Analytical Technologies in Biotechnology
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Module - 6
Spectroscopic Techniques
Lecture - 4
Circular Dichroism (CD) Spectroscopy

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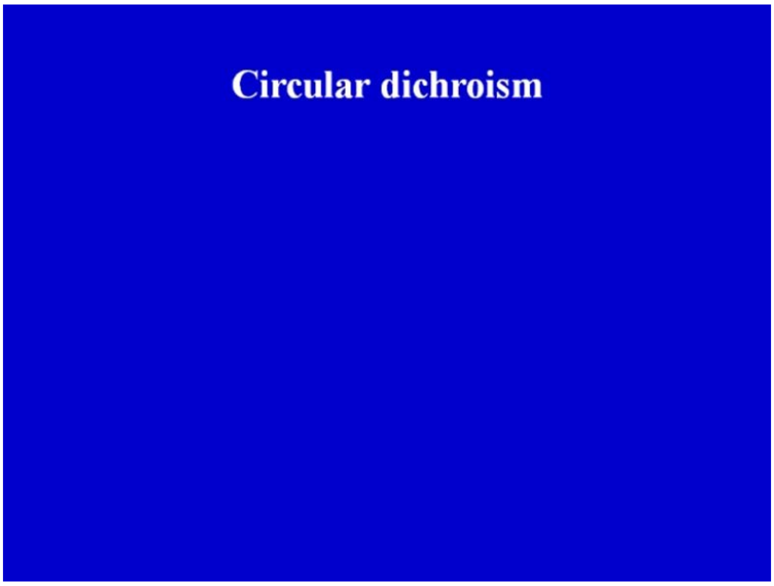

In this lecture, we are going to discuss about another important spectroscopic technique and that is Circular Dichroism. Circular Dichroism or in short CD refers to the differential absorption of left and right circularly polarized light. Now, this phenomenon was first discovered by Biot Fresnel and cotton in the first half of the 19 th century, it is exhibited in the absorption bands of optically active chiral molecules. When we say chiral molecules, it is a type of molecule that has a known supreme possible mirror images.

Dichroism is the word which is derived from Greek, which means two colors and because the sample under analysis has one color if eliminated with the right, polarized light and a different color if illuminated with the left one, so the color in fact, depends on the light absorption here. Now, we see there is a growing realization of the need to perform, structural, studies under the conditions in which proteins are actually operating, that is they are in solution. As well as under other conditions, and to provide measures of the rates of structural changes of proteins, which are often essential to their biological function.

As you can recall in previous lecture we were talking about the infrared spectroscopy, which to a certain extent helps in structural helps in providing the structural information. But, circular Dichroism has a lot of different parameters could be used and lot of different conditions you can work around and get lot of useful information. Remember these techniques, are not like NMR or X-ray crystallography, which gives you a three dimensional structure that is exact position of atoms or mean position of atoms.

But, rather these are an indicative of what kind of structure a protein or other macromolecules posses. As well as it can also give a various conformational changes in different conditions, which could be temperature p H or other conditions. So, the circular Dichroism has become increasingly recognized as a valuable structure technique, for addressing for these issues which are to obtain information, structural information here.

So, CD spectroscopy has a very wide range of applications in many different areas, and most importantly like the UV CD or ultraviolet CD ultraviolet region has been used to investigate the secondary structure of proteins. Then UV based we can say CD is an investigative charge transfer, for charge transfer transitions and other near infrared CD could be used to investigate, geometric and electronic structure by probing metal transitions.

Vibrational circular dichroism which uses light from the infrared energy region is used for structural studies of small organic molecules, and most recently proteins and DNA. So, there could be variations of these CD technique, as in the electromagnetic spectrum and for various applications now let us start like we said CD is differential absorption of right and left circularly polarized light let us talk little bit about polarized light.

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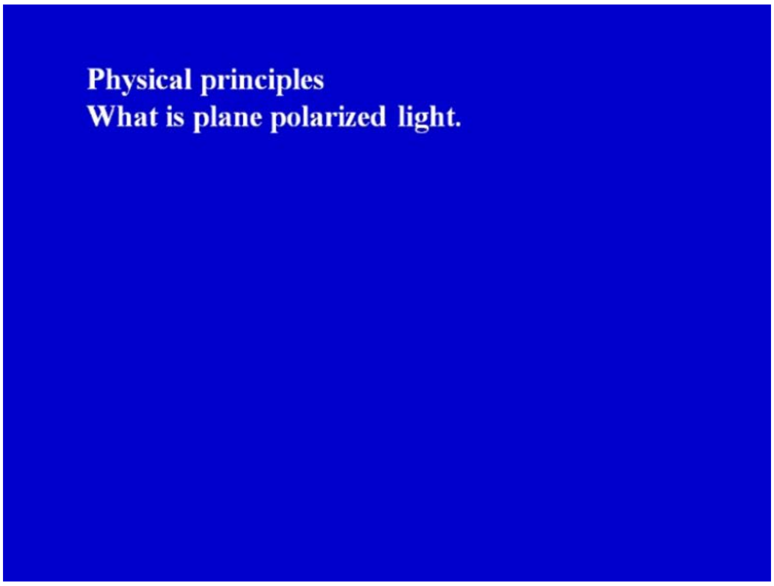

First things is what is plane polarized light? Now when you see light it is like as we have discussed it has two like light can be separated into two forms of plane polarized light parallel and perpendicularly. Parallel and perpendicular polarized light, and this like when we considered here, plane polarized light the plane of electric vector is taken.

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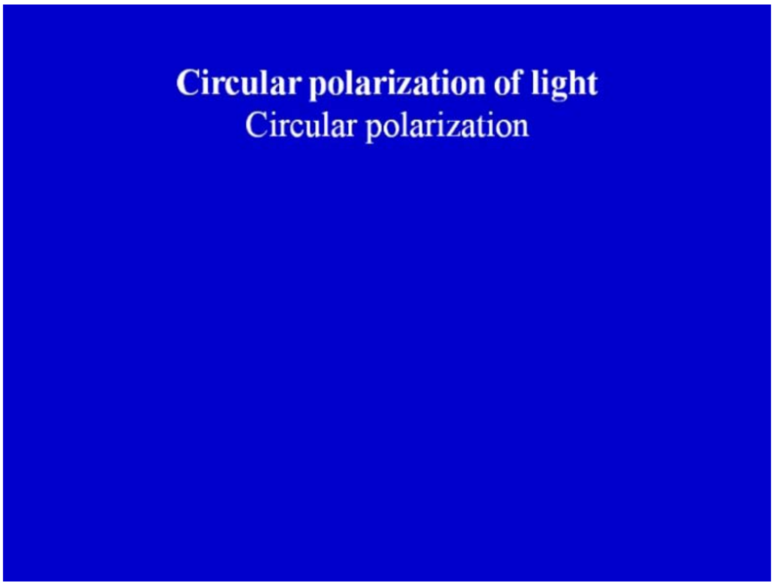

So, we come to circular polarization of light that is what we are going to discuss, now electromagnetic radiation is the form of energy. And it has both like I said electrical and magnetic characteristics, and electric and magnetic field that oscillate perpendicular to one another, and to the propagation direction x. So, while linearly polarized light occurs when the electric field vector oscillates only in one direction. So, here when we are talking about plane polarized light, we talk about electric vector and when it oscillates only in one plane, then it is a plane polarized light, whereas, circularly polarized light will occur when the direction of the electric field vector, rotates about its propagation direction. While the vector retains the constant magnitude, remember here the circularly polarized light is generated by combining two plane polarized light, and they are differ in say quarter waves length. Say plus 90 degree or minus 90 degree like they are oriented and when they combined there is particular resultant here, which is electric field vector which is rotating about it is propagation in a helical manner. And that is the you can say circularly polarized light is obtained.

