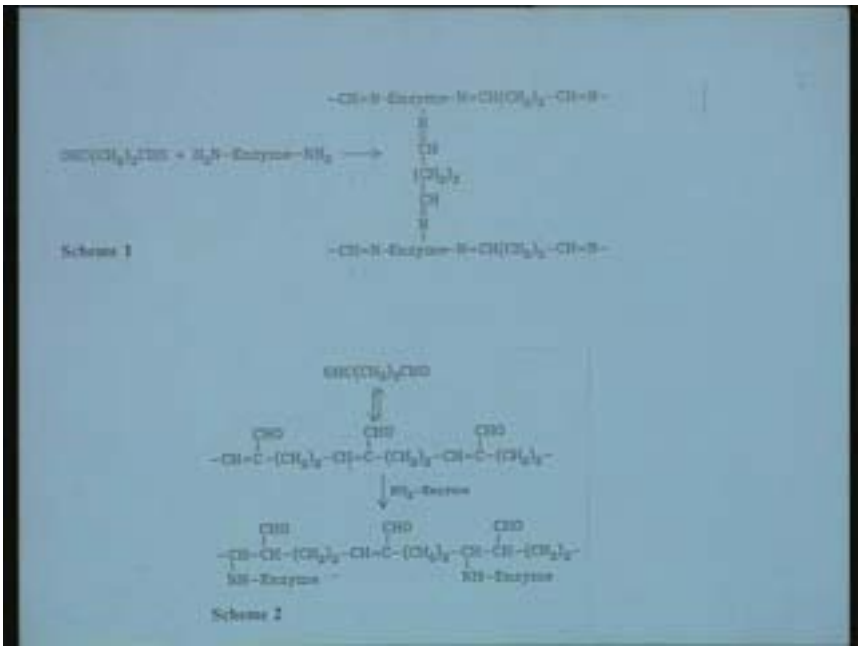

When you store the glutaraldehyde solution, it is present in two different forms. One as the free glutaraldehyde, a single glutaraldehyde molecule which contains two aldehyde groups separated by three $–CH_2$ groups and it also occurs in the form of a dimer, in this form. The two forms are present in equilibrium. It is available in this form as well in this form. From the two forms it gives you two different kinds of cross linked enzyme preparation. While in the case of the first, the monomolecular gluteraldehyde residues, they give you cross linking reaction between the enzyme molecule particularly amino groups of the enzyme molecules are involved in cross linking and you get a chain of the enzyme protein which gets aggregated and gets precipitated.

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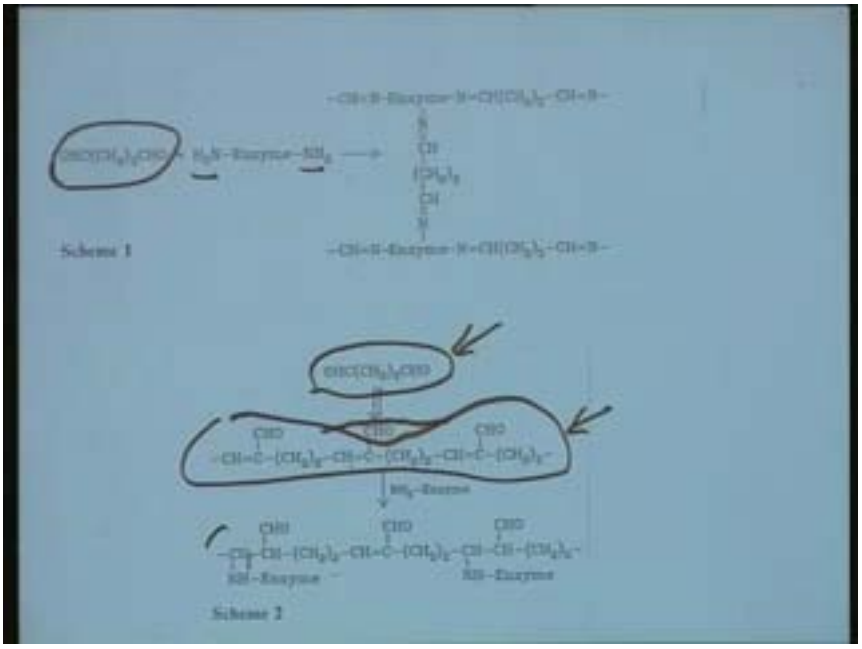

On the other hand the dimer of the gluteraldehyde forms an addition conjugate along with the enzyme and it again gives you a precipitated mass and both the forms remain catalytically functional. So on storage the concentration of this dimer increases. But the only difference will be that the reaction conditions that has to be employed, when I say reactions conditions, I am referring to the concentration of the gluteraldehyde, the ratio of gluteraldehyde to that of protein, the time of reaction and the temperature of reaction will vary in the case of two reactions.

