Enzyme Science and Technology
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Module - VII
Enzyme Substrate Interactions
Lecture - 32
Enzyme-Substrate Interactions (Part-II: Spectroscopic Methods)

Hello everyone, this is Dr. Vishal Trivedi from Department of Biosciences and Bioengineering IIT, Guwahati. And what we were discussing? We were discussing about the different properties of the enzyme in the course, Enzyme Science and Technology. And in the current module, we are discussing about the interaction of the substrate or the ligands with the enzyme.

And in this context, what we have discussed, we have discussed about the how you can be able to utilize the chromatography techniques to study the interaction between the substrate and the enzyme. And in today's lecture, we are going to discuss some more techniques which are related to the studying the interaction between the enzyme and the substrate. So, as you can recall that enzyme is actually recognizing the substrate utilizing the three important parameters.

One is geometric complementarity, second is electronic complementarity and the third is the stereospecificity. What is mean by the geometric complementarity is that the enzyme is going to recognize a typical three-dimensional conformation of the substrate. So, if the substrate is acquiring that particular type of three-dimensional conformation, it will go and fit into the active site right.

And then once it gets fit into the active site, it is actually going to make the different types of interactions. And based on these interactions, the enzyme is going to further confirm that this is actually the cognate substrate it was looking for. And then ultimately, the stereospecificity of the substrate is also important. For example, in most of the biological system, the L type of stereospecificity is more preferred over the T type of stereospecificity.

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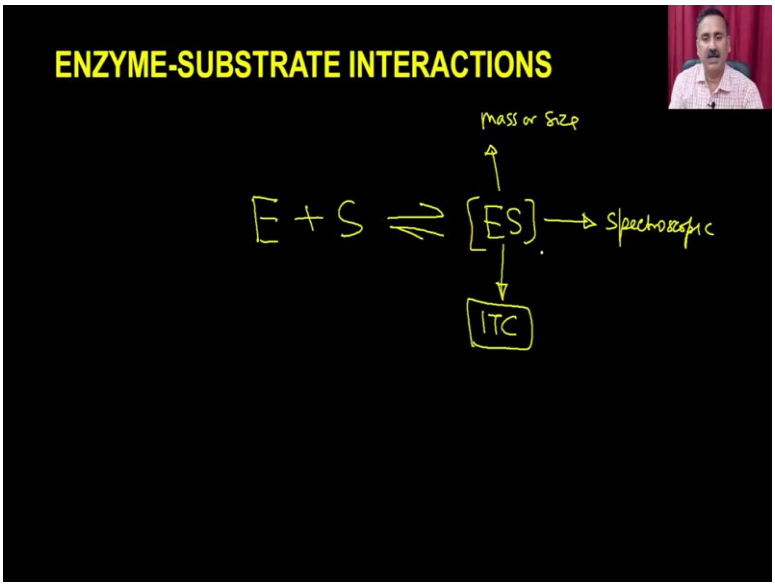

So, while the substrate is interacting with the enzyme, it is actually making the lot of modifications. So, when the enzyme is interacting with the substrates ok, and it is making a complex like the E S complex. And at this stage only, it the enzyme is substrate is interacting, is you know making lot of modifications into the enzyme structures.

It is changing the you know the mass or the size of the enzyme, it is also disturbing the electronic configuration of the groups, what are present on the enzyme. So, that can be mapped in the spectroscopic technique and then it can also be able to utilize that for studying the some of the other techniques like the there will be a change in internal in internal in heat of the enzyme substrate system and that can be mattered in the system called ITC.

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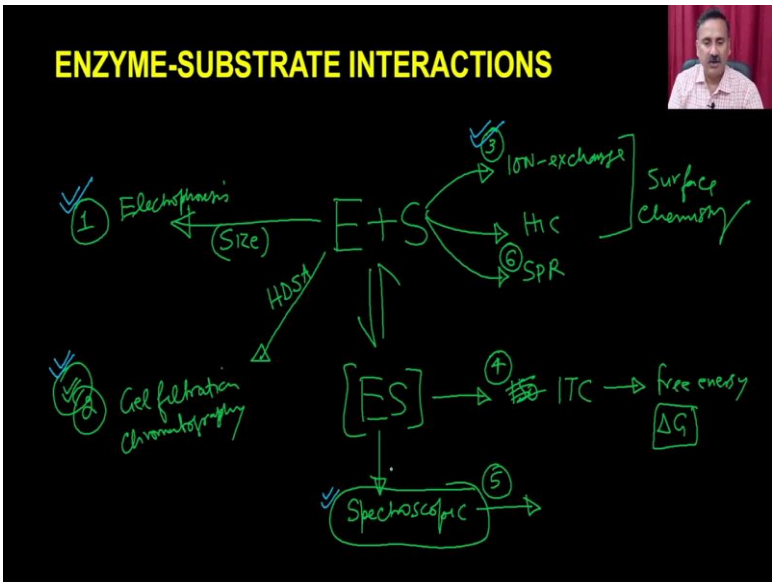

And we have discussed many of these study many of these techniques which can be used when you are mapping the enzyme substrate complex formations. Like for example, it is actually going to study it is going to change the size, size in terms of molecular weight and that can be mapped in the electrophoresis.

And then it can also be able to change the hydrodynamic surface area and that can be mapped in the chromatography. And then it also can change the surface chemistry and that can also be mapped in the ion exchange HIC or the surface plasma resonance. And then it can also be able to interfere or it actually can alter the different types of energy parameters and that can also be able to map into the isothermal titration colorimetry.

And then ultimately it also going to change the electronic configuration of the different groups onto the enzyme or the substrate and then that can be mapped into the spectroscopy. So, let us first start discussing about the electrophoresis methods. And in the previous lecture if you recall we could we have discussed about the ion exchange chromatography and as well as the gel filtration chromatography to utilize or to see the power of the chromatography system.

So, let us discuss about the electrophoresis how you can be able to use the electrophoresis to measure the size of the object.

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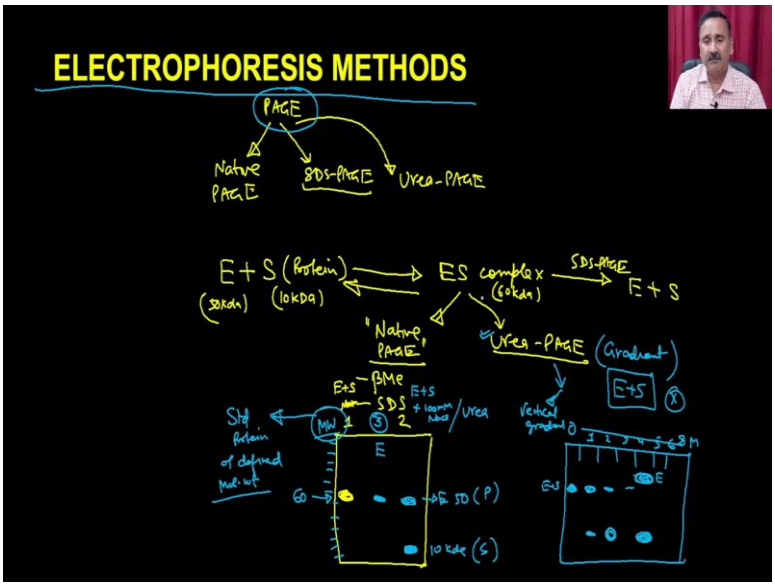

So, in the electrophoresis, electrophoretic system you have two options; one is called as the native page. And so, page can be of two types; it can be of native page, it could be SDS page and it could also be variation of the native page where you can have the urea page ok. And all of these three are can very very you know easily been used to measure the mass of the enzyme ok. Depending on the what kind of structure or what kind of you know the substrate is.

So, if the enzyme is interacting with a substrate and if this substrate is also a protein, then that is actually going to form the enzyme-substrate complex. And majority of these enzyme-substrate complex formations are reversible in nature, that means the enzyme and substrates are not covalently bound to each other right.

So, in that cases when you run the SDS page the enzyme-substrate complex when you run the SDS page it is actually going to be broken down. Because the SDS is actually going to denature the three dimensional structure of the enzyme and as a result it is actually going to break the enzyme-substrate complex; so, SDS page is not useful.

When you have the enzyme-substrate complex and it is not very robust then you what you have two options; one is you can run the native page and you can run the urea page. So, we will discuss both of these; so, that you will understand how you can be able to use that. So, native page means the polyacrylamide gel electrophoresis minus beta mercaptoethanol and minus FDS.

If you want to know more about the page and how you can be able to run the page for determining the or how what are different components of the page operators and all that thing. You can actually be able to follow some of my lectures from the other courses like the experimental biotechnology course. And that actually there where I have discussed in detail about the native page or the urea page.

So, we are not going to discuss that; whatever I going to discuss is that native page will actually going to be devoid of beta mercaptoethanol and devoid of the SDS page which means. It is actually not going to affect the enzyme-substrate complexes and how and if the substrate is proteinaceous in nature. For example, in the substrate is 10 kda and the enzyme is 50 kda, then the enzyme-substrate complex is going to be 60 kda right.

So, that is a very significant change in the in the size of the enzyme and that is how you can be able to measure that. So, in a native page what you are going to do is, you are going to run this under the two different conditions. So, in the lane one you are going to and in lane two right; so, in lane one you are not going to make any kind of modifications.

And so, you are going to have the enzyme plus substrate complex which you are going to run into the lane one; so, it is imagine that you are going to get a band here right. Now, in the lane two what you are going to do is you are actually going to make a change right. So, what you are going to do is in this one you are going to run enzyme plus substrate, but you are actually going to add suppose 100 millimolar of Nacl or you can actually be able to use the a urea ok.

So, Nacl is because mostly the substrate is always interacting with the enzyme with the electrostatic interactions; so, if you add the Nacl it is actually going to result into the breakdown. So, this is actually a 60 kda band ok and you can actually be able to know the size of these bands if you run a molecular weight marker.

So, if you run the molecular weight marker and if you do not know about molecular weight marker, molecular weight markers are actually the standard proteins of the defined size ok. So, it actually going to tell you which protein band is corresponding to which molecular weight ok; so, this is 60 kda.

Now, when you run this in the 100 millimolar Nacl and urea what will happen is that you are actually going to see the two bands. You are going to see one band which is corresponding to the enzyme; so, this is actually the enzyme. So, it is a 50 kda band and then you are going to see another band which is actually the 10 kda band and that is for the substrate; so, this is actually for the protein, this is for the substrate.

And that is how you can be able to say that the enzyme is substrate for interacting with each other. And you have already verified right, because you it was showing a 60 kda band and it is called still showing the 50 kda band. If you want you can actually run the in the entry you can just run the enzyme itself and that actually is going to be you know going to run in front of the this lane number two ok.

So, that will confirm that this is actually the enzyme band not the other any other kind of modifications. In the urea page what you can do is, you can actually be able to run the urea page either in a vertical gradient or in a horizontal gradient. So, you can actually be able to run the gradient urea page and the gradient urea page what it is actually going to do is what you are going to do is, you are going to vary the urea concentration from 0 to 8 molar in this direction ok; so, this lane is 0.

So, what you are going to do is you are going to make the enzyme substrate complex right and then you are going to load that into the different lanes ok. So, you are going to suppose you are going to have a lanes; so, this is 1 molar, 2 molar, 3 molar, 4 molar, 5 molar, 6, 7, 8 like that ok.

So, what will happen is that the first the enzyme is going to be like this; so, this is going to be enzyme substrate complex. And as you go towards the end of the urea right, what will happen is that you are going to start seeing you know the intensity of this band will small and then you will start seeing another band ok.

