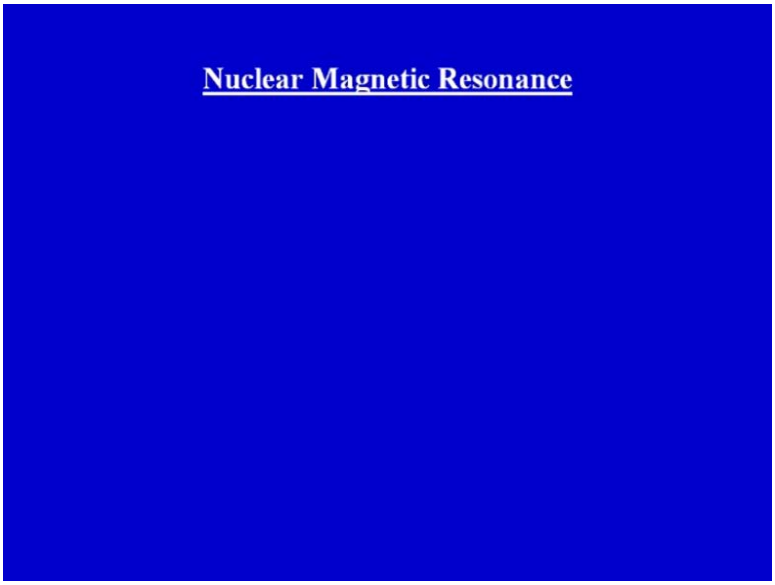
Analytical Technologies in Biotechnologies
Prof. Dr. Ashwani K. Sharma
Department of Biotechnology
Indian Institute of Technology, Roorkee

Module - 6
Spectroscopic techniques
Lecture - 5
Nuclear Magnetic Resonance (NMR) Spectroscopy and X-ray Crystallography

In this lecture, we are going to discuss about two most important and powerful techniques for determining three dimensional structure of molecules, may be large or small or organic molecules or macromolecules.

(Refer Slide Time: 00:41)



These techniques are nuclear magnetic resonance spectroscopy and X-ray crystallography methods. One technique, where we can do in nuclear magnetic resonance spectroscopy, the three dimensional structure could be solved in solution state, whereas in X-ray crystallography studies, you require crystals for determining the three dimensional structure. Now, we will without going in too much of detail, we will try to understand these two techniques the basic concepts of these two techniques.

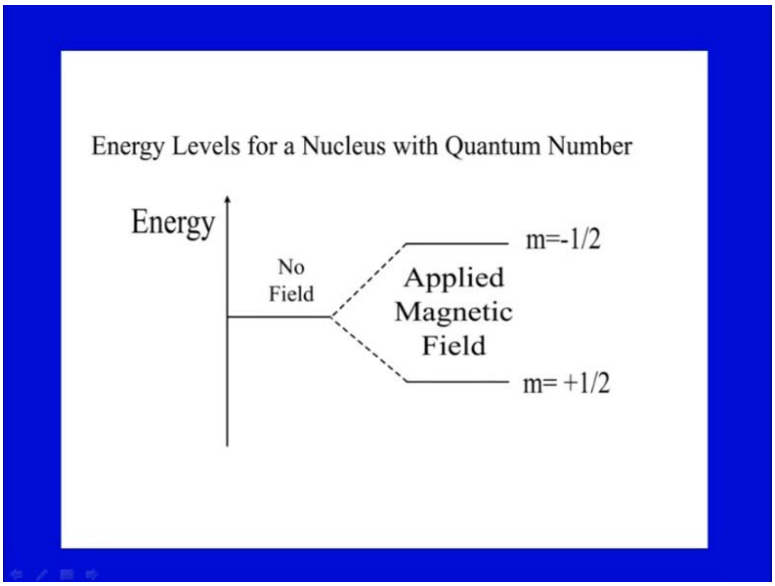
So, let us start with nuclear magnetic resonance here, NMR or we can say simply NMR. So, NMR relies on the ability of atomic nuclei to behave like a small magnet, and align themselves with an external magnetic field. So, when irradiated with a radio frequency

signal, the nuclei in molecule can change from being aligned with magnetic field to be opposed to it. Now, this is called nuclear for instrument works on stimulating the nuclei of the atoms to absorb radio waves.

And the energy of the energy frequency at which this occurs, can be measured and displayed as an NMR spectrum. So, this phenomenon is based on the fact that nuclei or of atoms have magnetic properties, like spinning charge acts as a tiny bar magnet and it has a particular angular momentum. And this could be utilized to yield chemical information, quantum mechanically subatomic particles like protons, neutrons and electrons have spin.

And in some atoms for example, say 12 C or 16 O or 32 S these spins appeared and cancel each other out in certain other. So, they will not have any overall spin, in certain other ones like 1 H, 13 C, 31 P, 15 N or 19 F the nucleus does possess an overall spin and to determine the spin of a given nucleus, one can use certain rules. Like say, number of neutrons and number of protons if both are even nuclei has no spin, if the number of neutron plus the number of proton is odd, then nucleus has a half integer spin. And if the number of neutrons and number of protons are both odd, then the nucleus has an integer spin that is 1, 2, 3 like that.

(Refer Slide Time: 03:28)



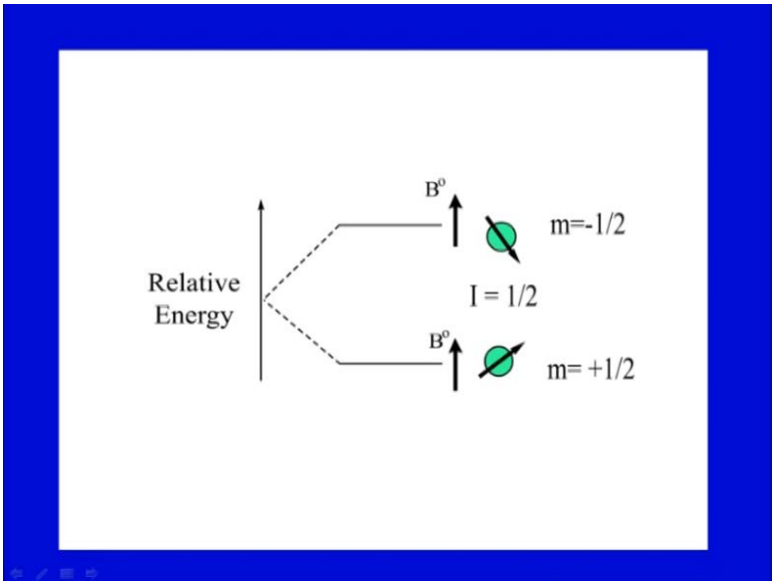
So, those atoms, which has half spin that is plus half or minus half, can be utilized in NMR technique. So, if you see in figure here, there are two like say we can take example

of proton, when there is no field they are like not, so much separated they are just oriented randomly. But, when external field is applied or external magnetic field is applied, then there are two species one having spin plus half, which aligned with in parallel to the applied magnetic field and there's minus half which aligned negative to the applied magnetic field.

So, this particular one is opposed that is anti parallel and other is parallel, as we say and they are referred to differently for this purpose. The rotational axis of the spinning nucleus, cannot be oriented exactly parallel or anti parallel with the direction of the applied field. So, it is mostly like what we say it presses about the particular field at an angle, which is angular velocity or angular momentum. And this particular property or magnetic movement of it is measured in terms of angular momentum.

The orientations that a nucleus magnetic moment can take against or external magnetic peaks are not of equal energy, and spin states which are oriented parallel to the external field are lower in energy, then the in the absence of external field. And in contrast spin states whose orientations opposed the external field are higher in energy than in the absence of an external field.

(Refer Slide Time: 05:21)

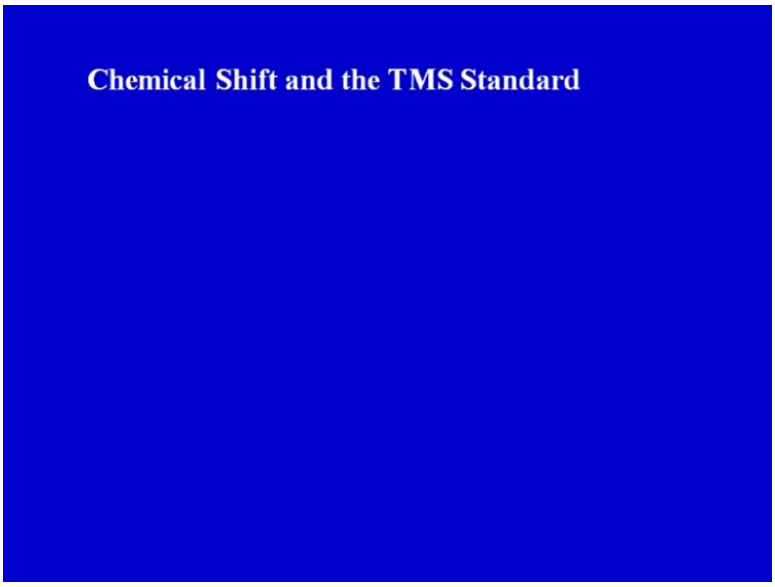


So, what you have is the two states which have particular spins, which plus half or minus half they are oriented in a particular manner, when applied magnetic field is there. Energy separations adjust in certain way, and these induce the transition between the

various spin states, like by irradiating the nucleus with electromagnetic radiation of the correct energy, which is in radio frequency range, nucleus with no low energy orientation can be induced to transition when orientation with higher energy.

Now, absorption energy absorption of energy during this transition will form the bases for NMR method. So, as we understand that when applied magnetic field is there, these nuclei have plus half and minus half, and they align are opposed to the applied magnetic field. And when an external electromagnetic radiation, most in form of radio frequency or radio waves is applied onto the sample, then the absorption will occur which will make a which induces a transition to an orientation with a higher energy of a particular nuclei from the low energy, and this is the basis of NMR method. So, this resonance is taken and this is recorded, now it is very important to understand like say for example, in proton NMR how the particular proton in a certain chemical environment is detected.

(Refer Slide Time: 07:14)



And that those particular differences which are obtained these are called chemical shifts actually, and they are taken in within a standard which is a TMS standard, lets discuss that. So, the chemically different protons which are present in different electronic environments, so what happens is their differences in the electronic environment, will cause the protons to experience slightly different applied magnetic fields, owing to the shielding and deshielding effect of the induced electronic magnetic fields.

