Biochemical Methods of Analysis

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Theory and Applications

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PREFACE

"No amount of experimentation can ever prove me right, a single experiment can prove me wrong".

Albert Einstein

Many outstanding advances in modern biology have been made as a result of invention and application of efficient methods. Choice of experimental methods in research are important to yield satisfactory results. There are many classical techniques that have become part of the standard equipment of biological research. A thorough understanding of the analytical techniques and equipments is an absolute requisite for any student of life sciences. Analytical science has developed an array of sophisticated instruments routinely in operation in most laboratories. This book deals with fundamental aspects of various biochemical methods of analyses. Each chapter has been treated as a unit by itself in this book. The theory given is largely descriptive with necessary equations. This is a text book which covers theoretical as well as practical aspects of biochemical investigations which are the pre-requisite for any type of research. This book can educate the scientists and technicians working in research laboratories and can serve as a good text book for undergraduate, postgraduate, and research students of various branches such as biochemistry, microbiology, pharmacy, botany, zoology, agricultural and food sciences etc. The authors solicit suggestions for improvement of this book from teachers as well as students.

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Neera Garg

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CHAPTER 1

DIALYSIS AND ULTRAFILTRATION

Dialysis is a separation process of substances in solution driven by concentration gradient and their varying diffusion rates through a semi-permeable membrane. Dialysis is employed to retain large molecules while exchanging small ones. Dialysis is a form of molecular filtration. It is a process that separates molecules according to size through the use of semi-permeable membranes containing pores of less than macromolecular dimensions. These membranes allow small molecules such as those of solvents, salts and small metabolites to diffuse through but prevents the passage of large molecules. Molecules having dimensions significantly greater than the pore diameter are retained inside the dialysis bag whereas smaller molecules and ions traverse the pores of such a membrane and emerge in the dialysate outside the bag.

A mixture of small and large molecules is placed in a dialysis bag (usually made by knotting dialysis membrane tubing at both ends) immersed in a large volume of aqueous solvent or buffer. The small molecules pass through the membrane into external fluid until equilibrium is reached. After several hours of stirring, the solution will be equilibrated and the macromolecules remain inside the dialysis bag. The mixture can be virtually freed of salt or other small molecules by dialysis against running water or by repeatedly changing the solvent/buffer.

The rate of dialysis depends on a number of factors, some of which are explained below:

1.1 THE MEMBRANE

Cellophane (made up of cellulose) is the most commonly recommended material for dialysis tubing or bag. A suitable size and length is selected and boiled twice in 0.1 M $NaHCO_3$ containing 10mM EDTA to remove heavy metal ions, thoroughly rinsed (inside and out) with distilled water, and stored at 4 degree Celsius either in 10mM EDTA or 20% (v/v) ethanol. Before use, dialysis tubing

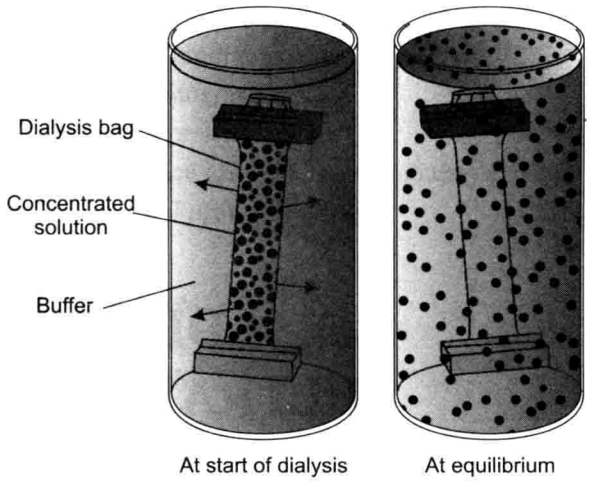

Fig. 1 Dialysis Protein molecules are retained within the dialysis bag, whereas small molecules diffuse into the surrounding medium.

