Essentials of Biomolecules: Nucleic Acids, Peptides and Carbohydrates
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Lecture-28
Modern Techniques For Biomolecules and Disease Diagnosis

Hello, everybody, and welcome back to the lectures. So today we will start a new module that is module 8 on modern techniques for biomolecules and disease diagnosis. So, in this module we will cover what are the useful technologies that we use nowadays for biological macromolecules as well as some organic molecules starting with the spectroscopy. So, to study various properties, functions of the biomolecules which can reconsider as organic molecules as well, only thing is that they are longer.

We use a lot of spectroscopic techniques such as very commonly used, UV visible spectroscopy, florescent spectroscopy, mass spectrometry, NMR sometimes, infrared spectroscopy, these are the characterization techniques along with other kinds of purification techniques such as HPLC, and all these things. So, plus some molecular probes in order to diagnosis a disease. So, we will cover some of these technological aspects in this module.

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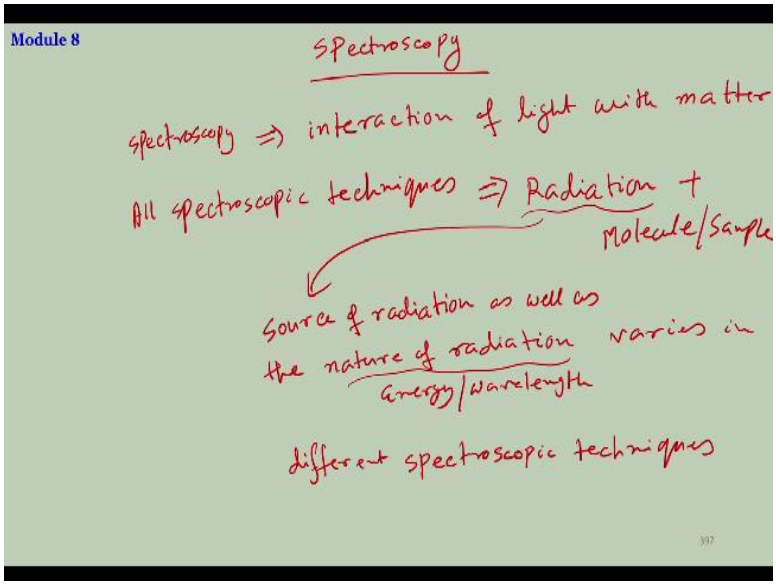

To start with spectroscopy because that is one of the most used equipment or one of the most use techniques, there are several spectroscopic techniques used in chemistry, biology and so on. So,

spectroscopy, I will first give you the very brief basic introduction, and then we will move on to the differences and how they can be applied in studying biological macromolecules.

So, the basic idea of spectroscopy or the basic principle of spectroscopy is that where given a radiation interacts with a matter. So, if you want to define spectroscopy, spectroscopy is the means interaction of light with matter, that is the basic definition of spectroscopy that we study the interaction of light or radiation with matter. Now, various techniques that are developed based on spectroscopy are so basically all spectroscopic techniques have the common feature.

That it has a source of radiation and there is a sample of your interest sample or I call it molecule. So, radiation plus molecule or sample, these are the 2 units you can say that is present in all spectroscopic techniques, radiation can vary. So, the various kinds of spectroscopic techniques that you know of today, they actually mostly vary in terms of the radiation. So, source of radiation or light as well as the nature of radiation.

Nature of radiation means here I am specifying these 2 it is wavelength or to its energy, which energy range this particular radiation is supplied. And that varies from one spectroscopic technique to the other spectroscopic technique. So, source of radiation varies as well as the nature of the radiation or the energy you can call it energy, you can call it wavelength. The range of energy or the range of wavelength that will varies in different spectroscopic techniques.

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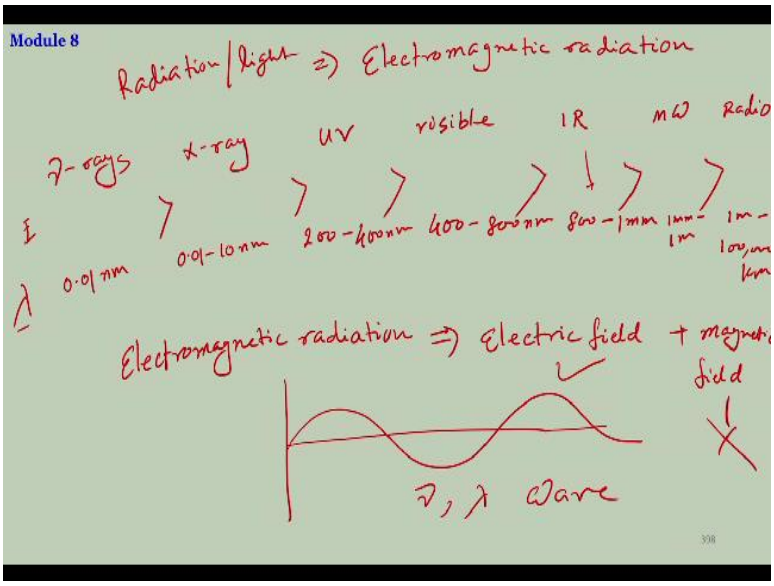

When we call a radiation or light, we mean electromagnetic radiation. All radiations are basically electromagnetic radiation. And if you want to see the range of different radiations in terms of energy. So, if you write down the electromagnetic radiations from the high energy to the low energy it will look like this, first will come gamma rays, then we will come x-ray with a very high energy, then comes the ultraviolet UV, then comes visible.