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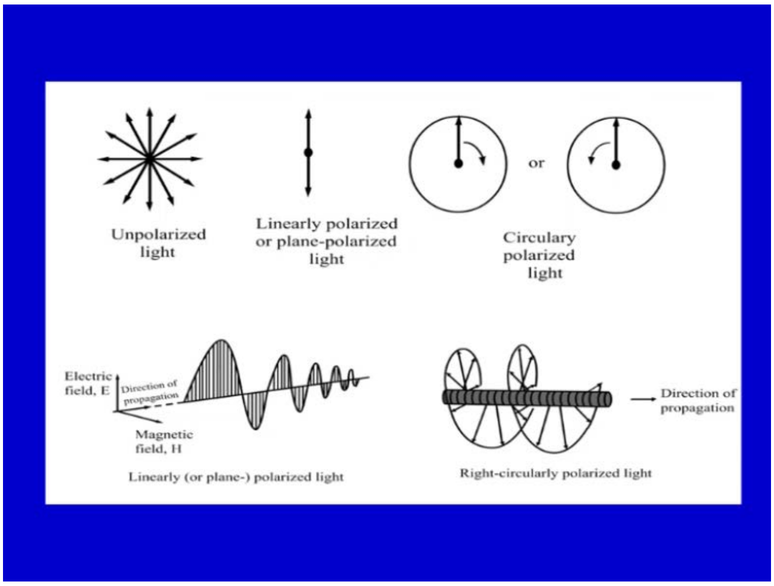

Just to understand here, if you see here this is what we called the un polarized light that is it is vibrating, if you take electric vector plane here. So, that vector or that is vibrations are in all different directions, so it is not a polarized light it is called un polarized light, then if through certain means you can make light to or electric vector to vibrate in only one plane then it is called plane polarized light. And then there is circularly polarized light, which could be in two directions.

Here you can see, this figure here it shows the plane or linearly polarized light or plane polarized light, which is it is oscillating in only one direction. And if you see here, this is the circularly polarized light and this is direction of propagation, so like it I said it could be obtained by combing two polarized light at 90 degrees.

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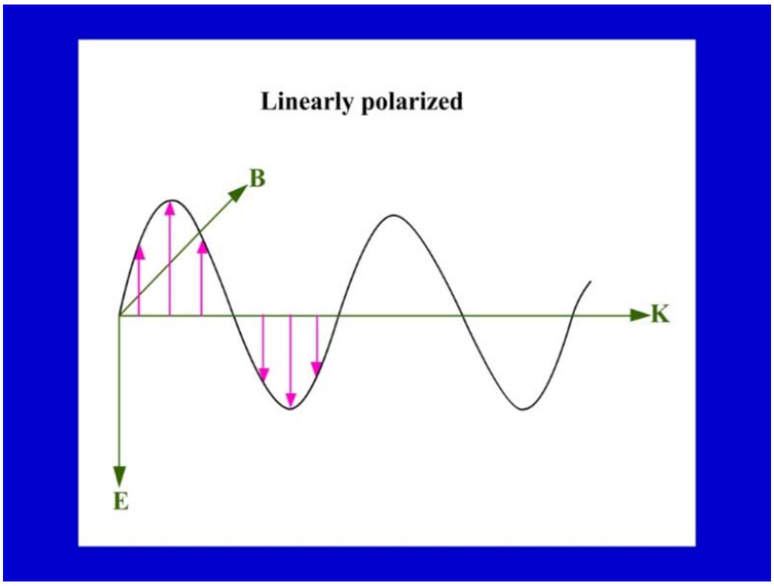

This is another representation here of linearly polarized light, now at a single point in a space, the circularly polarized vector will trace out a circle actually, over one period of the wave frequency hence the name has come from there. So, what we have seen that electric vectors of linearly and circularly polarized light, at one moment of time for a range of positions. We say the plot of circular polarized electric field vector forms the helix along the direction of propagation.

So, for left circularly polarized light with propagation towards the observer, the electric vector will rotate counter clockwise for the left circularly polarized light. For right circularly polarized light from direction of propagation to like as observer is seen, this is rotates clockwise. So, for left circularly it is counter clockwise and for right circularly like right circularly polarized light, it is clockwise as per the observers position.

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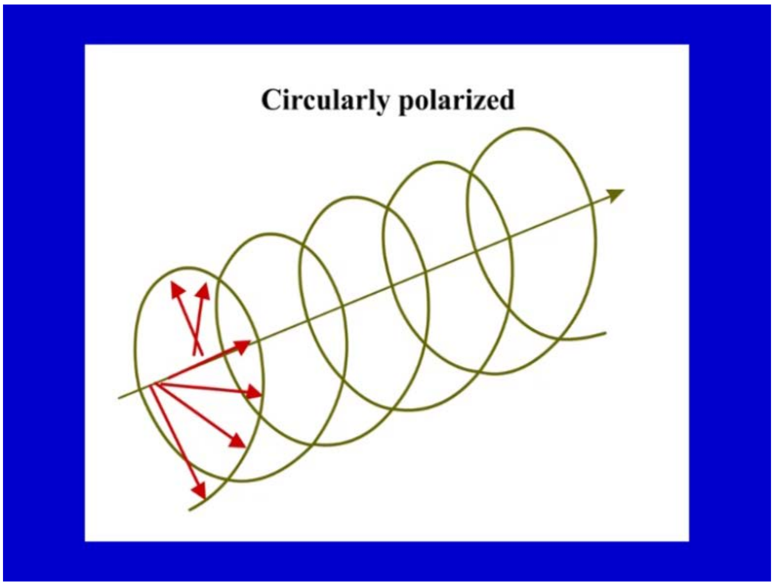

So, you can see this is in a particular direction and it will be set it is a circularly polarized light in propagating in a particular direction. So, this is how a circularly polarized light is produced, so there could be left circularly polarized light and there could be right circularly polarized light.