Very often it happens that some standard conditions given in the book do not exactly replicate in a laboratory experiment mainly because if the gluteraldehyde solution available is of different age they would have under gone dimerisation. It will behave in a different way. But both the products are crosslinked preparations and are useful. The crosslinking method also must involve or identify certain optimum conditions for the crosslinking. The optimum conditions depend upon the balance of parameters like concentration of the enzyme and cross linking reagent, pH, ionic strength, temperature and reaction time. Out of a large number of studies that has been carried out for cross

linking particular for gluteraldehyde usually protein concentration of 50 to 200 mg/ml is the usual range in which we operate for crosslinking. The gluteraldehyde to protein ratio is also used at about 10% weight by weight and gluteraldehyde concentration also varies over a large range from 0.2 to 0.6% solution. That means a very dilute solution of gluteraldehyde is required and at a ratio of about 1:10. That means 10% of gluteraldehyde on the basis of protein content in the mixture and that is allowed to react for time period ranging from say about one to three hours to twenty four hours. This time period is dependent on temperature and the temperature requirement is linked directly with the stability of the enzyme. Some of the enzymes which are fairly stable, thermally stable, they can be held at the room temperature and you can carry out the crosslinking say for example in one to two hours, where as certain other enzymes which are very sensitive to heat, they require to be reacted at 4^{o}C and then the reaction requires a time interval of at least twenty four hours. So all these parameters need to be optimized for a particular enzyme system to be able to get a gelatinous mass, which is crosslinked enzyme preparation. While cross linking may become a useful method in the laboratory in the form of the gelatinous mass for the practical reactor operation the gelatinous mass does not offer useful flow properties in the reactors for a continuous reactor. Therefore we usually use it along with the adsorption method. That means we take an adsorbent, along with adsorbent or after adsorption is over we add gluteraldehyde to it and ensure crosslinking of the enzymes that are adsorbed in the matrix and the crosslinked preparation then provides you a good immobilized matrix for use in the reactors.

The other method and which has very large variety is based on the formation of covalent bonds between the enzyme and the support matrix under mild reaction conditions.

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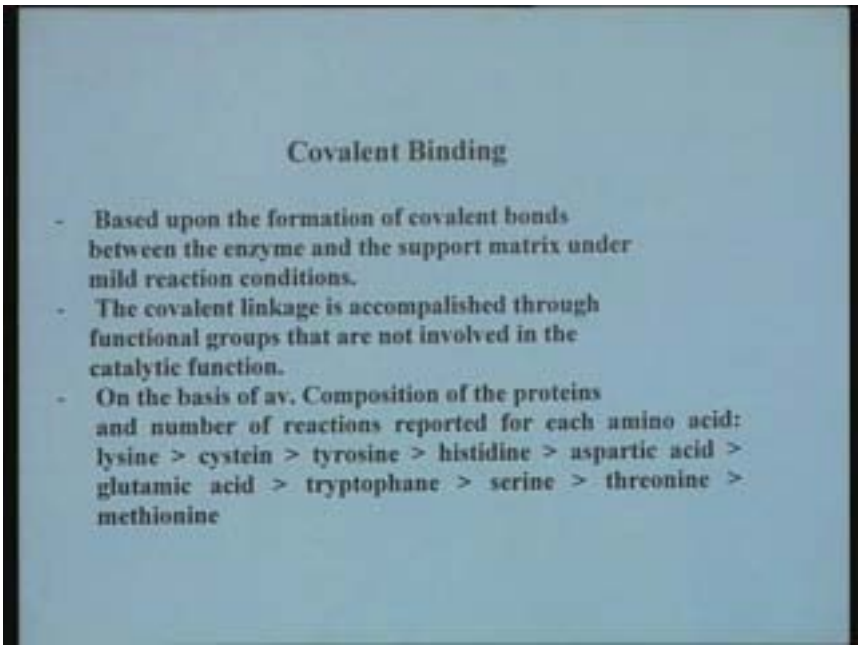

The term mild applies to the same feature and because we are talking about the formation of covalent bonds between the matrix and the enzyme molecule it brings in two major considerations. One is because of the covalent bond formation the immobilized enzyme preparation is very, very stable. Usually the covalently immobilized enzyme preparations will have a half life of few months when operated in a reactor and when stored under storage conditions it could go even for years without any loss of activity in terms of half life. But on the other hand because a covalent bond formation involves very reactive reagents for carrying out the reaction at room temperature or ambient conditions, it is bound to effect the confirmation of the enzyme protein. Although care need to be taken in covalent binding to ensure that the amino acid residues that are involved in the catalytic function are not involved in the covalent bond formation but still the reagents are not very specific. If suppose we design an experiment to make a covalent binding with n terminal amino groups of the enzyme protein, but if the reagents will act on n terminal amino groups, it will be very difficult to ensure that none of the amino groups of the lysine are involved. If lysine happens to be an amino acid residue at the active site there will be significant loss of activity. In practice while we get a preparation which is highly stable, we also get a significant loss of enzyme activity. Almost 40 – 60% loss of activity is a very common feature in covalently immobilized preparation. But if you consider the overall productivity of the immobilized enzyme that means the yield of the immobilization after considering the operational stability it still remains a desirable method. The choice of the method will again depend upon the carrier cost and the chemicals involved in the immobilization method and one of the disadvantageous part is that usually the carriers here cannot be regenerated. Because the carriers are functionalized they are attached with the protein and when enzyme confirmation is lost protein is still coupled to them in the deactivated form.