should be tested for leaks. The tubing should be firmly knotted, taking care to pull only on the short end so that tubing containing the sample is not subjected to strain. The sample is introduced and the free end of the tubing is closed, either by knotting or by use of a dialysis clamp. It should be noted that, depending on the concentration of protein, salt, glycerol etc. In the solution, water may be taken up by the solution leading to increase in volume. Therefore the dialysis tubing should be only partially filled leaving sufficient room for expansion and avoiding the risk of the dialysis bag bursting. The filled dialysis bag is placed in dialysis buffer in either a beaker, conical flask or measuring cylinder and stirred with a magnetic stirrer. Generally, the volume of buffer used should be about 100-fold greater than the sample volume.

According to Fick's law, the rate of diffusion v is given by the following expression:

$$v = -D \,.\, q \,.\, dc/dx$$

For a given diffusion constant *(D)*, the velocity is proportional to the surface area *(q)* and the concentration gradient *(dc/dx)*, from which it follows that it is advantageous to use dialysis tubing of narrow bore (and hence greater surface area) and to change the dialysis buffer periodically to accelerate the process. Usually, dialysis should be completeds in 12-24 hr. To ascertain whether dialysis is complete, the conductivity of the sample should be compared with that of the dialysis buffer. For small volumes, (< 1 ml) commercially available micro-dialysis chambers can be used. Alternatively, home made chambers can be prepared using Eppendorf tubes with the lid drilled out and a piece of dialysis membrane stretched across the end of the tube and fixed in place by the lid; to ensure that the membrane is held under the surface of the dialysis buffer, the tube should either be clamped or held upside down using a polystyrene float.

1.2 PERMEABILITY

The size of the molecule that is able to pass the membrane depends on its 'cut off value'. The commonly used cellulose dialysis membranes have a cut off around 10 kDa, meaning that globular proteins of Mr > 10 kDa diffuse through the membrane only very slowly. In practice, there is no sharp cut off point and larger molecules than this will diffuse through the membrane if dialysis is prolonged. There are, however, a number of commercial materials available with a high flow rate and well defined limits of permeability with cut-off values between 1-50 kDa which can be used for finer separations. They are mostly available in dry form on a roll and they must be re-hydrated before use.

1.3 ULTRAFILTRATION

It is also a separation technique defined in different ways:

- An advanced water treatment technique involving the filtration of water via membranes with pores that are some 10,000 times smaller than those of human skin. By removing all particles of a size greater than 0.01 microns (pollen, algae, parasites, bacteria, viruses, germs and cysts), it enables the production of ultra pure water.
- Filtration through a medium (as a semipermeable capillary wall) which allows small molecules (as of water) to pass but holds back larger ones (as of proteins).
- The process that uses membranes to achieve separation of various constituents; a typical ultrafiltration membrane allows water, ions and small molecules to pass through while rejecting large molecules and suspended solids.
- The removal of excess water from the blood.
- Removing of fluids through a membrane by exerting greater hydrostatic or osmotic pressure on one side of the membrane.
- A process for filtering water by forcing it through a screen with very small pores. Ultrafiltration falls between reverse osmosis and microfiltration in terms of the size of the particles removed, with ultrafiltration removing particles in the 0.002 to 0.1 micron range.
- Ultrafiltration is the filtration process in chemistry that can retain solutes with relative molecular masses in the order of 100s to 1000s. It is also used in haemodialysis to clean whole blood while keeping its composition intact.

Ultrafiltration and dialysis are related techniques, both depending on the separation of molecules according to size using membranes with defined pores. The difference is that ultrafiltration employs pressure, either positive or vacuum, to accelerate the process. In principle, ultrafiltration can be carried out using dialysis membrane fitted into suction bottle connected to a vacuum pump.

