S.Y.B.Sc. BIO-PHYSICS

-:Spectroscopy & Microscopy:-

Q.1 What do you mean by SPECTRA ? Give it's type and properties.

Ans : Spectra is a data obtained from spectroscopy. It represents energy verses mass or momentum etc. The information obtained from spectra is atomic and molecular energy levels, chemical bondings , molecular interactions etc.

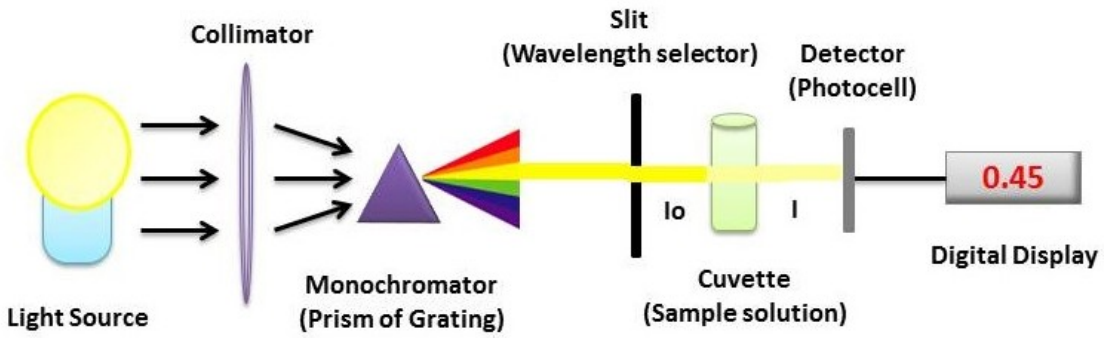
Types and Properties :

There are three main kinds of spectra that we have explored: continuous spectra, absorption spectra, and emission spectra.

Properties :

Spectra generates database .
It can explore information about atomic and molecular energy levels.
It explains molecular interaction briefly.
It helps to determine chemical composition of objects.
An unique spectra obtain for each atom and molecule.

Q.2. What is spectrophotometer ?
Ans: The spectrophotometer is an instrument which measures an amount of light that a sample absorbs. The spectrophotometer works by passing a light beam



Basic Instrumentation of a Spectrophotometer

through a sample to measure the light intensity of a sample.

Principle

A spectrophotometer is made up of two instruments: a spectrometer and a photometer. The spectrometer is to produce light of any wavelength, while the photometer is to measure the intensity of light. The spectrophotometer is designed in a way that the liquid or a sample is placed between spectrometer and photometer. The photometer measures the amount of light that passes through the sample and delivers a voltage signal to the display. If the absorbing of light change, the voltage signal also changes.

The basic spectrophotometer instrument consists of a light source, a digital display, a monochromator, a wavelength sector to transmit selected wavelength, a collimator for straight light beam transmission, photoelectric detector and a cuvette to place a sample.

The intensity of light is symbolized as l_{0} measure the number of photons per second. When the light is passed through the blank solution, it does not absorb light and is symbolized as (l) . Another important factors are Absorbance (A) and Transmittance (T) .

T = \frac{l}{l_{0}}

A = -log_{10} \ T

Here, we need to measure the intensity of light that passes a blank solution, and later measure the intensity of light passing a sample. Calculate the transmittance and the absorbance. For the measurement of absorbance, we can use an isosbestic point where the absorbance and wavelength of two or more species are same.

A number of protons transmit and absorb totally depended on the length of the cuvette and the concentration of the sample.

The transmittance and absorption relation is:

Absorbance \ (A) = -log (T) = – log(T) = -log\left (\frac{l_{t}}{l_{0}} \right)

The transmittance of an unknown sample can be calculated using the formula given below.

Transmittance \ (T) = \frac{l_{t}}{l_{o}}

Here,

l_{t} = Light intensity after passing via cuvette

{l_{0}} = Light intensity before passing via cuvette

Further, there are several varieties of spectrophotometer devices such as UV Spectrometry, atomic emission spectrophotometry and atomic absorption spectrophotometry and much more. It can also be classified into two types based on the range of light source wavelengths like IR spectrophotometer and UV-visible spectrophotometer

Q.3 What is U-V spectrophotometer ? Explain it briefly

Ans: UV spectroscopy is type of absorption spectroscopy in which light of ultra-violet region (200-400 nm.) is absorbed by the molecule. Absorption of the ultra-violet radiations results in the excitation of the electrons from the ground state to higher energy state. The energy of the ultra-violet radiation that are absorbed is equal to the energy difference between the ground state and higher energy states

