BMS COLLEGE OF ENGINEERING, BANGALORE-19
DEPARTMENT OF BIOTECHNOLOGY
Faculty : Dr. V. Saisha

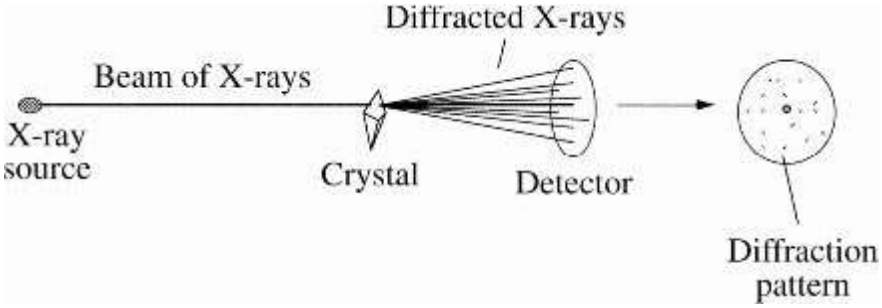
SPECTROSCOPIC TECHNIQUES

Topics:

Xray diffraction;structure determination via single crystal diffraction,fibre diffraction,neutron diffraction,XAFS,NMR spectroscopy (structure determination), Optical activity, CD, UV, IR ,Laser Raman, ESR/EPR.

X-RAY DIFFRACTION

The second widespread technique for determination of three-dimensional structure of biomacromolecules is based on the study of X-ray diffraction patterns by crystals of protein/DNA called *X-ray crystallography* The basic outline of this experiment is shown in Figure.



The technique is equally applicable to structure determination of small molecules such as those encountered in organic/inorganic chemistry and is based on the fact that atoms diffract X-rays in a pattern which is dependent on their location in three dimensional space. The reason X-rays are used is that their wavelength range is of the same order of magnitude as chemical bonds thus allowing us to 'see' at a resolution equivalent to interatomic distances (i.e. 0.8–2.5A°). To detect diffracted X-rays with high enough sensitivity, it is essential that many atoms contribute to the diffraction pattern obtained. In practice,this means that the molecule under investigation must

be present in the ordered three-dimensional array of a *crystal* so that many equivalent atoms in different molecules contribute to the diffraction pattern.In the case of biomacromolecules, it is often difficult to obtain crystals of adequate quality and this is a major limitation of this approach to structure determination. Inparticular, it means that biomolecules which *do not form crystals* (e.g. intrinsic membrane-bound proteins) are not immediately amenable to study by this technique. Not withstanding this limitation, X-ray crystallography has resulted in determination of three-dimensional structures for thousands of proteins and is still far more widely used than multi-dimensional NMR for high-resolution structure determination.It has made a number of major contributions to biochemistry such as the demonstration that enzymes are protein in nature, the Watson–Crick model of DNA (althoughdiffraction in this case was by DNA *fibres*) and understanding the molecular basis of phenomena such as allosterism and protein/DNA interactions. Moreover, structures derived from X-ray crystallography provide the major experimental link between structure and function underlying most modern biochemistry and molecular biology.

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X-ray diffraction experiment. A beam of X-rays ispassed through a crystal. Most of the beam passes straight throughbut some of the beam is diffracted by the systematic arrangement of atoms in the crystal. When a monochromatic X-ray beam is used, it is necessary to rotate or oscillate the crystal slightly during the experiment Diffracted beams are detected as a two-dimensional array of spots of particular position and intensity forming the diffraction pattern. The crystal is then rotated through a small angle (e.g. 1◦) and the measurement is repeated. At each angle some spots disappear, others appear and individual spot intensities change.this is called the diffraction pattern.

X-rays

X-rays are a form of high-energy electromagnetic radiation with wavelengths in the range 0.1–100 × 10−10 m X-rays were discovered in 1895 by the German physicist Wilhelm Conrad Röntgen and were so named because their nature was unknown at the time. X ray, invisible, highly penetrating electromagnetic radiation of much shorter wavelength (higher frequency) than visible light. The wavelength range for X rays is from about 10^{-8} m to about 10^{-11} m, the corresponding frequency range is from about 3×10^{16} Hz to about 3×10^{19} Hz.

Production of Xrays :Visible light photons and X-ray photons are both produced by the movement of electrons in atoms. Electrons occupy different energy levels, or orbitals, around an atom's nucleus. When an electron drops to a lower orbital, it needs to release some energy; it releases the extra energy in the form of a photon. The energy level of the photon depends on how far the electron dropped between orbitals.

X rays can be produced in a highly evacuated glass bulb, called an X-ray tube, that contains essentially two electrodes—an anode made of platinum, tungsten, or another heavy metal of high melting point, and a cathode. When a high voltage is applied between the electrodes, streams of electrons (cathode rays) are accelerated from the cathode to the anode and are bombarded.The energy of this bombardment is absorbed by the dislocation of electrons fromthe inner *K* and *M* atomic shells to outer atomic shells. When these electrons return to the ground state, X-ray radiation is emitted.Some of these electrons excite electrons from core states in the metal, which then recombine,producing highly monochromatic X-rays. These are referred to as characteristic X-ray lines.Other electrons, which are decelerated by the periodic potential of the metal, produce a broad spectrum of X-ray frequencies. Depending on the diffraction experiment, either or both of these X-ray spectra can be used. For crystallographic studies, radiation of $\lambda = 1.542 \times 10{-}10$ m (=1.5A° ; also-called *Kα* rays) are selected by passage through a graphite monochromator. *Kα* rays (λ = 1.542A°) are specifically selected by passage through a graphite monochromator or a nickelfilter.

A larger atom is more likely to absorb an X-ray photon in this way, because larger atoms have greater energy differences between orbitals -- the energy level more closely matches the energy of the photon. Smaller atoms, where the electron orbitals are separated by relatively low jumps in energy, are less likely to absorb X-ray photons.

X-rays having a single wavelength are desirable in crystallography because they give a single pattern of strong reflections. Two main laboratory-scale X-ray sources are widely used. *Sealed*

tube generators feature an evacuated glass tube which contains a filament (source of the electron beam)and a hollow target. They have the important advantages of ease of maintenance and replacement but produce beams of limited energy output (up to 3 kW) because too-powerful an electron beam could melt the target. *Rotating anode generators* consist of a target anode which can be rotated relative to the filament. This means that, since the electron beam impacts on a constantly-varied cool part of the filament,higher energy electron beams can be used which results in higher-power output (up to 12 kW). In addition to these,*synchrotron* sources are large-scale facilities which use a particle accelerator to produce a continuous spectrum of high-energy X-rays .

Diffraction

Diffraction is a wave phenomenon in which the apparent bending and spreading of waves occurs when they meet an obstruction. Diffraction occurs with electromagnetic waves, such as light and radio waves, and also in sound waves and water waves.

Diffraction of Waves by Crystals

The diffraction depends on the crystal structure and on the wavelength.At optical wavelengths such as 5000 angstroms the superposition of the waves scattered elastically by the individual atoms of a crystal results in ordinary optical refraction. When the wavelength of the radiation is comparable with or smaller than the lattice constant, one can find diffracted beams in directions quite different from the incident radiation. *The structure of a crystal* can be determined by studying the *diffraction pattern of a* beam of radiation incident on the crystal. Beam diffraction takes place *only in certain specific directions*, much as light is diffracted by a grating.By measuring the *directions of the diffraction* and the *corresponding intensities*, one obtains information concerning the *crystal structure* responsible for diffraction.

X-Ray Crystallography

X-ray crystallography is a technique in which the pattern produced by the diffraction of x-rays through the closely spaced lattice of atoms in a crystal is recorded and then analyzed to reveal the nature of that lattice. In order to determine molecular structure it is necessary to use X-rays because the wavelength of this radiation is of a similar order of magnitude as atoms and covalent bond-lengths The electrons of atoms are responsible for diffraction of X-rays. This is due to an interaction between the X-ray electromagnetic wave and the electron. The electric vector of the electromagnetic wave induces an *oscillation* in the electron of a frequency identical to that of the wave . This results in emission of secondary radiation of a wavelength and frequency identical to that of the incident X-ray but 180◦ out of phase with it which is known as *coherent scattering*. A related process called *anomalous scattering* can occur if the frequency of the incident beam happens to be near that of a naturally occurring oscillation in the electron. This latter phenomenon can be useful in determining the phase of scattered beams as discussed below .Since electrons are responsible for diffraction, there is a close relationship between the intensity with which incident X-rays are diffracted and the atomic number (Z) of the atom which diffracts them. This may be quantified by the *scattering factor*, f , which is related to the angle of incidence (θ) between the incident beam and the plane containing the diffracting atom and the X-ray wavelength. f is in fact a function of $\sin\theta/\lambda$ and has a maximum value of Z when $\theta = 0$.The scattering factor is a ratio between the intensity ofscattering of X-

rays by an atom and that of a single electron. heavy metals diffract with much greater intensity than other atoms and that hydrogen diffracts so weakly that it is usually not detectable.For this reason the actual structure we calculate from X-raydiffraction patterns is an *electron density map* representingthe location of C, N, S, O and other nonhydrogen atoms

In a diffraction experiment, the spacing of lines on the grating can be deduced from the separation of the diffraction maxima .The Information about the structure of the lines on the grating can be obtained by measuring the relative intensities of different orders. measurement of the separation of the X-ray diffraction maxima from a crystal allows us to determine the size of the unit cell and from the intensities of diffracted beams one can obtain information about the arrangement of atoms within the cell.

The diffraction pattern obtained contains the information we use to calculate this molecular structure. However, the diffraction pattern is a two-dimensional array of 'spots' of particular position and intensity In order to convert this into a three-dimensional structure itis necessary to collect a *data set* of many such diffraction patterns from a single crystal (the precise number required depends on the symmetry and other characteristics of the crystal).

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X-ray diffraction patterns may be detected on photographic film since Xrays ,like visible light, interact with film causing deposition of metallic silver. The intensity of each spot can be estimated relative to standard spots or by using a *densitometer*.The film is mounted in a camera which is orientated in a known angle relative to the incident beam and crystal. Alternatively, diffracted beams may be detected using *electronicdetectors* such as the *area detector*. These convert spot intensities and position to an electrical charge which is recorded Electronic detectors have a number of advantages over film; they are *more sensitive* which shortens the time necessary to collect a diffraction pattern (and hence shortens the exposure time of the crystal to the X-ray beam). Moreover, the data can be *stored electronically* in a computer-readable form which eliminates any requirement for a densitometer and again saves time. Once the crystalhas been exposed to the X-ray beam, the diffraction pattern is collected and stored electronically. The detector is then erased, the crystal rotated slightly and a second diffraction pattern collected. In this way, a data-set of diffraction patternsis built up for the crystal. Because most of the incident beam passes straight through the crystal without being scattered ,this would saturate the detector limiting its sensitivity .Accordingly, the detector is shielded from the direct incident beam by a circular piece of lead called a *beam stop*. More recently, detectors with an X-ray-sensitive phosphor screen coupled to a charge coupled device have become available.

Bragg's Law

As with all electromagnetic radiation waves, diffraction can result in interference between diffracted waves which results in the waves either reinforcing or weakening each other depending on their relative phases

These phenomena are characteristic of wave functions an are known as *constructive* and *destructive interference*, respectively.

In an X-ray diffraction experiment, a complicated pattern of scattering of X-ray beams is observed in which *both of these processes occur simultaneously*. The pattern of interference depends on the distribution of atoms in the sample through which X-rays pass. Since crystals

are highly ordered arrays of atoms, systematic interference patterns occur which may be thought of as 'encoding' information on relative three-dimensional atomic locations.In 1913 Bragg proposed that a crystal may be regarded as a series of planes which behave as 'mirrors' reflecting

X-rays. Incident waves are reflected from parallel planes of atoms in the crystal, with each plane is reflecting only a very small fraction of the radiation, like a lightly silvered mirror.wherin the angle of incidence is equal to the angle of reflection. The diffracted beams are found to occur when the reflections from planes of atoms interfere constructively.

When the X-rays strike a layer of a crystal, some of them will be reflected. We are interested in X-rays that are in-phase with one another. X-rays that add together constructively in x-ray diffraction analysis in-phase before they are reflected and after they reflected

Constructive interference between beams diffracted from successive planes occurs when there is an *integral* difference in λ between them (i.e. $n\lambda$, λ, 2λ, 3λ,etc.). this condition is met whenPQ + QR = $n\lambda$ where PQ and QR represent the distances shown. The relationship between these distances and the angle of incidence

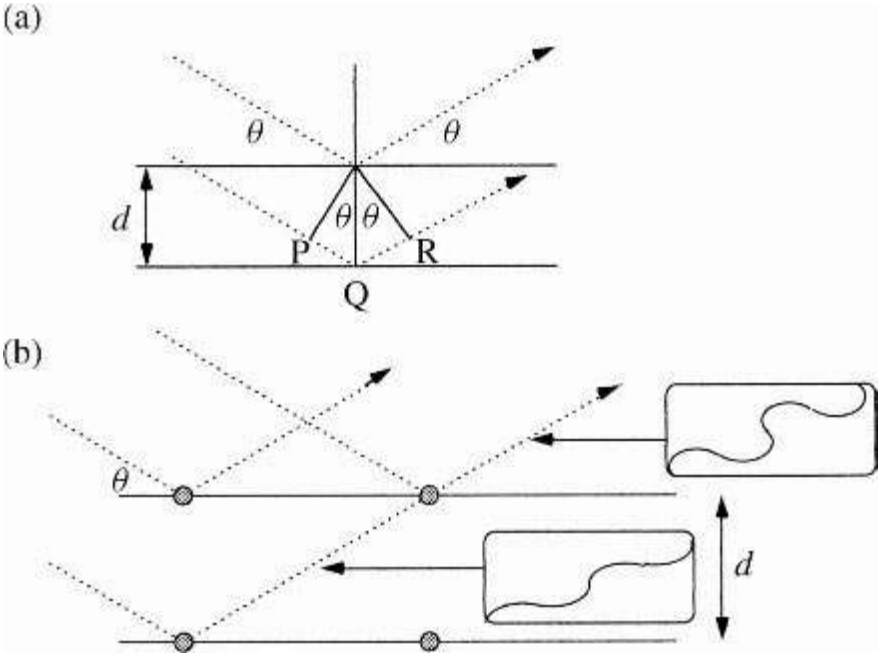


Figure. Bragg's law of diffraction.
(a) X-rays incident on successive lattice planes at an angle θ are reflected when $n\lambda = 2d \sin \theta$.where d is the distance between planes. (b) This is because successivelattice planes which obey Bragg's law give rise to constructiveinterference. Note that the diffracted wave from the upper latticeplane has greater amplitude than that from the lower plane as a result of constructive interference. of the X-ray beam (θ), is geometrical and is given byPQ = QR = $d \sin \theta$)Substituting Equation gives us *Bragg'slaw of diffraction*:$2d \sin \theta = n\lambda$.

In practice this means that the diffracted beams detected in an X-ray diffraction pattern (sometimes called *Bragg reflections*)are those originating from successive lattice planes which

obey Bragg's law and reinforce each other by constructive interference with a single reflection arising from each equivalent lattice plane (*hkl*) in the crystal. All other diffracted beams undergo some destructive interference and, since this would happen through many equivalent lattice planes in a real crystal, are so weakened in intensity that hey are not detectable. The only experimental variable in the Bragg equation is the angle of incidence, θ (since *d* isa fixed property of the crystal for a given hkl and λ is in most circumstances a fixed property of the X-ray beam).To label the reflections, Miller indices of the planes can be used. Miller indices allow identification of individual planes. They are designated in terms of (*hkl*, where *h = a/a_, k = b/b_, l = c/c_* representing points where the plane transects the unit cell (dashed lines). *Miller indices* provide a convenient way of identifying specific three-dimensional locations for such planes in the lattice. They are defined as the three intercepts (*h, k, l*) that a plane makes with the cell axes, in units of the cell edge. For example, if a plane intersects the cell axes *a−c* at points *a_*, *b_* and *c_*, the indices are given by *h = a/a_*, *k = b/b_* and *l = c/c_* Values for *h, k* and *l* are usually less than six for most crystals. beam corresponding to a value of n>1 could be identified by a statement such as 'the nth-order reflections from the (hkl) planes'.(nh nk nl) reflection

$$2\left(\frac{d}{n}\right)\sin\ \theta = \lambda$$

Rewriting the Bragg law for n-th order diffraction off (hkl) planes which makes n-th order diffraction off (hkl) planes of spacing 'd' look like first-order diffraction off planes of spacing d/n.Planes of this reduced spacing would have Miller indices (nh nk nl).Since Bragg's Law applies to all sets of crystal planes, the lattice can be deduced from the diffraction pattern, making use of general expressions for the spacing of the planes in terms of their Miller indices. For cubic structures

$$d = \frac{a}{\sqrt{h^2 + k^2 + l^2}}$$

Note that the smaller the spacing the higher the angle of diffraction, *i.e.* the spacing of peaks in the diffraction pattern is inversely proportional to the spacing of the planes in the lattice. The diffraction pattern will reflect the symmetry properties of the lattice Variation of θ is achieved by slight rotation of the crystal (approximately. 1◦) between exposures to the X-ray beam allowing detection of a distinct pattern of Bragg reflections for each angle of rotation. In a complete data-set, most atoms in the crystal lattice will contribute some Bragg reflections to some diffraction patterns.

Crystals contain considerable internal symmetry. A consequence of this is that there is identical though reciprocal symmetry in reciprocal space.This means that identical reflections may result at different crystal rotation angles. This has three useful consequences:1. Symmetry in the pattern of reflections allows identification and measurement of the lattice constants.2. All

reflections do not need to be collected for every crystal.3. Averaging of equivalent reflections gives more accurate estimates of intensities than measurement of single reflections alone.

