5.7 NMR SPECTROSCOPY

5.7.1 PRINCIPLE

NMR is the absorption of energy from a radiofrequency (approximately $\sim 10^8$ HZ) of EM radiation by a system containing unpaired nuclear spins in a strong static magnetic field. The separation of energy levels and hence the frequency of absorption depends on the strength of the magnetic field.

Like electrons spinning about their own axes, protons in a nucleus also spin either clockwise or counterclockwise about their axes. The positive spinning charge gives rise to a magnetic field with a magnetic moment. Pairs of protons have net magnetic moments of zero. However, an odd proton in the nucleus imparts a magnetic moment to the molecule, which can interact with an applied magnetic field. In an applied magnetic field H, it can exist either in a low-energy state aligned parallel to the direction of the applied magnetic field or antiparallel to it in a high-energy state. Upon absorbing energy from the radiowave region of the EM region, a proton can change from a low-energy state to a high-energy state, causing resonance to occur and giving rise to NMR, which is therefore also known as proton magnetic resonance (PMR). For reasons already explained, NMR can occur only in atoms containing an odd number of protons, for example, protons like ^{1}H, ^{13}C, ^{15}N, ^{19}F, and ^{31}P.

The resonance condition is given by

$$\upsilon = H/h\mathrm{I}$$

where

υ = frequency of EM radiation absorbed
μ = nuclear magnetic moment
H = magnetic field strength
h = Planck's constant
I = nuclear spin quantum number characteristic of an atom

The above relationship shows that the frequency of radiowaves absorbed during NMR depends on both the atom being studied (as described by I) and the strength of the magnetic field. It is common practice, however, to vary the magnetic field, H, and keep frequency constant in the radiowave region, rather than vice versa.

5.7.2 INSTRUMENTATION

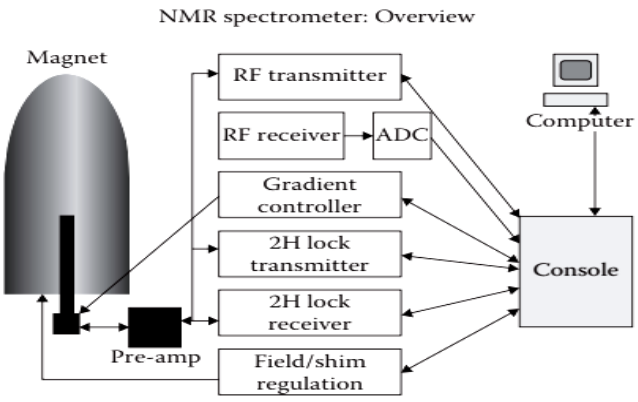
The instrumentation needed for NMR spectroscopy is as follows:

- Source is a radiofrequency transmitter to irradiate the sample.
- Sample cell containing a sample dissolved in a solvent that lacks the atom containing the unpaired electron that is going to be analyzed. For example, in PMR, D_2O or $CDCl_3$ is used.
- Electromagnets providing fields of 1–10 T, in conjunction with auxiliary sweep coils to vary the magnetic field over 1–100 mT.
- Radio receiver that serves as a detector of the absorption signal.
- Recorder that plots energy absorbed against the magnetic field strength applied (Figure 5.24).

5.7.3 CHEMICAL SHIFTS

The nuclear resonance of a particular atom is not always the same in a given applied static magnetic field. This is because the adjacent electron clouds interact with the applied field to give rise to small

(a)



(b)

induced magnetic fields that alter the effective magnetic field felt by each nucleus. Thus, the actual field experienced by a proton depends on its molecular environment; for example, the NMR spectra of ethyl alcohol show that it has three different types of protons: (1) those in the CH_3 group, (2) those in the CH_2 group, and (3) those in the OH group. If the induced field opposes the applied field, a higher applied field is required to make the nucleus resonate. Such nuclei are said to be shielded. On the contrary, nuclei are deshielded if the induced field augments the applied field. Such spectral shifts in different structural environments are called chemical shifts. The extent of the chemical shift is measured relative to the PMR of tetramethylsilane (TMS) and is called the tau (τ) value:

$$\tau = 10 - \frac{\left(\text{frequency difference from TMS}\right)}{\text{instrument frequency in Hertz}} \times 10^6$$

The chemical shift is influenced by

- The electronic configuration around the nucleus and therefore the molecular structure.
- The solvent in which the sample is dissolved. This is because the process of dissolution involves the bonding electrons of the solute and solvent.
- The temperature, in the case of molecules with hydrogen bonding, because it affects the strength of the hydrogen bond.