Generally, the most favoured transition is from the highest occupied molecular orbital (HOMO) to lowest unoccupied molecular orbital (LUMO). For most of the molecules, the lowest energy occupied molecular orbitals are s orbital, which correspond to sigma bonds. The p orbitals are at somewhat higher energy levels, the orbitals (nonbonding orbitals) with unshared paired of electrons lie at higher energy levels. The unoccupied or antibonding orbitals (pie* and sigma*) are the highest energy occupied orbitals.
In all the compounds (other than alkanes), the electrons undergo various transitions. Some of the important transitions with increasing energies are: nonbonding to pie*, nonbonding to sigma*, pie to pie*, sigma to pie* and sigma to sigma*.

Principle of UV spectroscopy

UV spectroscopy obeys the Beer-Lambert law, which states that: when a beam of monochromatic light is passed through a solution of an absorbing substance, the rate of decrease of intensity of radiation with thickness of the absorbing solution is proportional to the incident radiation as well as the concentration of the solution.
The expression of Beer-Lambert law is-
A = log (I0/I) = Ecl
Where, A = absorbance
I0 = intensity of light incident upon sample cell
I = intensity of light leaving sample cell
C = molar concentration of solute
L = length of sample cell (cm.)
E = molar absorptivity

From the Beer-Lambert law it is clear that greater the number of molecules capable of absorbing light of a given wavelength, the greater the extent of light absorption. This is the basic principle of UV spectroscopy.

Instrumentation and working of UV spectroscopy

Instrumentation and working of the UV spectrometers can be studied simultaneously. Most of the modern UV spectrometers consist of the following parts-

Light Source- Tungsten filament lamps and Hydrogen-Deuterium lamps are most widely used and suitable light source as they cover the whole UV region. Tungsten filament lamps are rich in red radiations; more specifically they emit the radiations of 375 nm, while the intensity of Hydrogen-Deuterium lamps falls below 375 nm.

Monochromator- Monochromators generally composed of prisms and slits. The most of the spectrophotometers are double beam spectrophotometers. The radiation emitted from the primary source is dispersed with the help of rotating prisms. The various wavelengths of the light source which are separated by the prism are then selected by the slits such the rotation of the prism results in a series of continuously increasing wavelength to pass through the slits for recording purpose. The beam selected by the slit is monochromatic and further divided into two beams with the help of another prism.

Sample and reference cells- One of the two divided beams is passed through the sample solution and second beam is passé through the reference solution. Both sample and reference solution are contained in the cells. These cells are made of either silica or quartz. Glass can't be used for the cells as it also absorbs light in the UV region.

Detector- Generally two photocells serve the purpose of detector in UV spectroscopy. One of the photocell receives the beam from sample cell and second detector receives the beam from the reference. The intensity of the radiation from the reference cell is stronger than the beam of sample cell. This results in the generation of pulsating or alternating currents in the photocells.

Amplifier- The alternating current generated in the photocells is transferred to the amplifier. The amplifier is coupled to a small servometer. Generally current generated in the photocells is of very low intensity, the main purpose of amplifier is to amplify the signals many times so we can get clear and recordable signals.

Recording devices- Most of the time amplifier is coupled to a pen recorder which is connected to the computer. Computer stores all the data generated and produces the spectrum of the desired compound.

Applications of UV spectroscopy

1. Detection of functional groups- UV spectroscopy is used to detect the presence or absence of chromophore in the compound. This is technique is not useful for the detection of chromophore in complex compounds. The absence of a band at a particular band can be seen as an evidence for the absence of a particular group. If

the spectrum of a compound comes out to be transparent above 200 nm than it confirms the absence of –
a) Conjugation b) A carbonyl group c) Benzene or aromatic compound d) Bromo or iodo atoms.

2. Detection of extent of conjugation- The extent of conjugation in the polyenes can be detected with the help of UV spectroscopy. With the increase in double bonds the absorption shifts towards the longer wavelength. If the double bond is increased by 8 in the polyenes then that polyene appears visible to the human eye as the absorption comes in the visible region.

3. Identification of an unknown compound- An unknown compound can be identified with the help of UV spectroscopy. The spectrum of unknown compound is compared with the spectrum of a reference compound and if both the spectrums coincide then it confirms the identification of the unknown substance.

