Organic Chemistry In Biology And Drug Development
Prof. Amit Basak
Department of Chemistry
Indian Institute of Technology, Kharagpur

Lecture - 04
Amino Acids: Separation and Detection, Electrophoresis and Ninhydrin Reaction

Welcome back to this session, last time we have discussed two important aspects of Amino Acids: one is isoelectric point and the other is the chirality. Chirality is very important to maintain, in order to develop drugs; the other point is the isoelectric point that is very important in order to separate not only amino acids, but also peptides and proteins. So, before we go on to any next topic, we start with a problem. So, suppose I have a solution containing a mixture of three amino acids.

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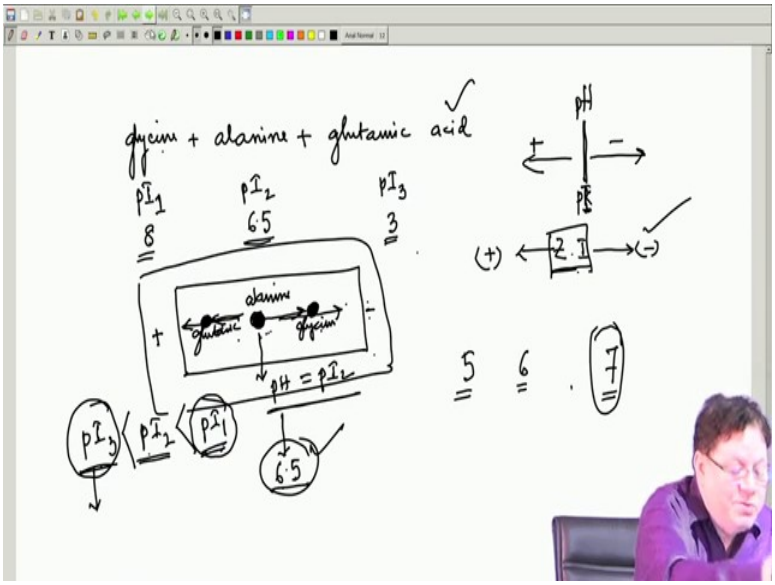

Glycine, alanine and glutamic acid. Suppose the isoelectric point (we are assuming, it may not be correct, we are not taking any value from the literature), of glycine is 8, the isoelectric point of alanine is 6.5 and the isoelectric point of glutamic acid is 3. So, that this pI 3 is less than pI 2 which in turn is less than pI 1. Now, I want to separate. So, what do I need to do? What I need to do is basically first select a matrix; matrix on which I can apply the solution of the amino acid.

What kind of matrix? It could be a piece of filter paper like the Whatman filter paper or it could be a gel like substance made up of poly acrylamide or agarose. So, these are the

different matrixes you can use and depending on the matrix we have different names of the process. The process is that we have the matrix and on two corners we put the positive and the negative electrode and apply the voltage. The process is called electrophoresis; electrophoresis is basically the mobility or the movement of a molecule under the influence of an electric field.

So, what I need to do? I need to first apply the solution containing the amino acids in the middle. And, the one which has got the pI in between the other two; that means, the middle one which is 6.5 (which is for alanine). So, I maintain the pH of the medium on which this paper is immersed or the gel is immersed, I put it, I keep the pH of that medium as the pI of the amino acid which is in the middle of the two (in between 8 and 3). So, 6.5 is the pH, I maintained for the buffer system here and then I apply the voltage.

Now what will happen? Alanine may be neutral at 6.5 and it will not move, it will remain here. And then what will happen to the other two? They will remain as charged species. Now, the question is what will be their charges? Now isoelectric point it is neutral. In this case, the pH maintained is 6.5 and the isoelectric point of glycine is 8.

So, if the pH is less than the isoelectric point, then that will be present as a positively charged species because, it has not crossed the isoelectric point 8. And, if the pH is greater than the isoelectric point then the species will be present as the anionic form. So, that means, the rule is that if this is the pH and this matches with the pI, then if you maintain the pH on this side, the species will be positively charged. And, if you maintain the pH on this side, the species will be negatively charged. So now, we have to decide; glutamic acid has a pI of 3, which is less than the pH that is maintained. That means, it is on which side then? It migrates to the anode because its pI is 3, but the pH is much more than that. So, it will be present as a negative form. So, the glutamic acid will move in this direction and the glycine will move in the negative direction (towards the cathode). Because, glycine's pI is still not crossed (as the pH maintained is 6.5), until you cross the pH 8 it will not be present as a negative species. So, below 8 it will be present as a positively charged species.