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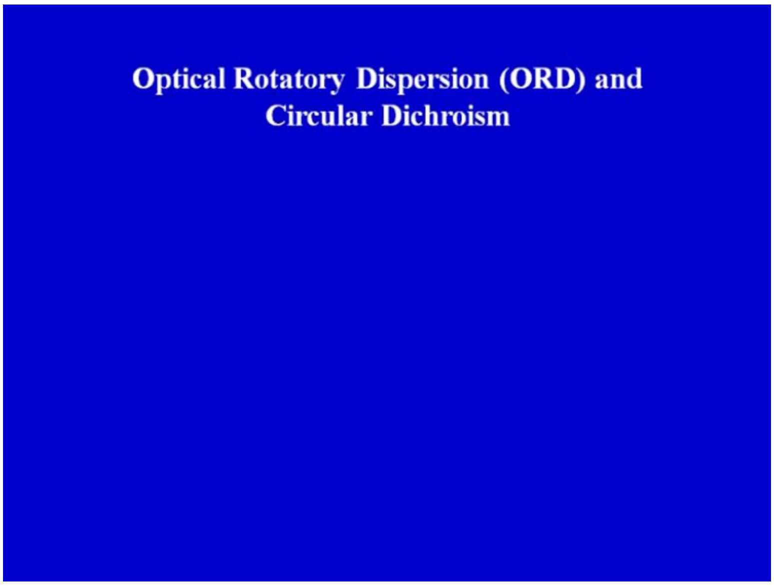

Now, there are two techniques here one is called Optical Rotatory Dspersion and another one which we were talking about is circular dichroism. Now, most of the biological components are optically active and thus able to rotate the plane polarized light. Now, here ORD that is Optical Rotatory Dispersion method measures the ability of optically active compound to rotate plane polarized light, as a function of the wavelength.

But...and a instrument which is utilized for ORD or which measures and produce ORD curves is called spectro polarimeter, which differs from polarimeter in that the later employs only one wavelength. Then there is another technique is circular dichroism, which where circularly polarized light is used, and circularly polarized light is obtained by like. I said supreme closing two plane polarized light of same wavelength and amplitudes, which are polarized in two perpendicular planes.

But, there is a phase difference between the two that is the quarter wavelength phase difference between them, and CD’s observed when optically active metal absorbs left and right circularly polarized light differentially or slightly differently. And this absorption is quantified by term known as molar extension coefficient, for optically active samples have distinct molar extension coefficient, for left and right circularly polarized light. So, in a CD experiment equal amount of left and right circularly polarized light of a selected wavelengths are alternatively radiated into chiral sample.

Remember only chiral samples are here one thing has to be remembered that these chirality can also come due to confirmations or structural particular kinds of structure, and also chirality of certain molecules. So, one of the two polarizations is absorbed that is either right or left, more than the other one. And these wavelength dependent difference of absorptions are measured in CD, which yields the CD spectrum of the sample. So, every sample will have a characteristic spectrum.

Now, due to the interactions with the molecule, the electric field vector of the light traces out an elliptical path, after passing through the sample. Now, it is important that the chirality of the molecule can be conformational rather than structure that is the for instant a protein molecule, with a helical secondary structures can have a CD that changes with changes in the confirmation.

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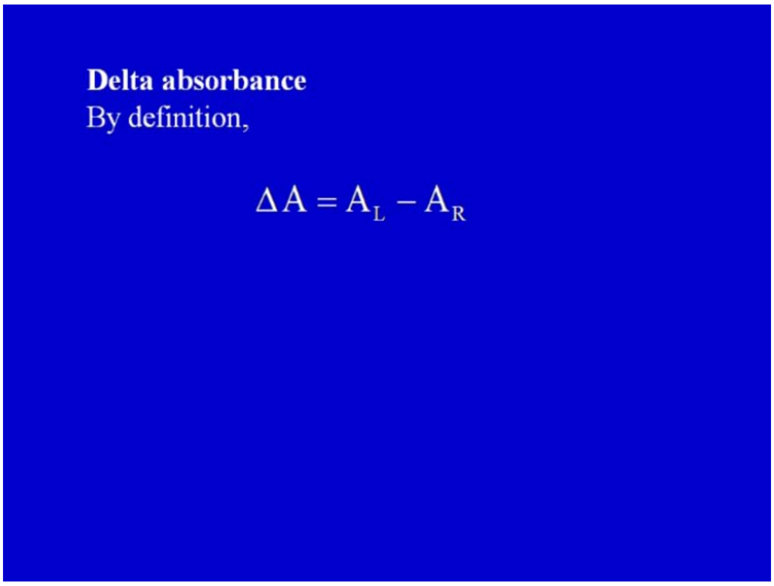

And, so when you like consider for recording this, this one is you record the delta absorbance or that is the changes difference in the absorbance, which is delta A equals A L minus A R which is A L is for left circularly polarized light, absorbance for left circularly polarized light and R is A R is for right circular polarized light. So, this difference is recorded or measured and this is a function of wavelength, so for measurement to be meaningful, the wavelength at which it is performed must be known that is very important. So, because every time if you change wavelength the absorption will be different, so it is a wavelength dependent absorption.

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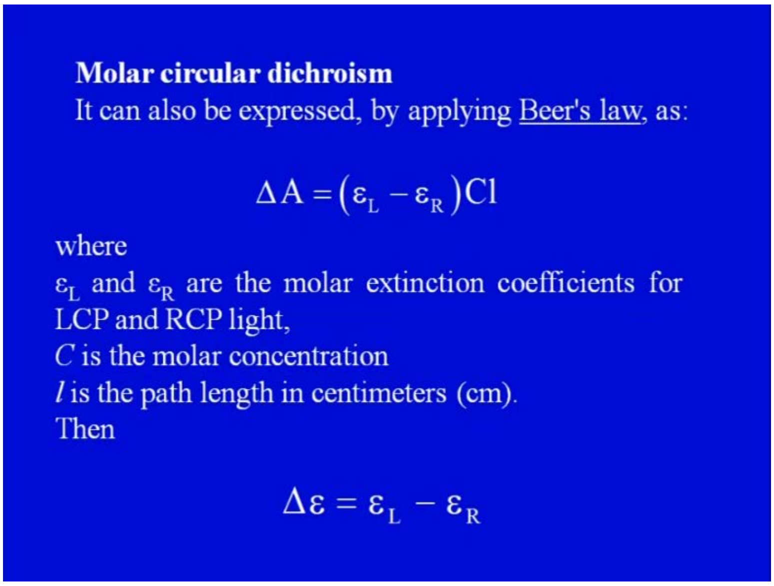

There is another term, which is called molar circular dichroism or it could be expressed in this form as beer's law we have seen that is delta a equals, the molar extinction coefficient which is difference between the two left and right circularly polarized light and concentration and path length. So, here if you say the delta e will be can be taken which is the difference in the molar extension coefficient, as we were discussing about molar extension coefficient if you could recall.

This will be like, here the difference between the two will be taken that is left and right circularly polarized light. So, this is the molar circular dichroism, and this intrinsic properties what is usually meant by the circular dichroism of the substance, so since delta epsilon or molar extension coefficient is a function of wavelength, a molar circular dichroism value must specify the wavelength at which it is valid.