4. Determination of configurations of geometrical isomers- It is observed that cis-alkenes absorb at different wavelength than the trans-alkenes. The two isomers can be distinguished with each other when one of the isomers has non-coplanar structure due to steric hindrances. The cis-isomer suffers distortion and absorbs at lower wavelength as compared to trans-isomer.

5. Determination of the purity of a substance- Purity of a substance can also be determined with the help of UV spectroscopy. The absorption of the sample solution is compared with the absorption of the reference solution. The intensity of the absorption can be used for the relative calculation of the purity of the sample substance.

Q,4 What is the difference between single beam and double beam spectrophotometer?

Ans:A double beam spectrophotometer compares the light intensity between two light paths, one path containing a reference sample and the other the test sample. A single-beam spectrophotometer measures the relative light intensity of the beam before and after a test sample is inserted.

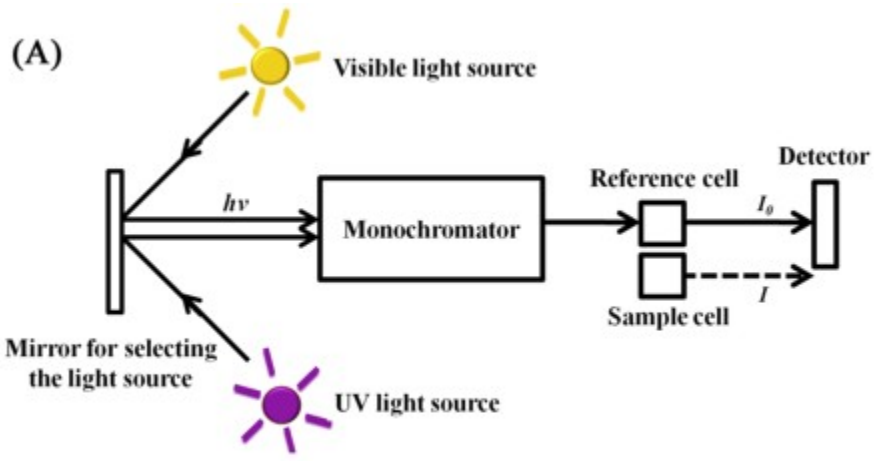
Q.5. Explain Single Beam spectrophotometer.

Ans :Single Beam Spectrophotometer (UV Visible) is used to determine the absorption of light from a sample and can be used as a detector for HPLC. A sample is placed in the UV/VIS beam and absorbance versus wavelength is measured.

Figure shows the schematic diagram of Single beam , The light enters the instrument through an entrance slit, is collimated and focused on to the dispersing element, typically a diffraction grating. The light of desired wavelength is selected simply by rotating the monochromator and impinged on the sample. The intensity of

the radiation transmitted through the sample is measured and converted to absorbance or transmittance (discussed later). Double beam spectrophotometers overcome certain limitations of the single beam spectrophotometers and are therefore preferred over them. A double beam spectrophotometer has two light beams, one of which passes through the sample while other passes through a reference cell (Figure 4.3B). This allows more reproducible measurements as any fluctuation in the light source or instrument electronics appears in both reference and the sample and therefore can easily be removed from the sample spectrum by subtracting the reference spectrum. Modern instruments can perform this subtraction automatically. The most commonly used detectors in the UV/Visible spectrophotometers are the photomultiplier tubes (PMT). Modern instruments also use photodiodes as the detection systems. These diodes are inexpensive and can be arranged in an array so that each diode absorbs a narrow band of the spectrum. Simultaneous recording at multiple wavelengths allows recording of the entire spectrum at once. The monochromator in these spectrophotometers is placed after the sample so that the sample is exposed to the entire spectrum of the incident radiation and the transmitted radiation is dispersed into its components.

The



advantages of single-beam instruments are the large dynamic range, simple optics, few moving parts, and compact design.

Applications:

These spectrophotometers are used in applications such as determining the concentration of analyte in solution by measuring the absorbance at a single wavelength.

It measures the amount of photons (the intensity of light) absorbed after it passes through sample solution.

