Experimental Biochemistry
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Lecture – 13
Fluorescence Spectroscopy

Today we start with our discussion on Fluorescence Spectroscopy in continuation of our discussion on UV visible spectroscopy in the last lecture. We have to understand that, the use of spectroscopy in experimental biochemistry is very vast and what we cover in the courses here particularly the theory part is very limited in its sense of the theory concerned. So, it is recommended that you would read the books related to the course, related to the specific experiments that we are going to cover during the experimental portion of the experimental biochemistry.

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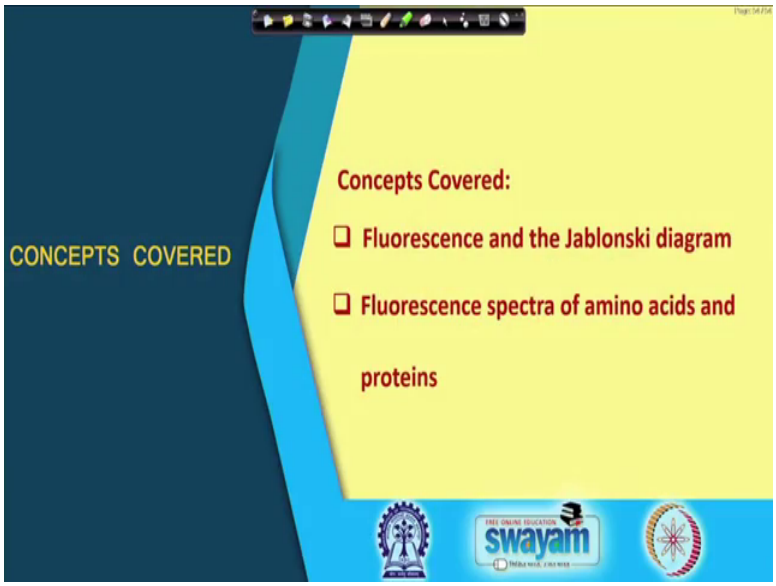

In our discussion on fluorescence, we will understand what we mean by fluorescence spectroscopy, what is meant by the Jablonski diagram and the fluorescence spectra of specific amino acids and proteins and what we are actually looking for, what is the information we can obtain if we take a fluorescence spectra. We will just revise some of the topics that we covered in the last lecture were we understood what we meant by a chromophore.

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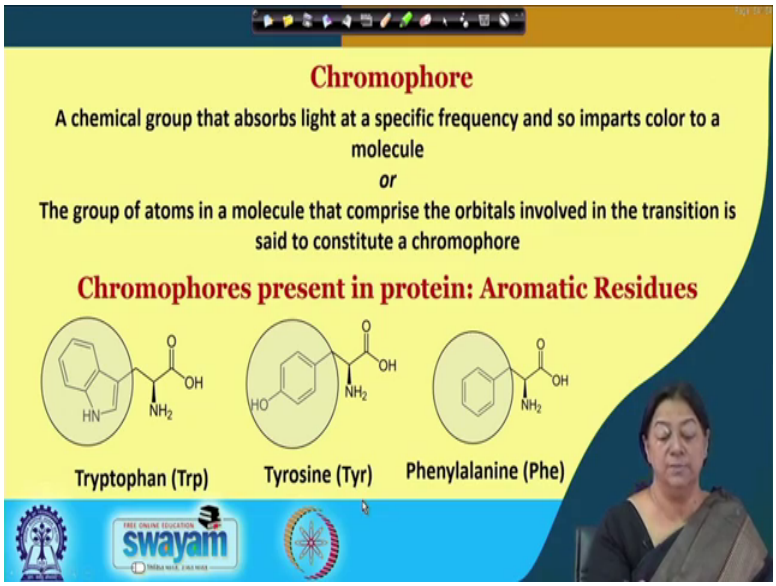

Which was a chemical group that absorbs light at a specific frequency and what it does is it imparts color to a molecule. Now in the electromagnetic spectrum we have UV, we have visible, we have infrared and as we mentioned in the last lecture it depends on the specific energy levels that we are concerned with the energy levels correspond to specific wavelengths. And we found out that when we are looking at the levels of the protein chromaphores which are the aromatic amino acid residues tryptophan, tyrosine and phenylalanine. When we are looking at these particular amino acid residues the chromophores are such that they absorb in the UV region. So, we look at the UV region for our study in proteins for our understanding of whether a protein is present or not in our solution.

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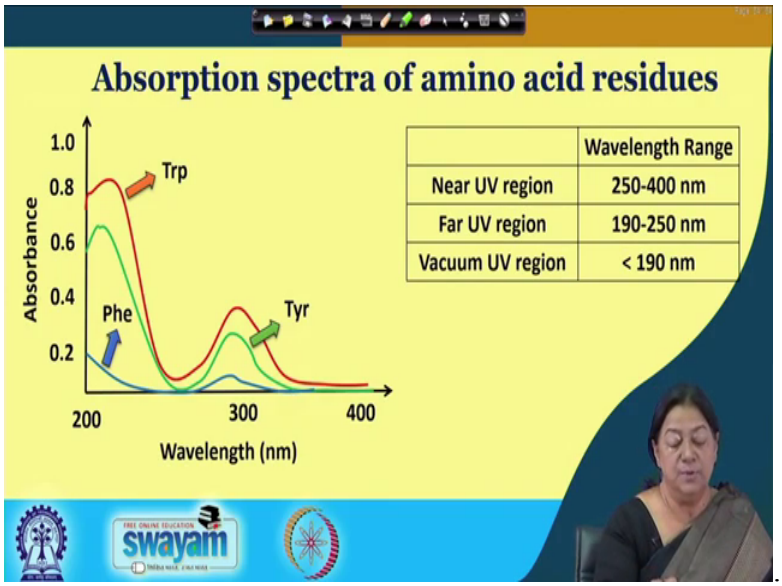

The absorption spectra of amino acid residues comprise these regions and as we mentioned in the last class we are most interested in whether we have a peak around this 280 nanometer region and this 280 nanometer region will tell us the presence of tryptophan, tyrosine and phenylalanine.

