Structural Biology
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Lecture – 32
UV-Vis and CD Spectroscopy

Hi everyone, welcome again to the course of structural biology. We are continuing with structural Biology techniques. Currently we are in the module of Spectroscopy. Today it is the second class of Spectroscopy we have talked about the general principles of Spectroscopy, how different parts of spectrophotometer work? What are the general principles? In today's talk we will concentrate on electronic transition with two techniques one is UV-visible and another is circular dichroism.

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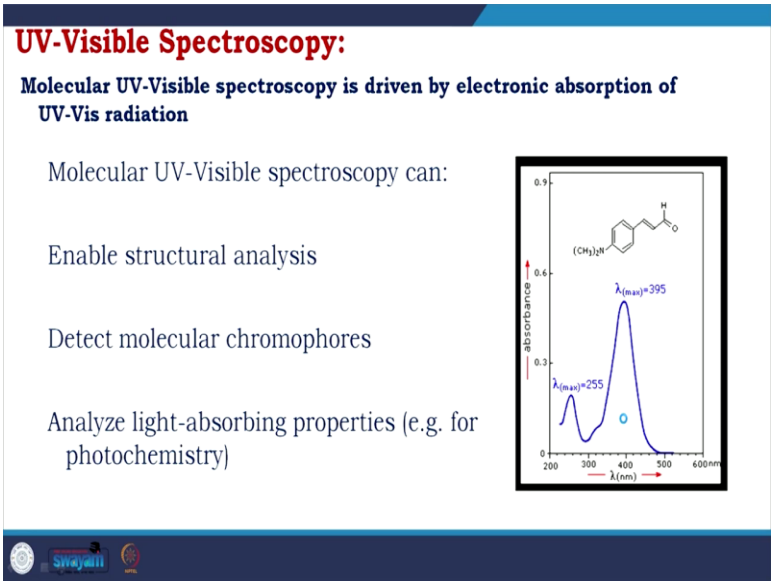

Start with UV visible spectroscopy: Molecular UV visible spectroscopy is driven by electronic absorption of UV radiations. Here the excitations affect the electronic level. Molecular UV visible spectroscopy can enable the structural analysis, detect molecular chromophores. The compounds which are specially showing difference, analyze light absorbing properties specially applicable for photo chemistry.

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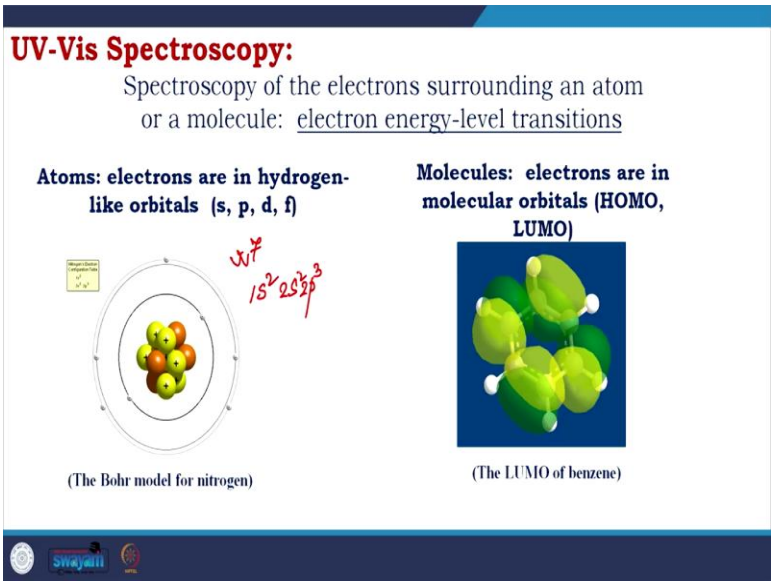

Spectroscopy of electrons surrounding an atom or molecule is the electron energy level transition. So atoms, electrons are in hydrogen like orbitals like s, p, d, f. In molecule electron are in molecular orbital HOMO, which is Highest Occupied Molecular Orbital, LUMO, Lowest Unoccupied Molecular Orbital. So this is the aspect the Bohr model of Nitrogen. Nitrogen have 7 electrons as you see and with electron distribution of $1S^2$ $2S^2$ $2P^3$. This is the lowest unoccupied orbital picture of benzene.

You see the delocalized electrons of the Pi system. So these types of systems are actually good and we will see the transitions which are allowed.

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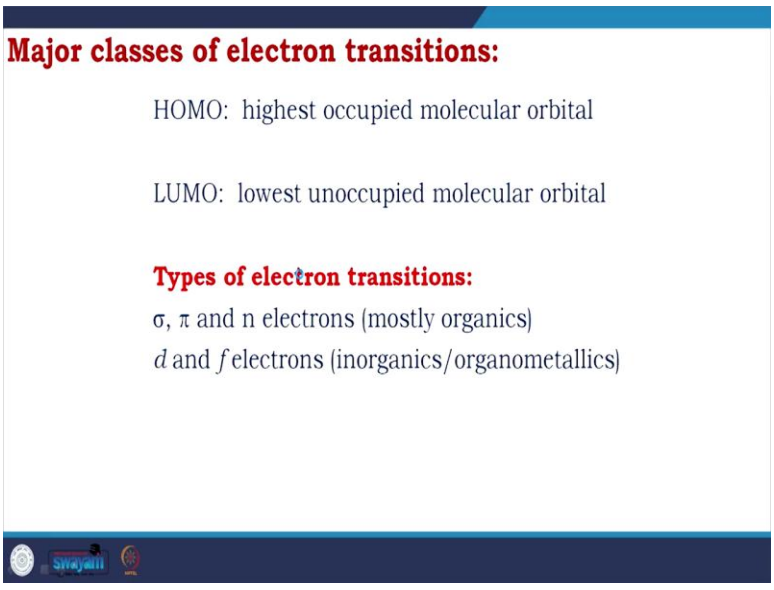

As I have told HOMO, the highest occupied molecular orbital, LUMO the lowest unoccupied molecular orbital. The type of electronic transition happened Sigma, Pi and n electrons. So, Sigma, Pi, n electrons are mostly apply to organics, d and f electrons are inorganic and organ metallic charge transfer for complexes.

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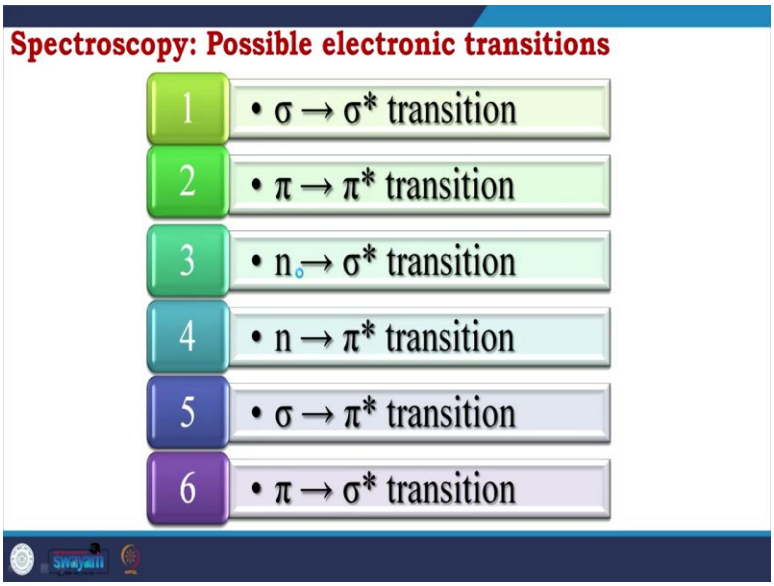

So possible electronic transition apply to any type of any form of Spectroscopy, σ to σ*, π to π* , n to σ* transition, n to π* transition, σ to π* transition and π to σ * transition.

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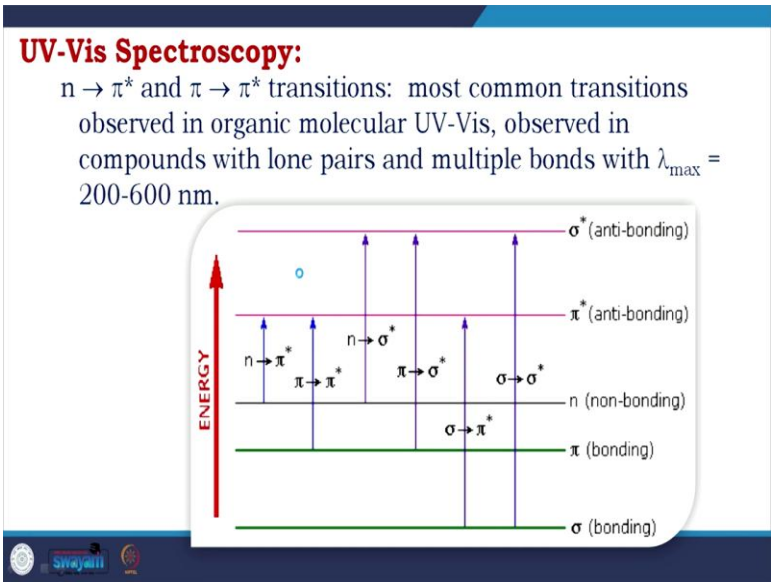

σ is for bonding, π is for bonding, n is for non bonding, π* star is for anti bonding and σ* is for anti bonding. Sigma is the most stable and sigma star is the most unstable. Pi is second stable Pi

star is the second most unstable, and in between there is nonbonding. These are the five layers. So as I told there are so many transitions possible from Sigma to Sigma star.

Sigma to Pi star these are allowable but then there is other one as I told Pi to Sigma star and then Pi to Pi star, n to Pi star, n to Sigma star. So n to Pi star and Pi to Pi star transition are most common transition of the organic molecule. UV-rays observed in compound with lone pairs and multiple bonds with Lambda Max within 200 to 600 nanometer or common or fragile for those electronic transition to be happened.

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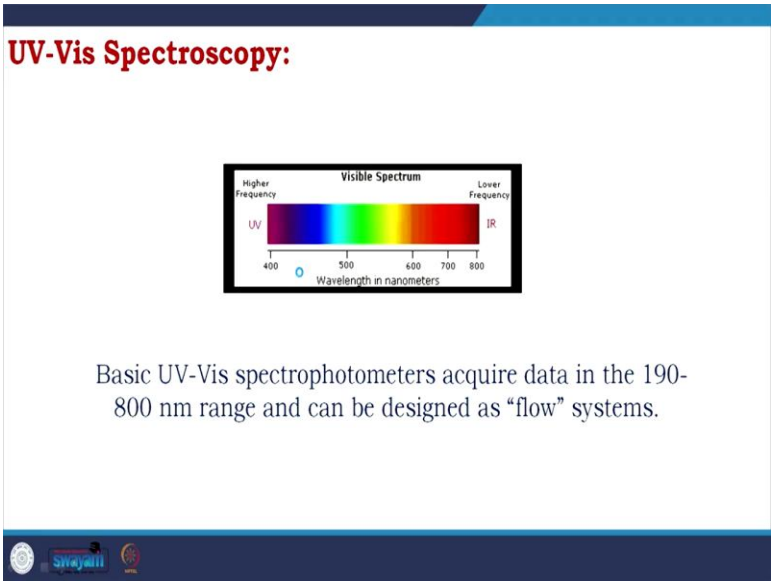

So we know that the three regions UV, then visible then IR. So UV visible spectroscopy is visible and UV. For basic UV visible spectrophotometer acquire data in the 192-800 nm which is covering even these range and can be designed as a flow system.