Then comes the infrared IR, then would be the microwave and then the radio. So, if you calculate the energy, this is the average has the highest energy, then comes x-ray, then comes UV, then comes the visible IR microwave. So, in decreasing order here, this has the highest energy, the radio frequency or the radio radiation has the lowest energy. In terms of wavelength, lambda, if you see an x-ray has 0.01 nanometer range, very small wavelength.

X-ray has wavelength of 0.01 to 10 nanometer. UV goes typically from 200 to 400 nanometers, visible light goes 400 to 700 or 800 nanometers actually 700 nanometers 800 is almost in the IR region and then the IR infrared is 800 up to 1 millimeter wavelength this IR, microwave would be 1 millimeter to 1 meter is the microwave wavelength pretty large actually, and then comes the radio from 1 meter to 100,000 kilometers.

So, radio has a very large wavelength and wavelength range also is very, very large. So, this is about the energy distribution of the radiation or light. Now, when you are talking about

electromagnetic radiation, that basically means that radiation has high electric field plus it has a magnetic field. Usually the electric field and magnetic field are perpendicular to each other, if you look at their wave propagation.

Now, magnetic field, the magnitude of the magnetic field is actually very low is close to 0. The magnitude of the magnetic field, I am not writing the equations, there are equations involved. If you look at the energy of the magnetic field, you will see that magnitude of the magnetic field is very, very low, and it is almost negligible. That is why we usually do not consider the magnetic field, we will only consider the electric field.

So, basically our radiation or a light, which in general is known as electromagnetic radiation. When we talk about spectroscopy, we mostly consider only the electric field of that radiation. But if you consider electric field, any electric field has a sinusoidal wave that will propagate in this manner. And of course, that has a fixed frequency and that has a fixed lambda L, which means that wavelength. In other words, an electric field actually behaves as a wave. So, any radiation will have a way property.

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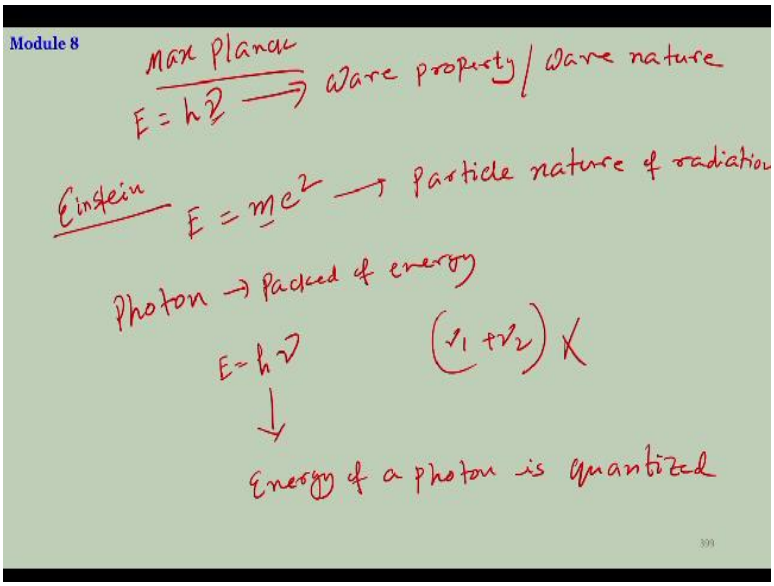

And this has been given by max Planck, according to max Planck the energy of radiation sorry wave would be equals to h nu, where nu is the frequency of the radiation, which obviously means that you are considering the radiation as a wave, otherwise you do not have the frequency

and the h is the Planck constant. So it is a constant quantity. So, according to max Planck, our radiation has a wave property or wave nature.

And later, according to Einstein, radiation can be considered as particle which means a matter and if you consider a matter that has a specific mass, if there is a particular will have a definite first. So, the energy of a radiation considering its particle nature should be equals m c squared, m is the mass of the particle, c is the velocity of light and this is considering the particle nature of electromagnetic radiation.

So, Planck has given the wave property to the radiation, Einstein has given a particle property to the radiation, be it the wave property, be it the particle property. One thing is very, very clear, that if you consider a wave nature, if you have a specific frequency, then energy is fixed. Similarly if you consider a particle which has a definite mass, then energy is constant, energy is fixed which means now this has been termed.

So, which basically means a particular radiation has a particular energy and that has been termed as photon. So, the idea of photon is photon or packed of energy which means it has a distinct energy and the idea is that you cannot break a photon, if you consider a photon has the energy h nu you cannot break the h nu, you cannot break the nu into nu 1 plus nu 2 no, it is quantized that is why the term is given the energy of a photon is quantized means fixed, you cannot break it.

And that is the basic of spectroscopy it is very, very important that whenever you are providing a light, you whenever you are providing a radiation, you have to always keep it in mind that the radiation has a particular energy and that energy you cannot break into pieces. So, why this is really that important.