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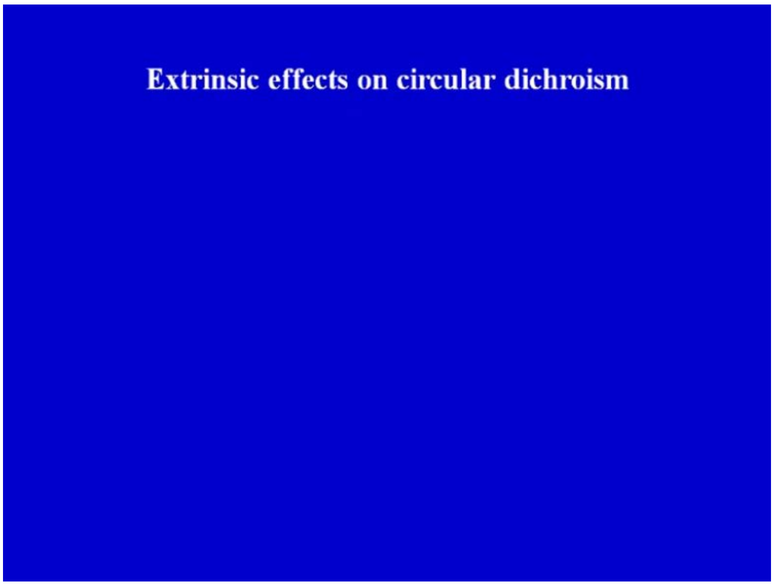

So, this delta epsilon value is very important here, in many practical applications of circular dichroism, as we discussed here the measured CD is not simply and intrinsic property of the molecule. But, rather depends on the molecular confirmation, and in certain in such a case the CD may also be a function of say temperature concentration, temperature it may be function of concentration, chemical environment around like say for example, solvents and other things present in there.

So, in the case of reported CD value one must also specify these other relevant factors in order to be meaningful for these values actually. So, that is very important that when we are reporting CD values as we measure, in terms dichroism or in molar extinction coefficient whatever terms. It is very important to specify other relevant parameters as well.

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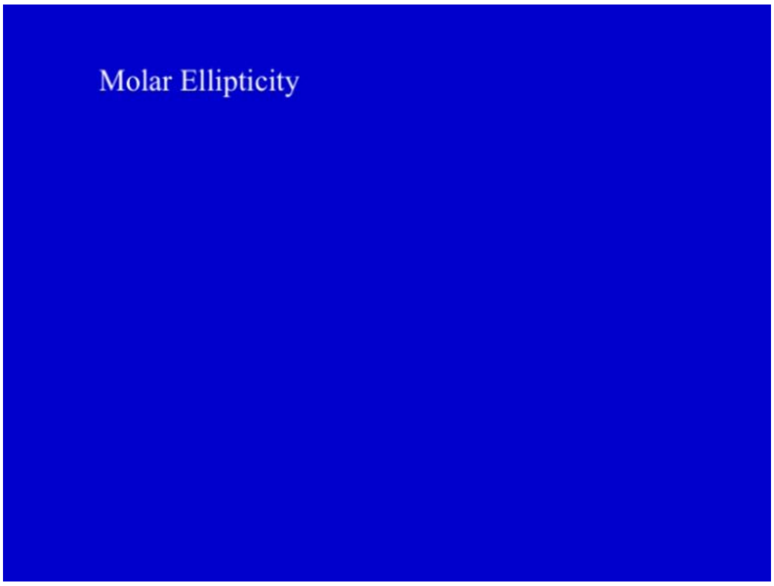

Now, there is term called molar ellipticity, so as we were discussing about delta A or absorbance differences in the absorbance, which is usually measured for different reasons. But, like most measurements are reported in degrees of ellipticity, and molar ellipticity is circular dichroism corrected for concentration, molar circular dichroism and molar ellipticity are readily inter converted.

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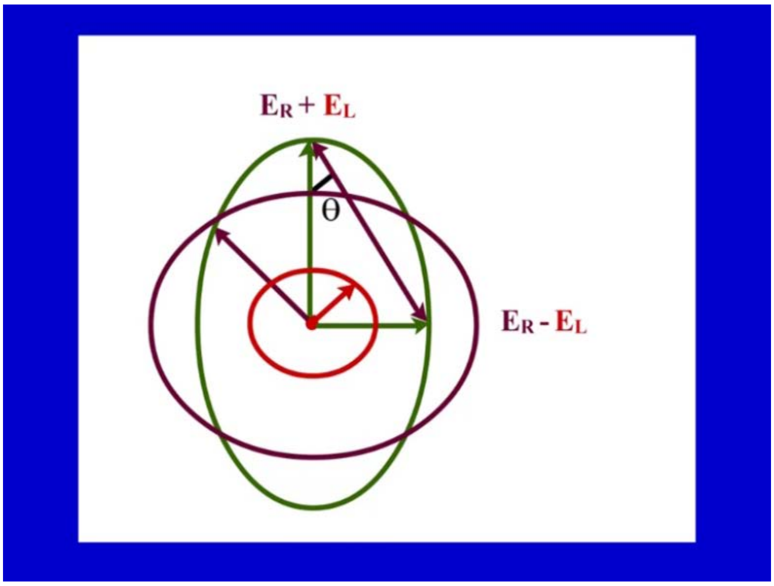

So, here if you see in this figure there are vectors E R plus E L and here E R minus E L this is the angle here for, so this is E R and this is E L here. So, this could be expressed in terms of elliptically polarized light or as we were saying that this could be converted like the two values molar circular dichroism, molar ellipticity could be converted by this particular equation here. So, elliptically polarized light say it is composed of unequal contribution of right and left circularly polarized light, and this relationship is derived by defining the ellipticity of the polarization in terms of this equation.

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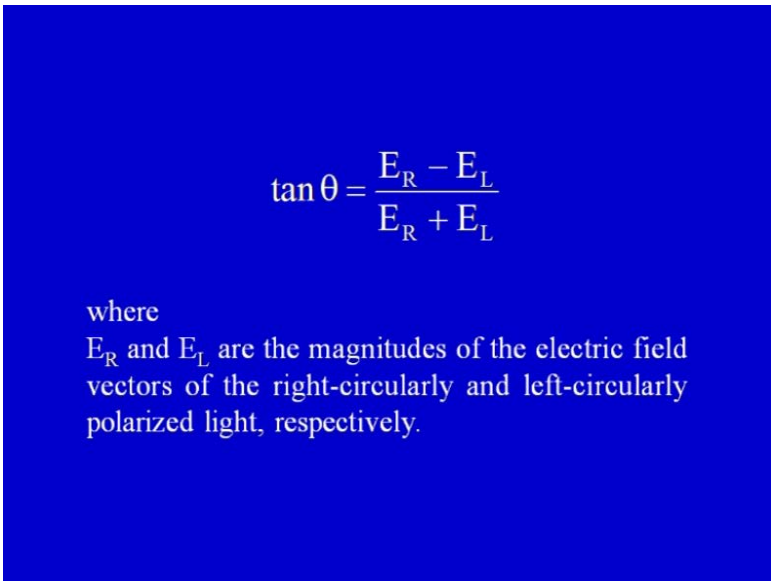

Now, here E R and E R are the magnitudes of the electric field vectors of the right circularly polarized and left circularly polarized light. Now, the units of molar ellipticity they are mostly expressed in terms of degree centimeter square, per decimole and to calculate molar ellipticity, the sample concentration cell path length, in centimeter sample concentration gram per liter. And the molecular weight gram per mole must be known. If the sample is the protein say the mean residual weight that is average molecular weight of the amino acid it contents is used in place of the molecular weight because essentially treating the proteins has the solution of amino acids.