There are many types of X-ray camera to sort out reflections from different crystal planes..

1. Laue photograph
2. Rotating crystal method
3. Powder photograph

The Laue method is mainly used to determine the orientation of large single crystals while radiation is reflected from, or transmitted through a fixed crystal. The diffracted beams form arrays of spots, that lie on curves on the film.The Bragg angle is fixed for every set of planes in the crystal. Each set of planes picks out and diffracts the particular wavelength from the white radiation that satisfies the Bragg law for the values of d and θ involved.

Back-reflection Laue Method

In the back-reflection method, the film is placed between the x-ray source and the crystal. The beams which are diffracted in a backward direction are recorded. One side of the cone of Laue reflections is defined by the transmitted beam. The film intersects the cone, with the diffraction spots generally lying on an hyperbola.

Transmission Laue Method

In the transmission Laue method, the film is placed behind the crystal to record beams which are transmitted through the crystal One side of the cone of Laue reflections is defined by the transmitted beam. The film intersects the cone, with the diffraction spots generally lying on an ellipse.*The symmetry of the spot pattern reflects the symmetry of the crystal when viewed along the direction of the incident beam. Laue method is often used to determine the orientation of single crystals by means of illuminating the crystal with a continuos spectrum of X-rays;*Therefore, the Laue method is mainly used to determine the crystal orientation.Although the Laue method can also be used to determine the crystal structure, several wavelengths can reflect in different orders from the same set of planes, with the different order reflections superimposed on the same spot in the film. This makes crystal structure determination by spot intensity diffucult.Rotating crystal method overcomes this problem.

Rotatıng Crystal Method

In the rotating crystal method, a single crystal is mounted with an axis normal to a monochromatic x-ray beam. A cylindrical film is placed around it and the crystal is rotated about the chosen axis.As the crystal rotates, sets of lattice planes will at some point make the correct Bragg angle for the monochromatic incident beam, and at that point a diffracted beam will be formed. Lattice constant of the crystal can be determined by means of this method; The reflected beams are located on the surface of imaginary cones.

By recording the diffraction patterns (both angles and intensities) for various crystal orientations, one can determine the shape and size of unit cell as well as arrangement of atoms inside the cell.

The Powder Method

$d = a$

If a powdered specimen is used, instead of a single crystal, then there is *no need to rotate* the specimen, because there will always be some crystals at an orientation for which diffraction is permitted. Here a monochromatic X-ray beam is incident on a powdered or polycrystalline sample.This method is useful for samples that are difficult to obtain in single crystal form.The powder method is used to determine the value of the lattice parameters accurately. Lattice parameters are the magnitudes of the unit vectors a, b and c which define the unit cell for the crystal. For every set of crystal planes, by chance, *one or more crystals* will be in the *correct orientation* to give the correct Bragg angle to satisfy Bragg's equation. Every crystal plane is thus capable of diffraction. Each diffraction line is made up of a large number of small spots, each from a separate crystal. Each spot is so small as to give the appearance of a continuous line.If the sample consists of some tens of randomly orientated single crystals, the diffracted beams are seen to lie on the surface of several cones. The cones may emerge in all directions, forwards and backwards. If a monochromatic x-ray beam is directed at a single crystal, then only one or two diffracted beams may result. A sample of some hundreds of crystals (i.e. a powdered sample) show that the diffracted beams form continuous cones. A circle of film is used to record the diffraction pattern as shown. Each cone ntersects the film giving diffraction lines. The lines are seen as arcs on the film.

Debye Scherrer Camera

A very small amount of powdered material is sealed into a fine capillary tube made from glass that does not diffract x-rays. The specimen is placed in the Debye Scherrer camera and is accurately aligned to be in the centre of the camera. X-rays enter the camera through a collimator. The powder diffracts the x-rays in accordance with Braggs law to produce cones of diffracted beams. These cones intersect a strip of photographic film located in the cylindrical camera to produce a characteristic set of arcs on the film.When the film is removed from the camera, flattened and processed, it shows the diffraction lines and the holes for the incident and transmitted beams.

Application of XRD

XRD is a nondestructive technique.

Differentiation between crystalline and amorphous materials;

Determination of the structure of crystalline materials Determination of electron distribution within the atoms, and throughout the unit cell;

Determination of the orientation of single crystals;

Determination of the texture of polygrained materials;

Measurement of strain and small grain size…..etc

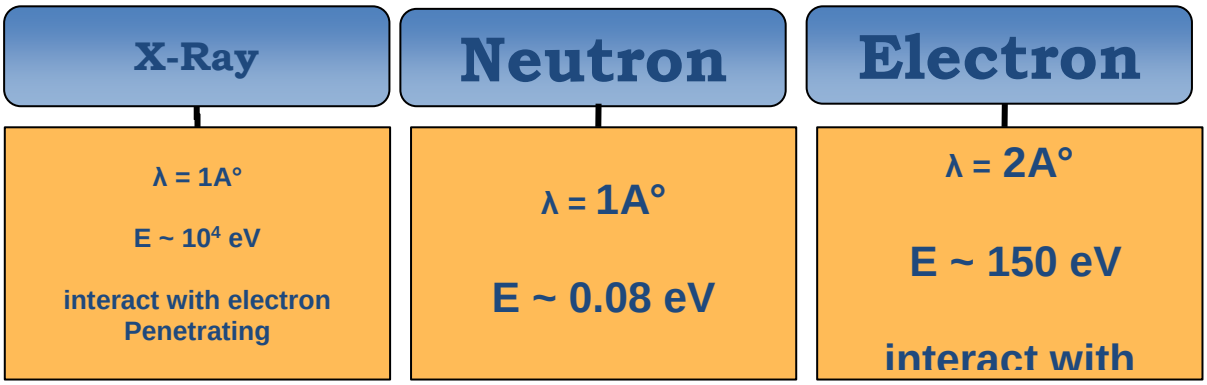
Advantages;

cheapest, the most convenient and widely used method.

X-rays are not absorbed very much by air, so the specimen need not be in an evacuated chamber.

*Disadvantage;*They do not interact very strongly with lighter elements.

Different radiation source of neutron or electron can also be used in diffraction experiments. The physical basis for the diffraction of electron and neutron beams is the same as that for the diffraction of X rays, the only difference being in the mechanism of scattering.



NEUTRON DIFFRACTION

Neutrons were discovered in 1932 and their wave properties was shown in 1936. The λ ~1A°; Energy E~0.08 eV. This energy is of the same order of magnitude as the thermal energy kT at room temperature, 0.025 eV, Just as electrons diffract X-rays, so a beam of neutrons can be diffracted by the nucleus of the atom. This diffraction also results in a 180◦ change of phase in the diffracted beam but, because nuclei have a fixed location in space compared to the relatively diffuse electron cloud, greater structural detail can be observed than in X-ray diffraction. Neutron does not interact with electrons in the crystal. Thus, unlike the x-ray, which is scattered entirely by electrons, the neutron is scattered entirely by nuclei

Although uncharged, neutron has an intrinsic magnetic moment, so it will interact strongly with atoms and ions in the crystal which also have magnetic moments. Neutron diffraction has several advantages over its x-ray counterpart;

Neutron diffraction studies can be complementary to X-ray diffraction in many ways as a result. It provides a means to locate the position of light atoms such as hydrogen which are not visible in electron density maps and also allows us to distinguish individual isotopes from each other. Moreover, since electron distribution around atoms is not necessarily symmetric, neutron diffraction can give more accurate estimates for bond lengths than those apparent in the electron density map and, for example, can help locate hydrogen bonds more precisely than X-ray diffraction. Neutron diffraction can also detect *thermal vibration* in crystals which is a major source of disorder and hence scatter in the electron density map. Major constraints on the

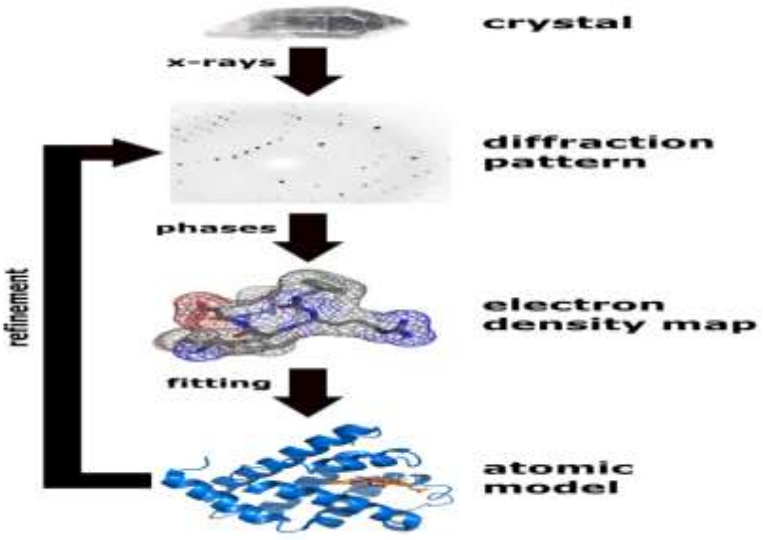
widespread use of this technique however, are the need for much larger crystals than required for X-ray or electron diffraction (~1mm3) and the requirement of time at a nuclear reactor to access a neutron beam.

ELECTRON DİFFRACTİON

Electron diffraction has also been used in the analysis of crystal structure. The electron, like the neutron, possesses wave properties Electrons are *charged particles* and interact strongly with all atoms. So electrons with an energy of a few eV would be completely *absorbed by the specimen*. In order that an electron beam can penetrate into a specimen , it necessitas a beam of very high energy (50 keV to 1MeV) as well as the specimen must be thin (100-1000 nm) low electron energies are used, the penetration depth will be very small (only about 50 A°), and the beam will be reflected from the surface. Consequently, electron diffraction is a useful technique for surface structure studies.

Electrons are scattered strongly in air, so diffraction experiment must be carried out in a high vacuum.

SINGLE CRYSTAL DIFFRACTION



The oldest and most precise method of X-ray crystallography is *single-crystal X-ray diffraction*, in which a beam of X-rays strikes a single crystal, producing scattered beams. When they land on a piece of film or other detector, these beams make a *diffraction pattern* of spots; the strengths and angles of these beams are recorded as the crystal is gradually rotated. Each spot is called a *reflection*, since it corresponds to the reflection of the X-rays from one set of evenly spaced planes within the crystal. For single crystals of sufficient purity and regularity, X-ray diffraction data can determine the mean chemical bond lengths and angles to within a few thousandths of an angstrom and to within a few tenths of a degree, respectively. The atoms in a crystal are not static, but oscillate about their mean positions, usually by less than a few tenths of an angstrom. X-ray crystallography allows measuring the size of these oscillations.

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The technique of single-crystal X-ray crystallography has three basic steps. The first—and often most difficult—step is to obtain an adequate crystal of the material under study. The crystal should be sufficiently large (typically larger than 0.1 mm in all dimensions), pure in composition and regular in structure, with no significant internal imperfections such as cracks or twinning.

In the second step, the crystal is placed in an intense beam of X-rays, usually of a single wavelength (*monochromatic X-rays*), producing the regular pattern of reflections. As the crystal is gradually rotated, previous reflections disappear and new ones appear; the intensity of every spot is recorded at every orientation of the crystal. Multiple data sets may have to be collected, with each set covering slightly more than half a full rotation of the crystal and typically containing tens of thousands of reflections.

In the third step, these data are combined computationally with complementary chemical information to produce and refine a model of the arrangement of atoms within the crystal. The final, refined model of the atomic arrangement—now called a *crystal structure*—is usually stored in a public database.

Sample preparation

Sample Selection and Preparation: Samples for single-crystal diffraction should be selected from unfractured, optically clear crystals. This can be determined by viewing the samples under crossed polars on a petrographic microscope.Crystals can be broken off a larger sample and the best fragment selected. Samples should be between 30 and 300 microns, with ideal crystals averaging 150-250 microns in size. To minimize absorption affects, equant crystals are preferred. Spherical crystals can be created using a small, air-powered crystal tumbler, however easily cleaved minerals can break during this process. Therefore, minerals lacking cleavage are the best choice for this step. If the sample is inequant, this must be corrected for during absorption corrections to the data

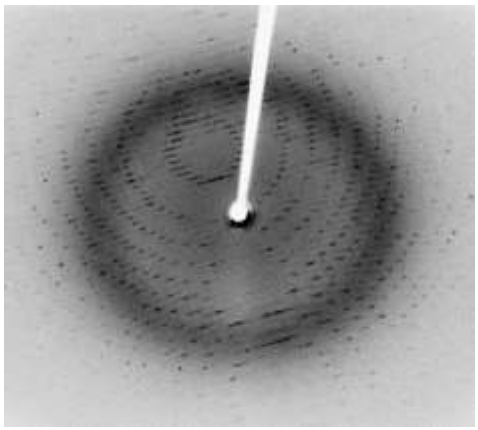
Sample Mounting: Samples are mounted on the tip of a thin glass fiber using an epoxy or cement. Care should be taken to use just enough epoxy to secure the sample without embedding it in the mounting compound. The fiber may be ground to a point to minimize absorption by the glass. This fiber is attached to a brass mounting pin, usually by the use of modeling clay, and the pin is then inserted into the goniometer head.

Recording the reflections When a crystal is mounted and exposed to an intense beam of X-rays, it scatters the X-rays into a pattern of spots or *reflections* that can be observed on a screen behind the crystal. The relative intensities of these spots provide the information to determine the arrangement of molecules within the crystal in atomic detail. The intensities of these reflections may be recorded with photographic film, an area detector or with a charge-coupled device (CCD) image sensor. The peaks at small angles correspond to low-resolution data, whereas those at high angles represent high-resolution data; thus, an upper limit on the eventual resolution of the structure can be determined from the first few images. One image of spots is insufficient to reconstruct the whole crystal; it represents only a small slice of the full Fourier transform. To collect all the necessary information, the crystal must be rotated step-by-step through 180°, with an image recorded at every step. However, if the crystal has a higher

symmetry, a smaller angular range such as 90° or 45° may be recorded. of reciprocal space. Multiple data sets may be necessary for certain phasing methods. A single crystal may degrade too much during the collection of one data set, owing to radiation damage; in such cases, data sets on multiple crystals must be taken.

An X-ray diffraction pattern of a crystallized enzyme.

The pattern of spots (reflections) and the relative strength of each spot (intensities) can be used to determine the structure of the enzyme.



Data analysis -the recorded series of two-dimensional diffraction patterns, each corresponding to a different crystal orientation, is converted into a three-dimensional model of the electron density; the conversion uses the mathematical technique of Fourier transforms.Each spot corresponds to a different type of variation in the electron density; the crystallographer must determine *which* variation corresponds to *which* spot (*indexing*), the relative strengths of the spots in different images (*merging and scaling*) and how the variations should be combined to yield the total electron density (*phasing*).Data processing begins with *indexing* the reflections. This means identifying the dimensions of the unit cell and which image peak corresponds to which position in reciprocal space. A byproduct of indexing is to determine the symmetry of the crystal. A full data set may consist of hundreds of separate images taken at different orientations of the crystal. The first step is to merge and scale these various images, that is, to identify which peaks appear in two or more images (*merging*) and to scale the relative images so that they have a consistent intensity scale

Strengths

No separate standards required

Non-destructive

Detailed crystal structure, including unit cell dimensions, bond-lengths, bond-angles and site-ordering information

Determination of crystal-chemical controls on mineral chemistry

With specialized chambers, structures of high pressure and/or temperature phases can be determined

Powder patterns can also be derived from single-crystals by use of specialized cameras.

Limitations

Must have a single, robust (stable) sample, generally between 50—250 microns in size

Optically clear sample

Twinned samples can be handled with difficulty

Data collection generally requires between 24 and 72 hours

Applications

Protein crystallography

X-ray crystallography has led to a better understanding of chemical bonds and non-covalent interactions. The initial studies revealed the typical radii of atoms, and confirmed many theoretical models of chemical bonding, such as the tetrahedral bonding of carbon in the diamond structure,

In material sciences, many complicated inorganic and organometallic systems have been analyzed using single-crystal methods, such as fullerenes, metalloporphyrins, and other complicated compounds.

- Single-crystal diffraction is also used in the pharmaceutical industry. Structure of penicillin was determined
- Determining structure of fatty acids which are important components of biological membranes
- Structures of vitamins such as vitamin B12.

FIBER DIFFRACTION

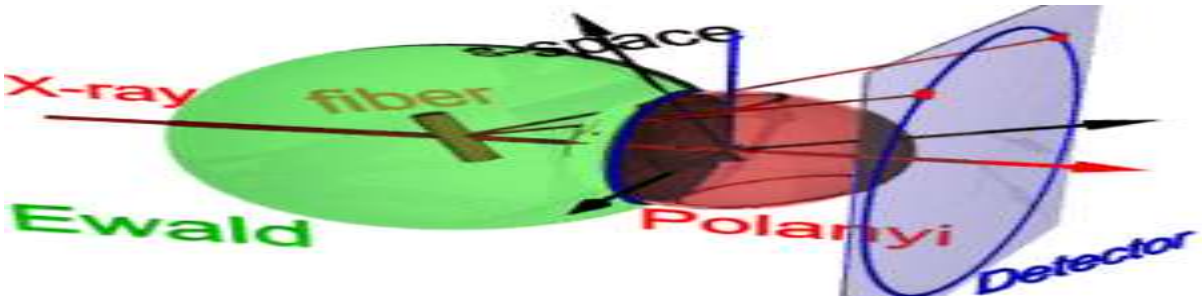
A single crystal has all its molecules and the atoms in the molecules arranged in well-ordered and repeating arrays. However, not all molecules can crsytallize X-ray diffraction of such structures is possible , if there is inherent symmetry within the molecule itself to cause constructive and destructive interference of the diffracted X-rays. Eg. in long helical biopolymer fibers.