So now, what will happen? If you apply the voltage for a certain amount of time? You will see here that the alanine and glycine may move somewhere here and it all depends on the molecular weight; the glutamic acid will be somewhere here. So now, these three

spots (three amino acids) are separated. I hope the principle is clear; the principle is that if you have 3 amino acids, you take the pH same as the pI of the amino acid which is in between the other two. Now, you can question me that if there are 4 amino acids what are we going to do? Ok.

Now, in that case also there is another point which is the mobility of these amino acids. As I said that molecular weight is an important issue and there are also amino acids which are having side chains that can form hydrogen bonds, which can form more interactions with the gel. See when you have applied an electric field there is a movement of the species because of the charge.

But, something is also dragging it behind. And, what is the dragging force? The dragging force is the interaction of the molecule; that means, in this case amino acids, it the interaction with the matrix. If it is a paper based matrix, it will be a cellulose (it is a carbohydrate which can have hydrogen bonded interactions with the amino acid side chains). So, we can easily pick out that if there is such an amino acid, if the choice is between glycine and serine, then serine will have a lower mobility than glycine under the influence of the same applied field.

So, even if you have 4 amino acids, their mobility is equivalent to which is called $R_{f;}$ in organic chemistry we also have R_f. That means the, the amount of movement related to the solvent front; in this case it is the differential mobility of the amino acids which can allow you to separate the amino acids. We can do so by applying this simple isoelectric point rule, ok. For 3 amino acids, you can take the middle one, but for 4 or 5, then you have to do electrophoresis for a longer time to separate them one after another.

So, that is the use of isoelectric point. Now, how do I know, where is the amino acids in this piece of paper because, the amino acids are not colored. So, although I have spotted the amino acid here to start with and they have separated into three spots, but the question is how do I know where are these spots? So that means, I should have something to visualize these amino acids.

Now, amino acids unfortunately do not have fluorescence, which means if you shine light, it will not fluoresce. But there are other chemical tests for which can have color for the amino acid and that will show the location of the amino acid at a certain place.

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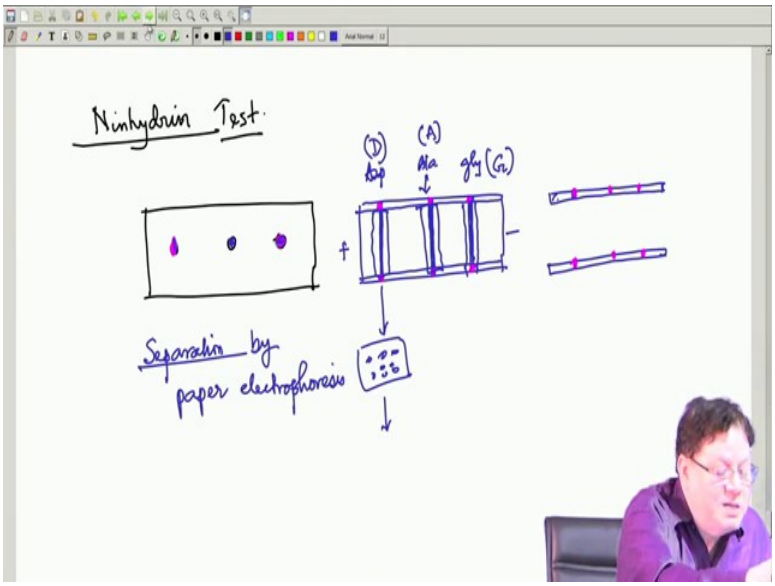

So, what is that reaction? The reaction is a well-known reaction which might be already known to you, ninhydrin test. What is this ninhydrin test? Ninhydrin is a reagent in which if you add an amino acid and then you heat it, you get a bluish violet color. You get a bluish violet color for all amino acids except proline. I will show you why proline does not give positive ninhydrin test; it gives some color, but which is different from other amino acids. But, all the other amino acids give a bluish violet color with the ninhydrin test.

So now, after the electrophoresis I have bands here. These are the spots of the 3 amino acids. How to know where are they? So, what I do is that I spray this ninhydrin reagent, it is just ninhydrin acetone solution. I spray it and then I heat the paper with a hot air gun. And, what I will see? I will see nothing, but that these spots will be somewhere bluish, see it is a mixture of something like this bluish violet. Yes, I think you cannot make this colored here, it is because it is a single color that it shows; slightly violet color will be there, something like this. So, this is the way you can visualize the amino acid.