And then you will see that this is actually decreasing and this is increasing, because more and more substrate is coming out from the protein and that is how it is actually going to be coming in this space. And ultimately what you are going to see is you are going to see that this sub particular band is actually going to disappear and a new band is appearing.

Because this is the new band for the enzyme alone and you are going to see a very high quantity of substrate. So, this is the place where actually the enzyme plus substrate is

broken down right. And by the help of the urea page, you can be able to determine whether the whether the enzyme substrate complex is formed or how stable the interaction is right.

So, SDS page is a very very robust technique to answer the question whether the substrate is interacting with the enzyme or not considering that the substrate is proteinaceous in nature. If the substrate molecular weight is very low; for example, you are talking about like glucose and hexose and hexo kinase. Then in those cases it is probably not be the best technique to answer the questions; so, this is all about the electrophoretic methods.

So, you can actually be able to run the page in the native page SDS page and as well as the urea page to answer the question whether the particular proteinaceous substrate is interacting with the protein or not and whether the how stable the complexes. So, we have discussed about the electrophoresis, we have discussed about the chromatography methods. And now let us move on to the next method and the next method is the spectroscopic method.

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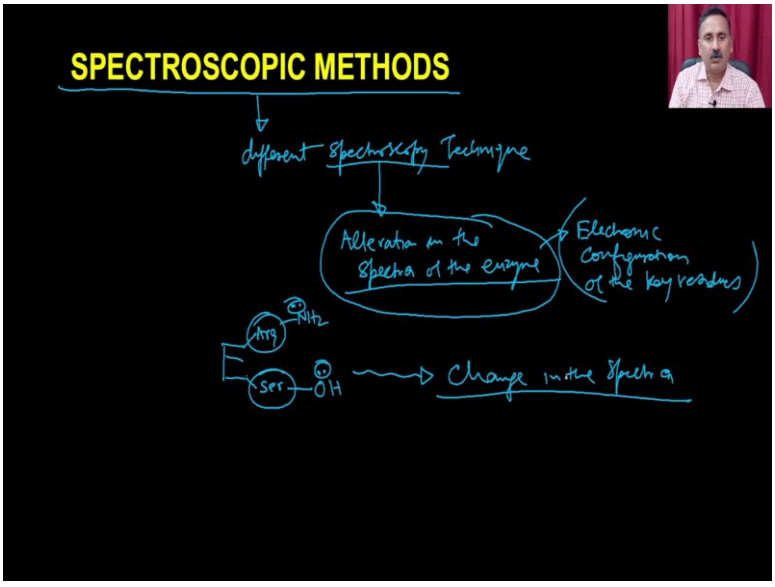

So, in the spectroscopy if spectroscopic method you are actually going to utilize the different types of spectroscopy techniques. So, different spectroscopy technique and as the name suggests right, spectroscopy means you are actually going to see alteration in

the spectra right of the enzyme or substrate right. And alteration in the substrate of the or a spectra of an enzyme can be possible by many ways.

One of the classical way in which you can be able to map that is actually going to change the electronic configuration of the key residues or key ok; so, electronic configuration is mostly being responsible. So, what will happen is that suppose you have an enzyme right and it has a arginine group right, it has an arginine amino acid at the active side or suppose it has a serine at the active side and so on.

So, serine is actually containing the hydroxyl group which actually contains the lone pair of electron; whereas, arginine also contains the amino group and that also contains the lone pair of electron. And these lone pairs of electron sometime get this delocalized or get interacted with the substrate and that is how they are actually going to show you a change in the spectra right.

And so, that is going to be mapped in the different types of chromatography technique. Apart from the electrons in the valence electron or the outer shell electrons you can also be able to map these kind of interactions with the different types of spectroscopy technique.

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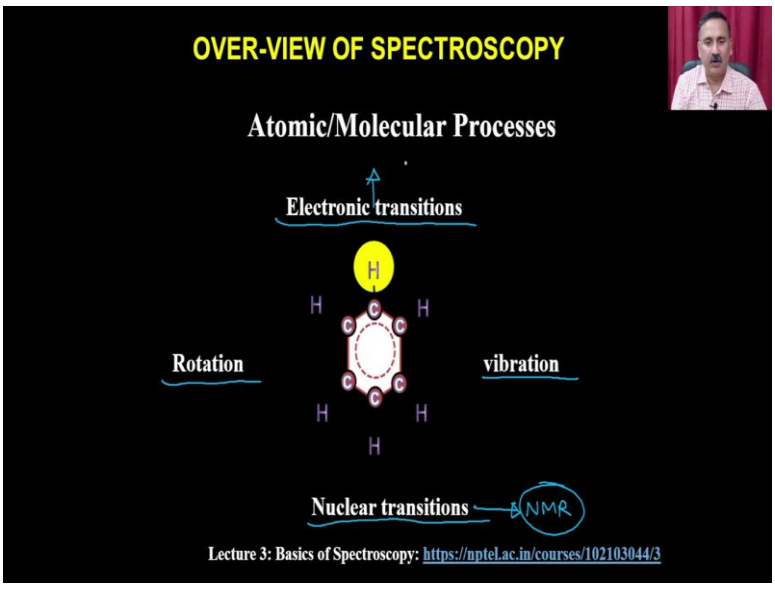

So, when we talk about the spectroscopy technique it can actually be able to work on the different types of molecular processes. For example, you can have the electronic

transitions, you can have the rotations, you can have the vibrations and you can have the nuclear transitions. In the electronic transitions you can be able to map that under the spectroscopy UV visible spectroscopy technique.

Rotations and the vibration can be mapped under the IR spectroscopy and the nuclear transitions can be mapped under the NMR spectroscopy. So, basically the most of these spectroscopy techniques can be exploited for studying the enzyme substrate interactions. But the electronic transitions are very very sensitive compared to the nuclear transitions and electronic transitions are easy to map. Because that can be done in the very easy technique called as a UV visible spectroscopy.

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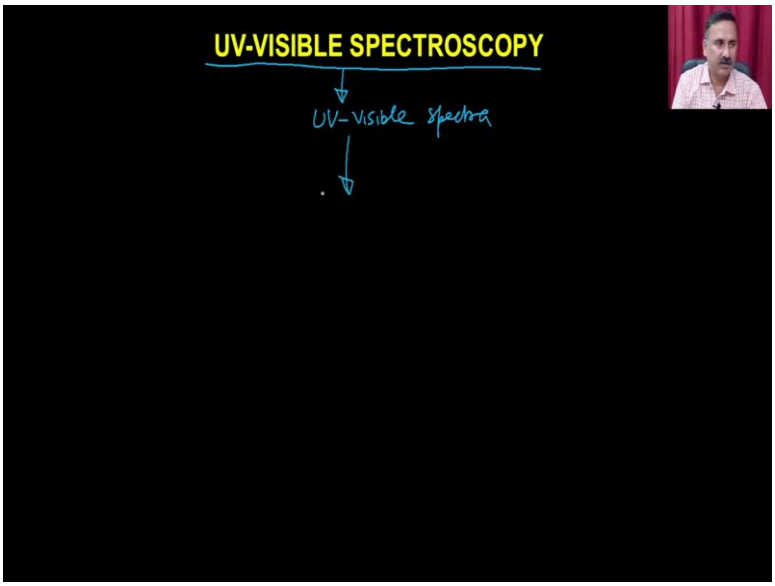

So, UV visible spectroscopy as the name suggests it is actually going to be a technique where you are actually going to see the UV the range in the UV visible spectra right of a compound or of an enzyme and you will see how it is actually going to altered. So, what is mean by the UV visible spectroscopy?

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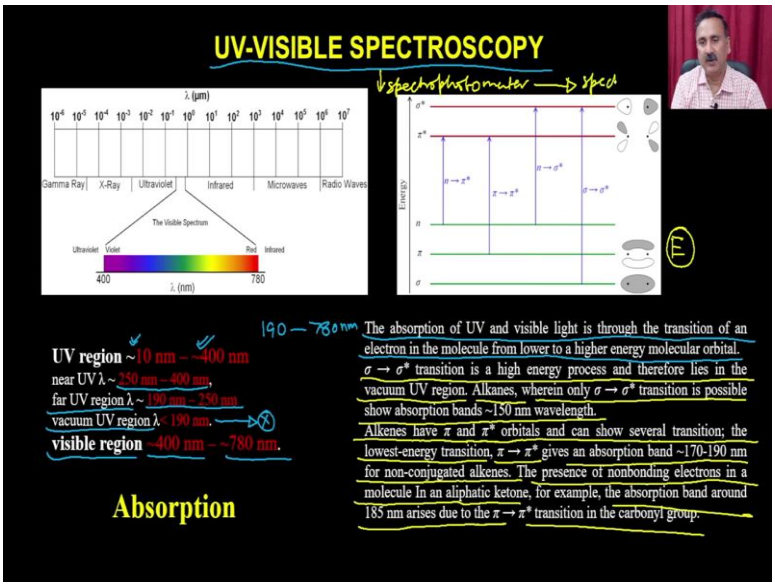

So, UV visible spectroscopy is a spectroscopy technique which where you are actually going to work under the visible range which where which starts from the UV range and it goes up to the visible range. So, UV range starts from the 10 nanometer to 400 nanometer and within the UV range you have the near UV range and as well as the far UV range.

So, near UV range starts from the 250 nanometer to 400 nanometer; whereas, the far UV range starts from the 190 to 250 nanometer. And you also have the vacuum UV range which is actually less than the 190 nanometer and that is actually going to be useless for any kind of molecular interaction studies. And then we also have the visible range; so, it can be from the 400 nanometer to 780 nanometers.

So, starting from the 190 nanometer starting from the; so, UV visible range is starting from the 190 to 780 nanometer; whereas, in which the 190 to 250 is the UV range and from 250 to or 400 to 780 is the visible range. So, the absorption of the UV and the visible range is through the transition of an electron in the molecule from lower to a high energy or molecular orbital right So, this is what it has been shown how the different types of UV visible spectroscopy happens right.

So, the absorption of UV and the visible light is through the transition of an electron in the molecule from the lower to a high energy state. So, when you are exciting a molecule right; so, when you are exciting a group what is present inside the enzyme, the electrons

of this particular functional group goes from their lower energy state to the higher energy state.

And then there will be a sigma to sigma pi in transition is a very high energy process and therefore, lies in the vacuum UV range. Whereas, the alkenes whereas, only the sigma to sigma pi transition is possible to show the absorption band around 150 nanometer. Alkenes have the pi to pi star orbitals and can show the several transitions, the lowest energy state is the pi to pi star giving absorption band around 170 to 190 nanometer for a non-conjugated alkenes.

The presence of the non-burning electron in a molecule is in an aliphatic ketone; for example, the absorption band is 150 nanometer arise due to the pi to pi transition in the carbonyl bonds. So, when you want to perform the UV visible spectroscopy you are actually going to use a spectrophotometer right, which is actually going to measure the UV visible spectroscopy you are going to measure the UV visible spectroscopy and it actually going to give you the spectra right.