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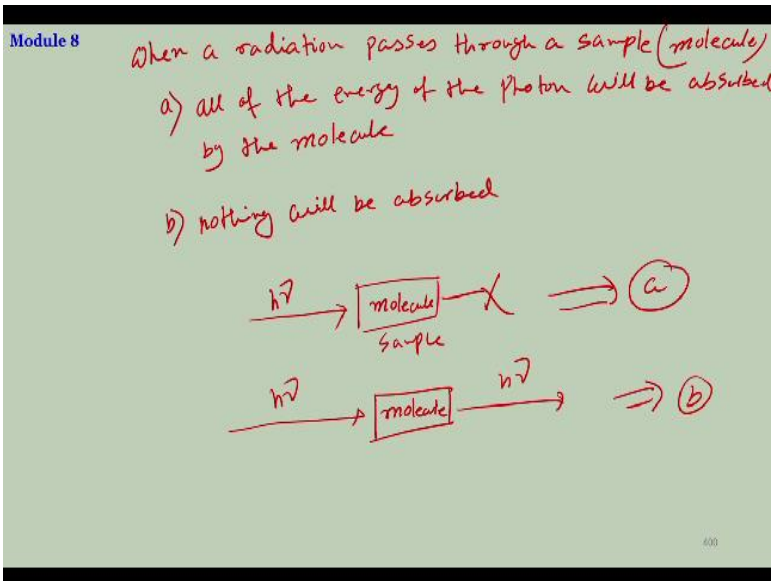

Because this basically means when you are considering that the energy is quantized means when a radiation passes through a sample basically, I am talking about molecules. Then there can be 2 cases, one is all of the energy of the photon will be absorbed by the molecule or nothing at all, nothing will be absorbed or interacted. This means if you have a molecule here which is called sample.

And you sign a radiation pass a radiation through it, which has energy of h nu. So, here I am considering only a single photon, only a single photon is there which has a energy of h nu. Then if the photon interacts with the molecule, then all of this h nu will be absorbed by the molecule and nothing will come out, there will be no photon coming out of it, that is the case a or same h nu is going through it.

And the molecule does not absorb anything, h nu does not interact with the molecule. Then what will happen all of this light will come out and nothing will be absorbed. So, this is case b, there cannot be anything in between for example, there cannot be that certain amount of h nu is absorbed by the molecule and others is released. No, it can be either the whole photon, whole energy or nothing at all. So, that is what is the basis of quantized energy so.

And this is very important spectroscopy is very important to remember. So, we have seen the according to Einstein, the radiation has a particle nature, according to max Planck the radiation

has wave nature and the question is then, how the spectroscopy was developed. We have given the definition of spectroscopy that the radiation interacts with matter. Now matter or molecule that has a specific mass or if radiation has a property, then it should not interact with molecule which has a mass and which is the particle nature actually.

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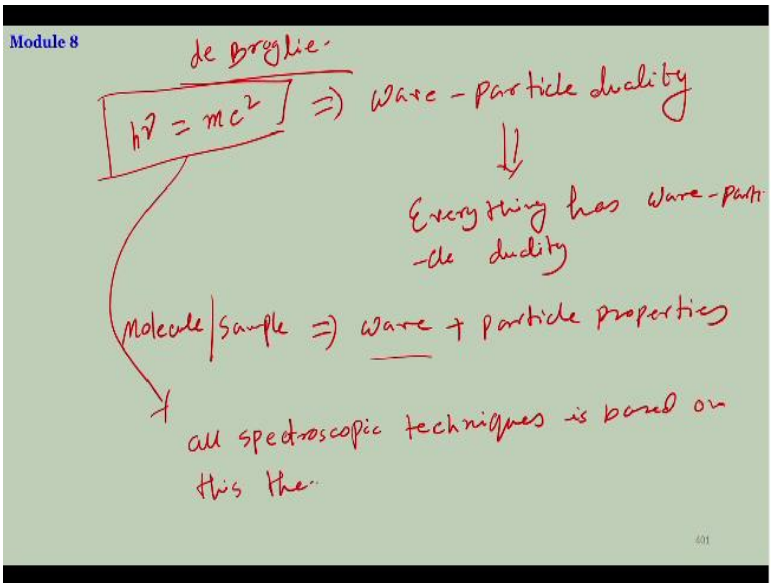

So, here came the de Broglie with his beautiful theory, he said that a given radiation has both a wave function, both wave property as well as the particular property. So, any radiation has a wave function as well as particle nature to it and he has equated this e equals h nu and e equals mc squared to h nu equals mc squared. This means a wave particle duality which means a radiation has a wave property and that radiation has a particle property as well.

And he did not stop here. He has extended his theory to all matters, all waves in this nature. So it is not only the radiation which has the particle duality, everything that is present in our nature, everything that is present in this universe has a wave particle duality. So everything has a wave particle duality**.** So how does it help us, this means when you are considering a molecule then the molecule also has or sample according to de Broglie.

Now, the molecule or sample also has wave property as well as a particle property, wave plus particle properties. And now you can explain the interaction between the light and a matter. So, if you consider light as radiation, and if you consider a molecule that has wave function. So,

radiation wave property of the radiation, wave property of the molecule, they may interact there is no problem head or influence collision out of phase collision.