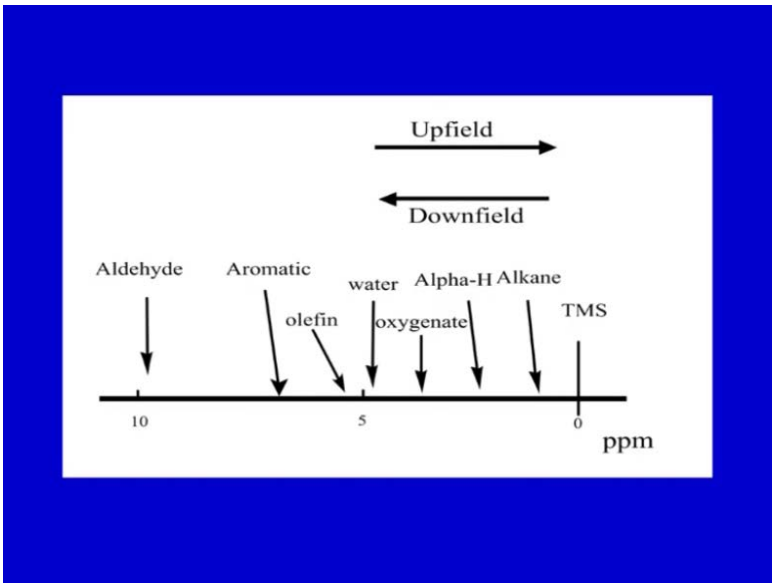
And over the years the NMR spectra have been obtained on every conceivable organic molecule in nature or synthesized in the lab. So, in order to standardize the NMR scale it is necessary to set 0 reference point, and to which all protons can then be compared, the standard reference that has been chosen is Tetra Methyl Silane or TMS. And this compound has 4 C S 3 methyl groups, single bonded to a silicon atom, so all of the protons on the methyl groups are in the same electronic environment therefore, only one NMR signal is generated.

So, the electro negativity of all carbon atoms is actually higher than the silicon atom to which they are bonded. So, here tetra methyl silane is taken as the reference molecule that is it is considered as 0, now result in the sigma electrons being shifted towards the carbon atom in the methyl groups. And the consequently the protons will be heavily shielded causing the one signal to be generated at a very high magnetic field strength setting, it is that signal that all other NMR signals of a sample are referenced to.

So, this association with the reference signal is called the chemical shift, and the shift is measured in terms of parts per million. So, in very simple terms tetra methyl silane is or TMS is taken as reference, and all other measurements or the protons in different environments, will be like calculated the positions will be calculated in the spectra as per the reference point. And these shifts which occur in reference to tetra methyl silane are called chemical shifts which are measured in parts per million.

Now, after the sample have been referenced to the TMS resonance at 0 PPM for actual NMR peak position, in hertz is divided by the resonance frequency of the spectrometer which is in megahertz. Thus 1 is dividing hertz by megahertz which is a part per million, so 1 PPM on a 58 megahertz NMR instrument is actually 58 hertz from the resonance position of TMS. While on a 300 megahertz NMR instruments, 1 PPM is 300 hertz for the TMS resonance position, so these are the differences here. Now, with the standardization and normalization in place, one can always ((Refer Time: 10:32)) say that all benzene protons, will resonate at around 716 PPM no matter what instrument is being used in the analysis.

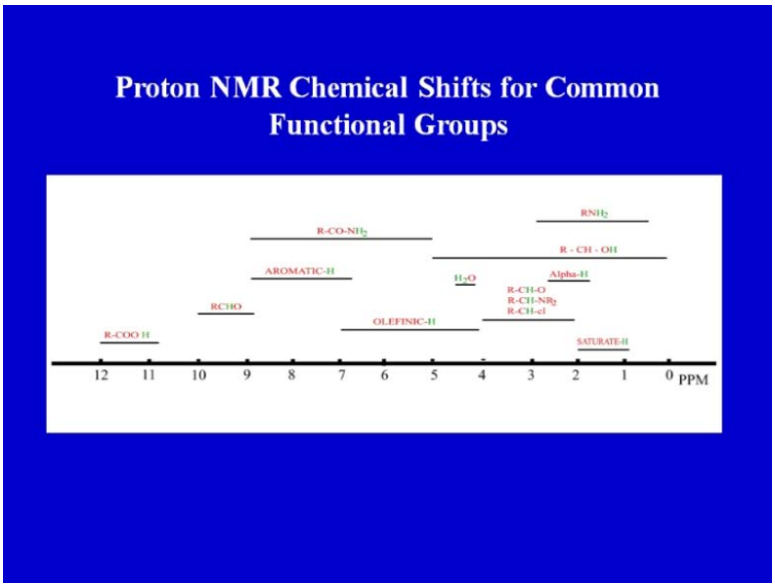
(Refer Slide Time: 10:43)



So, there is like in this here you have a down field, and there is a up field like you have a TMS which has a reference. And you have different positions, which proton will posses in different kinds of molecules, as you can see alkane, alpha H, water, aromatic, aldehyde depending on the chemical environment these peaks will appear. And they will be like from it is taken from 0 to 10 PPM in here, now NMR instrument like here TMS standards available to the reference one line spectrum.

So, the NMR peaks in the spectrum itself are used to reference the whole spectrum based on a well established knowledge of the process stream chemistry. The various aromatic groups in the spectrum can be a peak picked and assigned to their known chemical shift values, that have been locked in large database of animal NMR chemical shifts. So, there are databases available, and chemical shifts could be directly seen from or could be compared to those database, even if you are directly doing it.

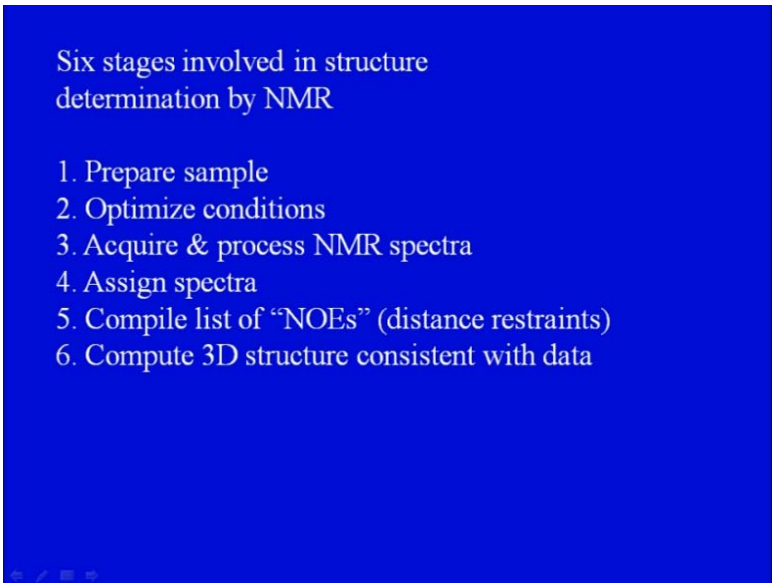
(Refer Slide Time: 12:02)



So, proton NMR chemical shifts for a common functional groups if we see, and we compare here you can see that depending on like different chemical environments. The protons will store different shifts actually or different chemical shifts, like starting from the 0 PPM TMS, if it is a saturate H it is somewhere around very close to the TMS and as the environment chemical environment changes. You can see the shift in the chemical, and which is highest around for carboxyl group in here proton in carboxyl group, then it is aldehyde group.

And finally, aromatic C O N H 2 group and likewise it is a like least here for R N H 2 or others, so or saturated H will have the least chemical shift. So, what is the basis here of all those things here, so electronegative groups are here de shielded and tend to move NMR signal from a neighbouring protons further down field to higher PPM or you can say to higher PPM values. Now, protons on oxygen or nitrogen have highly variable chemical shifts, which are sensitive to concentration solvent temperature. So, all these things will play a role in chemical shift, the system of alkenes, aromatic compounds and carbonyls strongly de shield attached protons, and move then to higher PPM values.

(Refer Slide Time: 13:53)



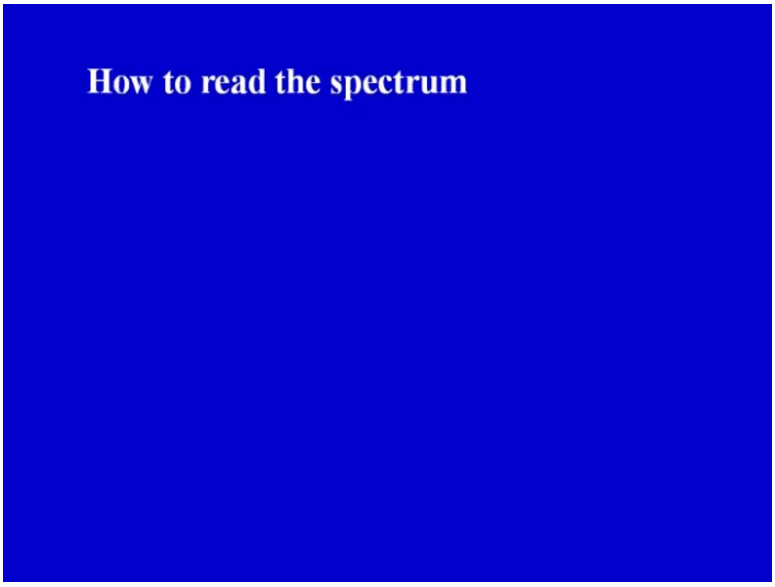
There are six if we compare if we see the NMR experiment, there are mainly six stages involved in a structure determination by NMR, 1 is to prepare the sample for NMR. So, sample could be done like by proton NMR, it has to be dissolved in proper solvents and if it does not contain radioactive nuclei, like we have discussed like 1 H or say for proteins 13 C or 15 N then those have to be put in there. Then optimized conditions has to be created for the experiment, after this the NMR signal is recorded on the machine and NMR spectra is obtained.

Now, this NMR spectra once it is obtained on the NMR spectrometer, then these spectra has to be assigned like peaks has to be assigned that is we call it assigning the spectra. In here what happens is that from like we discussed, different are selected and these different peaks which standard values are given in database, they can be picked from there. And according to the standard values, which could be compared the peak assignment could be done.

