Bio-Physical Chemistry
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Lecture – 40
Fluorescence Quenching

So, welcome to today's class. The topic we are going to discuss today is Fluorescence Quenching. But before you know you go into the topic let us look at what we have covered in the last class – a just quick recap.

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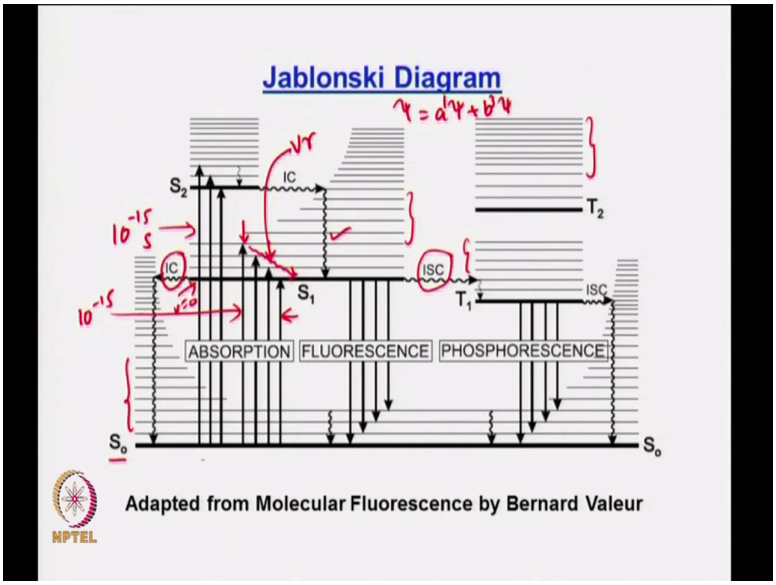

So, you know we started with the Jablonski diagram, because we are talking about fluorescence right. And we know there in the Jablonski diagram, we have these different

excited state processes. Say for example, this V r is corresponds to vibration relaxation, then ISC corresponds to intersystem processing.

Then if you have radiative transfer or other radiative de excitation from your triplets state to single state, then its phosphorescence. If it is on single state excited state in single ground state radiative, then it is fluorescence and now also you have host of non-radiative processes right.

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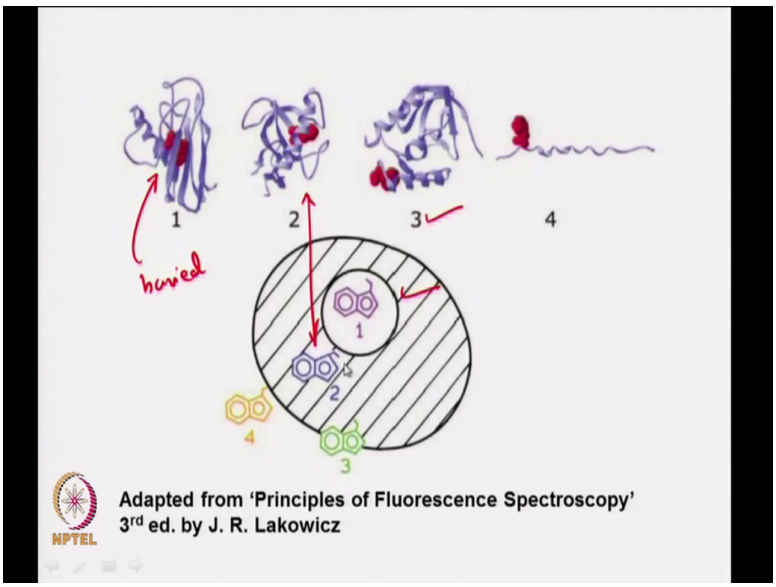

Now, because you know this is a course on biophysical chemistry and we are mainly focusing on proteins, so one of our goals always is to look at the application of fluorescence with respect to proteins alright. So, what we seeing out here is that there are four different proteins having you know different extends of exposure for the respective tryptophan residues.

Now, keep in mind this bit covered in the last class, but just kind of doing quick recap. So, in number 1, what is happening is, in number 1, your tryptophan which is in the red ball thing coloration is buried, right. In number 2, it is little more exposed. And if you can compare with this schematic which is a down here, so number 1 is in mostly in the interior of the protein, number 2 is the little less in the interior of the protein. Number 3 is kind of the interface; one part is interior the other part is exposed. Then number 4 which is this one, you can see this is number 4, and hence this is number 4 this is fully exposed.

So, what you expect? What we expect is and if you remember our discussion on fluorescence then for the buried tryptophan, because it is mostly in hydrophobic environment which is here for protein 1 that its fluorescence maximum would be most blue shifted. And for 4, it should be most red shifted, that means, it should be occurring at the highest wavelength among these four different proteins. Now, do we see that? That is exactly what we see right look at this number 1, position 1.

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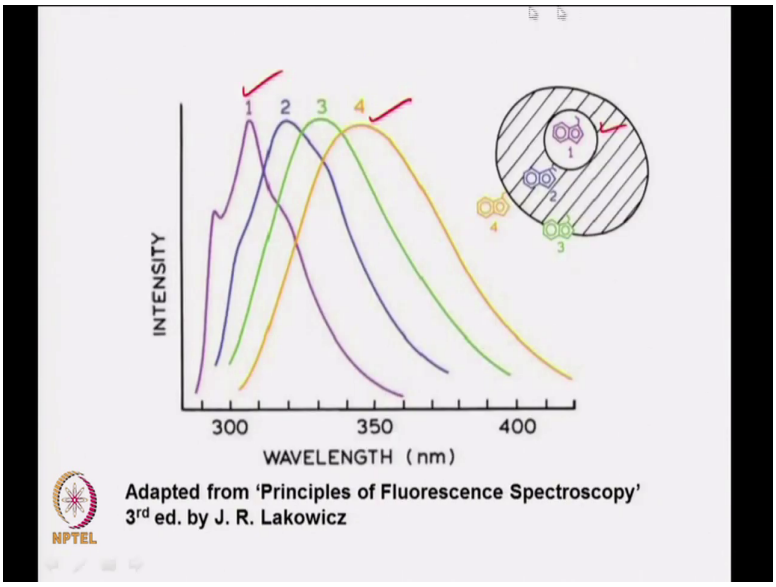

Position 1 was the case where it was solely in the interior of the proteins. So, you can see out here how blue shifted with respect to the other intensity. Then 2, it is a little less on the interior side; 3, so for 3 what is happened is kind of both facing towards the interior and a part of it is exposed. And number 4, it is fully exposed. And you can see how the wavelength are shifted from 1 to 4 towards the red side or the higher wavelength.

Now, this is typically what is observed so that means, if you take a protein and if you look at the tryptophan emission maximum, then you would be having an idea of to what extend is the tryptophan buried or to what extend it is exposed from the emission maximum.

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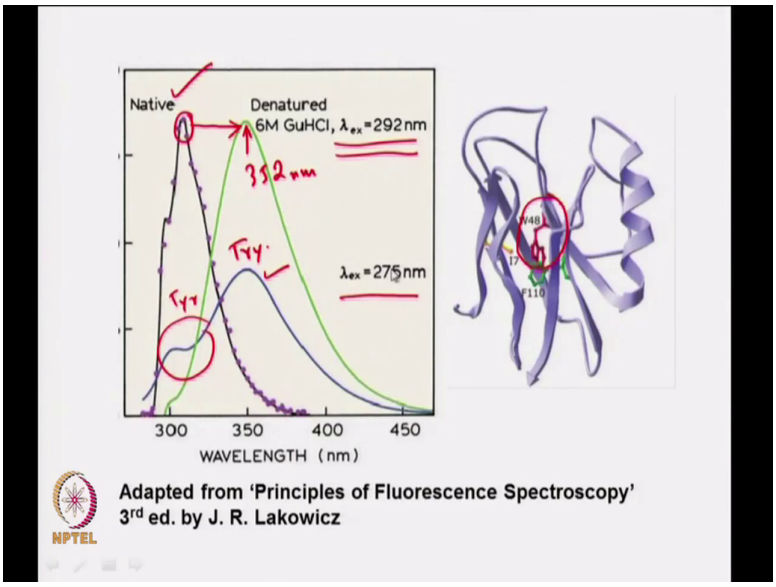

Now, this is another case where we are looking at the effect of denaturation on the fluorescence profile. So, you know without worrying too much about the fluorescence intensity right. Now, if you look at the native state the native state was is the one which is most buried. So, this is the tryptophan W48 we are talking about in this protein.

So, the native state has most blue shifted emission maximum, then when we denatured it, that means, you have added say guanidine hydrochloride chemical denaturing which is which has kind of denatured the protein to a huge extent. Then you see what has happened to this tryptophan intensity, this tryptophan intensity or other the emission maximum has shifted well to the red side or higher wavelength.

This is very similar to what we saw in the previous slide, but remember in the previous slide you were considering four different proteins right, that means, different exposures. In this

case, it is also an exposures case, but on for a single protein, that means, you started with protein, it was in the native state no denaturing the tryptophan was very much in the interior of the protein.

Now, when you start opening the protein hub with the help of denaturing guanidine hydrochloride what has happening is that means, your tryptophan is slowly coming out from the interior towards the exterior towards the expose site. It is getting exposed to the solvent molecules and hence its fluorescence is shifting to the red ok.

Now, what we have here? Now, please keep in mind here you can see this lambda excitation which has been underlined it is 292 nanometers. Now, the reason for this to taking this lambda excitation is this. Now, if you go back to a previous discussions in proteins along with tryptophan, we have two other fluorophores tyrosine and phenylalanine.

Now, tyrosine and tryptophan have very close absorption spectra. When it is a very close I mean that if you are exciting 285 or 280 what is going to happen is you are going to excite both tyrosine and tryptophan. But if you are exciting it 292 nanometers, what you will see is that 292, the absorption spectrum of tyrosine has almost died down, that means, the absorption is really low while there of tryptophan is still pretty high, that means you are solely or at least exclusively exciting tryptophan compared to tyrosine.

So, that is why if you look at the comparison of this lambda excitation is equal to 292 nanometers as compared to lambda exhibition equal to 275 nanometers, that means, we are exciting at 275, but tyrosine also has precial resolvance same for tryptophan. So, you can see you can you will see the tyrosine emission maximum out here or the tyrosine emission coming up close to 300 and then also for this is tryptophan which is you know still there ok.

So, this is kind of where we stopped in the last class right. And this is where we are getting introduced to how people use fluorescence for proteins. Now, there is one very important thing that people often use. Now, your question might be ok, we have talked about burial and exposure of these tryptophan residues. Now, you can ask me how would I identify or how

would I see, or how would I prove, how would I investigate to what extent it is buried, to what extent is it exposed? Right.

So, all suppose I am taking two different proteins, I do not know anything else, I do not know the structures of the crystal structure is not available. Can I do a simple set of experiment to figure out in which case the tryptophan is more exposed as compared to the other one? Obviously, one is fluorescence emission.

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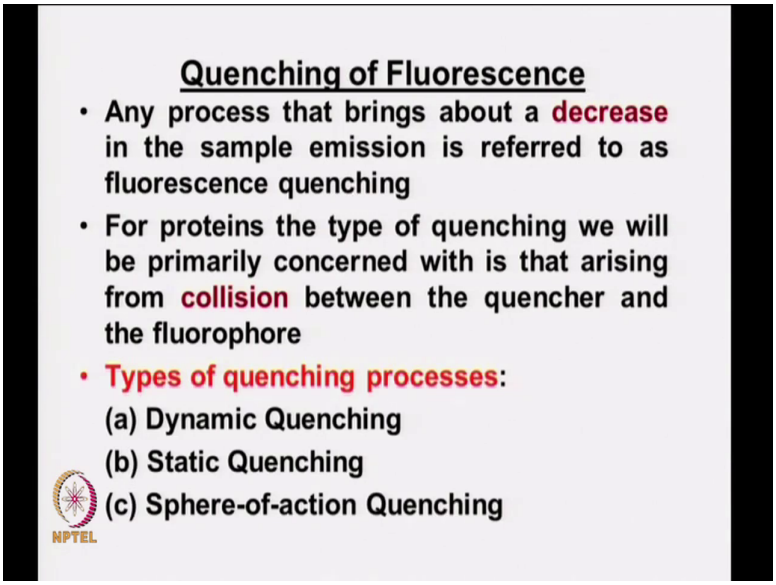

Now, the other case or the other type of experiments that people generally carry out is referred to as fluorescence quenching or quenching a fluorescence. Now, before going into the very specifics of this let us talk about this quenching processing in details. So, what is quenching? So, any process that brings about a decrease in the sample emission is referred to as fluorescence quenching that means, any process right it does not have to be a particular

process, it can be anybody any process by which you see that your fluorescence intensity has decreased.