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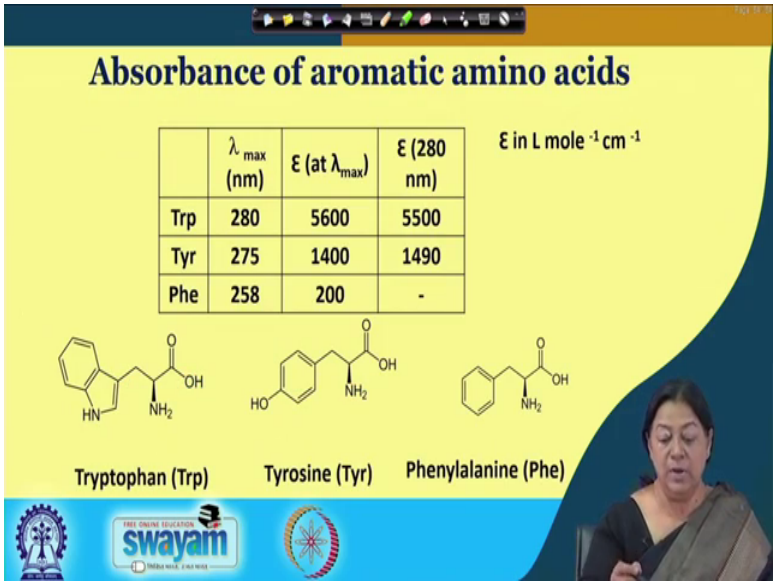

But as again we see that when we look at the specific molar extinction coefficients we see that the tryptophan has the highest value indicating that the presence of tryptophan will give a high absorbance to the molecule. And in addition to this we have the different

lambda max values what does this mean? This means, the wavelength at which we have the maximum absorbance for tryptophan, for tyrosine and for phenylalanine. Now when we go a step further in trying to understand that when we absorb when we shine light on the molecule now at 280 nanometers at around 275 nanometers and 285 nanometers these specific aromatic amino acid residues are going to be excited they are going to show an absorbance.

Now, since we have these specific amino acids already present in a protein, the protein having phenylalanine, tyrosine and tryptophan will give a specific absorbance.

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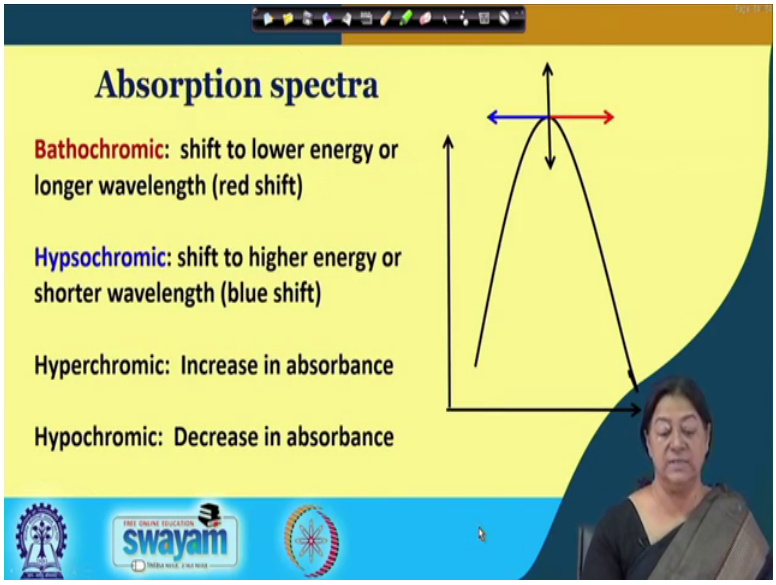

Now, when we try to understand the absorption spectra? If we consider this to be an absorption spectra we have absorbance on the y axis, the wavelength, on the x axis we want to see what happens to a spectra or spectrum when say if we have a protein and the protein unfolds we will do these experiments and an understanding of the theory involved in later lectures. But when we are considering a spectra we say that when we have a shift to lower energy a shift to lower energy means it is a shift going to longer wavelength, this is called a red shift.

When we have a shift to higher energy or a shorter wavelength it is called a blue shift or a bathochromic shift which means a red shift, a hypsochromic shift which means a blue shift. In addition what could happen to the specific peak in our spectrum it could be

enhanced where we could have a hyperchromic shift meaning and increasing in absorbance or we could have a decrease in absorbance.

Now when are we going to observe these we are going to observe these? When there are specific interactions in the molecules. What do we mean by this? We mean that if we have if the tryptophan is perturbed if the tryptophan is exposed we will see what these terminologies actually mean later on. We could have a red shift, we could have a blue shift, we could have an increase in absorbance, we could have a decrease in absorbance. So, these are the possibilities that could occur.

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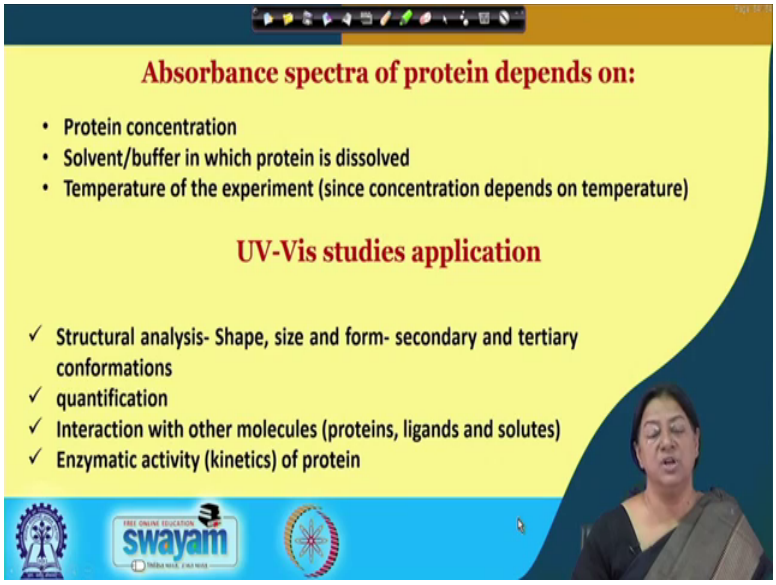

In our discussion there for on trying to understand what the absorbance of the protein depends upon we mentioned about the protein concentration, the specific buffer, the temperature of the experiment. And what is the information that we can get from the UV visible studies in trying to understand what happens when it interacts with other proteins, what happens when it interacts with other molecules, small ligands or even the solvent? We can quantify the protein using the UV spectroscopy that we have understood we can see whether there are any structural changes in the protein.