So, that is another important thing then compile the list of different like for example, if you are doing 1D NMR it is a simple proton NMR and it gives identifies different protons in various chemical or electronic environment. But, one can perform apart from one dimensional, it could be two dimensional, three dimensional spectroscopy. So, various distance restraints and interactions has to be like the 2 D or 3 D NMR spectra, are much more complex, and assigning the peaks is a very complex process in there.

Once this whole list of say certain peaks are assigned, then 3D structure needs to be computed which should be consistent with the data. And this is 3D structure is done on the basis of like different interactions, which is like there are lot of effects like correlation facts or nuclear over Hauser effects are there, which needs to be taken into consideration.

(Refer Slide Time: 16:39)

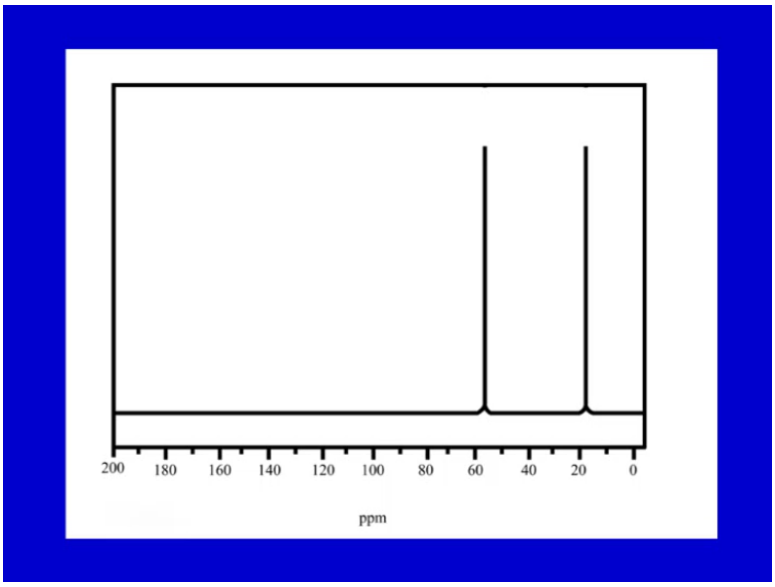


So, when you have to read the spectrum very important part of the overall experiment is reading the NMR spectrum. So, an NMR spectrum appears as a series of vertical peaks or signals, which are you can say lines or very it is a peaks as we have seen in other spectroscopy technique. The very fine peaks distributed along the x axis of the spectrum, as per the their chemical shifts actually, and each of these signals correspond to an atom within the molecules being observed.

Now, the position of the each signal in the spectrum gives information about the local structural environment of the atom, producing the signal. Say for example, if it is a 13 C NMR spectrum, then ethanol will be shown like where you have C H 3 and C H 2 the two carbons in ethanol are in different structural environments. And hence each produces a signal in the NMR spectrum, likewise the carbon attached to oxygen is de shielded due to the electromagnetic nature of the oxygen, and this shifts it is signal towards the left in the spectrum.

So, this is very important that whether it is a shielded or de shielded electrons whereas, the carbon bonded only to hydrogen's and carbons appears at the right of their spectrum.

(Refer Slide Time: 18:09)

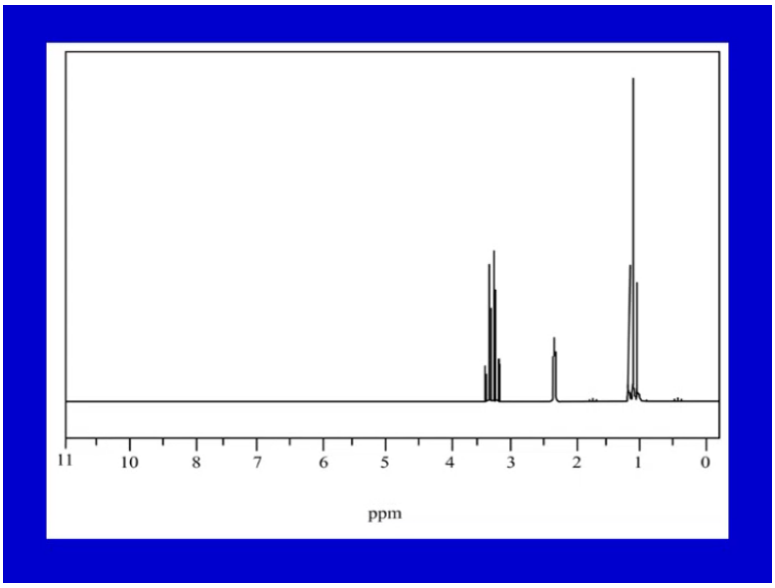


So, if it is there is a more de shielding of there is a electronegative atom then there's a shift actually like you can see, there are two peaks for two carbons in here. And one which is shifted to the left, which is connected to electronegative atom rather than the other carbons which are simple. So, these are the peaks which are obtained in here, and it depends on their electronic environment for carbon or say for NMR, if we say proton NMR where proton acts as the nucleus which is resonating.

Then in 1 H NMR spectrum say for ethanol, the two protons of the C H 2 group neighbouring the oxygen are further to the left, in the spectrum while the hydrogen of the C H 3 group that is most remote from the oxygen produces a signal towards the right. So, once which is closer to the oxygen will be having a higher chemical shift, as compared to the one which is connected to simply the carbons actually.

So, this is like signals in 1 H NMR spectrum do not necessarily appear in the single line because there is a splitting pattern as we have seen in here. And this gives information as to how many hydrogen's are present on the neighbouring carbon, so depending on the split it will tell about number of carbons and also integration of the 1 H NMR signal allows number of hydrogen's in each environment to be determined.

(Refer Slide Time: 19:35)



So, if you see here in this figure depending on how many hydrogen's, there is a splitting of the peaks occurs actually. And that is will be determined as per the number of atoms attached to a particular groups, so reading NMR spectrum is a complex process, but just in a very simple way as we have seen the chemical shifts, and as we have seen how a particular atom could be or a proton molecule. As we are doing proton NMR or carbon NMR that particular radioactive nuclei could be shielded or de shielded.

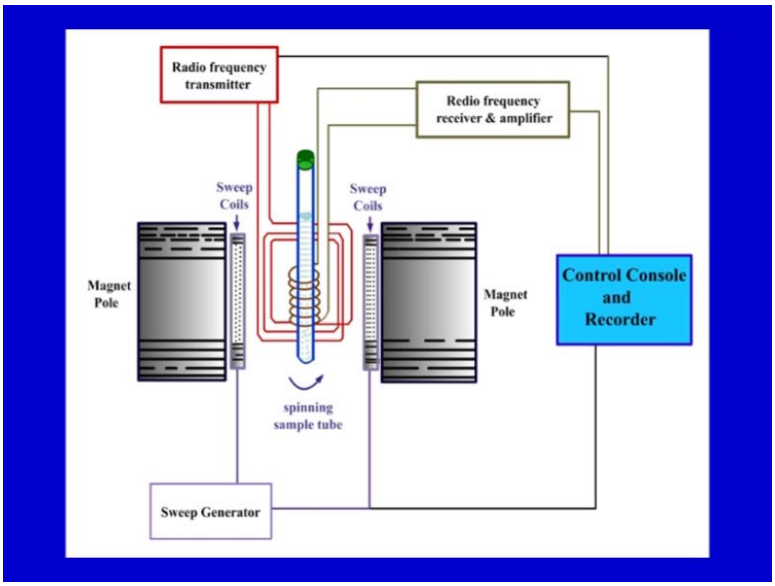
Depending on that, whether it is attached to a more electronegative or electron withdrawing group or to a simple saturated group, there will be a chemical shift. Like it was higher chemical shift for say atom connected to oxygen, and lower chemical shift for atom which is connected to say carbon group, where no such effect occurs.

So, accordingly this spectra will be obtained and according with reference to the database, it could be understood that whether this is present in say environment, which is having like say it is connected to carboxyl group or say simple carbon like in C H 3 or it is connected to aldehydic groups or say other groups. So, standard values yours your data needs to be compared to the standard values and peak assignment has to be done the instrumentation part of this NMR technique if you see here.

(Refer Slide Time: 21:21)

This is how this whole instrument looks like it is a big cylindrical vessel in here, where all the things are available.

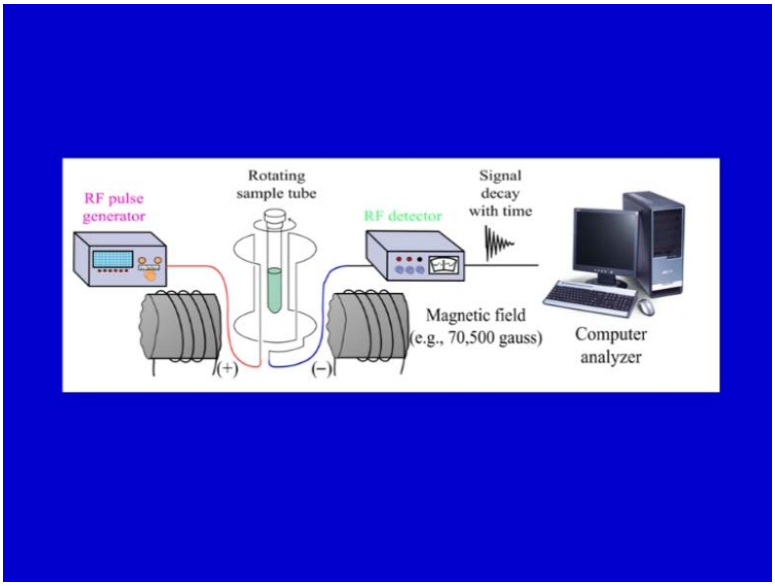
(Refer Slide Time: 21:28)



And if we see in detail here, if you see on your screen it is a like you are a spinning sample tube is in here, and it this has a coil which is around this sample. These are sweep coils, and there is a magnet pole or you can say very powerful magnets in here, there's a radio frequency transmitter which provides the radio frequency. And these are radio frequency receiver and amplifier which will collect the signal, so at correct resonant

frequency or radio frequency there will be signal, which is like transition of nuclei from one lower state to higher state. And the applied magnetic field is provided by this, and resonance will occur only when the frequency or the energy matches with the two resonating states. And that particular spectra will be collected, and then could be analyzed as we have discussed.

(Refer Slide Time: 22:34)



This is another presentation of the same thing where's RF pulse generator you have a rotating anode, and this spinning sample tube here there are two powerful magnets. And there's RF detector which is there and finally, signal is obtained in terms of peaks, and which could be analyzed on the automated system. With lot of different software's are available for computing the 3 D structure from say two dimensional or three dimensional NMR.

