Notes

18

SPECTROPHOTOMETRY, LIGHT EMISSION AND SCATTERING ANALYTICAL TECHNIQUE

18.1 INTRODUCTION

Measuring light emission, transmittance and scattering are few of the most important techniques used in modern biochemistry laboratory. They are used in spectrophotometer, colorimetry, chemiluminescence, ELISA etc. The ease and accuracy of measurement of light emission or transmittance makes it a method of choice in a large number of analytical techniques.

OBJECTIVES

After reading this lesson, you will be able to:

- describe the functional aspects of a colorimeter.
- describe Spectrophotometry.
- explain the functional aspects of chemiluminescence.
- explain the concepts of incident, transmitted, absorbed and scattered light.
- describe the Beer Lambert's law.

18.2 EXPLAINING THE TERMS USED

As we study further we will be encountering some terms such as incident light, absorbed light or absorbance, transmitter light or transmittance, scattered light etc. Here we try to explain the terms:

(a) Incident light: Incident light refers to the beam of light that is directed at the cuvette (or reaction vessel) from a light source.

(b) Transmitted light: Transmitted light is the light that passes through the cuvette to emerge on the other side.

(c) Absorbed light: Absorbance is the term used to describe the light that is absorbed by the reaction mixture and it depends on the intensity of the color of a reaction product in the reaction vessel.

Absorbance (A) = Incident light (I) – Transmitted light (T)

In conditions where scatterence is zero.

(d) Scattered light: It is the light that is not absorbed but is scattered or reflected back by opaque substances in the reaction vessel. This is used to measure turbidity in reaction such as immuno turbidity reactions.

18.3 COLORIMETRY

Colorimetry is a method employing the measurement of absorbance of a reaction mixture. It is the simplest method used and is employed by a large number of instruments such as a colorimeter, semiauto analyzer, auto analyzer. The most important thing for this method is that the metabolite or chemical we intend to measure should undergo a reaction in the cuvette to produce a colored product or there should be certain change in absorbance before and after the reaction as in cases of UV kinetic methods. The intensity of the color developed or change in absorbance will depend on the concentration of the metabolite in the sample. The method is based on passing a beam of light of a certain wavelength through the reaction vessel or cuvette containing the colored product of the reaction. The incident light as well as the transmitted light of the particular wavelength is measured and the absorbance calculated. The absorbance is plotted on a graph which has been drawn by plotting a curve of absorbance of the reaction mixture as against a serial dilution of the metabolite or chemical the reaction mixture contains.

Absorbance numerically is the log of the ratio of incident light upon transmitted light.

$$A = \log_{10}(I/ T)$$

This same principle is used in all colorimeters as well as autoanalyzers, semi auto analyzers. The difference being that in auto analyzers the machine itself transfers a pre determined amount of the sample and reagents into a reaction vessel and reads the absorbance and then plots the absorbance on a graph pre plotted during calibration while in a semi auto analyzer the reagent and sample

are mixed in a test tube and fed into the semi auto analyzer to be read and the value is plotted on a pre stored curve plot during calibration. In colorimeter the reading is taken manually and then the concentration is calculated by manually plotting the absorbance on a calibration curve.

In all these instruments the basic architecture is the same. A source of light is used from which the light is passed through a filter which blocks all wavelengths except the wavelength at which we need the sample to be read. This light passes through a cuvette which holds the sample post the color producing reaction. After passing through the sample the light beam falls onto a photo voltaic cell. This photo voltaic cell produced a current the strength of which is directly proportional to the amount of light falling on it which will be inversely proportional to the absorbance. In reality the photo voltaic cell measures incident light and based on this calculated the absorbed light or absorbance. The absorbance will be directly proportional to the amount of the colored particles in the reaction mixture and hence directly proportional to the metabolite or chemical we are trying to evaluate in the sample.