We can think of the molecules in oriented fibers as being aligned and therefore ordered in one dimension. If the molecules have a regular secondary structure ,then the inherent symmetry of the helices will generate repeating units that are aligned along the fiber axis. We can therefore treat an exact repeat of the helix as a crystalline unit cell with well-defined lattice points. The molecules within the fiber, however, are randomly rotated relative to each other. Thus, the unit cells are rotationally disordered and averaged across the width of the fiber.

In fiber diffraction the scattering pattern does not change, as the sample is rotated about a unique axis (the fiber axis). Such uniaxial symmetry is frequent with filaments or fibers. Fibers show helical symmetry rather than the three-dimensional symmetry taken on by crystals. By analysing the diffraction from orientated fibers one can deduce the helical symmetry of the molecule and in favourable cases one can deduce the structure.

Fiber diffraction patterns fall into two main classes: crystalline and non-crystalline. In the crystalline case (e.g. A-form of DNA) The long fibrous molecules pack to form long thin micro-crystals which share a common axis (usually referred to as the c-axis). The micro-crystals are randomly arranged around this axis. The resulting diffraction pattern is equivalent to taking one long crystal and spinning it about its axis during the x-ray exposure. All Bragg reflexions are registered at one time. The reflexions are grouped along "layer-lines" which arise from the repeating structure along the c-axis. However, particularly at high resolution the Bragg reflexions tend to fall on top of each other. In non-crystalline fibers (e.g. B-form of DNA) the long fibrous molecules are arranged parallel to each other but each molecule takes on a random

orientation around the c-axis. The resulting diffraction pattern is also based on layer-lines, which reflect the periodic repeat of the fibrous molecule. The intensity along the layer-lines is continuous and can be calculated via a "Fourier-Bessel Transform" of the repeating structure of the fibrous moleculeXray scattering from microcrystal can be given as :
Ftot.=Fm(S)Fl(S) ., Fm(S)-mol. Str. Factor., Fl(S)- sampling func. generated by lattice
Fiber diffraction geometry



It is based on the notions proposed by Polanyi. If the fiber is tilted away from the perpendicular direction by an angle β, as well the information about its molecular structure in reciprocal space (trihedron labelled *s-space*) is tilted. In reciprocal space the so-called Ewald sphere has its center in the sample. Its radius is 1/λ, with λ the wavelength of the incident radiation. On the surface of the Ewald sphere all the points of reciprocal space are found that are seen by the detector. These points are mapped on the pixels of the detector by central projection.

Applications

Determining structures of different nucleic acids and estimating structural differences among the different DNA molecules and other important intermediate molecules found occurring in various biochemical cycles. Eg. F actin.

XAFS (X-RAY ABSORPTION FINE STRUCTURES)

X-ray spectroscopy is a gathering name for several spectroscopic techniques for characterization of materials by using x-ray excitation.When an electron from the inner shell of an atom is lost due to some sort of excitation, it is replaced with an electron from the outer shell; difference in energy is emitted as an X-ray photon of characteristic for the element wavelength. Analysis of spectrum of emitted by specimen X-rays produces qualitative results about elemental composition of the specimen.

Two types :

Energy-dispersive X-ray spectroscopy :measures energy of incoming photons.

Wavelength dispersive X-ray spectroscopy.:single crystal diffracts the photons which are collected by a detector. Without any motion there will be just one wavelength detected. By moving crystal and detector, a wide region of spectrum is observed

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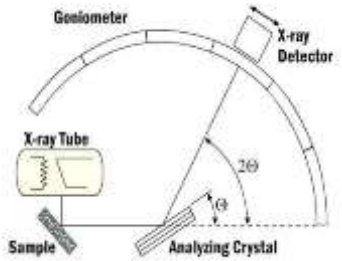
William Lawrence Bragg and William Henry Bragg, - pioneers in developing X-ray emission spectroscopy.

An X-ray spectrograph consists of a high voltage power supply (50KV or 100KV), a broad band X-ray tube, usually with a tungsten anode and a beryllium window, a specimen holder, an analyzing crystal , a goniometer, and an X-ray detector device. These are arranged as shown in the figure.



The continuous X-spectrum emitted from the tube irradiates the specimen and excites the characteristic spectral X-ray lines in the specimen. The strongest line, usually the Kalpha line but sometimes the Lalpha line, suffices to identify the element The existence of a particular line betrays the existence of an element, and the intensity is proportional to the amount of the particular element in the specimen. The characteristic lines are reflected from a crystal, the analyzer, under an angle that is given by the Bragg conditionThe crystal samples all cause the diffraction angles theta by rotation, while the detector rotates over the corresponding angle 2-theta. With a sensitive detector the X-ray photons are counted individually. By stepping the detectors along the angle, and leaving it in position for a known time, the number of counts at each angular position gives the line intensity. These counts may be plotted on a curve by an appropriate display unit. The characteristic X-rays come out at specific angles, and since the angular position for every X-ray spectral line is known and recorded it is easy to find the sample's composition

What is X-ray absorption spectroscopy (XAS)?

X-ray interacts with all electrons in matter when its energy exceeds the binding energy of the electron.X-ray excites or ionizes the electron to a previously unoccupied electronic state (bound, quasi bound or continuum). The study of this process is XAS. X-ray of sufficient energy can separate an electron from its atom. This electron propagates as a wave and scatters off of nearby atoms. The waves can then interfere .,at the site of the absorbing atom, the interference may be constructive, enhancing the chance that a given x-ray photon is absorbed, destructive, decreasing that chance, or somewhere in betweenThe kind of interference you get depends on:The energy of the incoming photon,The orbital of the absorbing electron ,The distance between the absorbing and scattering atoms,The electrical potential of the absorbing and scattering atoms, which in turn depends primarily upon which elements they are .Since the binding energy of core electrons is element specific, XAS is element and core level specific (e.g. Si K-edge at 1840 eV is the 1s electronic excitation threshold of silicon)

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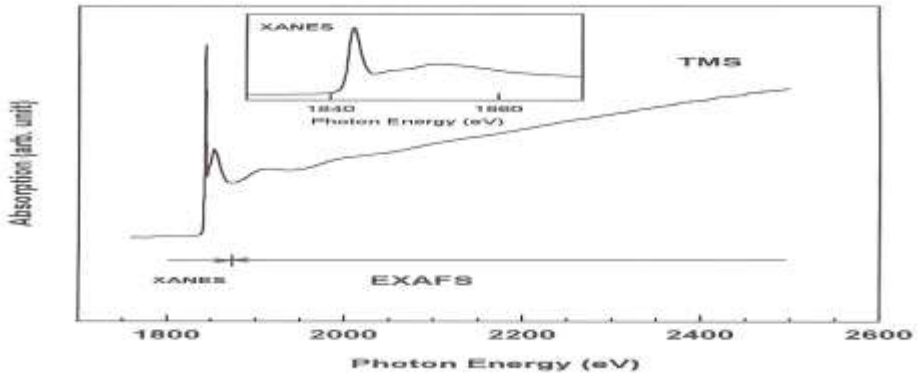
What is X-ray absorption fine structures (XAFS) ?

As core electron is excited with $h\nu \geq$ the threshold (E_o), it is excited to a final state defined by the chemical environment, which modulates the absorption coefficient relative to that of a free atom. This modulation is known the XAFS.XAFS contains all the information about the local structure and bonding of the absorbing atom .XAFS study requires a tunable X-ray source –synchrotron radiationXAS and XAFS are often used interchangeably, XAS is a general term, XAFS is specific to the modulation of the absorption coefficient by the chemical environment.

What does XAFS look like



X-ray Absorption spectroscopy is often referred to as

- NEXAFS for low Z elements (C, N, O, F, etc. K-edge, Si, P, S, L-edges) or

- XAFS (XANES and EXAFS) for intermediate Z and high Z elements.

NEXAFS (Near Edge X-ray Absorption Fine Structures) describes the absorption features in the vicinity of an absorption edge up to ~ 50 eV above the edge

XANES (X-ray Absorption Near Edge Structures), which is often used together with EXAFS (Extended X-ray Absorption Fine Structures) to describe the modulation of the absorption coefficient of an element in a chemical environment from below the edge to ~ 50 eV above (XANES), then to as much as 1000 eV above the threshold (EXAFS)NEXAFS and XANES are often used interchangeably.

Photoabsorption

Photoabsorption is a transition process between quantum states. It excites a core/valence electron into a previously unoccupied *bound states*, *quasi bound states* (excitation) or into the *continuum* (ionization, photoelectric effect).A photon can be regarded as an oscillating hammer of which the oscillating electric field acts as a perturbation to the system (*the hammer knocks the electron out of the core orbital*)Transition probability from a core level (partial absorption cross section) depends on the energy and symmetry of the initial and final states and the photon energy. Spectroscopy implication → *intensity*

At a given photon energy, all electrons in an atom with threshold energy less than the photon energy can be excited; the total absorption cross section is the sum of all the partial absorption cross sections of all levels involved.The *partial absorption cross-section* (transition probability) s, can be expressed as

$s \propto |\langle i|\varepsilon \bullet r|f\rangle|^2 r(E_f)$ $|f\rangle$ $i\rangle$: the initial state wave-function, ε: electric vector of the synchrotron r : the electric dipole vector $|f\rangle$: the final state wave-function, $r(E_f)$: the densities of states (occupancy: $|i\rangle$ bands, unoccupied molecular orbitals and continuum states). This expression is known as the Fermi's golden rule.

Scattering of photoelectron by the molecular potential – how the electron is scattered depends on its kinetic energy Low kinetic energy electrons - Multiple scattering (typically up to ~ 50 eV above the threshold, the region where bound to quasi-bound transitions take place); e is scattered primarily by valence and shallow inner shell electrons of the neighboring atoms - XANES region

•High kinetic energy electrons (50 -1000 eV) are scattered primarily by the core electrons of the neighboring atoms, single scattering pathway dominates - EXAFS regionBecause the absorption depends on the energy of the incoming photon, an x-ray absorption spectrum is given by a plot of absorption versus photon energy.

Regions of the spectrum

Edge It is sometimes used as a synonym for E_o.It is often used to represent the sharply rising part of the spectrum, although perhaps stopping before the white line

XANES.,NEXAFS.,EXAFS The portion of the spectrum from the beginning of the edge to 30 eV or so above the edge.NEXAFS tends to be used at energies below 1000 eV and XANES at higher energies. Extended x-ray absorption fine structure starts about 30 eV above the beginning of the edge. refers to the oscillations and other features visible within that region.

White line Some spectra show a sharp feature at the top of the sharply rising part of the spectrum. This feature is called the "white line."

Pre-edge:To identify the fairly featureless part of the spectrum before the sharp rise associated with the edge.

E_o:The point relative to which energies for this portion of the spectrum are measured.E_o is supposed to be the "energy origin" for the electron released when the sample absorbs an x-rayTherefore, in practice, E_o may be defined in any one of a number of reproducible, but fundamentally arbitrary, ways:At the first peak of the first derivative.,At the largest peak of the first derivative.,Half way up the steep rise.

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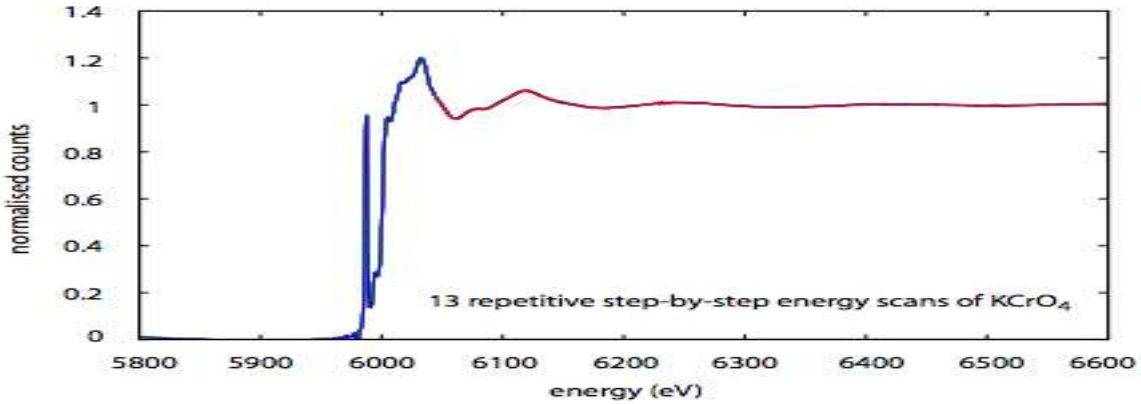


Why XAS?

Because EXAFS tends to die out within about 1000 eV of the edge and XANES is by definition limited to being near an edge, XAS is element specific; that is, the spectrum of one absorbing element can often be examined without much contamination from other elements in the sample

Because interference is affected by absorber-scatterer distances, XAS, particularly EXAFS can give information about bond lengths, disorder, and radial distribution functions out to more than five angstroms in some cases.

XAS, particularly XANES, yields information about the electronic structure of the absorbing atom, including valence.XAS can also yield information about the type, number, and arrangement of scattering atomsXAS works for a wide variety of samples: amorphous and crystalline; solid, liquid, and gas; magnetic and nonmagnetic, etc..

What do we measure in XAFS Spectroscopy?

In principle the measured quantity is the absorption coefficient μ [cm^{-1}]

The intensity I_1 of a beam with an initial intensity I_0 after passing through a sample with thickness d is:$I_1(E) = I_0(E)\, e^{-\mu(E)d}$ (Beer-Lambert law)

- => $\mu(e)d = -\ln(I_1(E)/I_0(E))$
- energy dependence of photoelectric mass attenuation coefficients. μ for a given element depends strongly on X-ray photon energy and shows additional sharp absorption edges

Experimental Set-up

Monochromatic X-rays ($DE / E \sim 10^{-4}$)The X-ray photon energy must be changeable over 1000 - 2000 eV

Position of incident beam on sample should be unchanged during energy scans

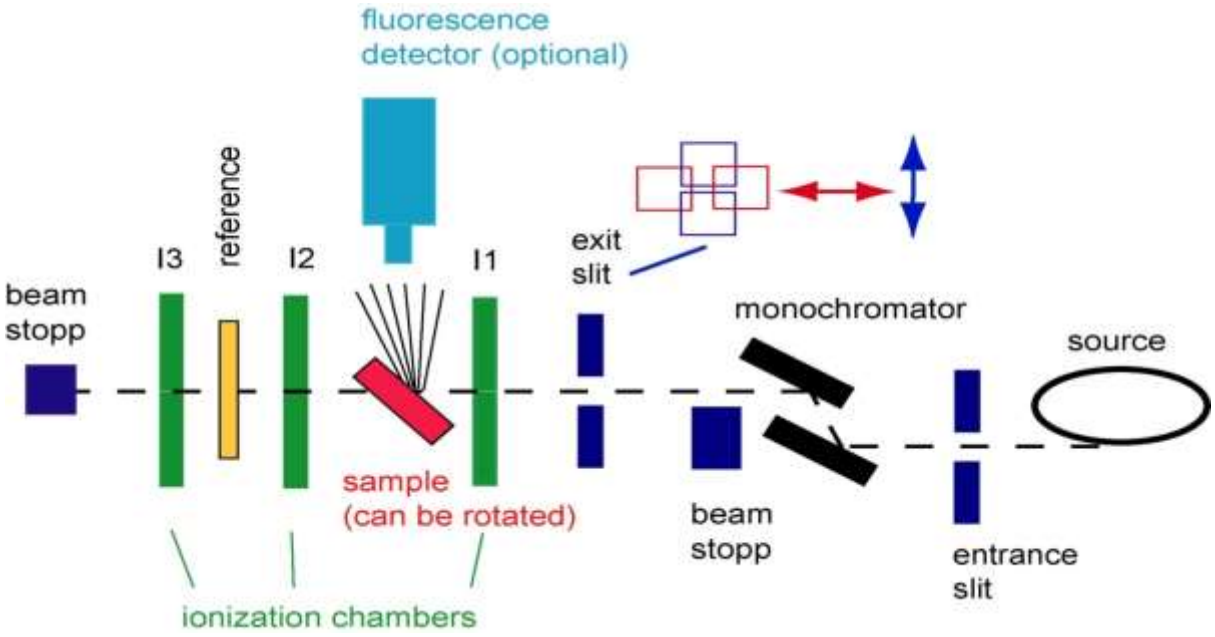
- Detectors with a large linear working range

Large number of photons (EXAFS oscillations are small, 10^{-3} - 10^{-2} effect) => 10^{6} photons/sec for statistical noise < 10^{-3} so => Synchrotron radiation necessary.

Bending magnet delivers 10^{8} photons/(sec x mm² x 10^{-4} BW) => flux from bending magnets is sufficient for normal transmission XAFS

Homogenous samples, concentrations of more than 1 wt% for element of interest

Standard XAFS set-up



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NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Nuclear magnetic resonance spectroscopy: commonly referred to as NMR, is a technique which exploits the magnetic properties of certain nuclei to study physical, chemical, and biological properties of matter. It is a physical phenomenon based upon the quantum mechanical magnetic properties of an atom's nucleus.It detects the absorption of radiofrequencies (electromagnetic radiation) by certain nuclei in a molecule. The nuclei of all atoms may be characterized by: a nuclear spin quantum number (I) .Only nuclei with spin number (I) ≠ 0 can absorb/emit electromagnetic radiation. These have an odd mass. If the number of neutrons plus the number of protons is odd, then the nucleus has a half-integer spin (i.e. 1/2, 3/2, 5/2). egI = 1/2 (^{1}H, ^{13}C, ^{19}F, ^{31}P), I = 3/2 (^{11}B, ^{33}S) & I = 5/2 (^{17}O). and if the number of neutrons and the number of protons are both odd, then the nucleus has an integer spin (i.e. 1, 2, 3). Eg. I = 1 (^{2}H, ^{14}N)If the number of neutrons and the number of protons are both even, the nucleus has no spin.Eg. I = 0 (^{12}C, and ^{16}O, ^{32}S) The spinning charged nucleus generates a magnetic field. which will have a magnetic spin moment, μ.proportional to the spin given as

$$\mu = \gamma p = \gamma \sqrt{I(I+1)}h/2\pi$$

If an external magnetic field (field-strength, B0) is applied to such a nucleus, it can orientate itself either with (parallel) or against (antiparallel). These two orientations are referred to as spin states and are distinguishable by their different spin quantum numbers, mI, which are, respectively, −1/2 and +1/2. When the energy of the photon matches the energy difference between the two spin states , an absorption of energy occurs. and cause the spinning proton to flip. This is called Resonance.