One dimensional NMR is only for identification of say proton NMR is only for identification of the chemical environment or electronic environment of a particular nuclei. So, like when a compound containing hydrogen is placed in a magnetic field, say a proton NMR we say the spin of the nuclei can be aligned parallel, to the lines of the magnetic field or to anti parallel, which is called lower energy is called alpha. And it is represented by alpha and higher by beta and the applied radio frequency energy from the generator will cause the spins to be promoted to the higher energy state.

And this will be the base you can record the NMR spectra, so we have not talked about quite lot of things in here, as these are like complex, these are like NMR process or NMR spectroscopy is a very complex and detailed discussion is required for this. But, as we understand very simple way that is one is there is a nuclear magnetic resonance or certain nuclei with half spins that is plus half and minus half, and like we said nuclei we have mentioned.

These when they are spinning around a particular axis, and they are processing you can say at a particular angular velocity and or you can say angular momentum. They act as magnets or tiny bar magnets, and when there's a applied, so they are oriented randomly one, but when there is a applied magnetic field. Then these will orient or they will be divided into two states, one is plus half which is aligned parallel to the external magnetic field, and which is at lower energy. And there another population will be with negative spin or anti parallel to the or opposed to the external magnetic field.

And which is again at a lower energy, so population in the higher energy is less than the lower energy, and as radio frequency or electromagnetic radiation in form of radio frequency waves is like, when sample is irradiated with that. Then at particular frequency that is equivalent to the resonating frequency of the nuclei, from lower state to higher state. Then some of these, will shift upwards or to higher state and this NMR spectra is recorded, which gives information about the electronic environment of that particular nuclei.

So, for example, a proton NMR when we are doing these environments are calculated in terms of chemical shifts, from reference molecule that is TMS. And TMS is taken as 0 parts per million, and then as per the shift chemical shift which could be farther from the TMS it is calculated. So, what you have is you like those protons which are attached to say electronegative atoms or other which are electron withdrawing groups, they will have like a shift higher chemical shift as compared to those which are attached to saturated atoms like carbon or others.

So, accordingly from database these things could be compared, and they could be peaks could be assigned and proper identification can be done. For two dimensional and three dimensional NMR, the NMR spectra will be obtained in two or three dimensions, and those NMR spectra will be very complex. And then peak assignment needs to be done,

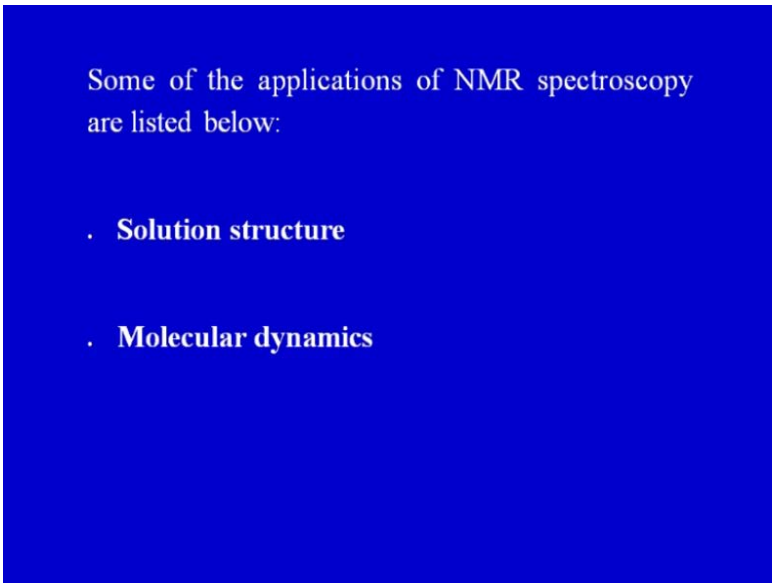
lot of things are automated, but still manual intervention and expertise is needed to work on these spectra, and to reduce the three dimensional structure.

Now, most of the organic compounds or any compound in solution could be taken for determining NMR structure, proteins molecules could be are solved here. But, there's a limit like very high molecular weight protein like above 30 k d a cannot be done in here, it becomes much more complex, and then proton NMR cannot be done on these complex protein molecules, as signal number of proton signals will be very, very high to really deal with. So, there is rather carbon 13 NMR or N 15 NMR needs to be performed on those to solve the protein structures.

So, this was in a very brief about NMR spectroscopy, now there are lot of applications of NMR as we were discussing, NMR has become a sophisticated and powerful analytical technique that has found a variety of applications. In many disciplines of scientific research, medicine and various industries, modern NMR spectroscopy has applications in bio molecular systems, and plays important role particularly in structural biology. There are methodologies which have developed, like it has become a versatile spectroscopic technique for analysis of lot of different kind of bio molecules.

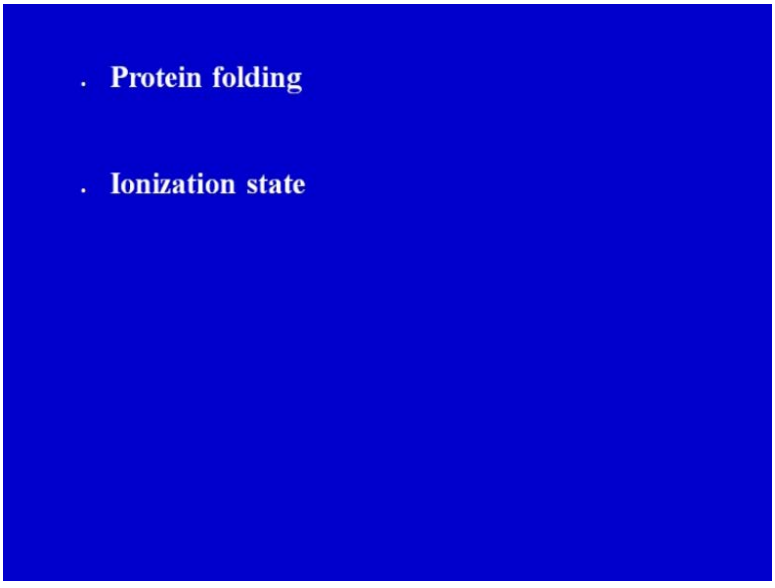
And like which could go up to very high, like you can have complexes interaction could be seen in here. And lot of different analysis could be performed, together with X-ray crystallography NMR spectroscopy is one of the two leading technologies for the structured, for the three dimensional structure determination of macromolecules at atomic resolution. In addition NMR provides unique and important molecular motion and interaction profiles, containing pivotal information on protein function, and this information is very critical to drug development and various other applications.

(Refer Slide Time: 29:46)



Some of the applications which are very important and very sure, one is the solution structure which could be done for various kinds of molecule. Then molecular dynamics could be performed, for say motional properties of bio molecules protein folding could be done which is very powerful for determining like intermediates or in folding.

(Refer Slide Time: 30:03)



Then ionization states could be determined that is chemical properties of functional groups in bio molecules are such as, ionization states of ionizer groups in active site of enzymes could be determined. And lot of other analysis could be done weak

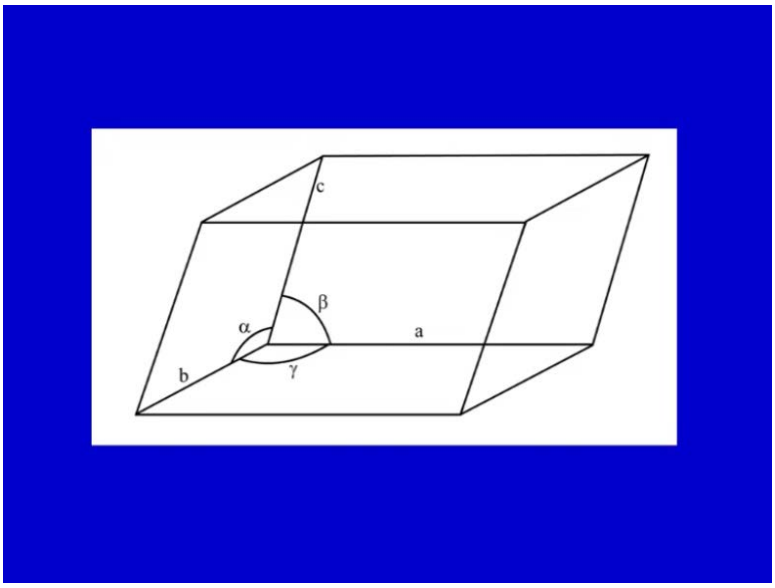
intermolecular interactions could be seen in here, and protein hydration to detect interior water and it is interactions could be done just one. Lot of this technique could also be used for direct detection of hydrogen bonding interactions.

And it is useful in identifying drug leads determining the confirmation of the compounds bound to the enzyme the receptors and other proteins. So, these are like in metabolic analysis also this technique is utilized, so there are whole lot of different applications of this, where chemical and different material analysis could be performed in chemistry, physics and biology. So, NMR spectroscopy is a very powerful and very important technique, which is utilized in various areas of biotechnology.

((Refer Time: 31:29)) Let us move to the next technique that is X-ray crystallography, now X-ray crystallography is one method where you require you can solve three dimensional structure of any molecule, provided there are crystals for that particular molecule. So, what you require is you require a crystal of a particular substance or material, and the basic very basic concept is that what you have is that when there is a crystal of a molecule is available. Then X-rays hit the crystals, and this crystal causes the beam of X-rays to spread into many specific directions, according to the diffractions.