Now, there are two questions that come from this. First of all if you treat this with ninhydrin your amino acid is lost, you have basically separated them. But, all the amino acids have reacted with ninhydrin and you cannot recover the amino acids. Secondly why does amino acid give color with ninhydrin? What ninhydrin is basically? What type of reagent it is? What is the structure?

Now, let us first answer one by one. First issue is that if I react all the spots with ninhydrin then the amino acid is lost. This will only demonstrate that the mixture has 3 amino acids, that much I can tell, but I am not getting to recover the amino acids from this. So, to bypass that what usually is done is that you take the same piece of paper, but instead of giving a spot; now you apply the solution as a band (like this) and then do the electrophoresis.

So now, what will happen? Alanine will remain here; I am taking the same example like earlier. And, there will be glycine somewhere here and aspartic acid depending on their mobility as I said. One more thing I should say that the mobility not only depends on the molecular weight, but it also depends on how far away is your pH from the isoelectric point. This is your starting point, this is the alanine, this is glycine and this is your aspartic acid.

Remember if you take the single letter symbol glycine is G, alanine is A, aspartic acid is D; aspardic acid (that is the way you try to pronounce it) so, D; so DAG. Now, what you do in order to isolate back the amino acids, the best way to do is you cut a strip; a small strip from this end and you cut a small strip from this end. So now, I have two strips here from these two ends and then I spray ninhydrin on both and then again heat with an air gun. So, what I will see? I will see a band here maybe I will take this, I will see a band here; I will see a band here; I will see a band here and the same thing I will see here.

Now what you do? You put those things again back to the place from where you have taken them. So now, I will see a band here, a band there, a band there; actually this is kind of bluish violet, there is no well-defined color; I cannot say that this is pink or this is magenta. But some books say that it is bluish violet or bluish pink type color, so this is the color.

Now, what you do? Once you reconstruct it, now take a piece of scissor and what do you do? You cut this piece of paper from here; cut this piece of paper from here, taking the width of here (the width of your band). And, then cut this piece of paper, just to make sure that you are taking all the amino acids, you take an extra width over there; little bit extra portion you should cut. Now, it is very easy, you take this piece of paper, cut into small pieces and put it in a flask; add water, these amino acids are mostly soluble in

water and then filter and then from the filtrate if you evaporate the filtrate you get the amino acid ok.

So, this is how the separation by paper electrophoresis is done. You cannot do it, if you have gel here; remember that it is not applicable for gel. For gel it is very difficult to cut this and then again reconstruct it. For paper this is very useful; you can use very big size filter paper to do it; even you can isolate milligram quantities of amino acids by using separation technique via paper electrophoresis. So, you see the importance of isoelectric point.

So, the first question is solved that is how to detect the amino acid and how can you isolate it back? So, that is done; next is what is the reaction involved? What is ninhydrin? Why does it give that bluish violet color?

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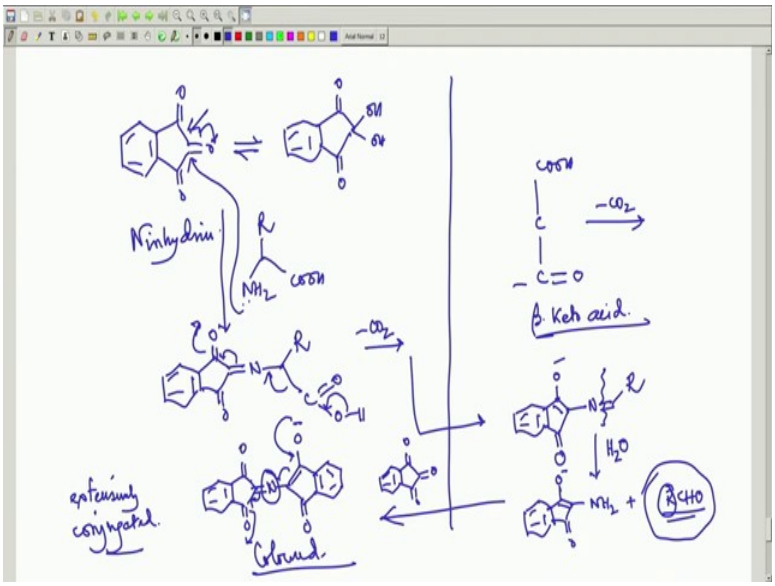

Now, ninhydrin has a structure which is like this; it is a trione, it is a benzene and a 5 membered ring with trione. However, from organic chemistry point of view, we know that if a carbonyl is flanked between 2 electron withdrawing groups, thenthis can exist in a form which is a geminal diol. So, it can exist as a geminal diol form (like chloral hydrate; there are several other examples like this). So, they are in equilibrium, diol with this ninhydrin. Now, you are taking an amino acid. So, write the amino acid NH_2 CO_2H and R.