The magnetic spin moment 'wobbles' or precesses around the axis of the external magnetic field by an angle,θ, and rotates around this axis with a particular frequency,ω, which is called the Larmor frequency given as . $w_L = g B_o$

The potential energies of the two spin states are given by Equations (1) and (2):1. (Low energy spin state; mI = −1/2)E = −μ · B0 · sin θ (1). (High energy spin state; mI = +1/2)E = μ · B0 · sin θ (2)The energy difference, _E, between them is therefore given by;

$\Delta E = 2\mu \cdot B0 \cdot \sin\theta$ The frequency corresponding to the transition is give as $v = 2\mu B_o \mathrm{Sin}\theta / h$The precise value of v depends on :

- Identity of nuclei
- Chemical environment

At room temperature the energy difference between the two spin states is very small and the low energy orientation is only marginally favoured over the high energy one. When radio frequency energy matching the Larmor frequency is introduced at a right angle to the external field, it would cause a transition between the two energy levels of the spin. In other world, the precessing nucleus will absorb energy and the magnetic moment will flip to its I = -1/2 state

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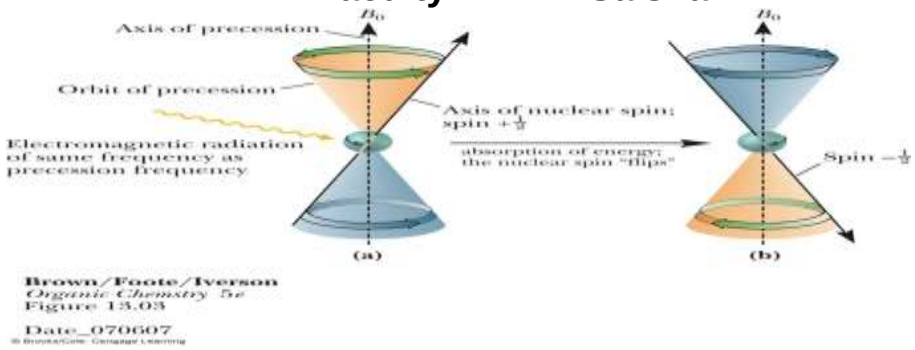


This is achieved when V of rotation of field B1 is equal to Larmor frequency The relationship is given as W= 2πν. NMR spectroscopy depends on absorption of electromagnetic radiation from the radiowave part of the spectrum causing the nucleus to undergo a transition from a low to a high energy spin state. The precise value of ν required for this transition depends on both the identity of the nucleus and on its precise chemical environment. Because of this, NMR spectra can yield precise information on the structure/composition of biomolecules and on processes in which they are involved(e.g. chemical reactions).

In order to undergo an NMR transition at a particular value of ν, a specific set of circumstances called the resonance condition needs to exist. The resonance condition is achieved when the frequency of rotation of the field represented by B1equals the Larmor frequency. This requirement for the resonance condition explains the inclusion of the word 'resonance' in 'nuclear magnetic resonance'.Energy difference is proportional to the magnetic field strength.$\Delta E = h\nu = \gamma B_0 / 2\pi$., Gyromagnetic ratio, γ, is a constant for each nucleus (26,753 $s^{-1}gauss^{-1}$ for H).NMR is especially informative, as the precise radiation frequency, ν, corresponding to the resonance condition for each type of nucleus at a given applied magnetic field strength can be affected by its immediate chemical environment.

Magnetic Shielding

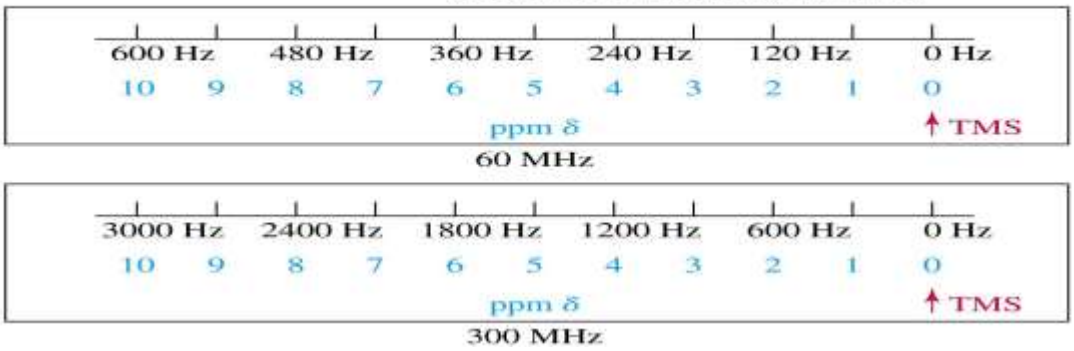
If all protons absorbed the same amount of energy in a given magnetic field, not much information could be obtained.But protons are surrounded by electrons that shield them from the external field.Circulating electrons create an induced magnetic field that opposes the external magnetic field.Magnetic field strength must be increased for a shielded proton to flip at the same frequency.Depending on their chemical environment, protons in a molecule are shielded by different amounts.A nucleus is said to be shielded when electrons around the nucleus circulates in a magnetic field and create a secondary induced magnetic field which opposes the applied field . For example, a proton forming part of a –CH2 group will achieve the resonance condition at a different radiation frequency to a proton forming part of a–CH3 group or an –NH2 group. This is due to the effect of the magnetic fields of nearby nuclei on that of the nucleus undergoing the transition and is known as chemical shielding. The effect is to alter the magnetic field experienced by the nucleus (Beff) such that :$B_{eff} = B0(1 - \sigma)$ where σ is a shielding constant.This phenomenon operates over short distances (approx.3–8A°) and allows us to identify characteristic frequencies corresponding to particular chemical groups. For example,a –CH2 group in one chemical structure (e.g. the amino acid serine) will achieve the resonance condition at a similarν to the –CH2 group of a monosaccharide. Intramolecular shielding effects like these can be used to determine the chemical structure of small molecules from their NMR spectra alone. 'Though space'shielding effects are also possible, however,

between adjacent chemical groups. For example, if a metal happened to be located near the nucleus under investigation or if two amino acid side-chains were brought into proximity in a folded protein, this would cause chemical shielding.

To standardize measurements between different NMR spectrometers and different experimental conditions, it is usual to include a reference compound(normally tetramethylsilane, TMS) with the sample to be analyzed. The frequency corresponding to the resonance condition for each transition in the sample is then expressed as the chemical shift, δ, in parts per million (ppm)as follows

$\delta = (\nu - \nu_{ref}) \times 10^6 / \nu_{ef}$., where νS and νRef are the frequencies of radiowave radiation corresponding to the resonance condition of the sample and reference nucleus, respectively. TMS has a chemical shift of 0 and each chemical group has a particular value-range. The chemical shift is given by the Delta Scale. i.e

$$\text{chemical shift, ppm } \delta = \frac{\text{shift downfield from TMS (in Hz)}}{\text{spectrometer frequency (in MHz)}}$$



- Chemical shift depends on :
- Electronegativity of nearby atoms
- Hybridization of adjacent atoms
- diamagnetic effects
- paramagnetic effects
- solvent effect
- More electronegative atoms de shield more and give larger shift values. Effect decreases with distance. Additional electronegative atoms cause increase in chemical shift.(table)

- A carbon-carbon triple bond shields an acetylenic hydrogen and shifts its signal to lower frequency (to the right) to a smaller value.Magnetic induction in the p bond of a carbon-carbon double bond deshields vinylic hydrogens and shifts their signal higher frequency.The magnetic field induced by circulation of p electrons in an aromatic ring deshields the hydrogens on the ring and shifts their signal to higher frequency

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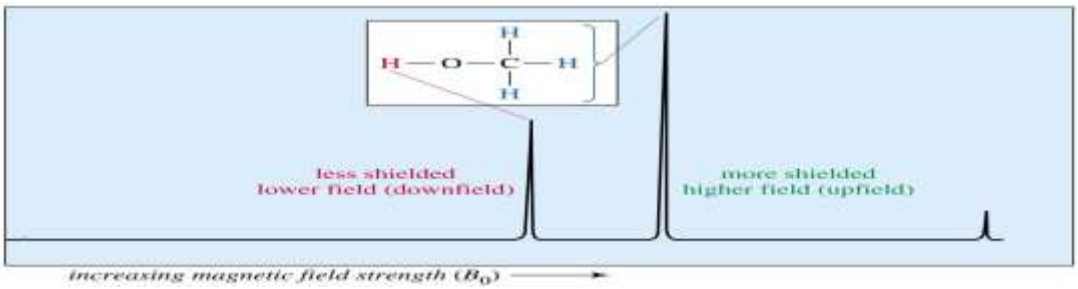
Type of Proton	Approximate δ	Type of Proton	Approximate δ
alkane (—CH_3)	0.9	>C=C<(—CH_3)	1.7
alkane (—CH_2—)	1.3	Ph—H	7.2
alkane (—CH(—)—)	1.4	Ph—CH_3	2.3
—C(=O)—CH_3	2.1	R—CHO	9–10
—C≡C—H	2.5	R—COOH	10–12
R—CH_2—X (X = halogen, O)	3–4	R—OH	variable, about 2–5
>C=C<(—H)	5–6	Ar—OH	variable, about 4–7
		R—NH_2	variable, about 1.5–4

Note: These values are approximate, as all chemical shifts are affected by neighboring substituents. The numbers given here assume that alkyl groups are the only other substituents present. A more complete table of chemical shifts appears in Appendix 1.

NMRspectra An NMR spectrum consists of a plot of the intensity of absorbance of radiowave radiation as a function of chemical shift .



- The number of signals shows how many different kinds of protons are present.
- The location of the signals shows how shielded or deshielded the proton is.
- The intensity of the signal shows the number of protons of that type.
- Signal splitting shows the number of protons on adjacent atoms.

Proton NMR

- ^{1}H experiencing the same chemical environment or chemical shift are called equivalent hydrogens.
- ^{1}H experiencing different environment or having different chemical shifts are nonequivalent hydrogens.
- Peak: The units into which an NMR signal is split; doublet, triplet, quartet, multiplet, etc.
- The area under each peak is proportional to the number of protons. Shown by integral trace.

Spin-Spin Splitting

- Nonequivalent protons on adjacent carbons have magnetic fields that may align with or oppose the external field.This magnetic coupling causes the proton to absorb slightly downfield when the external field is reinforced and slightly upfield when the external field is opposed. All possibilities exist, so signal is split.

  Signal splitting:
- Splitting of an NMR signal into a set of peaks by the influence of neighboring nonequivalent hydrogens.itfollows the (n + 1) rule: i.e If a hydrogen has n hydrogens nonequivalent to it but equivalent among themselves on the same or adjacent atom(s), its ^{1}H-NMR signal is split into (n + 1) peaks.

  Spin-Spin Coupling

- The coupling of the intrinsic angular momentum of different particles.. Spin-spin coupling between nuclear spin and electronic spin is responsible for hyperfine structure in atomic spectra.Such coupling between pairs of nuclear spins is an important feature of nuclear magnetic resonance (NMR) spectroscopy as it can provide detailed information about the structure and conformation of molecules
- J-coupling: also called indirect spin-spin coupling, is the coupling between two nuclear spins due to the influence of bonding electrons on the magnetic field running between the two nuclei. J-coupling provides information about dihedral angles, which can be estimated using the Karplus equation. It is an important observable effect in 1D NMR spectroscopy.The coupling constant, J (usually in frequency units, Hz) is a measure of the interaction between a pair of nuclei

  Range of Magnetic Coupling
- Equivalent protons do not split each other.
- Protons bonded to the same carbon will split each other only if they are not equivalent.
- Protons on adjacent carbons normally will couple.
- Protons separated by four or more bonds will not couple.

Coupling Constants

- Distance between the peaks of multiplet
- Measured in Hz
- Not dependent on strength of the external field
- Multiplets with the same coupling constants may come from adjacent groups of protons that split each other.

  Complex Splitting
- Signals may be split by adjacent protons, different from each other, with different coupling constants.Example: H^a of styrene which is split by an adjacent H trans to it (J = 17 Hz) and an adjacent H cis to it (J = 11 Hz).

  Stereochemical Nonequivalence
- Usually, two protons on the same C are equivalent and do not split each other.
- If the replacement of each of the protons of a $-CH_2$ group with an imaginary "Z" gives stereoisomers, then the protons are non-equivalent and will split each other.

Carbon-13 NMR

- ^{12}C has no magnetic spin.
- ^{13}C has a magnetic spin, but is only 1% of the carbon in a sample.
- The gyromagnetic ratio of ^{13}C is one-fourth of that of ^{1}H.
- Signals are weak, getting lost in noise.
- Hundreds of spectra are taken, averaged.

Fourier Transform NMR

- Nuclei in a magnetic field are given a radio-frequency pulse close to their resonance frequency.The nuclei absorb energy and precess (spin) like little tops.A complex signal

is produced, then decays as the nuclei lose energy. Free induction decay is converted to spectrum.

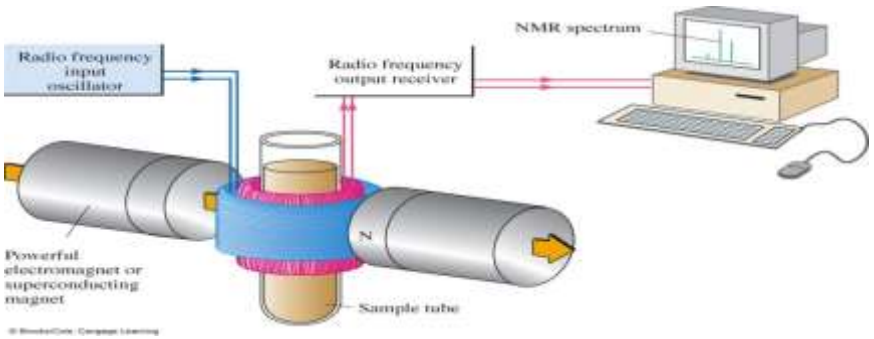
Differences in ^{13}C Technique

- Resonance frequency is ~ one-fourth, 15.1 MHz instead of 60 MHz.
- Peak areas are not proportional to number of carbons.
- Carbon atoms with more hydrogens absorb more strongly.
- It is unlikely that a ^{13}C would be adjacent to another ^{13}C, so splitting by carbon is negligible.
- ^{13}C will magnetically couple with attached protons and adjacent protons.
- These complex splitting patterns are difficult to interpret.
- To simplify the spectrum, protons are continuously irradiated with "noise," so they are rapidly flipping.
- The carbon nuclei see an average of all the possible proton spin states.
- Thus, each different kind of carbon gives a single, unsplit peak.
- ^{13}C nuclei are split only by the protons attached directly to them.
- The N + 1 rule applies: a carbon with N number of protons gives a signal with N + 1 peaks.

Interpreting ^{13}C NMR

- The number of different signals indicates the number of different kinds of carbon.
- The location (chemical shift) indicates the type of functional group.
- The peak area indicates the numbers of carbons (if integrated).
- The splitting pattern of off-resonance decoupled spectrum indicates the number of protons attached to the carbon.

Schematic NMR Spectrometer



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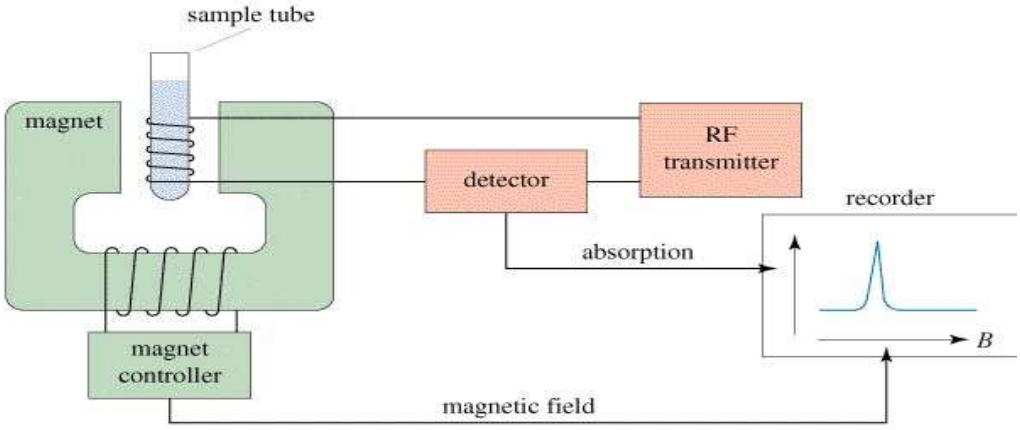

The first requirement is a magnet having the dimensions and strength required for the radiation frequency to be used. In the above instrument this consisted of a ceramic permanent magnet having a field-strength of about 1400 gauss. The second requirement is a coil to provide a small magnetic field that is adjusted to scan the range appropriate for the protons to be examined.

This coil can vary in complexity and in the original instrument includes a series of coils (called the Golay coils) that could also be used to adjust the field to a maximum degree of homogeneity.