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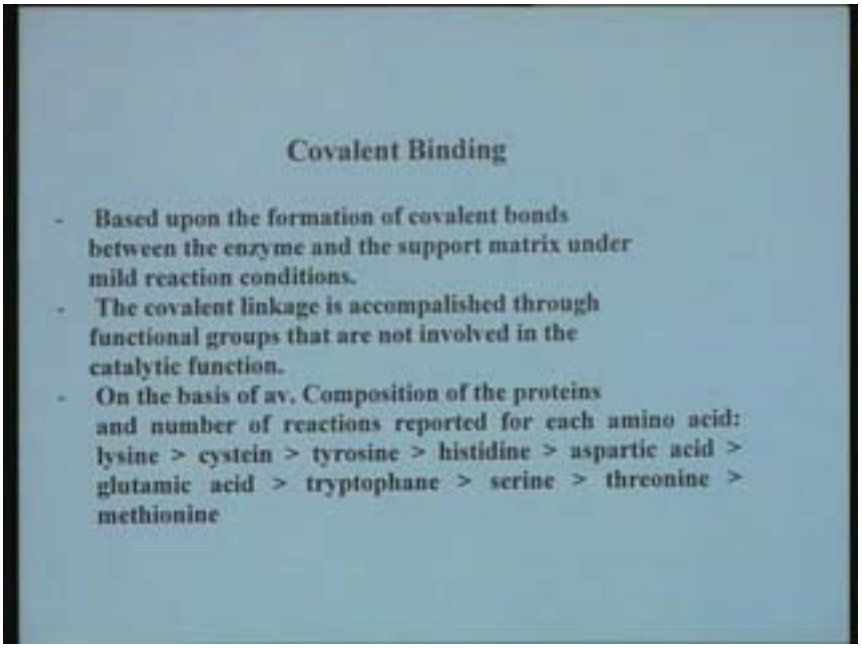

So the carrier cannot be regenerated. Therefore carrier cost becomes coupled with the cost of immobilization particularly in terms of chemicals it becomes quite high. So unless we want enzymes with a very high stability particularly when the enzyme cost is very high we don't want to lose them during the reaction and such methods are not preferred. Most of the chemistry of the covalent binding has been developed on the basis of the chemistry available for preparing the matrices for affinity chromatography. Because there also you couple a ligand on to a matrix by covalent binding to get a stable ligand bound matrix and almost similar chemistry because we have similar functional groups available here. It could be tertiary amino group of lysine or end terminal amino acid groups or a variety of other groups that are available on the enzyme protein.

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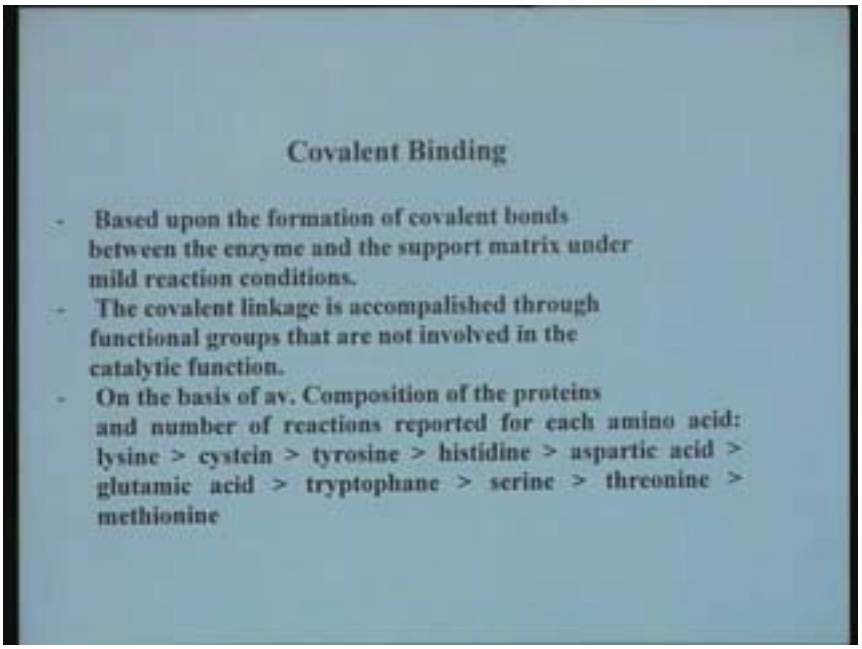

They could be, for example amino groups of lysine, terminal amino groups, thiol groups of cystein, carboxyl groups of aspartic or glutamic acid, carboxyl terminal groups, phenolic group of tyrosine, hydroxyl group of serine or threonine, it could be inert imidazole group of histidine. The functional groups that are involved in the covalent binding are same as that involved in the preparation of matrix for affinity chromatography. Almost similar functional groups on the ligands are used for making the matrices there. So almost similar chemistry is applied excepting that in those cases ligand is a thermally stable molecule. Methods available for choice is much broader compared to using enzymes where the stability of the enzymes become a key issue.

