Bio–Physical Chemistry
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Lecture – 39
Fluorescence

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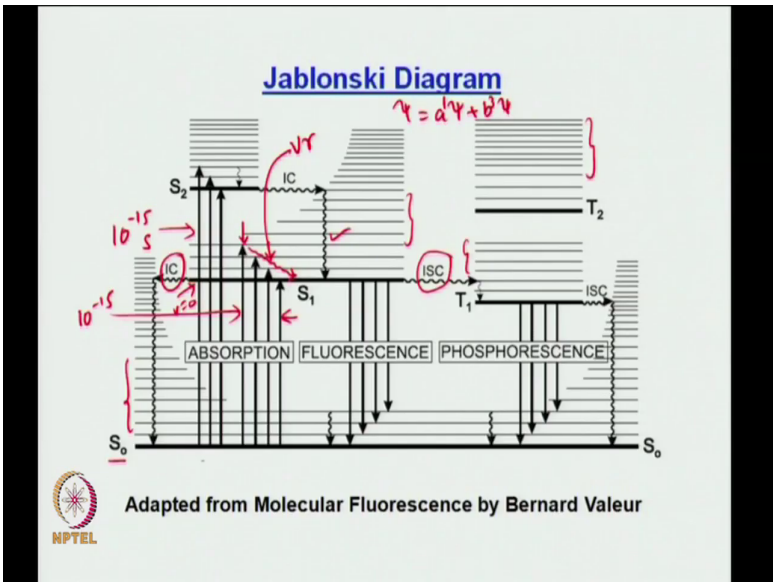

So, let us carry on with the discussion on fluorescence. Last class we started looking at the Jablonski diagram in some details right. We talked about, obviously we talked about absorption before, fluorescence we started talking about fluorescence. And before going to fluorescence, we talked about some of these non-radiative de excitation pathways right like internal conversion, intersystem crossing vibration relaxation, all these things right.

Now, just before I go forward, I will make a point and then come back to it later. So, the point is if you would remember that the vibration relaxation occurs in a times scale of ten to the

power minus 12 seconds right. Now, that is much faster than a normal fluorescence lifetime. And I will show you just based on a very simple expression the fluorescence lifetime is in the order of nano seconds or so, on an average right, that means, 10 to the power minus 9 seconds. So, you can understand that the vibration relaxation would be happening much faster than a fluorescence process would be happening, that means, the fluorescence de excitation.

So, then what would happen is if you would just consider two states S 0 and S 1 right not even S 2, I am not considering S 2 right now. So, if you would excite to a higher vibration level of S 1, what will happen is even before even before your fluorescence is happening what is happening? Your vibration relaxation is happening. When vibration relaxation happens, vibration relaxation is occurring within the vibrational manifold of the excited state S 1 right.

So, how far will it relax? It will relax to a point where it is v is equal to 0 of the excited state S 1. So, think about it. You have made some transitions like this to vibration levels right or the higher exited state S 1; but when the transitions are coming from S 1 to S 0, in most of the cases because of this time difference what you are having is or the time scale difference what you are having is they are coming from v is equal to 0 to different vibration states in S 0.

So, you can understand a small thing, the small thing is initially it was taking from v is equal to 0 in the ground state to vibration manifold in the excited state. Reverse case, it is happening from v is equal to 0 in the excited state to the vibration manifold in the ground state. But because v is equal to 0 is less, I mean v is because v is equal to v 0 has a low energy than the excited, v is equal to 1, v is equal to 2, v is to 3 of the S 1 manifold or the excited state manifold, then what is happening is you can see that you have already lost some energy right. Because you have lost some energy what has happened is losing energy means you are going towards the higher wavelength. Is not it?

The moment you have lost some energy, now you can understand if this was your absorption, and this is where your fluorescence is happening, see always have a gap in between your absorption band and your fluorescence band, and that gap is referred to as what?

Student: (Refer Time: 03:12).

It is referred to as stokes shift you know that is what strokes shift generally refers to right. It is a gap between the absorption band and the fluorescence band ok. So, this can be explained based on the Jablonski diagram so that is what we just did right now. I thought I would just mention this and I would come back to this again, because we are on this diagram.

Now, then we moved forward, and we talked started talking about the lifetime, and we even we even came across or we even derived some expression. So, the expression last time we we derived was we started from something like this. So, last class we started from something like this right.

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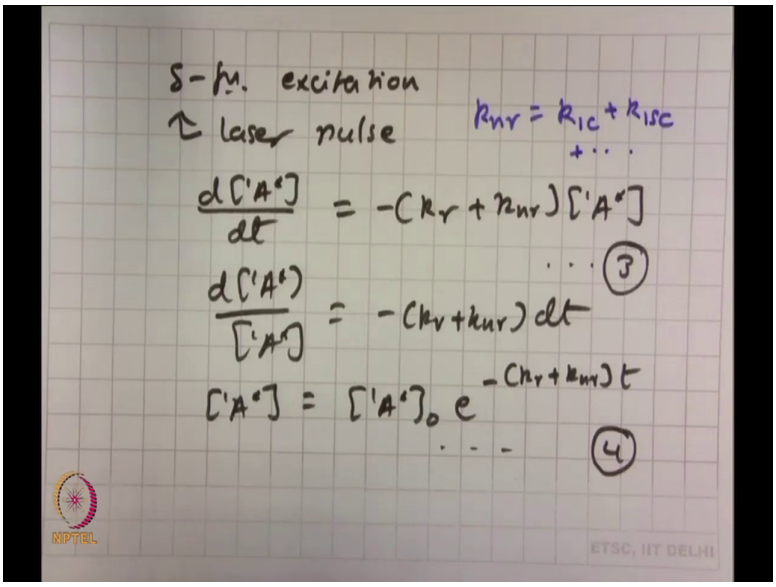

It was a I said it was a delta function excitation delta function means it is a very fast excitation that is essentially what you mean by that, right. And then from coming from laser pulse, and this is how decay is occurring K r plus K n r, these are the two broadly defined you know general rate constants. Now, remember when we are talking about K n r, when we talking about K n r, then what I can write is which I did not write yesterday here I can write that K n r is the sum of the rate constant of all your non radiative pathways right, all your non radiative pathways. So, just to make the point I can write this is K IC plus K ISC plus whatever else you have.

So, K IC is a rate constant for what internal conversion,

Student: Internal conversion.

K ISC is a rate constant for what?

Student: (Refer Time: 4:40).

Intersystem constant and so on. If you have any other de excitation pathway which does not read or which does not lead to fluorescence or radiated transition, then they are clumped in K n r ok, then they are clumped in K n r. Now, we went on with this derivation, and finally we came to an expression like this.

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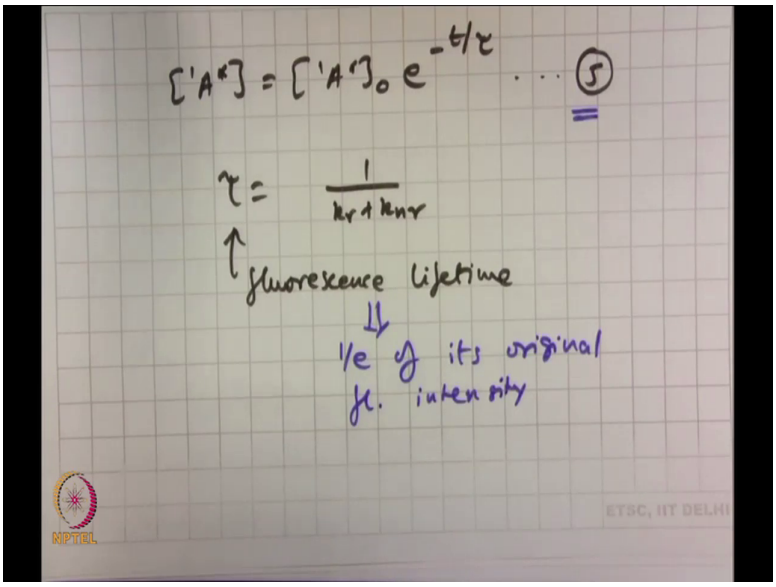

So, the expression look like this number 5, where the d K would be happening according to this exponential relation and tau is given by 1 by K r plus K n r ok. So, this is your fluorescence lifetime and this essentially tells you the time it takes to go to 1 by e of its original fluorescence intensity ok. So, fluorescence lifetime exponential relaxation time in this case is the 1 by e, the time it takes to reach 1 by e of its original fluorescence intensity that is the one it started with at t is equal to 0 right ok. So, this was tau for us.

And then and then what we also said was we defined one of the parameter called phi F which is the fluorescence quantum yield, and we say that phi F is a K r over K r plus K n r ok.

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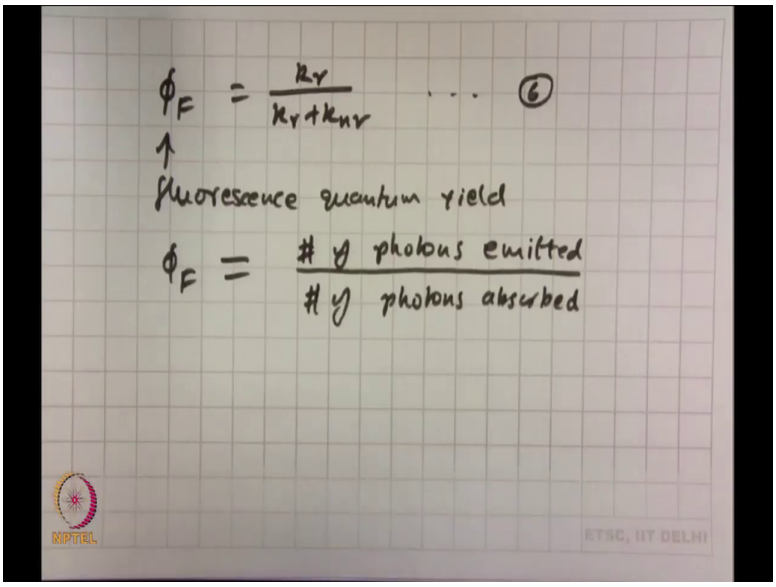

Which is essentially the number of photons emitted over the number of photons absorbed, ok. And this is a very important criterion or parameter. If you would be choosing a dye, or if you would choosing a fluorophore for anything this is something you will have to have the information about you have to have it in your hands before you can go ahead and do an experiment because this is one single parameter which is possibly of utmost important, so the most importance for a fluorophore right.