Now, for proteins in the type of quenching because we are going to mainly focus on proteins, the type of quenching we will be a primarily concerned with is that rising from collision between the quencher and the fluorophore. So, say the fluorophore is tryptophan, let us take the tryptophan for simplicity sake, and the quencher will be another molecule. And I will tell you what those molecules are as we progress through outer you know through with a lecture today.

So, what are the different types of quenching processes out there? One is dynamic quenching ok, this is essentially collision in nature that means, you have a quencher molecule, you have a fluorophore, again will soon come to this in couple of slides. And this quencher molecule will collide with the fluorophore and result in quenching ok. This is called dynamic quenching. Another one is called static quenching. This is also type of quenching, but it is slightly different from that of dynamic quenching the mechanism is. And the third one is known as the sphere-of-action quenching. But the two that we will mostly focus on are dynamic and static, and that is what we will discuss in some details.

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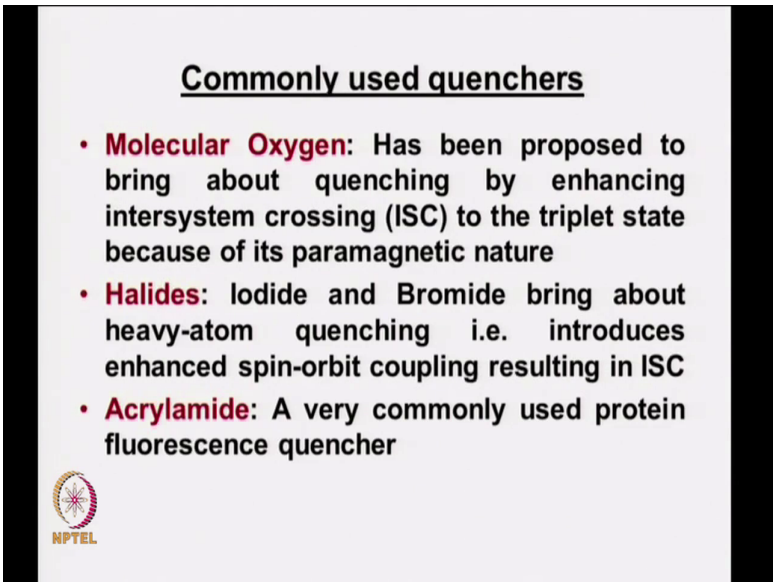

So, before going into the details of the mechanism or what you know dynamic and static quenching are, let us look at some commonly used quenches. So, one is molecular oxygen right. So, oxygen has been proposed to bring about quenching by enhancing inter system crossing to the triplet state because of its paramagnetic nature.

That means, if you have enough dissolved oxygen or if you have dissolve oxygen in a solvent which in most cases you have, and the amount of you know dissolved oxygen you have will depend upon the solvent you have proteins in, but your proteins are in water right, so our buffer. So, this oxygen what will happen is it will diffuse and collide with your fluorophore tryptophan say and bring about quenching.

So, you can understand that the more oxygen you will have, that means, the more dissolved oxygen you will be having, the more will be the quenching effect. So, even without you know

deriving anything or looking at an equation, you would you know try to guess that this extent of quenching would have a concentration dependence that is would be depend upon the concentration of the quencher in some way or the other, you know that is very straightforward to kind of realize or appreciate.

Another class of quenching molecules halides, iodide and bromide, they bring about heavier atom quenching. Now, this we have talked about before you know which results in spin orbit coupling, where is it you know this halides, this heavy halides or heavy-atoms introduce enhanced spin-orbit coupling resulting in inter system crossing ok. Now, this is also something we have discussed before right.

This is not new to you or it is just that now we are discussing it in the realm of fluorescence quenching. Now, there is also another class of molecules amides succinimide, acrylamide, acrylamide is the one I am going to refer to here this is also a very commonly used protein florescence quencher right.

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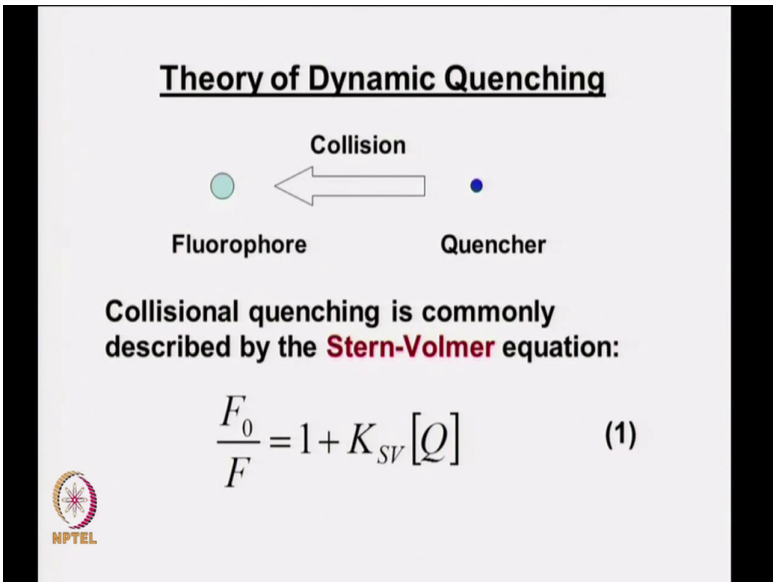

Now, what is a let us now let us go into the theory. What is the theory of dynamic quenching or collisional quenching? Ok. Now, there you have a fluorophore out here as a as a shown on out here, you know this is a fluorophore ok. Now, what you have, you have a quencher molecule. Now, not necessarily these you know these sizes are just schematic right they can be having the same sizes, the fluorophore can be a little bigger or bigger than the quencher and so on.

Now, if I am talking about dynamic or collisional quenching, what I am saying is this the quencher collides with the fluorophore. The moment it collides to the fluorophore what it what happens is, it de excites it from its excited state giving rise to or giving rise to a decrease in fluorescence. Now, this is typically what happens in case of dynamic quenching. So, collision quenching is commonly described by Stern-Volmer equation.

Now, let me again repeat the dynamic quenching is often referred to as collisional quenching because as you are seeing on the added certain collisions only when the collision is happening is the quenching seen right. So, that means, for the quencher to go to the fluorophore, it has it has to diffuse and then collide. So, diffusion you can understand would play a very important part in this collision or in this type of quenching.

So, the Stern-Volmer equation or the equations because, we will look we looking at a few more equations later. These are a classic set of equations, these are a classic set of equations that will describe most of the types of quenching that we encounter. So, what it says is F 0 I will tell you what F 0 by F you know F 0 F and these are, F 0 by F is equal to 1 plus K SV where SV stands for Stern-Volmer times concentration of Q now again.

Let me mention this or let me repeat this rather, remember we talked about the fact that it is easy to understand the higher the quencher concentration is the more will be the quenching, that means, there will be a dependence upon your quencher concentration, this is exactly what you see out here. Where Q is a quencher concentration, and you can see the F 0 by F this is a measure of your fluorescence quenching right is equal to 1 plus a certain constant which is K SV turns from a constant times the concentration of the quencher.

Now, let us expand on this equation, let us try to understand what these a different parameters mean.

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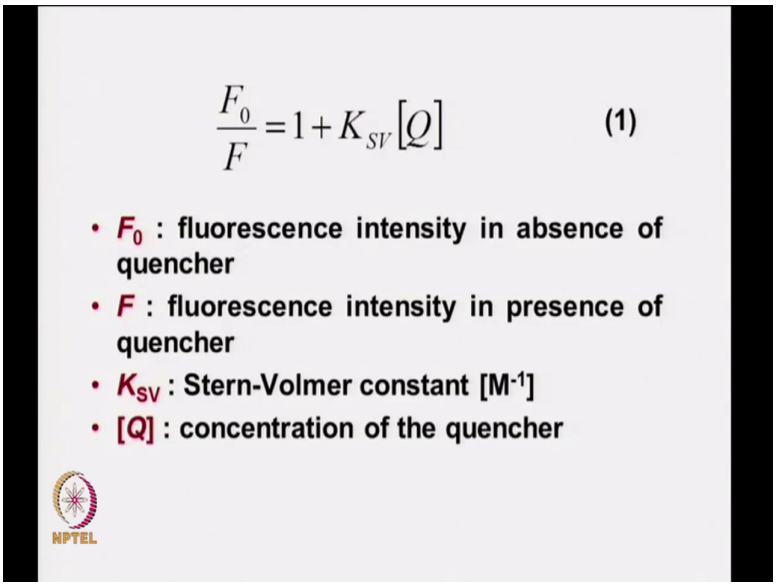

So, again looking at this equation. What is F 0? So, F 0 it is a fluorescence intensity in absence of the quencher that is what F 0 means. F 0 is essentially means that its F 0 quencher concentration, that means, this is the initial fluorescence intensity we have when you have no quencher molecule present in your sample solution right. Then what is F?

Obviously, there you can understand what F is, it is a fluorescence intensity in presence of the quencher right has to be because now we are putting a quencher and depending upon what concentration of quenching you have, and what quencher you have obviously, accordingly the fluorescence intensity of the corresponding fluorophore will diminish.

Now, also keep in mind if you are talking about protein right, if you are talking about a protein, then this amount of quenching will also depend upon the exposure of that tryptophan

residue, because we are mainly talking about tryptophans just to keep things simple right. So, you know quite a factors have to be kept in mind, you just not a one factor right.

So, next what is K SV? It is called the Stern-Volmer constant. And look at the unit, it is molar inverse ok. Now, generally concentration is expressed in molar concentration right. And hence if you look at this equation again, this is F 0 by F is equal to 1 plus K SV times Q. Now one obviously is dimension less. So, K SV times Q, so if Q is concentration moles or molar concentration, in case we has to have the inverse of the that, because you are adding this to a dimensionless quantity. So, this quantity also has to be dimensionless, so that is the easiest way of figuring out what the unit for K SV is right. So, if you ever have any you know doubts if you ever are confused, then just look at this equation and make a dimension comparison ok.

Now, also what is F 0 by F? See F 0 by F remember because we are talking about fluorescence quenching F is always less than F 0. Now, F 0 by F, that means, F is the fluorescence intensity in the presence of the quencher. So, what you can understand is F 0 by F will always increase because with increase in quencher concentration your F is decreasing, but F 0 was always the same right, so that means, you will see an increasing trend and it is mostly linear based on this equation right.

So, again what is Q? Q is the concentration of the quencher. So, to recap this part this is the Stern-Volmer equation for collisional quenching or dynamic quenching, where K SV is be the Stern-Volmer constant, F 0 is the fluorescence intensity in the absence of the quencher, F is a fluorescence intensity in the presence of the quencher, and Q is the quencher concentration. So, what you can understand is if I am going to plot F 0 by F versus the concentration of quencher, I am expecting a straight line is not it, and its slope is going to give me the value of K SV right.