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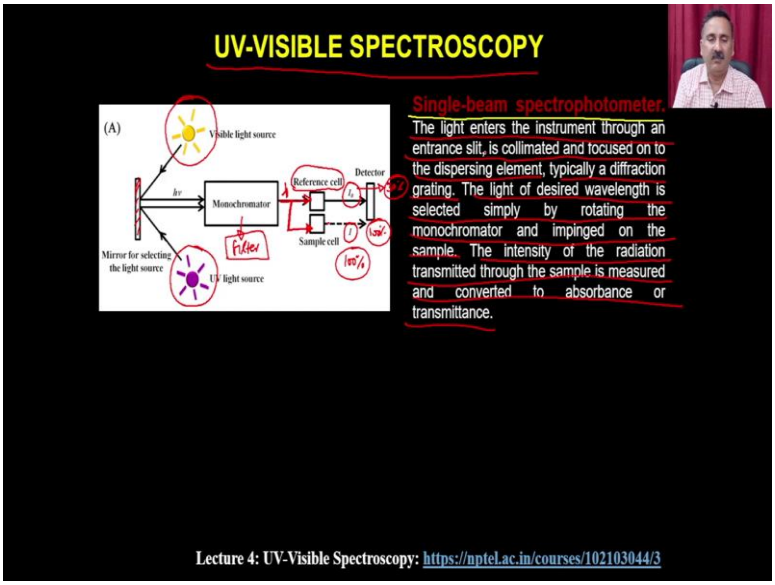

So, spectrophotometer can be of two types, it can be of single beam spectrophotometer or it could be a double beam spectrophotometer. So, in a single beam spectrophotometer you are actually going to have the one light; so, it is going to have the light source. So, for example, in a UV range or the visible rod; so, it is going to have two bulbs one for the visible range one another for the UV lamp.

And then both of these lamps are actually going to have the mirror right this mirror is going to reflect these lights and then they will go through the monochromator. The monochromator is actually a kind of a filter and that is actually going to filter the desirable wavelength.

So, it is actually going to give you the desirable wavelength; for example, if I selected the particular lambda then it is actually going to spin out all other molecules all other wavelengths, but it is only going to give allow the passage of the lambda. So, monochromator could be of different types, it could be a diffraction grating, it could be prism it could be anything.

And then it actually going to you know interact with the reference cells; so, in a single beam spectrophotometer you are going to have only one slot to keep the qubit ok. And then it is actually going to allow the absorbance of the light right, and then the unabsorbed light that is the I 0 is actually going to reach to the detector ok.

As a parallel beam ok; so, when the beam comes out it actually going to split into two part ok, one part will go and in can be received by the detector without any absorbance and the other one is actually going to go through the sample. And you can actually be easily be able to calculate what is the value of I 0 by I and that is how it is actually going to give you the value of absorbance.

Because, this is the 100 percent right this is the 100 percent light and this is the probably 20 percent or 30 percent whatever depending on how much is I been absorbed right. So, I 0 by I is actually going to give you the absorbance of that particular sample. So, the light enters the instrument through an entrance slit and it is collimated and focused on to the dispersing element.

Typically, a diffraction grating the light of desired wavelength is selected simply by rotating the monochromator and impinged on to the sample. The intensity of the radiation transmitted through the sample is measured and converted to the absorbance or the transmittance, then we have the double beam spectrophotometer.

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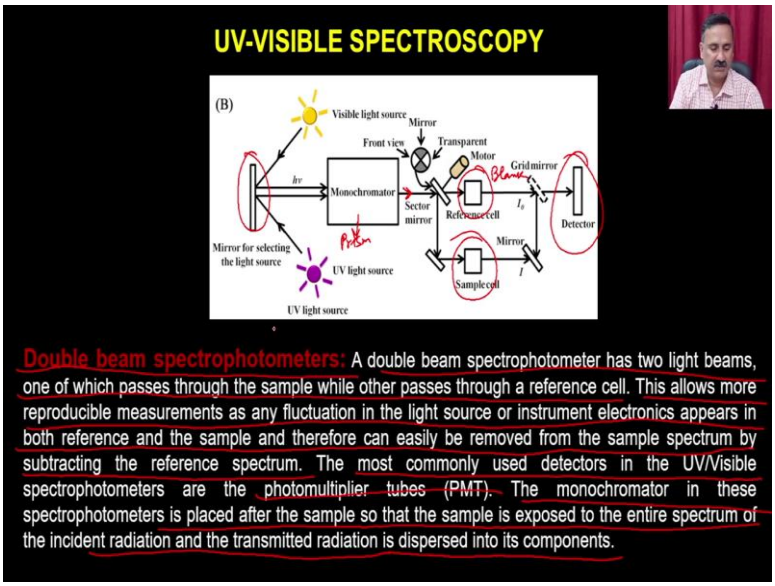

So, in a double beam spectrophotometer ah; so, remember that in a single beam spectrophotometer you only have one beam ok that one beam you are splitting into two and in this one that is all mathematically or you know virtually you are doing right. So, the second beam is directly going into the detector and its showing that this is the 100 percent light right, and this is the you know the absorb light ok.

So, based on that you can be able to calculate the absorbance and the transmittance, but if there will be any fluctuation in the direct light that you will not be able to map or measure. So, that is the one of the drawback of working with the single beam spectrophotometer and that is why you should have the double beam spectrophotometer.

So, in a double beam spectrophotometer has two light beams ok, one of which passes through the sample while the other passes through a reference cell. This allows the more reproducible measurement as an fluctuation in the light source or the instrument electronics appear in the both reference and the sample and thereby can easily be removed from the sample spectrum by subtracting the reference spectrum.

The most commonly used detector in the UV spectrum are photo-multipliers or PMT's, the monochromators in these spectrophotometer is placed after the sample. So, that the sample is exposed to the entire spectrum of the incident radiation and the transmitted radiation is dispersed into its component. So, it is exactly the same way that you have a

lights UV source you have a light source then it is actually going to you know fall onto a mirror and then this mirror is actually going to reflect.

So, depending on the and then there will be a monochromator this monochromator could be a diffraction grating or the prism. And from the diffraction grating you are going to have the single wing wavelength right. And then it is actually going to have the you know reflectors right and it is actually going to split into two beams one will go like this the other one will go just straight.

So, one will go into the reference cell, reference cell or it is also being called as blank; whereas, the other beam is actually going into the sample cell ok. And then both of these are actually going to be collected back and that is how you are actually going to detect the intensity of the beam, but comes from the reference cell and the intensity of the beam what comes from the sample cell.

And that is why if there will be any fluctuation into the intensity of these bulbs. For example, if there will be any reflection in the intensity of these bulbs it will reflect into the increase in intensity of this beam. And ultimately it is also going to reflect into the intensity of the both of these beam sources, because the all the both of these going sources are coming from the single beam right.

So, it and if there will be any fluctuation or any kind of modulation into the sample or the reference cell right that also can be mapped. So, that is why the errors are going to be reduced when you are going to talk when you are going to use the double beam spectrophotometers.

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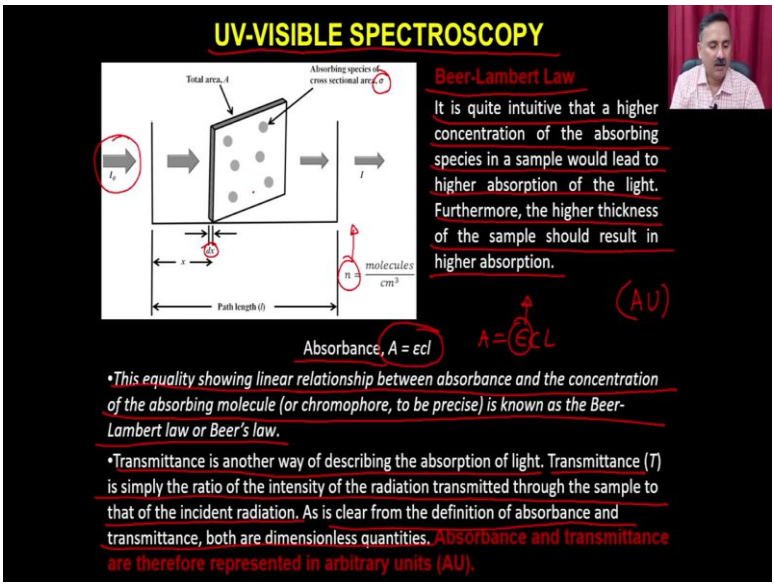

Now, when we are going to have the sample into the qubit it is actually going to absorb the material and that is how it is actually going to follow the Beers Lambert law. So, it is quite intuitive that the higher concentration of a absorbing species in a sample would lead to the higher absorption of the light.

Furthermore, the higher thickness of the sample should result in the higher absorbance and that is why the absorbance is directly proportional to the concentration and absorbance is directly proportional to the length. And that is why absorbance is going to be called as absorbance is epsilon CL and that is why epsilon is the molar absorptivity.

So, the equality showing linear relationship between the absorbance and the concentration of the absorbing molecule and that is known as the Beer Lambert law or the Beers law. Transmittance is another way of describing the absorption of light. Transmittance is simply the ratio of intensity of radiation transmitted through the sample to the incident radiation.

It is clear from the definition of the absorbance and transmittance that both are dimensionless quantities ok. So, absorbance and transmittance are both represented in a arbitrary unit or AU unit ok. So, what will happen is that if you are in illuminating a sample with the I 0 intensity and suppose this is the total surface area of the absorbing unit which has the thickness of dx and it has a surface area of this epsilon.

Then it is actually going to absorb the material according to this area and volume ok. And the path length is L then the number of molecules per centimeter is going to be given by the molecules by the centimeter cube. And that number of molecule is actually going to decide what how much the sample is going to absorb or how much less it is actually going to transmit.

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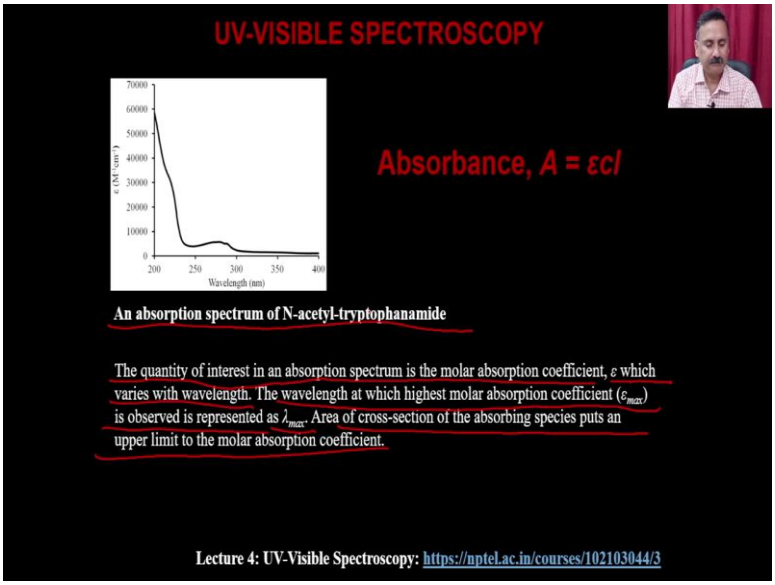

Now, take a simple examples right; so, an absorption spectra of N-acetyl tryptophanamide. So, what you will see here is the wavelength versus the absorbance right and the quantity of interest in an absorption spectra is the molar absorption coefficient epsilon which varies from the wavelength ok.

So, the wavelength at which the highest molar absorption coefficient is called as the epsilon maxima is observed in a representation as represented as lambda max. The area of cross section of the absorbing species put an upper limit to the molar absorption coefficients.