The third requirement is an RF coil that both supplies the radiation to the sample and also senses the energy absorption at the positions of resonance. Finally there is a tube to contain the sample that is attached to an air turbine to rotate the sample at high speeds.

The rotation of the sample is essential to ensure that the sample to be exposed to a magnetic field that is as homogeneous as possible. As the sample rotates, the net field experienced is the average of that swept out during a single rotation.

Applications

- Structural elucidation
  Proteins, DNA/RNA, Protein-DNA/RNA complexes, Protein-lipidcomplexes, and Polysaccharides
- Medicine: Magnetic resonance imaging (MRI)
- Chemistry: synthesis, pharmaceutical, quality control, structure, conformation, dynamics,kinetics, chemical exchange, equilibrium, molecular tumbling, etc…
- Drug design: Structure Activity Relationships (SAR)
- Petroleum industry: test water, oil, organic, inorganic composition.
- Process control: mining, polymer production, cool analysis, cosmetics.
- Food industry:drinks, solid foods, quality, contents, contamination..
- Environmental: fertilizers, pesticides, pollution, heavy metals.
- Natural product analysis: extracts, potential drug leads..

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ELECTRON SPIN RESONANCE(ESR) SPECTROSCOPY

ESR is identical in physical principle to NMR. ESR arises from the fact that an unpaired electron also possesses a spin magnetic moment when placed in a powerful magnetic field.Samples containing unpaired electrons are said to be *paramagnetic* and ESR is also sometimes referred to as electron paramagnetic resonance (EPR). Like many nuclei, this spin magnetic moment is quantized with allowed spins of only±1/2. However, the spin magnetic moment associated with an unpaired electron is approximately 1000-fold greater than that associated with 1H. The energy difference (_E) between the two spin states of the electron therefore corresponds to the microwave part of the electromagnetic spectrum rather than the radiowave associated with NMR .This has the important consequence that we can measure ESR spectra independently of NMR spectra and vice versa since each technique avails of a distinct part of the electromagnetic spectrum.

What causes the energy levels?

When an electron is placed within an applied magnetic field, B_o, the two possible spin states of the electron have different energies. This energy difference is a result of the Zeeman effect. The lower energy state occurs when the magnetic moment of the electron is aligned with the magnetic field and a higher energy state where m is aligned against the magnetic field. The two states are labeled by the projection of the electron spin, M_S, on the direction of the magnetic field, where M_S = -1/2 is the parallel state, and M_S = +1/2 is the antiparallel state. So for a molecule with one unpaired electron in a magnetic field, the energy states of the electron can be defined as:

$E = gm_BB_oM_S = \pm 1/2gm_BB_{o.,}$where g is the proportionality factor (or g-factor), m_B is the Bohr magneton, B_o is the magnetic field, and M_S is the electron spin quantum number. From this relationship, there are two important factors to note: the two spin states have the same energy when there is no applied magnetic field and the energy difference between the two spin states increases linearly with increasing magnetic field strength.

Measurement of ESR Spectra

Because of their common physical basis, ESR and NMR share several points of similarity. Both techniques depend on a frequency of electromagnetic radiation corresponding to a specific resonance condition. In both techniques, this frequency is determined partly by the identity of the chemical group containing the magnetic spin moment. In the case of ESR, individual chemical groups cause shielding of the unpaired electron, resulting in an altered value for g. Thus, experimentally-determined values of g may be used to identify the paramagnetic chemical group. Peak splitting can also arise in ESR as a result of spin coupling with nearby nuclei, especially the nucleus actually carrying the unpaired electron. The number of peaks we would expect is given by :Number of peaks = 2I + 1 where I is the spin quantum number of the nucleus The relative intensities of each peak also follow a binomial expansion as with NMR and may be determined from Pascal's triangle

if the unpaired electron was associated with two nuclei (A and B), the number of peaks resulting would be a product of the splitting induced by each:Number of peaks = $(2 \cdot n \cdot I + 1)A \times (2 \cdot m \cdot I + 1)B$where n andm refer to the number of each nucleus and IA and IB are their respective spins (Table 3.6). Thus, if an unpaired electron was associated with an N-H group, six peaks would be expected [that is $(2 \times 1 \times 1/2 + 1) \times (2 \times 1 \times 1 + 1) = 6$]. In practice, these six peaks would be organized as three doublets. As with NMR, the strength of coupling is related to the spacing between peaks which is called the isotropichyperfine coupling constant, a. The larger the value of a,the greater the coupling and the larger the spacing betweensplit peaks. Because of the limited number of combinations of nuclei found in biological ESR, peak splitting also helps unambiguously to identify the nature of the chemical group containing the unpaired electron if this is not previously known

.ESR spectra are measured in an ESR spectrometer which is generally similar in design to an NMR spectrometer The main differences are that ESR spectrometers expose samples to microwave rather than radiowave radiation and produce derivative rather than absorbance spectra. These differences mean that distinct spectrometers are required for each magnetic resonance technique. Despite the many similarities, ESR also shows severa lpoints of difference to NMR spectroscopy. Largely for instrumental reasons, ESR spectra are conventionally measured as the first derivative (dA) of the absorbance (A) as a function of frequency (ν).

An EPR spectrum is obtained by holding the frequency of radiation constant and varying the magnetic field. Absorption occurs when the magnetic field "tunes" the two spin states so that their energy difference is equal to the radiation. This is known as the field for resonance. As spectra can be obtained at a variety of frequencies, the field for resonance does not provide unique identification of compounds. The proportionality factor, however, can yield more useful information. For a free electron, the proportionality factor is 2.00232. For organic radicals, the value is typically quite close to that of a free electron with values ranging from 1.99-2.01. For transition metal compounds, large variations can occur due to spin-orbit coupling and zero-field splitting and results in values ranging from 1.4-3.0.

In addition to the applied magnetic field, unpaired electrons are also sensitive to their local environments. Frequently the nuclei of the atoms in a molecule or complex have a magnetic moment, which produces a local magnetic field at the electron. The resulting interaction between the electron and the nuclei is called the hyperfine interaction. Hyperfine interactions can be used to provide a great deal of information about the sample including providing information about the number and identity of nuclei in a complex as well as their distance from the unpaired electron. This interaction expands the previous equation to:

$$E = gm_B B_o M_S + aM_S m_I$$

where a is the hyperfine coupling constant and mI is the nuclear spin quantum number for the neighboring nucleus. It is important to note that if a signal is split due to hyperfine interactions, the center of the signal (which is used to determine the proportionality factor)

is the center of the splitting pattern. So for a doublet, the center would be half way between the two signals and for a triplet, the center would be the center of the middle line.

- Can be used to provide information
  - Number and identity of nuclei
  - Distance from unpaired electron
  - Interactions with neighboring nuclei

The rules for determining which nuclei will interact are the same as for NMR. For every isotope of every element, there is a ground state nuclear spin quantum number, *I*, which has a value of n/2, where n is an integer. For isotopes which the atomic and mass numbers are both even, *I*=0, and these isotopes have no EPR (or NMR) spectra. For isotopes with odd atomic numbers but even mass numbers, the value of n is even leading to values of I which are integers, for example the spin of ^{14}N is 1. Finally for isotopes with odd mass numbers, n is odd, leading to fractional values of I, for example the spin of ^{1}H is ½ and the spin of ^{51}V is 7/2.Isotopes with even atomic number and even mass number have $\boldsymbol{I = 0}$, and have no EPR spectra ^{12}C, ^{28}Si, ^{56}Fe, …Isotopes with odd atomic number and even mass number have ***n*** even ^{2}H, ^{10}B, ^{14}N, …Isotopes with odd mass number have ***n*** odd ^{1}H, ^{13}C, ^{19}F, ^{55}Mn,

Hyperfine Interactions

The coupling patterns that are observed in EPR spectra are determined by the same rules that apply to NMR spectra. However, in EPR spectra it is more common to see coupling to nuclei with spins greater than ½. The number of lines which result from the coupling can be determined by the formula:$2NI + 1$.,where *N* is the number of equivalent nuclei and *I* is the spin. It is important to note that this formula only determines the number of lines in the spectrum, not their relative intensities.

So a single nucleus with a spin ½ will split each energy level into two, as shown above, and then two transitions (or absorptions) can be observed. The energy difference between the two absorptions is equal to the hyperfine coupling constant The relative intensities of the lines is determined by the number of interacting nuclei. Coupling to a single nucleus gives lines each of equal intensity. If multiple nuclei interacting

- Distributions derived based upon spin
- For spin ½ (most common), intensities follow binomial distribution

Relative intensities of splitting patterns observed due to hyperfine coupling with a nucleus with $I = ½$. The splitting patterns are named similar to those in NMR If an electron couples to several sets of nuclei, then the overall pattern is determined by first applying the coupling to the nearest nuclei, then splitting each of those lines by the coupling to the next nearest nuclei, and so on. An example of this can be seen in the radical anion of pyrazine. Where coupling to two equivalent ^{14}N ($I = 1$) nuclei gives a quintet with the relative intensities of

1:2:3:2:1 which are further split into quintets with relative intensities of 1:4:6:4:1 by coupling to four equivalent hydrogens.

As NMR spectroscopy does not usually provide useful spectra for paramagnetic compounds, analysis of their EPR spectra can provide additional insight. Analysis of the coupling patterns can provide information about the number and type of nuclei coupled to the electrons. The magnitude of *a* can indicate the extent to which the unpaired electrons are delocalized and *g* values can show whether unpaired electrons are based on transition metal atoms or on the adjacent ligands.

- Examination of hyperfine interactions
  - Provides information on number and type of nuclei coupled to the electrons
  - Indicates the extent to which the unpaired electrons are delocalized

Instrumentation

Four essential components to build an ESR spectrometer:

A monochromatic microwave source

A waveguide for guiding the microwave power to the sample

A cavity designed to ensure a proper coupling between the sample and the incoming wave.

A detector for microwave power to detect the response of the sample to microwave irradiation. Microwaves are generated by the Klystron tube and the power level adjusted with the Attenuator. The Circulator behaves like a traffic circle: microwaves entering from the Klystron are routed toward the Cavity where the sample is mounted.

Microwaves reflected back from the cavity (less when power is being absorbed) are routed to the diode detector, and any power reflected from the diode is absorbed completely by the Load.

The diode is mounted along the E-vector of the plane-polarized microwaves and thus produces a current proportional to the microwave power reflected from the cavity.

Thus, in principle, the absorption of microwaves by the sample could be detected by noting a decrease in current in the microammeter. In practice, of course, such a d.c. measurement would be far too noisy to be useful.

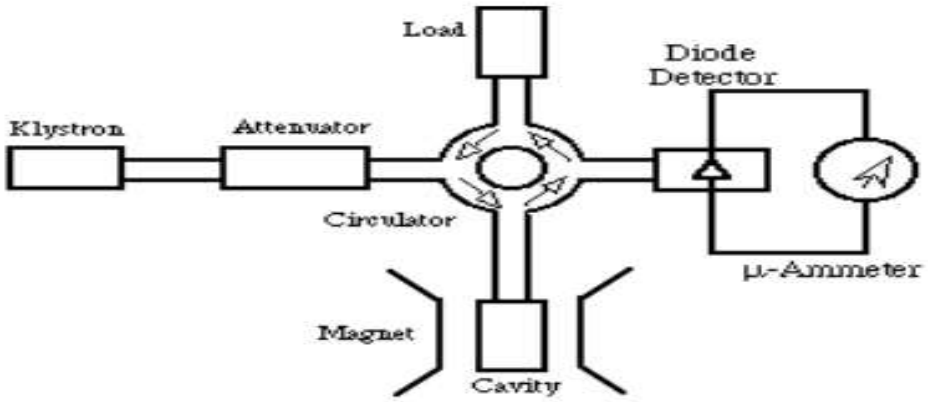


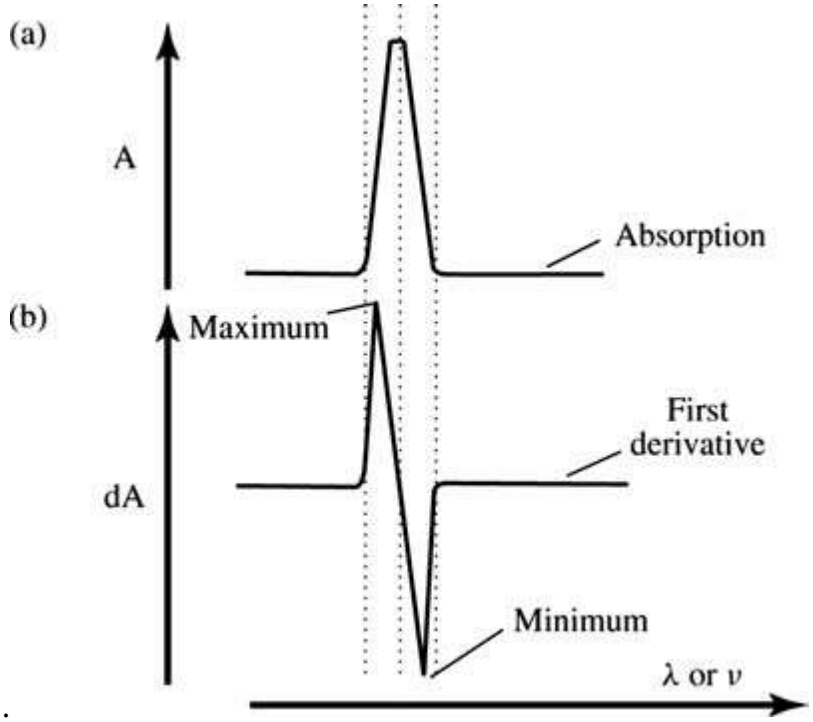
Figure 3.1. Block diagram of an ESR spectrometer.

ESR spectra are also considerably more simple and sensitive than NMR spectra. Species containing unpaired electrons are quite rare in biology which means that much fewer peaks are found in an ESR spectrum of a biological sample than in an NMR spectrum ofthe same sample. Good examples of paramagnetic species nclude molecular oxygen (O2), free radical intermediates formed during enzyme catalysis and metals forming part of metalloproteins. ESR spectra are measurable at micromolar concentrations while NMR spectra are usually obtained in the millimolar range.

Uses of ESR Spectroscopy in Biochemistry

ESR is a particularly useful technique in biological situationsinvolving unpaired electrons. Any process either involvingor producing such species may be conveniently followedby ESR. For this reason ESR has been widely used in studies of the production of free radicals and in biological processes involving transition metals. Good examples of these investigations include studies of the electron transport chain in mitochondria, kinetic mechanisms of metalloenzymesand the process of photosynthesis in plant cells.As well as studying naturally-occurring paramagnetic samples, it is possible to introduce nonbiological paramagnetic species into biomolecules. These spin probes are stable molecules which naturally contain unpaired electrons.If they can be attached at a unique site (e.g. ina protein or a membrane), they can act as reporter molecules.Unpaired electrons are especially sensitive to their freedomof movement. The more restricted the movement of a paramagneticcentre, the broader the ESR lines corresponding tothis centre Experimentally, movement can be restricted by crystallization lowering temperature, increased viscosity (e.g. by adding glycerol;) and by inclusion of the sample in a phospholipid bilayer. One of the most useful applications of ESR is therefore in identifying and quantifying dynamic mobility of molecules such as phospholipids, peptides, proteins and drugs in biological systems, especially in membranes. Changes in mobility,for example due to binding of a protein at the cell surface,can be detected as a result of effects on ESR spectra .ESR also provides an elegant means of identifying transmembrane domains in membrane proteins by site-directed spin labelling. Cysteine residues may be usedto replace individual amino acids in any protein by sitedirectedmutagenesis. Cysteine-specific spin probes can be attached at this cysteine in each mutant and the mutant proteins can then be incorporated in a phospholipid bilayer. A clear

band-broadening effect is seen in ESR spectra when the mutated residue becomes part of the transmembrane domain. Such experiments can provide important confirmation of predictions obtained from computer algorithms.



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ESR spectra are recorded as first derivatives of absorption.

(a) An absorbance spectrum is a plot of A versus λ or ν.(b) A first derivative plot of (a). This is a plot of rate of change of A.

Note that the maximum and minimum points of this plot correspondto the half-way points of (a).

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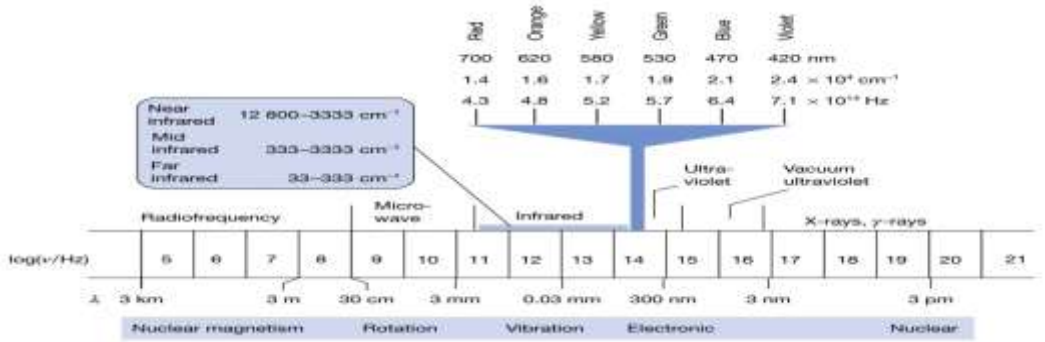
SPECTROSCOPIC TECHNIQUES.

Electromagnetic radiation spans a wide range of energy levels in a continuum called the electromagnetic spectrum. Each part of this spectrum may be characterized by a specific range of wavelengths. Depending on its energy content, radiation from different parts of the electromagnetic spectrum may interact with biomolecules in a wide variety of ways. Electromagnetic radiation therefore provides a useful means to probe the chemical structure of biomolecules.

Light is composed of electric and magnetic fields, which are mutually perpendicular and which radiate out from a source in all directions. It is therefore a form of electromagnetic radiation.