Now, let us you know move forward from here. So, what I will write down is if you remember this tau, the way we defined tau was 1 by K r plus K n r right, but there was also another tau we defined before, do you remember.

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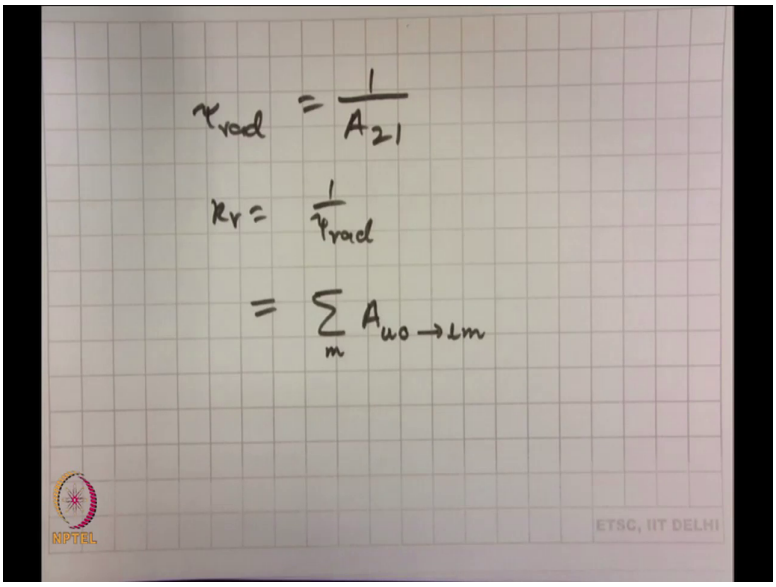

So, that tau was tau radiative which essentially tau r, what was it equal to?

Student: 1 by.

1 by was there A 2 1, where A was your spontaneous emission coefficient, so that was the tau radiative ok, no stimulated emission here right.

So, then what I can write is; then what I can write is K r is equal to 1 by tau radiative K r; that means, K radiative is equal to 1 by tau radiative; K r being the rate constant for the radiative process. Now, this can be further written like this ok. Now, remember what was A 2 1 when we consider A 2 1, we said it is a spontaneous emission from excited state 2 to excited state 1.

But see that time we were starting or discussion was spectroscopy and we did not consider any other levels what we said was only state 1 and state 2.

But now you know even if you have electronic state 1, electronic state 2, within that state, you will be having many vibration levels right. So, then when we are talking about A, when we are talking about A, it should not only be 2 1, what it means is it should also be from the respective vibration levels of excited state 2 to respective vibration levels of excited state?

Student: 1.

1. So, then what we should envelop or we should have is the envelop of all the transitions that is happening from the excited state to the ground state right.

So, then what you do is you do a summation that means your summation of all rate constants you have. So, the way you write is very simple you take a summation over what. So, this is A. And remember we just describe or we just discussed that because vibration relaxation is much faster than your fluorescence, then most of the emissions would be taking place from what the V is equal to 0 state of your upper excited state which is S 1. So, what I can write is if A is the spontaneous emission coefficient, I can write it is coming from upper state to lower state. So, I can write u which is the upper electronic state u, then I write 0. What does this 0 mean?

Student: (Refer Time: 08:51).

That means, the 0 vibration level of the upper electronic state right.

And then you are going to lower state right the lower electronic state. But in this lower electronic state, you can have a series of vibration levels then you write m ok. So, you can see what is the parameter which is now varying for you or the summation is over what?

Student: m.

m, because u and l are just upper and lower states 0 is defined for you ok, and this is what it is stands out to be. So, are you clear about this expression? So, essentially what it means is it is a spontaneous emission coefficient of whatever transition you have from your ground vibrational state in the upper electronic state to the vibration manifold of your low electronic state right ok.

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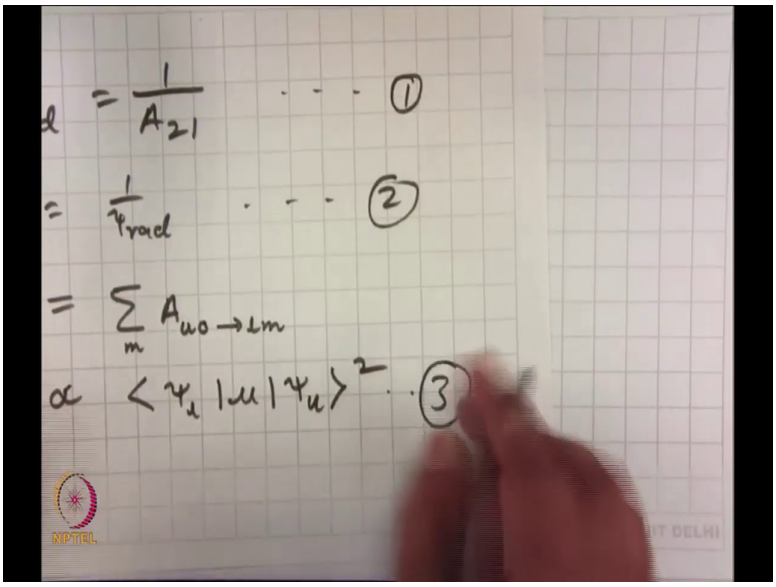

Now, this, this is proportional to, now also remember this if you are talking about spontaneous emission, whatever emission is going to happen, it is going to depend finally, on the vibration overlap integral, remember that Franck Condon overlap. So, if we the way we had define Franck Condon overlap, if it was the probability of a transition happening say absorption, fluorescence is essentially also the same thing right just a reverse of that. So, then in this case

also that one should play a role, that means, your A should be having a relation or should be in the certain way proportional to that transition probability or transition moment.

So, then what you can write is there is a proportionality relationship. We will not look at that. What we will just write is that this is proportional to psi l, then mu psi u square ok. Just with a small change, if you would have remembered the way we had written this transition moment integral for absorption, for absorption we were going from the ground state to the upper state right, that means, the lower state to upper state.

So, your operator was acting on what?

Student: Lower.

The lower state.

Student: Yes.

So, in that case, what we had is what we had the mu and the extreme right we had what psi lower, and then we had psi upper because it was going to psi upper. But now you are talking about the reverse process. So, what do we have is you have mu, then the extreme right it is operating on what psi upper. And after that it is going to?

Student: Psi

Psi lower which is coming here right. So, this is the way it is normally represented. Now, you can understand why it is the way it is represented is the final state comes before then the operator then initial state right.

Now, initial state for absorption is a ground state for its you know de excitation or the other way, emission is your upper state ok. And the reason this happens is because see for absorption if you take the example of absorption, only after your operator operates on the

ground state, you go to the excited state. And you know the operator acts on what not to the left, but to the right of it and that is the reason why you maintain this you know arrangement rather this sequence in the integral ok.

So, this is how it is maintained if if people are taking about transition moment integrals in spectroscopy, please stick to this. You will not get wrong if you revert it, it is not, not nothing is going to happen right, but it is just that it is good to go by the fundamentals. So, you that means you understand how the process is happening right.

So, again you can see that this is proportional to this square. And if you remember the Franck Condon overlap, this square was that Franck Condon factor, and that determines the intensity of the transitions the same thing is going to happen here ok. So, that was one.

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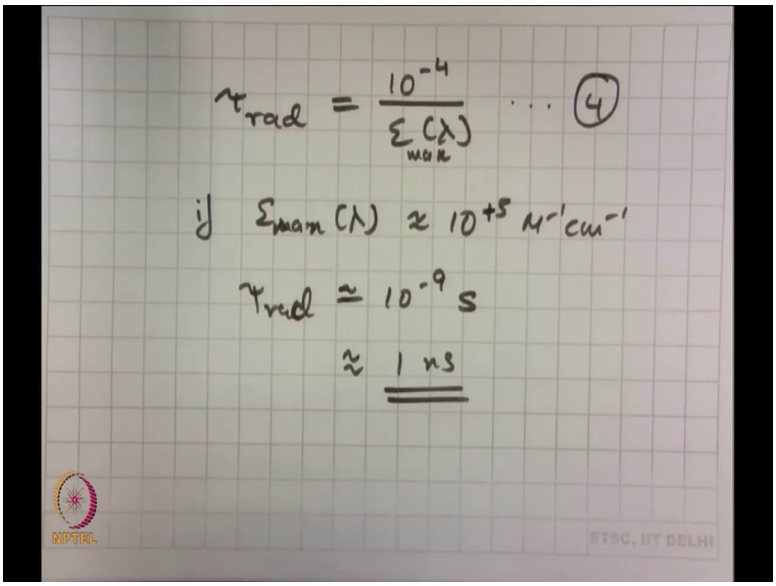

And the next thing is we will not derive it, but I will just give you another relation. So, what I can write is the tau radiative, it can be derived from whatever we have is generally or approximately can be written as 10 to the power minus 4 by epsilon ok. And this is max. So, let not writing any equation numbers, but if I write this to be 1, this to be 2, this to be 3, then I can write this to be 4. So, what did its telling you is that the radiative lifetime can be approximately calculated as by this ratio which is 10 to the power minus 4 over epsilon max.