5.7.4 Applications

NMR spectroscopy is of great importance due to chemical shifts, which make the spectra very precise and the ultimate in structural analysis. Chemical shifts are utilized for studying the following situations: The presence of neighboring aromatic rings can be detected due to abnormal shifts of a particular nucleus. Specific probes can be introduced to cause changes in the shifts making it possible to study the effect of the probes on molecular structure.

In a covalent bond, the electronic magnetic moment is zero because electrons are paired. However, the nuclear magnetic moment causes the electrons to be polarized slightly. This effectively transmits the direction of the spin of one nucleus to another. Such an interaction between like or different spins through the bonding electrons, called spin–spin interactions, cause the splitting of the NMR absorption peak already separated by chemical shifts. This splitting is called hyperfine splitting, and it is used to detect and identify the number and kind of chemical groups, bond angles, and isomers present. These can be extended to biomolecules such as nucleotides, hormones, peptides, and so on. Just like other spectroscopic techniques, NMR spectra is mainly used for studying the structure of molecules, conformational changes in macromolecules, qualitative and quantitative analysis, and at times for kinetic investigations (Figure 5.25).

7.33 Nuclear Magnetic Resonance Spectroscopy

In this technique, a sample is immersed in a magnetic field and bombarded with radio waves. These radio waves encourage the nuclei of the molecule to resonate or spin. As the positively charged nucleus spins, the moving charge creates what is called a magnetic moment. The thermal motion of the molecule—the movement of the molecule associated with the temperature of the material—further creates a torque or twisting force that makes the magnetic moment "wobble" like a child's top. When the radio waves hit the spinning nuclei, they tilt even more, sometimes flipping over. These resonating nuclei emit a unique signal that is then picked up on a special radio receiver and translated using a detector. This decoder is called the Fourier transform algorithm, a complex equation that translates the language of the nuclei. By measuring the frequencies at which different nuclei flip, one can determine the molecular structure, as well as many of the interesting properties of the molecule. Nuclear magnetic resonance (NMR) has proved as a power alternate to x-ray crystallography for the determination of molecular structure. NMR has the advantage over crystallographic techniques in that the experiments are performed in solution as opposed to the crystal lattice. However, the principles that make NMR possible tend to make this technique very time consuming and limit the application to small- and medium-sized molecules. NMR measures the distances between atomic nuclei, rather than the electron density in a molecule. With NMR, a strong, high-frequency magnetic field stimulates atomic nuclei of the isotopes H^1, C^{13}, or N^{15} (they have a magnetic spin) and measures the frequency of the magnetic field of the atomic nuclei during its oscillation period back to the initial state. The important step is to determine which resonance comes from which spin. The distance and the type of neighboring nuclei determine the resonance frequency of the stimulated atomic nuclei. The dependence on next neighbors is known as chemical shift (or spin–spin coupling constant) and reflects the local electronic environment and the information contained in the 1D NMR spectra. For proteins, NMR usually measures the spin of protons. The following reasons make the H^1 NMR spectroscopy the method of choice for biological H at many sites in proteins, nucleic acids,