Electric and magnetic fields are propagated through space as wave functions which may be characterized by wavelength, λ (the distance from one part of the wave to the corresponding position on the next wave) and frequency, ν (the number of times a wave passes through a fixed point in space every second). These parameters are related to the energy content of the wave, E, by Equations $E = h \cdot c/\lambda$ (1) and $E = h \cdot \nu$ (2).,where h is Planck's constant and c is the speed of light. These equations demonstrate that there is a fixed relationship between the energy of a particular type of electromagnetic radiation and its wavelength/frequency. High energy radiation is characterized by short wavelengths and high frequency while lower energy radiation is characterized by long wavelengths and low frequencies.

Electromagnetic radiation from the sun covers a wide range of wavelengths in a continuum called the electromagnetic spectrum.



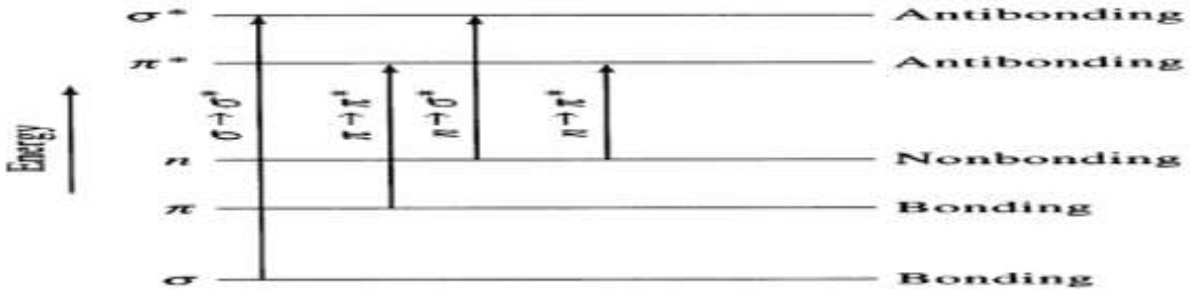
The Electromagnetic Spectrum

Wave-Particle Duality Theory of Light

The quantum theory of Planck and Einstein led to the suggestion that light had a particulate nature and could be regarded as consisting of tiny particles called photons. This particulate nature, however, appeared to contradict the diffraction of light and the fact that light passes freely through a vacuum which are consistent with a wave description of light. The competing wave and particulate theories were unified in 1923 by Louis de Broglie in his wave-particle duality theory. This postulated that light had both wave and particle natures. This is summarized in de Broglie's relationship: $\lambda = h/p$ (3.)_where p is the momentum (mass × velocity) of a moving particle such as an electron.

Absorption occurs when the energy contained in a photon is absorbed by an electron resulting in a transition to an excited state. The energy absorbed by an electron is equal to the diff. in

energy between the excited state and ground state of that electron. This amount of energy is released out when the electron returns to its ground state.



The below table gives a summary of various energy transitions occurring at diff.frequencies.

Table 12.3 Types of Energy Transitions Resulting from Absorption of Energy from Three Regions of the Electromagnetic Spectrum

Region of Electromagnetic Spectrum	Frequency (hertz)	Type of Spectroscopy	Absorption of Electromagnetic Radiation Results in Transitions Between
Radio frequency	$3 \times 10^7 - 9 \times 10^8$	Nuclear magnetic resonance	Nuclear spin levels
Infrared	$1 \times 10^{13} - 1 \times 10^{14}$	Infrared	Vibrational energy levels
Ultraviolet-visible	$2.5 \times 10^{14} - 1.5 \times 10^{15}$	Ultraviolet-visible	Electronic energy levels

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This phenomenon of transition between energy levels is not confined to electrons, however. For example, chemical bonds can occupy a variety of vibrational energy levels and atoms connected by single covalent bonds may rotate relative to each other through a number of rotational energy

Levels. These energy levels are also quantized ,having only a set number of allowed values. Transitions can occur between them corresponding to particular frequencies .

Because energy increments between vibrational energy levels are much smaller than those for electronic energy levels, the frequencies of radiation corresponding to these transitions are in the infrared part of the spectrum (i.e. longer λ and lower ν than the visible and ultraviolet). Similarly, energy increments between rotational energy levels are smaller even than those between vibrational energy levels and correspond to frequencies in the microwave part of the spectrum (i.e. longer λ and lower ν than the infrared). Transition between energy levels is a

common feature of the interaction between biomolecules and electromagnetic radiation. However, due to instrumental and experimental limitations, only some of the transitions are of use in the study of biomolecules.

ULTRAVIOLET/VISIBLE ABSORPTION SPECTROSCOPY

If light in the ultraviolet/visible part of the electromagnetic spectrum is passed through a sample in solution, some light energy may also be absorbed. Molecules (or parts of molecules) capable of absorbing light are called chromophores. This is known as an absorption spectroscopy experiment and it is of use because the particular frequencies at which light is absorbed are affected by both the structure and the environment of the chromophore. Each chemical structure absorbs slightly different frequencies of light as each has a characteristic electronic structure . The absorption phenomenon may be quantified by the Beer–Lambert law: log I0 / I= ε · c · l (4) where I0 is the intensity of incident light, I is the intensity of transmitted light, c is the molar concentration and l isthe length of the light path (usually 1 cm). ε is the molar extinction coefficient. The term [log I0/I] is the absorbance (A) at a particular wavelength or frequency. A plot of A or ε versus wavelength or frequency is known as an absorption spectrum .Wavelengths corresponding to maxima in such spectra are denoted by λmax.. Because each electronic energy level consists of several vibrational energy levels ,a range of wavelengths is absorbed rather than one fixed wavelength. Under standard conditions, this spectrum is a fixed property of a pure chromophore and may therefore be used in identification of previously-characterized molecules.

Moreover, since absorbance is directly dependent on molar concentration, it may be used as a measure of concentration provided a standard curve for that chromophore is also available. Absorbance is an especially appropriate scale for measuring interactions between light and molecules in solution in that, when no light is absorbed, this term has a value of 0. When light is absorbed, the absorbance value increases .In theory, the parameter is linear and can reach a maximum of infinity. But, since absorbances of 1, 2 and 3 correspond to 10, 1 and 0.1% of light transmitted, respectively, in practice it is usually not possible to measure absorbance accurately above 3.0. Moreover the accuracy of quantification measurements of absorbance are therefore obtained in the range 0–1.0. Some wavelengths are particularly useful in the study of biomolecules.eg. Amino acids have a strong absorbance around 210 nm and this is frequently used to detect peptides. The aromatic amino acids, tyrosine and tryptophan have relatively strong absorbance at 280 nm while nucleic acids absorb strongly at 260 nm. These wavelengths are therefore widely used in studies of proteins and nucleic acids, respectively.

The absorbance spectrum for a chromophore under standard conditions is only partly determined by its chemical structure. The environment of the chromophore also affects the precise spectrum obtained. The most important environmental factors affecting absorption spectra are pH, solvent polarity and orientation effects. These effects are especially important in studies of biopolymers such as proteins and nucleic acids where chromophores may act as reporter molecules which can give information about their immediate environment.

Protonation/deprotonation effects resulting from pH changes or oxidation/reductions., affect electron distribution in chromophores. This often results in dramatic differences between the absorption spectra of the protonated and deprotonated forms of the chromophore .

eg.Comparison of the spectra for protonated and deprotonated tyrosine reveal wavelengths called isosbestic points where absorbance of both forms of the chromophore are identical. This can be useful if it is desired to quantify the total chromophore in solutions of different pH. It is often possible to find a wavelength where only one form of the chromophore (prototonatedor deprotonated, reduced or oxidized) has a strong absorbance while the other does not. Measurements of absorbanceat this wavelength across a range of pH may beused to measure the pKa of the relevant group.

Solvent polarity also affects the absorption spectrum determined for chromophores Alternative solvents to water include aqueous solutions of dimethylsulphoxide, dioxane, ethylene glycol, glycerol and sucrose .Chromophores frequently give a slightly different spectrum in such solvents compared to water and this experiment is known as solvent perturbation. They are particularly useful in determining whether or not a chromophore is in contact with the solvent (e.g. on the surface of a protein or membrane or buried in the interior of the protein/membrane). Eg.The excited states of most $\pi \rightarrow \pi^*$ transitions are more polar than their ground states because a greater charge separation is observed in the excited state. If a polar solvent is used the dipole–dipole interaction reduces the energy of the excited state more than the ground state, hence the absorption in a polar solvent such as ethanol will beat a longer wavelength (lower energy, hence lower frequency) than in a non-polar solvent such as hexane The reverse is also observed if the excited state reduces the degree of hydrogen bonding. Here the transitions are $n \rightarrow \pi^*$ and the shift of wavelength is due to the lesser extent that the solvent can hydrogen bond to the excited state. Carbonyl groups in particular hydrogen bond to their solvent. For example changing from hexane to water as the solvent for propanone, the absorption maximum moves from 280 to 257nm .Ethene, containing only one double bond, has an absorption maximum at 185 nm (ε = 10 000). If the carbon chain length is increased this peak shifts to a slightly longer wavelength because the σ bonded electrons of the alkyl group interact with the π bond electrons in the double bond (ie the energy of the excited state is reduced).The shift in wavelength is small compared with the effect of increasing the number of double bonds, especially if the electrons in the π systems (the double bonds) can interact with each other. The simplest example is buta-1,3-diene, $CH_2=CH–CH=CH_2$. Buta-1,3-diene has an absorption maximum at 220 nm,with an absorption coefficient of 20 000 – ie both the wavelength and the intensity of the absorption have increased. This difference arises because instead of the double bonds absorbing in isolation of each other the π system extends over the length of the carbon chain – ie the system is conjugated (or delocalised) – and lowers the energy of the excited state The longer the conjugated carbon chain in the absorbing system, the greater the intensity of the absorption.

Absorption is advantageous in the acid-base indicators. A small change in the chemical structure of the indicator molecule can cause a change in the chromophore and it will absorb in different parts of the visible spectrum. Orientation effects are a spectroscopic consequence of the relative geometry of neighbouring chromophore molecules. A good example is the hypochromicity of nucleic acids . A solution of free nucleotides has a higher A260 (i.e. absorbance at a wavelength of 260 nm) than an identical concentration assembled into a single-stranded polynucleotide. Double stranded nucleic acids, in turn, have a lower absorbance at this wavelength than single stranded polynucleotides. For this reason, absorbance measurementsare useful in monitoring assembly or denaturation of nucleotides in vitro.

The effect of the various conditions which bring about a change in the absorbance of chromophore are of 4 types :

- Bathochromic shift (red shift) – a shift to longer l; lower energy
- Hypsochromic shift (blue shift) – shift to shorter l; higher energy
- Hyperchromic effect – an increase in intensity
- Hypochromic effect – a decrease in intensity

The absorption efficiency of an analyte generally not affected by:

- Other (low conc.) solutes
- Temperature (within reason)
- Concentration

This makes absorption spectroscopy one of the few bioanalytical methods where the signal intensity is directly proportional to the concentration

UV-Visible Instrumentation

Absorption spectra are measured in a spectrophotometer, the basic outline of which is shown in Figure. Electromagnetic radiation is generated by a lamp which contains a metal filament through which an electric current flows. For wavelengths in the visible range a tungsten-halogen lamp (290–900 nm) is used while deuterium lamps provide both ultraviolet and visible radiation (210–370 nm). The light emitted from these sources will consist of a wide variety of wavelengths. It is necessary to select a single wavelength by passing the light through a monochromator (diffraction grating) This monochromatic light is then used as the incident light in the absorption experiment.Transmitted light is detected by a photodetector (photomultiplier tubes and photodiode array detectors.) Photomultiplier tubes convert light intensity into an electric signal by amplifying the signal with a cascade of electrons. Photodiode arrays are cheaper alternatives to photomultiplier tubes and are composed of silicon crystals arranged in a linear array which are sensitive to light in the λ range 190–820 nm. Absorption of light by a photodiode produces a current proportional to the number of photons absorbed. Photodiodes allow extremely fast detection of light intensity.

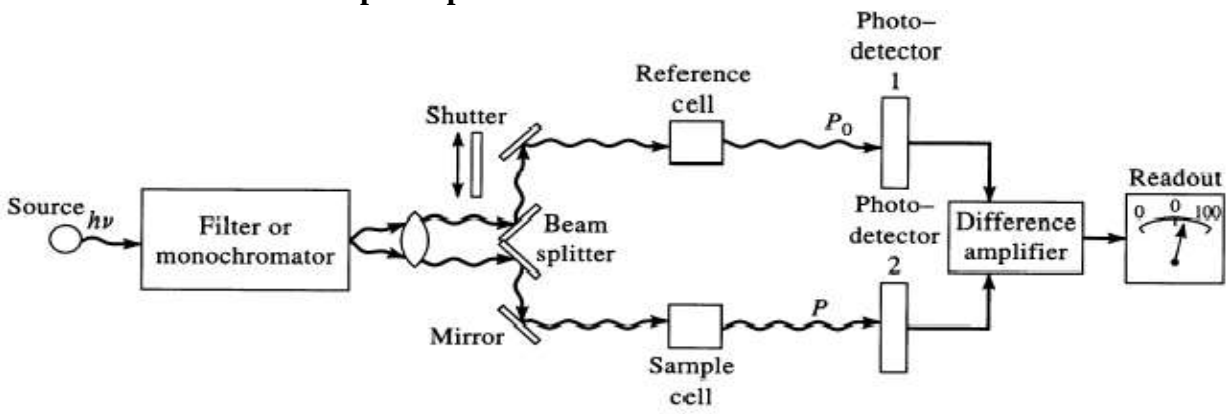
A variety of design formats are available for spectrophotometers including single beam, split (double) beam and dual beam instruments. Use of a single, fixed wavelength may be appropriate in some applications. This is achieved by replacing the monochromator with filters which allow a single wavelength of incident light pass through to the sample. Examples of this include microtitre plate readers (which are used to take readings from 96-well microtitreplates) and variable wavelength detectors of the type used in chromatography .

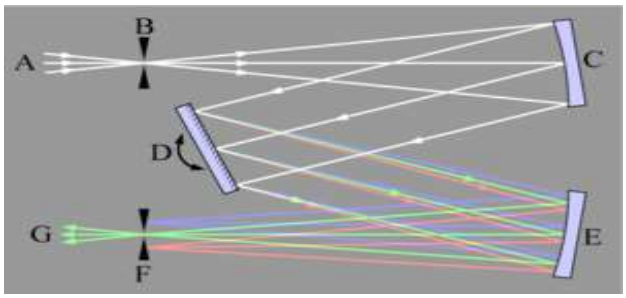
Sample is contained in tubes called cuvettes which may be constructed from a number of materials. Glass cuvettes are useful in the visible part of the spectrum but have the disadvantage that they may absorb ultraviolet radiation.Quartz cuvettes do not absorb in the ultraviolet but are fragile and expensive.

Disposable plastic cuvettes which do not absorb at wavelengths greater than 280 nm are now commercially available. Plastic cuvettes may be damaged by exposure to organic solvents which can limit their usefulness in certain types of experiments. Because the cuvette (and/or solvent) may have its own absorption spectrum, it is essential to determine a blank spectrum of cuvette plus solvent and to subtract this from that of solvent containing the chromophore to obtain a true spectrum for the chromophore .Frequently this is achieved in a dual beam

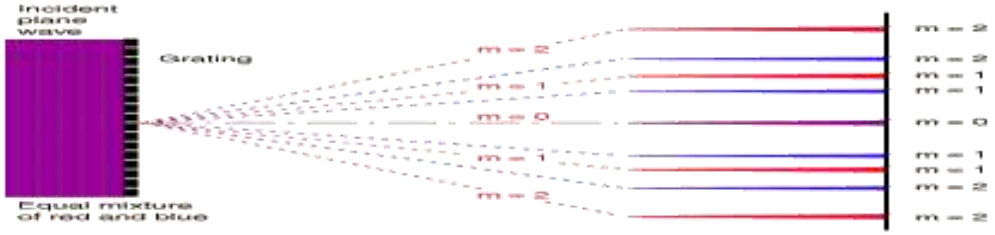
instrument with one beam passing through the blank cuvette and the other through the analytical sample.

Schematic of a UV/Visible spectrophotometer.