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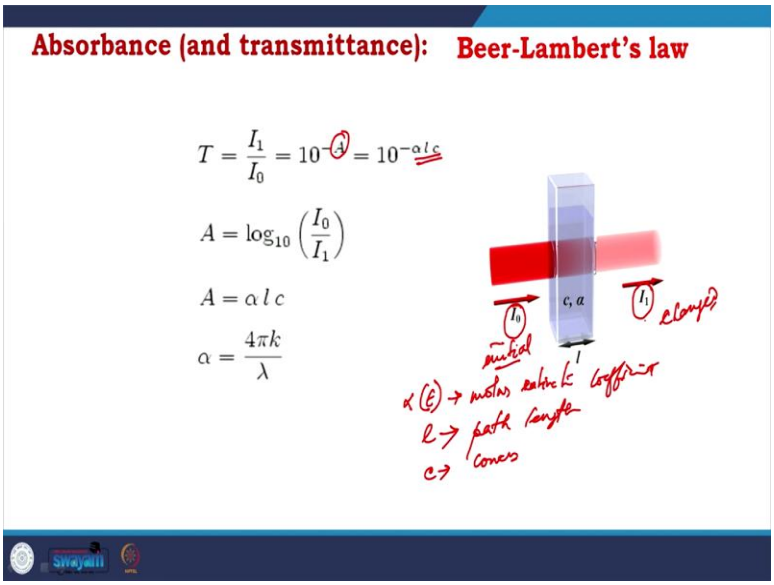

How it work? We have discussed about Beer-Lambert law, it is the main principle by which absorbance principle works.

So

Transmittance (T) = I_1 / I_0

I_0 is the initial intensity, and I_1 is the changed intensity. So this is equal to

Transmittance (T) = $I_1 / I_0 = 10^{-A}$

A is the absorbance

Transmittance (T) = $I_1 / I_0 = 10^{-A} = 10^{-\alpha lc}$

where α is the molar extinction coefficient, l is the path length and c is the concentration.

So this is the sample, the cuvette with the path length l, the concentration of the sample which you are putting here and using that we could have calculate all the possible parameters.

$\alpha = 4\pi k / \lambda$

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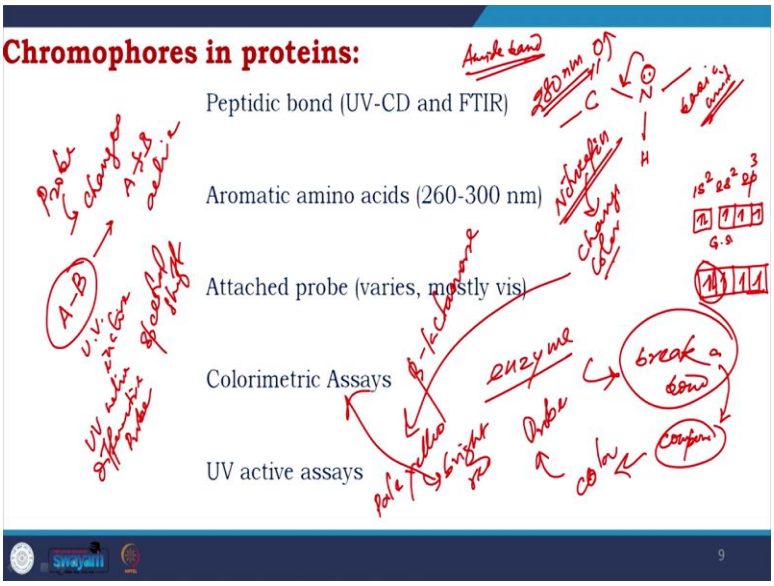

Chromophores in protein

Number one is the peptide bond; it could be the applied bond (amide bond). We know that amide bond has a lone pair because nitrogen is $1S^2$ $2S^2$ $2P^3$. So if you look at, in excited state it forms the SP^3 orbital where there is one lone pair, this lone pair is contributed and get a partial double bond in the amide of the peptide bond. This peptide bond is the basic unit of protein.

So we will discuss FTIR- Raman you will get specific amide band. In UV, it is also sensible we will talk about at giving characteristics spectra at 280 nm.

Aromatic amino acid give spectra at 260 to 300 nm, and then there are attached probes which are varies according to their property, and according to their binding. There are probes used for colorimetric analysis. When we say colorimetric analysis, then let us say you have an enzyme, and the enzyme could break a bond. If there is a compound which produce color when the bond breaks, then the compound would use for colorimetric analysis as a probe. Then there is a UV active assay, like a substrate having an A-B bond, and the bond breaks and this is UV active, specific spectral shift. These types of compounds are used as UV active or differentiative probe.

An example, we generally used is Nitrocefin. Nitrocefin is a compound which change colour when it is attacked by the enzyme called beta lactamase, the pale yellow colour change to bright red. So these could be used as a colorimetric probe in the visible range.

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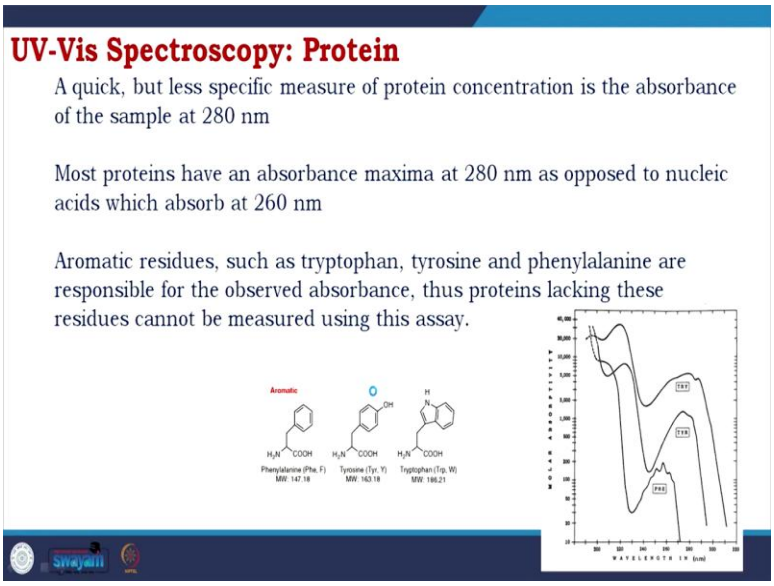

For protein a quick but less specific measure of protein concentration is the absorbance of the sample at 280 nm. As I was talking about, at around 280 nm, protein molecule shows peak, and then they have other peaks for aromatic amino acids, phenylalanine and tyrosine. Most protein has an absorbents Maxima 280 nm as opposed to nucleic acid at 260 nm. This property is utilized for quick determination of concentration of a protein when we are working in the laboratory.

Aromatic Residue such as tryptophan, tyrosine, and phenylalanine are responsible for the observed absorbance, thus protein lacking these residues cannot be measured using this assay.

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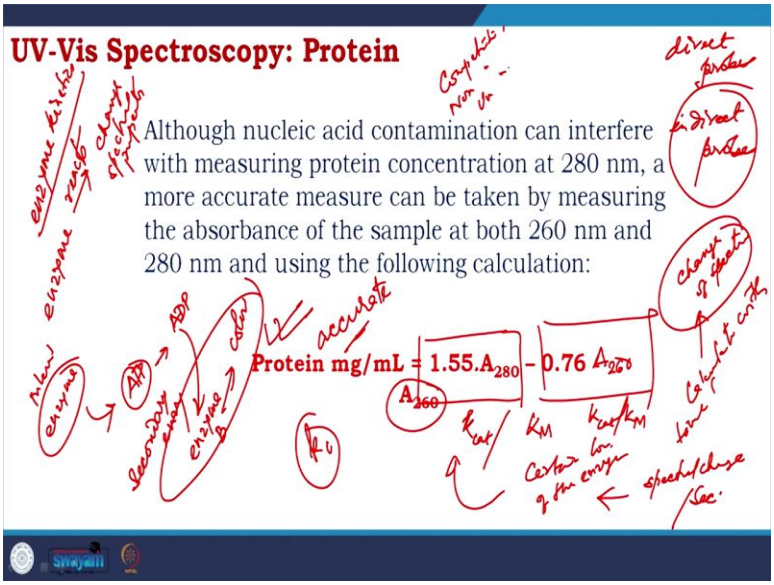

Although nucleic acid contamination can interfere with measuring protein concentration at 280 nm, a more accurate measure can be taken by measuring the absorbance of the sample at both 260 and 280 nm and using the following calculations.

So, protein concentration = $1.55. A_{280} - 0.76.A_{260}$.

This factor A minus factor B is very accurate calculation, that is usually used in laboratory condition.

One of the very good applications of Spectroscopy is enzyme kinetics. I was talking about the visible colour probes and UV based probes in the reaction, where there is a change in the spectral property. They are called direct probes.

But there are indirect probes, where you have one enzyme of interest, it makes a change to compound A, that leads to change of ATP to ADP (this is not colorimetric).

But this ATP changing can take part in a reaction with enzyme B which is making colour. Then this is used as a secondary enzyme in the assay and this is called indirect probe.

How the enzyme catalysis is measured? Change of spectra would be calculated with time. So now you have Spectral change, let us say per second with a certain concentration of the enzyme. In saturation kinetics, from there, you could measure kcat; you could also measure kcat / km, which is the measure of the efficiency of the enzyme.

You could also assay the inhibitors measuring ki. You could measure the effect of reversible inhibitors by different type of assays like competitive, non-competitive, and uncompetitive inhibition assay.

So UV-Vis Spectroscopy in a general set up; you have a sample holder and reference holder. But in the Spectroscopy instrument, which is used for Kinetic measurement there are multiple cuvette holders to fix with different concentration of substrate keeping enzyme concentration and other things fixed, and from the rate you could calculate kcat, km. Also, if you want to inhibit you could calculate in that way. UV visible spectroscopy is playing major role in enzyme kinetics, small molecule kinetics, photochemistry and many other things.