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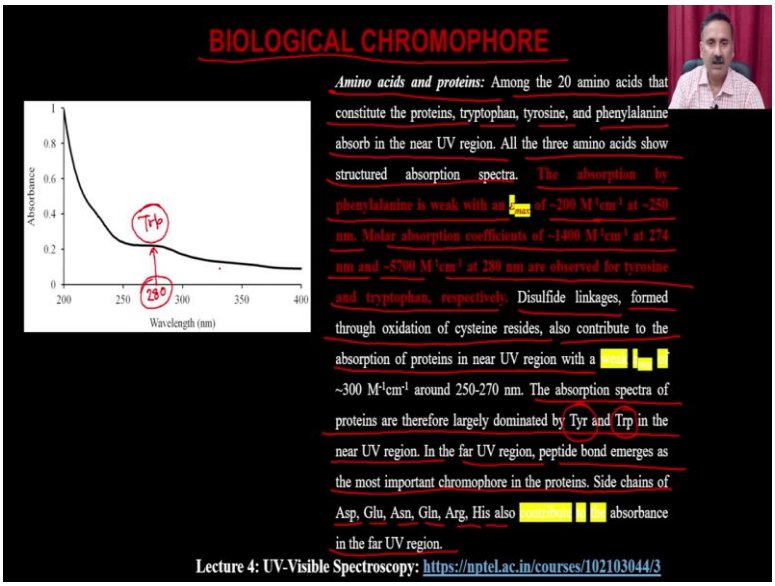

Then the UV visible spectroscopy can be used for mapping the different types of biological molecules, because most of the biological molecules are having the chromophores; for example, in the amino acids and the proteins. So, among the 20 amino acid that constitute the protein the tryptophan, tyrosine and phenylalanine absorbed in the UV range; whereas, all the three amino acids show the structure absorbance spectra.

The absorbance by the phenylalanine is V with an epsilon maxima of 200 per mole per centimeter at 250 nanometer. Whereas, the molar absorption coefficient of 1,400 is at 274 nanometer and 570 at 280 nanometer are observed for tyrosine and tryptophan respectively. Disulfide linkages formed through the oxidation of cysteine residue also contribute to the absorption of protein in the near UV range ok.

The absorption spectra of protein are thereby largely dominated by the tryptophan and as well as the tyrosine in the near UV range. And in the far UV range the peptide bond emerges as the most important chromophore in the protein. Side chain of the Aspartate, Glutamate, Asparagine, Glutamine, Arginine, Histidine are also absorbing in the far UV range.

So, what you will see here is that this is another (Refer Time: 30:32) spectra of protein and it actually shows a very intense peak at 280 nanometer ok. And this 280 nanometer is a summation of the different types of chromophore are present in this mostly in the tryptophan which is actually going to absorb at 280 nanometer and tryptophan. And

tyrosine are actually the predominant chromophore what are present in the protein molecules.

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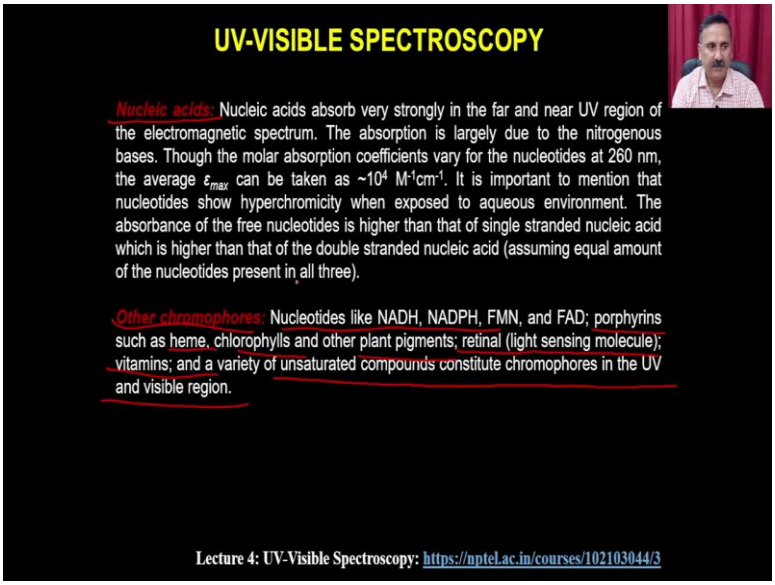

Apart from that you can also have the chromophore in the nucleic acids, you can also have the chromophore in the other biological molecules like the nucleotides. And you can have the porphyrins such as heme, chlorophylls and other plant pigments. Then we have a retinal which is a light sensitive molecule vitamins and variety of unsaturated compound which are having the chromophore in the UV and visible range.

And technically the UV visible spectroscopy can be done in two mode; one is called as the absorption mode and the other is called as the emission mode ok; so, other is called as the difference spectroscopy.

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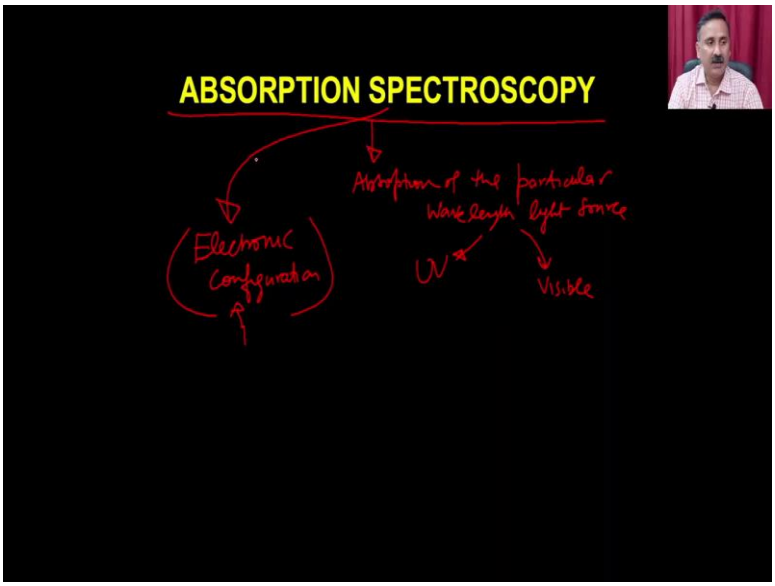

So, in an absorption mode what you are going to do is you are going to study the absorption of the particular wavelength ok particular wavelength light source. It can be in the UV range or it can be in the visible range which means it can be in the UV range. So, and as I said you know the absorption spectroscopy comes because it is actually going to be work on the electronic configuration of the molecule right.

So, if the electronic configuration is getting disturbed ok or if there will be any perturbation into the electronic configuration it is actually going to change how easily or how difficult our electron has is actually going to receive the energy from the system. And then it will actually go on to the higher energy state and from there it actually comes back right. And that is actually going to be variable from the one wavelength to another wavelength right.

Because most of these electrons are under the you know when they are rotating over when they are moving into their into the shell, they are associated with their specific amount of energy. So, when you are actually eliminating the object that it actually going to you know there will be a resonance between the between the energy of that particular electron and the energy what you are supplying from outside.

So, if there will be a resonance between these two then it is actually going to absorb maximally and that is how it is actually going to give you a specific and characteristic wavelength a characteristic absorption spectra.

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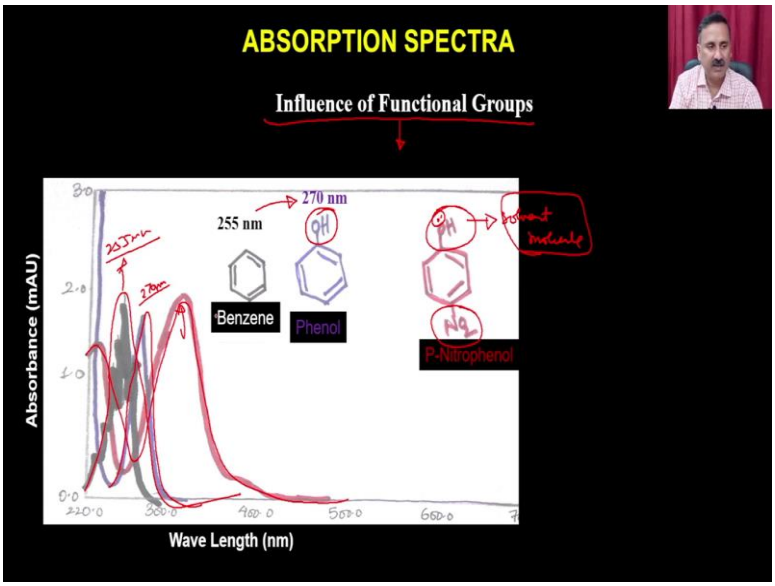

Let us see some of the examples; so, let us see how that functional group is actually going to decide, how the absorption is going to work. So, for example, I have taken three molecules one is benzene another one is that I have added the OH onto the benzene and then third one is the para nitrophenol; so, I have added the NO 2 on the front.

So, this one has no functional group, this one has one functional group, and this one has the two functional group and let us see. So, this is the benzene; so, this is the benzene spectra right; so, this actually goes like this right and it goes and give you a wavelength of 250 nanometer. So, at 250 nanometer it is actually going to absorb maximally, because at that particular wavelength the electrons are receiving the maximum amount of energy and that is how they are going into the high energy state.

And then when you added the OH right, you are actually shifting the wavelength and that is how it is actually absorbing like this. It is actually going to absorb slightly more right; so, it is actually absorbing a 270 nanometer. And then when you are putting the NO2 and OH it is further shifted right, it is further shifted towards this side and that is how it is actually. Because, you see now you have added the functional group and these functional groups are interacting with the solvent molecules.

And because of these interactions the electronic configuration or the electrons which are freely been available are you know which actually can go into the high energy state is

restricted. Because they are actually interacting with the solvent molecule and that is how these electrons are not seen for the moving into the high energy state.

And that actually goes up and that is how you are actually going to require higher energy wavelength. And that is how it is actually going to give you the you know the lambda max at a slightly more on more wavelength.

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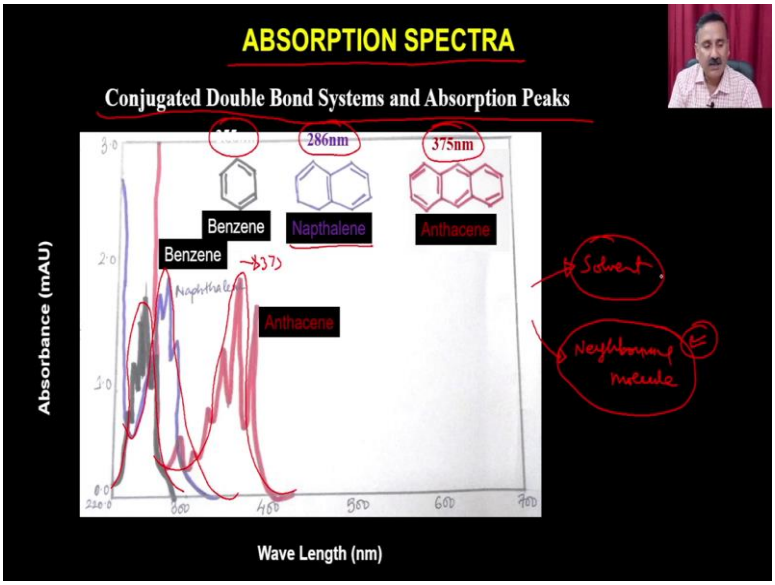

Then you can see the another system we have the conjugated double bond system and an absorption peak. So, in this case I have taken a 3 example one is benzene which is absorbing a 255 nanometer, then we have naphthalene; so, here you have one benzene ring, you have two benzene ring, here you have three benzene ring right. So, it is absorbing a 286 nanometer and this one is absorbing a 375 nanometer.