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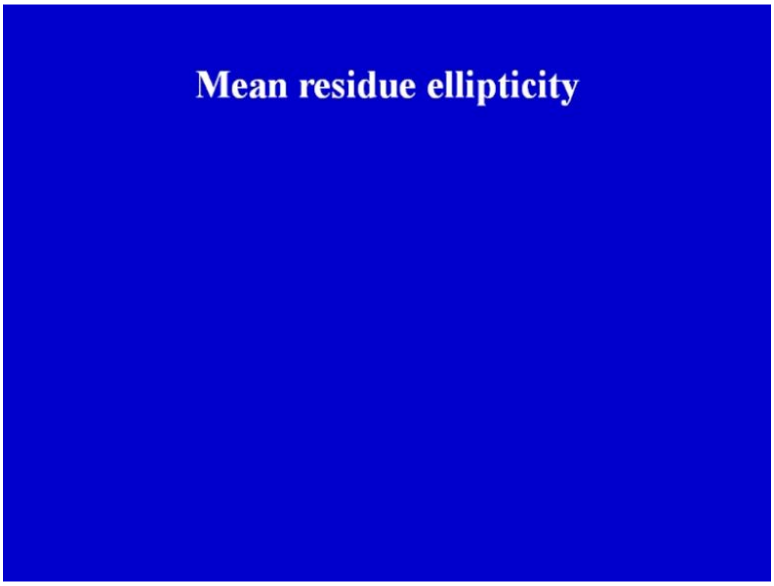

Mean residue ellipticity is another term, like for methods estimating secondary structures say in polymers like proteins, and polypeptides in particular it often requires that measured molar ellipticity spectrum, be converted into a normalized value. Specifically a value independent of the polymer length, so mean residue ellipticity is used for this purpose, and it is simply the measure molar ellipticity of the molecule, divided by the number of monomer units in the molecule. So, that way you can calculate mean residue ellipticity.

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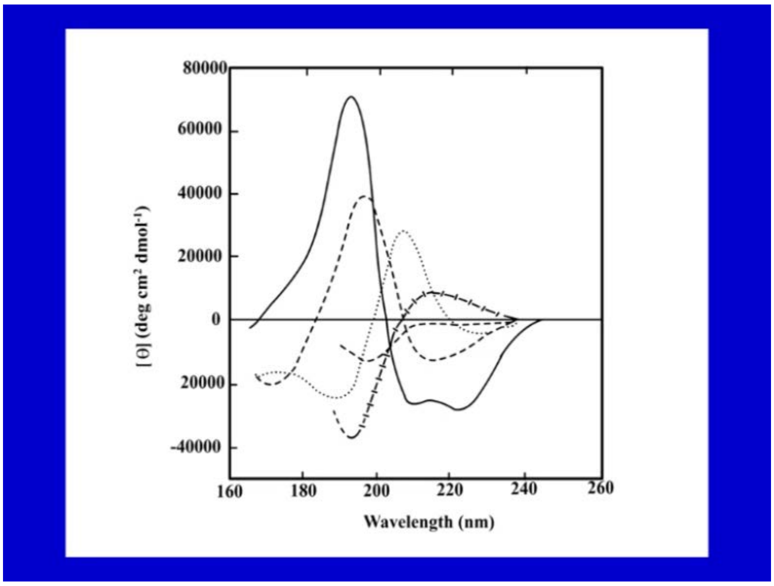

Now, this is a typical CD spectrum of protein for different kinds of confirmations, let us discuss them here 1 by 1. If you see here, this is solid lines here, this spectrum is for alpha helical confirmation in a protein, and if you can see there are two negative peaks here, which are around ((Refer Time: 19:14)) and 222 nanometer wavelength and there is a positive peak here which is around say somewhere around 19 or, so nanometer. Then there is, so this is for alpha helix, then there is this particular which is dashed lines the long dash lines are for beta confirmation.

Now, if you can see here there is a peak here around 218 nanometer and there is a positive peak around little less than 200 nanometer. So, for beta confirmation, so this clearly shows that beta sheets here they do not give a very clear like absorptivity, as compare to alpha helix. Then there is this curve here, which is for particular random structures, so dotted lines here gives 310 or other kinds of structures, this cross dashed lines produces like 310 or other helical structures, and there is a negative peak below 200 nanometer.

And, so this could be another structure here which is simple short dashed lines here, which is you can say irregular structure, which is present also like structure does not have any regularity. Then this will be not, so very significantly or clear peaks in here, so these are different like, if you have protein molecule and if you are observing it in CD spectroscopy. Then there are clearly signature patterns here available, like say for particularly for alpha helix here, there is very clear peaks negative peaks at 208 or 209 and 220 nano 22 nanometers.

Which are present, and for others like beta sheet also not, so clear, but still at two 218 nanometer, and then others are there which is for 310 or random coil structures, which are present. This one here one which is like dotted lines is more of beta turn type of structure, so these are indicative of the kind of structures, which are contained in a protein molecule alright. So, these like as we have seen characteristic CD spectra for protein, and kind of transitions here which happens in this spectra, which is like at U V spectra.

We can say and it ranges from around say 160 nanometer to 240 or 250 nanometer, if you observe that kind of transitions here, there are like transition, which are pi pi transition or they are negative at 195 nanometer in a random coil, which are n 2 pi transition and it is nonbonding to pi anti bonding transitions, for if you consider for beta sheet there are pi pi transitions at 218 nanometer that is negative peak as we have seen. And there is a positive peak at 196 nanometer, which has n that is non bonding to anti bonding pi transition.

If we consider these transitions for alpha helix there is pi pi transitions, which leads to the which leads to the positive 192 nanometer peak and there are pi pi transitions also for negative peak which are parallel at 209 and 222 nanometer. So, these are few like, so pi pi transitions you have like 192 and 209 nanometer, and negative at 220 nanometer is right shifted here. So, these are transitions which are present, which occurs during this particular CD in occurs in this during CD spectroscopy, when we expose the protein sample to in CD spectroscopy.