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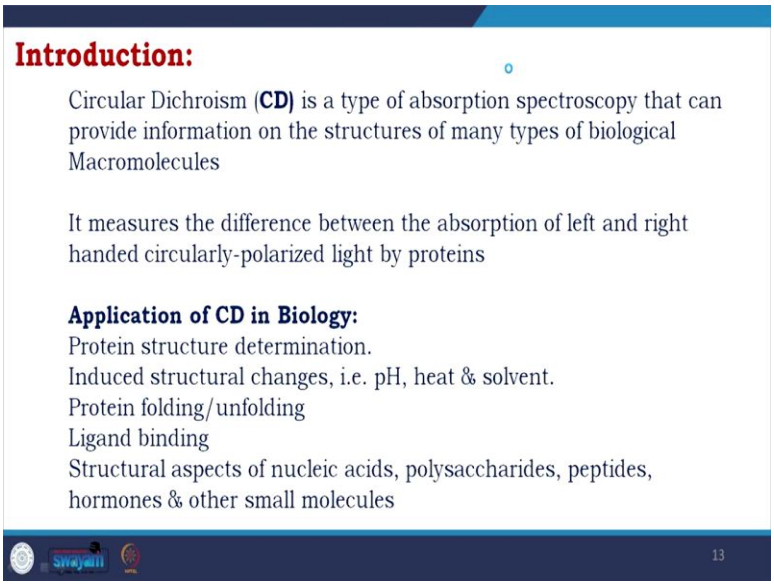

Coming to the Technique Circular Dichroism: To introduce Circular Dichroism, Circular Dichroism is a type of absorption spectroscopy that can provide information on the structure of much type of biological macromolecules. It generally measure the difference between the absorption of left and light handed circularly polarized light by protein. So this is a new thing because we had talked about change of intensity directly in a linearly polarized light.

We are introducing a concept which is called circularly polarized light and from there the technique is called circular dichroism.

Application of CD in Biology:

Protein structure determination; it is very good in determining the secondary structure of protein and associated changes. Changes are important because, high-resolution structures generally do not give you a lot of experiment with changes.

In the structure change associated with pH, with heat, with solvent, with denaturating agent, anything any changes you could trap in CD.

Protein folding and unfolding; Ligand binding; if there are induced changes after binding to the ligand then you could see that. Structural aspects of nucleic acids, polysaccharide, peptides, hormones and other small molecules are the applications of CD.

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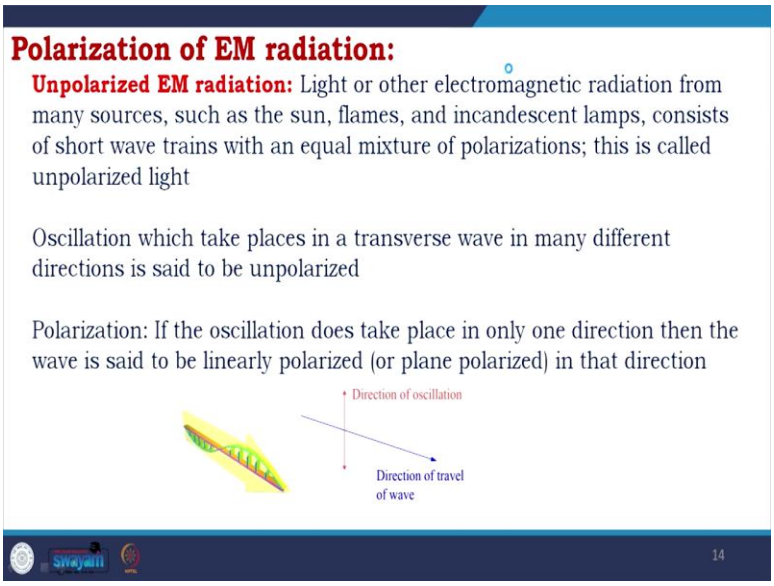

So we are talking about circularly polarized light. Let us start with unpolarized EM radiation. Light or other electromagnetic radiation from many sources such as Sun, flames and incandescent lamps, consists of short wave trains with an equal mixture of polarization. They have light going in all different direction is called unpolarized light. Oscillation which takes place in transverse wave in many different directions is said to be unpolarized.

Now if the oscillation does take place in only one direction, then the wave is said to be linearly polarized or plane polarized in that direction.

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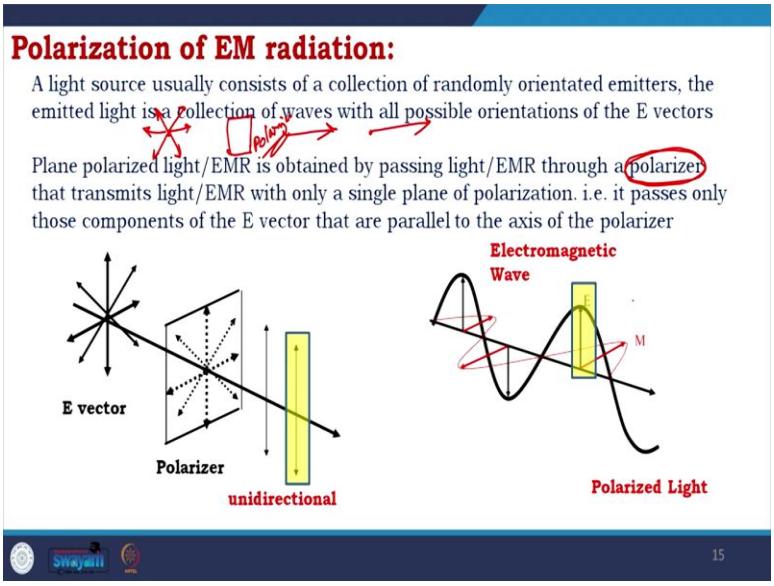

What is polarization? A light source usually consists of a collection of randomly oriented emitters in different direction. The emitted light is the collection of waves with all possible orientation of the E vectors.

Plane polarized light or electromagnetic radiation is obtained by passing that through a polarizer.

So you are passing the light through an instrumental setup called polarizer that transmits with only a single plane of planation.

So you see, this is the E vector they are going into many direction and polarizer takes them and convert them to travel in only one direction.

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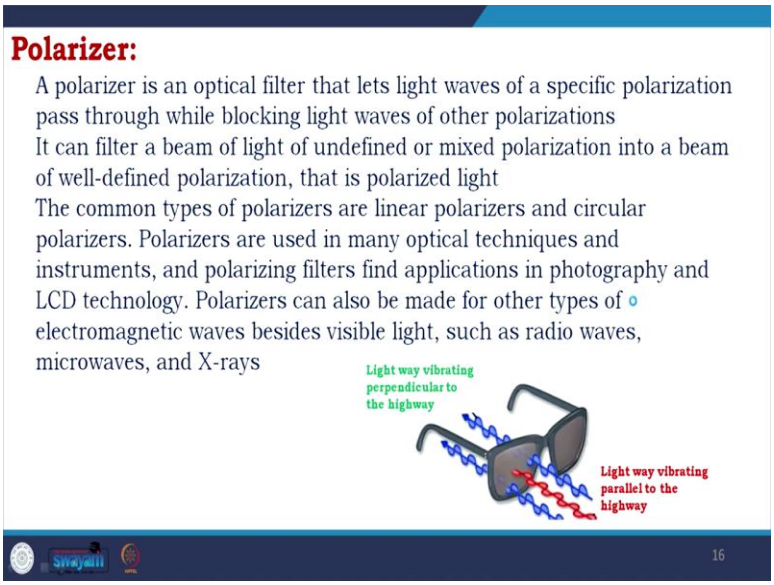

Polarizer is an optical filter that lets light wave of a specific polarization pass through while blocking light waves of the other polarization. It can filter a beam of light of undefined or mixed polarization into a beam of well-defined polarization and that is called polarized light. The common types of polarization are linear polarizer and circular polarizer. Polarizers are used in many optical techniques and instrument, and polarizing filters, and application in photography.

Polarizer can also be made for other type of electromagnetic wave besides visible waves such as radio wave, X-rays.

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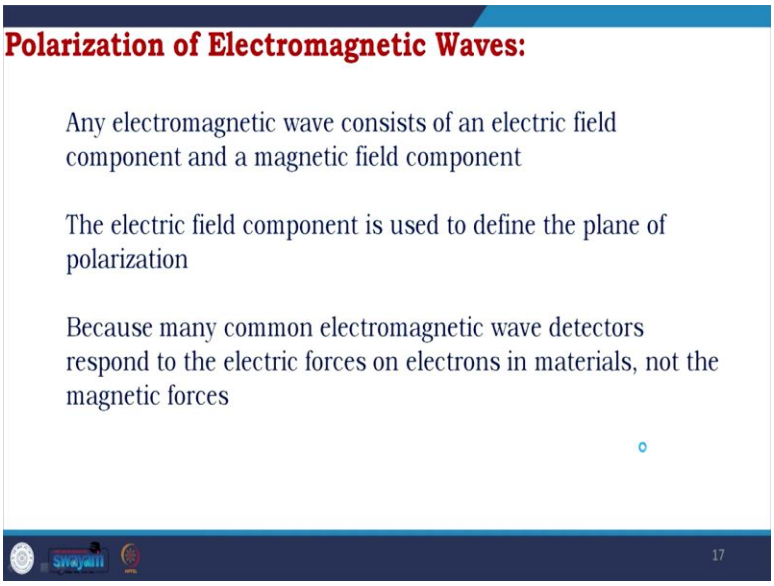

An electromagnetic wave consists of an electric field component and magnetic field component. The electric field component is used to define the plane of polarization. Because many common electromagnetic wave detector respond to electric forces on electron in material not the magnetic forces. So, generally we look at the electronic vector.

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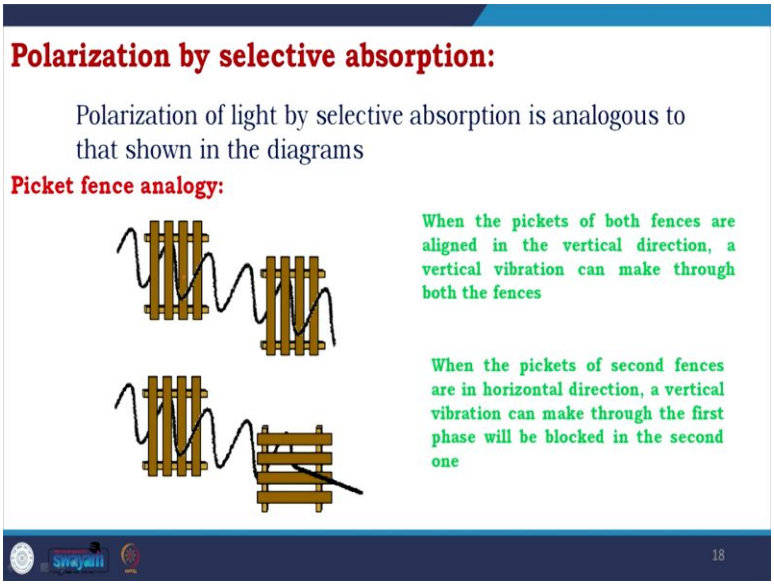

Polarization of light by selective absorption is analogous to the shown diagram which is Picket fence analogy diagram. When the pickets of both fences are aligned in the vertical direction, a vertical vibration can make through both the fences but in this case when picket of second fences are in horizontal direction a vertical vibration can make to the first phase and will be blocked in the second one.