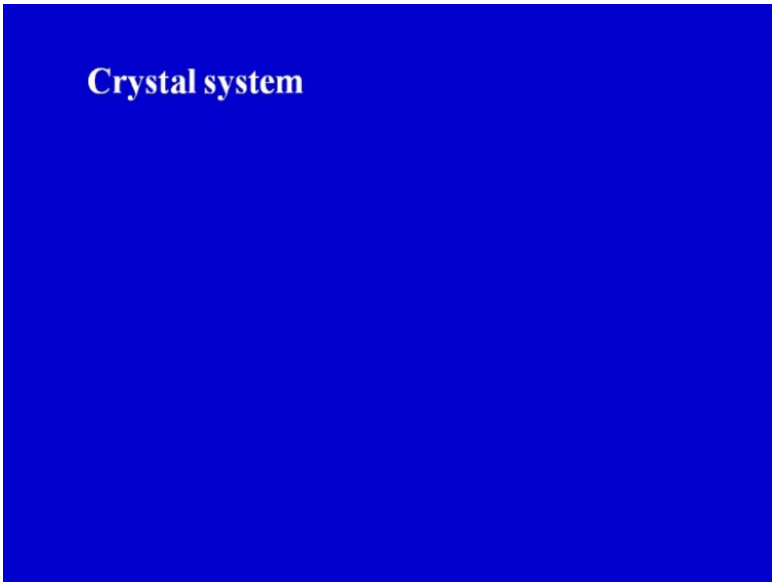
Like the X-ray the crystal is a periodic arrangement of molecules or atoms in the three dimension it is a regular arrangement, in the three dimension. So, there is a particular kind of diffraction pattern characteristic for each molecule, and from the angles and intensities of these diffracted beam, a crystallographer can produce a three dimensional picture of the density of electrons within the crystal. And from this electron density, the mean positions of the atoms in their crystal can be determined, and with certain knowledge their chemical bonds, disorders and other information as per the data is obtained. Crystals if you say, they are made of infinite number of unit cells, and unit cell if we say it is the smallest unit of a crystal that is the repeating unit of the crystal. And it repeats in all three directions, and will generate whole crystal.

(Refer Slide Time: 33:20)



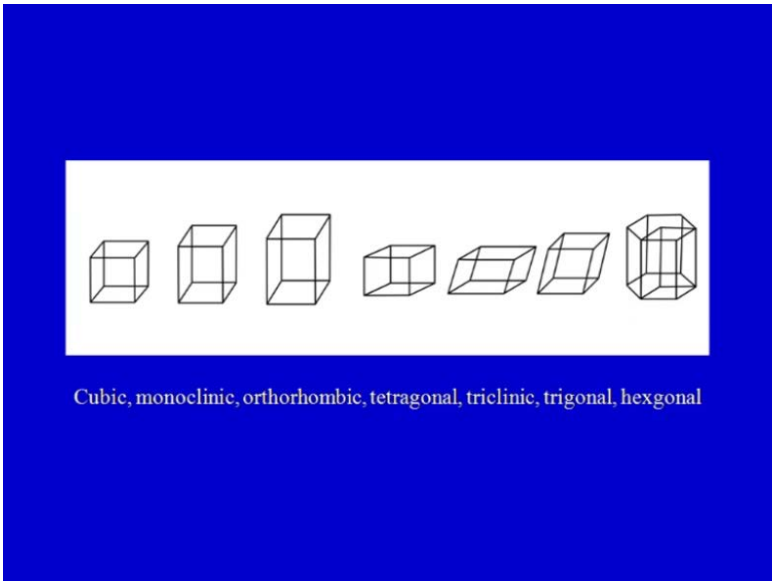
So, if I say what is the three unit if I say this is like say just for example, then it has a crystal or a unit cell in a crystal, which like it will be made like in all direction there will be lot of unit cells, which will make a crystal. And it has a six main things, one is three axis x, y and z or a, b, c you can say and there are, so we call it in this in proper crystal it is a, b, c. And there are three angles alpha beta and gamma between the three axis actually.

(Refer Slide Time: 34:01)



So, if we say we are not going too much detail, but crystal there are crystal systems which are grouped into seven crystal systems, according to the characteristic symmetry of the unit cell. Symmetry is very important part of crystallography because that is bases for determining the structure of the molecule, as particular symmetry will provide particular type of diffraction. And that gives lot of information and all different mathematical like, Fourier transform thing is based on in a part based on symmetry actually.

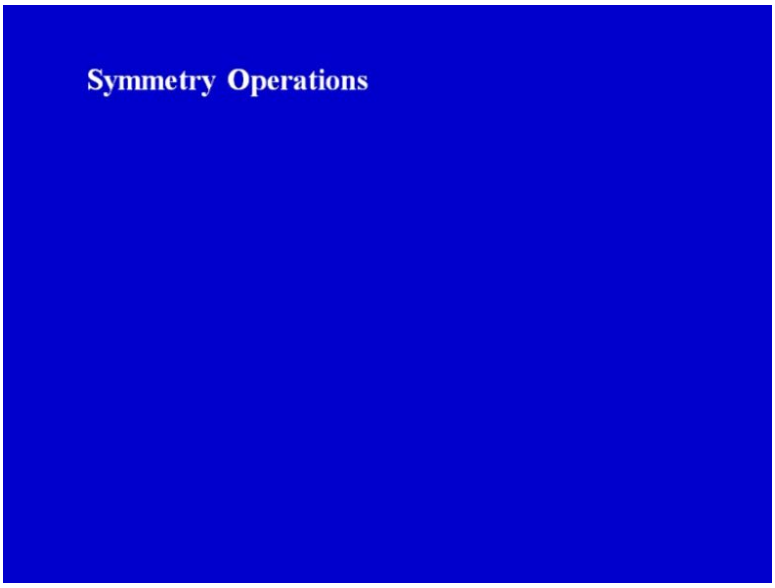
(Refer Slide Time: 34:47)



So, the characteristic symmetry of a crystal is a combination of many one or more rotations and inversions, and what you get is different types of like what I was calling crystal systems, which are these are if you see these are cubic, monoclinic, orthorhombic, tetragonal, triclinic then trigonal and hexagonal these are crystal systems, which are obtained just to give you an idea. And then from this combination of all available symmetry operations, there are 32 point groups.

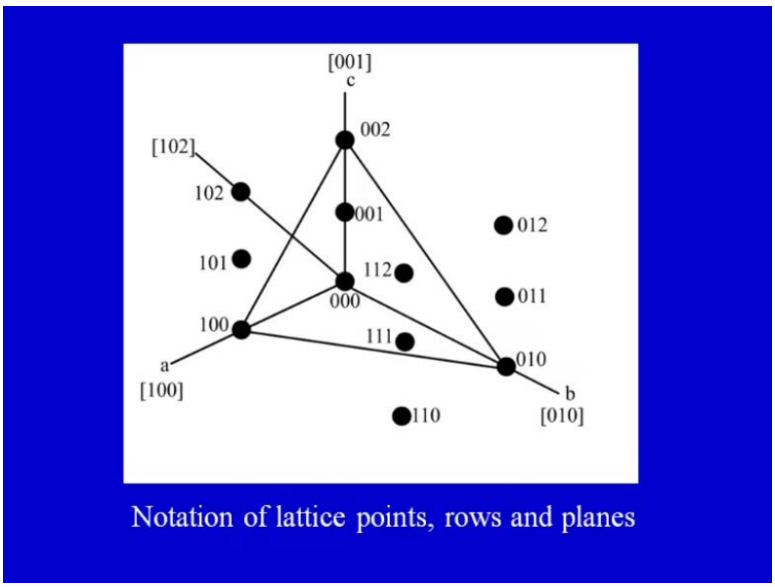
And with translation symmetry and within the all available lattices, which are 14 bravais lattices leads to 230 space groups, and these are described and arranged in like. You can say that space groups which that describe the only ways in which identical objects can be arranged in a infinite lattice. So, that is very important part these are the only possible space groups actually for to be arranged in here.

(Refer Slide Time: 35:47)



There are lot of symmetry operations, which could be a rotational, inversion, reflection and there are lot of lattice nominations which could be started like reference point from 0, 0 and axis directions which are we have shown a, b, c.

(Refer Slide Time: 36:08)



And these lattice points could be shown in here, like if you see there's a 0, 0 reference point and on all directions you can say, like they will be given certain. So, in a unit cell could be divided into different lattice points at different directions and positions will be defined by those points actually, which are in like. If you can say this is divided into 001,

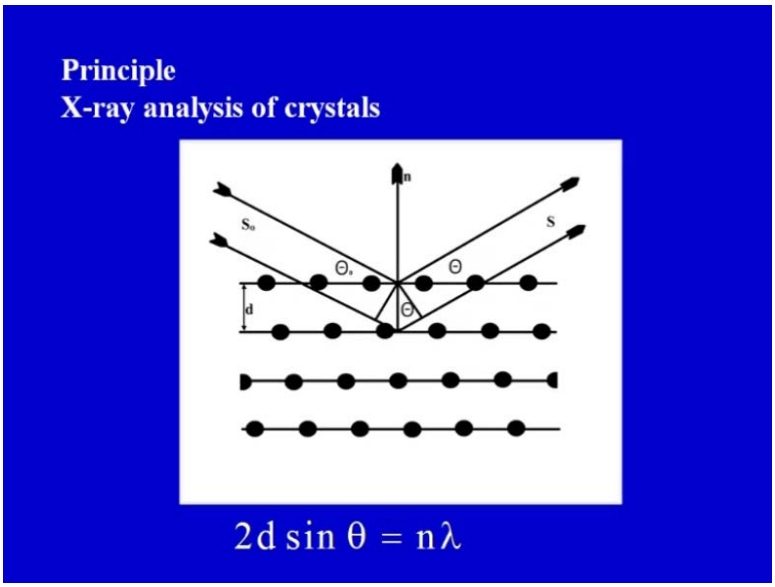
002 here, it is 102 and here it is like in another direction it is 010 depending on that these things are like, we are not going into detail, but just give you an idea.

Lattice planes are defined using miller indices, and calculated as reciprocal of the intercepts of the plane on the coordinate axis. So, the plane which might contain 100, 010, 003 these are three axis actually. Miller index is a series of co prime integers that is inversely proportional to the intercepts of the crystal phase or crystallographic planes within the edges of the unit cell. So, it describes the orientation of plane in 3D lattice with respect to the axis.

So, general form of the miller index is h k l where h k and l are integers related to the unit cell along the a, b, c crystal axis as I have shown you. So, this was little bit about symmetry and for detailed study one should go deeper into it, now X-rays were discovered like in 1895 by roentgen and likewise there was like crystal symmetry studied were being concluded at that time. And the field of X-ray crystallography of biological molecules took off like long like after long period like with Dorothy Hodgkin who is solving the structures of like vitamin B 12, cholesterol, penicillin etcetera.

And she got noble in 64 for this kind of work, crystal structure of proteins which are hundreds of times larger than you know smaller molecules, started with late 1950 with myoglobin by max Perutz, and Kendrew john Kendrew, and which they were awarded noble prize in chemistry in 62. So, basic principle on which this X-ray analysis or X-ray crystallography works is on the Bragg's law.