However, better performance is obtained using membranes which are thicker with large pores. Membranes developed for ultrafiltration are constructed of mechanically strong synthetic or natural polymers of pore sizes ranging from very low (0.5 kDa) to high (200 kDa) cut-off values. The ultrafiltration membrane is clamped in a pressure chamber, whose volume can range from a few millimeters to several litres. Small molecules are driven across the membrane by the application of pressure, which is usually supplied by gas from nitrogen cylinder, or from compressed air if the sample is not susceptible to oxidation. It is usual for the solution to be stirred mechanically to ensure that the membrane does not become clogged as the protein accumulates during the ultrafiltration process.

Although ultrafiltration can generally be considered as a gentle process, partial inactivation may occur with particularly sensitive proteins. A further problem is that particulate contamination arising from 'wear' of some membranes can interfere with fluorescence and light-scattering experiments, particularly when very dilute solutions have been concentrated. Irreversible absorption of protein on the membrane, with consequent loss of yield, can occur during ultrafiltration depending on the nature of the membrane and the composition of the solution.

Ultrafiltration can be carried out using a centrifuge to generate the force needed to drive the solution through the membrane. Disposable tubes with integral dialysis membrane are available commercially for use in fixed angle rotors to concentrate sample volumes from a few ml to less than 100 ul in a relatively short time (minutes to hours).

Dialysis and ultrafiltration should both be considered for small molecule separation since it is somewhat easier to calculate the selectivity of ultrafiltration. The basis for discrimination with a permeable membrane is obvious when the particles being filtered are relatively large. But as the particle size becomes smaller, in the range of molecular dimensions, other factors come into play. The size exclusion effect becomes overshadowed by a host of other effects, most of which stem from molecular interactions. A serious difficulty in ultrafiltration derives from the fact that the pores are somewhat distorted by pressure and becomes more or less plugged as filtration proceeds, thus altering the effective pore size. Dialysis is free from this disadvantage because only diffusional activity would tend to cause a particle to enter a pore. If the particle is too large to pass through, the diffusional activity of the solvent in the reverse direction should be effective in dislodging it.

1.4 APPLICATIONS

Dialysis is a standard procedure in preparation biochemistry used across a broad range of activities like desalting-removal of high or low molecular weight solutes; removing ammonium sulphate from protein precipitates.

Concentrate: Removal of solvents, enrichment of macromolecular solutes or suspended matter. Dialysis can also be used to concentrate protein solutions by dialysis samples against 10-20 %(w/v) polyethylene glycol (high molecular weight; MW = ca 7.5 kDa); care should be taken that too much water is not removed from the sample since this can lead to precipitation. More simply, the dialysis bag can be laid in a bed of powdered polyethylene glycol or Sephadex G-200, both of which take up water (and salts) on swelling. Alternatively, the dialysis bag can be suspended in a pressure vessel through which dry clean compressed air is passed. Again, care should be taken with these methods that concentration does not exceed so far.

Fractionate: Separation of macromolecular mixtures; partition of free microsolutes from bound microsolutes.

Equilibrium dialysis is an analytical technique which is usually performed with commercial apparatus. In this technique, the two dialysis, separated by a membrane, are filled respectively with macromolecule solution and with ligand, which is usually radioactively labelled. After equilibrium has been reached, samples are removed from the two chambers; the concentration of free ligand is determined from one sample, and free plus bound ligand from the other. Parameters characterizing the binding equilibrium can be determined by appropriate analysis of the date. Equilibrium dialysis is a more quantitative approach often used in studies of ligand – macromolecule interactions. Equilibrium dialysis and ultrafiltration are used for measuring the extent of protein binding of drug in plasma and changing buffers.

Ultrafiltration is more applicable for highly concentrated protein solution or tissue homogenate. It can also be used for desalting.