Epsilon max is what? Epsilon max is the maximum extension coefficient to have for a given molecule right. Now, this is for you just estimate even before you do something if you know the epsilon what the fluorescence lifetime might be. I am not saying that it will be this, because remember this is tau radiative. You always have competing pathways. What do you have? You have the non-radiative transitions, and you never measure tau radiative.

What do you measure is actually the tau which is equal to 1 by K r plus K n r. Because non-radiative transitions are always there to a certain extent depending upon external conditions, depending upon the molecule, depending upon your solvent conditions right ok, but anyway just to have the flavor of it because I I said that vibrational relaxation happens 10 to the power minus 12 seconds, this one happens sorry, the fluorescence happens in about 10 to the power minus 9 seconds.

So, you can see, if epsilon max lambda says is 10 to the power minus 5 mole inverse centimeter inverse ok, and that is what it is, then you can see the tau radiative would be equal to what? Sorry, this would be 10 to the power plus 5, make it plus 5, it should be 10 to the power 9, minus 9 seconds, its 1 nano seconds ok. So, this is just an approximate, I mean this is Ballpark idea. You know this can be derived, let me tell you this can be derived, we are not just going into the derivation. Based on certain equations, this can be derived, and that is how you know that the fluorescence lifetime is typically in the order of a nanosecond or so without doing any experiments right.

So, you know this was what you need to know about fluorescence at least in terms of the de excitation pathways and all these things, now listen there is one more thing possibly you guys

would realize, when you are doing any experiments say you are doing fluorescence experiment, I am talking about because here we are discussing fluorescence. When we are doing a fluorescence experiment no matter what you do depending upon the solvent you always have some dissolve oxygen in it ok.

Now, oxygen, oxygen is a quencher of your fluorescence ok. It is also quencher of your phosphorescence, but since you are discussing fluorescence let us stick to that, it is a quencher of fluorescence.

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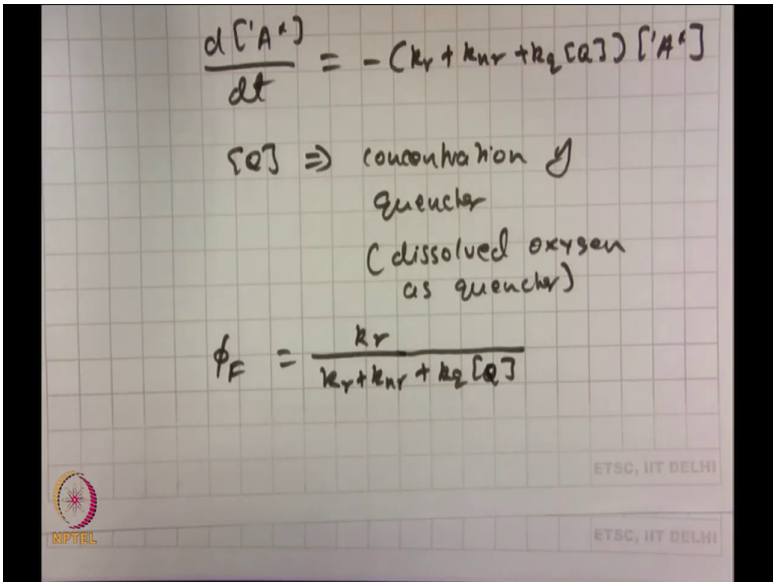

Now, what it will what will happen is, so when I wrote before d of 1 A star over d of t, see what we wrote was K r plus K n r right, these are the two ways it can depopulate that the excited state can depopulate. But now think about this; is another factor. Suppose, your suppose your solvent has dissolved oxygen on the order of milli molar level right that is typical

how what it is in some of your solvents, then what will happen is this oxygen would collide with the fluorescence molecule.

The moment it would collide with your fluorescence molecule it would take away some of the energy and that those photons rather that that energy will not be available to you in terms of a radiative photons right, that means, you are losing those photons in a separate non-radiative pathway this non-radiative pathway is coming is due to the collision of oxygen with your fluorophore. So, this is, so they here oxygen is referred to as something known as quencher, that means, it quenches the fluorescence of your fluorophore.

So, there therefore, if you have dissolved oxygen I can write it as plus K q times concentration of Q 1 A star, where Q is a concentration of quencher. And here we are talking about dissolved oxygen as a quencher ok. Here we are talking about dissolve oxygen as a quencher ok. So, that means, apart from K r and K n r, you have another rate constant which is coming in. So, now, what will the fluorescence quantum will be the expression? It will still be the same only with what K r over K r plus K n r plus K q times Q is it not, because this is the extra process you have.

And simple it is K q times Q, because you have the rate constant which depends upon the concentration of oxygen right that is what you have. And K r plus K n r always in terms of time; that means, inverse of time. So, K q what is a; well what is the unit of K q now? Obviously, it would be time inverse;

Student: Yes.

That means second inverse or whatever. What is the other?

Student: Concentration

Concentration inverse 2 right because dimension has to amended and that is why you have K q times Q. So, the concentration cancels out ok.

So, you can now realize if you have an additional collisional quenching term what would happen its quantum who would further decrease ok. Now, this brings us to a very important point. The point is that you dissolve oxygen; obviously, is always there. So, the way what you can do is you can try to remove dissolve oxygen, that means, you can purge your sample solution with argon nitrogen or whatever, and try to remove that that is one way of doing it.

Now, I am not sure whether you have heard of this concept of fluorescence quenchers. So, there is some compounds which are quenchers. Now, we have talked about one type of quenching not exact in terms of quenching, but in terms in terms of inter system crossing, we talked about effect, what effect was that?

Student: Heavy atom.

We talked about heavy atom effect right. Now, heavy atom effect, remember there were two types; one was internal, internal means iodide I would rather this was a part of the molecule itself; and the other one was external, external means it was with the solvent right like iodo ethane or something like that we said.

Now, similar to that, but not exactly similar, suppose you have a fluorophore suppose you have a fluorophore in a solution right and you add potassium iodide right. And let me tell you that the fluorophore is a charged molecule ok. Now, when you add potassium iodide, what will happen is potassium iodide is also charged and say the fluorophore is positively charged. So, this iodide will have an affinity for the charge species anyway. Now, you also know that iodide favors what spin orbit coupling, so it will favor what inter system crossing. And the more it favors inter system crossing what will it happen? What will happen is the fluorescence quantum would decrease right.

So, essentially that is what happens is then you can use iodide as a fluorescence quencher. So, here we talked about oxygen, but exactly in the same way other fluorescence quenchers, other quenchers.

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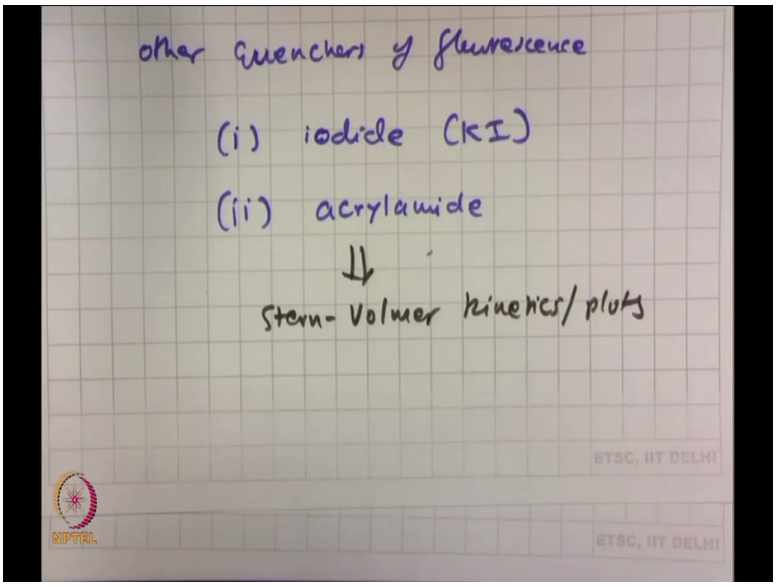

So, other one is your iodide ion. So, people use potassium iodide. The other one is a compound known as acrylamide; the other one is a compound known as acrylamide ok. So, these having very I mean these are very commonly used quenchers. You can understand a difference iodide is charged right; acrylamide by the way is neutral, it is not charged. So, if you have; if you have a scenario where you do not have much charge-charge interactions out there, that means you are talking about an environment say a protein where the interior is essentially hydrophobic. So, because it is non-polar, iodide will find it very difficult to access the interior of the protein.

See if you would like to look at a quenching of a tryptophan residue which is in the interior of a protein, you would not use iodide. What would you use?

Student: Acrylamide.

You would use acrylamide, because acrylamide does not have a charge it is neutral. And in that sense it would possibly having a little more accessibility to or for the tryptophan or for the fluorophore inside the protein hydrophobic site ok. So, this leads to completely separate chapter on quenching. And this can be referred to as Stern-Volmer kinetics or plots, Stern-Volmer kinetics or plots right, I mean just know this we will not discuss it right now ok.