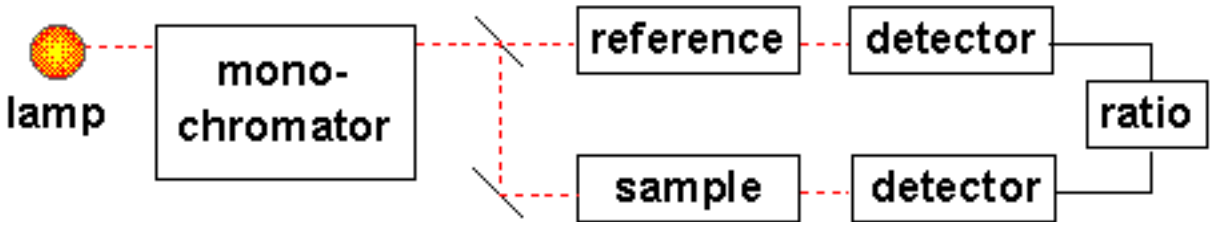
The amount of a known chemical substance (concentrations) can also be determined by measuring the intensity of light detected .

Q.6 Explain DUAL Beam spectrophotometer.

Ans: the purpose of this instrument is to determine the amount of light of a specific wavelength absorbed by an analyte in a sample. Although samples can be gases or liquids, an analyte dissolved in a solvent.

Double beam spectrophotometer compares the light intensity between two light paths, one path containing a reference sample and the other the test sample.

The light from the source , after passing through the monochromator, is split into two separate beams, one for the sample and other for the reference.



The purpose of this instrument is to determine the amount of light of a specific wavelength absorbed by an analyte in a sample. Although samples can be gases or liquids, an analyte dissolved in a solvent is discussed here. [In the infrared, solid pellets using an IR. transparent matrix (like a high purity salt such as Kr) can be used for solid analytes. Thin disks are made using a pellet press and the disk suspended in the sample cell through which the sample beam passes.]

The starting point is the light source. Depending on the wavelength of interest, this can be an electrically powered ultraviolet, visible, or infrared lamp. Not shown in the animations that accompany this page is the spectrophotometer's monochromator which selects the analytical wavelength from the source lamp's broad spectrum containing many wavelengths of light. The analytical wavelength is chosen based on the absorbance characteristics of the analyte. Monochromators are instruments whose sole purpose is to allow polychromatic (that is many wavelength containing) light into the entrance slit of the monochromator and only allow a single (or at least very few) wavelength (monochromatic light) out via the exit slit. This exiting, well-shaped, narrowly-defined beam now contains a small region of the electromagnetic spectrum. The spread, or band-pass, of the wavelengths depends on the slit settings of the monochromators (usually adjustable) and the quality of the light dispersing element in the monochromator (usually a grating in most modern monochromators).

here the source lamp's beam is alternately diverted at right angles by a rotating disk with three distinct panels. One sector allows the beam to past straight through the disk, another has a mirror surface, and a third is black. When the beam passes though the disk it shines directly into the sample cell. If the sample is a liquid then this cell contains a cuvette and is made of a transparent material, such as quartz, that does not absorb light in the spectral region of interest. The analyte is dissolved in a solvent held in the cuvette. When the source light is reflected at 90 degrees by the rotating disk instead of striking the sample cuvette it passes through a cuvette in the reference cell which contains ONLY solvent.

During the third sequence, when the black sector blocks the source beam, NO light passes through the disk. And as can be seen below, therefore no light arrives at the phototranducer. This part of the cycle is used for the computer to digitize and measure the dark current--the amount of light produced by the phototransducer circuit when no light impinges on the phototransducer. The dark current can be subtracted from the overall light measurements made by the system.

After travelling through either the sample cell or reference cell the light that was not absorbed--by far, most of the beam-- is directed onto the phototransducer or light detector. This component converts the arrival of photons into an electrical signal. By the way, the light path through the spectrophotometer need not be in a straight line since the light beam can be redirected using mirrors as can be seen here. Sometimes, lenses are also used to collect and collimate the light.

The alternating light signals, from either the reference beam or sample beam generate alternating electrical phototransducer signals. A computer, sampling those signals, can now determine the analyte absorption in two ways. Some instruments merely subtract the sample beam signal's digitized light intensity from that of the reference beam. The difference is a measure of the amount of light absorbed by the analyte.

Q.7 What are the types of MICROSCOPY?

Ans:Microscopy is the technical field of using microscopes to view objects and areas of objects that cannot be seen with the naked eye (objects that are not within the resolution range of the normal eye). There are three well-known branches of microscopy: optical, electron, and scanning probe microscopy.