So, what you see here is this is the benzene spectra right and benzene is absorbing a 255 nanometer. And then when you are putting two benzene ring, it is shifting towards this side and that is how it is actually absorbing at ah. So, this is a naphthalene it is absorbing a 286 nanometer and then you also have the anthracene which is actually three benzene ring. So, that is actually absorbing at even more on this sides and that is how it is absorbing a 375 nanometer.

So, absorption spectra is very sensitive for two things, one it is actually very sensitive for the solvent molecule and it also very sensitive for the neighbouring molecules ok. So, it

which means you can be able to use the absorption spectra to measure or to see how it is actually going to affect the enzyme structure.

So, if you take the Bessel level of enzyme structure or Bessel level of enzyme absorption spectra it will actually going to show you one spectra. And then when you add a substrate and if the substrate is actually going to interfere into the electronic configuration of the some of the key residues or some of the key molecules.

Or if it is actually going to experience any kind of alteration in the solvent content, then it is actually going to show you the deviation in the absorption spectra; let us take an example of this.

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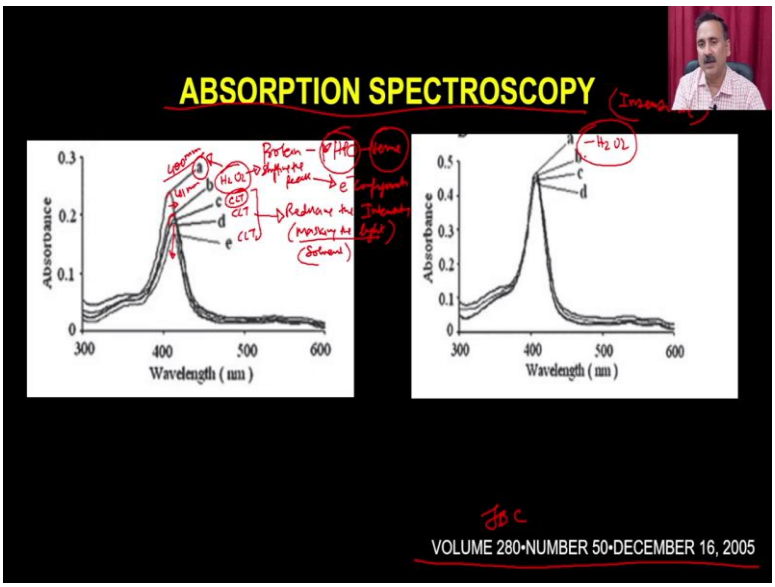

So, we have taken this examples and this I have taken from one of the paper ok from JBC right. So, what you see here is that here I have taken a protein ok which is called HPO right; so, it is called hemoparasites. And then what you and this is actually a protein which contains the heme as the functional group or heme as a cofactor.

So, what you see here is that this is actually the native enzyme; so, when you have the native enzyme which is actually going to show you that absorbance at 400 nanometer right. And when you add the H 2 O 2 ok; so, H 2 O 2 will go and bind with this particular enzyme in basically binds next to the heme more residues and that is how you will see that there will be a shift in the absorption peak.

So, it is actually shifts now at 411 nanometer ok and when it is shifting and then you are adding the different types of substrate or different types of inhibitors. So, for example, in this case I have added the inhibitor like Clotrimazole and what happened is that as soon as it shifts you add the Clotrimazole.

And then Clotrimazole actually goes and bind and that is how it is now reducing the absorbance, this means Clotrimazole basically this one is shifting the peak right. So, it is shifting the peak which means it is actually changing the electronic configuration of some of the free residues right. And that is how it is actually shifting the peak; whereas, the Clotrimazole is not shifting the peak it is reducing the intensity right; so, it is reducing the intensity right.

So, it actually goes down like this right this means it is actually masking the sample or masking the light ok. This means it is actually not allowing the molecule to absorb the light very strongly, this means it is actually protecting the system ok and this is actually being done always by the solvent molecules ok.

So, because the inhibitor is in the vicinity of the enzyme it is actually behaving just like a solvent and it is not allowing the enzyme to absorb or enzyme this the functional groups present at the active side to absorb, because it is you know having the kind of a curtain outside.

This is the same data what I have shown where I have removed the hydrogen peroxide. So, when you do not have a hydrogen peroxide this kind of binding is going to be compromised. Now, what is the major issue with the absorption spectroscopy is that it is actually going to show you a alteration and it is actually going to show you a alteration because of the interaction of the substrate.

But the absorption spectroscopy is very very insensitive compared to the difference spectroscopy ok. And that is why mostly people are not using the absorption spectroscopy for studying the interaction of the substrate with the protein enzyme molecules mostly people are using the difference spectroscopy.

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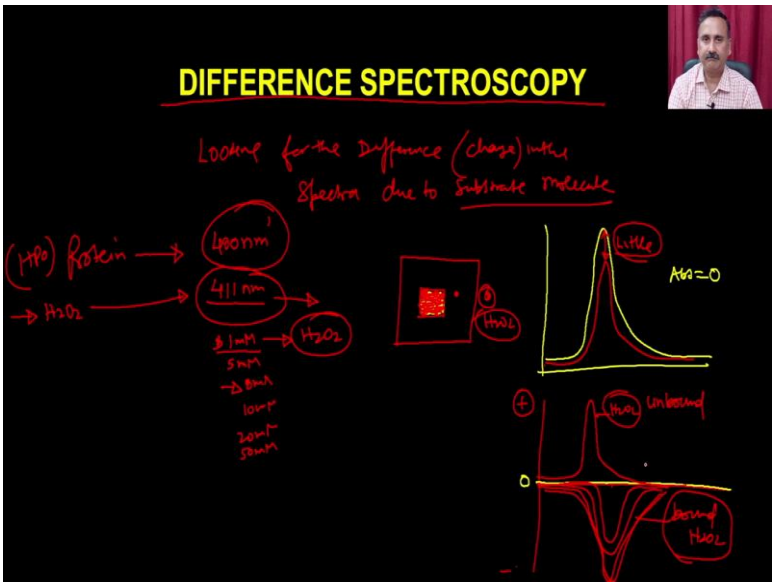

So, what is mean by the difference spectroscopy? Difference spectroscopy means that you are looking for a difference right. So, you are looking for the difference or you are going to say that the change in the spectra due to substrate molecule right. And so, for example, if I have a protein ok; and for example, HPO right we have taken an example right and it absorbs at 400 nanometer right.

Now, if I add a substrate for example, if I add the H 2 O 2 right what will happen is that H 2 O 2 is going to take up some of the intensity from hydrogen peroxide from the protein right and that is why it is actually going to absorb at 400 nanometer elements right. So, now the energy what is going into the protein will actually go into the complex.

And as a result, it is actually going to show you a difference in the spectra. And if you understand this more in a dramatic way; so, imagine that this is the protein right. And this is the portion of the protein which is responsible for binding of the substrate right. Now, if I fill this space ok if I suppose I fill this space with a yellow dye ok; so, this you can imagine that this is the area which is actually being responsible for the giving you the absorption spectra.

So, what will happen is that it is actually going to show you an absorption spectra like this and because of this it is actually having the absorption spectra right. Now, if I have added the hydrogen peroxide right; so, what will happen is that at low concentration of

hydrogen peroxide for example, if I add like 1 millimolar. For example, if I add 1 millimolar it actually going to give you some amount of molecules.

So, hydrogen peroxide molecules will go and build bind right, suppose they have captured this much area right. So, if they have captured this much area of the active side, this means they have reduces the area of protein to be absorbed under this right. So, as a result what will happen is that it is actually going to show you a change in absorption.

Now, this change what you see here is actually this is I am showing in absorption spectra right, this is absorption spectra. But this change is very subtle ok this change is very little to be mapped in the absorption spectra. Instead, if you do the different spectroscopy; so, what you can do is you can just make this routine spectra as the zero spectra.

So, in that case what I will do is I will take the routine spectra; so, I will what I will do is I will take the protein spectra right. So, I will make the protein spectra as absorption 0 ok; so, what will happen is that this is 0 right; so, protein will show me an absorption like this ok. Now, if I add the hydrogen peroxide ok; so, what will happen is that it is actually going to withdraw some of the substrate right some of the these in molecules right.

So, it is actually going to go like this; so, this much is now been amplified ok, because it is showing the more into the dip ok. And this is side this side is minus side this side is a positive side; so, this is a positive signal this is a negative signal ok. Now if I add some more like for example, if I make it 5 millimolar right, then it will go further down.

How long it will go? Because it is actually going to now capture some more amount of active side; so, if I do third time it is going to capture more. So, it is going to go like this it is going to keep capturing until this whole chromophoric side is going to be captured. So, for example, if I do another injection, another range like 8 and a millimolar 10 millimolar, 20 millimolar like that ok.

So, it will be keep going keep going like this and ultimately it is actually going to get saturate and it will going to stop going right. And that is very very interesting, because once you go beyond this for example, if you add 50 millimolar ok. So, 50 millimolar will actually go will and show you a separate molecule; so, ok and in that case, you will see a absorbance of the hydrogen peroxide.

So, this is a hydrogen peroxide molecules absorbance; whereas, this is the withdrawal or this is the subtraction of the absorbance because of the hydrogen peroxide binding into the enzyme. So, once the value will go beyond this binding side it will actually go and be present as a substrate molecule.

So, this is actually going to be present as a substrate molecule and that is why it is actually going instead of showing you difference spectroscopy, it will actually going to start showing you absorption spectroscopy. So, this is actually unbound hydrogen peroxide; whereas, this is a bound hydrogen peroxide and that is very very robust and important tool to be exploit in the difference spectroscopy.

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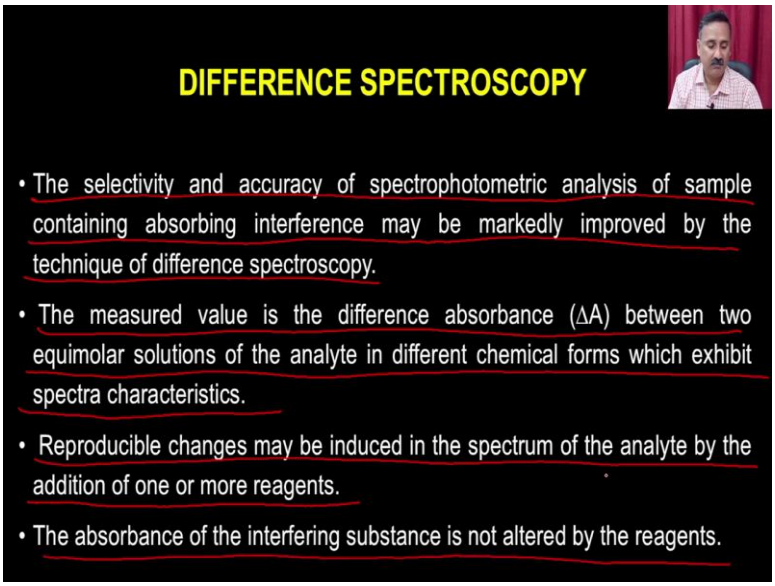

So, the selectivity and the accuracy of spectrophotometric analysis of sample containing absorbing interference may be markedly improved by the technique of difference spectroscopy. The measured value is the difference absorbance between the two equimolar solution of the analyte in different chemical forms which exhibit spectra characteristic.

Reproducible changes may be induced into the spectra of analyte by the addition of one or more reagents. The absorbance of the interfering substance is not altered by the reagents.