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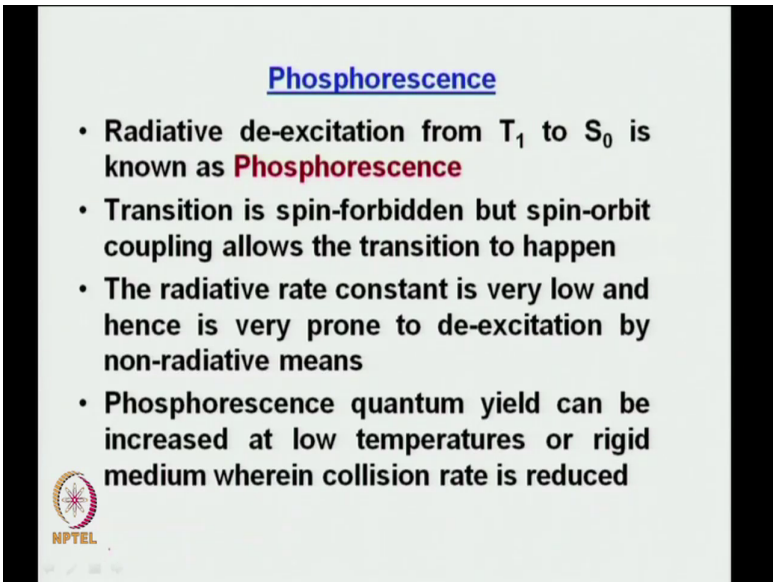

So, now, let us go back to the slide. We have talked about fluorescence at you know bit. Let us talk about the other radiative process which is phosphorescence right. So, we are talking about phosphorescence now. So, it is a radiative de-excitation from T 1 to S 0 right. The transition is spin-forbidden, but spin-orbit coupling again allows the transition to happen right. The radiative rate constant is very low, remember it is forbidden right. So, the radiative rate

constant is low. And hence is very prone to de excitation by non radiative means, now try to understand the significance of this.

We just said that a fluorescence lifetime is on the order of nano seconds 10 to the minus 9 seconds. Now, let me tell you if you remember a table which I which we had discussed last time the phosphorescence lifetime in the order of micro seconds or so, above ok. So, you can see there is a huge gap between fluorescence and phosphorescence. So, which state is long lived, is it fluorescence or is it phosphorescence?

Student: Phosphorescence.

Phosphorescence is. Now, suppose you have these two, suppose you have these two states right hypothetical situation you have fluorescence and you have a phosphorescence right, you know that this one is going to come down first and then this one would follow. Now, I take this in a system where I have many quenchers available say oxygen, say oxygen right or any other quencher which would quench both, oxygen quenches both. Now, tell me which one would be more affected?

Student: (Refer Time: 23:58) phosphorescence.

Well.

Student: Phosphorescence.

How many of you are for phosphorescence? 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, man and the rest are for fluorescence, because rest is the other half of the class ok. You know this is a collisional quenching with oxygen. So, it will depend upon diffusion right. If you remember that Smoluchowskis you know diffusion relation what we had said, so it is typically in the order of nano seconds, remember its typically on the order of nano seconds right ok, the collisional quenching constant rate constant.

So, you think about this your fluorescence has almost the same rate constant as that of your quenching ok. So, depending upon which one happens faster, if fluorescence is happening faster than the quenching, then quenching would not be effective that much. If the fluorescence is slower than the diffusion of the quencher molecules, then obviously fluorescence would be affected, so that means, in fluorescence you have a chance of a it not getting that affected by it will get affected by, but not getting that affected, because now you are comparing between two similar rate processors right just start with.

But think about phosphorescence now. Does phosphorescence have any other option its starts from micro second, because it is always there right. So, no matter what it is your quencher molecule is always going to hit the molecule the phosphorescence state before it can come back. So, essentially what it does is, essentially what it does is, it decreases the quantum yield of phosphorescence.

Do you understand why now? It decrease the quantum yield of phosphorescence, because it is a longer lived. And because it is longer lived it is more prone to collisions with what your quencher molecules that is why phosphorescence is actually very hard to have a very high quantum yield of phosphorescence. This is the reason.

If you have to have a high quantum yield of phosphorescence, there are two ways a couple of ways you can do it one is you reduce the diffusion, that means, say you go to higher viscosity or you remove the quenching molecules by some way by bubbling gases or some way or the other ok, so that is why phosphorescence is always more affected as compared to fluorescence ok. So, that is what we meant by this.

So, that is now this is the other thing I was telling you phosphorescence quantum yield can be increased at low temperatures or rigid medium where collision rate is reduced. So, essentially if you reduce collision rate by one way or the other, then you can increase the phosphorescence quantum yield ok.

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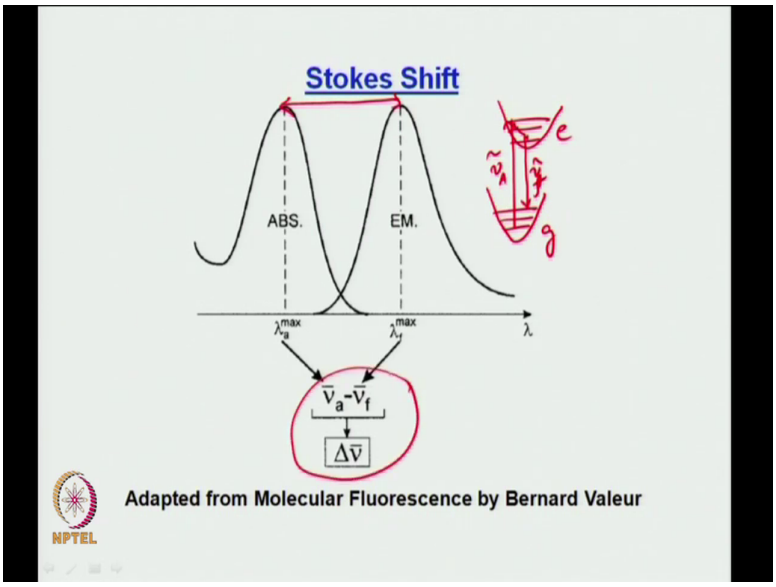

One more thing, so there was phosphorescence for you. Let us talk about the Stokes shift right. So, this is what we said. We said that you have these, this is excited state, this is ground state right. You do excitations so it goes from the ground state to here ok. So, this is the absorbing energy, so which is nu A say, it is nu A; nu A means in wave number ok, your energy of absorbance.

But then before it can come down from here, what it does is, it does a vibration relaxation to here and then its starts coming down from here ok. So, you can what has happened is, you can already see that this energy nu A is higher than nu f. So, then what will happen is there will always be a gap always be a gap between the absorption maximum and the fluorescence maximum specially in the condensed phase.

If you were go to the gas phase, see you would not be having these many interactions. So, vibration relaxation would be reduced and they would be far closer to each other ok. So, the Stokes shift arises from the fact that you have rapid vibration relaxation before the molecule can come down to the ground state from the excited state ok, and that is why this you can see this difference as it is said here at the bottom is referred to as your Stoke shift ok.

And generally Stoke shift is referred in terms of referred to in terms of wave numbers ok, because Stokes shift always has something in terms of energy. And I will tell you why I will tell you why because where do hear Stokes shift from first when you are doing spectroscopy, did you hear from phosphorescence or did you hear from some something else?

Student: (Refer Time: 28:26).

Raman right, now you have Stokes lines and anti-Stokes lines. So, Stoke line is what? Stokes line is very it moves to a lower energy as compared to your Rayleigh scattering. So, you know that is essentially how stokes comes around. So, your emission is always at the lower wavelength than your absorption right that is your stokes shift that happens because of this now, because there are many other reasons why your emission can be ata much low wavelength then your absorption right. Now, anyway this is the difference, this, this is one you have always have to keep in mind ok.

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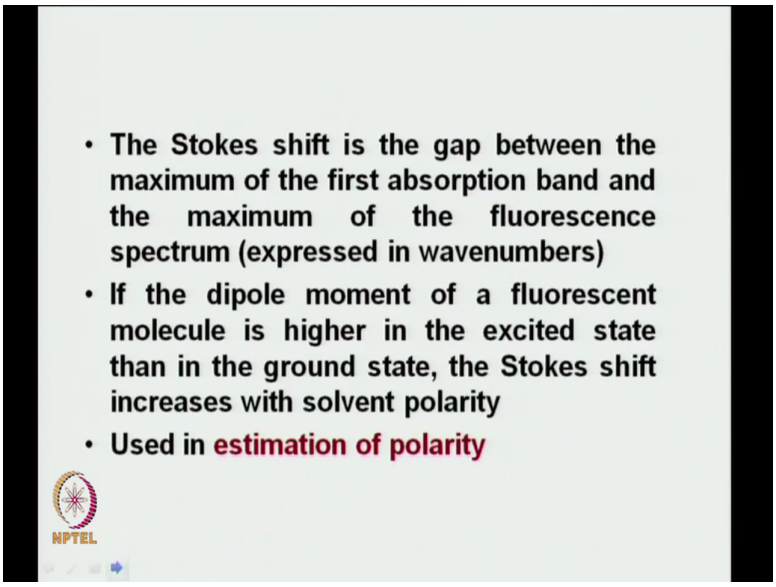

Now, if you, if I give you an example, well, first the Stokes shift is a gap between the maximum of the first absorption band and the maximum of the fluorescence spectrum expressed in wave numbers this is what I said. Now, if the dipole moment of a fluorescent molecule is higher in the excited state than in the ground state, the Stokes shift increases with solvent polarity ok.

Now, just keep this in mind, I will show you a diagram, where you would understand how solvent effects fluorescence, and then you would see that how this Stoke shift will depend upon the polarity of the medium right, because Stokes shift will not be constant, it will depend upon some factors one of this fact is being the solvent polarity. So, the as I said it is used in estimation of polarity of the corresponding solvent.

So, for example, if you would take a fluorophore, you would take it in solvent A and solvent B. If you take in solvent A, if you would see that the Stokes shift is small, and in solvent B we see the Stokes shift is huge, then you would say that the solvent B is more polar than the solvent A, and that that is how you can estimate it ok, and there are other scales too right.