Now, if there are aromatic residue here, aromatic residues held in space then it is environment is asymmetric and it will exhibit circular dichroism. Aromatics have allowed they allow pi pi transition, and that are directed in the plane of pi bonding systems, and are orthogonal to each other. Now, phenylalanine has a small extension coefficient because of high symmetry and it is also the least sensitive to alteration here like tyrosine has lower symmetry than pi.

And therefore, has more intense absorption bands it has absorption bands around 276 nanometer and shoulder at 283 nanometer. So, the hydrogen bonding to the hydroxyl group leads to the right shifts of up to 4 nanometer, then there is tryptophan which has most intense absorption band centered around 282 nanometer, and hydrogen bonding to the N H can shift the 1 L A band that is particular band by as much as 12 nanometer. Then there are disulphide spectra have a broad band at 250 to 300 nanometer with no vibrionic structures.

So, there are like these residues, which is phenylalanine, tyrosine and tryptophan depending on their symmetry and asymmetry can have like absorptions, maxima at different wavelengths. Because, of their interactions and their properties, now if we considered the CD instrument these are known as spectro pyrometers, the measure the dispersion in absorbance between the left and right circularly polarized components. But, we will generally report this in terms of ellipticity in degrees.

So, there are various methods by which CD effect can be measured in the spectro pyrometer, which could be modulation in which the incident radiation is continuously switched between the L and R component. There could be direct subtraction in which the absorbance of the two components are measured separately, and then subtracted for each other. And there could be another way which is like ellipsometric way, in which the ellipticity of the transmitted radiation is measured.

In such a CD instrument plane polarized light is split into the left and R components by passing through a modulator, and the modulator normally used consist of piezoelectric quartz crystal and the thin plate of isotropic material like se fused silica tightly coupled to the crystal. As the transmitted radiation is switched between L and R components these are detected in turned by photomultiplier. Now, origin of the CD fact the left and right circularly polarized components of plane polarized radiation.

If we see here as I will show you, where the two components of the same amplitude and when they combined they generate plane polarized radiation. When the components are of different magnitude, the resultant will be elliptically polarized also when we consider absorption that is relationship between absorption and CD spectra. And what we see is that the positive there will be absorption will be in the same way seen, like as in absorption spectra.

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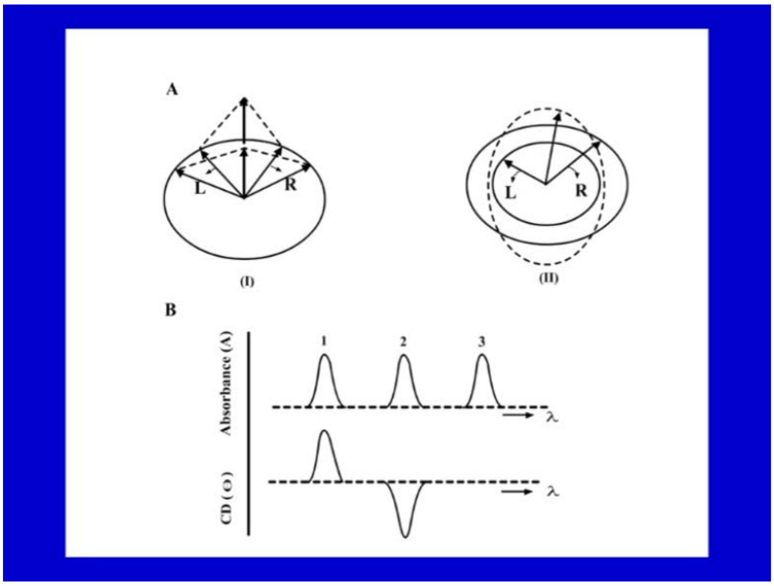

But, CD spectra will be different, so if we see here in terms if you see here there is one like both components if they are of equivalent, then there is a plane polarized light. Otherwise, there is a elliptical or you can say like if two components they are not same or there are different magnitude then there will be elliptically polarized as in seen in here, they are elliptically polarized. Likewise, if you absorption and CD spectrum these are two different absorption spectrum every time shows the absorption.

If you see here the first one this is absorption, but this is left circularly polarized light in positive way, this is right circularly polarized light and here there is no signal because this a chiral chromophore. So, here like in here right is more absorbed then left, left here left is more absorbed than right and this is a chiral, so these kind of patterns are seen in CD as compared to the absorption spectrum. And if these components are different, then certainly elliptically polarized light is obtained alright. So, if we see the simple CD instrument, it is looks like a spectrophotometer, like instruments with computer controlled, with computerized there which contains software to control the instrument.

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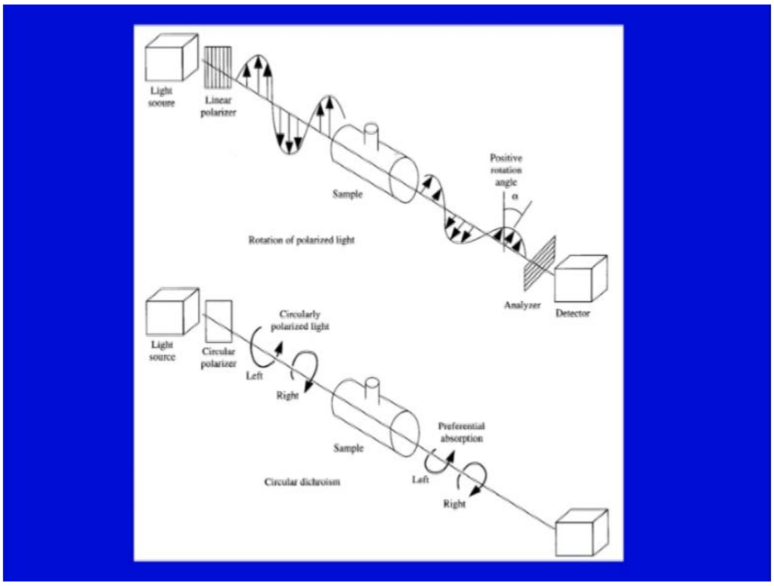

In very simple terms if it is ORD, if you see here on your screen if it you are working with plane polarized light, then what you have is a light source linear polarizer. There is a linearly polarized or plane polarized light, which passes through the sample depending on the sample there will be positive rotation angle or differently it will react and finally, it will be detected after passing through analyzer.

But, in circular dichroism what is done is there should be a circular polarizer or circularly polarized light has to be, which will be like left and right circularly polarized light. And as we said alternatively there could be a pattern, where left and right circularly polarized light I put in or there will be different ways, as this light is passes through there will be a preferential absorption for one of them and that will be detected in here.