So that is how you could have blocked some specific waves or in other word, you could have allow some specific waves to go and when you have blocked almost all the waves and allow one single this is called plane polarized light.

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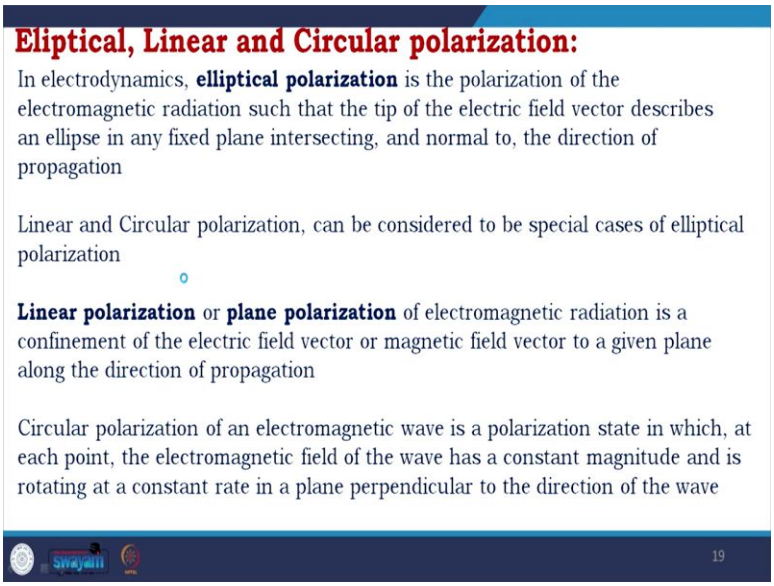

Elliptical, linear and circular polarization: Elliptical polarization is the polarization of the electromagnetic radiation such that the tips of the electric field vector describe an ellipse in any fixed plane intersecting and normal to the direction of the propagation. In elliptical polarization there are two special cases one is linear polarization and another is circular polarization.

So, linear polarization or plane polarization of electromagnetic radiation is the confinement of the electric field vector and magnetic field vector to a given plane along the direction of the propagation. We have already talked about this circular polarization of the electromagnetic wave. It is a polarization state in which at each point, the electromagnetic field of the wave has a constant magnitude and is rotating at a constant rate in a plane perpendicular to the direction of wave.

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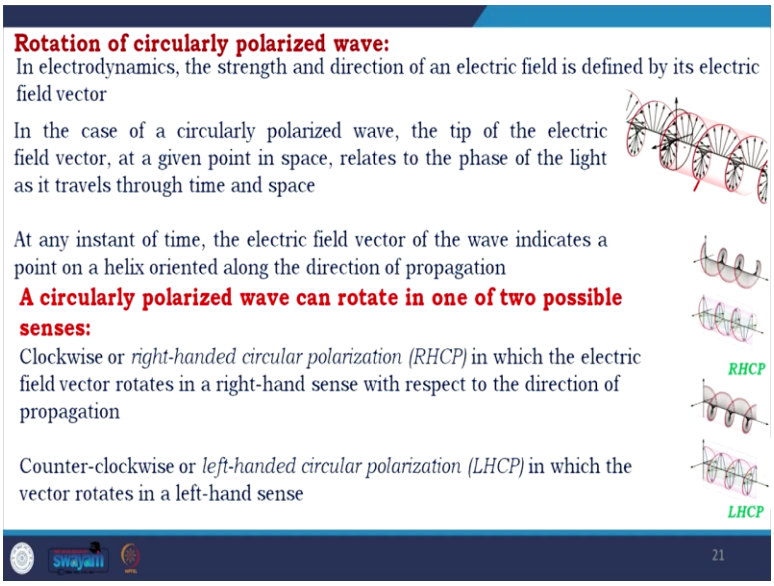

So, rotation, here you see the rotation in electrodynamics the strength and direction of an electric field is defined by its electric field vector. In the case of circularly polarized wave, the tip of the electric field vector, at a given point in space, relates to the phase of the light as it travels through time and space. At any instant of time the electric field vector of the wave indicates a point on a helix oriented along the direction of the propagation.

If you see here, it shows the development of a helix. A circularly polarized wave can rotate in one of the two possible senses. Clockwise or right handed circular polarization (RHCP), in which the electric field vector rotate in a right hand sense with respect to the direction of the propagation. Counterclockwise or left handed circular polarization (LHCP) in which the vector rotates in a left-handed sense.

If you look at this point, you could understand that they are working in two different directions. And the interesting point here if you apply the rotation and you get a differentiation between the right-handed circular polarization and the left and right circular polarization. You will get it for optically active molecule. Optically inactive molecule is called a Chiral molecule.

Now if you understand protein, you know that protein have 20 amino acids among this 20 amino acid 19 amino acids are chiral. So that is what circular dichroism in becoming so popular and so interesting TO study of protein molecule.

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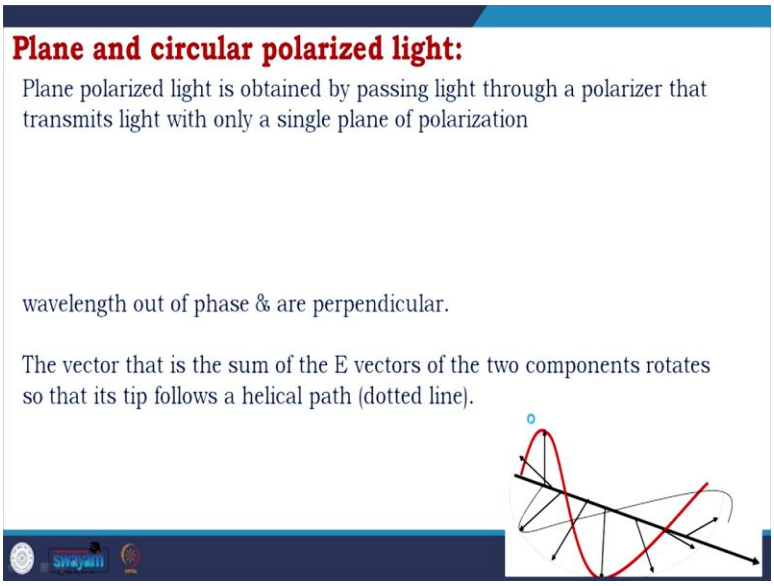

So the plane polarized light is obtained by passing light through a polarizer that transmits light with only a single plane of polarization. It passes only those components of the E vector that are parallel to the axis. Circular polarized light: the E vector of electromagnetic wavelength out of phase and are perpendicular. The vector that is the sum of the E vector of the two-component rotates so that its tip follows a helical path. You will see that in the dotted line.

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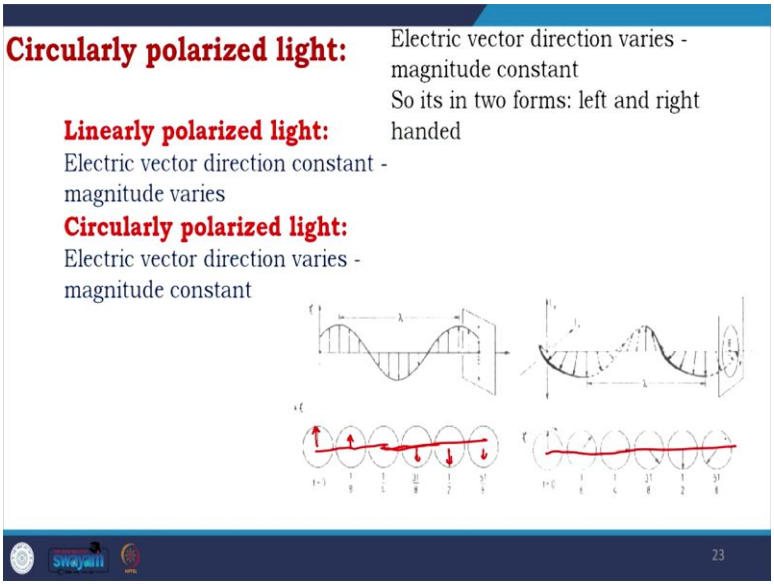

And coming to the linearly and circularly polarized light comparison: For the linearly polarized light the electric vector direction constant and magnitude varies. For Circularly polarized light the electric factor direction varies and magnitude constant. So here you see this is the rotating around.

So the electric vector direction varies, the magnitude is constant, so it is in two form left and right handed.

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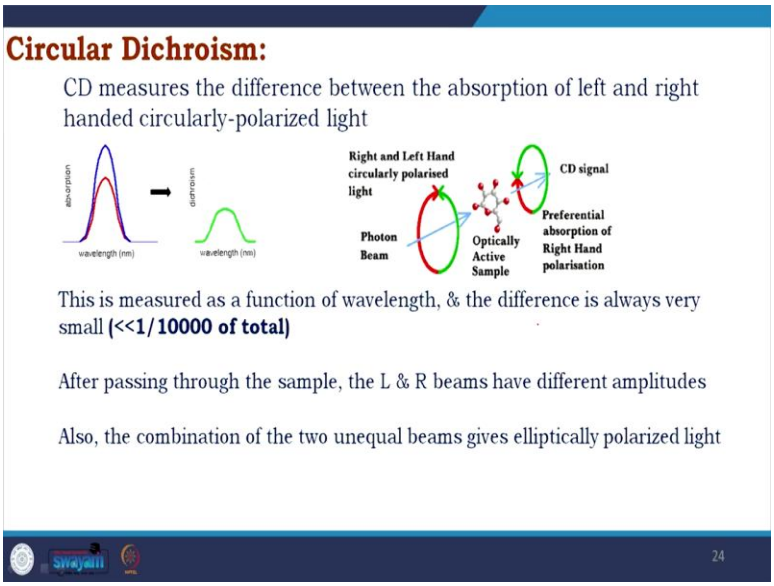

The CD measures the difference between the absorption of left and right handed circularly polarized light. This is the photon beam when you put the optically active compound you get the CD signal where the preferential absorption of the right hand polarization and left hand polarization which would be different.

One of the problem with this technique is, this is measured as a function of wavelength and the difference of the wavelength between left and right is always very small one by 10,000 (1 / 10000) of the total. After passing through the sample the left and right beam are of different amplitudes. Also the combination of the two unequal beams gives elliptical polarized light, which we talked about. Hence CD measures the elipticity of the transmitted light. The light that remains not absorbed.