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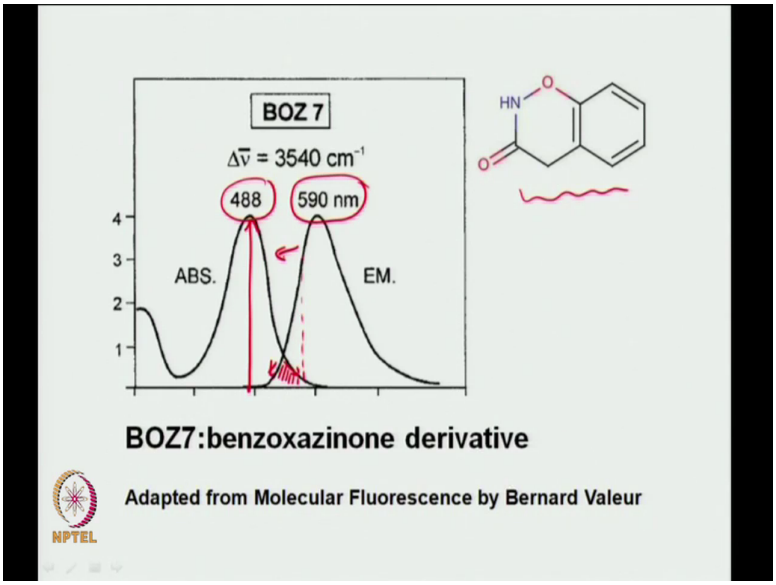

So, this is an example. So, this is of a derivative of this compound, it is called benzoxazinone derivative. Now, you can see out here the absorption is at 488 right; this is the absorption out here. And this is the emission peak at 590 nano meters. And the Stoke shift is of the order of 3540 centimeters inverse. And you know that is not bad, that is pretty huge.

Try to realize this, try to realize this, what will happen is this is a case where your fluorescence is pretty well separate from your absorption spectrum ok. So, suppose you are going to excite somewhere here, say suppose you are going to excite here right, that means, you have excited

this benzoxazinone derivative BOZ7 at this 488 nano meter right because here it absorbs the most now because you have excited it.

Now, you can see effectively, effectively, you can collect the fluorescence you can collect the fluorescence from any point from any point say only the fluorescence which is exclusively fluorescence from any point say here, because here the absorption is scaling down.

But keep one thing in mind there is in this case there is a small overlap between your absorption spectrum and your fluorescence spectrum. Can you see that? So, this is the absorption spectrum which is coming down, and this is your fluorescence spectrum. So, this shaded area is the overlap between the absorption and the fluorescence spectrum ok.

Now, this is a huge consequence when you know specially have you are doing fluorescence resonance energy transfer and all these things, because this really matters, but anyway. The point is that instead of this if you had a compound which had a smallerStokes shift right what would happen, suppose, suppose you take another benzoxazinone derivative which is still absorbing at 488, but it has a smaller Stokes shift. So, where it will go?

So, this one would move on this side towards this side right, because it would move towards this side what would happen, a larger amount of fluorescence would overlap with your absorption. Now, that is always not welcome ok. Right now I cannot go into the details, but this just the these are just the ramifications of having a largest Stoke shift or a smaller Stokes shift ok.

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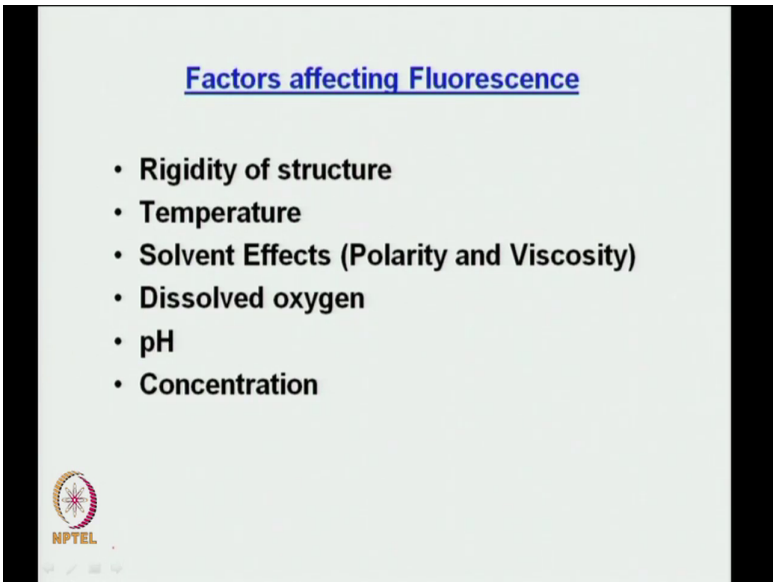

So, what are the factors affecting fluorescence? One is the rigidity of structure, the other one is temperature, solvent effects – polarity viscosity, dissolved oxygen, this we have discussed, pH, concentration and many other factors ok. But these are you know the broadly defined factors that we can look into.

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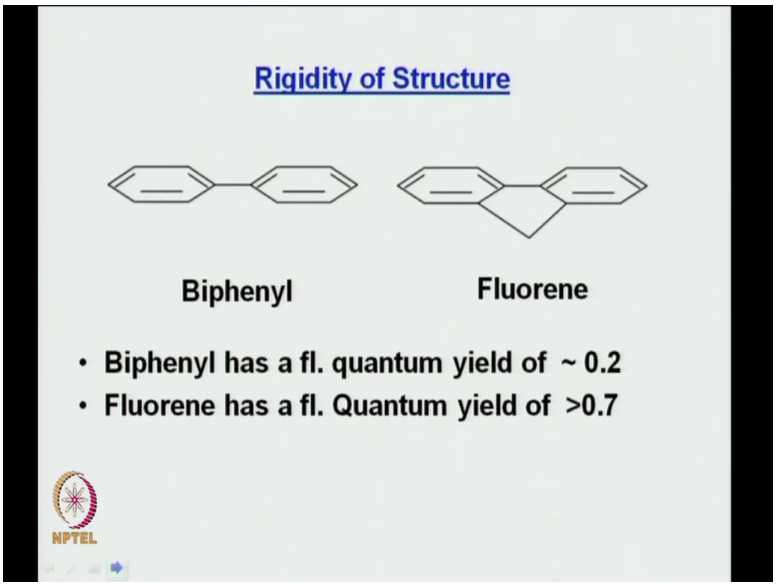

If you look at the first one the rigidity of structure, look at these two compounds, one is biphenyl and one is fluorene ok. Now, before giving you the answer on the slide itself, tell me which one is supposed to have higher quantum yield the first one or the second one?

Student: (Refer Time: 33:00) second one.

Second one? Any other thoughts? Well, the other thought will be the first one anyway right. No one is going for the first one, none? Ok, excellent, all of you are right. Now, tell me why.

Student: (Refer Time: 33:20).

More rigid where rigidity of structure ok, it is more rigid ok. Then what happens if it is more rigid?

Student: Conjugation will be more; Conjugation will be more.

Conjugation will be more, but it is not about conjugation I am talking about, it is about something else. What is the difference between a flexible molecule and a molecule which is rigid? Think about in terms of your de excitation rates which one is more fluxional which one is more flexible? Biphenyl.

See, if you would move a lot like this right, so before because it will it will have a large amount of motion, it would be having higher chances of colliding with some other stuff, because it has higher chance of colliding with other stuff, which one would have a higher chance of coming down without radiating photons, the biphenyl one, that means, the one which is more flexible. The one which is rigid does not have too many ways to go around right. So, it is you know pretty much stuck. So, this is your rotational mobility essentially you are talking about right. This is what your rigidity of structure is.

So, as you said the biphenyl has a fluorescence quantum yield of 0.2; fluorene has a fluorescence quantum yield of what 0.7 ok. So, this is one very important factor. So, that means, if you would take two molecules of similar structure, one which is more flexible than the other, even by closing your eyes without doing any experiments you can say which one would be having a higher quantum yield and which one you would rather take for doing some fluorescence studies if you have to use one of those.

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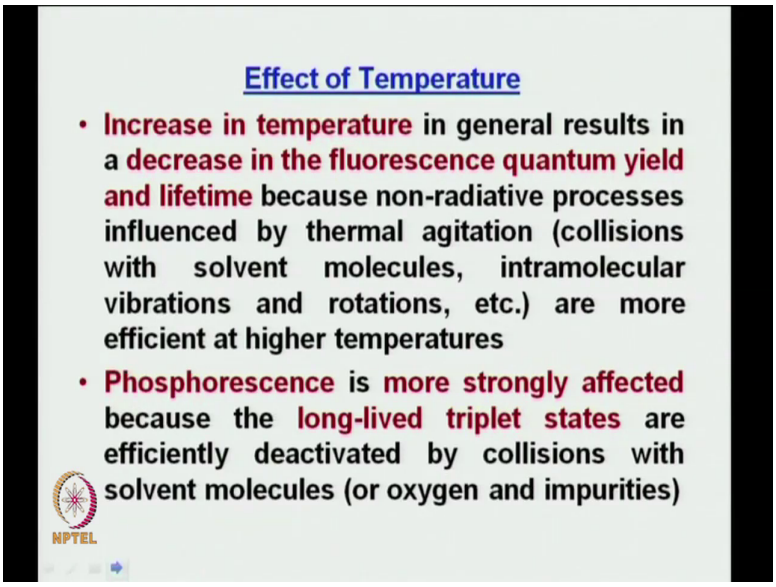

The next one is the effect of temperature. Now, what do you thing that the effect of temperature would be, would it increase the fluorescence quantum or would it decrease?

Student: (Refer Time: 35:06).

It would decrease fluorescence quantum yield, why because with temperature what will happen?