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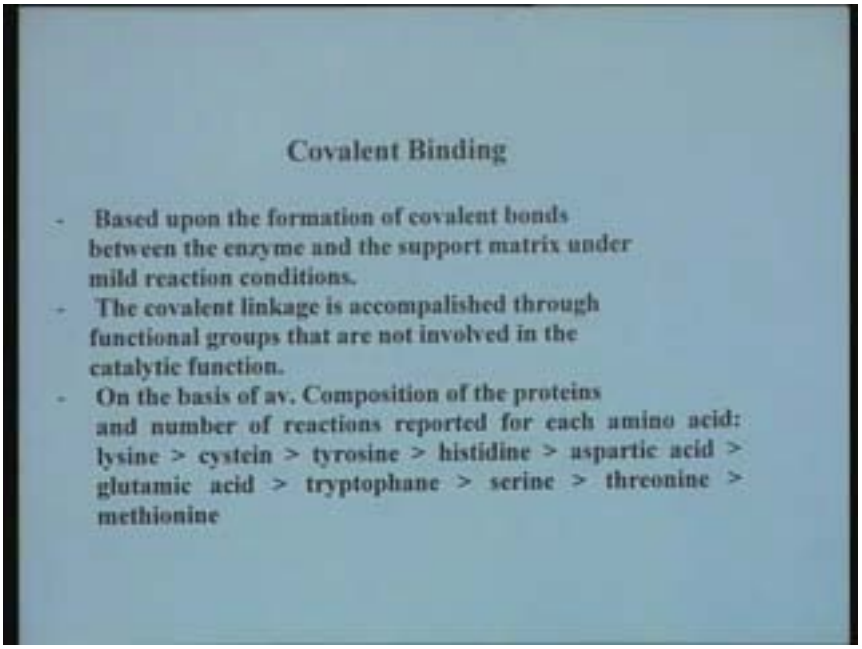

The covalent linkage is accomplished through functional groups that are not involved in the catalytic functions, a very standard prerequisite for any immobilization method. A very important general observation is on the basis of the average composition of the proteins. That means for the availability of the different amino acid residues in the protein molecules and the number of reactions that are reported for each amino acids you notice that this the kind of sequence. Lysine has been most commonly used for covalent binding, next followed by cystein followed by tyrosine, histidine, aspartic acid, glutamic acid, tryptophane, serine, threonine and methionine as the rare case. So that kind of a series is based on large number of methods which have been reported by people in the literature and also the average composition of protein. This kind of a sequence is the observation which is followed.

If you look at some general principles of covalent coupling we can probably consider all covalent bond forming immobilized enzyme preparations on the basis of three classes of methods. The A class refers to grafting of the enzyme to a pre-formed water insoluble functionalized polymer.

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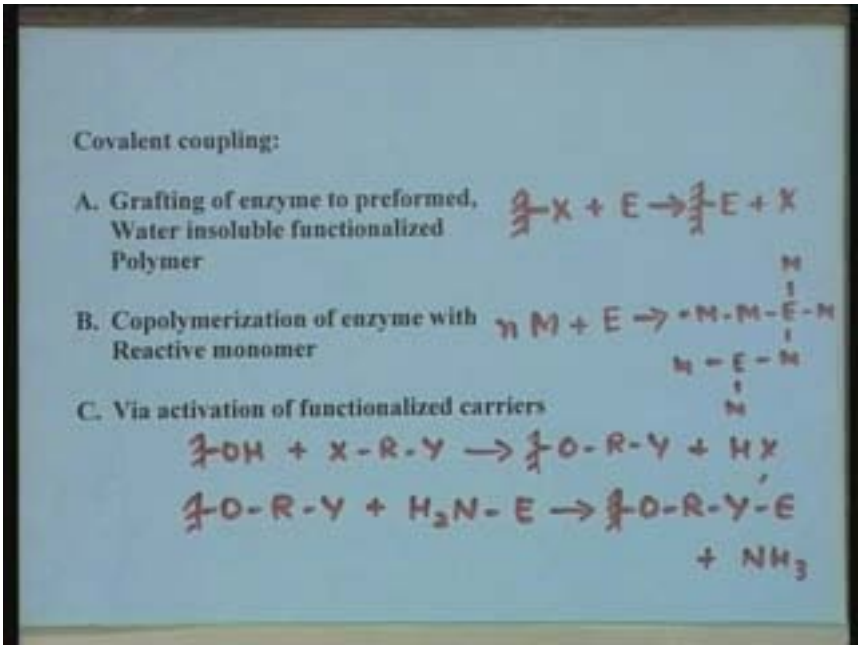

That means the polymeric matrix is already functionalized and has a reactive functional group which under ambient conditions can react with the functional group of the enzyme protein and give you the product. A typical reaction is shown here. A matrix containing functional group X reacts with the functional group on the enzyme and the grafting takes place. The enzyme gets grafted on to the matrix and some other molecule is released. Another class of method is co-polymerization of an enzyme with the reactive monomers. That means you don't start with the functionalized polymer. You start with polymerization reaction starting with monomer and copolymerize in the presence of enzyme. That means some of the functional groups of the monomer and the enzyme have to be common so that in the polymeric network at some places instead of the monomer unit the enzyme is inserted and you get a polymeric mass which contains enzyme molecules distributed in the polymeric network in that kind of method.