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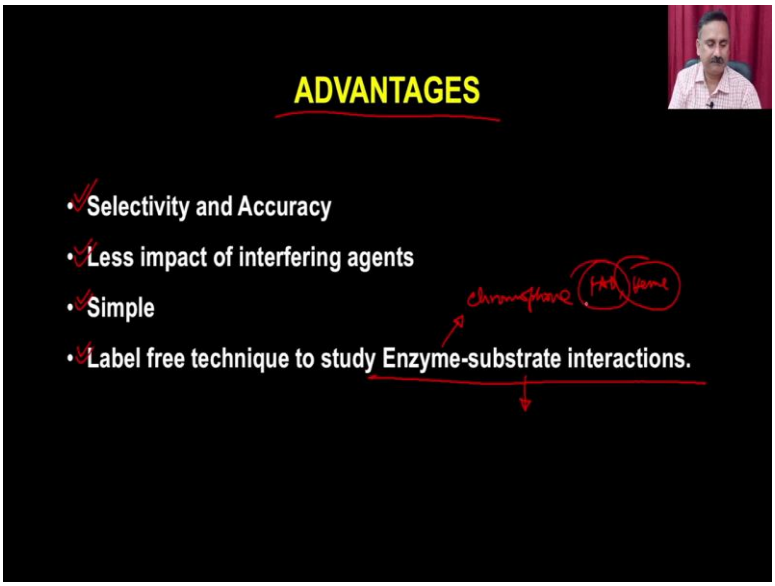

What are the different advantages? Advantages is that it is selective and accurate, it is having the less impact of the interfering agents. Then it also have a simple and this is one of the very cheap and reliable label free technique to study the enzyme substrate interactions. The only condition is that this substrate will go and interact with the enzyme and the enzyme should actually if it is always being easy if the enzyme has some chromophore.

Which means if it has some you know colored cofactor present; for example, fad or heme then it is easy because then you can actually be able to monitor this.

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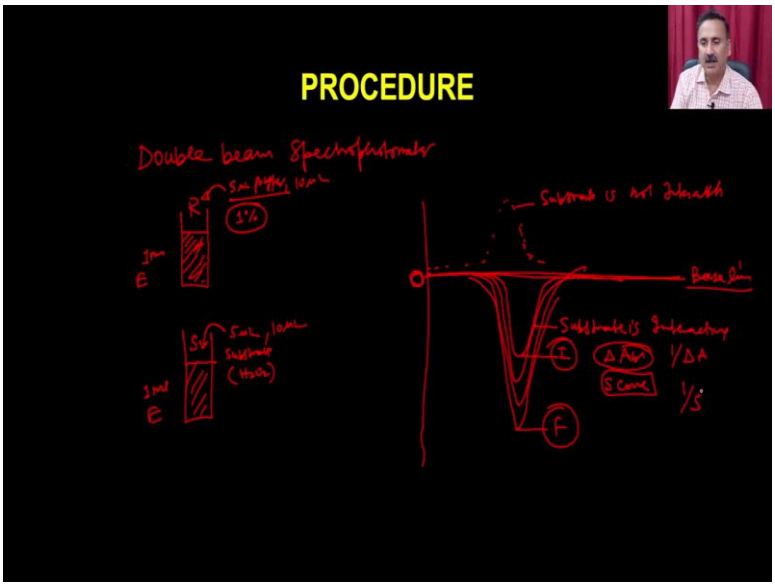

How we are going to do this? We are what we are going to do is we are going to take a double beam spectrophotometer right and in a double beam spectrophotometer you are going to prepare the sample ok. So, in a double beam spectrophotometer you are going to have two qubits right; so, this is going to be a reference qubit this is going to be a sample qubit.

Now both are actually going to have the one ml of your protein sample ok; so, one ml of protein sample of same amount ok. So, for example, you fill the both the qubits with one ml of enzyme solution one ml of enzyme ok. Now, in the reference qubit you are going to add for example, 5 microlitre of buffer ok, and in the sample qubit you are actually going to add the 5 microliter of your substrate; for example, in this case we are doing it with the H 2 O 2 ok.

So, what will happen is and then when you are actually being done with the 1 ml of enzyme what you are going to do is you are going to collect one spectra, ok. So, it is actually going to show you a single line ok, because the value because the sample and as well as the reference qubit are actually going to have the same amount of proteins right.

So, it is actually going to show you a straight line with a 0 absorbance ok. Now, when you add the 5 microliter of buffer into the reference qubit and 5 microliter of substrate into the sample qubit, it will if the substrate is interacting with the enzyme, it will actually going to show you a dip.

If the substrate is interacting; so, if the substrate is interacting it is actually going to you know mask some of the chromophores and that is why it is actually going to show you the dip in the spectra. If the substrate is not interacting, then it is actually going to show you a an increase in spectra right. So, this is actually going to be when the substrate is not interacting; so, this you have to continue after 5 microliter you can add 10 microliter.

So, here also you have to add 10 microliter, the only condition is that your dilution should not go beyond 1 percent ok. This means you can actually be able to do not do like a lot of dilution otherwise you will see this dip and your baseline is also going to do right; so, this is actually called as baseline ok the zero absorbance.

And then when you keep increasing it is actually keep going like this right and it will keep going like this and ultimately it is actually going to stop ok. So, then you what you are going to do is you are going to show this is the initial and this is the final right. So, this is the final spectra, this is the initial spectra and what you are going to do is you are going to start calculating the delta absorbance right, how much absorbance it is lost right and at what considerations.

So, you are going to have the substrate concentration and you are going to have the delta absorbance ok. And then what you are going to do is you are going to calculate 1 upon delta absorbance and 1 upon substrate ok and that is what you are going to do in the next step.

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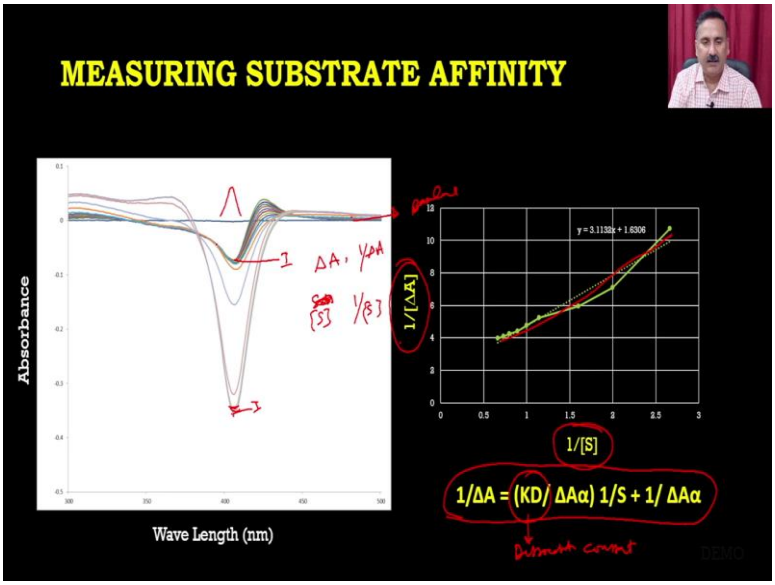

So, once you collected this data the data will look like something like this ok. So, this is actually going to be a baseline; so, this is the baseline. And this is actually the final data this is the final curve and this is your initial curve ok. And from each curve you can be able to calculate the delta A which means how much is the dip of the absorbance and then you can calculate the 1 by delta A.

And similarly, you can add the substrate calculation; so, you can calculate the substrate computation. And you can calculate the 1 by substrate calculation and then you can just make a plot between 1 by delta A and 1 by delta S ok and that is actually going to give you a linear curve ok.

And then taking this into account you can be able to use this formula ok and you can be able to calculate the KD which means you can actually be able to calculate the dissociation constant ok. Because after this if you if; so, for example, you see the all the substrate all the spectra that coming here if you add more, it will actually going to show you more ok and this is what we are going to show you, this is another data right.

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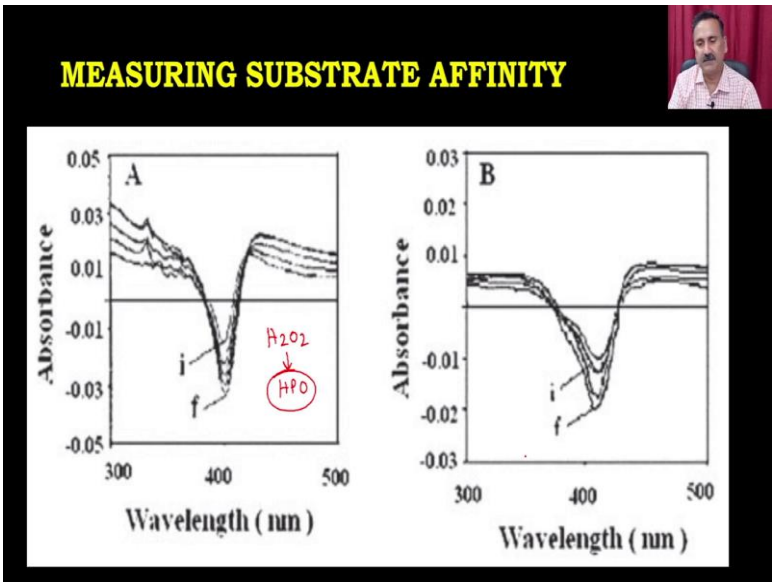

So, this is the H 2 O 2 binding of the protein into the hemoprotein ok; so, this is the data what you are going to see when you are going to do the H 2 O 2 binding to a real protein; so, these are the real samples. So, we have prepared a small demo clip just to explain you each and every of these steps how you can be able to perform the different spectroscopy.

And how that can be how you can do the calculations and other things and I hope that this demo clip will actually help you to perform these experiments in your laboratory.

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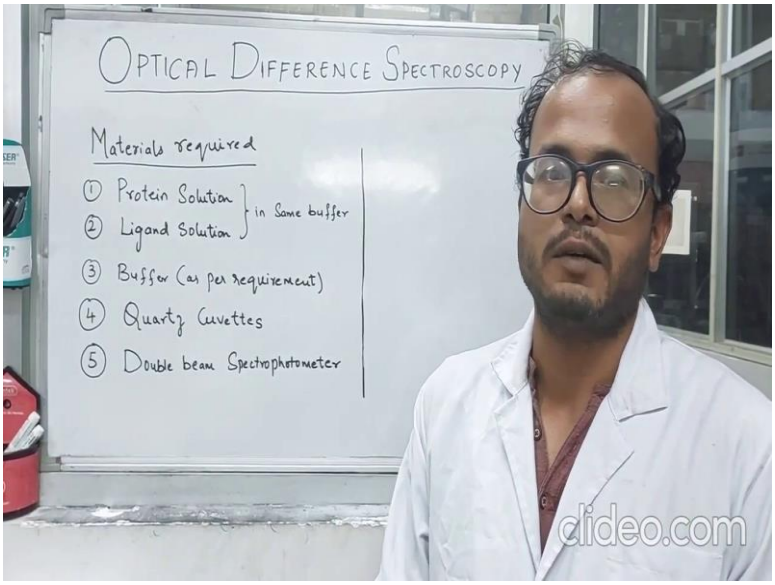

Hello everyone, I am Alok Kumar Pandey, a PhD scholar and under Professor Vishal Trivedi in Department of Bioscience and Bioengineering Engineering IIT, Guwahati. In this video I will be performing a demo on the optical difference spectroscopy experiment. So, for this experiment we have taken a protein and ligand as example and we will use this experiment to find out the KD value of this ligand binding to the protein.