Student: (Refer Time: 35:12).

Right you collision frequency would increase right that exactly what happens. So, increase in temperature in general results in a decrease in the fluorescence quantum yield and lifetime,

because non-radiative process is influenced by thermal agitation which are collisions with solvent molecules, intramolecular vibrations and rotations are more efficient at high temperatures right.

Now, phosphorescence, look at this sentence, phosphorescence is more strongly affected because of the long-lived triplet states which are efficiently deactivated by collisions with solvent molecules right. Now, you can understand as we were discussing before which one is more effected phosphorescence or fluorescence?

Student: Phosphorescence.

Phosphorescence because phosphorescence is more long-lived right.

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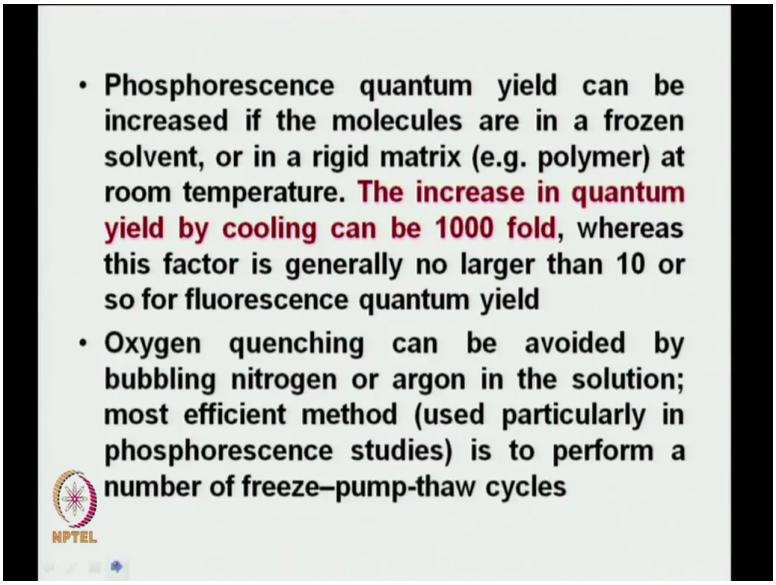

See if you have to increase the quantum yield of the phosphorescence, what you would do is this. If molecules are in a frozen solvent or in a rigid matrix ok, that means, they are not moving around a lot collisions, collisions frequency is decreased. You decrease the temperature. You decrease the temperature what will happen is you can see by cooling, that means, decreasing the temperature the phosphorescence quantum yield can be increased by about 1000 times as compared to a fluorescence which is only affected about 10 times or more so right. So, this is how phosphorescence is much more sensitive to your temperature quenching right even other collisional quenching as compared to your fluorescence. Just because of the fact that it occurs at a much later time, it is a delayed radiative process.

Now, oxygen quenching as I said can be avoided by bubbling nitrogen or argon in the solution. The most efficient method used particularly in phosphorescence studies is to perform a number of freeze-pump-thaw cycles. That means, you freeze a solution you thaw it you freeze it again you thaw it you do it 4 to 5 times, and that is one of the best freeze of getting rid of all dissolved gases you can have in the system and then you use it ok. Now, this is also something which is normally done for you know specially done by groups who really have to worry about this oxygen quenching right ok.

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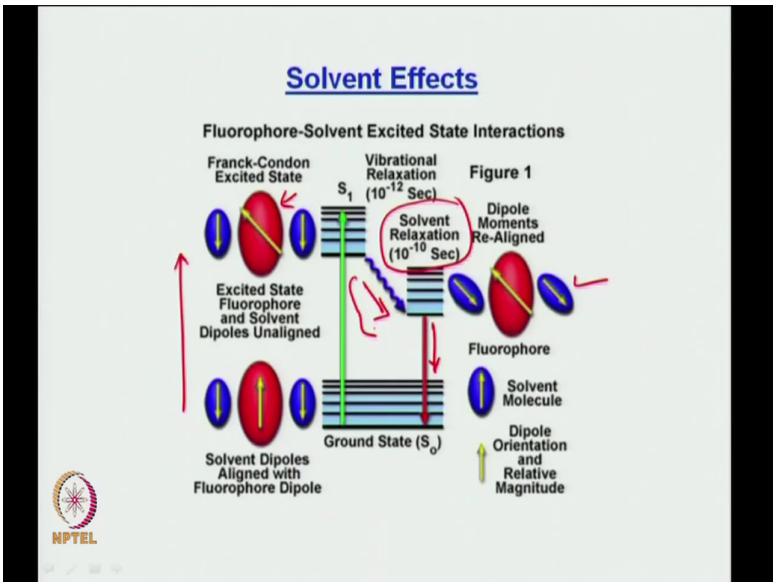

Now, the solvent effect, I was talking about the solvent effect. See, what happens. Suppose, you have a ground state molecule right. So, this is a ground state molecule. Now, do not worry about the excited state right now, this is the ground state molecule. So, your solvent molecule you have taken as a dipole right. So, the way at equilibrium before doing any excitation, your water molecules, so you have taken it in water or any solvent, your solvent molecules will also orient them since along with the dipole to stabilize the system to give you the lowest energy, so that is a ground state electronic energy right along with the vibration levels.

Now, think about this. The issue is you make a transition. The moment you make a transition you bringing about electron redistribution right. And in many cases, there is a large enough change of a dipole moment. See, for example, if you take tryptophan which we have you know

talked about, you have heard about a lot. Tryptophan has about a change of 4 to 5 debyes when it goes from the ground state to the excited state – it increases ok.

Now, because it increases, tell me the ground state there was not much of charge separation now the charge separation is increased in the excited state right. Now, because it is increased in the excited state, now see what is happening, when it increase when it is excited, the solvent molecules have not been able to respond why because this was ten to the minus 15 seconds. And we said the nuclei follow much later than the electronic excitation.

Now, you are talking about solvent molecules, we shall be even later right because these are kind of much heavier than the electron. So, but because you have given rise to non equilibrium situation, what will happen is solvent molecules will finally equilibrate to the new situation and solvate it.

The, now the way you solvate it is because it goes up like this, this is your absorption, remember, this is your absorption which has happened before any solvent reconfiguration has taken place right. But after equilibrium, this levels slowly comes down here. Why, because you have a charge separation, the solvent molecule depending upon the polarity of the solvent say if it is water, it will reorient to stabilize this and it will decrease the energy of this electronic state. Now, this is the equilibrium at the electronic state, that means, the after your solvent reorganization has taken place. Are you with me up to this part? So, you understand. So, here where no solvent relaxation, no solvent reorganization, the energy is pretty high.

After that the solvent stabilizes the excited electronic state which has a change in the dipole moment, it comes here right because this you know polar interactions. So, now, tell me your absorption was from here to here, your ground state has not changed ok, just keep it like that this one has been stabilized more right. So, what will happen to the fluorescence now, is it of high energy or low energy?

Student: Low energy.

It is of low energy right. Now, remember Stoke shift I said it will also depend upon solvent polarity. So, what would happen, depending upon the solvent polarity the more polar the solvent is the more it will stabilize this electronic state. So, the more if you come down to a lower energy, the lower the solvent polarity is, the less it will stabilize the electronic state, so the high the energy of that stabilized state would be. So, that means, the solvent polarity would be having a telling effect a defining effect on the observed Stokes shift. Is it clear or not? Ok. So, that means, solvent polarity is a very important issue.

That is why you will see that if you take dyes, if you take dyes specially with a tryptophan, I will show you tryptophan that is how we will end the class, you would see that. If you change solvents, say if you say you if you take a dye say in hexane which is the non-polar solvent, you go to water which is a very polar solvent you see that Stokes shift that gap between the absorption fluorescence has changed a lot right. Then in many cases remember absorption spectrum is not that affected; in some cases, it is affected ok, so that thing you also will have to keep in mind. Whatever discussion we are having right now is we are taking the assumption that the absorption spectrum is not affected that much ok.

So, then that is what I said. So, you can see here initial equilibrium situation, your solvent dipoles were reconfigured in equilibrium along with the molecule dipole. You go to the excited state now. It says here the excited state fluorophore, this is the excited state fluorophore its dipole direction has changed you see ok, because it is a new dipole, it is in a different direction. But the solvent molecules because they are massive, they have not been able to respond to it, but still pointing in the same direction. So, it is a high energy state, it is a non-equilibrium situation.

Now, what will happen is, you have given time, the solvent molecules will try to reorient. So, now, you can see this is the next stage where it is fully reorient. So, the energy has decreased. Now, this process is referred to as you can see out here, it is referred to as something known as solvent relaxation ok. This is a very interesting topic ok. It has been I mean people have looked at it like anything people are still looking at it. So, solvent has brought down the

energy of the excited state, the initial excited state to a lower energy level. And now your fluorophore is taking from here to the fluorescence to the ground state.

So, this difference which is your Stokes shift then it will depend upon your solvent polarity. So, this is how your fluorescence spectrum is affected because of the solvent polarity ok. Now, the other thing is viscosity your fluorescence can be affected by viscosity. How would viscosity play a role? Viscosity would possibly increase the quantum yield why because it would decrease the collision rate right, so that is how viscosity plays a role right and very simply speaking ok.