Optical microscopy and electron microscopy involve the diffraction, reflection, or refraction of electromagnetic radiation/electron beams interacting with the specimen, and the collection of the scattered radiation or another signal in order to create an image. This process may be carried out by wide-field irradiation of the sample (for example standard light microscopy and transmission electron microscopy) or by scanning a fine beam over the sample (for example confocal laser scanning microscopy and scanning electron microscopy). Scanning probe microscopy involves the interaction of a scanning probe with the surface of the

object of interest. The development of microscopy revolutionized biology, gave rise to the field of histology and so remains an essential technique in the life and physical sciences.

Q.8 Explain Electron Microscopy

Ans : It is a type of microscope in which instead of light beam, a beam of electrons are used to form a large image of very small object. These microscopes are widely used in the field of engineering and medicine.

Principle:

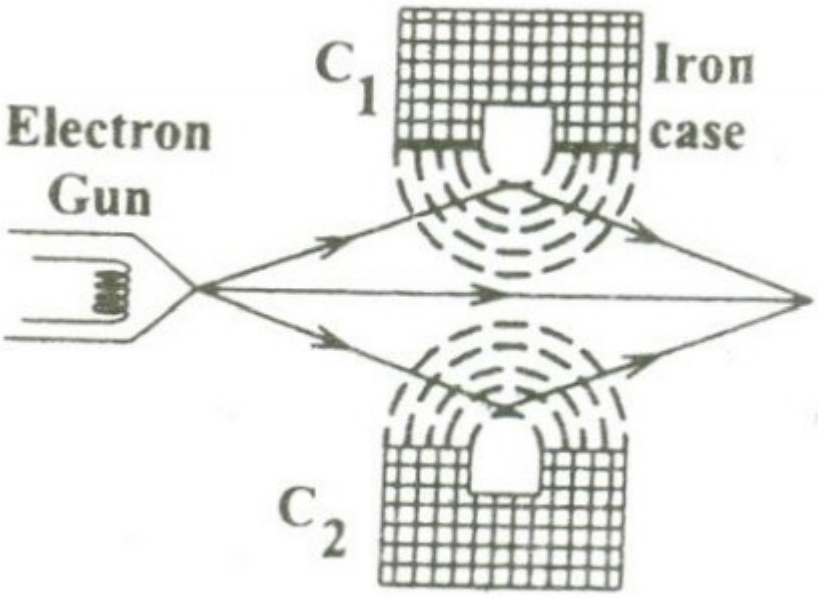
A stream of electrons is passed through the object and the electron which carries the information about the object are focused by electric and magnetic fields.

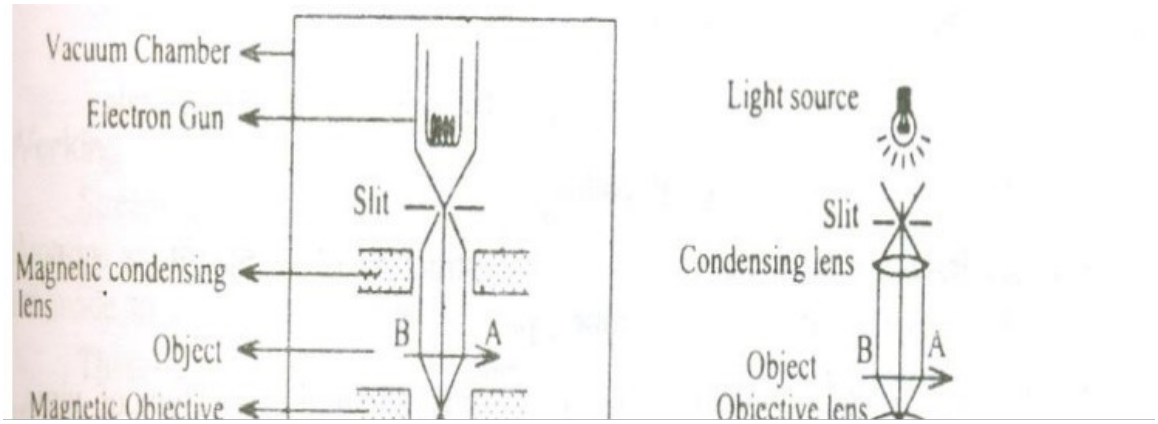
Since the resolving power is inversely proportional to the wavelength, the electron microscope has high resolving power because of its shorter wavelength.