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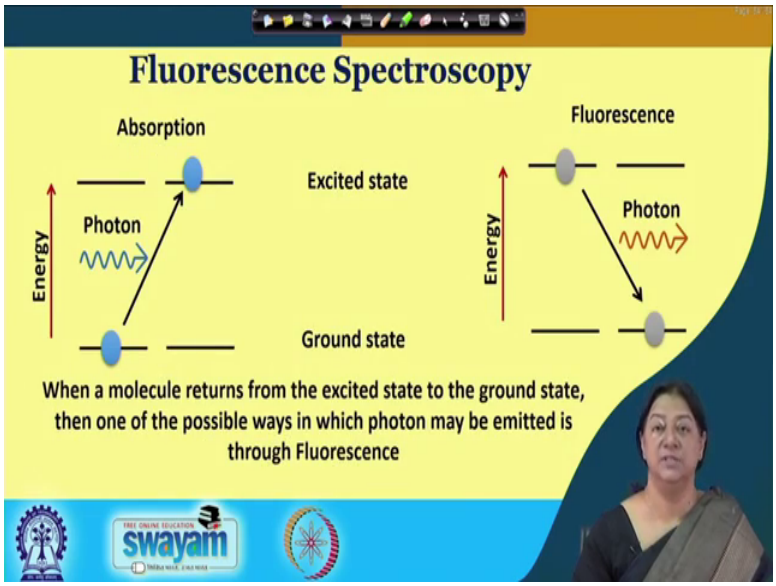

Now, when we come to fluorescence spectroscopy we are talking so far we have been talking about absorbance of light is absorbed. Now when light is absorbed and the molecule is in the excited state it is going to lose this energy. Now how does it lose its energy? We can have the loss in energy either by a non radiative path or a radiative path. If it is a radiative path it means it emits light. Now if it does emit light this will happen when we have fluorescence this will also happen when we have phosphorescence. So, when we so, once the light is absorbed it will lose this energy. Now if it loses it in a fashion where there is emission it could be fluorescence emission or phosphorescence emission or it could be just a non radiative loss of energy.

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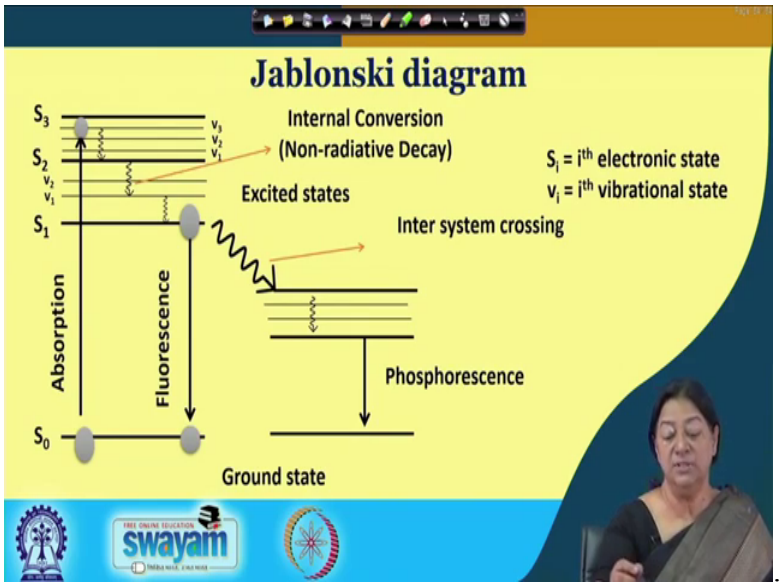

Now, in the Jablonski diagram this is this gives us a whole picture of what can actually happen light is absorbed. So, these different levels given in the dark lines here are the electronic levels, within the electronic levels we have the vibrational levels. Now when we have, we have different spectroscopy related to UV to visible to vibrational spectroscopy which is something that you will study later on. So, when we have absorption now this molecule is in the higher electronic state or a higher vibrational level belonging to a higher electronic state. It will gradually you loose this energy dissipate and come down to the lowest electronic state whereby it can radiatively lose the energy which will amount to fluorescence, this is non radiative decay. When it comes to this state this is the triplet state, in the triplet state it will again lose its energy.

So, we have in this case a singlet to triplet transfer which is called inter system crossing which as I mentioned in the beginning is something that you should read from the book to get a better idea of what we mean by these specific processes. Here we have internal conversion, here we have inter system crossing, growing from a singlet state to a triplet state and the loss of energy in a radiative fashion in this case is phosphorescence. So, we have fluorescence and we have phosphorescence, we will be confining our discussions to fluorescence alone.