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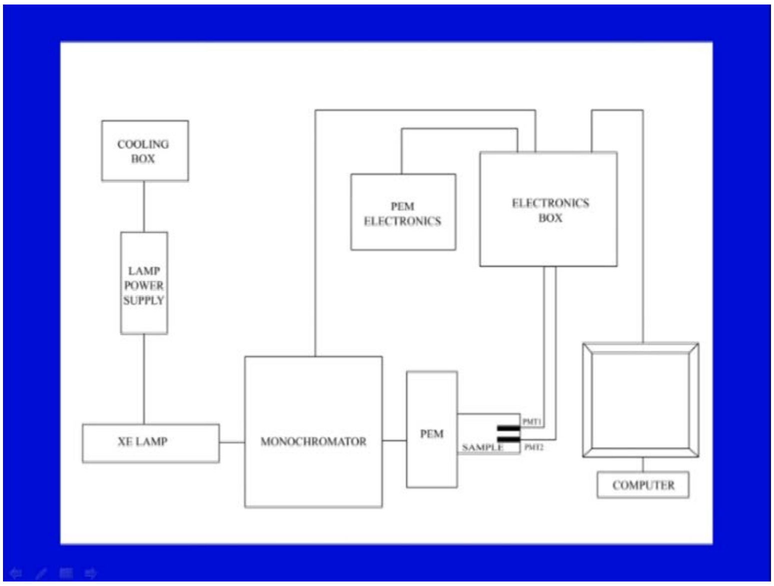

This is very simple schematic of different things which are present in here, there were power supply with the cooling box in here, mono chromator this is the sample holder, this is PEM electronics here. And then finally, we have computer system and detector in here, so these are like very simple way to like these are photomultiplier tube, which is for each reference cell as well as for sample. There is two PMT tubes in here, and it will be data value processed and finally, will be analyzed on the computer.

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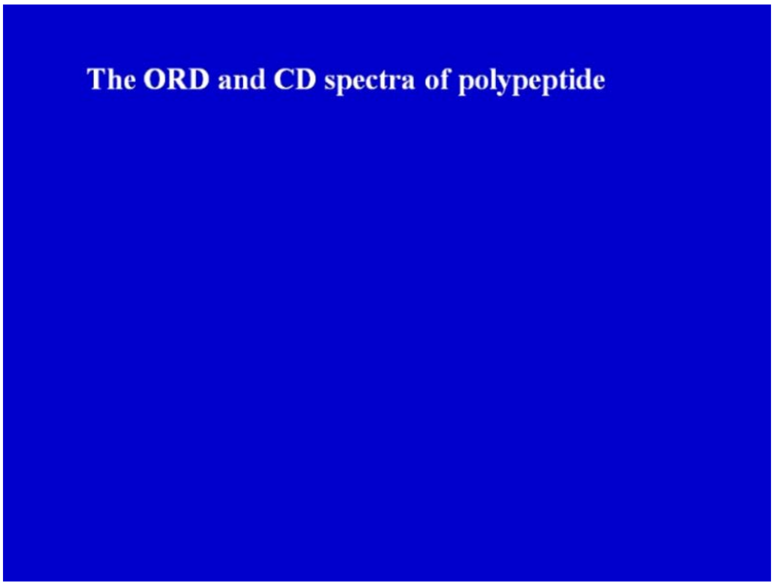

So, these are very simple schematics, the ORD and CD spectra if we compare say of a polypeptide, polypeptide which may be random has a CD spectrum which is similar to that of simple amid. But, will bigger in magnitude, but the spectrum will displays the small positive, and to pi transition and that is transitions at approximately 230 nanometer, and the large single pi pi transitions at around 195 nanometer. Like I have shown you in there, the spectrum of alpha helix shows the large negative and 2 pi transitions at 220 nanometer.

And pi pi transitions, which is split into two transitions because of exciton coupling, these transition has a negative band at approximately 208 or 9 and a positive band at approximately 192 nanometer. So, 220 nano 2 nanometer band is due to the negative band is due to n to pi transition that is non-bonding to anti bonding pi transition whereas, 209 or 208 nanometer, and 192 nanometer band is due to pi pi. Transitions the CD spectrum of the anti-parallel plated sheet as we have shown you is pi pi transitions, how about the split of the transitions are different then in the case of the helix.

The spectrum of anti-parallel beta structure, as we have shown has a negative banded 280 nanometer, so what we have shown in earlier in the spectra protein CD spectra of proteins. The negative band mostly is for anti-parallel beta and there will be positive band for 195 nanometer.

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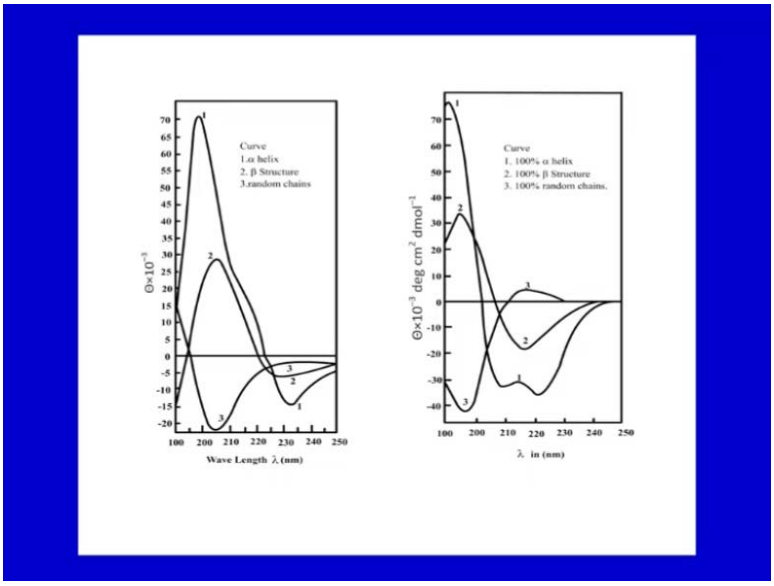

So, if you consider ORD and CD spectra in here, then as you see here like these diagrams they are clearly the there are much clear bands in CD spectra like for this is for alpha helix. And this is for beta sheet, and this is for random coil structure, if you see that for alpha helix there are two peaks in here, but does not seem to be in ORD here. Likewise, there are like the peaks which are much more clear and distinct in CD spectra as compared to ORD spectra. So, CD spectra is now most of the time it is utilized for different applications, particularly structural or conformational determination of macromolecules.

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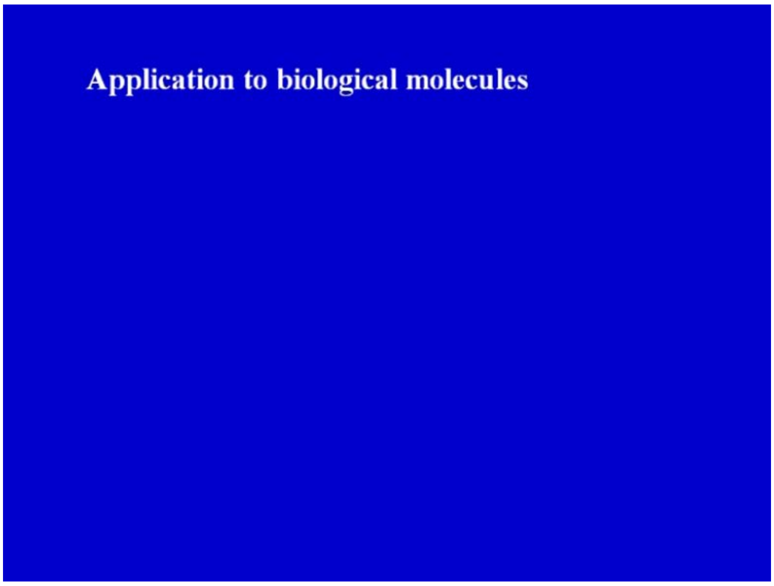

And other molecules also now, if you consider certain applications of CD in biology or biological molecules, one is as we were discussing and showed these spectra also that is determination of proteins secondary structure that is alpha helical and beta sheet content. So, that could be determined or random coil content through signature spectra, and in a standard conditions that could be determined that what is percentage content of say alpha helix and protein. Then one can determine the optical purity of the biological molecules, also analysis of the tertiary structure of proteins and confirmation changes can be worked in here.