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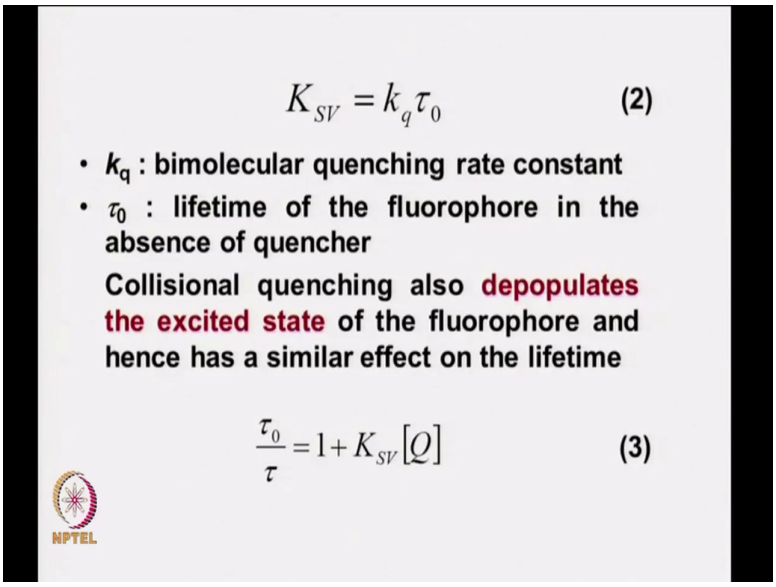

Now, K SV we will come to that graphical interpretation later, but what is K SV? K SV is also can also be written as k Q times tau 0. Now, here see all this time we have been talking about the steady state fluorescence intensity right, this is what we measure using a normal steady state terminator of I mean fluorometer. But here you see K SV has a different dependence that means, it is also dependent upon the lifetime tau 0.

So, what is tau 0? Tau 0 is the lifetime of the fluorophore in the absence of the quencher right. So, here is tau 0, it is a lifetime of the fluorophore in the absence of the quencher like F 0. F 0 was the intensity of the fluorescence intensity of the fluorophore in the absence of the quencher, tau 0 is a lifetime in the absence of the quencher.

What is k q? k q is a bimolecular quenching rate constant. What do you mean by bimolecular quenching rate constant? It should be easy to conceive because remember we are talking about

a collision and hence collision has to between two molecules hence bimolecular right. So, collisional quenching, now why did we bring in this lifetime issue. So, collisional quenching also depopulate the excited state of the fluorophore and hence has a similar effect on the lifetime ok.

I repeat collisional quenching also depopulates the excited state of the fluorophore and hence must be having a similar effect on the lifetime. Now, this is easy to understand. Now, suppose you have a fluorophore right and you have excited the fluorophore to the excited state right. Now, you have a quencher, whatever the quencher molecule is. The quencher molecule collides, the moment it collides what did happens what happens is it deexcides, that means, it gives rise to another non-radiative pathway in case of this collisional dynamic quenching.

Moment you have another non-radiative pathway what will happen is the quantum yield of the fluorescence will decrease, because remember that k n r this quenching process deexcitation would add to that k n r right that is k non-radiative remember what we talked about before in case of lifetime. And hence it would also decrease your lifetime and so your fluorescence quantum yield. Hence you see an effect on lifetime and you also see an effect on your steady state intensity ok.

So, here this is what you see again that is why tau 0 by tau now is equal to 1 plus K SV times Q. So, again having the same meaning see 1 plus K SV times Q is the same thing you saw for F 0 by F that was equation 2 for you or rather equation 1 in the previous slide tau 0 by tau, tau 0 we have just said what it is in this slide. While tau, what it is? Tau is now the lifetime of the fluorophore in presence of the quencher; it is very similar to what we had said for F right.

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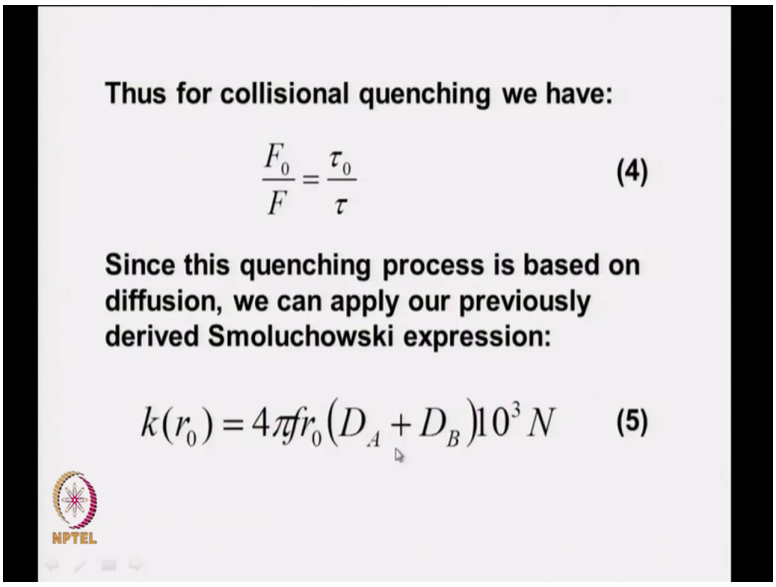

So, thus for collisional quenching, if you can summarize this is what we have. So, F 0 by F f has to be equal to tau 0 by tau that means, the ratio of the steady state intensity has to be equal to the ratio of the lifetimes. Since this quenching process is based on diffusion, remember we talked about this right this is a collisional quenching for collision these two have to collide and for them to collide they will have to diffuse towards each other. So, then we can apply a diffusion theory based on the Smoluchowski expression.

If you would remember what we had you know talk something back about the Smoluchowski expression we derived it, so this is what it looks like. So, this k r 0 is equal to 4 pi f r 0 D A plus D B times 10 to the power 3 times N, where N is the Avogadro's number. Now, what is D A plus D B? Simple, if you remember D A and D B, so D is the respective diameter, and A

and B are the respective you know diffusing species. Here the if the fluorophore is the part of the protein, and it is a for the protein, and in that case it is a other case it is the quencher.

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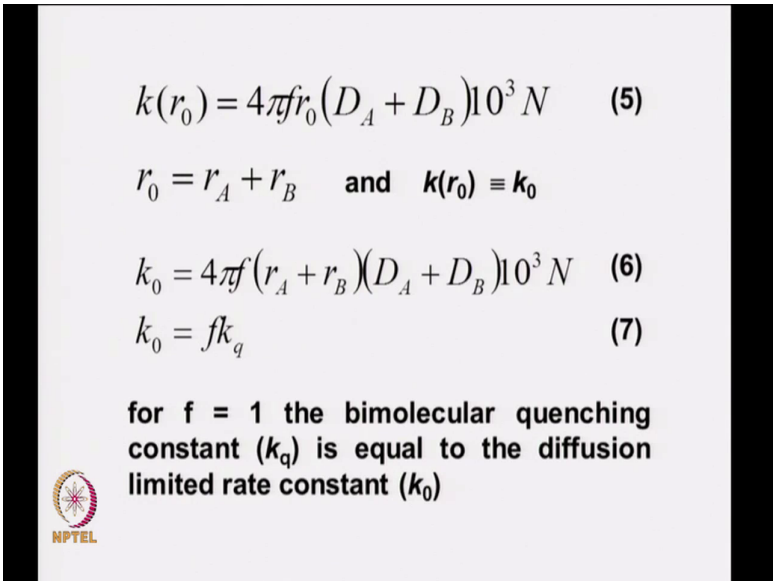

So, we can now for the right this k r 0 as r 0 equal to r a plus r b, where r a plus r b now just you know to go back just missed one thing. So, this D which is I said to the diameter is actually it is not right, this D is the diffusion constant right. So, these two are moving towards each other, so D is the diffusion constant of species A, and D is the diffusion constant of species B, it is, it is not the diameter. Now, r where the distance comes in r 0 is equal to r a plus r b this is the radius of that particular species right, whether it is of the protein or the quencher depending upon what do you have.

What would do now is we just replace k r 0 by k 0 ok. Now, what we have is then, k 0 is equal to 4 phi f times r a plus r b times D A plus D B then the rest of the expression. And

hence we can write this as k 0 is equal to f times k q right, where k q is the whole of the expression apart from f. Now, what is f mean? See f is essentially the frequency of collision.

So, what it means is suppose I am I am having 100 collisions right. And if out of 100 collisions all the collisions bringing about quenching, then my success is 100 percent, that means, f is equal to 1. However out of 100 molecules if only 50 collisions, that means, of 100 collisions if 50 collisions bring about quenching right, then my f would be 0.5. So, f is essentially related to the frequency or the effectiveness of the quenching process.

So, then what we have is for f is equal to 1, the bimolecular quenching constant k q remember this is what k q we saw before, where K SV is equal to k q times tau 0 this is equal to the diffusion limited rate constant k 0 right. So, k 0 is a diffusion limited rate constant at if each and every collision is effective f is equal to 1, then k q is equal to k 0. Now, that is what this expression is telling you.

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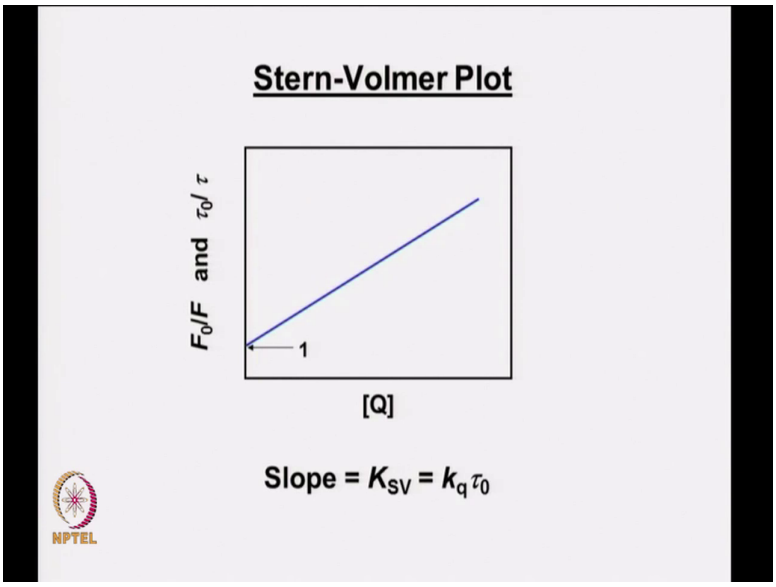

Now, as promised let us look at Stern-Volmer plot. So, what does it say? So, whether we have we are plotting F 0 by F or tau 0 by tau on the y-axis against the concentration of Q, so what is going to happen is, you can see it always starts from 1, the intercept is 1, and then it goes on increasing in a linear fashion. Now, why does it start from 1, it is easy to understand because at 1, that means, at this place the quencher concentration is 0. So, at the quencher concentration being 0, F, this F in the denominator would be F 0 and F 0 by F 0 is 1; same for tau 0 by tau 0 is equal to 1 that is why it starts from 1, and then it increases.

Now, this loop is essentially your K SV that is what it says. So, your K SV is equal to k q times tau 0 so ok. So, then for collision quenching, there is two ways of looking at it. One is you can look at the steady state fluorescence in density changes, and the other one is you can

also look at the lifetime right you know both of these are similar if it is a dynamic quenching F 0 by F should be equal to tau 0 by tau as we just discussed.

So, you can use either of these, right. But then there is reason why we are going through this or why we discussing this equivalence of F 0 by F is equal to tau 0 by tau, it will be very instrumental when we try to differentiate between different types of quenching ok.

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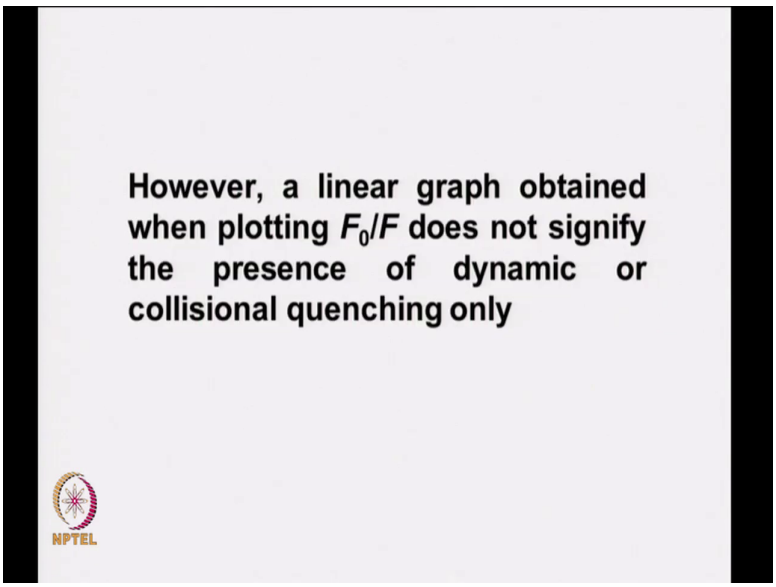

So, now as you saw this was a linear graph, but there is a linear graph guarantee that, you are having dynamic or collisional quenching. So, it says however, a linear graph obtained when plotting F 0 by F does not signify the presence of dynamic or collision quenching only. Now, this is extremely important. Why? Because remember when we talked when we are talking about types of quenching, we have talked about different types of quenching one was dynamic – the one we just discussed, the other one was static and there we had sphere of action ok.