So, that means, the light can interact with matter. Similarly, on the other way around if you consider the particle nature of the wave and the particle nature of the molecule and particle, particle can interact as well. So, that explains the spectroscopy and from here actually spectroscopy was developed that radiation can interact with matter. So, basically all spectroscopic techniques is based on this theory.

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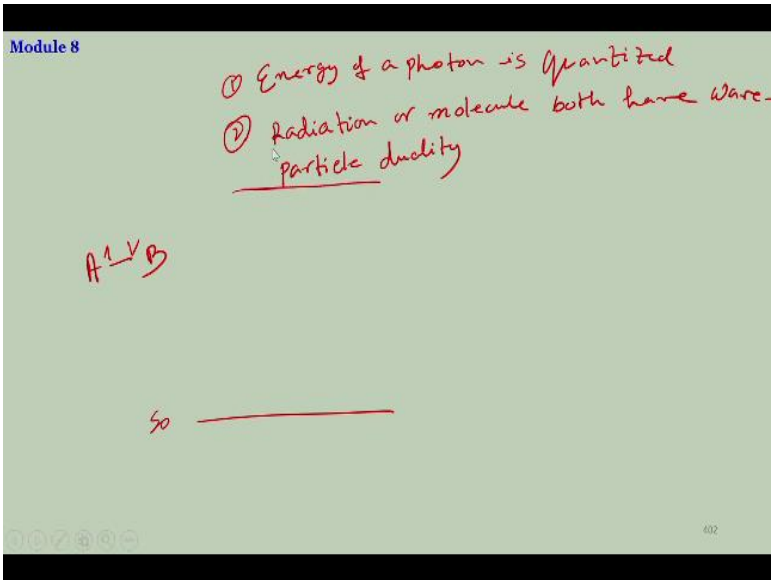

So, the key point of this is, first one is that energy of a photon is quantized, that is very important of photon is quantized you cannot break it, second point is radiation or molecule both have wave particle duality, radiation also has wave particle nature, molecule also has wave particular nature. So, these 2 are extremely important to remember for spectroscopy. So, now coming back to what will happen.

So, far we have been talking about the radiation, the nature of the radiations properties of the radiations. Now come back to molecule, what the radiation will do to the molecule. So when you consider a molecule, let us consider a very simple molecule at first, A-B, which has a bond. Let us consider organic molecules. So, this is a covalent bond. Now, a molecule can undergo various kinds of movements or transitions.

In other words, the electron we are restricting ourselves for the timing with the electrons. So when there is a bond, there is a pair of electrons. So this electrons can move can have different kinds of transitions. The first one is when you are forming a bond, that means the electrons are paired of and they are in the ground electronic state. So STO. So if you have the electron now those electron let us go to the next phase and need more space.

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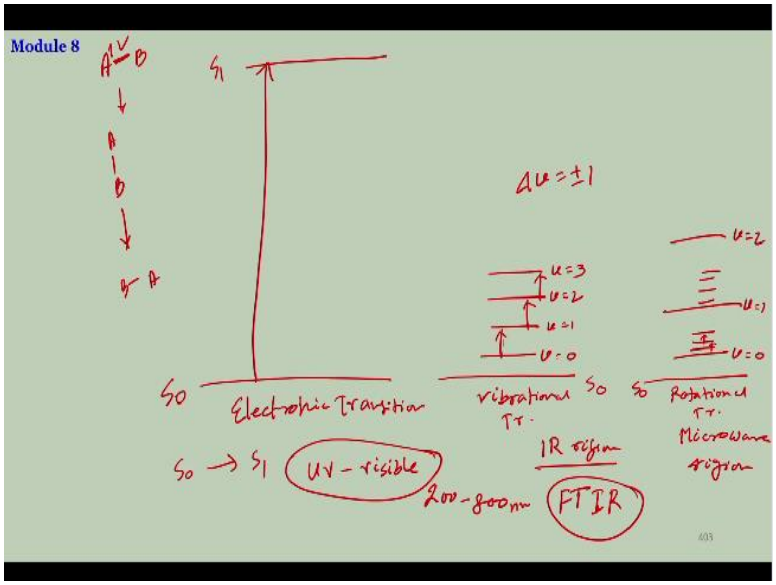

A-B ground electronic state and then you have the excited electronic state. So, here we are considering the electrons, then the electrons can move from one from the ground electronic state to the excited electronic state. This is called the electronic transition. Electronic transition from S 0 to S 1. This is S 1, now electronic transition, the gap of the electronic transition is very high the energy gap.

So, it has this much of energy gap, now what are the other transitions or other movements that are possible when you are considering your bond, one is of course, the electrons can go from one level to the other. Second one is your bond and bond can vibrate. If a bond vibrates, then it can create several different vibrational levels. And those vibrational levels are all happening within the same electronic level, within the same electronic ground state.

So if you consider the electrons to be in the ground state to, they are still both electrons are in the ground state. But the bond is vibrating. Sometimes it is getting extended. Sometimes it is getting squeezed. So bond length changes, and accordingly the energy levels also gets changed. So due to vibrational motion there would be several vibrational levels created, but they are all within the same electronic level.