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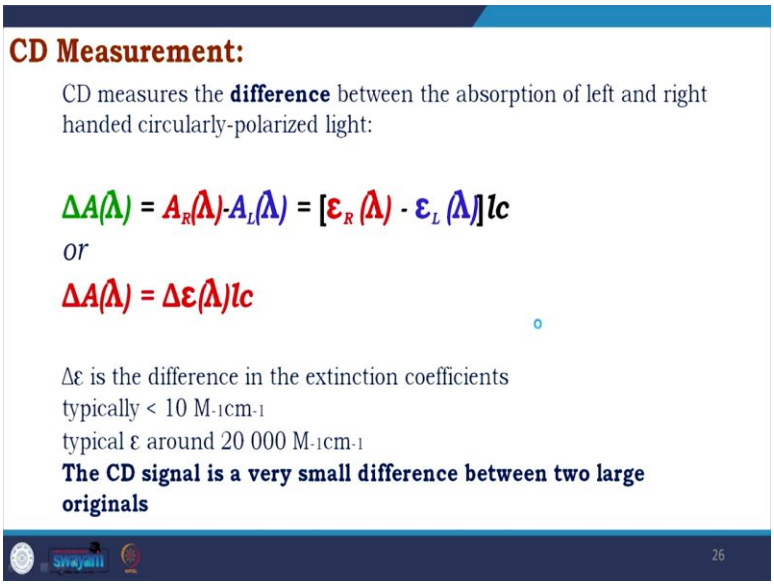

So again, the measurement here is also absorption spectroscopy, though CD could operate in other region too. The measure the proportion absorbed as a function of wavelength equal to log I 0 by I. I0 is the initial intensity and I is after the absorbents. So the Beer-Lambert law is applicable here. A equal to Epsilon of alpha lc. E equal to extinction coefficient observance is directly proportional to concentration c of absorbing species in the sample and path length of the light l though the sample. The longer the path or more concentrated the sample the higher the observance with the same principle we are talking about in UV. So, CD measures the difference between the absorption of left and right handed circularly polarized light. So

$\Delta A\ (\lambda) = A_R(\lambda) - A_L(\lambda) = [\epsilon_R\ (\lambda) - \epsilon_L\ (\lambda)] / lc$

Or you could also said

$\Delta A\ (\lambda) = \Delta\epsilon\ (\lambda)lc$

$\Delta\epsilon$ is the difference in the extinction coefficient typically less than 10 $M^{-1}cm^{-1}$.

Typically ϵ is around 20,000 $M^{-1}cm^{-1}$. The CD signal as we have discussed earlier also is a very small difference between two large originals.

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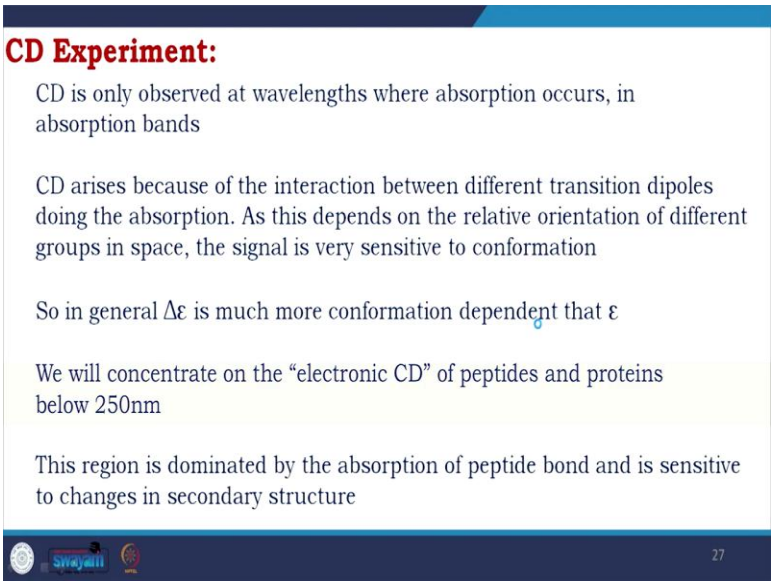

CD is only observed wavelengths where absorption occurs, in absorption bands. CD arises because of the interaction between different transition dipoles doing the absorption. As this depends on the relative orientation of different groups in space, the signal is very sensitive to the confirmation and that is why it is very critical for studying protein. So in general $\Delta\epsilon$ is much more confirmation dependent then ϵ.

We will concentrate on electronic CD of peptide and protein below 250 nm. This region is dominated by the absorption of peptide bond and is sensitive to changes in secondary structures,

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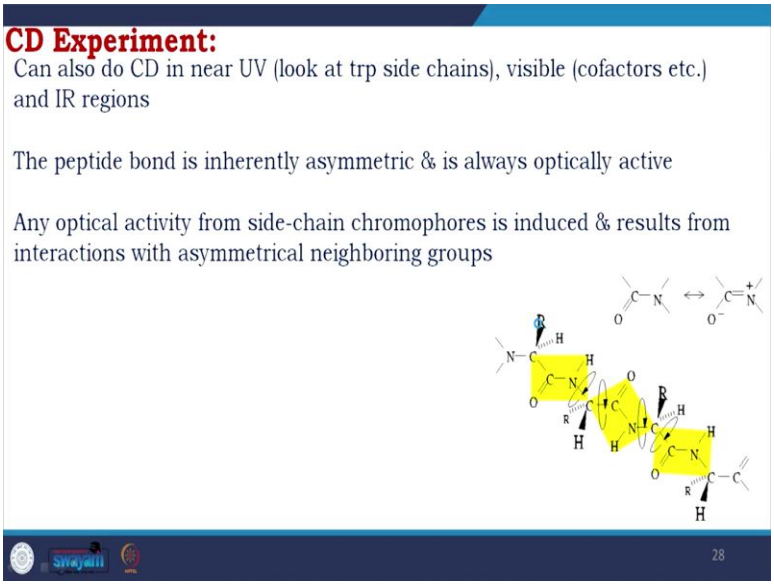

We can also do CD in near UV to look at trp side chains visible (for the cofactor) and higher regions. The peptide bond is inherently asymmetric and always optically active. Any opticaly active from side chain chromophores is induced and results from the interaction with asymmetrical neighboring groups.

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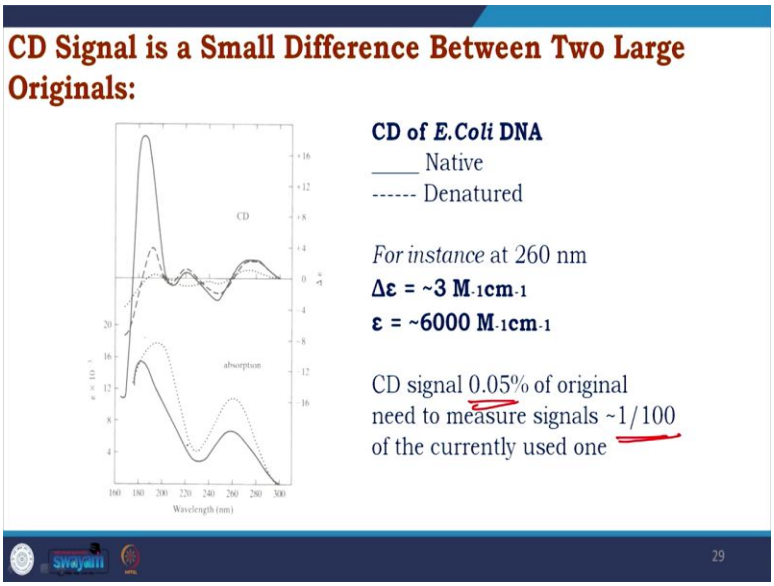

Coming to some examples, CD signal is small difference between two large original:

This is the circular dichroism of E. coli DNA. The bold one is for native DNA and dotted one is for denatured DNA. So as we told in case of nucleic acid at 260 nm

$\Delta\epsilon = 3\ M^{-1}cm^{-1}$ whereas

$\epsilon = 6000\ M^{-1}cm^{-1}$

So, CD signal is 0.05% original need to measure signals at 1 / 100 of the currently used one. If you see that, here the absorption is high, whereas when you are doing the CD, the difference is not at all high ($\Delta\epsilon$) and that is why we need different setup.

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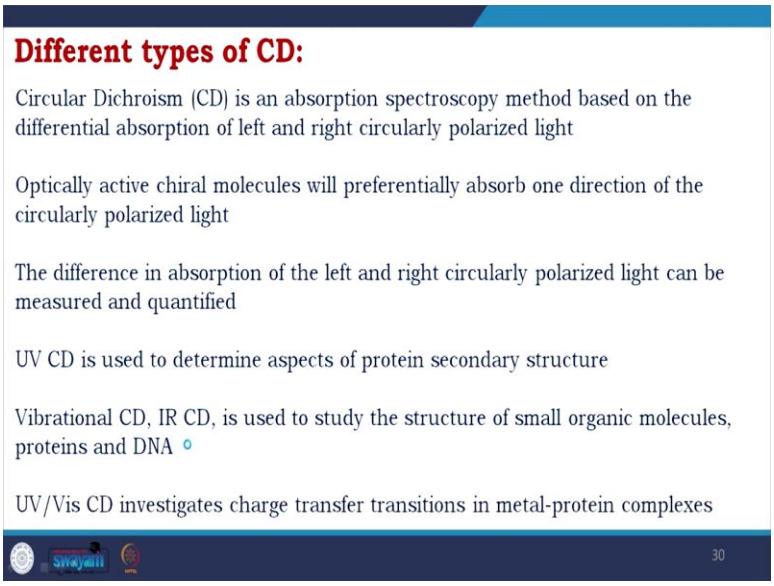

There are different types of (CD) circular dichroism. CD is an absorption spectroscopy method based on the differential absorption of left and right circularly polarized light. Optically active chiral molecules will preferably absorb one direction of the circular polarized light. The difference in absorption of the left and right circularly polarized light can be measured and also could be quantified.

UV CD used to determine aspect of protein secondary structure.

Vibrational CD, IR CD is used to study structure of small organic molecules, proteins and DNA.

UV Vis CD investigates charge transfer transitions in metal protein complexes.