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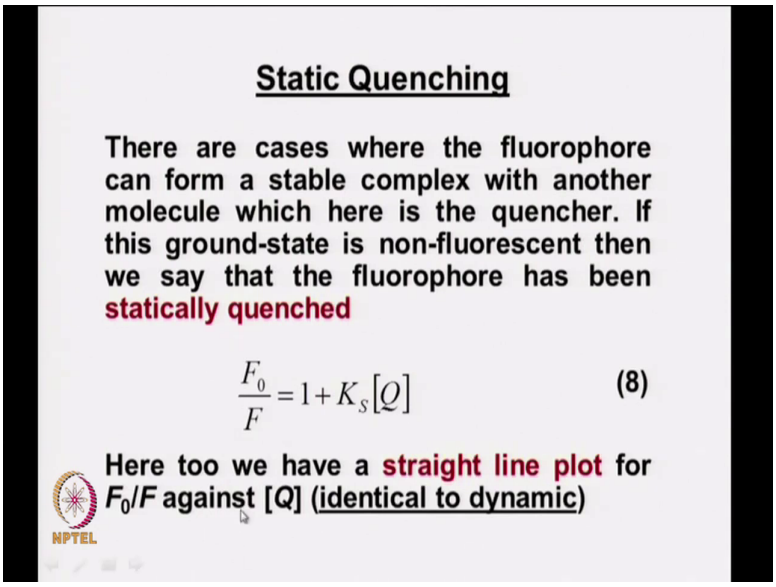

Now, leaving as a sphere of action, and then what is static quenching how do I you know how is it different from dynamic quenching that is why we have to take look at it. So, here we start discussing was static quenching. So, what is static you know what you mean by static, means you know when can you see static quenching? So, there are cases where the fluorophore can form a stable complex as written here with another molecule which here is the quencher because we are talking about a fluorescence about fluorescence quenching. Now, if this ground-state is non fluorescent, then we say that the fluorophore has be statically quenched.

Now, please keep this difference in mind when you are having a dynamic quenching, you are not forming any ground ground-state complex. What are you looking at you know only looking at the collision, collision between these two different molecules either the fluorophore by itself, or the fluorophore when it is a part of a fluorophore protein molecule and the quencher molecule right. Then in case of static quenching how what is happening. So, the

quencher molecule has to form a complex in the ground state with that of the fluorophore ok, so that means, it comes and binds and forms a complex.

So, here you will be having something known as a complexation constant or equilibrium constant, because it is forming a ground-state complex. Yes, it definitely has to collide it will form the complex after it you know colloids, but then in case of dynamic quenching after collision it would never stay at (Refer Time: 25:33) you know it will always move in collide and move out, but in case of static quenching what it comes it stays and forms a complex right. This is the difference. There will be more differences later as you will see. So, here also here also the Stern-Volmer relation goes like F 0 by F is equal to 1 plus K S times concentration of Q.

Now, please keep this in mind in the previous case, we said K SV which was the Stern-Volmer constant, in this case it is K S ok. It is a static quenching constant. And what is K S? Let us take a look at it. But before you know going to the next side, let us see what we have written here. Realize this fact that here too if you plotting F 0 by F against quencher concentration.

Then we are going to have a straight line identical to dynamic quenching, but as we just discussed it is not a dynamic quenching right, it is static quenching. Then how do we differentiate between these two? Because both these if they are occurring individually, both these would give rise to respective straight plots or you know straight lines, or linear plots right. So, the in that case how would you figure out ok?

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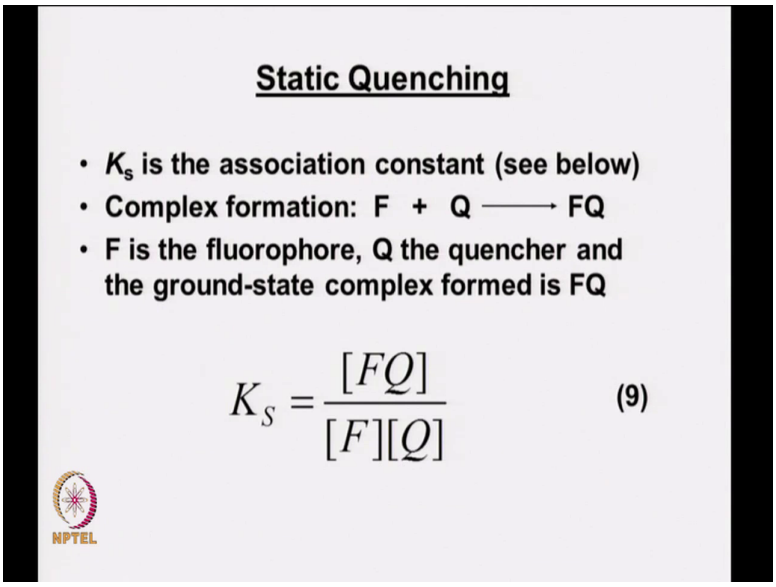

So, looking at static quenching again K S as I said is a association constant, that means, the constant with regards to the complex formation. So, the complex formation is F plus Q giving F Q, because it forms a complex. Then F is the fluorophore, Q is a quencher and the ground-state complex, and the ground-state complex form is F Q ok. So, then the moment I have this, then I can give rise to or you can write down an equilibrium constant expression. So, that means, K S is equal to concentration of F Q over concentration of F times concentration of Q right. Now, this is expression for K S the static quenching constant as opposed to the dynamic quenching constant K SV which was equal to k q times tau 0.

So, look at this now. In the dynamic quenching constant that K SV that K SV could be equated to k q the biomolecule quenching constant times the lifetime tau 0 in absence of the quencher ok. That means, that is why you could have the equi that equivalence between the

lifetime and statistical intensity, but here no lifetime is coming into play. So, possibly we are not going to have that.

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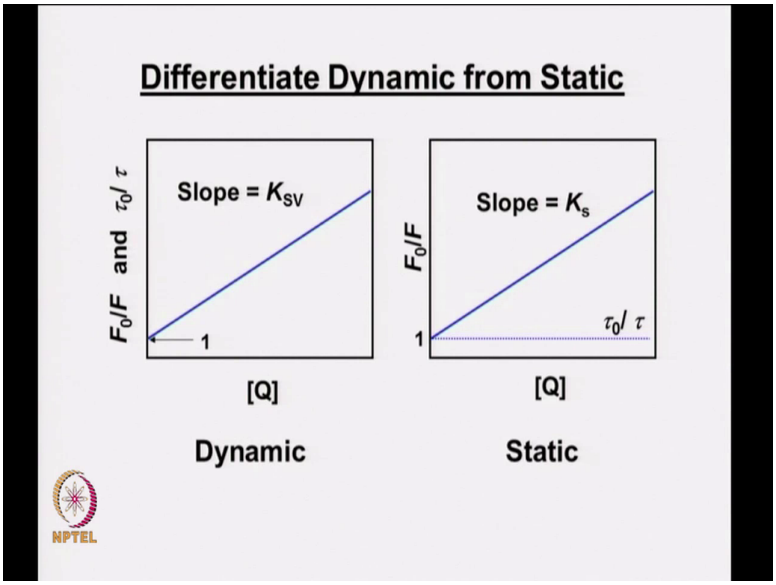

So, how do we differentiate dynamic from static? Now, this is what I was telling you before, you look at this. First look at the right hand side right. On the right hand side, what we are seeing is on the y-axis that means, on the right plot, on the y-axis, you are plotting F 0 by F. And by this time we are familiar with what F 0 by F means right against concentration of quencher.

So, here you can see we are having a straight line right, so that means, F 0 by F against concentration quencher is having is giving you a straight line. But the same is obtained for dynamic quenching too right if you look at the if you look at now at the left hand side or the left plot. But just see on the y axis of the left figure or the left panel what we are writing out

here is F 0 by F and tau 0 by tau, now because F 0 by F and tau 0 by tau they were equal F 0 by F is equal to tau 0 by tau, then it does not matter which one we plot, you are going to get the same straight line right like this. It is a linearly increasing plot.

However, look at the right panel now. On the y-axis, I only have F 0 by F we have we do not have tau 0 by tau. However, if we do try to plot tau 0 by tau, and if this is a case of static quenching only, that means, there is no other quenching involved only a case of static quenching, and then you can see how it is out here.

So, this is tau 0 by tau. The tau 0 by tau, however, does not follow the F 0 by F that means, tau 0 by tau is always equal to 1. In other words, what it says is, that means, in case of static quenching, the lifetime does not change ok, that means, of the lifetime of fluorophore is not affected. You found the ground state complex, the fluorescence is totally quenched, you never observe fluorescence from those molecules.

On the contrary, whatever molecule is not complexed your exciting and you are getting the fluorescence from that molecule. So, that means, that tau 0 or that tau is always equal to tau 0, because whatever molecules are exciting they are coming down fluorescing because they are not complexed right. So, this is the major difference between these two. So, if you would, if you would be getting straight line plots, how would you differentiate or how would you know try to answer the question whether it is dynamic or static?

So, what you would do is to see its dynamic, you have to plot tau 0 by tau, that means, have to do lifetime measurements that is means one of the easiest ways of doing it. If lifetime measurement that is tau 0 by tau follows F 0 by F just like your first panel or left hand panel, then you know it is a case of collisional quenching or dynamic quenching. However, if tau 0 by tau follows this plot that means it is almost shows no change then it is a case of static quenching.

So, this is one of the best ways of trying to figure out which one is static or which one is which process is static quenching process, which process is a dynamic quenching process. But

this is the only way we can differentiate? Well, this is one of the best ways definitely, but not the only way.

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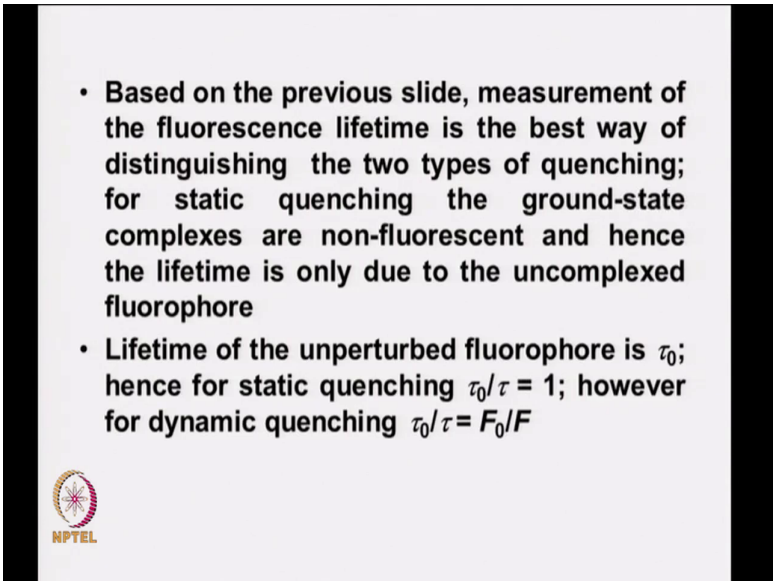

Let us look at another way, but just to you know kind of go through it again. So, based on the previous slide, slide we were just discussing, measurement of the fluorescence lifetime is the best way of distinguishing the two types of quenching. For static quenching, the ground-state complexes are non-fluorescent and hence the lifetime is only due to the uncomplexed fluorophore that means, that fluorophore which is free which is not complex with Q. Now, the lifetime of the unperturbed fluorophore that is tau 0, you know it is tau 0, right.