So, we call it v 0, v 1 these are vibrational states, v 2, v 3. And if you go through the selection rules of the spectroscopy, then you will see not all vibrational transitions are allowed only delta v equals plus minus 1 that is allowed, which means your electrons can move from v 0 to v 1. It can move from v 1 to v 2, it can move from v 2 to v 3, v 0 to v 3 straight not possible. Selection rule does not allow them.

And every time I am repeating, that all these things are happening within the same electronic level. Therefore, if you compare the energy that is required for the electric transition is much, much, much higher compared to the energy that you would require a for vibrational transition. Now, so, this is vibrational transmission and let me just tell you the third one that is now, when it has a certain vibrational level which means that it has a certain bond length.

If it vibrates, then it will be elongated or squeezed. But if it stays in the given vibrational range, for example, we equals 0, then it has a static bond length and within that level, molecule can rotate. So, it can stay like this. It can stay like this next phase, it can go like this B-A molecule can rotate. Keeping the same vibration level, which means keeping the same bond length. So, that is called the rotational motion of the molecule.

And all those rotational motions would be happening within a given vibrational level. So, this is v equals 0, this is v equals 1, this is S 0, electronic state. Similar to next state, within that you have the vibrational states v 0 v 1 v 2, within every vibrational state, there are rotational levels. And almost similar selection rule is applied to the rotational energy levels that only plus minus 1 transition is possible.

So, if you consider the energy now for a rotational transition that is even less compared to a vibrational transmission. So, that is where is coming the energy distribution, electronic transmission request a very high energy, vibrational transmission request smaller energy, rotational transition requires even a smaller energy. And after that there is transitions which happens at room temperature.

So, I am not talking about it that is because of the translational motion of the molecules. So, now, why we are talking about these is that, according to all spectroscopic techniques, spectroscopic techniques have been developed depending upon these factors. So, if you look at the electronic transmission, the energy that you would be needing for electronic transmission falls under UV visible range either in UV range or UV visible range.

So, basically 200 to about 800 nanometers is the wavelength range that will supply you this energy and that falls under the UV visible range. So, whenever you are talking about electronic transition, you are basically talking about UV visible spectroscopy or the other way around, if you are talking about UV spectroscopy, if you are talking about UV visible spectroscopy, that basically means, you are talking about electronic transmission.

Similarly, the energy that would be required for a vibrational transition falls under infrared region, IR region and that is why the vibrational transition or spectroscopy is known as infrared spectroscopy FTIR. This is free transform FTI full air transformed infrared spectroscopy. Whenever you are talking about FTIR, that means to deal with vibrational transitions only, not electronic transitions.

Similarly, UV visible spectroscopy, we will talk about the electronic transition only. And then comes the rotational transition, this energy falls under the microwave region. So small that it falls under microwave region. So, there are many kinds of spectroscopic techniques have been developed based on the energy requirement and based on the nature of the transitions. So, if you remember, if you talk about microwave spectroscopy, or microwave transitions, then of course you would remember the microwave that you use in the kitchen.

So, it functions in the same way that it is responsible whenever you are citing microwave radiation in the kitchen microwave, then basically it provides the energy that activates the molecules to rotate and this rotation speed is so high that it generates heat very quickly. So, the rotation is so high that because of this rotation heat is generated and that is the idea of cooking using the microwave radiation.

So, now we have UV visible spectroscopy. Now, we have the FTIR spectroscopy and in all these cases, if you look carefully, I have been talking about the transitions from down to the up ground state to the excited state, which means the absorption, I am talking about absorption. So, all these 3 spectroscopic techniques I have talked about are absorption spectroscopy. So, in practice what we use we do a visual study and we do IR in FTIR studies, those are basically absorption spectroscopy.

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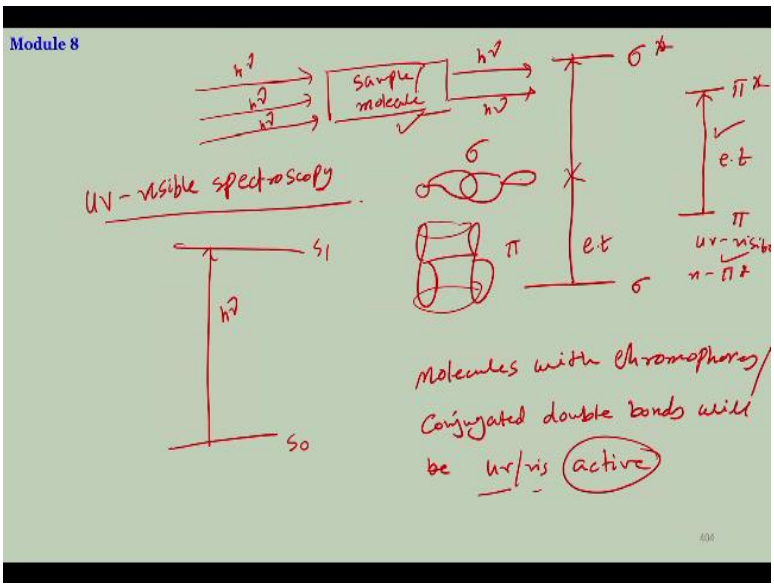

Now, when we have talked about photon is that, so if you have a sample or molecule and then you pass through radiation, let us say we are talking about the UV visible spectroscopy, which means you have supplied radiation in the range of UV visible, and each of them has a particular wavelength, all of them have h nu, same energy, h nu, h nu, if you consider a monochromatic light, then all of them have the same energy h nu, but there will be many numbers of them.