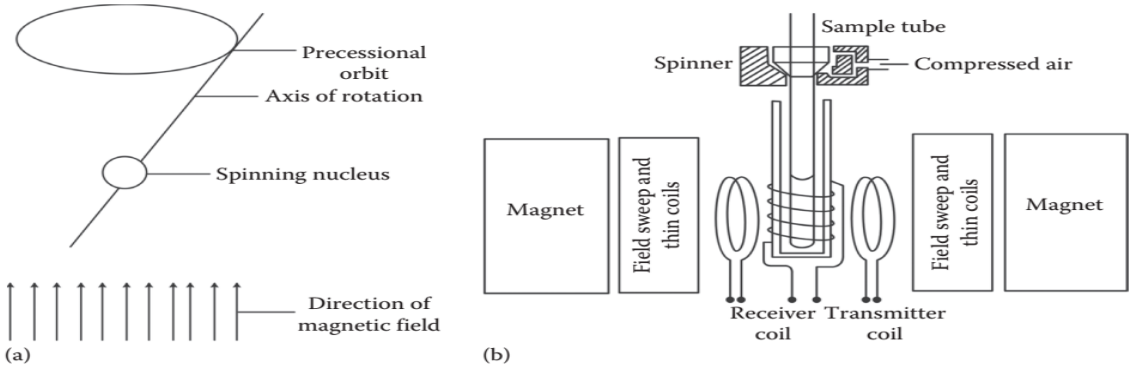


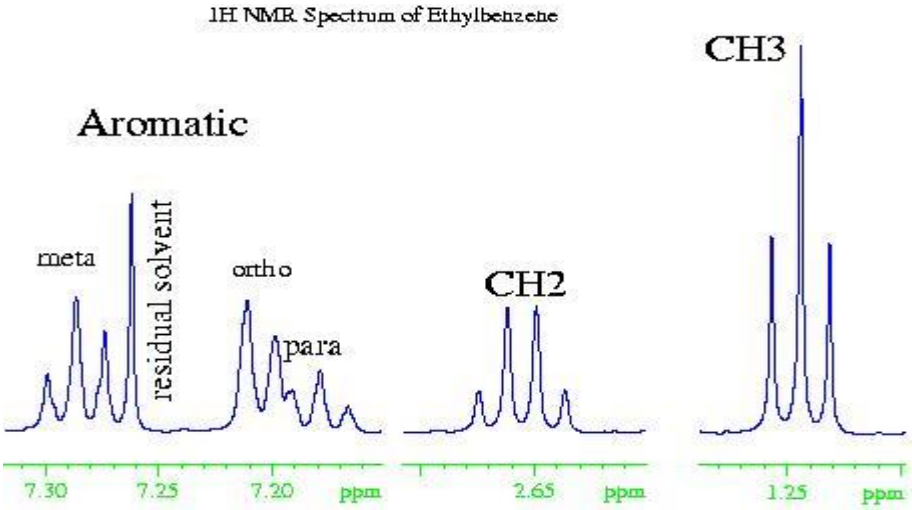
Figure 7.34 (a) Nuclear spinning in external magnetic field and (b) NMR spectrometer.

TABLE 7.3 Comparison of NMR and X-Ray Crystallography

NMR Spectroscopy	X-Ray Crystallography
Short time scale of protein folding	Long time scale, static structure
Solution, purity	Single crystal purity
<20 kD, domain	Any size, domain. Complex
Functional active site	Active or inactive
Domains	Domains
Aromatic nuclei, chemical bonds	Electron density
Resolution limit 2–3.5 Å	Resolution limit 2–3.5 Å
Primary structure must be known	Primary structure must be known (except if resolution is 2 Å or better for every single residue)

and polysaccharides since H has a high abundance for each site and the H nucleus is the most sensitive to detect (Figure 7.34 and Table 7.3).

The 1D spectra contain information about all the chemical shifts of all the H in the protein. The frequency resolution is often not enough to distinguish individual chemical shifts. The 2D NMR solves these problems by containing information about the relative position of H in molecular structures. The 2D NMR spectra contain information about interaction between H that is covalently linked through one or two other atoms (COSY or correlation spectroscopy). Alternatively, pairs of H can be closed in space, even if they are from residues that are not close in sequence (NOE spectra or nuclear overhauser effect). A complete structure can thus be calculated by sequentially assigning cross-peak correlations in the 2D spectra. Currently, the size limit for proteins that are amenable to NMR solution structure analysis is about 200 amino acids. An important feature of the identification of cross-peaks is that regular patterns that stem from secondary structure elements such as alpha helices and parallel or antiparallel beta sheets can be recognized, because they contain typical hydrogen bonding network.