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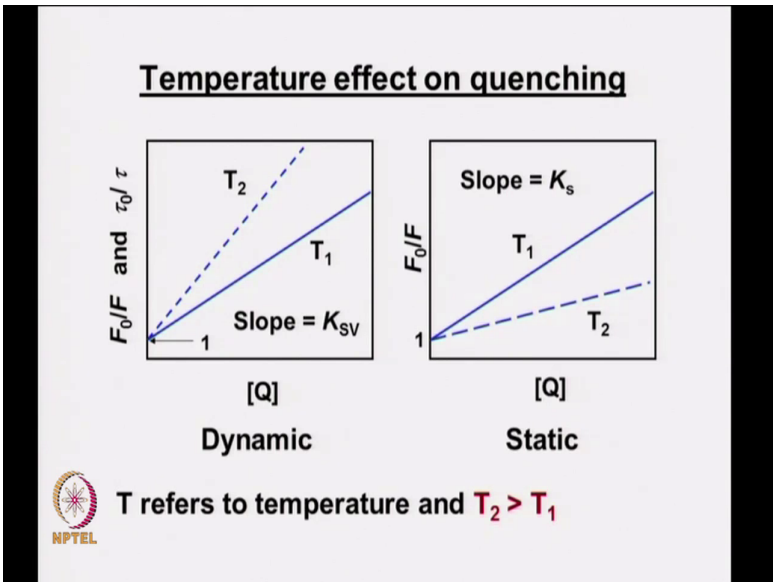

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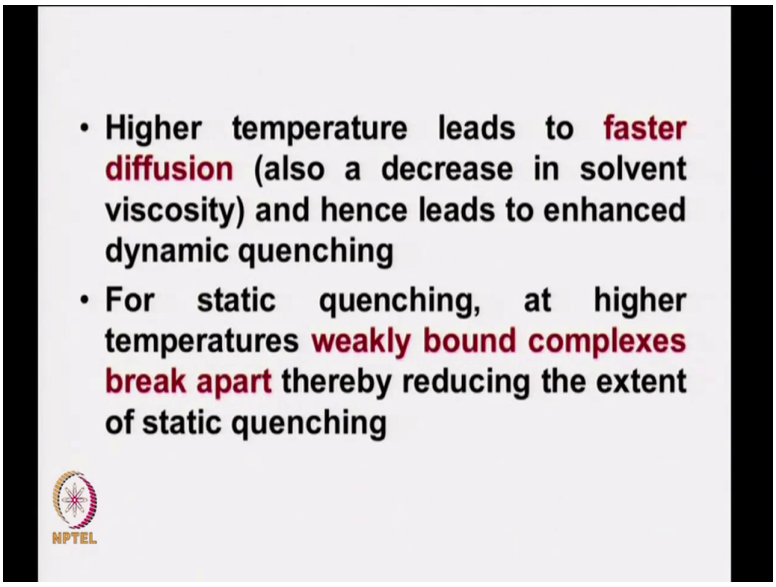

So, because it is unperturbed, so let me go back. So, lifetimes of the unperturbed fluorophore is tau 0 that is what we had said. And hence forth static quenching tau 0 by tau is always equal to 1, because that tau in the denominator, the tau in the denominator is also tau 0 because you are only looking at uncomplexed fluorophore fluorescing. However, for dynamic quenching, what is happening for dynamic quenching, we have seen tau 0 by tau is equal to F 0 by F. So, again this is one of the best ways of trying to figure out the difference between these two right or which one is which.

Now, as I said is this the only one or is this the only way to differentiate? No, there is another effect the temperature effect. Now, try to realize what is going to happen. Again look at the left curve. In the left curve we are having dynamic quenching. So, T 1 and T 2 are the different temperatures, where T 2 is greater than T 1. Now, think about this. When you go to increase

temperature, you are going to put in you are going to increase thermal energy of the molecules, and hence is going to increase the diffusion rate.

The moment it increases the diffusion rate, what will happen is it will collide at a higher frequency at a higher rate. And hence if your dynamic quenching is purely or rather your quenching is purely dynamic, then the effect of quenching or the rate of quenching will increase, that means, the slope the slope would get steeper, and slope would increase. So, that is exactly what you are seeing out here. You can see the slope is K SV for dynamic quenching. This is T 1, this is T 1; this is T 2. T 1, T 2 is greater than T 1. So, because it is a higher temperature and I said diffusion increases right and hence you have much more quenching in case of dynamic.

However, the reverse is seen for static quenching. Now, remember we are talking about a complex. Now, generally what happens is when you would if it is you know now if it is a loosely formed complex and if you would be increasing the temperature, what would happen what would happen is a complex would break apart, so this is exactly what you are seeing out here. So, here for static quenching at T 1 this is a slope you are looking at.

But at T 2 what has happened is the slope is decreased, why because a quenching has decreased, why because the complex are formed here are forming at the high temperature is not as stable as it was at the low temperature, and hence you can see the quenching has decreased ok. So, this is also another way by looking at the temperature difference, you can figure out which one is which, that means, which one is dynamic which one is static right.

So, as we just you know discussed higher temperature leads to faster diffusion, remember higher temperature also brings out a decrease in solvent viscosity, and hence leads to enhanced dynamic quenching. For static quenching at higher temperatures weakly bound complexes which these are you know these static quenching complexes are they break apart thereby reducing the extent of static quenching ok. So, now, you have two ways of looking at it, one is by measuring lifetime changes, and the other one is by measuring you know the temperature dependence ok.

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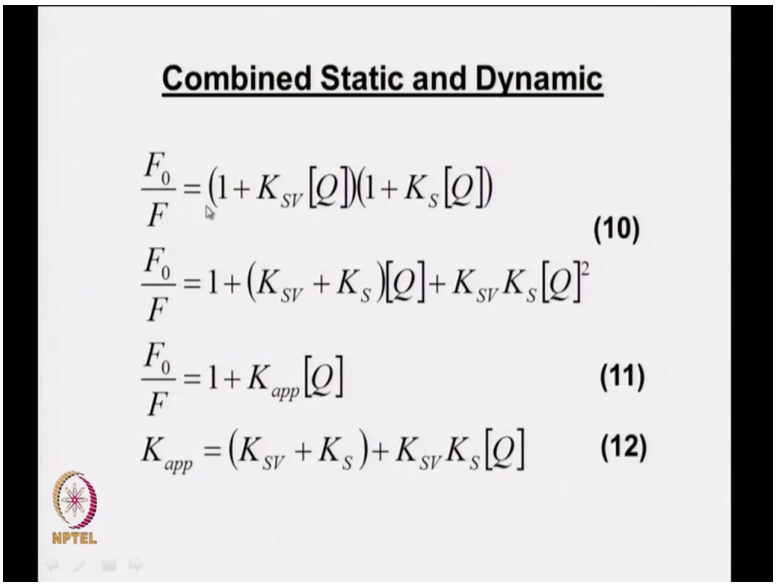

Now, there is also just we before I go to this slide let me tell you one thing. So, when in many cases it has been found that if you would look at the absorption spectrum, in case of a static quenching, because it forms a ground-state complex, right then the absorption spectrum it would be different than that of the uncompressed flurophore in case of a static quenching case. However, in case of dynamic quenching, you do not have any complex formation, and hence you would not be having any such changes in absorbance, good.

So, we have talked about two individual types of quenching, one is static, one is dynamic. We started with dynamic and then we said static is different from dynamic, but both exhibiting linear plots if they are occurring if they are occurring individually. Now, you can have cases right, you can have cases where both are occurring together. So, it can be shown in that case that the F 0 by F you know which we are familiar by now is equal to the product of the two,

that means, 1 plus K SV times Q which is the dynamic part, and 1 plus k S times concentration of Q which is a static part.

And so if you go on and expand it, you will see that you have a quadratic right in Q with; so, here you have the here you have the Q squared term. Now, this can be simplified as tried out by yourself, where F 0 by F is equal to 1 plus K apparent times concentration of Q. And this K apparent has this expression. You know just simple algebra manipulation, and you will get this expression right.

So, what you can see now is here if you are going to plot F 0 by F for a case where you have a combined static and dynamic quenching, that means, both are occurring simultaneously, you would not be getting this straight line, other on the contrary you would be getting a curve plot because of this quarter independence is that what you see.

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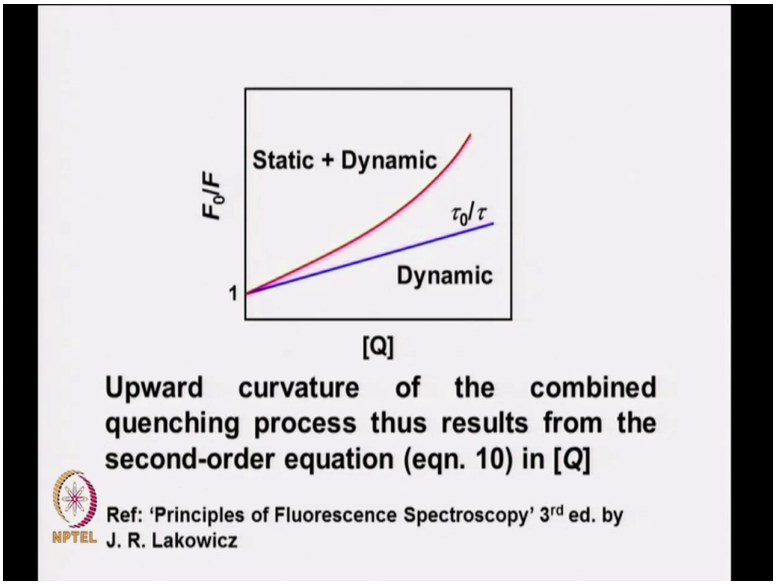

Let us see this is what you see right. This is F 0 by F again against concentration of Q. You can see how this plot is curved. This is a case of static plus dynamic. And this curvature you can understand is arising from that quadratic term ok. However, if you talk about the dynamic, then this is a word it should look like.

So, what is this you know, so what is this graph telling you, let us go through it again. The red curve, the red plot or the red curve is a combination of two types of quenching process static and dynamic. Now, suppose you are asked to figure out what is the contribution of dynamic quenching here, what would you do?

Now, you know that this is a combined case, but also what you know is the static quenching, there is no life time dependence; in case of dynamic quenching the lifetime just follows the same strength as the fluorescence intensity. So, if you would do this tau 0 by tau, then what you would figure out is you would be able to extract, you would be able to extract the dynamic part from the combined static and dynamic. So, this that is essentially what has been shown here.

So, this red plot is again the combined one and the blue plot is a tau 0 by tau which is telling you that this is the dynamic part of that combined plot. Remember if you were plotting tau 0 by tau for the static one, it should be just parallel to the x-axis that means it should be 1. So, as I just said the upward curvature of the combined quenching process, this results from the second order equation, this equation 10 in Q right. So, quenching plots with downward curvatures.

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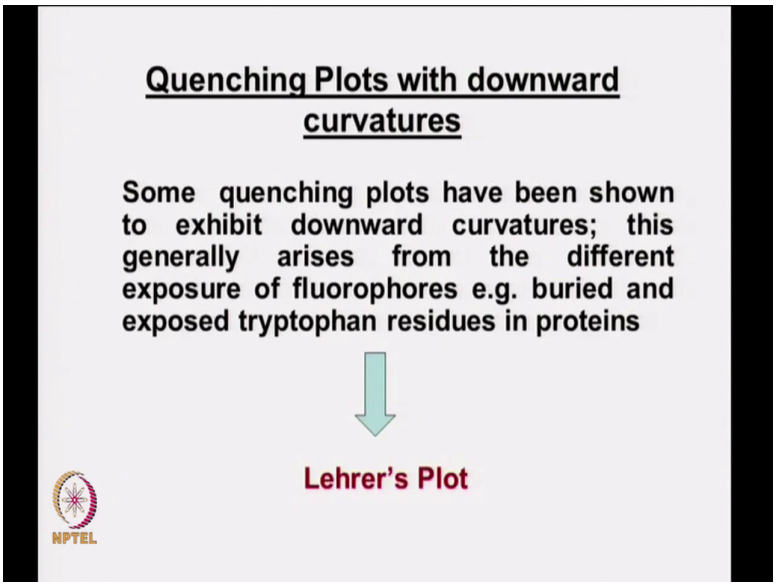

Now, quick recap what did we start from, we started from dynamic quenching, linear plot, you looked at static quenching, it was also a linear plot. How do we differentiate? In one case there is a lifetime (Refer Time: 39:03), in the other case the lifetime is unperturbed. Here the next is temperature, by temperature because also differentiate. Then also there are cases where your plot is on linearity, it is curved.