The third and very commonly employed method is via activation of the functionalized carrier. You don't take a preformed carrier but some carrier with some functional groups. Activate those functional groups by certain reagent and then the activated functional groups or activated matrix can react directly with the enzyme functional group to give you an immobilized preparation. Here a hydroxyl group containing matrix reacts with a bi-functional reagent. Both the X and Y could be aldehyde like in the glutaraldehyde. X and Y could be different also.

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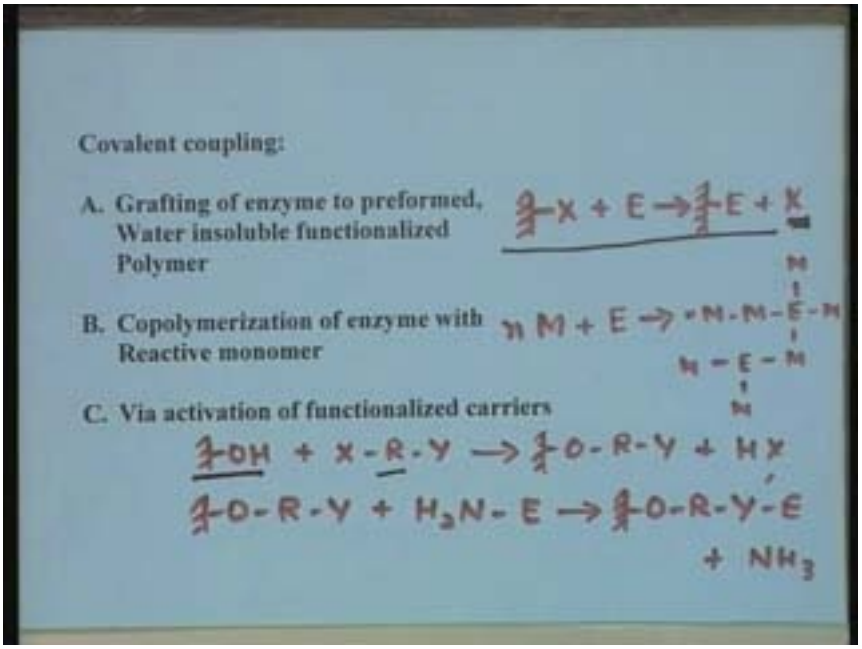

You get an activated matrix and the activated matrix then can react with the amino group of enzyme and give you the enzyme bound to it and the ammonia can be released.

A variety of methods that are available in literature for covalent binding will fall into one of these three categories. The interesting part is that if you look into literature probably during first part of the seventies a very large number of the reactions have been reported for immobilization of the enzymes on to polymeric matrices by covalent binding. Not that all of them are industrially useful because sometimes you synthesize an immobilized enzyme preparation but the cost considerations or the other factors which we discussed earlier may not be so attractive industrially. But still the technique is available and for some other application probably that method might become useful.

I will take a few examples to illustrate and the first and the simplest method is diazo binding. You get a diazonium derivative of the solid support by treating the support containing aromatic amino groups with sodium nitrite. Under acidic conditions you get a diazonium derivative. This diazonium derivative is highly reactive and can react with number of amino acid residues that are present on the enzyme molecule. For example if you take this and react with a phenolic group of the enzyme molecule, it gives you a enzyme bound at the phenolic group. Similarly it can also react with histidine group. It can also bind to histidine group and similarly give you an immobilized preparation with diazonium bond.

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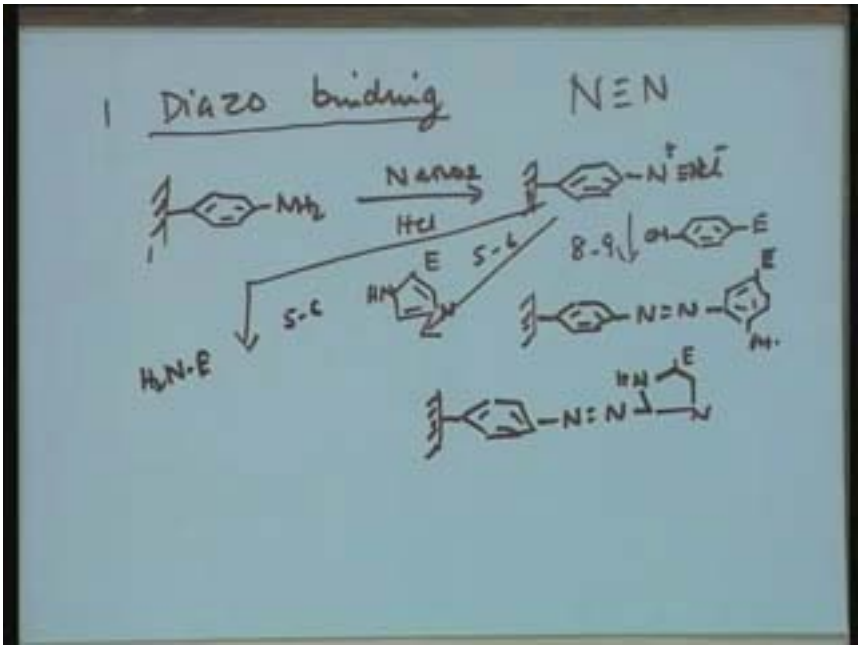