SUGGESTIONS FOR FURTHER READING

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CHAPTER 2

COLORIMETRY AND SPECTROPHOTOMETRY

One of the earliest studied characteristics of chemical compounds were their colors. Color intensity is the basis of the most widely used set of biochemical assay procedures involving colorimetry. Things are colored because of their ability to absorb or remove certain components of light that impinges upon them. For example, if one looks at white light through a glass of wine, the wine appears red. It appears red because all the blue and yellow components of white light were removed as it passed through the wine, leaving only a red component to be detected by the eye (Fig. 1).

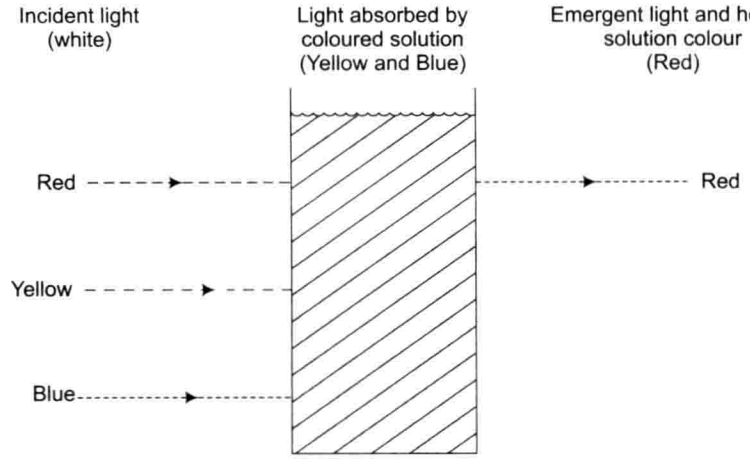

Fig. 1 Why solutions appear coloured

When white light falls onto colored material, the material either reflects or transmits only those wavelengths which we see. The wavelengths that are not reflected or transmitted are absorbed by the material and the color we see is white

light minus what is absorbed. Thus grass looks green because the chlorophyll pigments absorb a range of wavelengths in the blue region of the spectrum (400 – 500 nm) and another range in the red region (600 – 700 nm) which leaves a range of mainly green wavelengths (Fig. 2). The precise range and intensity of the wavelengths give the different shades of green.

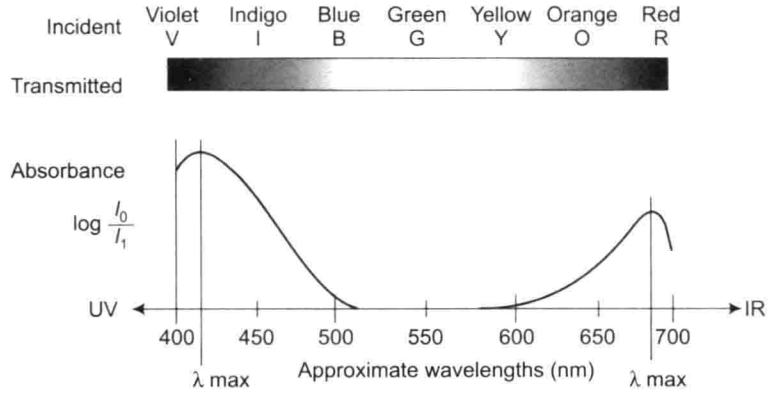

Fig. 2 The absorption spectrum of chlorophyll

Many compounds are not themselves colored but can be made to absorb light in the visible region by reaction with suitable reagents. These reactions are often fairly specific and in most cases very sensitive, so that quantities of material can be measured in very small concentration (mmole / l). The main advantage is that the complete isolation of the compound is not necessary and the constituents of a complex mixture such as blood can be determined after little treatment. The depth of color is proportional to the concentration of the compound to be measured, while the amount of light absorbed is proportional to the intensity of the color and hence to the concentration.