So, when ligand binds to the protein it creates a difference in the characteristics absorbance of that protein and that difference we will exploit using a formula to calculate the KD value of this ligand binding to this protein. So, for this experiment the materials which we required require are a protein solution and a ligand solution of known concentrations in the same buffer the buffer and two quartz cuvettes and the double-beam spectrophotometer.

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So, now we will start the experiment these are the two cuvettes both contain the protein solutions; now, we will put them in the spectrophotometer.

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So, one cuvette will put in the sample cell and the other one we will put in the reference cell and after this we will scan this to get a baseline.

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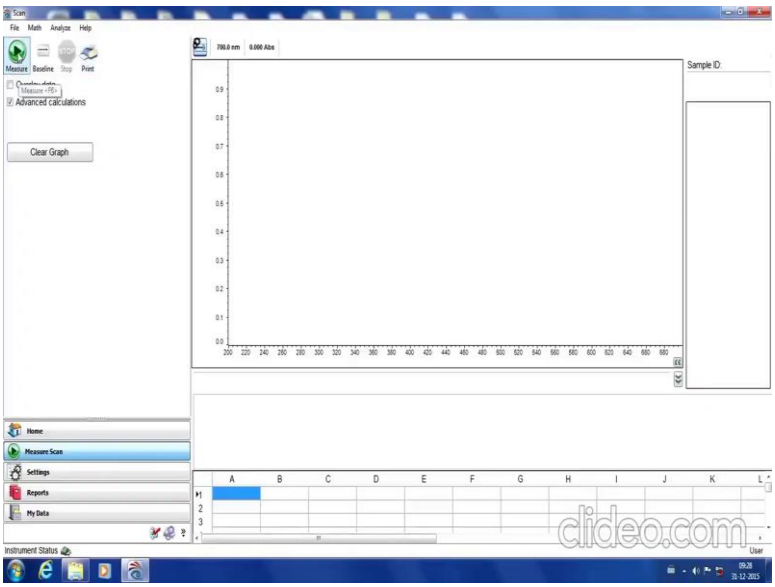

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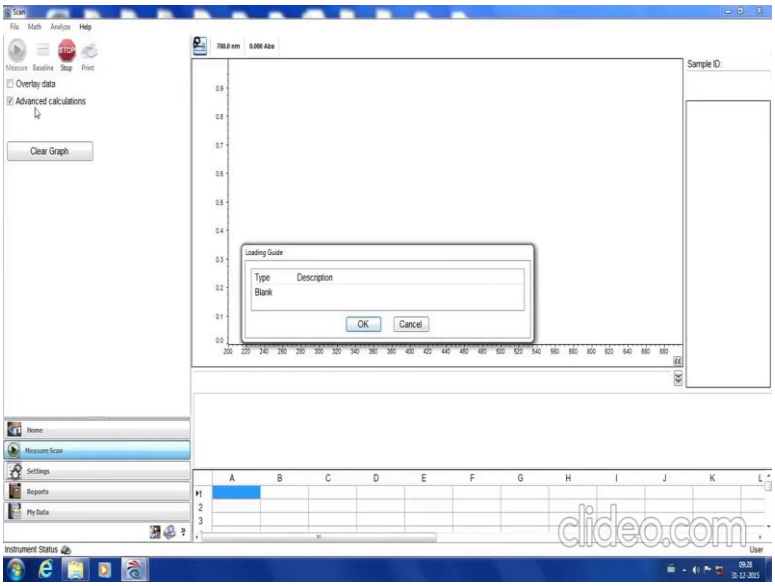

So, here we will click on measure to find out the baseline and this sample is a blank sample; so, we can name it as blank. And now it is taking the baseline nothing is visible because it is just a blank sample; so, it will not show any line. But after we obtain the baseline, we will add our ligand to the protein in the sample cell.

And we will observe any dip in the peak at around 400 nanometer which is characteristics for this protein and constantly we will add increasing concentrations of the ligand the ligand.

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So, this is the ligand solution, we will take 2.5 microliter from this.

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And then we will add this 2.5 microliter in the sample cuvette and the same amount of buffer we will add in the reference cuvette to neutralize the dilution caused by adding that ligand.

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So, same amount of buffer we will add in the reference cell and after adding this again we will scan this.

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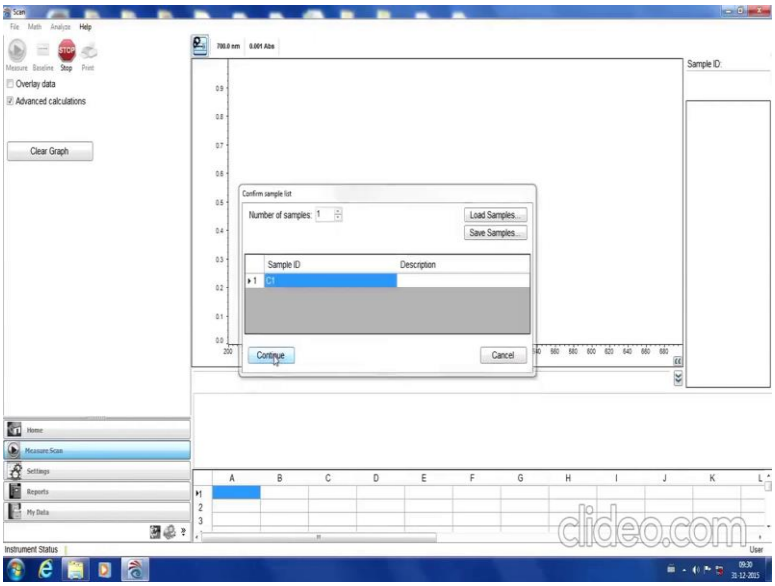

So, now after adding the ligand we will measure its spectrum we can name this as C 1 concentration 1.

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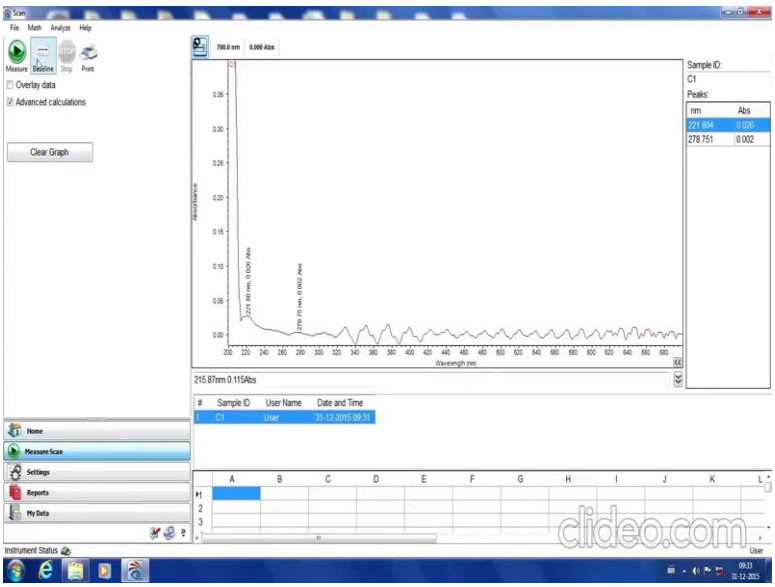

And this is the spectrum it is given for the for this concentration of ligand; so, we will we are expecting a dip in the peak, but the concentration is less. So, we will get a less dip or we may not observe in the smaller concentrations, then we will add further increasing concentrations of the ligand.

So, here we can see around 400 we are not getting any significant dip in the peak; so, we will add another concentrations in the same way as I have shown and we will.

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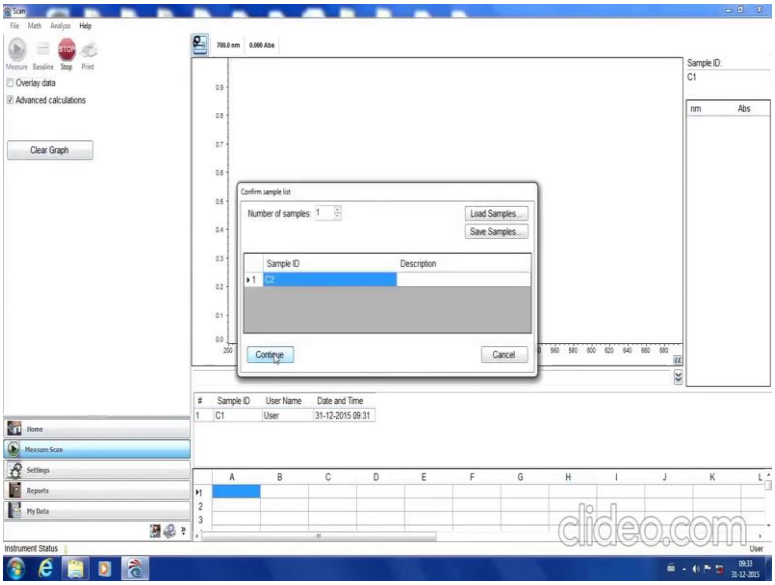

So, now, I have added the ligand 2.5 microliter of the ligand more and we will take the spectrum with the next concentration as C 2.

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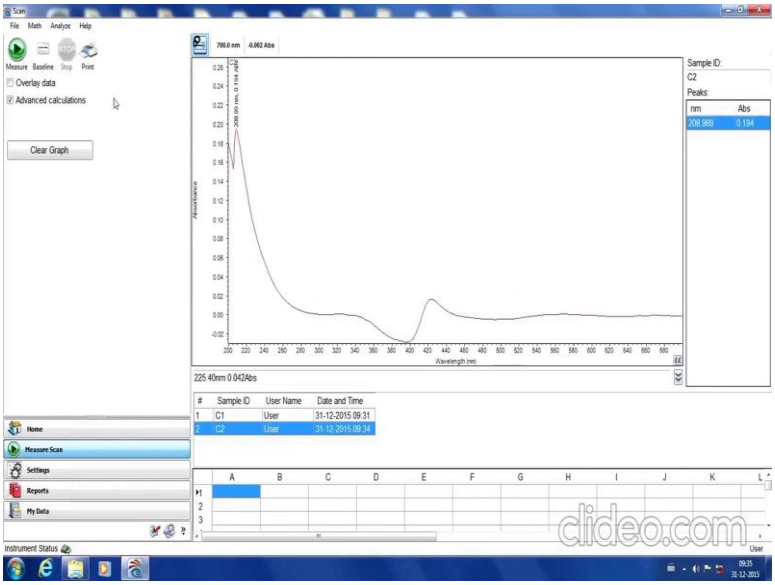

We will level this concentration as C 2 and this is the spectrum for the for this concentration of the ligand, again same we will be expecting a peak at around 400. See this at this concentration we got a nice dip in the peak at around 400 nanometer. So; now,

we can see we will use few more concentrations of the ligand and watch the spectrum and dip in the peak.

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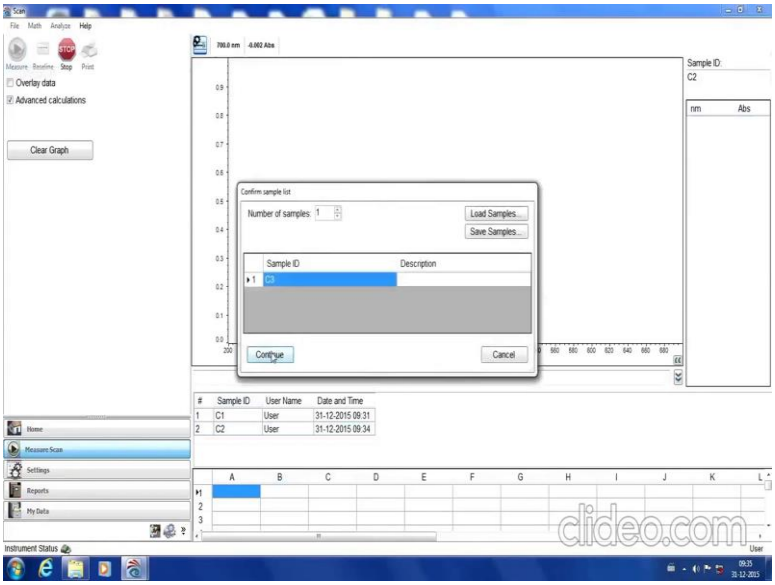

So, now, we will take the spectrum for next concentration, we will level this concentration at C 3 and measure the spectrum.