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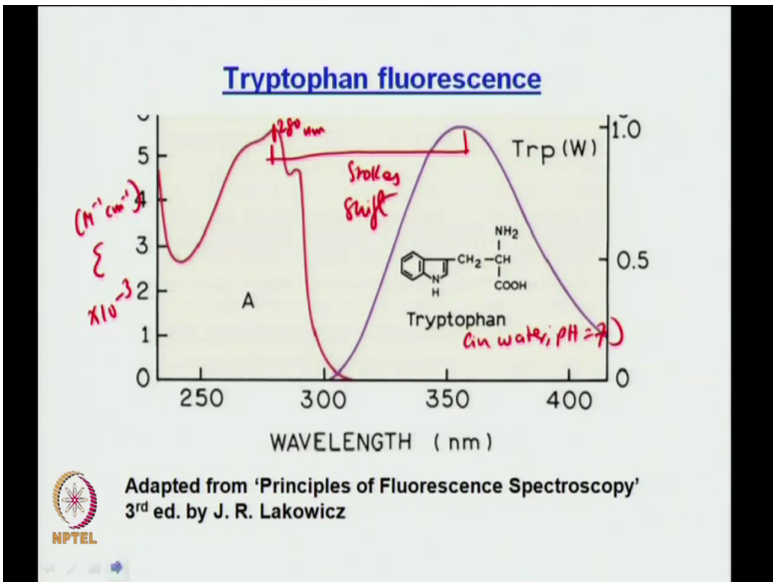

Now, let us end the class by looking at some of your protein fluorescence right I mean so that you can have some feel about how people use these things in their bio physical studies. Now, this is in I have generally taken I mean taken this pictures from this figure from this book on

Fluorescence Spectroscopy by Lakowicz. This is really good book. If you are interested in fluorescence you should definitely have a copy of that and look at look that up.

Now, what you can see here this is the tryptophan fluorescence. On this side is your absorption spectrum which is epsilon to the left times 10 to the power minus 3, that means, whatever epsilon value you have in terms of remember what was the unit mol inverse centimeter inverse was it not, right, that was a unit times 10 to the power minus 3. See, if you look at the 280 was the peak right of tryptophan. So, you see this is 280, I do not know what is 280, I guess this say this is 280 nanometers, you can see it corresponds to something close to 5.

What was the epsilon max of tryptophan? Do you remember was there any 5500, 5600 or so? So that means, here it is kind of 5.6 10 to the power 3 times, 10 to the minus 3 which is giving you 5.6 right, so that is why it says epsilon times 10 to the power minus 3 ok.

Now, this is your spectrum as absorption spectrum. Now, look this is your emissions spectrum. This is emission spectrum in tryptophan in water, in water at pH 7, equal to 7 ok. Now, you can realize can you see the shift between the absorption maximum and the fluorescence maximum ok. So, this is essentially your Stokes shift ok, good.

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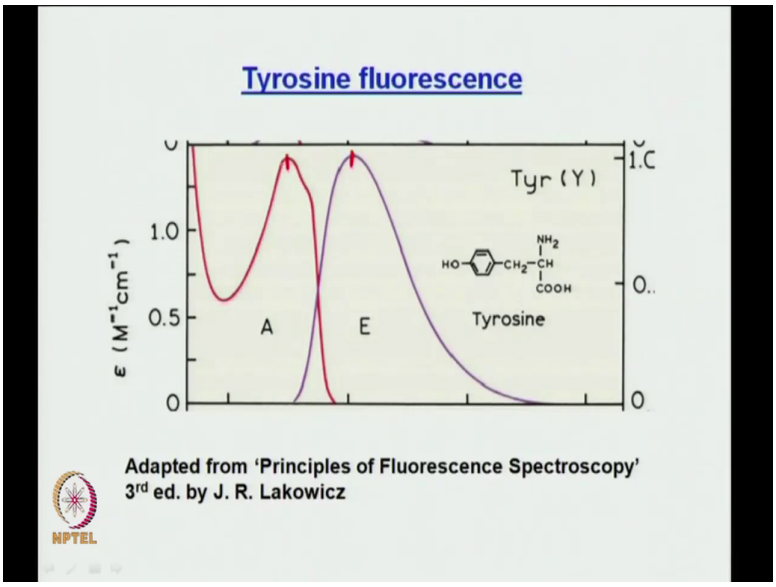

Now, let us look at the other one. This is tyrosine. Tyrosine, the absorption maximum was where 274 ok. So, this is epsilon it is written out here. Now, you can see here if you would observe properly, this Stokes shift of this is actually not as high as that of tryptophan right.

And personally you can understand that I said tryptophan as a high dipole moment change of about 4 Debye units or 5 Debye units, tyrosine might not be having that that much of a change, so that is why this Stokes shift might not be that large right, but anyway. So, this is the fluorescence of tyrosine when you are exciting in the absorption spectrum.

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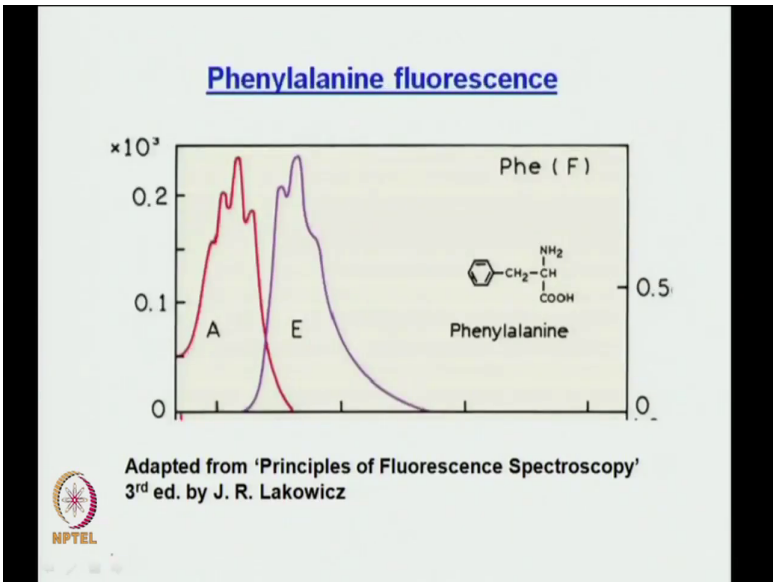

The other one is phenylalanine ok. Now, one thing you can realize phenylalanine is not used that much because first of all its epsilon value is really low; and its fluorescence quantum yield is also not that high ok. So, I forgot to write down the fluorescence quantum yields out here but for tryptophans in water it is like a zero point, it is close to 0.2, 0.15, 0.16 like that ok, it has the highest among all the amino acids right.

So, this is the three typical fluorescence spectra along with absorption spectra of the amino acids that we are concerned with when we are talking about spectroscopy of proteins. Now, remember these are parts of your proteins. So, these are called intrinsic fluorescence. Intrinsic fluorescence means it is already a part of the proteins; it is internal to the protein you do not have to add fluorescence from outside ok.

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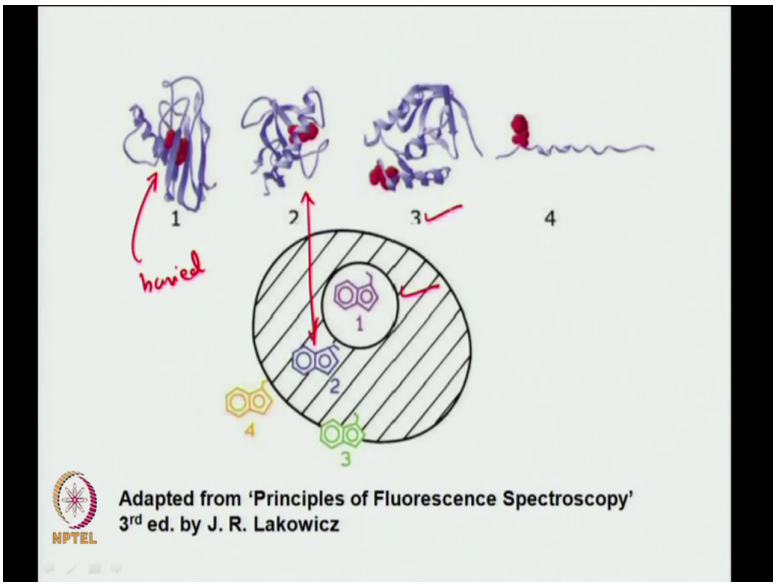

Now, let us take this case remember we talked about solvation. When we talked about solvation, what did we talk, we talked about the polarity of the environment right. And if you are talk about if you are talking about the polarity of the environment now think about the situation. You are given four proteins. In one protein, I do not know whether it is visible in one protein, you have a tryptophan is the red one is the tryptophan and one protein. So, in number one, the tryptophan is buried. You can see it is very much inside the hydrophobic core, so that means, it is inside and it is surrounded by all the protein residues. So, it is very it is not actually it is not accessible to water, because it is inside.

Now, if it is not accessible to water, then what is the interior environment? Hydrophobic, good. Now, look at number 2. So, here this is number 1, this was number 1. You can see it is very much sitting in the interior. Let us look at number 2. So, number 2 is a little less buried than number 1. So, this is what we are talking about this is number 2. Now, look at number 3,

number 3 is kind of a interface, a part of is buried, a part of it, is not buried right. So, this is number 3 and number 4 what is happening?

Student: (Refer Time: 47:15).

It is fully open. So, you have four situations, four situations where a four where a four different degrees of polarity right. The buried case has a lowest polarity, because it is mostly hydrophobic right, almost fully hydrophobic. The open case which is fully exposed to what is one which is facing the most polar environment right. So, then if you think about if you then if you think about that Stoke shift, think about the Stoke shift which one would be having the highest amount of Stoke shift?

Student: 4.

4, and the one which is lowest would be number 1. So, let us hope this is maintained when you do an experiment right.