NMR also requires the knowledge of the amino acid sequence, but the protein does not have to be in the ordered crystal, yet high concentrations of solubilized protein must be available (NMR structures are therefore also called solution structures). In biopolymers, the primary structure (sequence) logically breaks up the molecule into groups of coupled spins, normally one or two groups per residue. This is true not only for proteins but also for nucleic acids and polysaccharides.

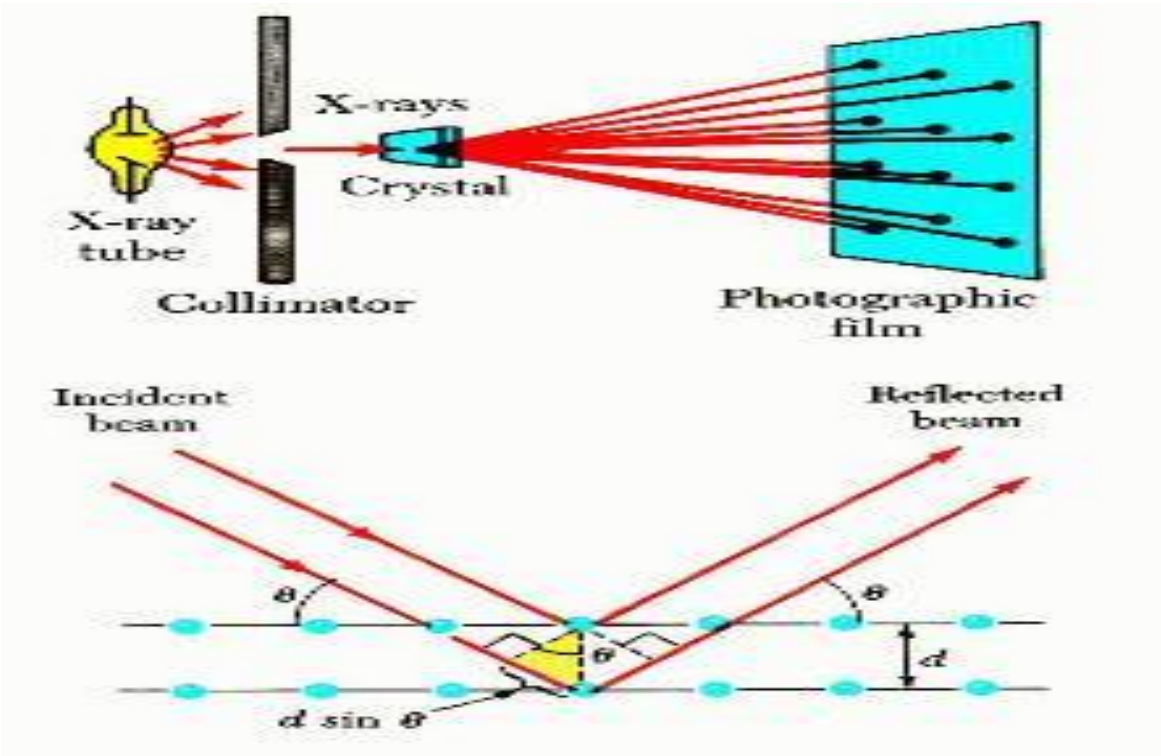
X-Ray differaction

X-ray diffraction is the elastic scattering of x-ray photons by atoms in a periodic lattice. The scattered monochromatic x-rays that are in phase give constructive interference. Figure 1 illustrates how diffraction of x-rays by crystal planes allows one to derive lattice spacings by using the Bragg's law.

8.5.2 Principles of X-Ray Crystallography

Crystals, including those of globular proteins, consist of repetitions of a basic structural component called a "unit cell," which may be a single molecule or a symmetrical arrangement of several molecules. Thus, each atom in a crystal must lie in a specific position with respect to all other atoms in the crystal, enabling the structure to be determined by x-ray diffraction analysis. This consists of directing a beam of x-rays of a single wavelength on a crystal and studying the characteristics of the emerging rays. Most rays pass straight through the crystal without being affected, but the rays that come into contact with an atom in the crystal are scattered by the clouds of electrons surrounding it. More precisely, these electrons act as secondary sources of x-rays, which then radiate out from the atom in all directions. The intensity of the x-rays leaving an atom of high electron density, such as a heavy metal, is much greater than that of x-rays leaving an atom of low electron density, such as hydrogen; thus, areas of high electron density can be said to scatter x-rays more strongly than areas of low electron density.