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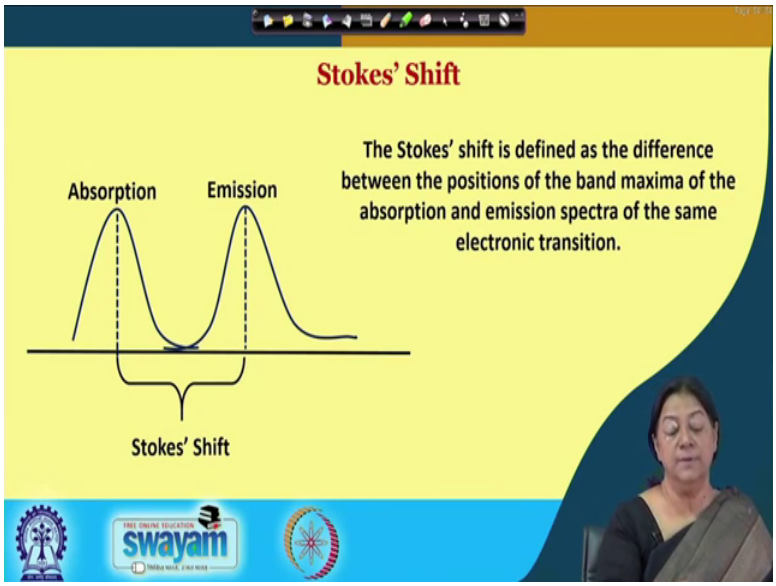

In another terminology that we have is we looked at an electronic transition. Now when we have absorption and we have emission in terms of the fluorescence spectra that we are going to talk about. The stoke's shift is something that is defined as the difference between the positions of the band maxima of the absorption and the emission spectra of the same electronic transition. Now there is one thing that needs to be noticed here, we have the absorption here and the emission to the right in terms of wavelength which means that we have a red shift. What do we mean?

When we have a red shift we learnt that if we have a red shift it means it is at lower energy, if you look at the Jablonski diagram now you will see that the energy difference between the singlet states, the ground singlet state and the singlet state from which the fluorescence emission is occurring is the energy difference is less than the energy of the absorption which means that the wavelength is longer it is red shifted. So, when we look at this fluorescence spectroscopy what else do we look at, what else or what other information can we get?

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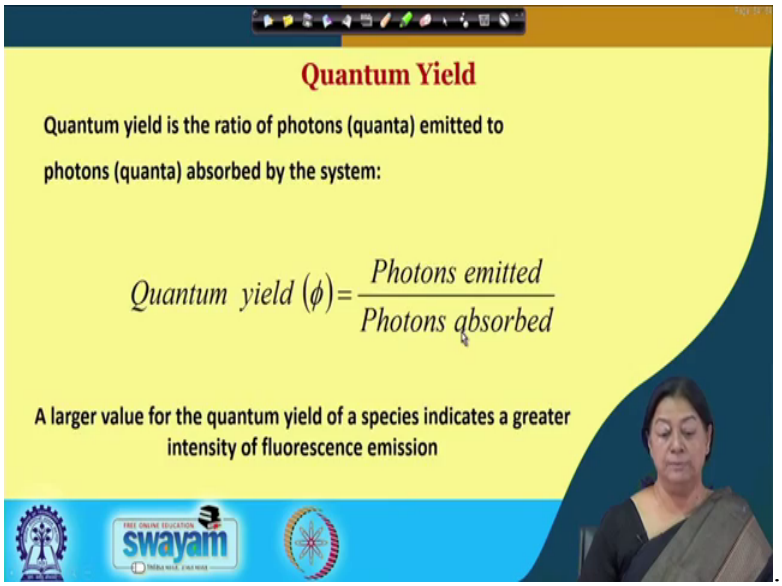

Quantum yield is the ratio of the photons the quanta emitted to the photons absorbed by the system. So, quantum yield corresponds to the ratio of the photons emitted to the photons absorbed and a larger value for the quantum yield indicates a greater intensity of the fluorescence emission because we have a larger number of photons emitted to the ratio of the photons absorbed.

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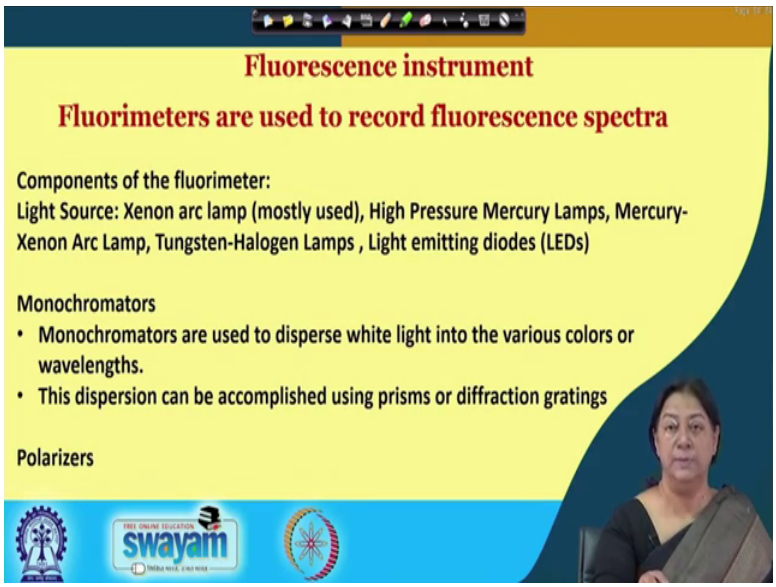

Now, if we look at the fluorescence instrument a fluorimeter which is used to record fluorescence spectra their specific components to the fluorimeter. What are these components? These are a light source of monochromator, polarizers.

And in addition we can have other components in addition to more sophisticated instrumentation that will of course, give us more information about the molecule of interest. The light source is usually a xenon arc lamp which is mostly used the monochromators are used to disperse the white light into different specific wavelengths that are of interest and the dispersions are usually accomplished by prisms or diffraction gratings.