No. Sorry this is N-Cl. I think this is N$\equiv$NCl. There is already N there. Right. Diazonium derivative will have N triple bond N. Similarly it can also react with amino group of the lysine and give an immobilized enzyme preparation. That is one method which is used very commonly particularly because of its versatility. Only difference is the pH requirement in the three cases. The pH requirement here is alkaline 8-9. In the case of histidine it is 5-6 and if you consider through amino group, the pH requirement is also 5-6. Under control for pH conditions one can use different functional groups of the enzyme protein depending on the amino acid groups that are available at the active site to produce an immobilized enzyme preparation by azo binding methods.

A large number of methods for covalent coupling are based on peptide bond formation. The same bond which is available in the protein molecules and a very simple reaction. If you take an acid anhydride that can react with the amino group of the enzyme molecule and give you an immobilize preparation with –CONH-enzyme and -COOH. One of the typical factors here is that in addition to the enzyme binding a carboxylic group is also generated which means an additional charge, a free charge on the immobilized enzyme preparation and this negative charge gives sometimes undesirable properties to the preparation and sometime it can effect unfavorably the stability of the enzyme. To overcome this disadvantage we usually neutralize some of the negatively charged species by addition of diamines. At the end of the reaction certain diamines are added so that this carboxylic group which is generated is neutralized and gets bound with the amino group and the preparation is neutral.

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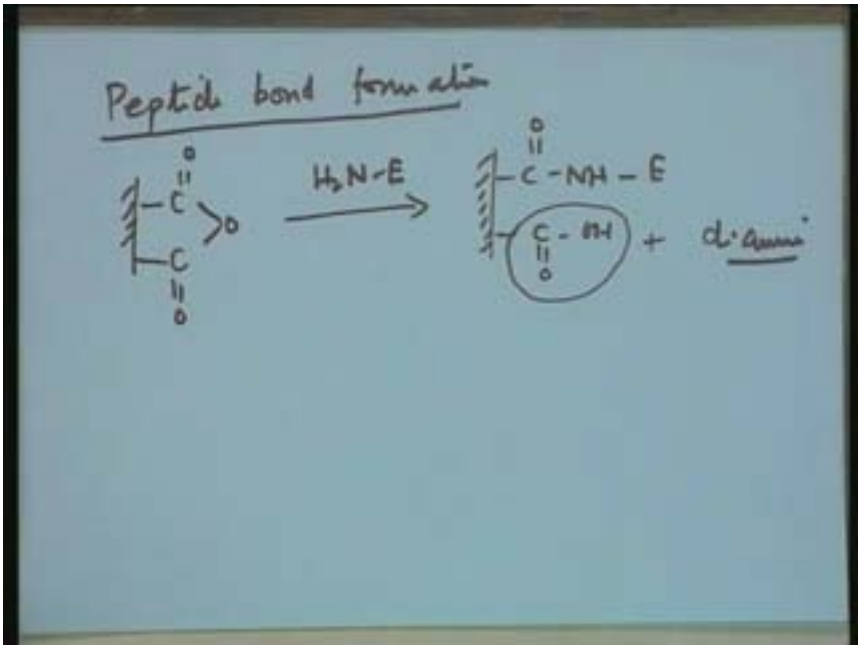

No. It cannot form a peptide bond. Electrostatic interaction it can bind if you have a negatively charged protein. But it cannot form the peptide bond under the mild reaction conditions at which we are talking it will be only feasible, if you have an acid anhydride group. Take a carboxylic group containing matrix put into enzyme solution it won't make a covalent bond because you need an activated matrix. This functional group has to be active or the reaction should be feasible under conditions which are mild in the sense that they are usually very close to the ambient conditions. Yes. You are right that this free carboxylic group will be available for adsorbing some proteins which are negatively charged and that's why we try to remove this charge group so that it doesn't interfere during the reaction.

Another method for peptide bond formation which is very commonly employed is by the use of certain condensing reagents, usually the group of reagents defined as RNCNR$'$. There are two R's – R and R$'$ are the alkyl residues. These reagents can combine or they can activate the carboxylic group containing matrices to give, under acidic conditions, usually pH 4-5, ortho acyl iso urea derivative. That is –COOCNHRNHR$'$. If it is reacted with the amino group of the enzyme at pH 4-5 you get a preparation CONH-enzyme. Again a peptide bond is formed. This is not a single reagent. These reagents which are commonly called as carbodimide are available in a whole range of these derivatives R and R$'$, which also makes its solubility properties. Some of the reagents are soluble in aqueous medium, some are insoluble in aqueous medium and you require a non-aqueous or a more non-polar medium.