And typically in those cases you know that it is the case of mixed quenching happened that is what you just saw. How do we extract the dynamic counterpart, we take the lifetime ratio, and you can understand to what extent is the dynamic contribution having its effect with the total plot.

Now, what about you know quenching plots with downward curvatures? Now, why do you have a downward curvatures to start with? The curvature you saw in the previous slide was

upward right. It is moving out up like this, but that means, downwards means it would be coming like this ok. So, some quenching plots have been shown to exhibit downward curvatures. Now, this generally arises from the different exposure of fluorophores example buried and exposed to tryptophan residues in proteins right ok.

So, immediately what I tell you is this. I can have a protein where taking the simplest case I have two tryptophan residues. One tryptophan residue is well exposed, and one tryptophan residue is very much in the interior of the protein. Now, if there is the case, if that is the case that means you have two different you have the same fluorophore, but two different subpopulations. One population is the one which is exposed and the other one which is buried, that means, you have two different types of accessibilities to the quencher molecule.

Now, if that is the case, this is this is a typical situation where you would possibly see quenching process with downward curvatures because you have two different subpopulations of fluorophores, so one less accessible, one much more accessible or more accessible. So, this has been described by a certain type of plot or this process has been described by certain type of plot known as Lehrer's plot.

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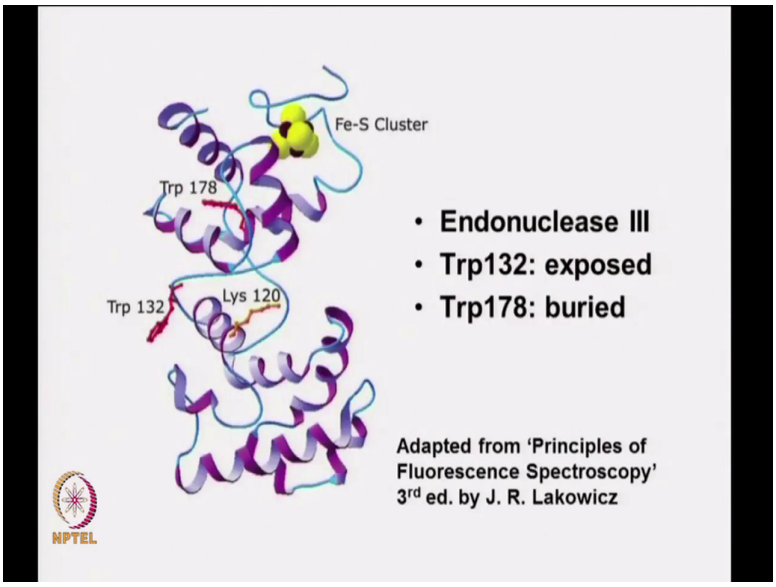

So, before going to the actual you know theory on the Lehrer's plot. Let us look at a protein, so as I was telling you about this you know accessible, non-accessible. So, this is a protein called endonuclease III right, I adapted this from Lakowicz. So, endonuclease III you can see this is an F e iron sulfur cluster and you look this two cases, you have two tryptophans Trp 178 and Trp 132. Now, Trp 132 you can understand is out here which is exposed right, the Trp 178 you can see it is between the two helices out here and it is buried, so that means you already have two different populations.

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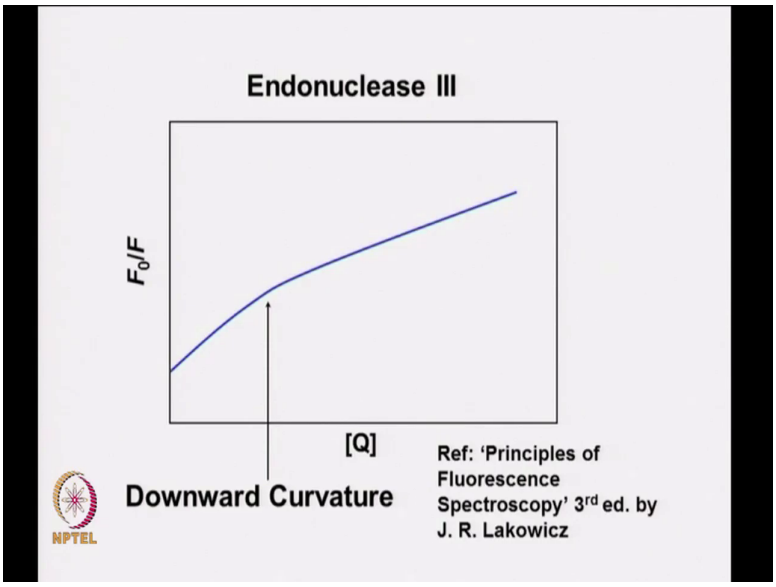

Now, this is what the plot would look like if you would be having F 0 by F against Q you can see as opposed to the upward curvature we had seen before, here you can see at definite download curvature, so that means, this is being shown for endonuclease III.

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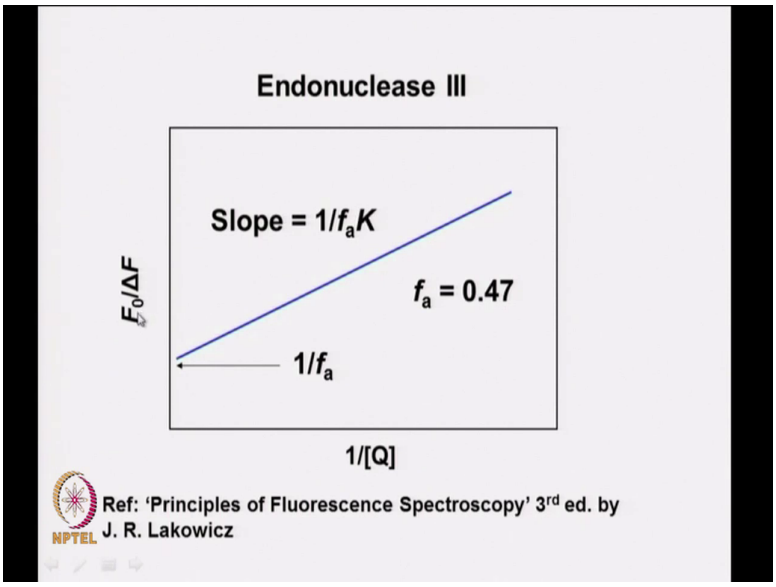

So, endonuclease III it has been shown that F 0 by delta F; its not F it is delta F and I will tell you sorry, let us go going back to the previous slide by this F 0 by F its not delta F; F 0 by F against Q, it gives a downward curvature. Signify immediately that F, two different populations; one more accessible, one less accessible. Now, how do you derive the theory for it? So, let us try to do that so now I will use the sheets a little bit, so we will be talking about Lehrer's plot.

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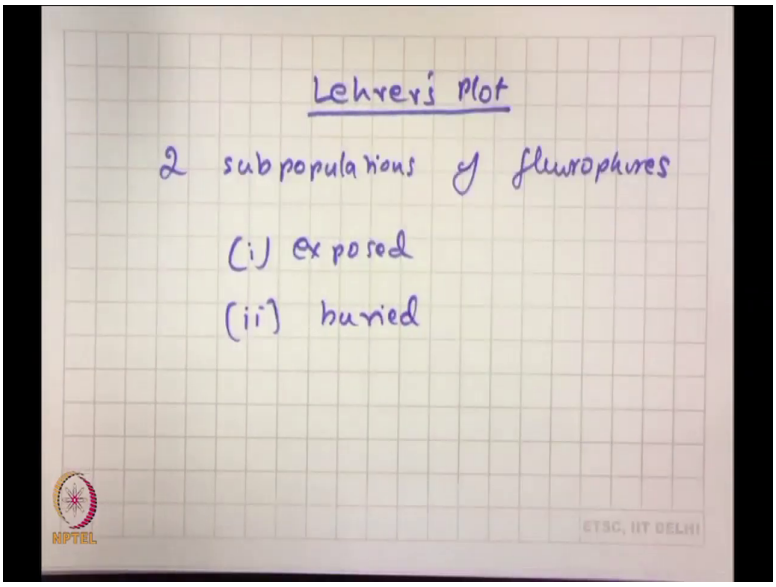

Now, when we talk about Lehrer's plot, what we meaning is this, what we meaning is we are considering two subpopulations of fluorophores. Two subpopulations of fluorophores, one is exposed and the other one is buried; now this is essentially what we considering is very simple ok, two sets ok. Now, what we say is so this is how we start with the derivation.

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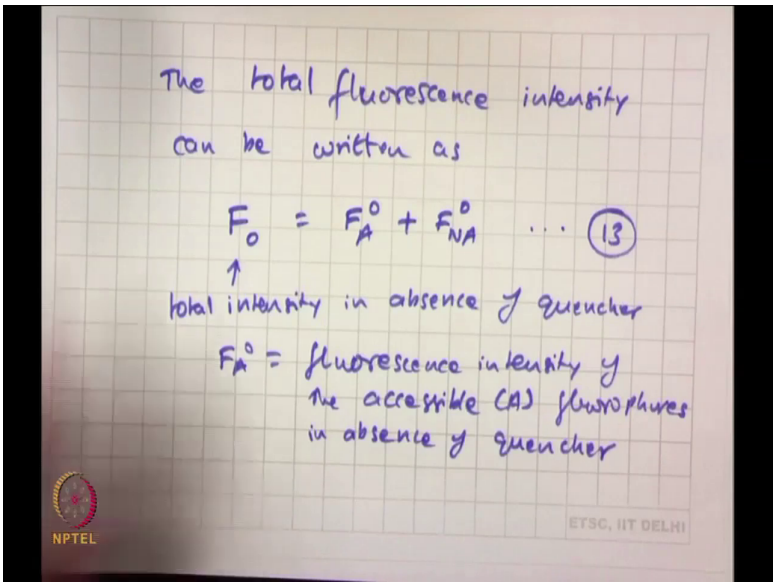

What we say is the total the total fluorescence intensity can be written as can be written as F, can be written as F 0 by this time you must be knowing that F 0 means that in absence of the quencher is equal to F A 0 plus F N A 0 and this is equation 13 ok; I guess the last one was 12, but if I am wrong please do correct it accordingly.

Now, F 0 obviously is the total intensity in absence of any quencher ok, right. Now, what is F A 0 then F A 0 is the fluorescence intensity, the fluorescence intensity of the accessible which is A fluorophores of the accessible fluorophores in absence of quencher right.

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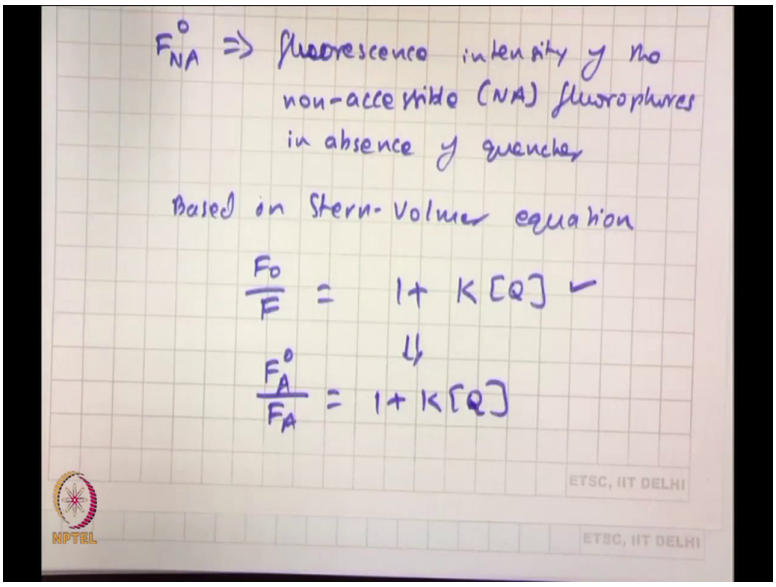

So, again F A 0 or F A naught is the fluorescence intensity of the accessible, A stands for accessible fluorophores in absence of quenchers, so then you can easily understand. What will F N A naught stands for naught this will stands for, fluorescence intensity of the non-accessible that is why its N A fluorophores in absence of quencher ok, so it makes sense right.