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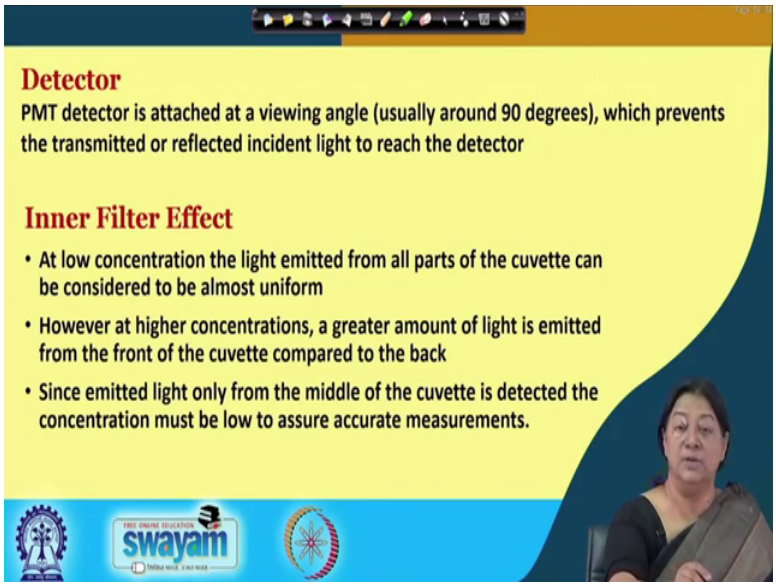

Now, a specific instrument will have also a detector a photomultiplier detector that is attached at a specific viewing angle that is around 90 degrees and why is it at 90 degrees? Because, it prevents the transmitted or the reflected incident light to reach the detector. Now at low concentration the light emitted from all parts of the cuvette can be considered to be almost uniform.

So, we saw what a cuvette was and in the case of the fluorescence cuvette it will look different than a UV cuvette. However, at higher concentrations a greater amount of light is emitted from the front of the cuvette compared to the back. Since the emitted light only from the middle of the cuvette is detected the concentration must be low to get

accurate measurements. So, we do not want too higher concentration because that will affect our measurements and each instrument has a specific sensitivity by which it works.

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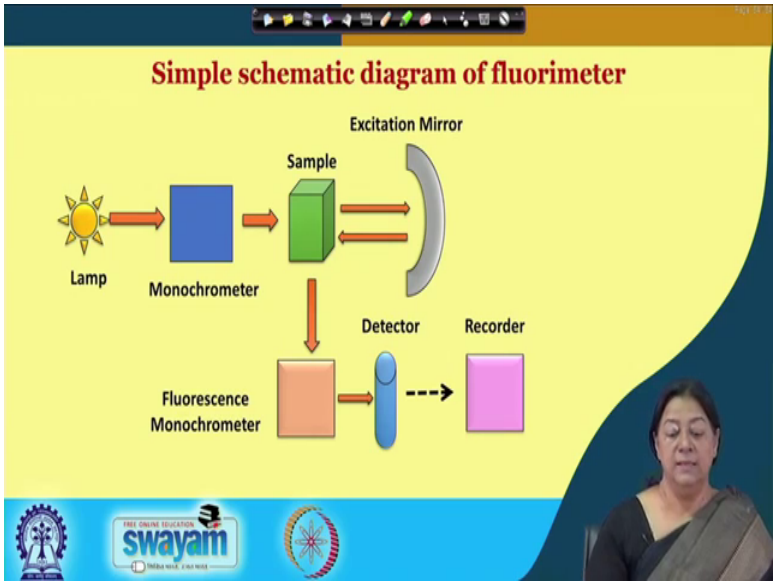

This is a simple schematic diagram where we have the lamp, the monochromator, the sample, the excitation mirror, the fluorescence monochromator, the detector and the recorder. We will be showing you how to record fluorescence spectra and what differences can occur when we look at this fluorescence spectrum scoping.

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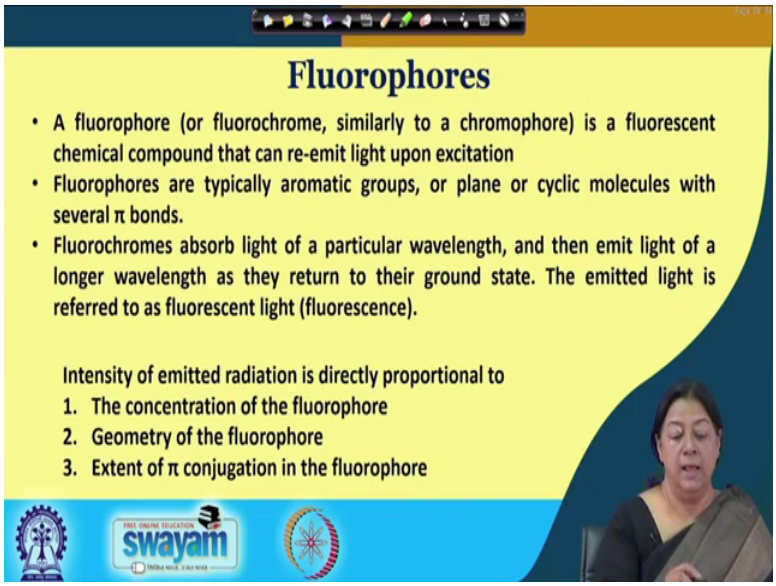

So, now we had a chromophore when we are looking at UV visible, now we have a fluorophore. What is a fluorophore? A fluorophore is going to be a fluorescent chemical compound that after excitation can re emit light. Fluorophores are usually aromatic compounds these are plane or cyclic molecules with several pi bonds to them and they absorb light of a particular wavelength and then emit light of a longer wavelength as mentioned before as they return to their ground state.

So, if it is at a longer wavelength it is red shifted the energy is less. The emitted light is referred to fluorescence or the fluorescent light. Now what is this dependent upon? This is dependent on the concentration of the fluorophore, the geometry of the fluorophore and the extent of the pi conjugation. Now, when we consider these specific fluorophoric moieties in proteins we will understand that we have in most importantly the phenylalanine, the tyrosine and the tryptophan which have specific lambda max values which we learned previously.