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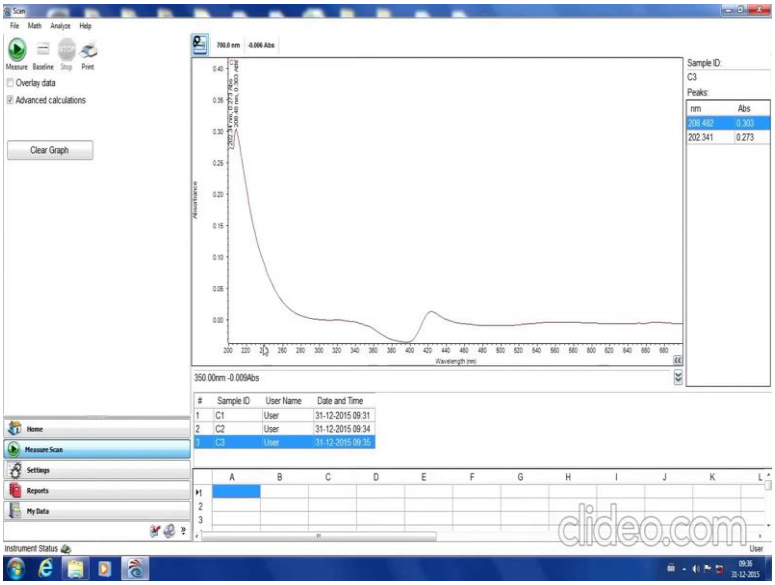

Again, we will we will see the a dip in the peak which will be more than it was on the previous concentrations. And similarly, we will keep increasing the concentration of the ligand until we see that there is not a significant difference between the dips of the peak.

And then after that we will use this difference in the absorbance to calculate the KD value of this ligand binding to this protein.

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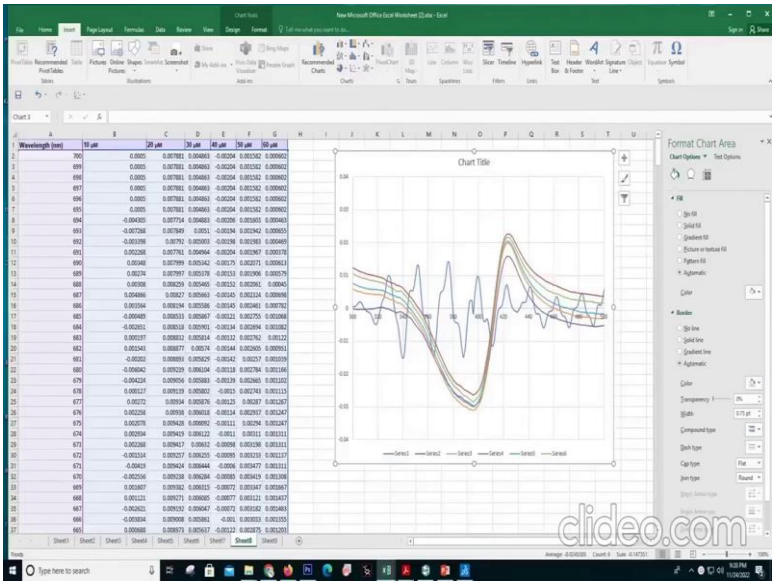

So, now we will come to the calculation part of the experiment, this is the data which we have obtained from the instrument. And these are the absorbance values at different ligand concentrations, the ligand concentrations are calculated using the volume of the ligand which was added from the stock solution of the ligand. And on the x-axis these are the wavelengths at which we took the spectrum.

So, a graph we can plot a graph between wavelength and absorbance, here we can see in the graph that at the first ligand concentration that is 10 micromolar there is not much dip in the peak. But after that at 20, 30, 40, 50 and 60 micromolar we have got significant peak and the change in the peak is in the peak values is not too much; that means, it is saturated and we can use this these values to calculate the KD value.

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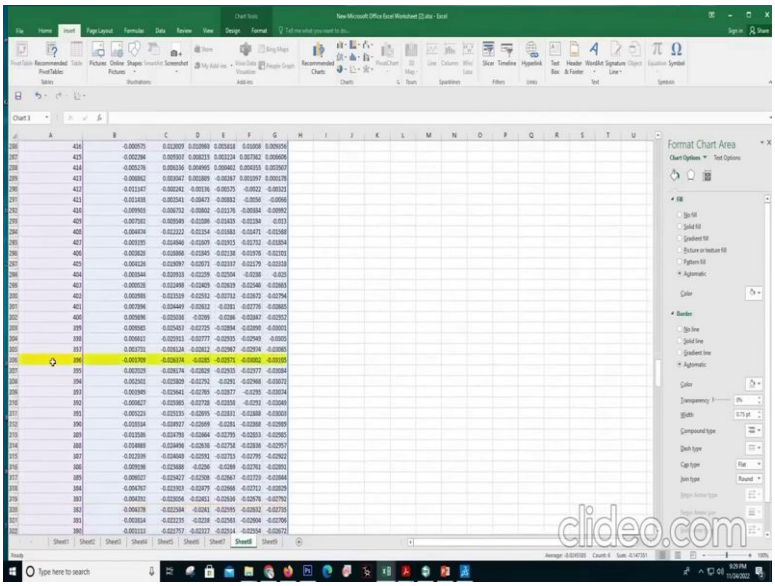

Also, in the data we can find out the wavelength at which the peak is there. So, if we see it is the we have got the peak at 396 nanometer as this is the these are the most negative values. So, now we are going to use this absorbance values at 396 nanometer to calculate the KD value.

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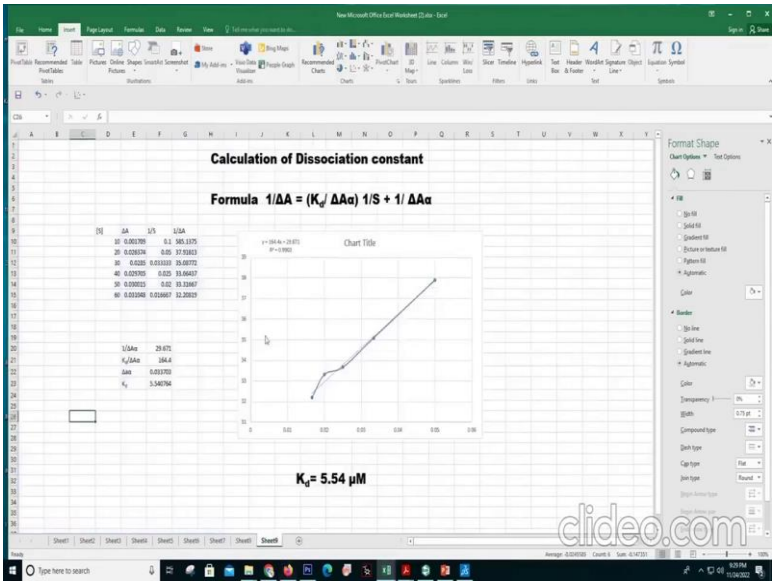

So, to calculate the KD value the formula is 1 divided by delta A is equals to KD divided by delta A alpha into 1 by S plus 1 divided by delta A alpha. Here delta A is the absorbance difference which we have got in the experiment and S is the concentration of

the ligand. So, this equation this formula is a straight line equat ion like y equals to m x plus C, C where y is 1 by delta A and x is 1 by S.

So, now from the data which we have got we have taken this these are the ligand concentrations this is the delta a value at 396 nanometer and from this we can calculate 1 by delta S and after that we can calculate 1 by delta A. So, now, we can plot a graph between 1 by S and 1 by delta A 1 by S on x axis and 1 by delta A on y axis.

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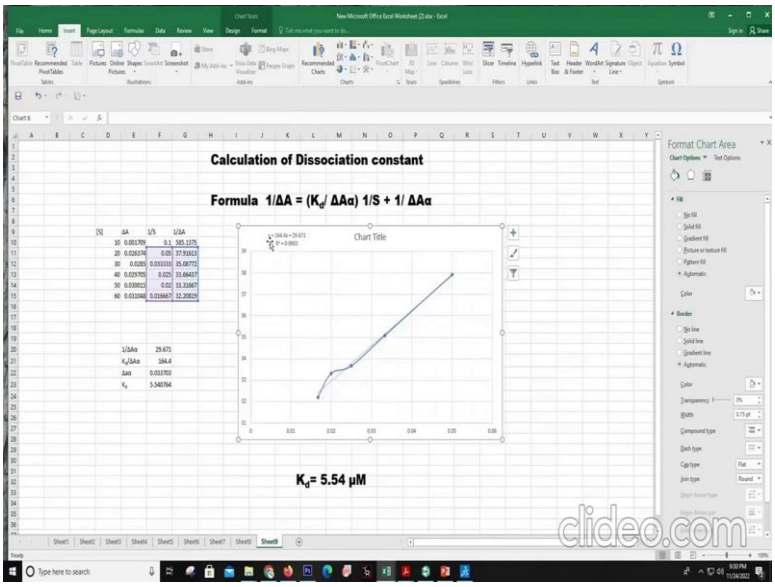

So, here is the graph we can see we have got a straight line and the equation for this line is y equals to 164.4 x plus 29.671. So, the intercept of this line is 29.671 which will be equal to 1 divided by delta A alpha. Here we can see 1 divided by delta alpha alpha is 29.671 and the slope of this line is 164.4 which is will be equal to KD divided by delta A alpha.

So, KD divided by delta delta a alpha is 164.4; so, from this 1 by delta A alpha value we can calculate the value of delta A alpha. So, delta A alpha is equals to 1 divided by this value which comes to be 0.0337. And now the KD value if we see the slope KD divided by delta A alpha is equals to 164.4, here we can put the value of delta A alpha which we have calculated and then find out the KD value; so, here the KD value comes to be 5.54.

So, the KD value for this ligand binding to this protein is 5.54 micro molar. So, this is this is how we can use optical difference spectroscopy to calculate the KD value of a

ligand binding to a protein. It can also be used for other interactions also, but in this video, we have taken a protein and a ligand interaction as examples, thank you.

So, this is all about the potential of spectroscopy in measuring the or mapping the enzyme substrate interactions. So, far what we have discussed? We have discussed about the electrophoretic techniques and how you can be able to use the electrophoretic techniques to map the enzyme substrate interactions. The only condition is that the substrate should be proteinaceous in nature and it should give you a significant change in the molecular weight.

And subsequent to that we have also discussed about the spectroscopic techniques and we have discussed about the absorption spectroscopy as well as the different spectroscopy, how you can be able to use that for measuring the substrate interaction studies.

So, in a subsequent lecture we are going to discuss some more techniques; so, that you can be able to use them for studying the substrate enzyme substrate interactions; so, with this I would like to conclude my lecture here.

Thank you.