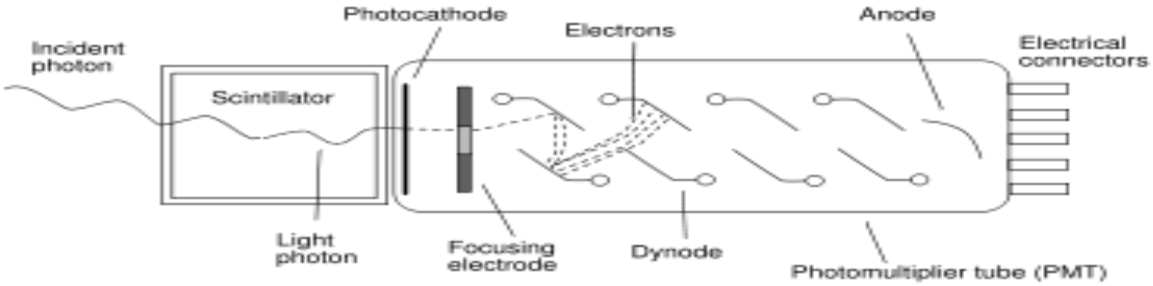


- Monochromators eg. Czerny-Turner setup
- Monochoromatic beam of light is obtained by passing narrow beam of light through reflectors
- 

Diffraction gratings



- After passing though a slit (or bouncing off a ridge) the angle at which the light leaves is given by $a\sin\theta = n\lambda$
- Photomultiplier



In photomultipliers, light hits a photocathode, releasing a small number of electrons, which are then made to collide with a series of dynodes, each more positive than the last Each collision produces more and more activated electrons.Sensitive, but noisy. Pretty much needed for low energy (IR) photons

Applications

- commonly used for concentration measurements or validation:Protein concentration with dyes (Bradford) and without (A_{280} - Tryptophan)
- Purity of protein or nucleic acid preps (A_{260} /A_{280})
- Quantitative measurements.: If the value of ε is known, it is possible to calculate concentrations directly from absorbance readings at specific wavelengths.eg. In enzyme assays the concentration of either substrate or product may be measured to allow calculation of rates of continuous (i.e. the reaction is monitored continuously in the light-beam of a spectrophotometer) or stopped (i.e. the reactionis halted at various time-points and spectroscopic measurements are performed separately from the reaction producing product or consuming substrate). For example, reduction of NAD to NADH + H+ by oxidoreductase enzymes is conveniently followed by continuous measurement of A340. NADH has a strong λmax at this wavelength while NAD does not .
- structural studies of biopolymers such as proteins and DNA. These are complex biomacromolecules and their assembly and unfolding are of considerable research interest. Chromophores, which are part of these molecules, are very sensitive to their immediate environment. We can often follow assembly/denaturation processes in such molecules, therefore, simply by monitoring absorbance changes at particular wavelengths, sincethese processes will alter the precise environment of thechromophore
- Under standard conditions, absorption spectra are characteristic for specific biomolecules. Such spectra may therefore provide a useful chemical ‘fingerprint’ for comparison This makes possible confirmation of identity between molecules perhaps purified from different sources. Conversely, differences between spectra are good evidence for structural difference.

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INFRARED SPECTROSCOPY

Absorption is the process by which the energy of a photon is taken up by the matter, and this process plays a key role in IR spectroscopy. IR radiation does not have enough energy to induce electronic transitions as seen with UV and visible light. Absorption of IR is restricted to excite vibrational and rotational states of a molecule..For a molecule to absorb IR, the vibrations or rotations within a molecule must cause a net change in the dipole moment of the molecule. Polar molecules are those which have a permanent dipole moment. The alternating electrical field of the radiation interacts with fluctuations in the dipole moment of the molecule. If the frequency of the radiation matches the vibrational frequency of the molecule then radiation will be absorbed, causing a change in the amplitude of molecular vibration.

Physical Basis of Infrared Spectroscopy

When atoms come together to form a covalent bond, they undergo an electronic rearrangement which involves two competing sets of forces. The positively-charged nuclei of the two atoms tend to repel each other (electrostatic repulsion)while the nucleus of each atom is attracted to then egatively-charged electrons of the other (electrostatic attraction).The mean distance settled on between the atoms(i.e. the bond length) is a reflection of a point of balance between these competing attractive and repulsive forces and ,in general, will be characteristic for a particular chemical bond. For example, in polypeptides Cα–C bonds are 1.52 °A while N–Cα bonds are 1.45A° . It is possible to plot the energy of a molecule as a function of the interatom distance,r, in a Morse diagram Most molecules in a population at rest are at the minimum of this diagram, but individual bond lengths can vary by±0.5 °A and bond angles by ±5◦ at room temperature.We can think of each chemical bond, therefore as existing in a particular vibrational energy level characterized by a particular bond length, bond angle and electron density.However, if radiation of an appropriate frequency is passed through the sample, it is possible for the molecule to undergo a transition to a higher vibrational energy level by absorption of radiation. As described earlier,vibrational energy levels are quantized in the same way as electronic energy levels although the _E values associated with vibrational transitions are much smaller than those associated with electronic transitions. A vibrational transition from the ground state to the first excited state due to absorption of infrared light is called a fundamental absorption and the frequency, ν, associated with this is called the fundamental frequency. Whilst other transitions are also possible (e.g. from the ground to the second or third excited state), they occur much less frequently and represent weak absorbance.

What is a vibration in a molecule?

Any change in shape of the molecule- stretching of bonds, bending of bonds, or internal rotation around single bonds .Asymmetrical stretching/bending and internal rotation change the dipole moment of a molecule. Asymmetrical stretching/bending are IR active.Symmetrical stretching/bending does not. Not IR active.A non-linear molecule having n atoms may have many different vibrations. Each atom can move in three directions: 3n. Need to subtract 3 for translational motion and 3 for rotations thereforer the no.of vibrations = 3 n – 6 (n = number of atoms in non-linear molecule)

The below table gives stretching frequencies of some bonds.

Table 12.4 Infrared Stretching Frequencies of Selected Functional Groups

Bond	Stretching Frequency (cm^{-1})	Intensity
O—H	3200–3650	Weak to strong (strongest when H-bonded)
N—H	3100–3550	Medium
C—H	2700–3300	Weak to medium
C=C	1600–1680	Weak to medium
C=O	1630–1820	Strong
C—O	1000–1250	Strong

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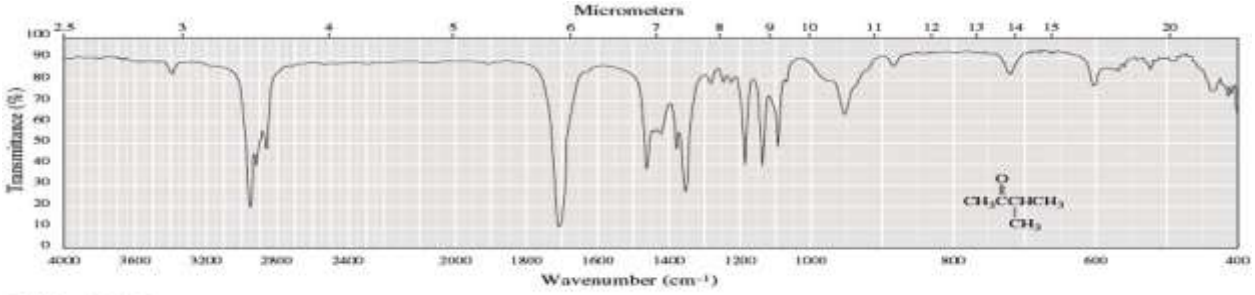
Infrared spectrum



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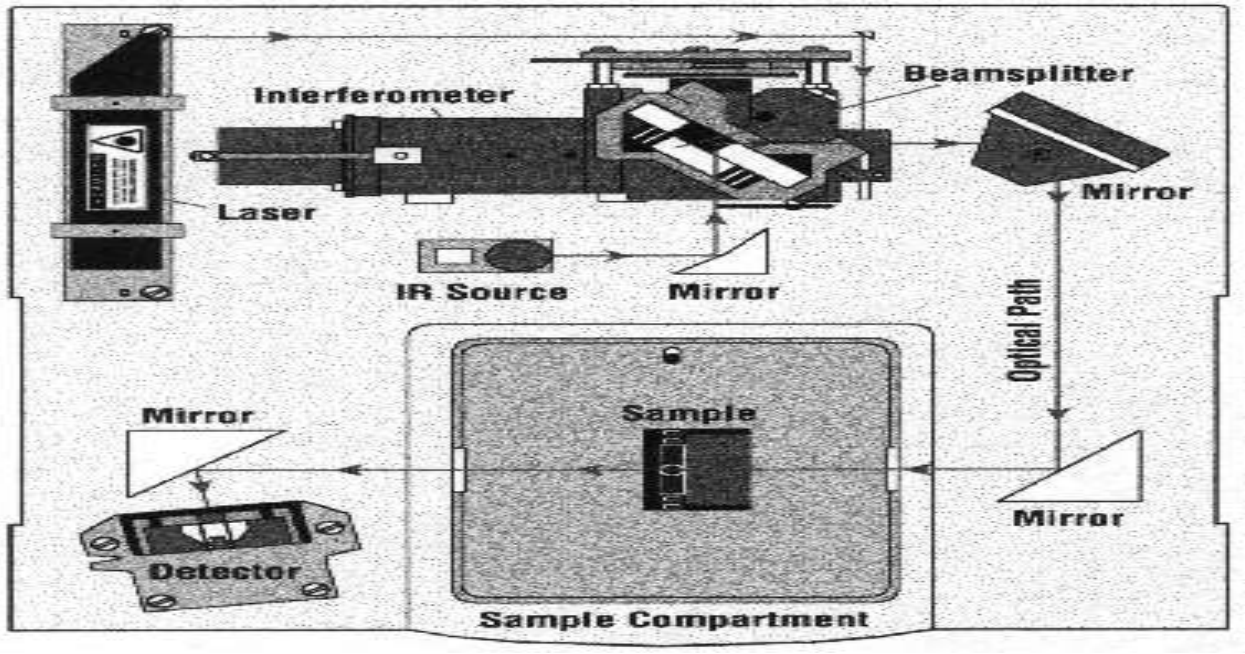
An infrared spectrum consists of a plot of absorbance versus frequency or wavenumber (1/λ). In comparison with absorbance spectra in the ultraviolet/visible range, infrared spectra of small molecules consist of narrow lines rather than broad peaks . However, because of the large number and variety of bonds in macromolecules, infrared spectra of proteins and DNA consist of a small number of broad peaks.It is often possible to assign the peaks in the 1600-3600 cm^{-1} region by consulting tables or databases of IR spectra.where the type of bond and the type of vibration, e.g. O-H stretch or C-H bending vibration. is given. The most useful regions are as follows:

- 1680-1750 cm^{-1}:C=O stretches the type of carbonyl group can be determined from the exact position of the peak.
- 2700-3100 cm^{-1}: different types of C-H stretching vibrations.
- 3200-3700 cm^{-1}: various types of O-H and N-H stretching vibrations.
- Too many bonds absorb in the region of 600-1600 cm^{-1} to allow confident assignment of individual bands. However, this region is useful as a fingerprint of a molecule, i.e. if the spectrum is almost identical to an authentic reference spectrum then the structure can be assigned with some confidence.

Water gives a strong infrared spectrum because of its high absorption coefficient in the infrared range and high concentration (55 M). For this reason, infrared spectroscopy may be carried out either on non -aqueous samples prepared as dry films or on samples dissolved in alternative solvents such as D2O or chloroform. It is also possible to subtract the spectrum due to the water solvent by difference spectroscopy but this requires high concentrations of protein or DNA (5–20%). These conditions are generally unsuitable for the study of biomacromolecules in their native state and this limits the practical application of simple infrared spectroscopy to their

study. However, the technique has become standard for the study of low molecular mass biomolecules and yields structural information complementary to that available from other methods such
as mass spectrometry CD and NMR .

Infrared Instrumentation



The source : 1) Nernst glowers: are constructed from a fused mixture of oxides of zirconium, yttrium and thorium molded in the form of hollow rods 1 -3 mm in diameter and 2 – 5 cm long. These glowers are fragile.
2) Globar: bar of sintered silicon carbide 6 – 8 mm in diameter and 50 mm long has characteristics intermediate between heated wire coils and the Nernst glower. It has an operating temperature near 1300C
Monochromator: Prism ./diffraction grating

- According to wavelength it should be made up of CaF2, KBr , Nacl. The surface should be smooth.Grating monochromator: They are very popular. They are made of Al and are not attacked by moisture

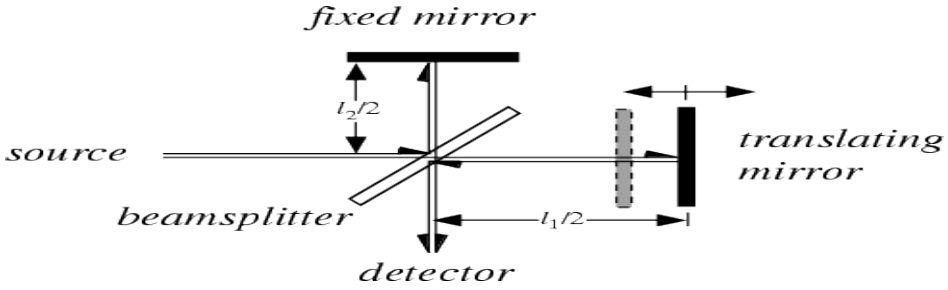
Cuvette: made up of NaCl.
Detector: At the short wave length below 1.2 μm, the preferred detection methods are the same as those used for Visible and Ultraviolet radiation.

- The detectors used at longer wavelengths can be classified into two groups ie, thermal detectors and photon detectors.

Fourier transform Infrared Spectroscopy

- Instead of recording the amount of energy absorbed when the frequency of the IR radiation is varied by a monochromator, the IR radiation is guided through an interferometer. The purpose of the interferometer is to have a beam of IR radiation, split it into two beams, and make one of the beams travel a different (optical) distance

than the other in order to create alternating interference fringes. A diagram of a Michelson interferometer is shown below.



Michelson interferometer

Advantages of FTIR over other methods

- FTIR spectrometers are cheaper than conventional spectrometers because building of interferometers is easier than the fabrication of a monochromator.
- In addition, measurement of a single spectrum is faster for the FTIR technique because the information at all frequencies is collected simultaneously.
- This allows multiple samples to be collected and averaged together resulting in an improvement in sensitivity.

Applications

- Structure determination
- The FT-IR analysis of microorganisms allows a fast and reliable identification of microorganisms
- forensic analysis for example in identifying polymer degradation.
- biomolecules like proteins, lipids, carbohydrates and DNA/RNA can be identified and quantified very sensitively

RAMAN SPECTROSCOPY

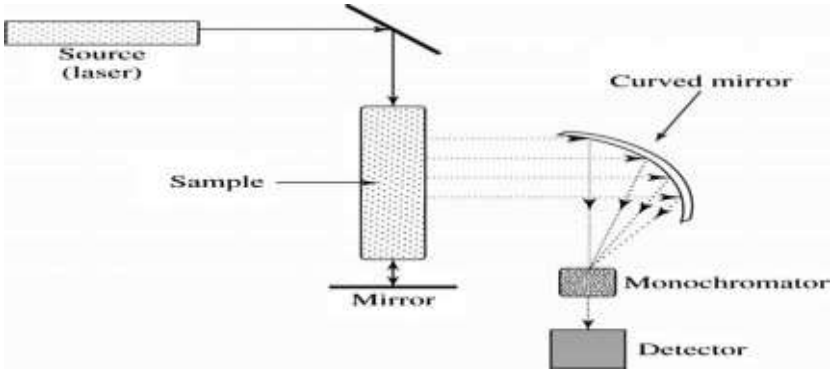
In 1928, the Indian physicist C. V. Raman discovered that the visible wavelength of a small fraction of the radiation scattered by certain molecules differs from that of the incident beam The shifts in wavelength depend upon the chemical structure of the molecules responsible for the scattering. This phenomenon results from the same type of quantized vibrational changes that are associated with infrared absorption. The difference in wavelength between the incident and scattered visible radiation corresponds to wavelengths in the mid-infrared region. Thus the Raman scattering spectrum and infrared absorption spectrum for a given species often resemble one another quite closely.

Scattering of monochromatic light is of two general types Rayleigh scattering occurs when the frequency of the scattered light is the same as that of the incident light. Most of the light scattered by a sample results from this process. Much less commonly,the frequency of the scattered light may be greater or less than that of the incident light. This is a phenomenon known as Raman scattering and it forms the basis of Raman spectroscopy.

Both of these phenomena occur with light of all frequencies and if the incident beam is composed of visible light then the scattered light will also be in the visible range. Because

Raman scattering occurs with such a low probability, it is usual to use a high-energy laser emitting light . The relevance of Raman spectroscopy to infrared spectroscopy lies in the fact that the energy-differences between incident and Raman scattered light corresponds to vibrational transitions. Raman spectroscopy is therefore a means of studying vibrational transitions, even though the light used is usually in the visible part of the spectrum.Moreover, since the phenomenon is based on scattering rather than absorption, it is possible to measureRaman spectra in aqueous solutions without the problems encountered due to water absorption in dispersive infraredspectroscopy.

Instrumentation



Raman spectrometer. Monochromatic light is passed through a concentrated sample. Scattered light (dashed lines) iscollected with the aid of a curved mirror. This is then diffractedthrough a monochromator which allows the identification of frequencies due to both Rayleigh and Raman scattering. Note that the lower mirror reflects transmitted light back through the sample todouble the intensity of scattered light.

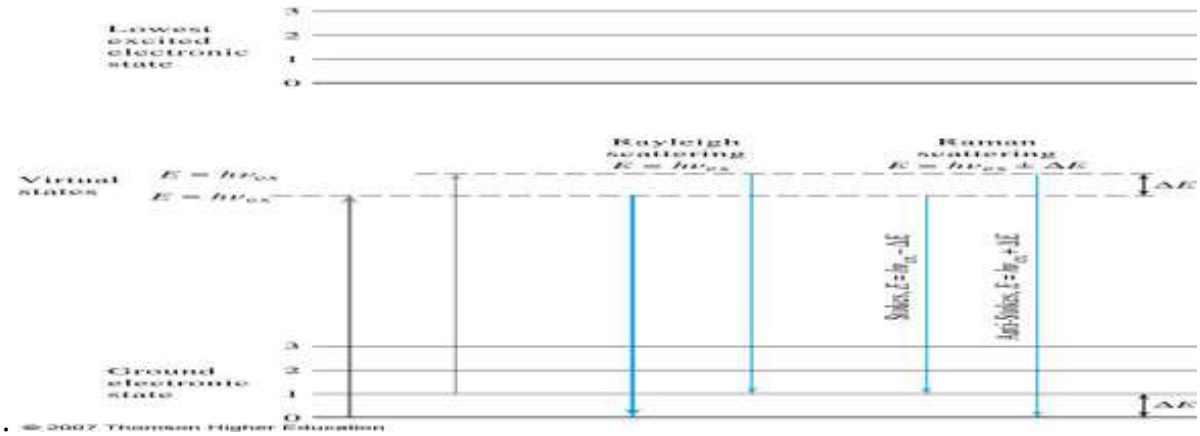
The experimental apparatus used to measure Raman spectra is shown in Figure. Since the effects measured occur with such low probability, it is necessary to use highly concentrated samples (i.e. in the range 20 mg/ml) to detect scattered light. Moreover, it is essential that the sample does not aggregate under these conditions as this may change the properties of the spectrum obtained.