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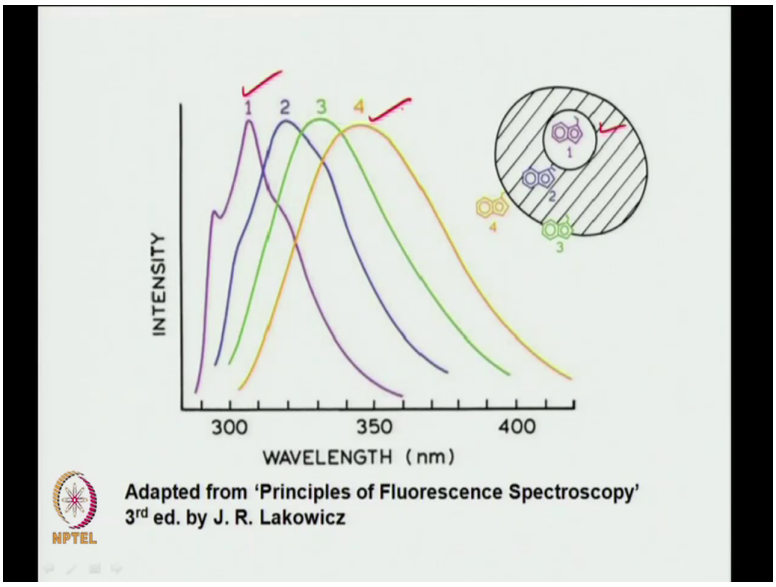

So, let us look at the spectra right. Now, now, see does it follow what we had predicted? Look at number 1. Number 1 is the one which is most buried right this is the one which is the lowest wavelength. What does it mean by lowest wavelength? Means highest energy; that means, the energy gap between the ground state and the excited state, that means, the relaxed excited state is higher ok, and 4 is the one which is the lowest ok.

Also if you try to realize one thing, because if you are already here though we are not talking about this you know feature of the spectrum too much. But tell me another very important difference between say 4 and 1 looking at the spectrum.

Student: Broadening.

Broadening, very good. What is this broadening due to?

Student: (Refer Time: 48:44) diode exposure (Refer Time: 48:47).

Diode exposure. So, diode exposure to the solvent molecules right. So, now, you can understand that because it is in 4 because it is so exposed to solvent molecules you will be having so many different types of interactions hydrogen bonding interactions and all these things, hence the number of different transitions would be lot higher and this the spectrum becomes broad. So, this is called broadening ok, this is called broadening.

And, obviously, if the same fluorophore is inside, the in the interior of a protein rather it is in the interior of the protein, then it would not be accessible to solvent molecules. So, you would not be having that many collisions with the solvent molecules, and hence its broadening would be less ok. So, that is typically how a fluorescence spectrum would vary right. If you take a tryptophan in the interior of a protein and if you take the tryptophan and put it on the surface of a protein, this can easily be figured out ok.

Now, guys, think about this. Suppose, I start with a protein where the tryptophan is in the interior like a case which is 1, so that means, it would be having a very blue shifted fluorescence, that means, a fluorescence which is at a low wavelength ok. Now, I start denaturing the protein. When I start denaturing the protein, what am I doing? I am slowly opening it up. If I am opening it up, what is happening to this fluorophore, is it getting more exposed or less exposed to the solvent?

Student: More exposed.

More exposed. So, if it is getting more expose to solvent, what is going to happen? See, it is the fluorescence maximum is going to shift; it is going to go over towards the red side. So, this is typically what you would see when you take up a protein ok, so that means, if you are

unfolding a protein this is general case if you are unfolding a protein this is what you would observe ok.

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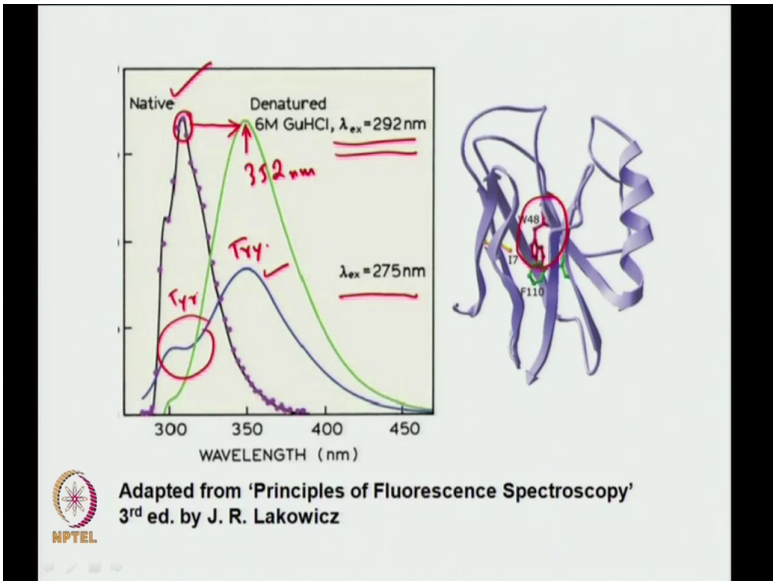

So, see this is what you observe here. One is this is a protein called azurin right. This is a protein called azurin. So, you can see there is a one tryptophan here, one tryptophan protein. This protein azurin, in its native state, so this is the native state, in its native state, what has happened is you can see it is very similar to what you had before, it is close to what like 305 or so, something like that.

But the moment you denature with 6 molar guanidine hydrochloride, see how much it is shifted by it has shifted by huge amount right. Now, guys remember all those Stokes flow kinetics we talked about and we talked about a certain changes a signal the spectroscopic signal right. Now, think about that kinetics and think about an experiment you are doing,

suppose you looking at the refolding of azurin right. If you are looking at the refolding of azurin you can do it in two ways, two ways you can do it is. That means, when azurin is fully unfolded, say in a high constant of denaturing, its emission maximum would be close to what here. So, this is about say 352 nanometers or so.

Now, the moment you refold it as a function of time, what would happen to this, this intensity at 352 would decrease, because it is moving slowly to the left right. So, what you can do is, you can monitor this change in intensity at 352 nanometer of the protein as function of the time, that means, you mix it by Stoke flow or continuous flow or whatever. And then you tell your detector to monitor the change in intensity for me at 352 nanometers because that is where the unfolded state was. So, what will happen is its fluorescence as a function of time at 352 nanometer would decrease right. And the last point it decreases when it reaches equilibrium; that means, if reach the folded state.

What is the other way of looking at it?

Student: (Refer Time: 52:31).

Observing the?

Student: (Refer Time: 52:35).

So, the other way right, so the other way is I can look at this one, is it not? One is looked at the decay or the decrease in intensity at the denatured state fluorescence. The other way is I can look at the increase in the fluorescence intensity of the folded state which is a 305. So, in that case your fluorescence intensity would increase, and finally, stop when you have reached. So, this is exactly what we will do right. And that is why you have to know fluorescence before you can do that, because you have to know that at what point I have I will have to absorb or rather observe ok. And you select out always the emission maxima.

You select out always the maxima because these are the places where the change is because these are maxima right. So, the change is happening to the most at this places that is why it will always help you a lot in terms of its signal to noise, because under given set of conditions at the maximum point you always have the maximum number of photons coming out. And that is where it is going to help you a lot, is it clear? Or how the strokes flow fluorescence studies are done, so this is how Stokes flow fluorescence is done ok.

See, if you have a change in fluorescence, you park a detector, you say a detector to take the fluorescence from this wavelength or this wavelength that you decide depending upon the system, and then you monitor the change as a function of time ok.

Now, there is one more thing in this figure. If you look at this spectrum, this blue one right. If you look at the blue one, what is going to happen is this. The first two; that means, the first two spectra you can see that the lambda excitation, this lambda excitation is 292 nanometers ok. Now, lambda excitation is two ninety two nanometers means I am using a stedy state photometer, I am heating the sample with an excitation light which has wavelength of 292 nanometers. Now, why I have selected 292 nanometers can you tell me? We have done this before.

Student: (Refer Time: 54:23).

To excite only what?

Student: (Refer Time: 54:25).

Tryptophan, because tyrosine at that place if you look at the absorption spectrum has very low intensity, so that means, exclusively you would be exciting tryptophan. So, whatever emission you would monitor is from tryptophan ok. Now, if you would do the same excitation at 275, here what would happen though is you would be exciting both, that means, you would be exciting both tyrosine and both tryptophan. If you are exciting both of these, now do you

realize where this one is coming from? So, this one is here tryptophan pick. And this one is your?

Student: Tyrosine.

Tyrosine ok. So, for a protein having tryptophan and tyrosine, if you change your excitation wavelength like this right and scan for your emission, these are the changes you are going to observe ok. And there is that is that is that is why people selectively use 290 or 295e. So, that they can look at tryptophan only right.

So, I think that is pretty much what I had to discuss. You know there are lot of other things that can be done with fluorescence. The good thing about fluorescence is fluorescence is very sensitive. You can do experiments with low concentration of samples right. See if you would doing in absorbance, remember absorbance would typically involve taking protein concentration at very high level. But you would do fluorescence, fluorescence can be done at very low levels right, so micro molar level little high micro molar level so on.

Now, what it helps is in this, one is obviously, your sample consumption is very low ok, no problem. The other issue is there is a phenomenon known as protein aggregation ok. So, if you have a very high concentration of proteins, they have a tendency to aggregate. So, that means, if you were doing any absorbance measurements where you need high concentration of proteins, that means, you also have a high chance a decent chance of the protein aggregating for you, if you the protein is aggregating, then it is no use doing the experiment because your sample is already bad, that means, you cannot use the sample anymore for your experiment ok. So, your results would not be conclusive because you also have contribution from protein aggregates.

But fluorescence, see if you are doing a let say 1 micro molar or 5 micro molar or so, most of the proteins in generally most, most, most of the proteins in general would not be aggregating at that lower concentration, and hence you can easily do fluorescence without wandering

about this completing process which can which can have effect on your experimental data or your experimental findings ok.