(Refer Slide Time: 38:57)



So, if we consider the crystal here if we see, the molecules which are arranged in different planes actually. If you see these are molecules or you can say atoms, these are diffraction planes which are arranged in here, and this is the distance between the two planes or diffracting planes. So, when X-rays hit the plane then it is diffracted or you can say reflected here, and this is at a particular angle which is like this and then when it is diffracted on another plane, then or just particular one could be related by this whole there will be, you can say phase difference will occur.

And, so there will be constructive and destructive interferences occur, and this whole relationship is given by 2D sin theta and lambda that is the Bragg equation. So, here like as we explain d is the spacing between the diffracting planes, theta is the incident angle n is simple integer, and lambda is the wavelength of the beam, which is for X-rays like copper one, which is generated from copper anode is 1.54 angstrom, which is very similar to the bond angles. These specific directions appear as a spots on the diffracting patterns, but we got reflections actually.

So, X-ray diffraction results from an electromagnetic wave that is X-rays, impinging on a regular array of scatters that is repeating arrangement of atoms within the crystal. And Bragg's laws here interference effects are observable only when, so according to the Bragg's law interference effects, which is could be both constructive or destructive. These are observable only when radiation interacts with physical dimensions that are approximately the same size, as the wavelength of the radiation.

So, only diffracted beams that satisfy the Bragg conditions are observable, which is constructive or destructive. And diffraction can thus be treated as a selective reflection, and n is an integer which is in order, lambda is the wavelength of the radiation, and d is the spacing as between the lattice planes, and theta is the angle between the incident and reflected beam incident or beam and the lattice planes actually. So, X-rays for chemical analysis are commonly obtained by simple rotating anode generators, which could be in-house generators or it could be synchrotron radiation facility.

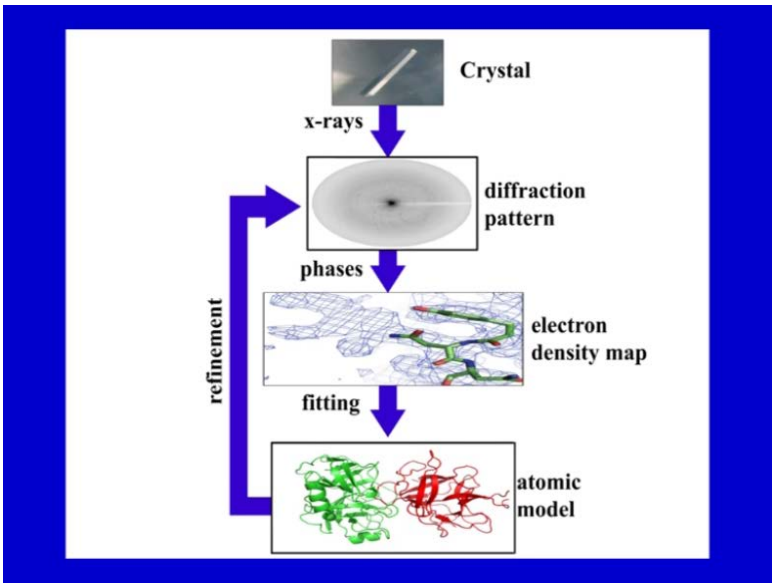
In rotating anode generators or rotating metal target is bombarded with high energy, electrons and that knockout core electrons. An electron in an outer shell fills the whole as we have discussed earlier in X-ray spectroscopy introduction, and then inner shell and emits the energy difference between the two, as an X-ray photon. Now, as x rays are diffracted by electrons, so just to understand that molecules or atoms are arranged in crystals, and there is an electron environment around each atom, atom is made up of proton, neutron and surrounding electrons.

So, X-ray diffraction occurs due to the electrons and this is the elastic diffraction, and due to the constructive and destructive interferences. One will get the intensity patterns due to like if there is amplitude are is phase, then you will get high intensity, if there is not in phase then low intensity spots. And this electron this set is produced, and this X-ray diffraction data set produces an electron density map, by Fourier transform method.

Why Fourier transform one of the very important part of X-ray crystallography is phase solution as we will discuss. Instrumentation for X-ray diffraction, if we consider is the very simple one is that X-ray source, which could be like synchrotrons, radiations source or rotating anode tubes as we have discussed. Now, X-rays are used to produce the diffraction patterns because their wavelength is typically the same order of magnitude 1 to 100 angstroms. And spacing between the planes, in the crystals the X-rays scattering is determined by the density of electrons within the crystals.

So, more higher the packing order in the crystal or they are more densely packed higher will be the diffraction, and since the energy of an electron is very much greater than that of valence electron, the scattering may be modelled as Thompson scattering, the interaction of an electromagnetic ray with a free electron.

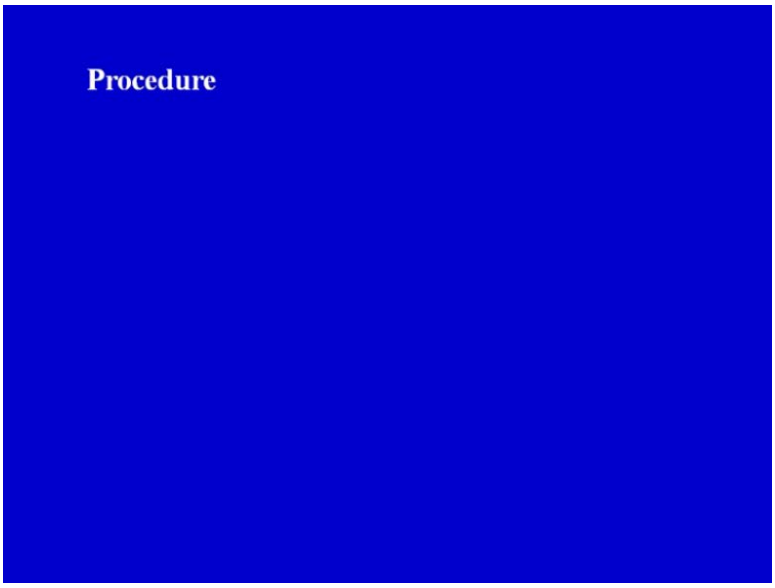
(Refer Slide Time: 44:15)



So, major aspect here if we go through the procedure here, what you have got is the crystal, and crystal X-rays are focused on the crystal. And the X-rays are diffracted by the crystal what you get a diffraction pattern in forms of intensity, this diffraction pattern is a characteristic of that particular molecule. And this, so what we are doing is like in microscope what you have is objective and IP's and finally, you see the image, but in here there are no lenses to focus the X-rays.

So, what is done is these the pattern is recorded in between actually, and these pattern which is like not focused in image formation is not taking place. The image formation is done, by going backwards through Fourier transform method, and Fourier transform finally, makes an electron density map, which is we called phase solution actually. And the problem is called phase problem, electron density map is the map of the electrons surrounding the atom. So, the electron density map and with the knowledge of the molecule, you can generate this web like structure, which is electron density map. And finally, from electron density map atomic model or the atomic positions and from that whole structure is derived.

(Refer Slide Time: 45:38)



So, procedure as we have discussed it is a very simple, where you have a single crystal X-ray crystallography has three steps, one is most difficult and the bottleneck is to get good diffracting crystals. So, adequate crystals has to be prepared from the material which needs to be analyzed, crystals should be sufficiently large say 0.1 millimetre or above and they should have homogenous composition. They should be regular in structures that is good diffracting crystals, with no significant imperfections.

Second step is the crystal is placed in intense beam of X-rays, like single wavelength which is monochromatic X-rays producing the regular pattern of reflections. And crystal is rotated at 1 degree or, so that reflections appear like many frames are collected at different like angles. So, that every like all symmetry like it is collected all full data is collected, as per the symmetry operations for determining the symmetry first single image could be collected, and from diffraction data symmetry of or a space group could be calculated.

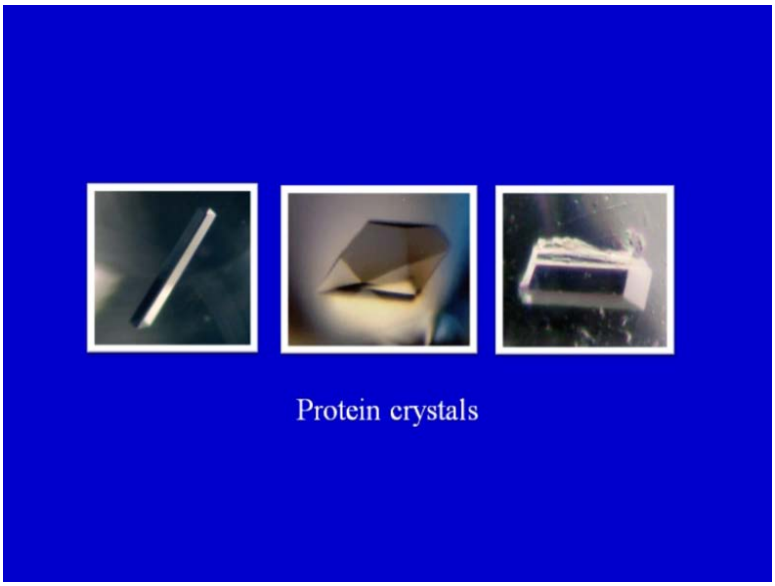
And then one can decide how many reflections or how many frames like whether it has to be rotated to 180 degree or like as per the space group, the number of reflections will be or number of rotations will be performed. The intensity of every spot is recorded at every orientation of the crystal, and multiple data sets may have to be collected with each set covering slightly more than half of full rotation of the crystal. And typically

containing tens of thousands of reflections, so remember as per symmetry operations actually you can determine the number of reflections.

And once you know the space group, then like say depending on if it is a cubic symmetry then if you do 180 degrees you are repeating the data. But, if it is a triclinic then you need to collect in full at 180 degrees, with 1 degree rotations the crystal data has to be collected. In the third step, the data are combined computationally with complementary chemical information, and to produce and refine a model of the arrangement of atoms within the crystal.