If you are considering a light source having a fixed energy, then there will be millions and millions of photons in that, that would be passing through the sample. So, let us consider that there are 3 photons that is passing through the sample. Now, what can happen, if this energy matches exactly with this energy, then only this energy would be absorbed by the molecule. And if this light is absorbed by the molecule, you cannot see that here.

So, let us consider out of 3 photons, 1 photon has been absorbed by the molecule and then therefore, the 2 other photons will come out of the source. Now, if you measure the intensity of light at the beginning here, and intensity of the light here, then you can figure out how many photons have been absorbed by the molecule. And accordingly, you can plot, accordingly you can say that this what particular frequency has been absorbed by the molecule and in this much number.

So from there you can find out about the concentration of a given sample as well, as well as which range the sample would adsorb. So this is considering the absorption spectroscopy. So, let us start with the UV visible spectroscopy. Here we will see 2 things. One is the UV visible absorption, and then we will see the fluorescence, that is emission spectrum. So, you already know that from a ground state to the higher excited electronic states.

This movement, this jump, or this transition will be named as the electronic transition. Now if you consider a molecule that has different types of bonds, it has a sigma bond molecule has a pi bond. So, if it has a sigma bond there will be corresponding sigma star bonding antibonding and this would be the energy gap. If you want to have a transition between sigma to sigma star, but this energy gap is so high that it does not fall under the UV visible spectrum region.

So, when you see electronic transmission in UV visible spectroscopy that is not because of sigma, sigma star transmission. So, what is it for. Now, considering the pi bond, pi bonds are weak bonds because okay whenever you are forming a sigma bond I am just rapidly reminding you. This is the interface collision. So head on collision basically, this is the sigma bond. Whenever you are considered a pi bond you are basically considering lateral overlap, side by side overlap and obviously side by side overlap, it is weak compared to a head on collision.

So, pi bond have less energy. Therefore, pi star also have lesser energy compared to sigma star. Since the bond is weak, the transition becomes easier. So, pi pi star transition is also very visible and this energy gap actually falls under the UV visible range. This is also electronic transmission et, this is also electronic transmission. But sigma, sigma star electronic transmission does not fall under UV visible pi pi star falls under UV visible.

Similarly, n-pi star transition also falls under UV visible range. So, whenever you see a spectra in the UV visible spectroscopy, that means your molecule must be undergoing through a pi pi star transition or through n-pi start transition. In other words, it means the molecule must have pi electrons, the high pi electron density and that is why we call that every molecule that absorbs in the UV visible region must have a chromophore.

So molecules with chromophores or conjugated double bonds will be UV or visible active means they can absorb UV or visible radiation. So, all molecules that will absorb that will show absorption spectra in the visible spectroscopy must have a chromophore for most type conjugated double bonds.

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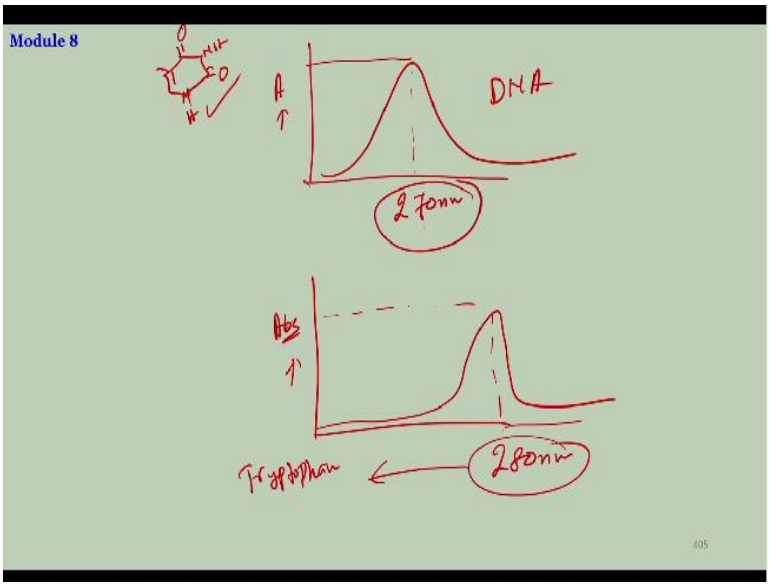

So, if you consider of the nucleobases, for example, if you write the structure of thiamine because of the automatic form and because of the long pair of electrons on the nitrogen, that is

automatic system, so, that is a pi electron rich system and also that has conjugation in it. So, that is why we have seen that in the UV visible range, you see a spectra like this and this is roughly I forget what the exact wavelength.

So, this probably is, let us say 270 nanometers this and this is your amount of absorption. So, this much of light has been absorbed, which has this particular frequency 270 nanometer by this molecule, that is what is the absorption spectroscopy. So, using that absorption spectroscopy, in this case the UV visible we can study or you can identify the presence of the biomolecules such as DNA, because it has chromophore, so, this is a DNA.