2.1 ABSORPTION OF LIGHT

Theory: At room temperature, most of the electrons in a molecule are in a ground state and when a compound absorbs UV (200 – 400 nm) or visible (400 – 700 nm) radiations, both the bonding and non-bonding outer electrons undergo excitation from lower energy level (ground state) to higher energy level (excited state). The most common type of these excitations are of delocalized electrons such as bonding π to π^* electrons which give intense absorption bands. Aromatic and conjugated compounds show these excitations. This technique exploits property of the compound to absorb light of particular wavelength (s) which defines its absorption spectra, and it is measured with a colorimeter and spectrophotometer.

The absorption of light is described quantitatively by the Beer-Lambert Law which states that quantity of light absorbed is proportional to its intensity (L) and to the concentration of absorbing species. When a ray of monochromatic light (Fig. 3) of initial velocity I_o passes through a solution in a transparent vessel, some of the light is absorbed so that the intensity of the transmitted light I is less than I_o. There is some loss of light intensity from scattering by particles in the solution and reflection at the interfaces, but mainly from absorption by the solution.

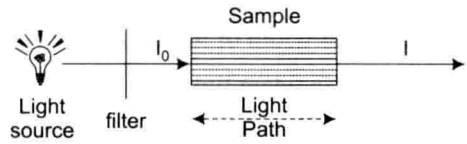

Fig. 3 Transmission of light through a solution

The proportion of transmitted light can be represented by the ratio of intensity of incident light and the intensity of transmitted light after traversing through the solution. Thus

$$T = \frac{I}{I_o}$$

Where

T = Transmitted light or transmittance

I_o = Intensity of incident light (Photons cm^{-2} sec^{-1})

I = Intensity of transmitted light

Percent transmittance can be expressed as:

$$\% T = \frac{I}{I_o} \times 100$$

For a completely non-absorbing transparent solution I will be equal to I_o and hence T will be 100 %. Conversely for an opaque solution which does not allow any light to pass through it, value of I will be zero and hence T will be equal to 0%. Thus transmittance can vary over a range of 0-100 % Absorbance (A) of light through the solution is inversely proportional to $\log_{10}$ of % T. Thus,

$$A = \log_{10}\left(\frac{I}{T}\right) = \log_{10}\left(\frac{I_o}{T}\right)$$

Absorbance has no units and varies from 0 (100% transmittance, hence $\log_{10}(I/I_o) = \log 1 = 0$) to $\propto$ (0% transmittance, $\log_{10}(I_o/I) = \log 0 = \propto$).

Lambert's Law: It states that light absorbed by a solution is directly proportional to the length of light path through the solution. Hence

$$A = \log_{10}\left(\frac{I_o}{I}\right) = \varepsilon.l$$

where

A = Absorbance

ε = Molar absorbance coefficient

l = Light path or width of liquid in cell or cuvette in cm

I_o = Intensity of incident light

I = Intensity of transmitted light

Beer's Law: According to this law, the amount of light absorbed is directly proportional to the concentration of absorbing solute in the solution, thus

$$A = \log_{10}\left(\frac{I_o}{I}\right) = \varepsilon.c$$

where

ε. = Molar absorbance coefficient

c = Concentration of the solute in moles / l.

Combining the two laws, we get

$$A = \log_{10}\left(\frac{I_o}{I}\right) = \varepsilon.c.l$$

If a standard cuvette with light path of 1 cm is used, this is a general practice, then

$$A = \log_{10}\left(\frac{I_o}{I}\right) = \varepsilon c$$

This indicates that absorption of light by a solution depends upon the molar absorbance coefficient and concentration (moles/ l) of the absorbing medium. A plot of absorbance against concentration gives straight line passing through the origin; whereas a plot of % transmittance against concentration gives a negative exponential curve (Fig. 4).

Molar absorption coefficient also called molar extinction coefficient is the extinction given by one molar solution of the solute using a standard cuvette of 1cm path. If this value of a compound is known, then its amount can directly be calculated by estimating absorbance of the solution against an appropriate blank. Otherwise, a standard curve can be plotted by taking known amounts of standard