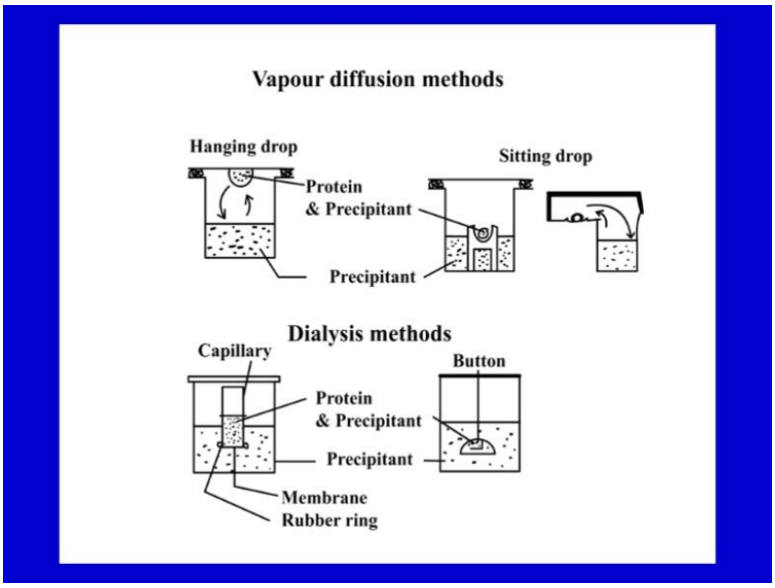
The finally, refined model of the atomic arrangement is called crystal structure, and it is refined to a particular value which is acceptable, which is called R factor or reliability factor and it is comparison of observed data and the experimental data. ((Refer Time: 48:41))

(Refer Slide Time: 48:44)



Now, crystallization which is very important or bottleneck you can see crystals looks very simple, it is depiction of how crystals might look like. They could be different shapes sizes of the crystals there could be various types of there could be thin sheet like or there could be rectangular or different shapes could be obtained.

(Refer Slide Time: 49:05)



To obtain the crystals there are various methods, which is hanging drop or sitting drop or dialysis methods. And if you can see here in the figure, in hanging drop this drop of the say protein or any material is hanging, and this whole chamber is sealed and because of this precipitants which are at higher concentration. Than in the drop they will be evaporation of from the protein drop to precipitant.

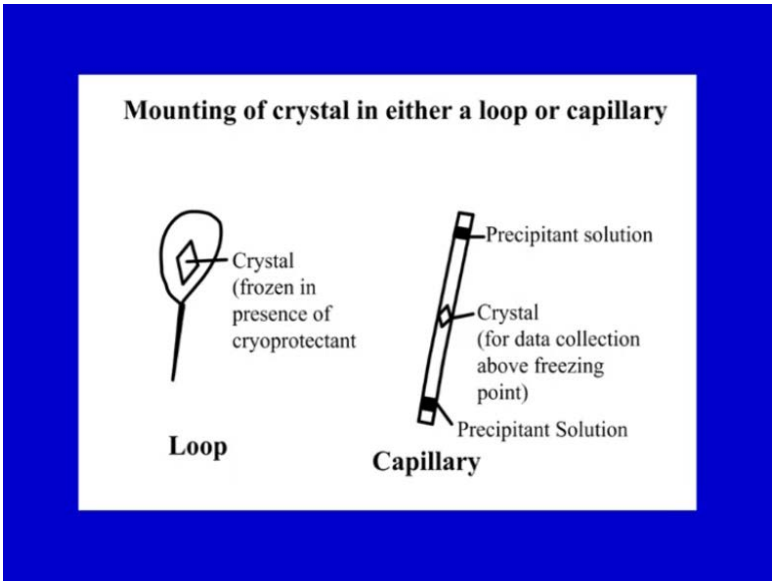
So, crystals will occur it is like you are taking the protein solution or any analyte solution to super saturation, and in that process two things can happen one is protein or things could precipitate or they could form crystals. So, that is a very tricky situation and many times it is a very hard to get crystals, this is hanging drop which is here named because drop is hanging. Then there is a sitting drop where a drop is put in here, and this is precipitant or reservoir solution.

And exchange of solvent occurs in here, again this is brought to super saturation by keeping it for some time, and crystals might form in here. Then there is a dialysis method where through a filter a membrane which is dialysis membrane, the solution or the protein, and solution or reservoir solution or precipitant is like, they are interacting. And due to difference in their conditions that is a precipitants conditions, water will be extracted out from the protein solution, and will be brought to the higher or you can say super saturation stage to yield crystals.

So, these are all methods, but there's no definitely one cannot say that crystals will grow or not this is like you have to it is a hit and trial method, and many attempts with different conditions has to meet, like for concentration differences or p H differences it has to be performed. So, crystal growth is characterized by let us say nucleation of a macroscopic crystalline, possibility having only 100 molecules followed by growth of the crystal. And then diffraction quality crystals are obtained, and that is the main aim of the crystallographer to obtain a good quality crystal.

So, like many times you have to screen or there are lot of screens available in the market, to like put your put your protein or analyte, and in different conditions like it could be 100 to 50 or may be more types of conditions are there in a particular screen. And possibly you might get a crystal in one of the conditions.

(Refer Slide Time: 52:08)



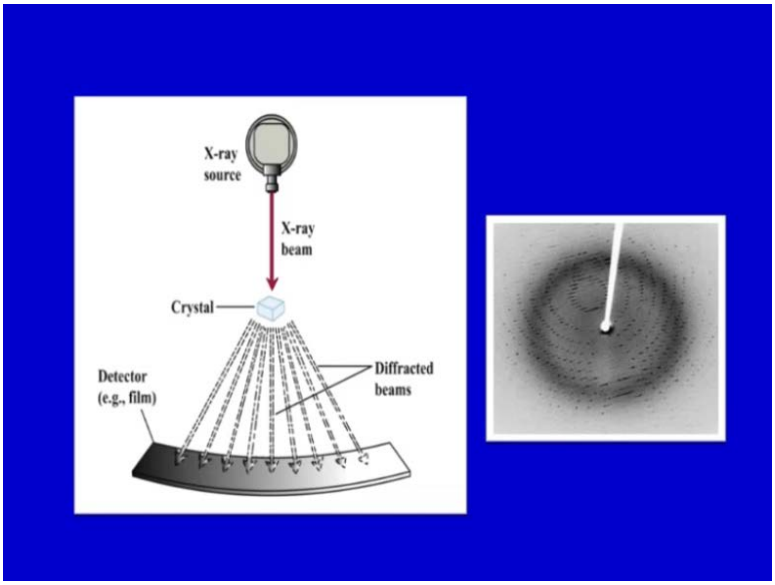
Once crystal is obtained the crystal is in this in the drop, and this crystal is picked up either it could be mounted in a loop or in a capillary. So, this is picked up surface tension it could be picked up, the crystal comes in the loop then it could be frozen with liquid nitrogen or you can do it with liquid propane, and then can put in liquid nitrogen this could be stored also for a long time in proper condition. So, once crystal is picked this could be put in front of the X-rays and then data could be collected.

Now, capillary or loop which is like once crystal is in capillary or loop, the this will be mounted on a goniometer head, and this goniometer allows it to be positioned accurately

within the X-ray beam and rotate it. So, you can position in front of the beam and properly centre your crystal, so that it is bathed by it is in X-ray direction, since both the crystal and beam are often very small, the crystal must be centred within the beam.

So, that within 25 macrometer accuracy, and this would aided by there are camera and there are systems available, so that you can do that. Then mounted crystal is then irradiated with the beam of monochromatic X-rays, the brightest and most useful X-ray source are synchrotrons, where even modulation of frequency or modulation of different wavelengths could be performed. And you get better resolution there, they also make it convenient to tune the wavelength of the radiation in synchrotron facility, which is useful for multi wavelength anomalous dispersion method phasing. Synchrotrons are generally national facilities, and there are dedicated beam lines actually for collection of the data.

(Refer Slide Time: 54:04)

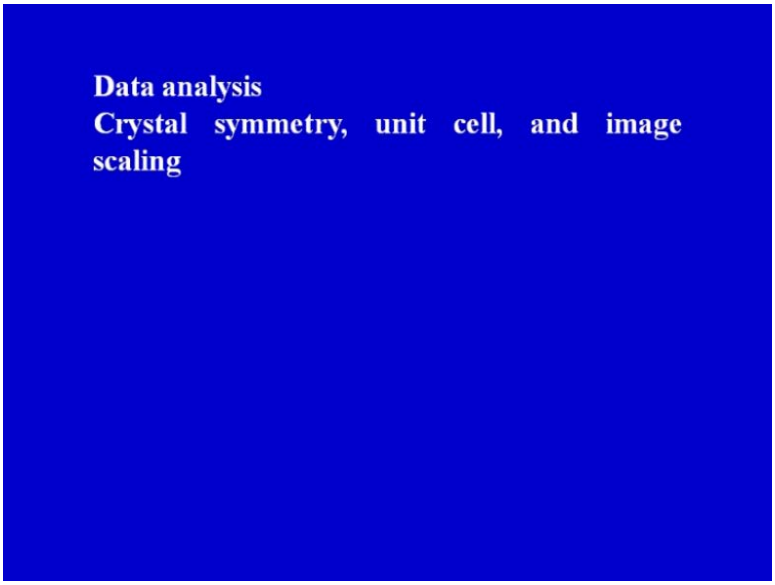


So, what you have is necessarily if you summarize there's a X-ray source, the crystal mounted in a loop, it is irradiated diffraction pattern, crystal diffracts as per it is packing. And other parameters you can have, so it is diffracted, now remember higher like as we have discussed in microscopy also earlier, higher the angle of diffraction that is more outer diffraction from the centre axis that centre it is not diffracted. So, as you go along this angle, higher the angle or higher the value then more will be the resolution actually.

This is a screen or you can say this could be a photographic X-ray film or it could be a phosphorescence screen, as in imaging plate scanner or there could be other CCD

detectors and other detectors could be there. And finally, what you get is the resolution pattern, now if you can see this is the centre and as you go here to the periphery, more is the diffraction towards this side, more is the resolution. That is we get higher resolution it means if the lower is the value if we say one angstrom resolution then it is higher resolution, if we get two angstrom or three angstrom then it is a lower resolution, so depending on how much information that is two atoms which are closely spaced, if they could be resolved. Then it is the higher resolution and it is will be obtained when there is a higher reflection or diffraction at higher angle actually.

(Refer Slide Time: 55:44)



Data analysis is done as per the crystal symmetry, unit cell and image scaling, recorded series of diffraction patterns in two dimension. And like I said many frames are collected, and all this data is combined and this is like through Fourier transform converted into three dimensional model of the electron density map. And this is done through mathematical technique called Fourier transform, each spot corresponds to a different types of variations in the electron density.