Now, out of these 3 carbonyls the most reactive carbonyl has to be this middle one, as this is the most electrophilic carbonyl carbon since this carbonyl is flanked between two electron withdrawing carbonyls. So, the NH_2 is going to react with this carbonyl. So, if that reacts then what is the reaction between a carbonyl and a primary amine? It forms the imine. NH_2 lone pair of electrons first attack the carbonyl, that goes there, oxygen takes the hydrogen. And, then in the next step, nitrogen lone pair again flies back to eliminate water and it give this form which is called an imine.

I am just skipping that step . However, this is not very stable; this is a very basic concept from organic chemistry, that if you have a carboxy group COOH then a carbon and then one electron withdrawing group like a carbonyl, then what happens? It loses carbon dioxide very easily, that is called the decarboxylation of β keto acid. So, β keto acids are very unstable, they lose carbon dioxide. That is why when we buy acetoacetic acid, we buy it as the ester form (as CO_2Et), in order to block the release of the carbon dioxide.

Now, if you look at this structure, it is very similar, this is carboxy, then a carbon, here there is a carbon, then nitrogen, then a double bond and then another electron withdrawing group. So, this is even more facile for decarboxylation; this structure is such that it will facilitate the release of carbon dioxide, like the arrow that I am showing. So, it loses carbon dioxide and as it loses carbon dioxide, this O minus is formed and then you have this N. So, this is a very good way of decarboxylating amino acids.

Because, the carboxylation is not very easy; usually you need a lot of energy to decarboxylate a carboxylic acid. But, here ninhydrin is acting as an electron sink; see what is happening; what is an electron sink? When electrons are going towards the direction, that direction means there must be something like a black hole that it is trying to absorb whatever is coming out. Here it is electrons; electrons are going to this direction; that means, this is electron sink. So, what is being formed is an imine, like this, ok. Then there is a hydrolysis of this imine, if there is hydrolysis, then what will happen? Imines are not very stable; unless they are aromatic (you must be knowing that). So, you have this C double bond O, this is O minus and then that will become NH_2 and you have generated an aldehyde. What is the aldehyde R? The aldehyde R is the same R of the amino acid, but now that has no function.

Now, what will happen? This amine that has been generated, reacts with another molecule of ninhydrin, because this amine is now a new nucleophile that is formed. So, this new nucleophilic amine is going to attack this carbon followed by expulsion of water to form this highly conjugated molecules..

So, that will be lost and this will form double bond N, that means water is gone and then double bond N and then you have the other ninhydrin system ok. This is the other ninhydrin system; now you have O minus here, you have this double bond.

So, this is the product which is the colored compound. Why it is colored? Because, you see the extensive conjugation here, this comes here, that goes there, that can go there, that can come there or it can go there, that can go there. So, it is an extensively conjugated system.

So, that is why you see the color; why do you see the same color for all amino acids? Because, ultimately the color is not due to the R group, the color is basically due the compound generated between the 2 ninhydrin molecules which has a nitrogen in-between. This nitrogen actually is the nitrogen from the alpha amino acid.

So, that is why all amino acids give the same color except proline. Now, what happens with proline? Let us see what happens during the ninhydrin reaction of proline. Now, proline being a secondary amine, so we expect some difference and that is the only secondary amine. And, indeed it gives a brownish yellow color with ninhydrin. Now, why is that? Because the reaction stops at an intermediate step so, I will write the reaction along with the mechanism.