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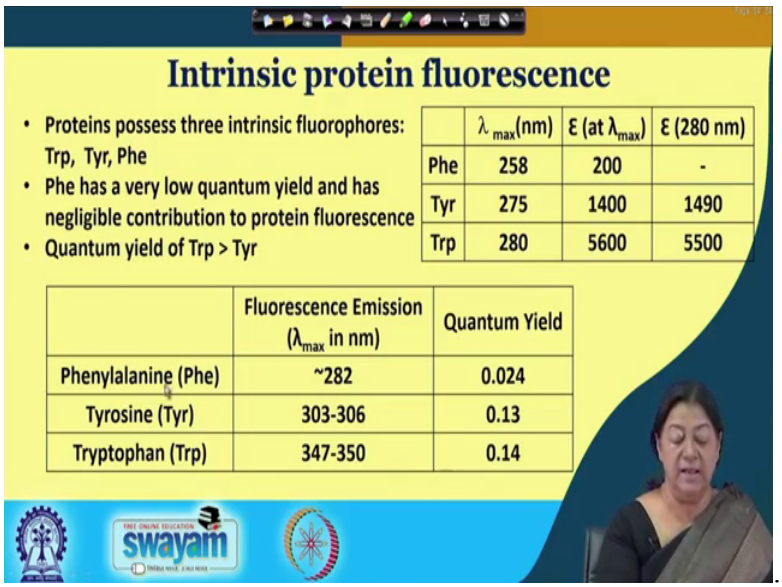

These lambda max values mean that at this particular wavelength this amino acid has the highest absorbance which means that it is going to have absorb the maximum amount of energy at that specific wavelength.

So, once we have this excitation, once the excitation takes place then this is going to lose its energy in the form of fluorescence it is going to fluoresce. Now if we look at the specific quantum yields we will see proteins that have these three intrinsic fluorophores

what do we mean by intrinsic fluorophores? Fluorophores that are already present in the protein. So, if we have a protein chain we know that the protein chain is made up of amino acids. So, in these amino acids if we have phenylalanine, tyrosine and tryptophan we have intrinsic fluorophores.

We can also have additional extrinsic fluorophores which we are molecules that we add to the protein which we can monitor which we will also tell you about. Phenylalanine is the very low quantum yield and has negligible contribution to protein fluorescence as you can see. The quantum yield of tryptophan is higher than that of tyrosine and when we look at tryptophan the lambda max is 280 nanometers what does this mean? that means, if I do if I consider an excitation at 280 nanometer then I try to monitor the emission which I know is going to be red shifted I start monitoring the emission at a wavelength more or less 10 nanometers away from the lambda max.

So, I start monitoring the fluorescence emission say for tryptophan which I have excited at 280 nanometer and we look at the tryptophan emission at 347 to 350 nanometer where we get the fluorescence emission maximum. Now, we have to be very careful in case of proteins when we excite the molecule. Because, if we want to excite the tryptophan alone we have to be careful that the tyrosine is not excited. So, if we use 280 nanometer if we remember the spectra the UV spectra that has an overlap at 280 nanometer with the tyrosine then we will also be exciting the tyrosine molecules or the tyrosine amino acids.

So, what normally what is used is for tryptophan an excitation of 295 nanometer is used this excitation will prevent tyrosine from getting excited and whatever information we obtain from the emission spectra will be from tryptophan alone and from the tryptophan residues that are present in the protein. So, we have fluorescence emission that we monitor as I mentioned further down that is at a longer wavelength. So, we excite our specific molecule of interest in this case a protein say at 295 nanometer where we are sure that the tryptophan moieties as we call them the tryptophan amino acid residue is getting excited and we start monitoring the emission spectra at say from 305 nanometers onwards and we see that we get an emission peak at 347 to 350. This will be shown to you in the experiment on fluorescence spectroscopy.

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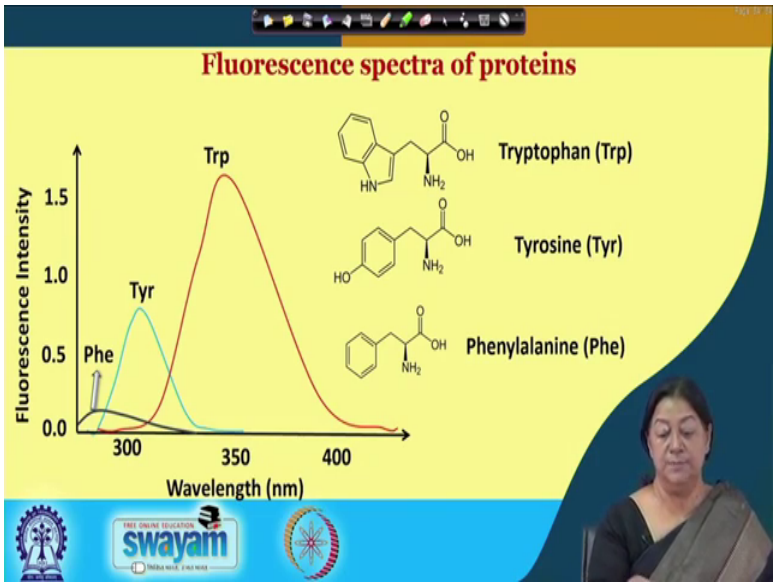

So, if you look at the fluorescence spectra of proteins you can see that this is where we have the phenylalanine fluorescence intensity, this is the tyrosine fluorescence intensity and this is the tryptophan fluorescence intensity. Which we can see the tryptophan fluorescence intensity and accounts for the high quantum yield of tryptophan which means that there are larger number of photons emitted for photons absorbed compared to tyrosine and compared to phenylalanine.

Now when we are looking at this region this is where we see now a fluorescence maximum. This is where we have an intensity maximum from the tryptophan the tyrosine intensity maximum is here and the phenylalanine is here which is relatively weak because of the low quantum yield. Now if you want to look at the specific structure of the amino acid or the specific location of an amino acid in a protein and see what happens.

So, if this is the fluorescence emission spectra for tryptophan that is in a particular position in a protein, any perturbation or any change in the environment is going to result in a change in the fluorescence emission here. So, we can have a quenching of fluorescence what do we mean by a quenching of fluorescence? We were going to have a loss in fluorescence intensity. So, if a protein is bound to a ligand is bound to some other molecule that blocks the fluorescence emission of tryptophan then we will not have a

higher fluorescence intensity there will be a drop in fluorescence intensity that is called quenching.