Also if you have a protein sample that will also show you around 280 nanometers with a maximum, the highest absorption would happen at 280 nanometer. This is because of the presence of tryptophan amino acid in the DNA, if you look at the structure of tryptophan that is aromatic structure 6 membered ring fused with a 5 membered ring, so aromatic structure, which means it has multiple pi bonds, chromophore unit is there, therefore, that will absorb radiation.

So, this is for the UV of the full spectroscopy. So, far we have talked about the absorption right that here this is the absorption Abs amount of light that has been absorbed by the molecule that you are studying here.

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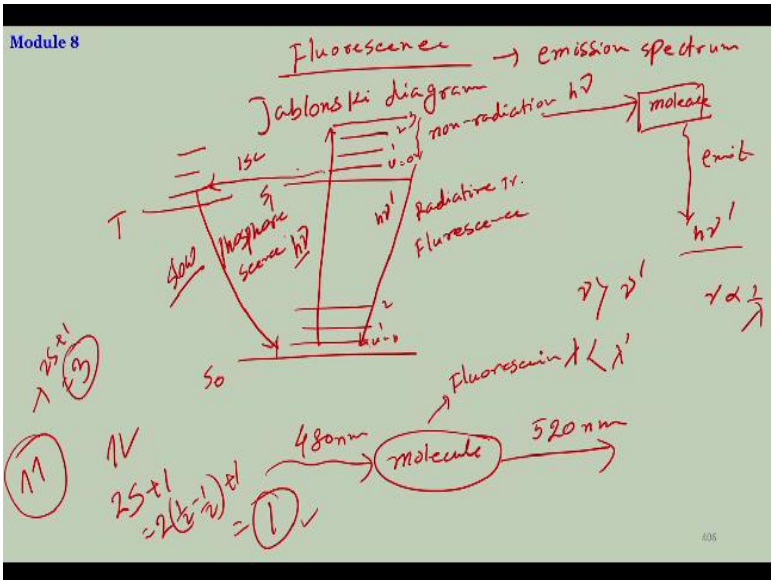

Now coming to a very important application in biology at least is the fluorescence spectroscopy, if you want to know what is happening inside the biological cells, biological body you have to send some molecules or how to send something into your cell, into the body to see the interaction or to study its functions. The question is how would you know what is happening inside. So that is why most of the times a fluorescent tag is being used.

So you need a tracker. It is like a GPS tracker. If you have a GPS in your mobile, other people can look at you where exactly you are. Similarly, if you have a fluorescence molecule attached to whatever you want to do in that probe, and you send it to the body, then you can see where is your molecule wait. So fluorescence molecule or fluorescent molecules has huge applications, in biology or in the studying biomolecular properties.

And so, the basic idea of fluorescence can be explained by this Jablonski diagram. I know that most of you already know what is the Jablonski diagram, but still I will just give you a brief intro. So fluorescence spectroscopy basically means it is an emission spectroscopy, emission spectra or spectrum. So, if you have seen it is an absorption spectra, fluorescence is the opposite emission spectra, which means, if you have a sample and you send light through it h nu.

Then the molecule will absorb this light, it will do certain structural change or certain physical change in the excited state and then it will radiate or emit another frequency h nu prime. So, you have excited the molecule with what kind of radiation but the molecule will emit a different kind of radiation. So, that is what is the fluorescence is emission spectroscopy. So, if you have the ground electronic state S 0 that has many vibrational stages.

And now you have here the higher excited state S 1 that also has vibration levels 0 1 2, 0 1 2. Now let us say you have a bond and your electrodes are present in the v 0 of the down electronic state here. If we excite if you sign with the UV radiation and if the energy matches, so, this will go through there will be electronic transition absorption spectroscopy here to the certain vibration rich level.

Now, what can be the freight after it reaches the excited state, what can be the freight of the electron or what can be the freight of the radiation, freight of the electrode can be that from here once it reaches here it can come back it has to come back to the because excited state nobody wants to stay everybody wants to cool down. So electrons or the radiation are to be cooled down. Let us say this radiation has a frequency of h nu.

Once it reaches the excited vibrational state, it will come down to this v equals to 0 for example, to the ground vibrational state v equals 0. So, this is a non radiative process which means, when this is coming down it has to lose energy and this energy does not come in terms of the radiation, it will be released in terms of heat, that is called non radiative, non radiative release of energy. So, the radiation would come from here to the down vibrational state in a non radiative wave and from there it can come to the down state through a radiative transition.

So, this is radiative is discrete. So, fluorescence basically means emission of radiation from the lower vibrational state transition. And when something is coming down, it is of course, a spontaneous process. If you fall from sky, it will be a freefall is spontaneous process. Same thing from here to there it is a spontaneous process and that is what is called the fluorescence. So, it is a spontaneous process nothing of the excited electronic level.

And obviously, you can see, if you look at the energy, this would be different or better this, this would be of low energy, low energy means it has to be different frequency. So, nu would be higher than nu prime. In other words, if you convert it into wavelength, nu equals hc by lambda, so, nu is inversely proportional to lambda wavelength. So, lambda would be less than the lambda prime or the wavelength for the fluorescence will usually be higher than the excited radiation.