The total fluorescence you see, in absence of quencher should be equal to the sum summation of the fluorescence coming from two different subpopulations. One is the fluorescence of the accessible portion, F A naught in absence of the quencher; and if fluorescence of the not accessible portion, F N A naught in absence of the quencher right.

Now, suppose you are adding quenchers what will happen, because we taking the non-accessible portion to be non-accessible, totally non-accessible let us take the most

extreme case. Then if you are adding quenches, the quenches would actually quench the intensity of the accessible quenches only right. So, then what I can write is based on Stern-Volmer equation, what we can say is F 0 by F is equal to 1 plus K times concentration of Q ok, so this is our familiar Stern-Volmer's equation.

However, what I will do here is instead of writing this what I will write like this is this I can write F A naught over F A is equal to 1 plus K times Q ok. So, just to make the point, so this one you have already seen you have already encountered and this is the one where F A is the fluorescence intensity accessible part, because simply speaking as we just said the non-accessible portion is not being accessed by the quenches and hence it is not being quenched, good.

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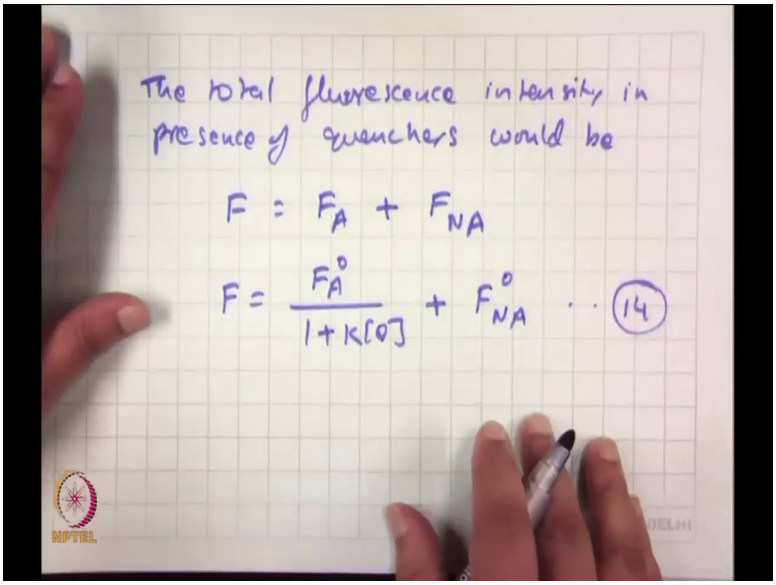

So, this is one part, so then how would are combined intensity look like. So, if I now say, then the total fluorescence intensity in presence of quenchers, the total fluorescence intensity in presence of quenchers would be what; I can write F, I can write F this is the total fluorescence quenching intensity or the fluorescence intensity in terms of quenchers is equal to F A right plus F N A, ok.

Now, will make one more substitution please keep this in mind, this is in presence of quencher, this is also in presence of quencher, this is also in presence of quencher ok. For one thing we already have what we can right now is F is equal to if you go back to what we had done just in the previous sheet of paper, F A naught by F A is equal to 1 plus K times Q. So, here I already have an expression where F A is equal to F A naught by 1 plus K times Q. So, then what I can write out here is I can replace this by F A naught 1 plus K times Q plus now see what I am writing, I am writing this one to be F N A naught ok.

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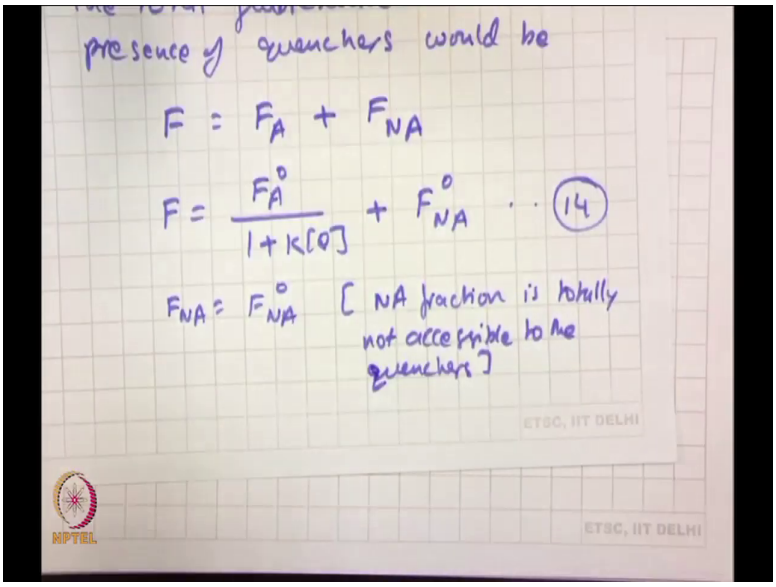

Now, why am I writing this to be F N A naught remember our assumption was that F N A is equal to F N A naught why, because the N A fraction is totally not accessible to the quenchers and hence it is not been quenched. So, then if it is not being quenched, it is not going to have any changes its in its fluorescence intensity; so F N A in presence of quenching it should be equal to F N A naught which was the same in the absence of the quencher.

So, now having said that what we can do now is for this simplify, so how we will simplify find what we will do is we have these two equations. So, we have these two equations; one is this number 14 and one is what we have what we had what we had started with before number 13.

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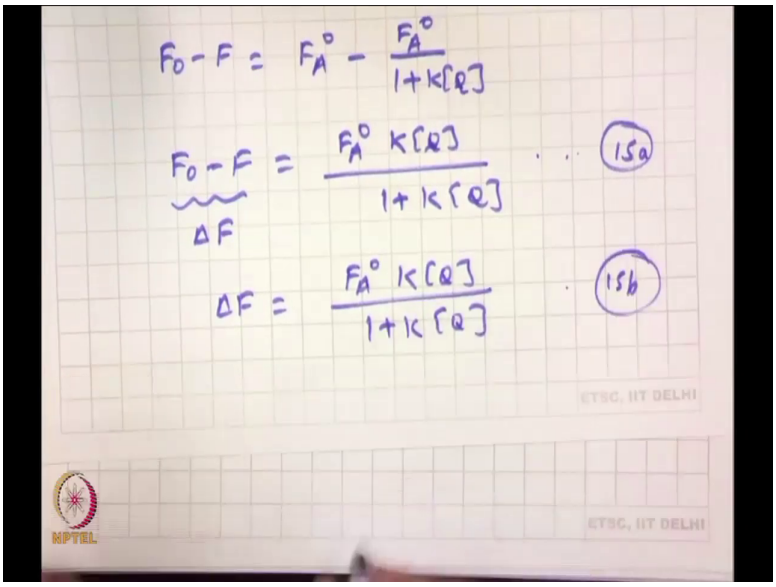

So, now what we can do is we can say equation 13 minus 14 gives us what, remember we are trying to get a very simple equation how simple is it we will see later, but how do we get it. So, then this will tell me that F 0 minus F is equal to remember F 0 is equal to F N naught plus F N A naught. Then I can write this one as F A naught minus F A naught 1 plus K times Q right, and the concentration or rather the fluorescence intensity of in F N A cancels out why, because F N A is equal to F N A naught right.

So, then on for further simplification I can write F 0 minus F is equal to what can I write, I can write F A naught can do this K times Q over 1 plus K times concentration of Q; so let this be number equation number 15 ok. So, we already have an equation like this, where this F 0 minus F can be replaced by delta F or I can write delta F is equal to F A naught K Q 1 plus K

times Q, ok; so let us this be let us this be 15 a, let this be 15 b. Now, where do we go from here one more simplification we need.

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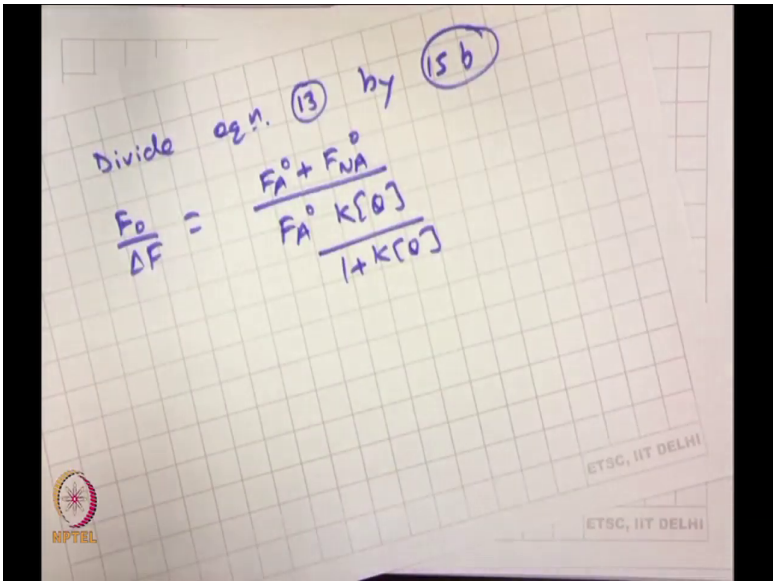

So, what we do now is we divide equation 13 by 15 say b; does not matter which one we are taking whether it is 15 a or 15 b. So, then what we are having is F naught by delta F is equal to this is what we have for F naught plus F N A naught, then what we just derive for this delta F, this is F A naught K times concentration of Q over 1 plus K times concentration of Q, ok.

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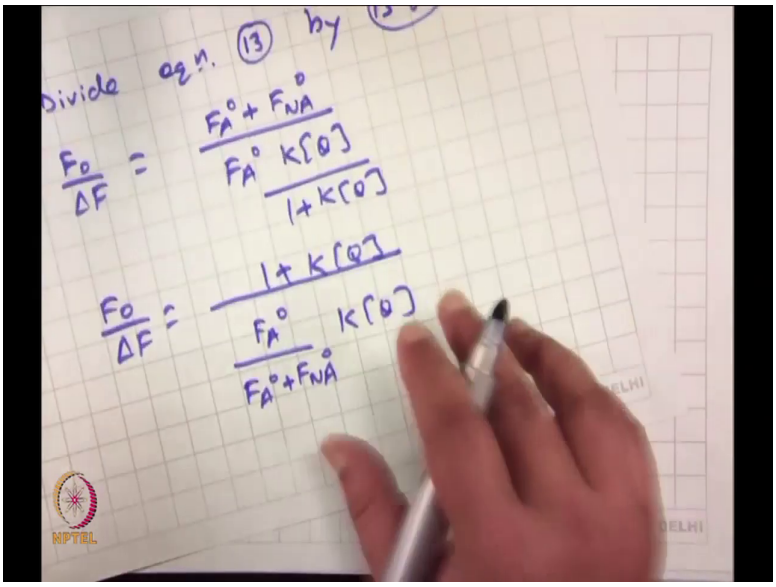

Now, we do a minus simplification, so that is what we do is we get F 0 by delta F is equal to this F A naught plus F A F N A naught I bring down. So, I can have 1 plus K times concentration of Q over F N A F A naught over F A naught plus F N A naught times concentration of Q, still not done.