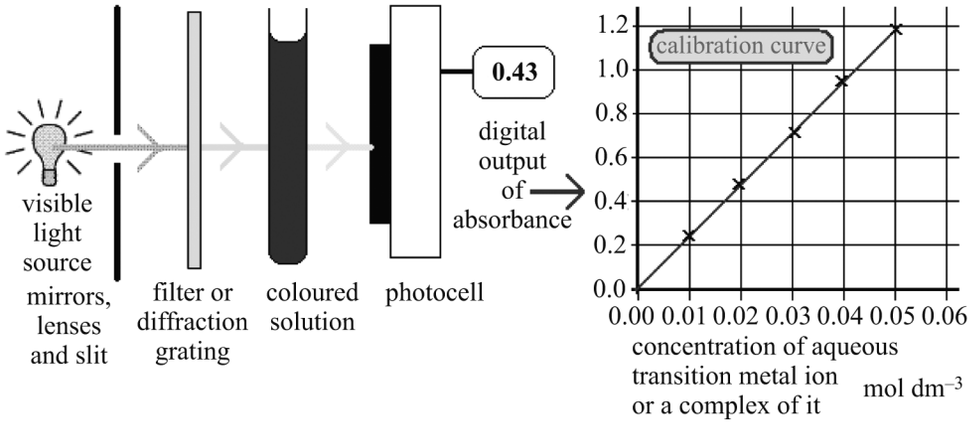


Fig. 18.1: A Simplified diagram of a colorimeter

18.4 BEER LAMBERT'S LAW

The Beer Lamberts law describes the co relation between the intensity of incident light, the path the light has to travel and the absorbance of the medium it passes through.

Absorbance is directly proportional to length of the path travelled by the light beam and the optical density of the colored solution. In a colorimeter the length of the path is fixed and this factor is negated when we set zero in the colorimeter

with only distilled water in the cuvette. Then the only factor affecting the absorbance is the optical density of the solution in the cuvette.

$A = \varepsilon b c$

A = absorbance (-)

ε = molar absorbtivity with units of L /mol/cm

b = path length of the sample (cuvette)

c = concentration of the compound in solution, expressed in mol / L

18.5 SPECTROPHOTOMETRY

The difference between colorimetry and spectrophotometry is that in spectrophotometry instead of taking reading at a set wavelength we read the absorbance over a range of wavelength taking readings at every 5 or 10 nm range. It instead of giving a specific absorbance reading gives a data stream which contain absorbance for every wavelength made incident on the sample. Or it can also give a spectra reading or a graph that plots absorbance against the increasing wavelength of incident light.

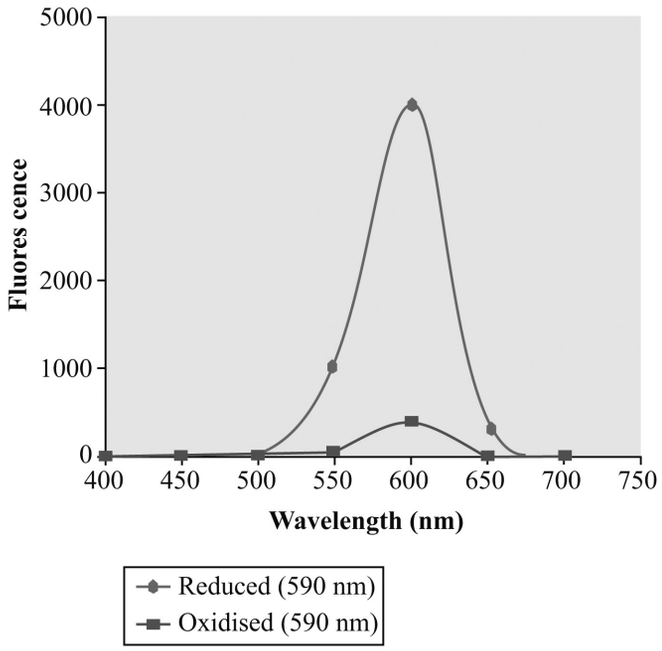


Fig. 18.2: Spectophotometry

This is an example of spectra reading where the absorbance of the sample is plotted over a wavelength between 400 to 750 nm.

18.6 LIGHT EMISSION TECHNOLOGIES OR CHEMILUMINESCENCE

Chemiluminescence is the production of light during a chemical reaction. This is seen in nature in various natural reactions such as the one happening in fire flies.

The chemical reaction can be displayed like this:

$$[A] + [B] \rightarrow [\blacklozenge] \rightarrow [\text{Products}] + \text{light}$$

One such reaction is the reaction between luminol and hydrogen per oxide:

$$\text{luminol} + H_2O_2 \rightarrow \text{3-APA}[\blacklozenge] \rightarrow \text{3-APA} + \text{light}$$

- where 3-APA is 3-aminophthalate
- 3-APA[♦] is the excited state fluorescing as it decays to a lower energy level.