So, if you consider a fluorescent molecule and of course that has to do with the nature of the molecule. So, molecules should have the capability to absorb this radiation go to the higher vibrational level, comes back from there to the lower vibrational level and then freefall emission, that is the property of the molecule. So, if you excite it, for example, with 480 nanometer light, if your molecule is a fluorescent, fluorescent is a fluorescent molecule, you can look up the structure of it, then, the emission spectra usually comes at around 520 nanometer.

So, that is about the fluorescence spectroscopy. Now since you were at fluorescence spectroscopy, you can explain the phosphorescence that you see also. Some molecules are phosphorescence, even here you can see phosphorescence, if you have tube light in your room, if you turn off the light, the light, after that even you can see a faint radiation coming through the light, that is the phosphorescence.

So, the phosphorescence is basically that once this electron comes down to the lower vibrational energy level, if that vibrational energy level matches with the triplet energy step, so this S 0 S1 that is why I have taken the nomenclature S 0 S 1 that means the singlet state, this means the triplet state, similar statements where the spin multiplicity of the electrons is 0. So if you have 2 electrons with opposite paired, then the spin multiplicity 2S plus 1 that will become 1 because 2 into S plus half and then minus of opposite plus 1.

So, that is coming as 1. So, when you have a bond, the electrons are paired off. So, the spin multiplicity is 1 and therefore, the electronic transition would be from the singlet state to the singlet state. If they are, if the electrons have the same spin plus half or plus half, plus half or plus half then the spin multiplicity 2 S + 1 would be 3, that is called the triplet. And this is the triplet, triplet will also have this is electronic state.

So, it will also have the vibrational energy state. Now, if this energy level matches with some of this energy level, then there can be inter system processing. The electrons will come from the singlet state to the triplet state may happen and from here, you will have the transmission, the emission. So in this case it is a delayed emission because your electron is moving from singlet to the triplet and triplet to the singlet back again.

And singlet, triplet steps has a higher lifetime. So this process is slow process. Usually, and that is why this happens little bit later. Fluorescence is a spontaneous process, phosphorescence is not a spontaneous process or it is a delayed process. So, this is what is phosphorescence. So, some of the fluorescence molecules that we use in biochemistry or biology or organic chemistry is fluorescent.

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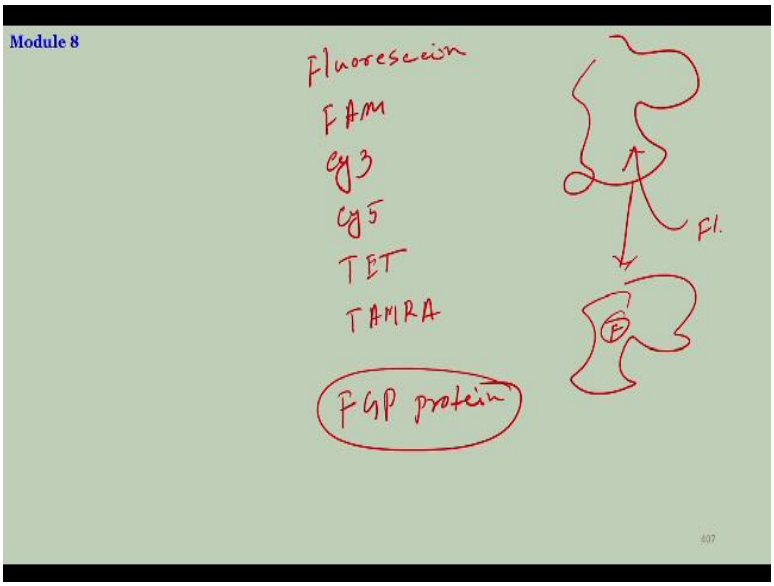

FAM, you can look up the structures. I am not writing the structure FAM. Then Cy3, Cy5, TET, TAMRA and all this. There are many other variations are there, which shows fluorescence all of these molecules actually they vary in their excitation and emission spectra. So, they require different excitation wavelength to go to the higher exeter state and they also emit different wavelengths of light.

So, according to your requirements, you can choose which one would be suitable for you which one fluorescence molecule will be suitable for you. And that fluorescence molecule you can use as a tag to protein for example, if you have a protein molecule and you want to see where the protein molecule is going in the body, then you can always tag this protein molecule with fluorescence, the fluorophore molecule.

So that you will have a fluorescence molecule inside the protein. And wherever the protein is going, you can track it, you can keep it a track. So that is how the different taggings are work. So these are the organic molecules I am talking about, there are biological fluorescence molecule also, their particular protein called GAP protein. GAP protein shows huge fluorescence. GAP is isolated from fish, fluorescent fish.

So that was all, that was big news when it was first isolated and the person also I forgot the name. The person also received Nobel Prize for his GAP protein. And this protein actually used a lot in biology to tag other proteins to tag other biomolecules such as DNA, RN, mRNA and so because since this is a protein, it is easy to get into the cell and cell do not mind to take it up. So, yeah, that is about the fluorescence molecule.

At next class I will talk some more about another very useful technique that is used to study biomolecular properties is the FRED fluorescence reference energy transfer and then we will move on to the other non spectroscopic techniques. Thank you.