In Raman scattering, a small amount of energy is transferred from the incident light (frequency, νi) to promote the sample molecule from the ground state to an excited vibrational level. In other words light energy causes a vibrational transition. Since E =hμ .,this loss of energy causes a small decrease in the frequency of the scattered light to a value ν. This decrease can be expressed as _ν:_ν = ν − νi where ν is the frequency of Raman scattered light. Passage through a monochromator allows diffraction of scattered light into its various frequencies and a Raman spectrum is frequently a plot of light intensity versus _ν .Most of the scattered light has the same frequency as the incident light, νi, that is _ν = 0. This is called the Rayleigh line of the spectrum. However, other lines in the spectrum have measurable _ν values which depend on the structure of the sample molecule. These are called Stokes lines .A second way in which the sample molecule and incident light can interact is where some molecules existing in the first excited vibrational energy level impart energy tothe incident light thus decreasing the frequency of Raman scattered light. This gives rise to lines in the spectrum ofopposite sign but

identical _ν to the Stokes lines which are called anti-Stokes lines. Because most molecules in a population

are in the ground state, vibrational transitions leadingto a decrease in frequency are statistically much more likely than those leading to an increase in frequency. Therefore,anti-Stokes lines are usually much weaker than Stokes lines. Therefore a sample molecule will give a Raman spectrum characteristic for its chemical structure.

Since this spectrum is due to vibrational transitions, the information contained in such a spectrum is no different to that contained in a dispersive infrared spectrum. However,Raman spectroscopy has the important practical advantage that it is not affected by solvent water. In addition to studies on samples prepared in solution, it isalso possible to measure Raman spectra on samples prepared as dry fibres or as single crystals. Thus it is possible to compare Raman spectra collected in the presence and absence of solvent water. Raman spectroscopy therefore provides a convenient means of confirming structural data obtained from X-ray crystallography.



The heavy arrow on the far left depicts the energy change in the molecule when it interacts with a photon. The increase in energy is equal to the energy of the photon hν.

The second and narrower arrow shows the type of change that would occur if the molecule is in the first vibrational level of the electronic ground state. The middle set of arrows depicts the changes that produce Rayleigh scattering. The energy changes that produce stokes and anti-Stokes emission are depicted on the right. The two differ from the Rayleigh radiation by frequencies corresponding to ±ΔE, the energy of the first vibrational level of the ground state. If the bond were infrared active, the energy of its absorption would also be ΔE. Thus, the Raman frequency shift and the infrared absorption peak frequency are identical. The relative populations of the two upper energy states are such that Stokes emission is much favored over anti-Stokes. Rayleigh scattering has a considerably higher probability of occurring than Raman because the most probable event is the energy transfer to molecules in the ground state and reemission by the return of these molecules to the ground state. The ratio of anti-Stokes to Stokes intensities will increase with temperature because a larger fraction of the molecules will be in the first vibrationally excited state under these circumstances

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Instrumentation

Components of a Raman spectrophotometer :

A laser source, a sample illumination system and suitable spectrometer.The sources used in modern Raman spectrometry are nearly always lasers because their high intensity is necessary to produce Raman scattering of sufficient intensity to be measured with a reasonable signal-to-noise ratio. Because the intensity of Raman scattering varies as the fourth power of the frequency, argon and krypton ion sources that emit in the blue and green region of the spectrum have an advantage over the other sources.

Sample handling for Raman spectroscopic measurements is simpler than for infrared spectroscopy because glass can be used for windows, lenses, and other optical components instead of the more fragile and atmospherically less stable crystalline halides. In addition, the laser source is easily focused on a small sample area and the emitted radiation efficiently focused on a slit. Consequently, very small samples can be investigated. A common sample holder for non absorbing liquid samples is an ordinary glass melting-point capillary.

Raman spectrometers were similar in design and used the same type of components as the classical ultraviolet/visible dispersing instruments. Most employed double grating systems to minimize the spurious radiation reaching the transducer. Photomultipliers served as transducers. Now Raman spectrometers being marketed are either Fourier transform instruments equipped with cooled germanium transducers or multichannel instruments based upon charge-coupled devices.

Applications Of Raman Spectroscopy

The Raman technique is often superior to infrared for spectroscopy investigating inorganic systems because aqueous solutions can be employed. In addition, the vibrational energies of metal-ligand bonds are generally in the range of 100 to 700 cm^{-1}, a region of the infrared that is experimentally difficult to study. These vibrations are frequently Raman active, however, and peaks with $\Delta\nu$ values in this range are readily observed. Raman studies are potentially useful sources of information concerning the composition, structure, and stability of coordination compounds.

Raman spectra are similar to infrared spectra in that they have regions that are useful for functional group detection and fingerprint regions that permit the identification of specific compounds. Raman spectra yield more information about certain types of organic compounds than do their infrared counterparts.

Raman spectroscopy has been applied widely for the study of biological systems. The advantages of his technique include the small sample requirement, the minimal sensitivity toward interference by water, the spectral detail, and the conformational and environmental sensitivity.. Raman spectra tend to be less cluttered with peaks than infrared spectra. As a consequence, peak overlap in mixtures is less likely, and quantitative measurements are simpler., Raman sampling devices are not subject to attack by moisture, and small amounts of water in a sample do not interfere..

Despite these advantages, Raman spectroscopy has not yet been exploited widely for quantitative analysis. This lack of use has been due largely to the rather high cost of Raman spectrometers relative to that of absorption instrumentation

Resonance Raman Spectroscopy

Resonance Raman scattering refers to a phenomenon in which Raman line intensities are greatly enhanced by excitation with wavelengths that closely approach that of an electronic absorption peak of an analyte.

The most important application of resonance Raman spectroscopy has been to the study of biological molecules under physiologically significant conditions; that is, in the presence of water and at low to moderate concentration levels.

Eg.The technique has been used to determine the oxidation state and spin of iron atoms in hemoglobin and cytochrome-c. In these molecules, the resonance Raman bands are due solely to vibrational modes of the tetrapyrrole chromophore .

OPTICAL ACTIVITY AND CIRCULAR DICHROISM.

Electromagnetic radiation consists of mutually perpendicular electric and magnetic vectors .In a beam of radiation originating from the sun or any other light-source, the electric vectors are randomly orientated around the beam axis If, however, the electric vectors in one plane could be

selected a beam of *plane polarized* or *linearly polarized* light would result. It is possible to achieve this by passing unpolarized light through a filter called a *polaroid*. Plane polarized light may be thought of as being composed of two circularly polarized vectors of opposite direction but of equal strength. These are called, respectively,*left* and *right circularly polarized* components of plane polarized light. The superposition of a left circularly polarized wave and a right circularly polarized wave with equal amplitudes and wavelengths gives a plane polarized wave.Can also be put in the following way:Any linearly polarized light wave can be obtained as a superposition of a left circularly polarized and a right circularly polarized light wave, whose amplitude is identical.

Molecules which are capable of rotating the plane of plane polarized light are said to chiral molecules .this phenomena is called optical activity.

Chirality in Biomolecules

Atoms which have four different groups attached to them are said to be chiral molecules and show optical activity.e they can exist as different isomeric forms which can rotate the plane of polarized light either to left or right. Accordingly they are either dextro rotatory if they rotate the plane polarized light towards the right (represented as d) or laevorotatory if they rotate the plane polarized light towards the left(represented as l).The no. of stereo isomers formed can be given as 2^n., where n is the no. of chiral atoms.

. Eg.Carbon has four valencies and, when bound to four distinct chemical groups, has two possible isomeric forms which may be referred to as d and l .

Pairs of isomerswhich are *non superimposable mirror images* of each other are called *enantiomers* or *optical isomers*.Some of these structural variants are obviously stereoisomers but are not related to each other as enantiomers (i.e. nonsuperimposable mirror images of each other). These are referred to as *diastereoisomers*.

The existence of enantiomeric pairs is known as *chirality* and molecules capable of existing as enantiomers are called *chiral molecules*. 'Chiral' derives from the Greek word for hand (*cheir*) because hands are good examples of nonsuperimposable mirror image objects. l- and d-enantiomers are conventionally assigned by comparison with the reference compound l-glyceraldehyde. An alternative notation called the RS convention gives the *absolute* configuration of enantiomers .

The phenomenon of chirality is especially important in biochemistry because many biomolecules are chiral. In biological systems there is usually a selection for one of a pair of enantiomers. For example most amino acids in living systems are l-enantiomers with d-enantiomers occurring only very rarely (e.g. in antibiotic peptides). Similarly, most monosaccharides are d-enantiomers with l-enantiomers occurring rarely. By contrast, most chemical reactions carried out in solution result in a 50 : 50 mixture of d- and l-enantiomers.

Chirality has several important consequences for the structure, shape and functional properties of biomolecules.For example, l-amino acids result in the right-handed helices observed in proteins. d-amino acids result in left handed helices which are themselves mirror images of right handedhelices while heteropolymers of a mixture of d andl-amino acids cannot form helices at all.

Proteases are capable of hydrolyzing peptide bonds in polypeptides composed of all-l-amino acid substrates but cannot hydrolyzethose of all-d composition. Conversely, synthetic proteases which can be made chemically with all-d-amino acids (e.g. the HIV protease) can cleave all-d polypeptide substrates but not those of an all-l amino acid composition .Indeed, the synthetic HIV protease is itself a mirror-image of the natural all-l enzyme showing that chirality is maintained throughout the hierarchy of protein structure .It has also been discovered that a pair of enantiomers may display distinct toxicity to humans with one enantiomer being toxic while the other is nontoxic. A well-known example of this is the fertility drug *thalidomide* which was widely prescribed in the late 1950s. One enantiomer of this compound was nontoxic while the other was *teratogenic* leading to deformity or lack of limbs in babies born to patients treated with the drug. For this reason there is considerable interest in the pharmaceutical industry in the possible use of enzymes for *enantioselective synthesis* of new drugs.

Circular Dichroism (CD)

In the early nineteenth century the French physicist Jean Baptiste Biot observed that solutions of some organic molecules appeared to *rotate* the plane of polarization of plane polarized light, a phenomenon referred to as *optical rotatory dispersion*

. Light passing through a chromophore solution may interact with the sample in two main ways. The light may be *refracted* or delayed on passage through the solution or it may be *absorbed*. Refraction is quantified by the *refractive index*, n, of the solution while absorption is quantified by the *molar extinction coefficient, ε.* If the light is plane polarized and the sample is optically active, each enantiomer may interact *differently* with the left and right circularly polarized

components of the light beam.ORD arises from the fact that there is a specific refractive index for left (*n*L) and right (*n*R) circularly polarized light :*n*L _= *n*R .The difference in refractive index at any wavelength maybe expressed as _*n*. An ORD spectrum is a plot of _*n* against wavelength (*λ*).

Similarly, optically active samples have distinct molar extinction coefficients for left (*ε*L) and right (*ε*R) circularly polarized light. This is called *circular dichroism* (CD):*ε*L _= *ε*R

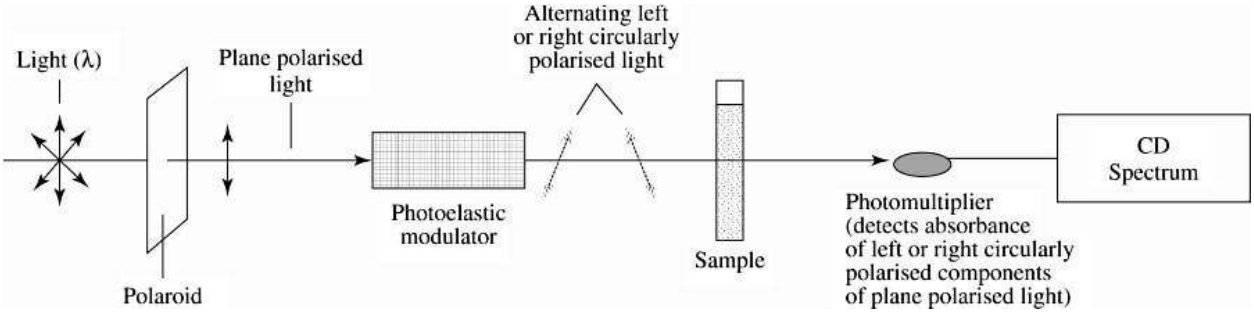
The difference between *ε*L and *ε*R may be expressed as _*ε*.

If _*ε* or _*A* or *ellipticity* (*see* below) is plotted against wavelength (*λ*), a CD spectrum is obtained. The CD spectrum of one enantiomer is a mirror image of that of the other and is related to the corresponding ORD spectrum (and *vice versa*) by a mathematical transformation called the *general Kronig–Kramers transformation*.

Both ORD and CD spectra are evidence for optical activity in the sample and both reflect structure of molecules in the sample, especially of chiral biopolymers such as proteins and nucleic acids In practice, ORD has now largely been superseded by CD spectroscopy.

Instrumentation

CD spectra are measured in a special type of spectrophotometer called a *CD spectropolarimeter* of which an outline design is shown in Figure below.



. Since CD depends on differential absorbance, a means of selectively exposing sample to left and right circularly polarized light is necessary. This is achieved by passing a beam of plane polarized light througha *photoelastic modulator* which is normally a quartz crystal subjected to an oscillating electric field. The effect of this is to vary the circular polarization of the beam passing through the modulator alternately from left to right with a frequencyof some 50 kHz whilst maintaining a constant light intensity.

Differential absorption of left and right circularly polarized light is detected at a photomultiplier and converted into *ellipticity*, *θ*, which has units of milli degrees. This term arises from the fact that selective absorption of one of the circularly polarized components of plane polarized light has the consequence that the resultant electric vector traces an elliptical path around the axis of the beam and is said to be *elliptically polarized*

In a CD spectropolarimeter, the two light beams are not in fact recombined but a photomultiplier detector converts incident light intensity into an electric current composed partly of alternating current (AC) and partly of direct current (DC) components. The DC component is related to total light absorption by the sample while the AC component is a direct measure of CD. This arrangement facilitates separate absorption measurements of the right and left circularly polarized components of plane polarized light.. If this light is passed through a chiral sample, one circularly polarized component is selectively absorbed. This lowers the

amplitude associated with that component (R in the sample above). When recombined ,the resultant electric vector now traces an elliptical path.The degree of ellipticity is a measure of circular dichroism. Ellipticity may be converted to units of absorbance using the following equation

:_$A = \theta$32 982

Applications

- Determination of the secondary structure of the proteins that cannot be crystallized.
- Investigation of the drug binding onto the secondary structure of the protein.
- Study of the environmental effects on a protein.
- Study of the ligand introduced conformational changes.
- Study of the temperature and chemical introduced conformational changes of proteins.

Structural determination

Measurement of CD spectra in proteins and peptidesof unknown secondary structure is often used (by analogywith such homopolypeptides) to estimate empirically their percentages of common secondary structure features

Eg.The synthetic homopolymer poly-l-lysine adopts a randomcoil structure at neutral and acid pH values. However, athigh pH values, a mainly α-helical conformation is adoptedwhich may be converted to predominantly antiparallel β-sheet by gentle heating. Each of these three forms of polyl-lysine gives a characteristic CD spectrum in the range190–240 nm and the fact that similar spectra are obtained forhomopolymers of other amino acids suggests that they arise predominantly from asymmetry of the polypeptide backbone.

In practice, CD spectra provide a generally useful indexof structure in proteins (Changes in CDstructure. For example, binding of a ligand to a proteinor protein folding/unfolding can be conveniently followedby CD spectroscopy. A particular advantage of CD in this regard is the short time-scale of the CD measurement compared to that of other spectroscopic measurements.

CD measurements may be expected to detect ligand protein effects of shorter duration than those detectable by NMR.

Even though many ligands may be intrinsically achiral,their unique orientation on binding to a protein coupled with their specific interaction with the protein can causet hem to acquireCDproperties for the duration of the interaction.This phenomenon is known as *induced* CD. Examplesof this phenomenon include CD spectra induced on binding of bilirubin to bovine serum albumin and of dicumarol derivatives to α-1 acid glycoprotein.

The second major class of biopolymers which has beenstudied by CD is the nucleic acids. Of the nucleotide's structural components, only the pentose sugar is chiral. Mainly as a result of the presence of this sugar, nucleotides are intrinsically chiral structures and give measurable, albeit weak, CD spectra. As the level of structural order increases, however (e.g. polymerization of nucleotides into polynucleotides followed by assembly into duplex structures such as double-stranded DNA and tRNA), the asymmetry of the system and hence the strength of the CD

Differences are observed in CD spectra due to variation of nucleotide sequen ce, GC composition,stacking of bases in different forms of DNA (i.e. A-, B- andZ-DNA), formation of macromolecular assemblies such asribosomes and nucleosomes and ligand binding to DNA.As with proteins, induced CD is also possible with DNAsuch as when anthracene-9-carbonyl-*N*1-spermine binds to oligonucleotides of defined sequence.
Many chromophores associated with biomacromolecules are themselves chiral and CD measurements of their spectra can give information on their interactions with proteins, DNA or macromolecular complexes.

References:

1. Physical Biochemistry-Principles and applications by -David Sheehan ., Wiley publications.
2. Biological Spectroscopy by J. D. Campbell and R. A.Dwek, Plenum Press.
3. Biophysical Chemistry by Cantor R., and Schimmel P.R.,Freeman
4. Principles and Techniques of Practical Biochemistry –Keith Wilson ., Cambrigde Univ. Press.
5. Principles of Physical Biochemistry Kensal E. van Holde, W. Curtis Johnsonand P. Shing Ho., Pearsons publications.
6. Web Sources