Construction:

An electron microscope is similar to that of an optical microscope. Here the focusing of electrons can be done either by magnetic lens or by electrostatic lens. Normally in electron microscope magnetic lenses are used for focusing.

In general, the magnetic lenses are made of two coils C1 and C2 enclosed inside the iron cases which have one hole as shown.





The electron microscope consists of an electron gun to produce the stream of electrons. Similar to the condensing lens, objective and eye piece in an optical microscope here three magnetic lenses are used.

a. Magnetic condensing lens

b. Magnetic objective lens

c. Magnetic projector lens

The whole arrangement is kept inside a vacuum chamber to allow the passage of electron beam.

Working:

Stream of electrons are produced and accelerated by the electron gun. The electron beam is made to pass through the center of the doughnut shaped magnetic condensing lens. These electrons are made as parallel beam and are focused on to the object.

The electrons are transmitted more in the less dense region of the object and is transmitted less (i.e.,) absorbed by the denser region of the object.

Thus the transmitted electron beam on the falling over the magnetic objective lens, resolves the structure of the object to form a magnified real image of the object. Further the image can be magnified by the magnetic projector lens and the final image is obtained on the fluorescent screen.

In order to make a permanent record of the image of the object, the final image can also be obtained on a photographic plate.

Advantages:

a. It can produce magnification as high as 1, 00,000 times as that of the size of the object.

b. The focal length of the microscopic system can be varied.

Disadvantages:

It has a very wide area of applications (e.g) in biology, metallurgy, physics, chemistry, medicine, engineering etc.

a. It is used to determine the complicated structure of the crystals.

b. It is used in the study of the colloids.

c. In industries it is used to study the structure of textile fibers, surface of metals, composition of paper, paints etc.

d. In the medical field it is used to study about the structure of virus, bacterial etc which are of smaller size.

Q.9 Explain Scanning Electron Microscopy.

Ans: Scanning electron microscope is an improved model of an electron microscope. SEM is used to study the three dimensional image of the specimen.
It produces images of a sample by scanning the surface with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that contain information about the sample's surface topography and composition. The electron beam is scanned in a raster scan pattern, and the beam's position is combined with the detected signal to produce an image. SEM can achieve resolution better than 1 nanometer. Specimens can be observed in high vacuum in conventional SEM, or in low vacuum or wet conditions in variable pressure or environmental SEM, and at a wide range of cryogenic or elevated temperatures with specialized instruments.

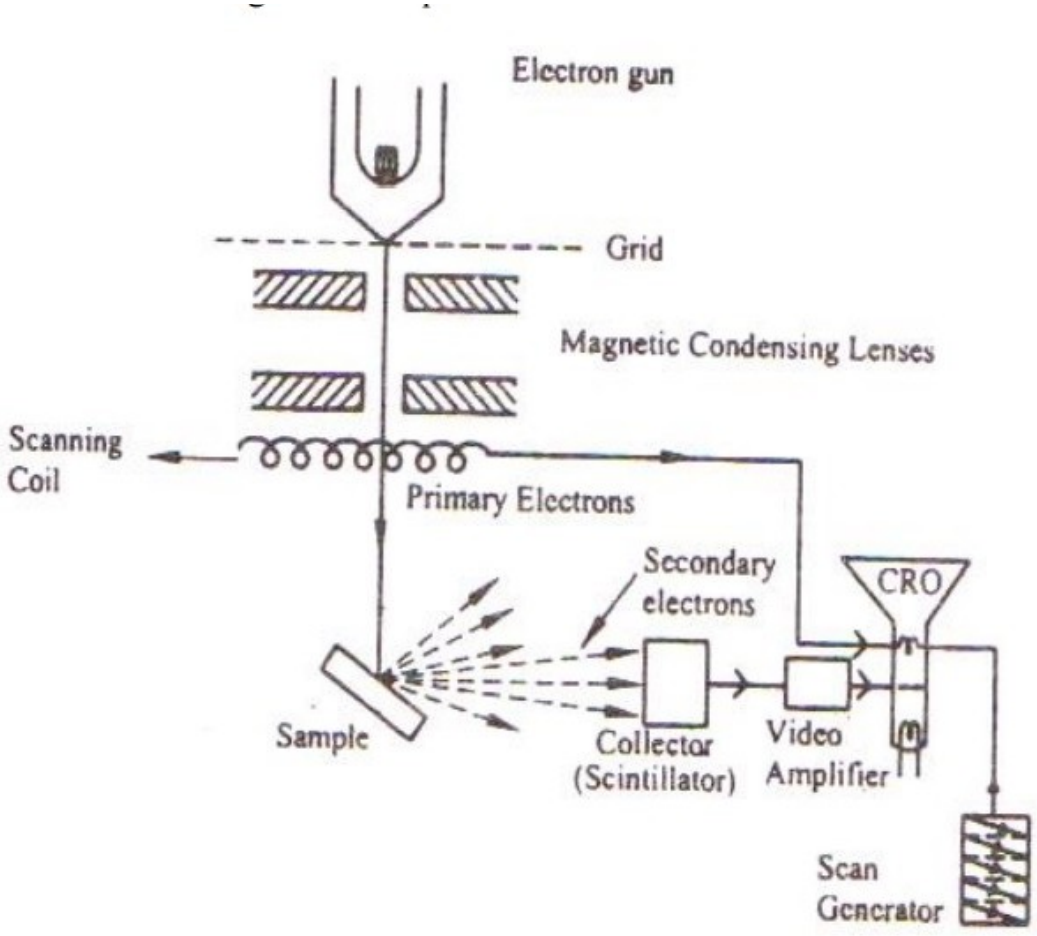
The most common SEM mode is detection of secondary electrons emitted by atoms excited by the electron beam. The number of secondary electrons that can be detected depends, among other things, on specimen topography. By scanning the