X-rays, similar to other forms of electromagnetic radiation, are best regarded as waves of characteristic lengths and amplitudes (Figure 8.5); the intensity of a ray is proportional to the square of its amplitude. If two rays of identical wavelength are traveling on a common path so that they are exactly in phase, that is, the crests and troughs of the waves correspond exactly, then they will combine to give a ray of the same wavelength and phase but greater amplitude. The amplitude, and hence intensity, obtained under these conditions will be the maximum that can be obtained by any combination of these two rays. If the two rays are one quarter of a cycle out of phase, the intensity of the combined ray will be about one-quarter of the maximum possible value, and the phase of the combined ray will



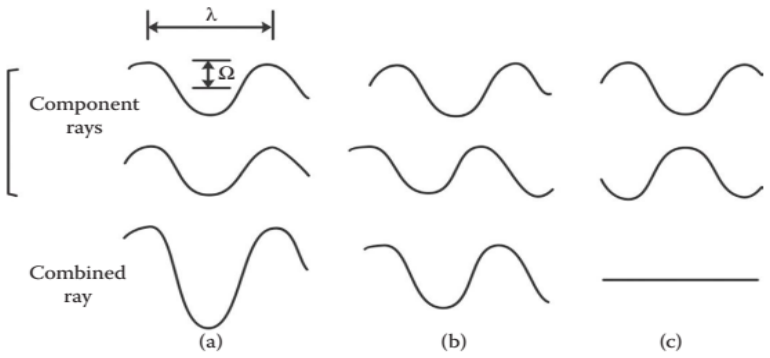


FIGURE 8.5 The combination of rays of identical wavelength (λ) and amplitude (Ω) when directed along a common path. The component rays are (a) exactly in phase, (b) one-quarter of a cycle out of phase, and (c) half a cycle out of phase.

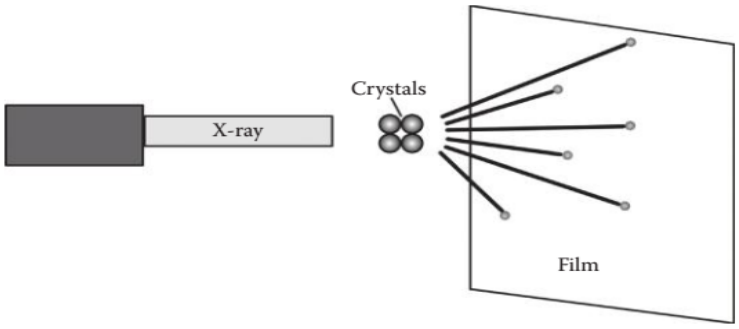


FIGURE 8.6 Diffracted x-rays.

be a combination of the phases of its component rays. If the two rays are exactly half a cycle out of phase, the waves will cancel each other out and the intensity of the combined ray will be zero.

The scattered x-rays emerging from a crystal can combine in the following way: Rays emerging at certain angles to the incident ray will combine to give rays of maximum intensity, whereas those emerging at other angles will cancel each other out. The results can be observed by placing a photographic plate behind the crystal to register the impact of emerging rays. In general, development of the plate will show a spot at the center caused by the undeflected x-rays, which will be in phase; this is surrounded by a pattern of other spots corresponding to the angles where emerging rays combine to give intensity maxima. The overall effect is known as a "diffraction pattern" (Figure 8.6).