And these variations corresponds to which spot or you can say indexing is done, the relative strengths of the spots in different images. And how the variation should be combined to yield the total electron density, all these things are very complex procedural. Now, it is quite automated and many different advanced methods of phasing, and indexing are available, so that better results could be obtained. Now, data processing

starts with indexing the reflections that is when you have collected many frames at say you rotate the crystals by 1 degree.

Than those it is like you have identify, so what is done is here indexing means, identifying the dimensions of the unit cell, and which image peak corresponds to which position in reciprocal states. So, there is a direct space and reciprocal space, direct space is the crystal space and reciprocal space is diffraction space, so a by product of indexing is to determine the symmetry of the crystal that is it is space group. So, like once it is done, reflection symmetries is formed there are 65 space groups of 230 possibles which are allowed for protein molecules.

And almost always chiral indexing is generally accomplished using an auto indexing protein, once it is done having assigned the symmetry data is then integrated, this converts the hundreds of images containing thousands of reflections or diffraction pattern as we stay into single file. And it records the miller index of each reflection that is positions in the unit cell, and remember we are not talking about direct space it is reciprocal space. Each reflection and the intensity for each reflection, reflections are in reciprocal state.

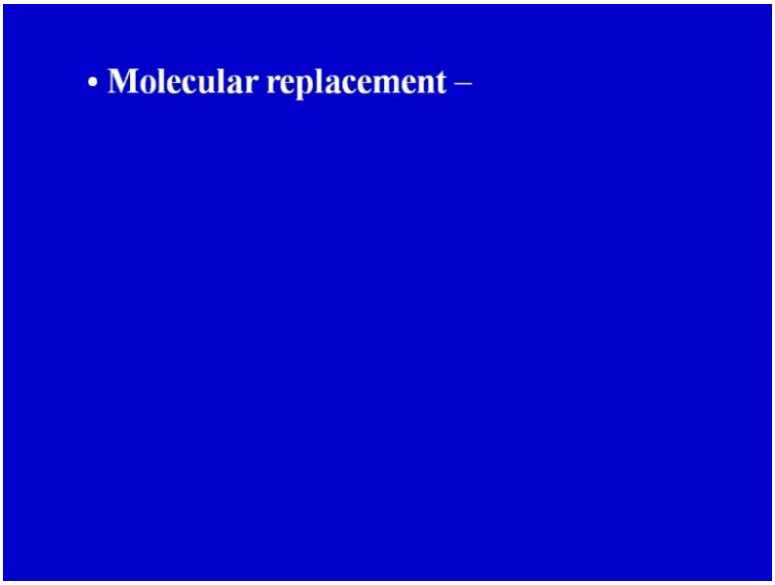
And finally, full data set will be with hundreds of reflections will be done, there will be lot of repetitions also which is calculated for degeneracy. And once it is done, then phasing is done, in phasing data collected from diffraction experiments and reciprocal space is a reciprocal space representation of the crystal lattice. The position of each diffraction spot is governed by the size and shape of the unit cell, and the inherent symmetry within the crystal.

The intensity of each diffraction spot is recorded, and this intensity is proportional to the square of the structure factor amplitude. The structure factor is a complex number containing information relating to both amplitude and phase of the wave, and in order to obtain an interpretable electron density map, both amplitude and phase must be known. And, so for this phase cannot be directly recorded during a diffraction experiment, and this is known as the phase problem as I was talking that you are collecting data in between.

So, the estimates has to be done by various ways like for a small molecules there are direct method available, which is like due to certain information you can say phase

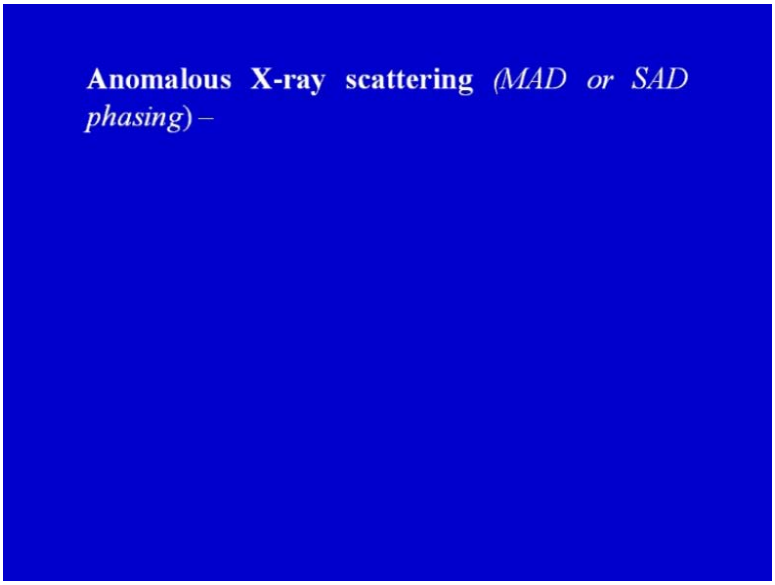
information is like in direct methods is exploited. And for a like if the resolution of the data is better than 1.4 angstrom and it is a small method a small molecule direct methods could be done.

(Refer Slide Time: 1:00:00)



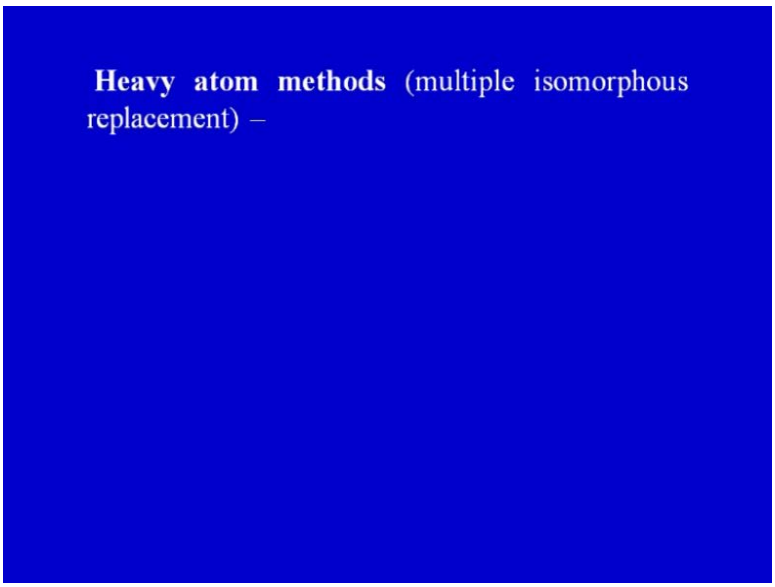
But, for bigger molecules there you have to have either methods are like molecular replacement in molecular replacement, if a related structure is known, then that could the phases of that could be taken for generating the electron density of your molecule.

(Refer Slide Time: 01:00:14)



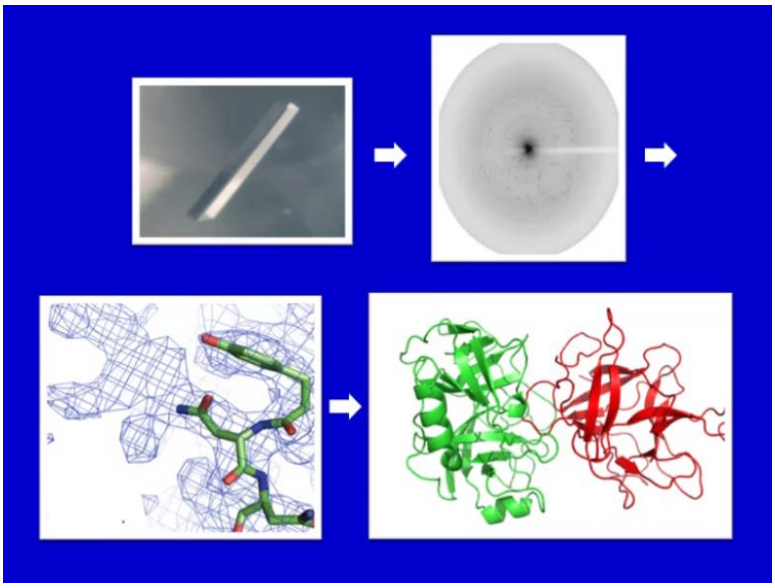
There could be anomalous X-rays scattering, this is another different method where anomalous X-ray scattering could be utilized, here X-ray wavelength may be scanned past an absorption edge of an atom, which changes the scattering in an known way. And by recording the full sets of reflection at three different wavelengths, one can solve for the structure of the anomalously diffracted atoms, and hence the structure of the whole molecule.

(Refer Slide Time: 1:00:49)



Then there could be heavy atom methods that is native crystal data is solved is taken, and then the native structure is soaked with heavy atoms, like say a platinum or gold or mercury. And then data is collected, the phases of heavy atom are calculated, and then those phases are utilized for solving the structure.

(Refer Slide Time: 01:01:15)



So, these are different methods, so what you get in the end is if to summarize this is a crystal you get diffraction pattern, by exposure of crystals to X-rays, there is electron density. And finally, you get the model by different phasing statistics, so finally, like not all the things could be covered, both for NMR and X-ray the crystallography in here. But, in essence X-ray crystallography the three important factors is, one is to get the a crystals, then second is to collect the good data high resolution data.

And third is to solve the structure by solving the phases actually that is to solve the phase problem, as we have discussed with different methods. Once the phases are known of the waves, then the positions of the atoms or electron density could be generated, and electron density maps once are obtained, then a specific position of the atom in the electron density, and with the knowledge about the structure or the composition of the molecule.

Like say for example, we know the protein sequence, then it will be easier to put in the amino acids as per the sequence. And they could be arranged in electron density and could be compared like for example, side chain of aromatic or for say arginine will have longer density as compared to alanine, and those things could be compared. And then refinement could be performed, to get the solution to as close or as reliable as possible.

As per there are different methods, different various validation methods for say calculating bond lengths, bond angles. And various other parameters where whether an

atom or whether an amino acid is in allowed or disallowed space as per the Ramachandran plot. Like phi psi values are taken, and that is how the whole structure could be solved, lot of things we could not cover because of certain constraints, but this will certainly initiate and make you aware about, these two powerful techniques to solve the three dimensional structures.

Thank you.