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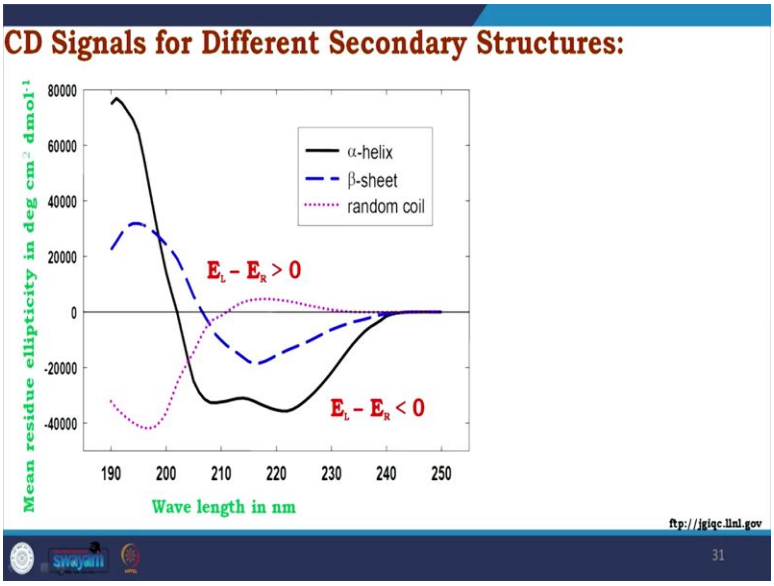

So, looking at CD signals of different secondary structure, so this is a graph, the x-axis is wavelength in nanometer and the y-axis is mean residue ellipticity in degree cm^2 $mole^{-1}$. In the plot you see two part, when $E_L - E_R$ is less than 0 and $E_L - E_R$ is greater than 0. So, black bold is alpha helix, the blue dotted a beta sheet and the dotted line is random coil.

These are the Fasman standard curves for polylysine in different environment.

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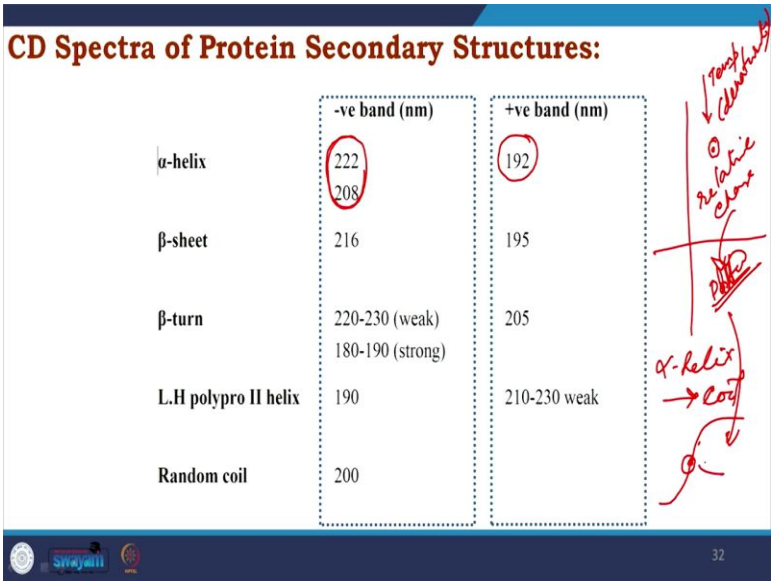

If you see there are two types of bands negative band and positive band. For Alpha helix, 222 nm and 208 nm as negative band. 192 nm is positive band. For beta sheet 216 nm is negative band, 195 nm is positive band. For beta turn 220 to 230 is the negative band (that is weak) 180 to 190 (is strong) and positive band is at 205. So for poly Pro II helix special type of polypropylene type helix, 190 negative band 220 to 230 weak positive. For random coil in negative band at 200 nm, so these are Fingerprint characteristic bands for different secondary structures.

For example, we know that for alpha-helix. We have negative Banda 222 and 208 and positive band at 192. So let this positive band is here. Now if and temperature there would be denaturation. By denaturation the Alpha helix would be converted to more or less random coil. So there would be change. And the relative change could be plotted to get a curve like that and the middle would be melting temperature. So in that way the fingerprints could be used for other work.

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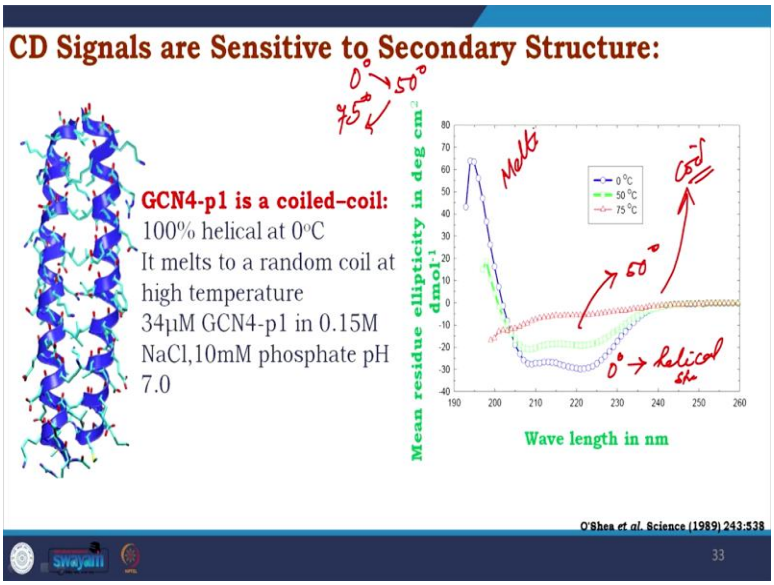

Let us take an example where CD signals are sensitive to secondary structures. So this is GCN4p1 it is a coiled coil. It is 100% helical at 0 degree centigrade (maintained that secondary structure). It melts to random coil at high temperature. So, this Alpha helical structure is converted to random coil when you provide temperature.

For doing this experiment this 34 μM of GCNP1 in 0.15 molar NaCl in 10 mM phosphate and 7 pH is being maintained. It kept at zero degrees centigrade; it is heated at 75 degree centigrade and 50 degree centigrade. So, 0, 50, 75 and when it was plot against wavelength and mean elipticity, you can see that at 0 degree it maintained the helical structure. Whereas at 50 degree it deviated, and at 70 it is mostly random coil. So by this if you take more points you could actually calculate the melting temperature which I am talking about. So this is a very important experiment because this is not only for this protein. It could be done in any protein and because this is based on the fingerprint. So you could have very accurately calculated them.

Same experiment can be performed with pH or anything that could deviate the structure that could defeat the confirmation.

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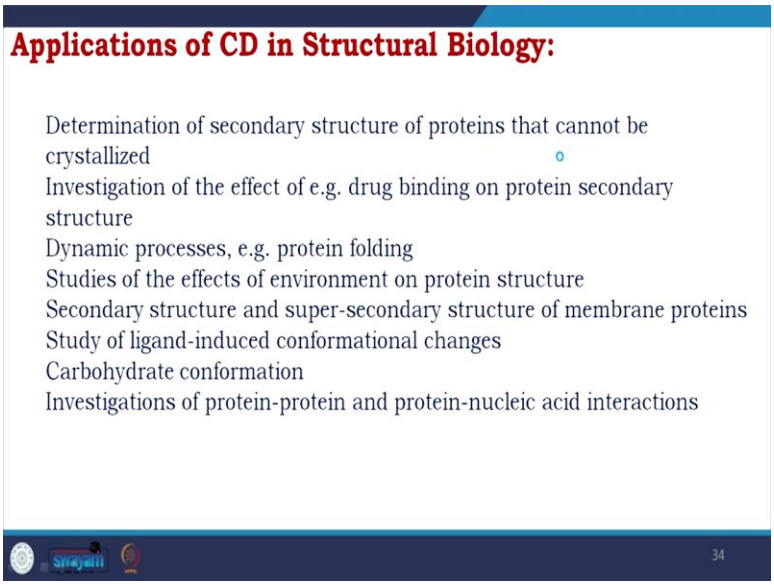

So, application of CD in structural Biology, it is helping in determination of the secondary structure of the protein that cannot be crystallized or you do not get NMR structure. Investigation of the effects of drug binding on protein as drug binding change the confirmation, so you could study that. Dynamic process of protein folding with increase in temperature and pH. Similarly you have an unfolded protein and you are kind of removing the condition which is causing the unfolding then it would start refolding and you will get to see the change in the confirmation. Studies on the effect of environment of protein structure. Secondary structures, super secondary structure of membrane proteins. Studies of ligand induce conformational change of the protein. Carbohydrate conformations, carbohydrate because of their forming of straight chain bond, branch chain bond. So straight chain, branch chain and when they form the folded structure, it could be of any combination and permutation. So is very difficult to detect or predict it. The investigation of protein-protein and protein-nucleic acid interaction, interaction makes changes, especially when macromolecules are interacting they make changes in the confirmation that change is reflected on the CD spectra.

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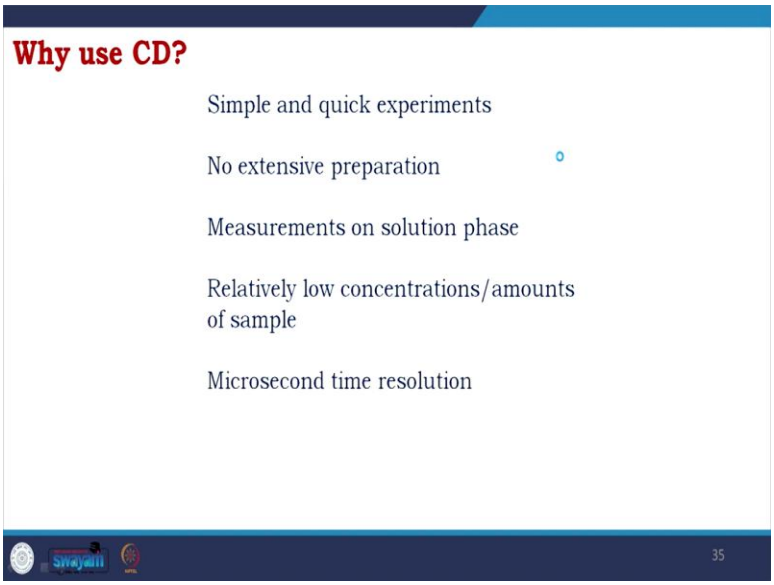

Why use CD? First very simple very quick experiments. No extensive preparation. You just have a pure protein you put in proper buffer and do the experiment. Measurement of solution phase throughout the experiment was at the course, when you are getting a protein at a specific state like a crystal that is not giving you the information which is biologically relevant. Crystallography definitely gives the co-ordinate of a protein, its best thermodynamics form, but it is not as good as solution structure.

CD could be stored in solution phase, so that is another advantage. Relatively low concentrations, microsecond time resolution, any sizes of macromolecules (size-independent) can be evaluated.

However electron microscope you are going to study will not work with smaller molecules. X-ray cannot work if crystallization does not happen, CD anything and everything.