Before discussing x-ray crystallography in more detail, let us consider why we cannot observe the atoms in a molecule by the use of optical or electron microscopy. Vision consists of two processes: The beam of light (another form of electromagnetic radiation) that strikes an object is scattered by the atoms, and these scattered rays are brought back together (focused) by the lens in the eye to produce an image of the object on the retina. A magnified image may be produced by the use of further lenses (optical microscopy), enabling one to clearly distinguish (resolve) features that are too close to be seen separately by the unaided eye. The limit of resolution in microscopy depends on the wavelength of the type of electromagnetic radiation used and the focusing properties of the instrument. With optical microscopy, the limit of resolutions is about half the wavelength of the light used. Hence, individual atoms separated in a molecule by distances on the order of 1–2 Å (1 Å = 0.1 nm) cannot be resolved by an optical microscope, since the wavelength of visible light is in excess of 4000 Å.

Electron microscopes give a much greater resolving power than optical microscopes, but despite the very low wavelengths of electron beams it is still not possible to visualize individual atoms using an electron microscope because of the generally poor performance of the electromagnetic lenses used in electron microscopy. Similarly, x-rays have wavelengths much smaller than those of light rays; in fact they are of the same order of magnitude as inter-atomic distances. However, no procedure has yet been devised for focusing x-rays, therefore no image can be produced using them. Nevertheless, the detailed structure of a crystal scattering x-rays can be deduced from the diffraction patterns obtained.

4.8 Fluorescence Spectroscopy

4.8.1 Fluorescence

Some compounds not only absorb radiation but also emit some of the energy in the form of fluorescent light. Energy is absorbed in the UV region of the spectrum and molecules are elevated from the ground state to a high energy level. The excited molecules then return to the ground state with the consequent emission of visible light. The wavelength of the emitted light is always higher than that of the absorbed radiation.

The requirements for a compound to fluoresce are an absorbing structure and a high resonance energy. Aromatic compounds in general are often capable of fluorescence, particularly if the substituent in the ring is electron donating.

4.8.2 Quenching

However, fluorescence is not as common as absorption due to quenching. Molecules containing Br, I, NO_2, and azo groups show little fluorescence because of this. Quenching decreases the quantum yield so that the absorbed energy is used for competitive electronic transitions with excited molecules or for breaking weak bonds instead of being emitted as fluorescent light. Quenching can also occur by interaction with the solvent and other molecules in solution. In some cases, the quenching reactions are fairly specific and can be used to identify a particular fluorescent compound.

5.5.1 Principle

Fluorescence is a phenomenon whereby a molecule, after absorbing the radiation of a particular wavelength, emits radiation of a larger wavelength. This is called the Stokes shift. When the emitted wavelength falls in the visible region, a glow can be seen. Measurement of the intensity of this glow with respect to the intensity of incident radiation is called fluorescence spectroscopy, or simply fluorometry (Figure 5.16).

Absorption and emission are almost instantaneous, with a time lag of only seconds, approximately during which a molecule exists in an excited state. Most organic molecules in their ground state are singlets (paired); on absorbing radiation, they are excited to a higher energy state without a change of spin. These are called excited state singlets. An excited state with the lowest energy is the first excited singlet (Figure 5.17).

Fluorescence occurs when the first excited singlets relax to the ground state. The intensity of fluorescence (I_f) is related to incident radiation (I_0) by