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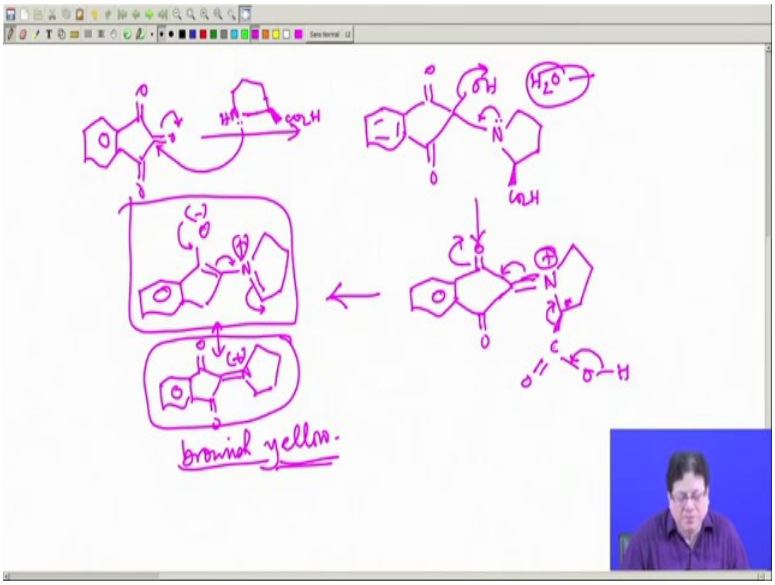

So, the ninhydrin is here, it's a tricarbonyl compound. And, we know that it also exists in equilibrium with the geminal dihydroxy form because it is flanked between the 2 carbonyls. Now, proline has a structure which is represented by this in the correct stereochemistry having CO_2H in β-bond. But this reaction does not depend on the stereochemistry, it gives the same color irrespective of whether you use L amino acid or D amino acid which is an important point.

But, here we are showing either the D amino or the L amino acid. This is 1, that is 2 and that is 3, hydrogen being α so, this is S configuration. Now, the first reaction is the same like the earlier cases where primary amine is used. So, that becomes OH and the nitrogen loses the hydrogen. So, then you have an intermediate which is like this: a carbonyl and then you have OH, you have this carbonyl and that is linked to the proline nucleus. Now, what will happen? This nitrogen will utilize its lone pair and remove the OH as water while the OH minus takes up hydrogen from water.

And, in the process, unlike in the previous cases, here the nitrogen remains as a positively charged species. So, you have a double bond with the nitrogen and it is an iminium ion, now OH and this is plus. But, still there are two electron withdrawing carbonyl groups and this is present at the β position. So, this double bond is under tremendous stress being linked to positively charged nitrogen and also to electron withdrawing carbonyl groups.

So, there is now scope for decarboxylation like the previous cases and you have this relay process of electron shift. And, that gives you the decarboxylation of proline. So now, the proline has just become a pyrrolidine (a 5 membered heterocyclic nitrogen containing ring is what is called a pyrrolidine).

So, it becomes a pyrrolidine and you have an intermediate like this, O minus and this is N and that is the pyrrolidine. So, you see the stereochemistry does not matter because all these ninhydrin reactions are associated with liberation of carbon dioxide, destroying the stereochemistry of this stereogenic center.

So now, there is a double bond here and this is the one. So, that becomes a pyrrolidinium ion and this is brownish yellow in color that I was talking about; because this has got a resonance structure like this. So, because of this resonance, you get a color because extended conjugation is the main cause of giving coloration and this is the hydrogen. But, the nitrogen remains positively charged and this is brownish yellow colored.

So, proline behaves little bit differently because it is not able to produce the NH_2 attached to the ninhydrin. Earlier primary amine replaces the carbonyl with NH_2 and that reacts with another ninhydrin. But, in this case, only 1 ninhydrin is associated with the pyrrolidinium ion and this pyrrolidinium ion is coming from the decarboxylation of the proline ring, so this is brownish yellow. So, that is the situation with ninhydrin reaction; remember all amino acids except proline give a blue violet color.

The color is same for all amino acids except proline; this is because the R group is not there in the final conjugated product since decarboxylation leads to liberation of primary amine along with the release of R as aldehyde. And so, it is the amine ultimately that forms the color while it reacts with another ninhydrin.

So, the color is independent of the nature of the side chain. In case of proline, we have a slight difference because, it cannot liberate the primary amine and so, it stays in these two resonating forms, so that becomes the brownish yellow. So, that ends up the detection issues of amino acids. Next, we will go to the synthesis of peptides, how these building blocks are added one after another.