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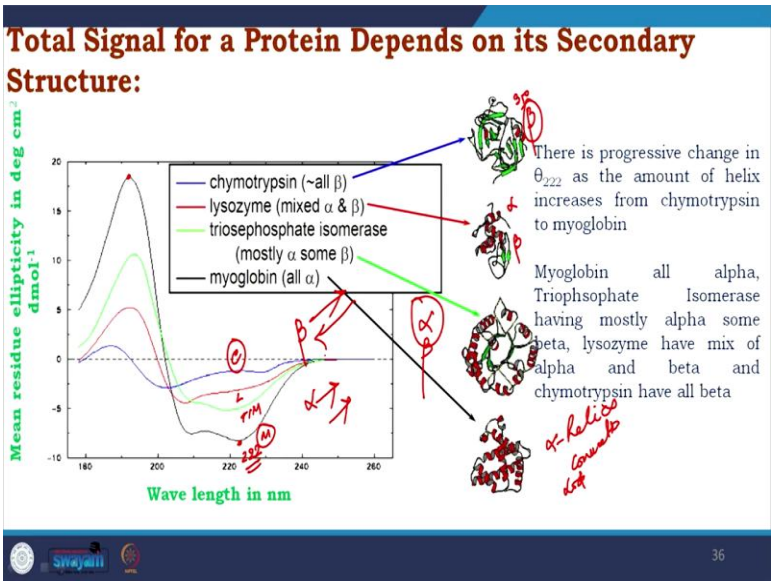

This is where we are shifting from a particular secondary structure to a proper protein. So four proteins are taken. What are the four proteins? One is myoglobin which you see is all Alpha, you could see here only Alpha helices connected to loop. Triophosphate isomerase having mostly alpha and some beta. Lysozyme has mix of alpha and beta, chymotrypsin has all beta. So when you plot them you see a characteristic for all alphas. So there is progressive change in molar elipticity at 222 nm when amount of helix increases from chymotrypsin into myoglobin.

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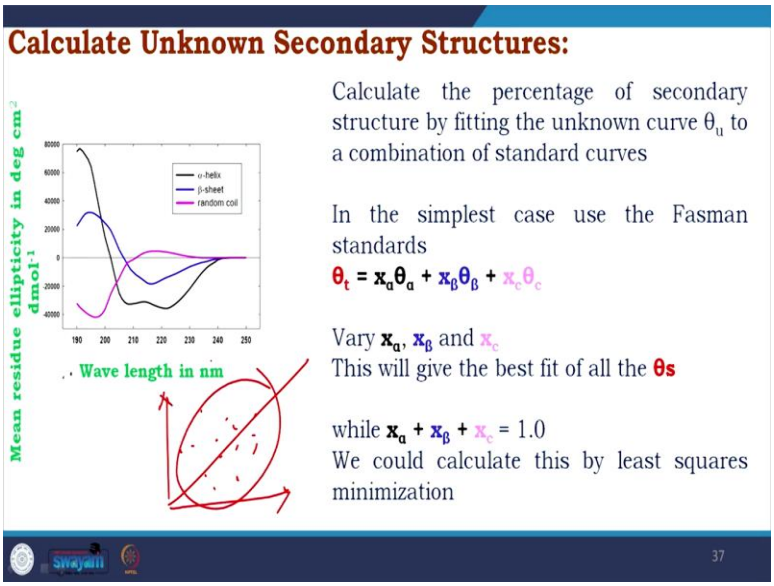

And these types of data are actually calculated and now they are used for determining unknown secondary structure. This particular graph calculate the percentage of secondary structure by fitting the unknown curve θ_u to a combination of standard curve. In the simplest case you use the

Fasman standard, you already know in Fasman standard, poly lysine curve and many other curves are actually used.

So the formula used

$\theta t = X\alpha\ \theta\alpha + X\beta\ \theta\beta + Xc\theta c$

Where, α is the alpha helix contribution and β is beta sheet combination and c is the random coil. Now there would be varying alpha, beta and coil and this will give the best fit of all the θ.

And if you consider

$X\alpha + X\beta + Xc = 1$

we could calculate this by least square minimization.

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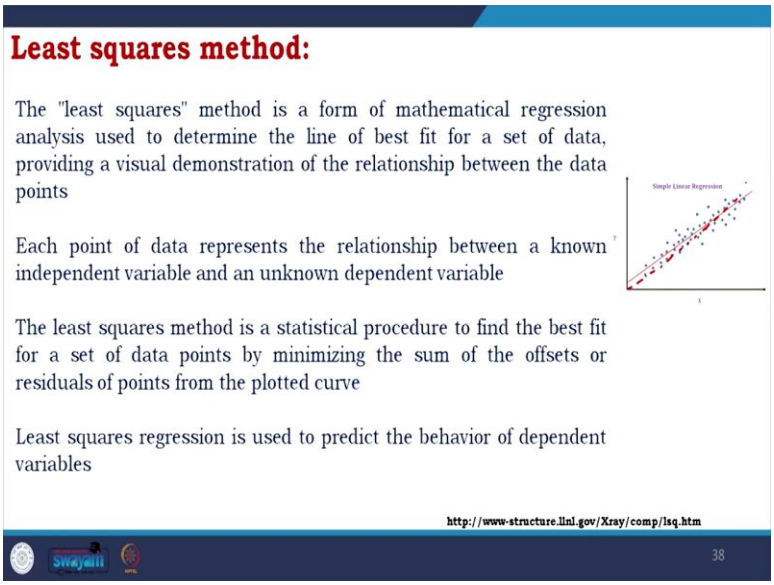

The least square method is a form of mathematical regression analysis used to determine the line of best fit for set of data providing a visual demonstration of the relationship between the data points.

Each point of data represents the relationship between a known independent variable and an unknown dependable variable. The least square method is a statistical procedure to find the best fit for the state of data points by minimizing the sum of the offsets or residual of points from the plotted curve. Least square regression is used to predict the behaviour of dependent variable. So here we get a lot of standard data put them and then put our unknown and determine their.

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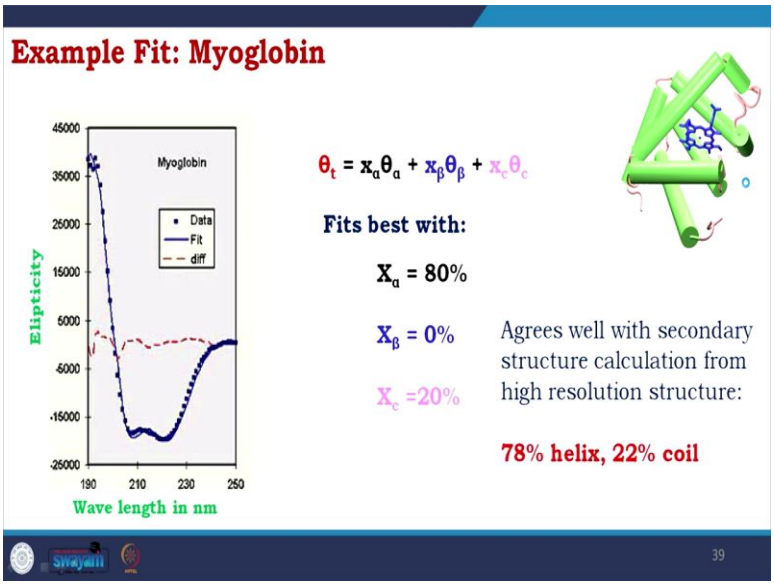

So, example we take myoglobin. The formula is

$\theta t = X\alpha\ \theta\alpha + X\beta\ \theta\beta + Xc\theta c$

The fit gives us best point where x alpha is equal to 80, x beta is equal to 0 and x coil is equal to 20. So from the linear regression calculation this was found and this is myglobin.

So let us see what is the actual prediction from the high-resolution structure? It agrees well with the secondary structure calculation from high-resolution structure. 78% helix and 22% coil so this x alpha 80 is very close to 78 and 22 is also close to x coil and there is no beta.

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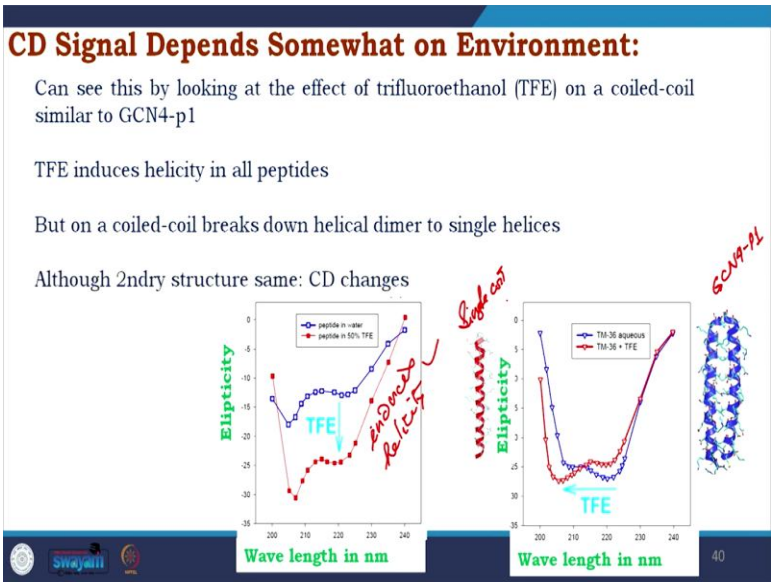

CD signal depend somewhat on environment will discuss that point. We can see this by looking at the effect of trifluoroethanol TFE on a coiled-coil similar to GCN4-p1. TFE induces helicity in

all peptides. So if you take any peptide and you introduce 50% TFE, TFE is making a change from this peptide to more helical.

Though TFE induces helicity in peptides, at the same time coil-coil breaks down helical dimer to single helices, and major we have seen that they maintain same secondary structure.

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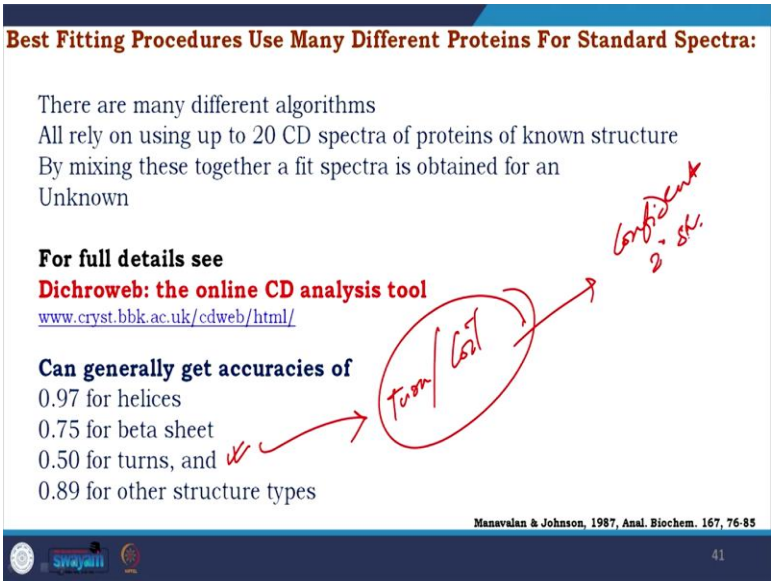

There are many different algorithms rely on using up to 20 CD spectra protein of known structure. By mixing those together a fit spectra is obtained for an unknown. So there are different software I recommend for these to go for Dichroweb the online CD analysis tool. The link is given here, one can generally get accuracy for 97% of helices. For helices calculation is always easy because of their stability. 75% for beta sheet, 50% for turn, and 89% for other structure type.