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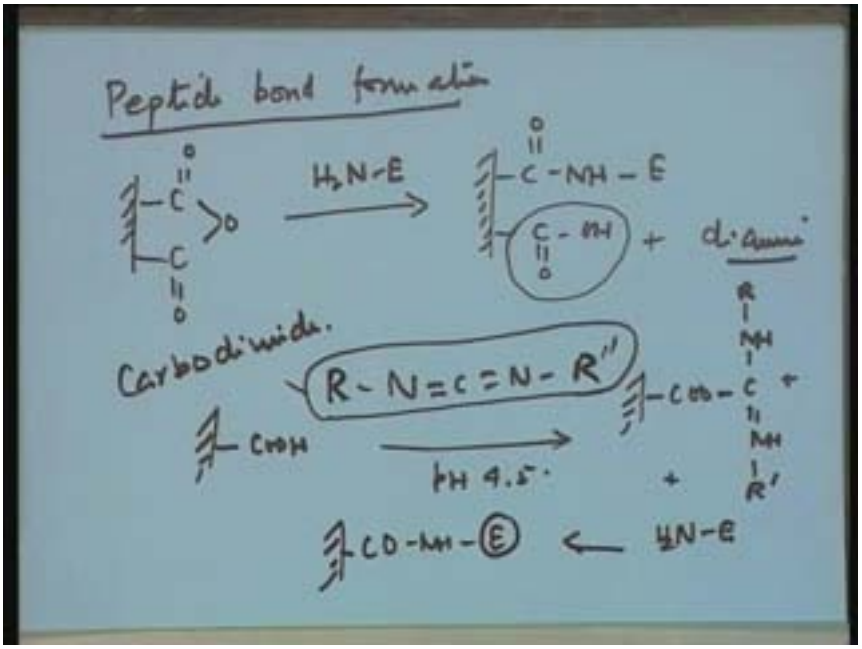

I would like to introduce you to another method involving the use of cyanogen bromide not only from the point of view of preparing the immobilized enzyme preparation but it also has an additional property that is the cyanogen bromide method is used in the case of carriers that have hydroxyl groups. The typical example is cellulose. If you react with the cyanogen bromide, you get an imino carbonic acid and this imino carbonic acid then can react with the amino group of the enzyme, at pH 8-9 under alkaline conditions, to give -CH-O-CONH-CHOH. Enzyme is coupled here. One of the characteristic features of this reaction and for which it has been used and valued is that you can adjust the distance of the enzyme from the matrix what we often name as a spacer arm.

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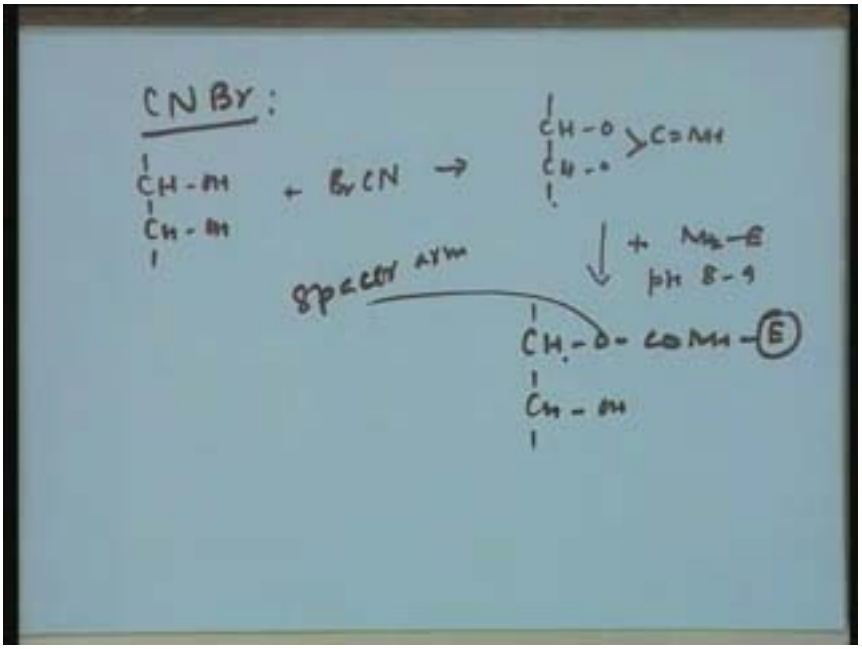

We had yesterday also discussed that in the method of metal salt linkage by repeated use you can increase the distance of the enzyme molecule from the support matrix. Similarly here this imino carbonic acid complex instead of reacting with the enzyme molecule can react with hexamethylene diamine which provides you free amino groups and the hexamethylene diamine will attach here or instead of giving an immobilized enzyme, you will be getting -CHOH–CH-O-CONH-$(CH_2)_6$–NH_2. You can use any length of the hydrocarbon chain. So you get the hexamethylene diamine derivative of the cellulose where you have already separated the matrix by six CH_2 groups. Therefore in most cases where the enzyme experience steric hindrances, this method can give you a preparation where by the steric hindrances are totally over come.