Now, remember that is not like it you obtained tertiary structure, but the conformational changes in the tertiary structure could be detected by CD experiments. Then comparison of the secondary and tertiary structure of say wide type and mutated proteins could be performed in here, doing far U V and near UV CD nucleic acid structure and changes upon say binding or melting could be monitored, at a particular wavelength. Like say DNA melting curves could be obtained in CD.

And like we also discussed in U V based where you know, at particular wavelength if you monitor then like G plus C content and other things could be known actually. So, in general this phenomena will be examined in absorption bands of any optically active molecule. So, circular dichroism is exhibited or this particular property which is exhibited by biological molecules, because of their patterns like dextrorotatory levorotatory components. So, therefore, this like alpha helical content of protein or the double helix content of nucleic acids have a CD spectra, which is we can call signature patterns of these structures.

And there could be very much utilized for knowing the knowing the structure of say many different biological micro molecules. Now, the capacity of CD to give representative structural signature, makes the very powerful tool in modern biochemistry with applications that can be found virtually in every field of the study. CD is generally considered to be more advanced and it is advantage is apparent in the data analysis, structural elements are more clearly distinguished. Since they are recorded bands do not overlap, extensively at particular wavelength as they do in the ORD or Optical Rotatory Dispersion.

So, there could be different kinds of analysis which could be performed in CD experiments, there are far UV CD experiments like ultraviolet region 195 to 250 nanometer. CD spectrum of proteins can reveal important characteristics in far UV of their secondary structure, and in this case the chromophore in this area this particular region is peptide bond. CD is the valuable tool specially for showing changes in the confirmation.

Like for example, if you want to study the secondary structure changes in a molecule as a function of temperature or as a function of p H or the concentration of the denaturing agents, like say urea gadolinium chloride. This could be monitored by comparing the spectra, at in different conditions and that can reveal very important thermodynamic information, about the molecule. And lot of experiments for say enthalpy or Gibb's free energy of denaturation, could be done and they like lot of kinetic experiments could be performed in CD, which is like binding experiments or like monitoring at different conditions, environmental conditions, and the secondary structure content all these things could be performed in here. Then there is near UV spectro region, which is 250 to 350 nanometer and here the chromophores as we were discussing is aromatic amino acids, and disulphide bonds. And we have discussed about like phenylalanine tyrosine and tryptophan, they absorbed at different wavelengths and then disulphide bonds can give.

So, the patterns which are obtained for phenylalanine, tyrosine or tryptophan can give an important information about, the changes in confirmation in tertiary structure. Because, of certain shifts in their absorbing patterns actually, then there is visible CD spectroscopy is also very well powerful technique to study metal protein interactions, and can resolve individual DD electrons transition as separate bands. So, CD gives it will not give you a specific structural information in terms of three dimension, which could be done by more X-ray crystallography and protein NMR or NMR technique.

And both give atomic resolution data, but CD spectroscopy is very useful it is a quick method, which does not require large amounts of proteins or extensive data processing it is a very direct, very convenient method for like analyzing very quickly the structural content of a sample. And it is mostly utilized for to study protein solutions, and under various conditions to see the changes, to see the conformational changes occurring. So, this is like there are whole lot of applications for CD, experiments and there are lot of variations in experimental ways, which one can perform and get obtained useful information.

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There are certain experimental limitations also of CD experiment, like CD has also been studied in carbohydrate, but with very limited structures actually. Because, CD spectra which is for carbohydrate is like around 100 to 200 nanometer, where the corresponding CD bands of un substituted carbohydrates lie, and substituted carbohydrates with bands above this region which could be measured, but otherwise it is very hard to measure. So, measurement of CD's also complicated by the fact that typical aqueous buffer shape systems often absorb in the range.

So, where structural features exhibit differential absorption of circularly polarized light. So, only certain buffers could be utilized, like phosphate buffer is very common or say carbonate acetate state buffers are, so here if you say phosphate and other buffers could be utilized, certain buffers are totally like not like. If you say phosphate buffer also, phosphate buffer or carbonate this should be utilized in only very small amount or concentrations. Otherwise at high concentrations, they are also unacceptable, tris buffer is completely avoided while performing UV CD.

So, there are like certain buffers cannot be utilized at all, but other buffers could be utilized which are at low concentration. The usual light source in these instruments is a high pressure salt arc xenon lamp, and ordinary xenon arc lamps are unsuitable for use in the low UV region. So, light from synchrotron sources also has been with a much higher flux at short wavelengths, and has been used to record CD down to 160 nanometer.

Recently, the CD spectrometer at the electron storage ring facility ISA at the university of Aarhus in Denmark was used to record the solid state CD spectra, down to 120 nanometer. So, at the quantum mechanical label the information content of circular dichroism, and optical rotations are mostly identical, so these are certain limitations which one could overcome by taking the proper precautions here. And can have successful CD experiments to obtain useful data.

So, this was about CD spectroscopy and as we have seen the CD spectroscopy is a very useful technique and this is based on differential absorption of right and left circularly polarized light by a sample, which is a Chiral sample. Like, we have seen alpha helix and or protein secondary structure has a very has signature spectra or signature patterns, and this could be very useful tool for determining the secondary structure content of unknown protein, by you can take in database standard proteins, whose crystal structures are solved could be taken.

And these CD data could be collected for those standard proteins, and then they could be standardized for particular peaks and various conformational states. And then unknown spectra from unknown sample can be compared to those, and relative secondary content in terms of percentage could be calculated. So, it is a very useful technique many times you can, also one can compare the protein confirmation or monitor the protein confirmation in various environmental conditions, under denaturing conditions or otherwise.

So, biological macromolecules and other different kinds of molecules could be studied in here, binding studies could be performed kinetics at different temperatures could be performed. So, there are whole lot of different applications of this technique, and this could be a substitute or as a quick technique it could be utilized for secondary structure determination, before one does or performs X-ray crystallography or NMR. So, this completes our section on circular dichroism, and in the next lecture we are going to discuss about NMR and X-ray crystallography. Also we will be discussing about atomic absorption and flame emission spectroscopy, plus little bit about mass spectrometry.

Thank you.