Immunochemilumnisence is a method in which a chemiluminescence reaction is used by conjugating an enzyme, catalyzing the chemiluminescence, reaction to an antibody specific to another antibody which is bound to the antigen of interest. This enzyme converts a substrate to a product and during the process releasing light energy. This photon of light is detected by a sensor and an accurate measurement of the number of chemiluminescence reactions occurring during a specific time period can be made. This directly proportional to the number of enzyme molecules present which is directly proportional to the antigen molecules present in the sample.

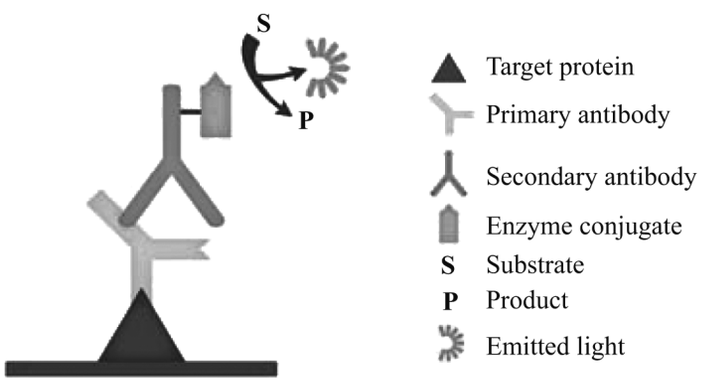


Fig. 18.3: Chemiluminescence

Chemiluminescence is an extremely accurate method with its accuracy reaching to the gold standard tests such as Radio immune assays (RIA). It is used to measure hormone levels in serum.

18.7 FLUORESCENCE

It is the ability of a substance to emit visible light after absorbing light from the visible or UV range. When these substances absorb light in the UV range of the spectra and emit light in the visible range, this property is called UV florescence and is responsible for certain substances like Vitamin B_2, quinine, certain minerals glowing in the dark.

The principle behind fluorescence is that of excitation of an electron. The incoming light commonly UV light provides the electron in an orbital energy hence helping it shift to a higher energy orbital. Some of the energy in the incident light is used up in this movement of electron. After sometime the electron returns to its original lower energy orbital and the remaining energy is released in form of light. This light has lesser energy than the incident light and hence has a larger wavelength. This results in the phenomenon of high energy UV light being absorbed by a substance and low energy visible light being emitted in its place.

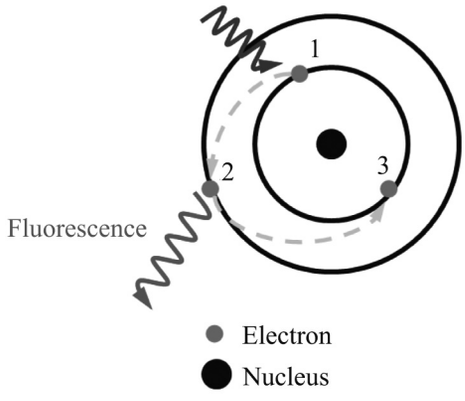


Fig. 18.4: Fluorescence

Principal of fluorescence

In laboratory fluorescence spectroscopy is used to analyze organic compounds. Tryptophan is an aromatic amino acid which displays fluorescence. It is present in a large number of proteins. The fluorescence of the tryptophan residue of any protein is effected by the environment of the tryptophan residue. Hence in cases of aberrant proteins or in cases of aberrant folding of the amino acid chain in any protein the tryptophan residue's immediate environment is changed and hence a measureable change in fluorescence is seen.

The fluorospectrometer consists of a light source, a filter, a monochromatizer, a sensor to measure the fluorescent light placed at 90% to the incident beam of

light to prevent reflected light from reaching the sensor. A quartz cuvette is used as a high grade quartz cuvette does not absorb the light in the wavelength range of fluorescence.