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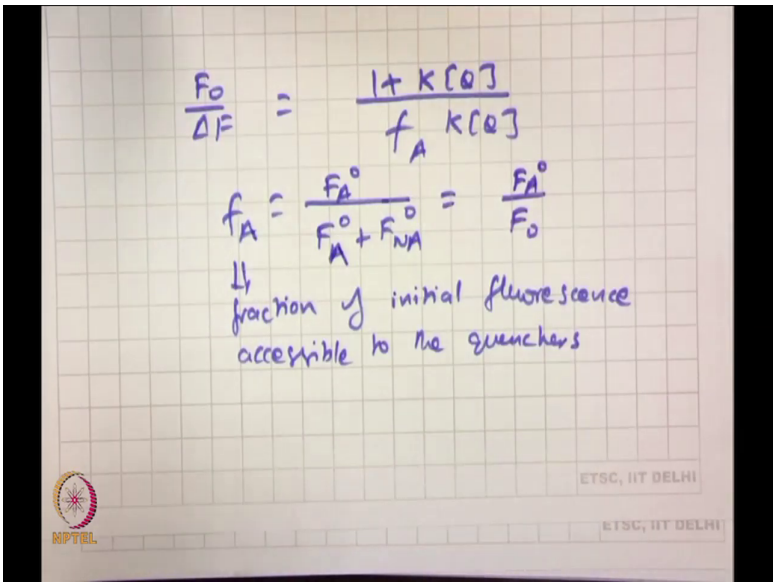

So, what we do after this is the way we express this we can write it like, this F naught over delta F is equal to 1 plus K times Q, this is f A times K Q. Now, what is f A? So, f A is equal to F A naught over F A naught plus F N A naught. Now, this is important to understand what f A is, f A is then you can realize what f A is, f A is the fraction of initial fluorescence accessible to the quenchers.

Now, this is what is important what you are saying is see f A, this is equal to F A naught plus F A naught plus F N A naught; now F A naught plus F N A naught is equal to F naught or F 0 and this F A naught, so this can be written as F A naught over F 0 is not it. So, it immediately says if my total intensity is F 0, then F A naught over F 0 would be the fraction of the initial fluorescence which is already accessible to the quencher molecules, ok.

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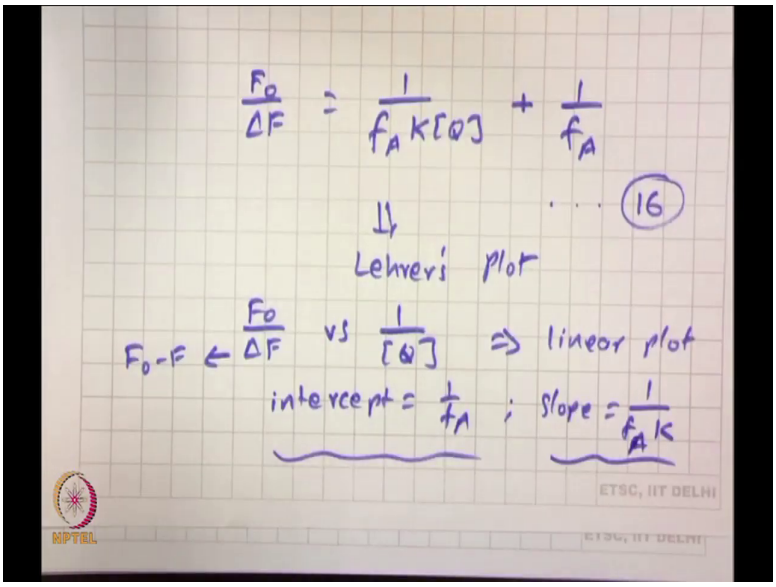

So, now going further going further using this equation, using this equation going further what I can write is I can write F 0 by delta F is equal to 1 by f A times K concentration of Q plus, now you can see already have 1 by f A right; so let this be equation number 16. This is commonly referred to as the Lehrer's plot; this is commonly referred to as Lehrer's plot.

So, what you can see now, so what you can see now is if I plot that means, F 0 by delta F and remember this delta F is equal to F naught minus F verses 1 by concentration of Q, what am I going to get I am going to get a straight line right, I am going to get a straight line. Now, If I am going to get straight line, then see straight lines are always similar to intercept, now this is why we went to this derivation I told you that we are going to get a simpler expression at least something which we can deal with a little more easily.

So, if this is what we have then you can understand from here from the straight line plot is a linear plot, this is a linear plot. So, the intercept is equal to 1 by f A and the slope is equal to 1 by f A times Q ok, these two are very important you know parameters of this equation. So, if you have to write it now, if you have to write it now or if you have to look at the plot, this is how the plot will look like and now let us look at the slide again.

So, this is typically how it looks you look at endonuclease, you look at endonuclease again. So, this is this was a plot before right this was the downward curvature plot, where I said two different fluorophore accessibilities.

Now, you can the same plot you can see what I have done is I have plotted F 0 by delta F you know, the thing we just derived and this is the plot you are looking at here this is 1 by f A which is the intercept and this is 1 by f A times K which is a slope; here f A is 0.47. What is what it means is remember this was a fraction accessibility, what it means is out of the total initial intensity whatever it was close to 50 percent or 0.47 or 0.5 fraction was accessible to the quenching molecules right.

So, you know but remember one thing, this is just a case or an example where we have taken two different quenchers or two different populations; not quenchers, populations fluorophore one which is fully exposed and the other one which is fully buried, but then you are not going to have the same thing all the time.

So, what might be happening is one might be partially buried, partially exposed; the other one might be you know more buried, less buried well you can be having a combination of many things. So, you can understand that this plot will always not be so easy to interpret, ok.

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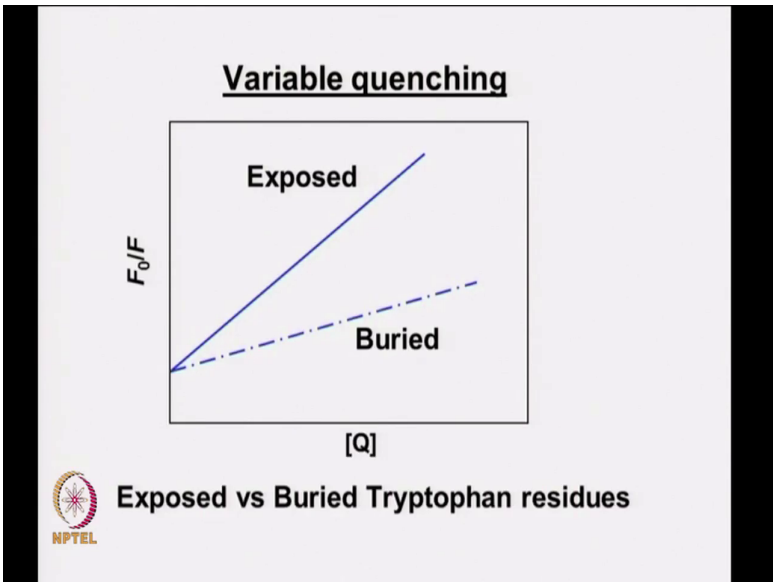

But anyway this is one way you can simplify it, right. As you just to you know end the class today, what I will tell you is this you have gone to these different types static, dynamic or dynamic, static; I have not talked about sphere of action, but then we have talked about this Lehrer's plot or this accessibility situation where you would be having a different you know quencher accessibilities, right.

Then let us look at a case of variable quenching in variable quenching what I mean by variable quenching is at this it means now let us not talk about Lehrer's plot ok, just forget let us consider very simple linear plot either static or dynamic does not matter what it is.

Now, suppose you have a protein which has a tryptophan at the very exposed portion and another protein which is tryptophan at a very less exposed portion, then what is going to happen, now I am talking about single tryptophan now. And if you are going to plot F 0 by F

against concentration of Q, then you can see the exposed portion would be more quenched and hence the slope is higher, the buried portion would be less quenched and obviously the slope is lower.

So, this is how you can figure out remember we talked about this; how can we figure out between explosion and buried, this is what it is this is how you can figure out, ok.

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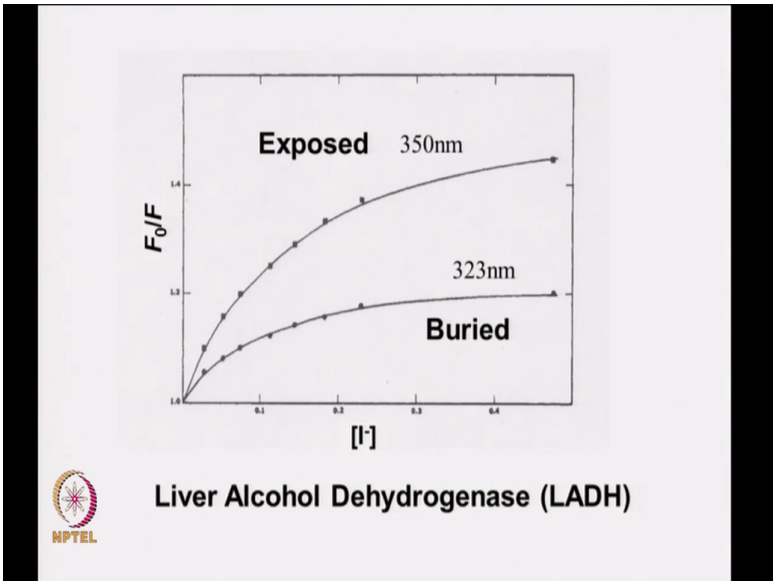

So, you can again see out here, this is the case for a liver alcohol dehydrogenase. Now, here is a case of Lehrer's plot again. You can see the downward curvature, the downward curvature. So, this is this enzyme liver alcohol dehydrogenase. The exposed portion and the buried portion you can see, the exposed portion has a higher slope, sorry exposed has a higher slope, and the buried portion has a lower slope. Not only that how do you figure it out, or how did the people who did experiment figure it out?

So, what did they did was, remember the buried tryptophan has a lower wavelength emission maximum than the exposed tryptophan. So, that is what you see the buried tryptophan for monitoring the buried tryptophan, what they did was, they monitored the emission at 323 nanometers, and for the exposed monitor 350 nanometer, and that is how they came out you know they came around to plotting these two different graphs. For the buried the slope is smaller; for the exposed the extent of quenching is much more and that is understood.

So, in a nutshell this is where I am going to stop today, but in a nutshell we have discussed quenching. Now, quenching is used a lot for you know studying protein folding specially where we are trying to look at the accessibility right. And you know this is also very unique. Why it is so unique? Because if you are you know talking about circular dichroism which we were going talk about later this, what it tells you is this circular dichroism or even you know to a certain extent FTIR or infrared it gives you idea it gives you an idea about the total change in the structure.

But here when you are doing is quenching, remember you are looking at the quenching of tryptophan, when you are looking at the quenching of the tryptophan it does not matter to you what has happened to the rest of the protein, instead the information you are getting is very local. That means, you are getting almost a residual specific information not all most you all getting residue specific information because you looking at the quenching of tryptophan only.

However as you must have realized not every protein has only one tryptophan. Some proteins might not be having tryptophans, then we then possibly we try to look a tyrosine of a proteins might be having many tryptophans, then obviously, this analysis does become a little bit complicated, so not so straightforward. But still, but still in terms of exposure in terms of figuring out the quenching constant, the extend of quenching your experiments are still valid and you can still do that. It is just the interpretation that sometime becomes a difficult one.

Now, people nowadays are getting smarter. So, what they are doing is, if you have say multiple tryptophans you know more than two more than three or even more than one, what they are doing is they are mutating one tryptophan to something which is equivalent so as not

to change the gross structure. Hence since they have only one tryptophan now they can figure out which one is which, that means, to what extent is this tryptophan contributed to the quenching, to what extent is the other tryptophan contribute to the quenching by mutation right.

So, this is typically all I had to tell you about quenching. Next class, now what I will do is, I will change the topics a little bit though fluorescence is not over, because we still have to talk about this widely used fluorescence resonance and energy transfer method. So, I am going to do is I am going to change case a little bit come out of fluorescence because we have already spent a lot of time of fluorescence, look at to all the techniques one is infrared and the other one is circular dichroism which are very frequently used in the biophysical studies of proteins.

And after that we are going to come back to fluorescence and look at some advanced techniques, and the application of fluorescence resonance energy transfer or fret faster resonance energy transfer ok.