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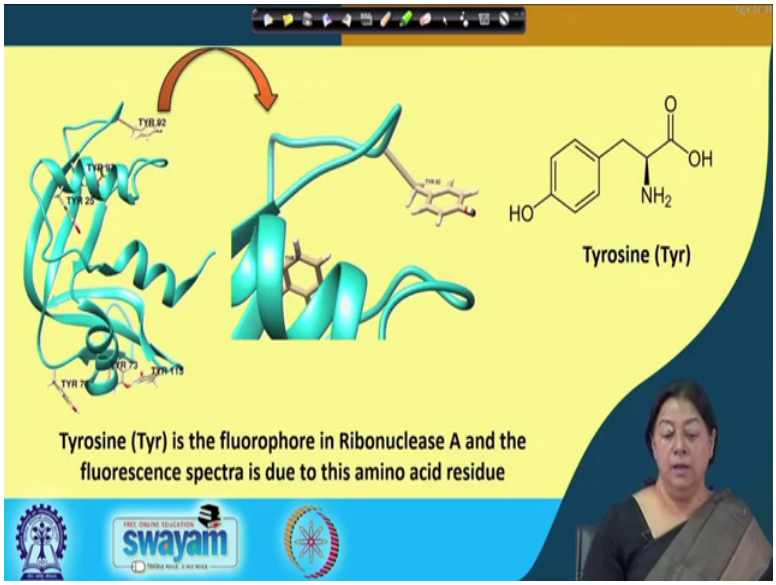

For example if we look at a specific protein this is the protein Ribonuclease A, it has a specific tyrosine here it actually has six tyrosine atoms or six tyrosine amino acid residues and we just if we just blow up this region we can see that there is a specific tyrosine. Now these tyrosine amino acid residues since this specific protein does not have a tryptophan the fluorescence that we monitor for this protein will be due to tyrosine alone.

So, any changes in the structure of the protein so, if we consider we will look at the structure of protein in a bit detail in further lectures. So, if this protein gradually unfolds then what is going to happen is the tyrosine is going to get more exposed there is going to be greater fluorescence intensity and so, this is where we are amino acid tyrosine is going to show up in the fluorescence intensity spectrum.

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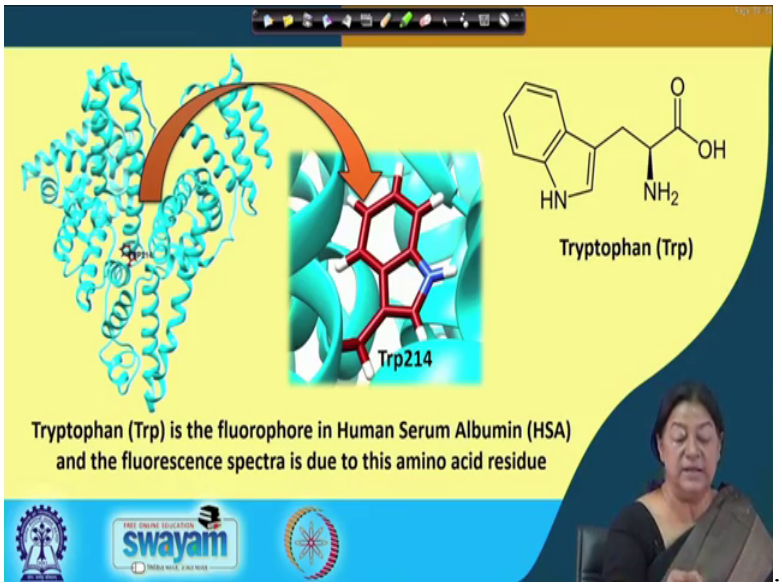

If we look at another molecule this is human serum albumin. Human serum albumin has many tyrosines. But it has a single tryptophan, one tryptophan at position 214. Now this means that if we look at this shape of it is a heart shaped molecule, if we look at where the location of the tryptophan it is the fluorophore in human serum albumin because there are many tyrosines, but since we have a tryptophan as I mentioned before we will excite the molecule at 295 nanometers and what we are going to do is we are going to excite the tryptophan.

Then we are going to monitor the fluorescence spectrum. Now if we add a specific compound so, here is our tryptophan at this position here. If we add a specific compound to human serum albumin in our cuvette in our sample preparation we allow it to incubate or we keep it for a while and then we measure the fluorescence or we take a fluorescent spectrum again and then we compare it with the previous fluorescence spectrum that was taken. What is most likely going to happen is if the molecule interacts with or sits in this pocket.

So, this is kind of a pocket as I mentioned it is a heart shaped molecule, if we have the molecule come and sit around where this tryptophan is what is going to happen to the fluorescence intensity? The fluorescence intensity is going to be quenched. What does it mean? It means there is going to be a reduction in the fluorescence intensity the

fluorescence spectra is going to have lower intensity for the emission spectrum that we see.

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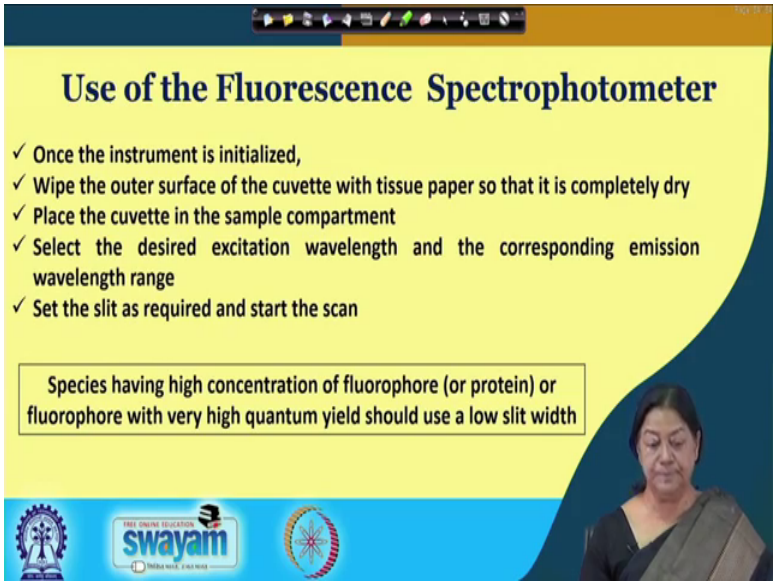

Now, how do we use this? Before the machine is switched on we check the room is sufficiently cold, we check the humidity is low we turn the lamp on and we wait for the machine to get initialized we have to ensure that the ladies clothes and the compartment is kept empty during the initialization procedure. Once the instrument is initialized we have to wipe the outer surface of the cuvette with tissue paper.