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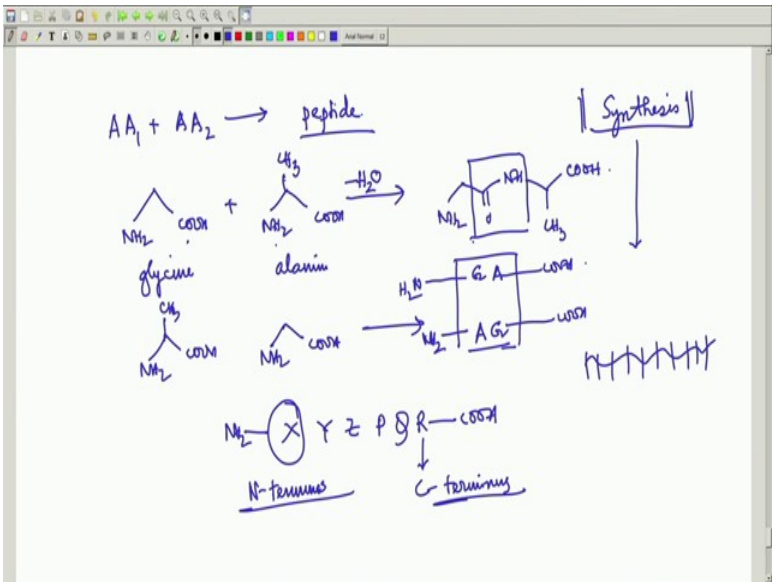

We know that amino acids (AA_1 and AA_2) combine; that means, 2 amino acids are combining and that gives what is known as a peptide, ok. Let us give some example; suppose glycine and say alanine (NH_2, CO_2H and there is a CH_3). Now, they react with each other and form a new bond that is between the carboxy of the glycine and the NH_2 (amine) of the alanine and you get what is called a dipeptide. Now, the point to notice here that this is a very facile reaction; it is so because there is elimination of water. A stable molecule of water that is being eliminated, so that means, its ΔG is highly negative.

So, it should be a spontaneous process; however, it is not a spontaneous reaction. Thermodynamics says that this reaction should go, but as you know that ΔG negative does not immediately say that the reaction will take place under ordinary conditions. To have this reaction taking place in a test tube, you have to provide what is called activation energy. We have to do something to ensure that the activation energy is lowered and the reaction takes place. So, this amide bond is very stable and this reaction is very thermodynamically favored because of the loss of water.

However, the reaction does not take place if you mix an amine and an acid because of high activation barrier. The other point to note is that that this is alanine and this is glycine; they can react in two ways. One that they can form a peptide which is G A, now we should start writing the symbol G A glycyl alanine or that could be the other way

around, i.e. the alanine carboxy reacting with the glycine amine . Then you get what is called alanyl glycine, this is glycyl alanine, this is alanyl glycine.

So, 2 amino acids can react to make two different peptides, they are not same. In this case there is this amine functionality which is free and the carboxy which is free at this end. And, in this case, the glycine carboxy is free and the amine is free on this other side. Now, when you write a peptide, then the traditional way of writing is that on the left side (which is the amino acid), the amine is free and on the right side (the extreme right side) the carboxy is free.

That means if I have an amino acid suppose X, Y, Z, P, Q (whatever; just an arbitrary sequence), if I write this; suppose these are all amino acids, then immediately it says that this R has carboxy free and this has got NH_2 free. And, this will be called N terminus amino acid and the other will be called the C terminus amino acid; C terminus or the carboxy terminus. And, the other important point (although this is very simple), is that a dipeptide has a notion, sometimes the students get confused.

Dipeptide means there should be 2 peptide bonds, tripeptide there should be 3 peptides bonds; that is not the case. Dipeptide means a peptide containing 2 amino acids, it is not the number of peptide bonds. The number of peptide bonds is always less by 1 than the number of amino acids that are reacting. Of course, there is one intricate point here that we are only talking about linear peptides, we are not talking about any cyclic systems. Because, the whole scenario will change, if I start making cyclic peptides which are also possible.

We are only talking about linear peptides; so, if there are linear peptides then what happens? The number of peptide bonds will be one less than the number of amino acids that are reacting. So now, there are two things which are very important; one is how to synthesize peptides, how do I do this reaction? I said this is thermodynamically favored but kinetically disfavored. This has got very high activation barrier. So, one issue is the synthesis, and we are talking about chemical synthesis. We are not talking about biosynthesis because if you think of the biosynthesis; that means, how proteins are made in the body.

At that time activation comes from somewhere else because, in the body you cannot provide high energy, high temperature, high thermal condition; that is not possible. So,

by synthesis, we mean chemical synthesis. The other point is that if I have a peptide, then how do I know the sequence of the different amino acids. So, these are the two challenges, that the protein chemist faced; firstly how to know the sequence of amino acids? By the way that is what is called the primary structure of proteins and the other point is; how to synthesize proteins as per a design?. The next lecture we will tell about that.