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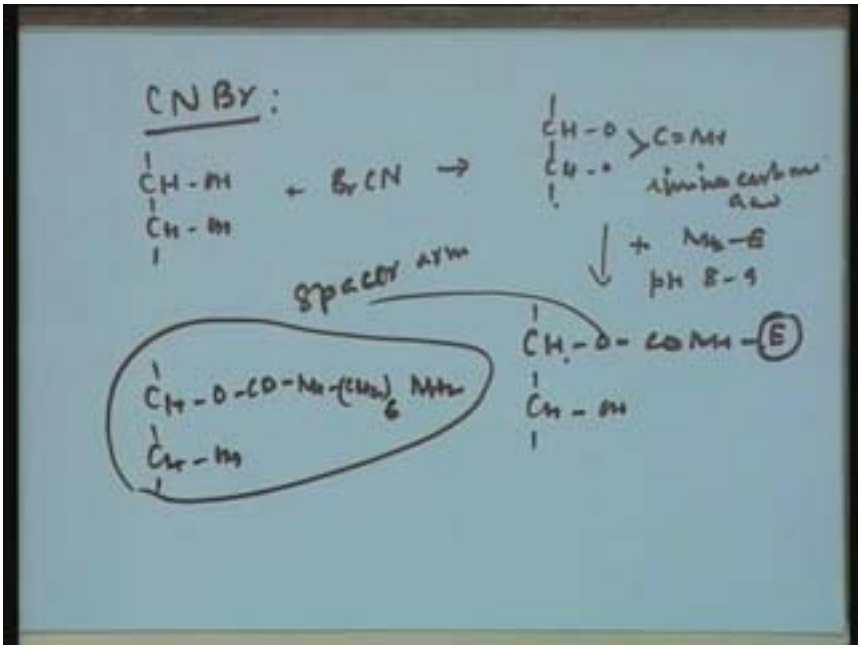

After cyanogen bromide treatment, you get an imino carbonic acid. Then you can react with amino group of the enzyme molecule. Instead of amino group of enzyme molecule you react this material with hexamethylene diamine or any compound which has amino group as the functional group and a chain of methylene groups. So again the reaction will take place with the amino groups and the hydrocarbon chain will be inserted in between. This amino group containing derivative can be used for immobilizing enzyme by any method which is applicable for amino group containing material.

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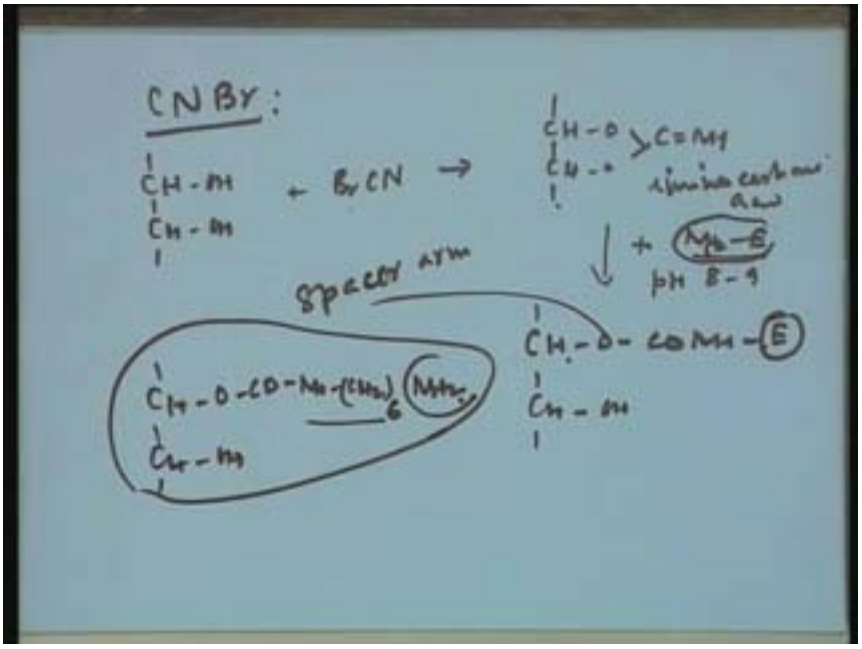

Therefore you can get immobilized enzyme preparation which is free of any steric hindrance.

There are large variety of covalent bond formation methods available in literature. But the choice of the method will depend on what enzyme we are trying to immobilize because we must know the functional groups that are involved in that enzyme molecule so that any method which is based on coupling of that particular functional group which is involved in the catalytic function must be avoided. The second thing we have to know is the stability of that enzyme so that the reactions that we are using must also not be carried under conditions under which the enzyme catalytic function is poor. If the enzyme is more stable under alkaline pH, then we can take a method which is based on alkaline pH because if you find the whole range of method, you can get in the whole range of pH conditions, whole range of temperature conditions and temperature usually we do not go higher than ambient for all these kind of preparations and the immobilized enzyme preparations. One of the major advantages in the case of covalently bound enzymes is their stability. They can be used over a long period of time with very high operational stability but the cost of preparation usually is much higher than all other methods. I think we will stop at this point today.