So, it is completely dry place the cuvette in the sample compartment and then select the desired excitation wave length we usual excitation wavelength selected for a molecule is the lambda max value of absorbance. Which as I mentioned for tryptophan should have been 280 nanometer, but because we know that we do not want to excite the tyrosine we shift it a bit 2 to 295 nanometer where we will not have any interference from tyrosine.

And we select the desired excitation wavelength and the corresponding emission wavelength range which I mentioned is going to be 10 nano meter away from the excitation wavelength because we know that our fluorescence emission spectra is going to be red shifted we set the slit width as required and start the scan. So, species having high concentration of fluorophore that is protein or fluorophore with a very high quantum yield should use a slow low slit width because it is going to have a higher fluorescence intensity.

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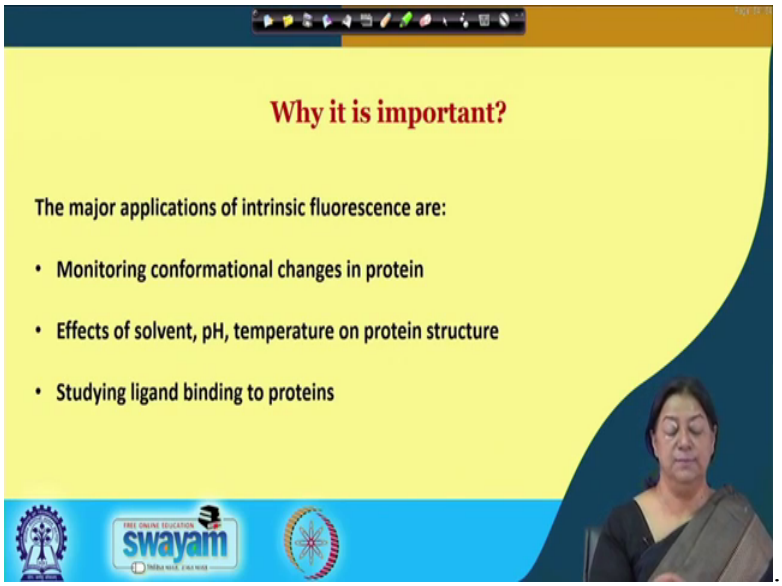

Now, why is this important? The major applications of intrinsic fluorescence are monitoring the conformational changes in the protein because if we see a change in the protein structure it means that the tryptophan is going to get exposed the tryptophan is probably going to show up in the fluorescence spectra. The effects of the solvent pH temperature on protein structure can also be studied when we look at fluorescence spectra. If we add if we increase the temperature what happens to the fluorescence spectra if we change the pH what happens to the fluorescence spectra?

If we change it from a non polar solvent to a polar solvent what happens to the structure of the protein? All of this can be monitored through fluorescence spectroscopy and of course, ligand binding which I just mentioned in the case of human serum albumin where there is a single tryptophan. A single tryptophan that may interact with the molecule that is going to sit in the pocket where the tryptophan is located what are we going to see in that case we are going to see a reduction in the fluorescence intensity or fluorescence quenching as it is called.

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The references; this is a book Joseph Lakowicz, the Principles of Fluorescence Spectroscopy that practically covers everything related to fluorescence spectroscopy apart from many references present in the book itself.

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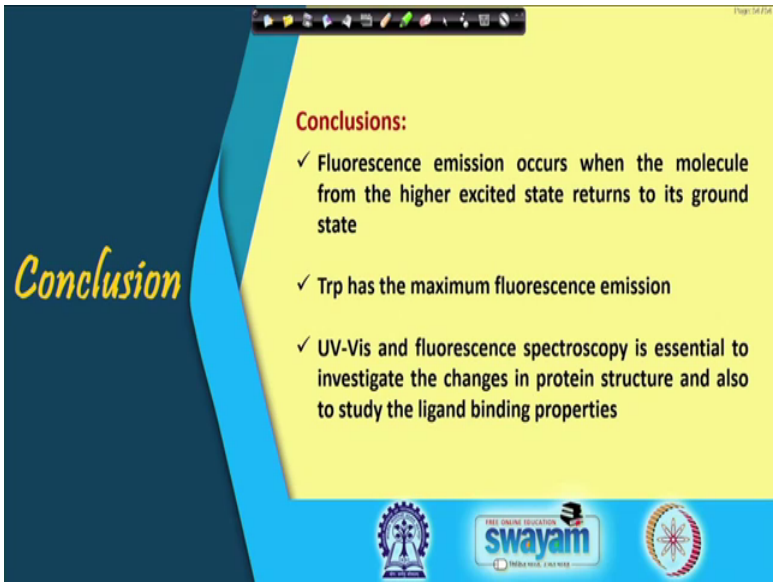

So, what we do learn is that the fluorescence emission occurs when the molecule from the higher excited state returns to its ground state. So, instead of having non radiative emission we now have radiative emission in terms of fluorescence. Tryptophan has the maximum fluorescence emission what does this mean? It has a high quantum yield, a

high quantum yield means that the photons emitted are going to be larger in number and the ratio of the photons emitted to the photons absorbed is a high value.

So, UV and fluorescence spectroscopy is essential to investigate the changes in protein structure and also to study ligand binding properties. We will show you specific experiments where we will understand how the UV spectra change, how the fluorescence spectra change due to addition or due to interaction due to temperature changes or due to solvent changes in the course of the experimental part of the lectures.

Thank you.