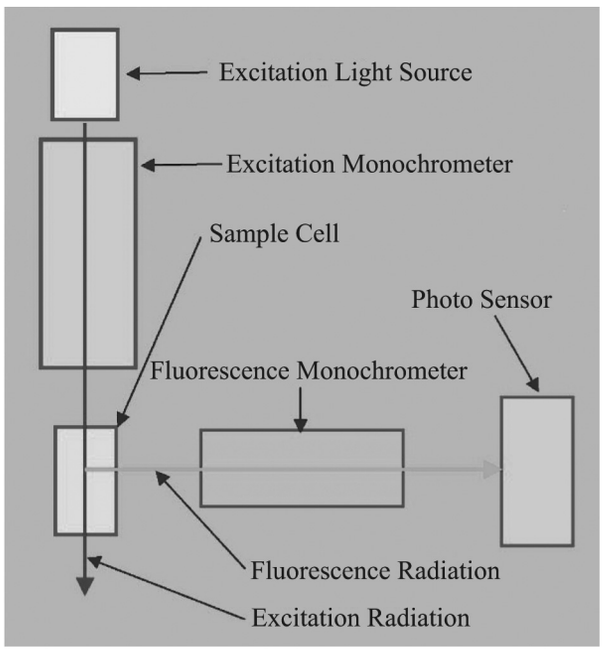
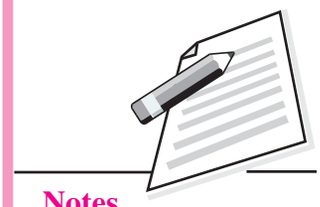


Fig. 18.5: Simple diagram of the architecture of a fluorescence spectrophotometer

Apart from this spectro photometer the flame photometer also works on the principal of excitation of an electron so as to move it out of its orbit and then read the visual spectra of the light emitted when the electron moves back to its original orbit. The only difference being that the excitation energy is provided by the flame into which the metal ions are sprayed into in form of a fine spary or mist.

INTEXT QUESTIONS 18.1

1. Beam of light directed to the cuvette is .....
2. Absorbance of a reaction mixture is measured by .....
3. ..... is used to measure turbidity in reaction
4. Absorbance over a range of wavelength is taken in .....
5. The production of light during a chemical reaction is .....



WHAT HAVE YOU LEARNT

- Measuring light emission, transmittance and scattering are few of the most important techniques used in modern biochemistry laboratory.
- They are used in spectrophotometer, colorimetry, chemiluminescence, ELISA etc.
- The ease and accuracy of measurement of light emission or transmittance makes it a method of choice in a large number of analytical techniques
- Incident light refers to the beam of light that is directed at the cuvette (or reaction vessel) from a light source.
- Transmitted light is the light that passes through the cuvette to emerge other side.
- Absorbance is the term used to describe the light that is absorbed by the reaction mixture and it depends on the intensity of the color of a reaction product in the reaction vessel.
- Scattered light is the light that is not absorbed but is scattered or reflected back by opaque substances in the reaction vessel and is used to measure turbidity in reaction such as immune turbidity reactions.
- Colorimetry is a method employing the measurement of absorbance of a reaction mixture. It is the simplest method used and is employed by a large number of instruments such as a colorimeter, semiauto analyzer, auto analyzer
- The difference between colorimetry and spectrophotometry is that in spectrophotometry instead of taking reading at a set wavelength we read the absorbance over a range of wavelength taking readings
- Chemiluminescence is the production of light during a chemical reaction
- Immunochemilumnisence is a method in which a chemiluminescence reaction is used by conjugating an enzyme, catalyzing the chemiluminescence, reaction to an antibody specific to another antibody which is bound to the antigen of interest
- Fluorescence is the ability of a substance to emit visible light after absorbing light from the visible or UV range

TERMINAL QUESTIONS

1. Enumerate the various diagnostic methods that use emitted, transmitted or generated light for measurement.

2. What is the beer lambert law? What are the correlations derived from it.
3. What is the principle of colorimetry? Draw a simple labelled diagram of a colorimeter.
4. Write a short note on spectrophotometry.
5. What is chemiluminescence? What are its uses in diagnostic?
6. What is fluorescence? How is it used in diagnostics?
7. What is the principal of a flame photometer?

ANSWERS TO INTEXT QUESTIONS

18.1

1. Incidence light
2. Colorimetry
3. Scattered light
4. Spectrophotometry
5. Chemiluminescence