sample and collecting the secondary electrons that are emitted using a special detector, an image displaying the topography of the surface is created.

Contents

Principle:
When the accelerated primary electrons strikes the sample , it produces secondary electrons . these secondary electrons are collected by a positive charged electron detector which in turn gives a 3- dimensional image of the sample.
Construction:

It consists of an electron gun to produce high energy electron beam. A magnetic condensing lens is used to condense the electron beam and a scanning coil is arranged in-between magnetic condensing lens and the sample.



The electron detector (Scintillator) is used to collect the secondary electrons and can be converted into electrical signal. These signals can be fed into CRO through video amplifier

Working:

Stream of electrons are produced by the electron gun and these primary electrons are accelerated by the grid and anode. These accelerated primary electrons are made to be incident on the sample through condensing lenses and scanning coil.

These high speed primary electrons on falling over the sample produces low energy secondary electrons. The collection of secondary electrons are very difficult and hence a high voltage is applied to the collector.

These collected electrons produce scintillations on to the photo multiplier tube are converted into electrical signals. These signals are amplified by the video amplifier and is fed to the CRO.

By similar procedure the electron beam scans from left to right and the whole picture of the sample is obtained in the CRO screen.

Q.10. Explain Transmission Electron Microscopy.

Ans:
Principle:

Electrons are made to pass through the specimen and the image is formed on the fluorescent screen, either by using the transmitted beam or by using the diffracted beam.

Construction:
It consists of an electron gun to produce electrons. Magnetic condensing lens is used to condense the electrons and is also used to adjust the size of the electron that falls on to the specimen. The specimen is placed in between the condensing lens and the objective lens as shown.

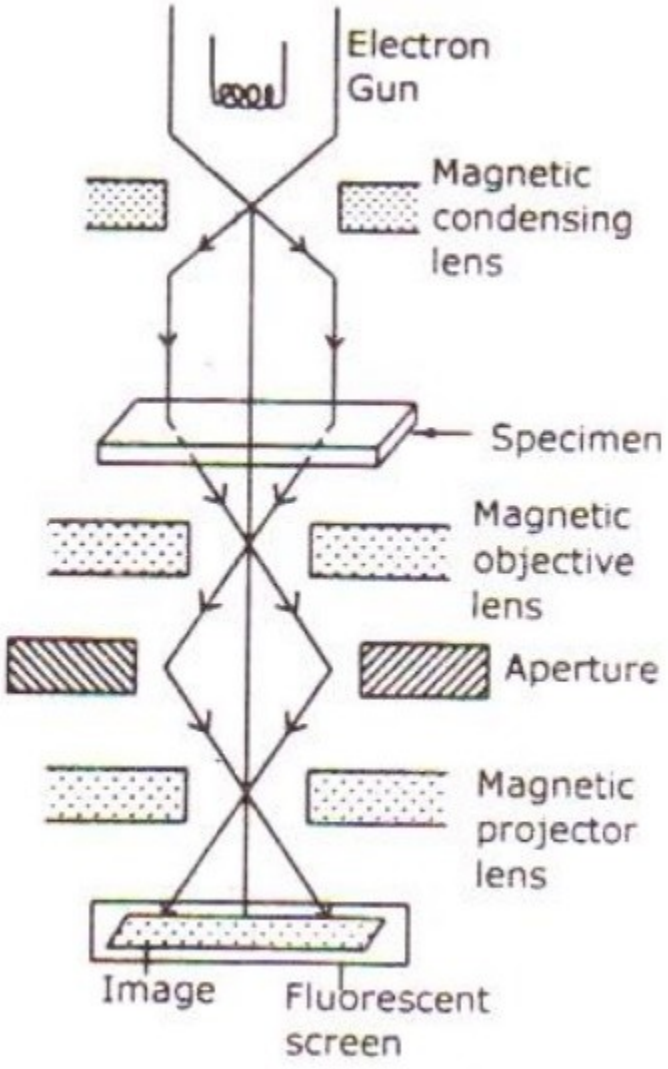
The magnetic objective lens is used to block the high angle diffracted beam and the aperture is sued to eliminate the diffracted beam (if any) and in turn increases the contrast of the image.