$$I_f = I_0 2.3e\lambda cdQ$$

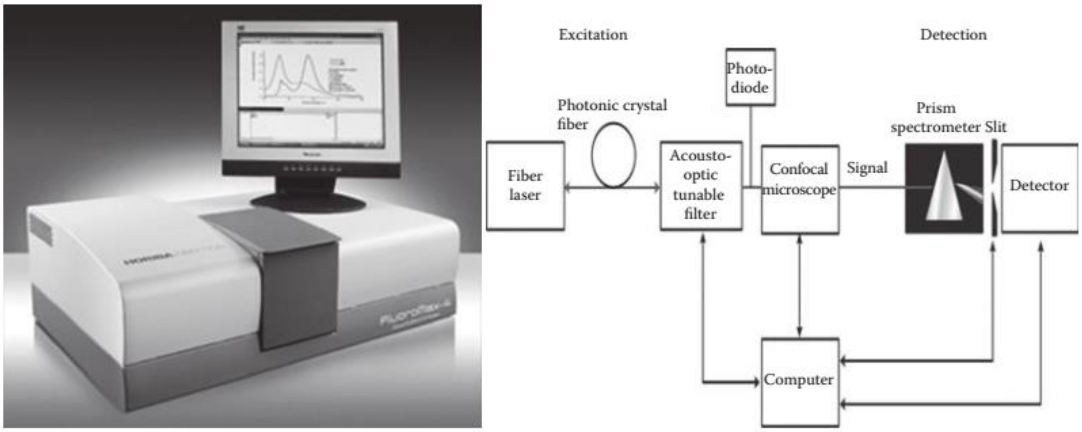


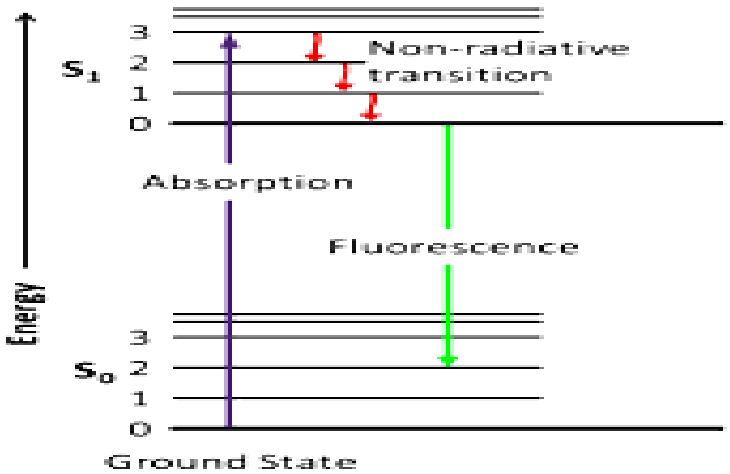
FIGURE 5.16 (a) Fluorescence spectrophotometer and (b) functional view.

where

c = concentration of fluorescing solution (M)
d = light path in the fluorescing solution (cm)
ε = molar absorptivity coefficient for the absorbing material at wavelength λ ($dm^3 \cdot mol^{-1} \cdot cm^{-1}$)
Q = quantum efficiency that is equal to the number of quanta fluoresced divided by the number of quanta absorbed

If the initial absorption generates a higher excited state, this will relax quickly and nonradiatively to the first excited singlet (decay), which may then fluorescence. The nonradiative relaxation mechanisms include:

- Thermal relaxation transfer of energy to molecular and submolecular motions like collision, rotation, and vibration.
- Photochemical reactions. When such processes win the competition with radiative energy loss, quenching of fluorescence is said to occur.



The efficiency of these nonradiative processes depends on the environment of the molecule; therefore, so does fluorescence intensity. Thus, the fluorescence of an emitter is a probe (indicator) of its environment.

5.5.2 INSTRUMENTATION

Figures 5.18 and 5.19 show the main components of a spectrofluorimeter. The source is a mercury lamp or xenon arc. The M_1 monochromator is for selecting a chosen wavelength of irradiation. The M_2 monochromator enables determination of the fluorescence spectrum of a specimen. The photocell detector and recorder are the same as in a UV–visible spectrophotometer. The fluorescence from a sample is emitted in all directions but is examined at right angles so that the transmitted light does not interfere.

If the sample lacks intrinsic fluorescence, it can be made to bind to a fluorophore or probe and the so-called extrinsic fluorescence can then be measured. Some extrinsic fluorophores include dansyl chloride, l-anilinonaphthalene-8-sulphonate (ANS), fluorescein, ethidium bromide, and so on.

4.8.3 Applications

Fluorescent compounds are used extensively in biochemical investigations as they can be detected at very low concentrations and with a high degree of selectivity. The absorption and fluorescent spectra of a compound are quite characteristic so that when the maxima are selected by filters or monochromators on the incident and emitted beams, the fluorescent compound can be detected and measured even when other fluorescent compounds are present. Some of the applications include the use of fluorescent compounds as membrane probes, substrates for sensitive enzyme assays, and immunofluorescence.