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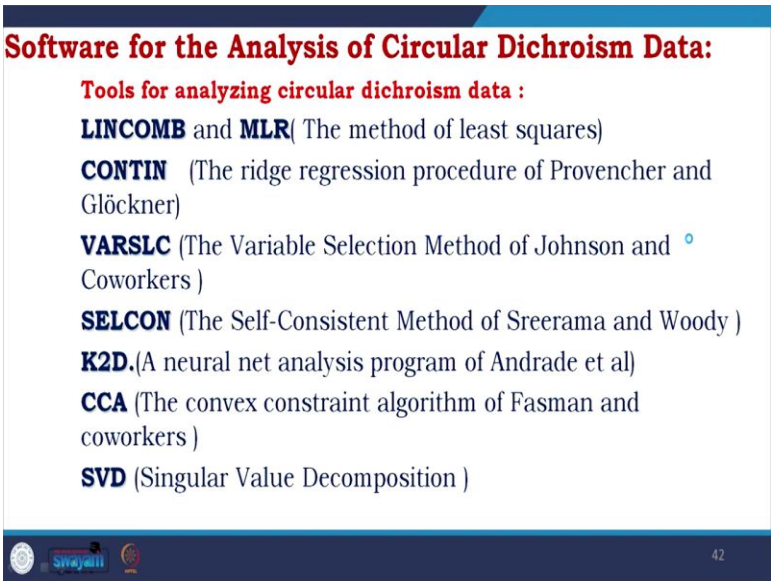

There are tools for analysing circular dichroism data. LINCOMB and MLR are the method of least squares. CONTIN the Ridge regression procedure of Provencher and Glockner. VARSLC the variable selection method of Johnson and Coworkers. SELCOM the self-consistent method of Sreerama and Woody. K2D dot neural network program of Andrade et al. CCA the convex constraint algorithm of Fasman and Coworker. SVD singular value decomposition.

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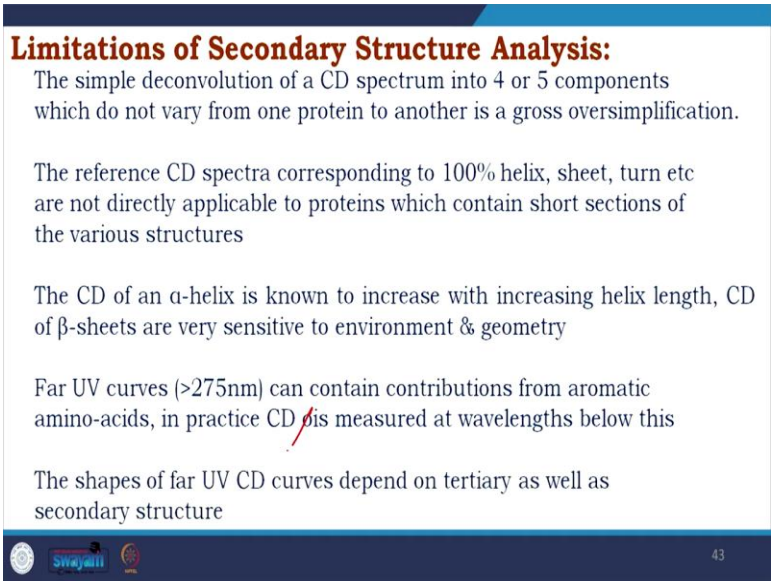

There are limitations of the CD method in Secondary Structure Analysis: The simple deconvolution of CD spectrum into four or five components which do not vary from one protein to another is a cross-over simplification. The reference CD spectra corresponding to 100% helix, sheet, turn etc are not directly applicable to protein which content short section of the various

structures. The CD of an Alpha helix is known to increase with increasing helix length. CD of beta sheet are very sensitive to environment and geometry and their CD spectra calculation never works.

For UV curves greater than 275 nanometer and content contribution from aromatic amino acid, and in practice CD is measured at wavelengths below this (near UV CD). The shapes of far UV CD curves depend on tertiary as well as secondary structure.

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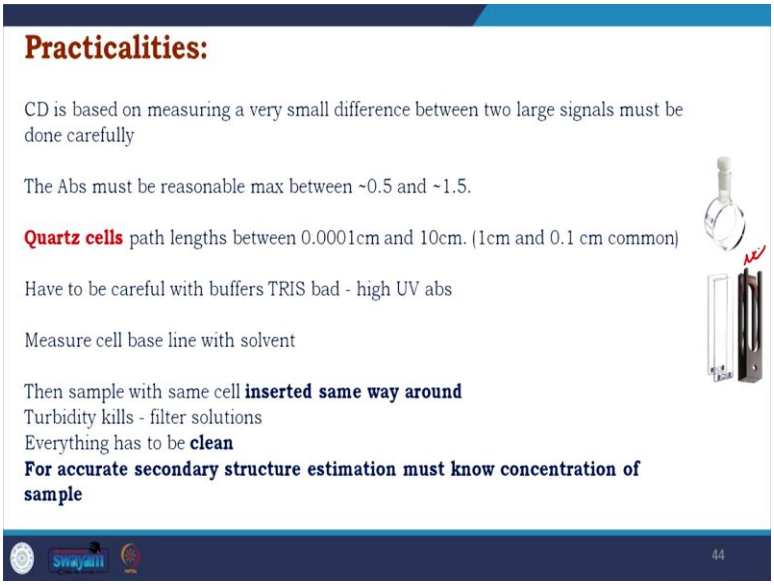

Some practicalities to work CD is based on measuring a very small difference between two large signal must be done carefully. The absorbances must be reasonable Max between 0.5 to 1.5. Quartz cell which is shown here, path length between 0.0001 cm and 10cm (1cm and 0.1cm are common). Have to be careful with buffers (TRIS bad- high UV absorption). So TRIS buffer you cannot use in the CD Spectroscopy.

We generally used phosphate buffer, but you could you use other buffers too. Measures cell baseline with solvent is very critical. Then sample with same cell inserted same way around. Turbidity kills and filters the solution. Everything has to be very, very clean. For accurate secondary structure estimation must know the concentration of the sample.

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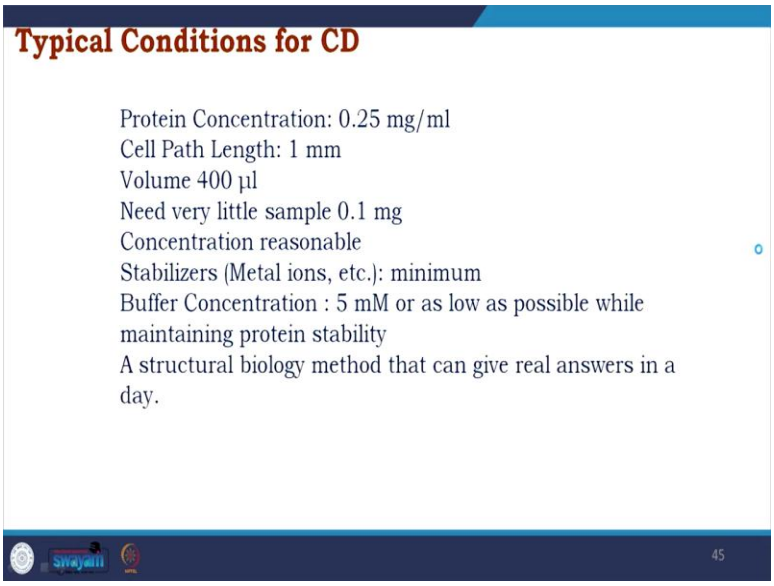

Some typical condition for getting good circular dichroism; Protein concentration 0.25 Milli gram per ml, cell path length 1 millimetre, volume 400 microlitre, but depending on the CD. Cuvette need very little sample 0.1 milligram concentrations are pretty reasonable, stabilizer metal ions etc should be used minimum, Buffer concentration 5 millimolar for as low as possible while maintaining protein stability. If you do not maintain the protein stability, your experiment is not going to work.

Protein stability is the most important thing, you have to maintain the protein stability. But while you are maintaining the protein should not forget that these other stabilizers have their own CD property, Structural Biology method that can give real answers in one day.

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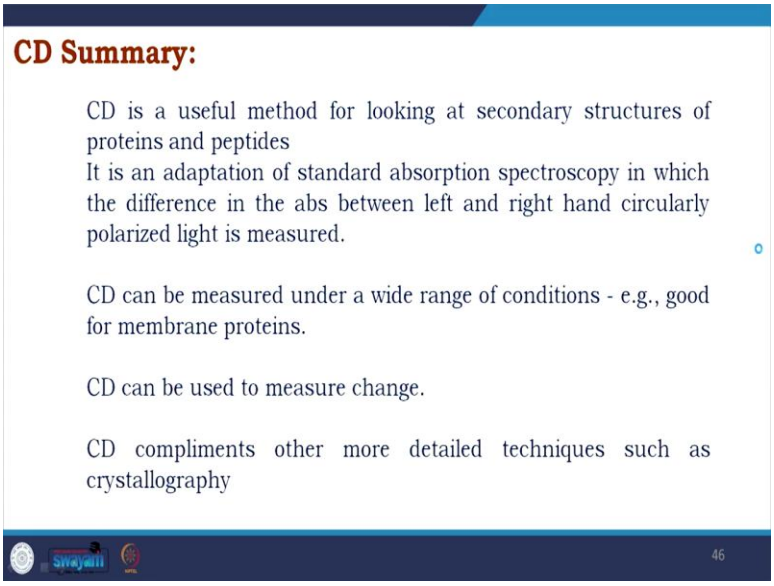

Summarizing CD is a useful method for looking at secondary structures of proteins and peptides. It is an adaptation of standard absorption spectroscopy in which the difference in the absorption between left and right hand circularly polarized light is measured. CD can be measured under a wide range of conditions. It is very good for membrane proteins we did not discussed about membrane proteins in this module but CD is very differently applied and as membrane proteins are difficult to study. So this is one of the very interesting parts of CD.

CD can be used to measure change, CD complement other more detail techniques such as x-ray crystallography the findings in x-ray crystallography would be very well coordinated with the CD structure. So with this I have talked about UV absorption, UV visible absorption and CD. These are techniques which have taken a very important part in determining different properties of protein. As I told UV vise especially important for operating a regulating reactions in enzymes acid, enzyme catalysis.

CD is good because it different approaches taken here. Where all the Spectroscopy is best known linearly polarized light here, circularly polarized light and their difference is taken. So specially for protein CD is very critical and CD experiments are really informative. With that I would finish today's talk. As I told earlier keep listening to the classes and keep asking questions to us. Thank you very much.