The magnetic projector lens is placed above the fluorescent screen in order to achieve higher magnification,. The image can be recorded by using a fluorescent (Phosphor) screen or (CCD – Charged Coupled device) also.

Working:
Stream of electrons are produced by the electron gun and is made to fall over the specimen using the magnetic condensing lens.

Based on the angle of incidence the beam is partially transmitted and partially diffracted. Both these beams are recombined at the E-wald sphere to form the image. The combined image is called the phase contrast image.

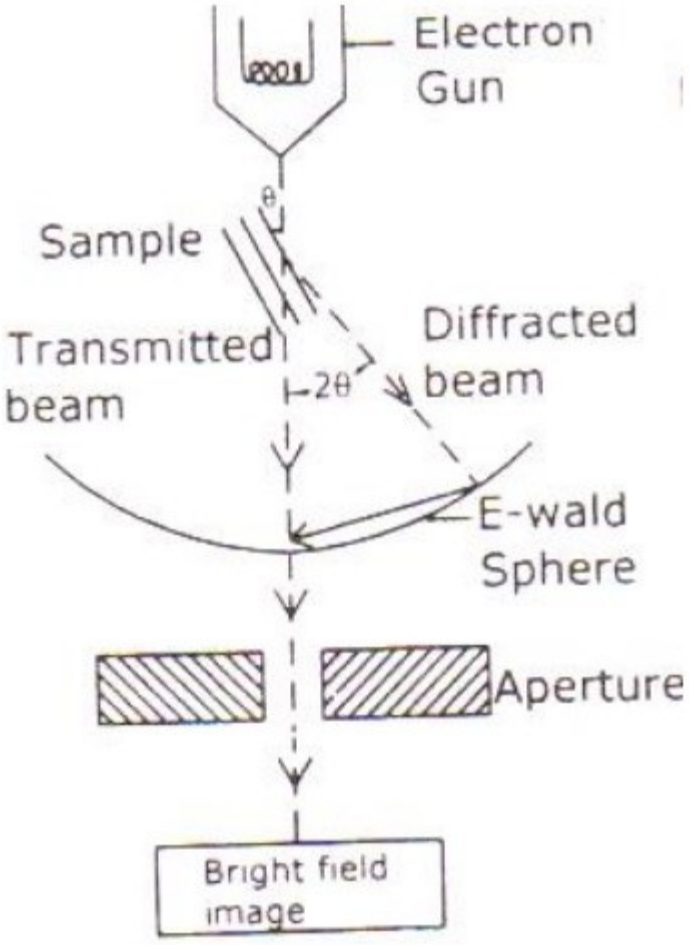
In order to increase the intensity and the contrast of the image, an amplitude contrast has to be obtained. This can be achieved only by using the transmitting beam and thus the diffracted beam can be eliminated.



Now in order to eliminate the diffracted beam, the resultant beam is passed through the magnetic objective lens and the aperture. The aperture is adjusted in such a way that the diffracted image is eliminated. Thus, the final image obtained due to transmitted beam alone is passed through the projector lens for further magnification.

The magnified image is recorded in fluorescent screen or CCD. This high contrast image is called Bright Field Image.

Also, it has to be noted that the bright field image obtained is purely due to the elastic scattering (no energy change) i.e., due to transmitted beam alone



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