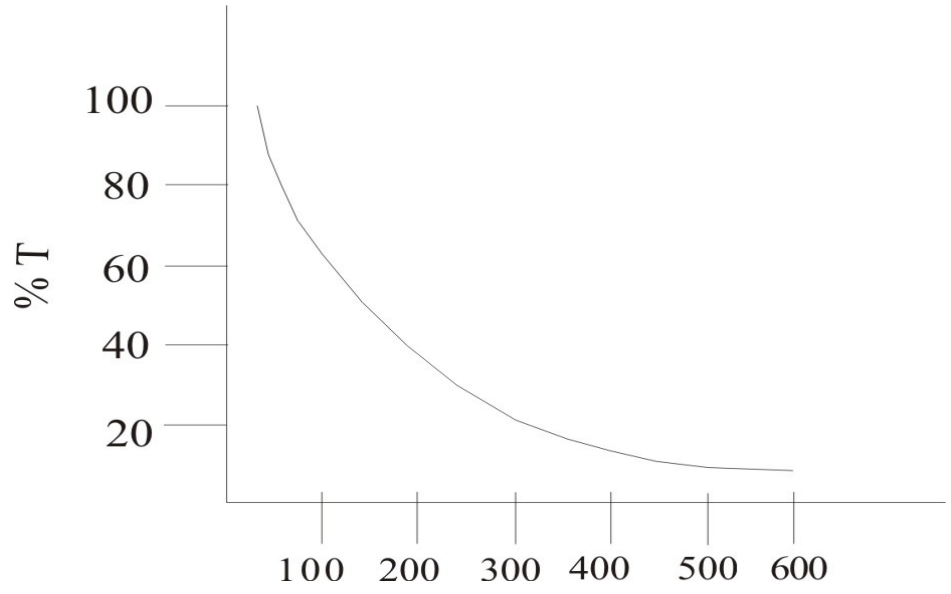
COLORIMETRY & SPECTROPHOTOMETRY

The measurement of concentration of coloured substances in solution forms the basis of colorimetric analysis, many substances of biological and medical interest are either coloured or form coloured derivatives when entered into chemical reactions.

When a ray of monochromatic light of initial intensity (Io) passes through a coloured solution, some of the light is transmitted with intensity (I) and some is absorbed.

The intensity I/Io (usually expressed as percentage) is called 'transmittance T' of the solution. As the concentration (C) of the compound is increased, the transmittance decreases inversely and logarithmically, as in figure (1):



Concentration

Fig (1)

With modern photoelectric equipments, the colorimeters and spectrophotometers, another property of the coloured solution is measured and is called absorbance 'A' which increases as the concentration of the solution increases.

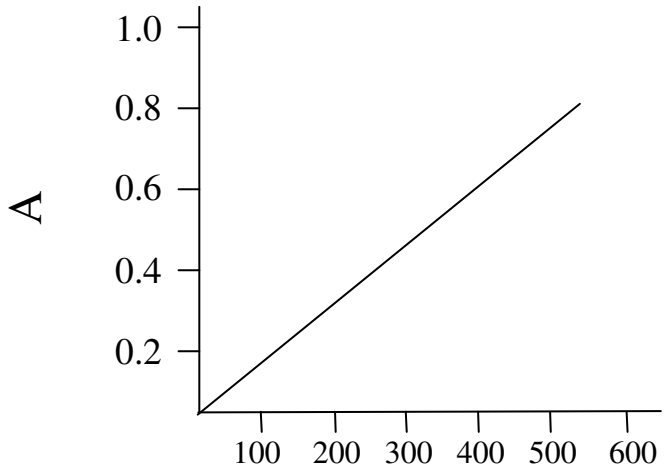


Fig (2)

Transmittance and Absorbance are related to each other by the following relationship:

$A = \log_{10} [100/T] = 2 - \log_{10} T$

Where T = percentage transmittance (%T)

A = absorbance

Beer's Lambert law

Under suitable conditions, if a coloured solution is illuminated with monochromatic light, its absorbance (A) will be directly proportional to the concentration (C) of the coloured substance multiplied by the depth (I) of the solution in the light path thus:-

$$A \alpha C \times I$$

Or $A = K \times C \times I$ where K is constant

This relation is known as the Beer's-Lambert law which is used to compare the concentration of unknown test solution with a standard solution measured in the same way, then:

$$A_{test} = K \times C_{test} \times I$$

$$A_{St} = K \times C_{st} \times I$$

$$\text{Then } \frac{A_T}{A_{St}} = \frac{C_T}{C_{St}}$$

$$\text{Or } C_T = \frac{A_T}{A_{St}} \times C_{St}$$

If at any step of the experiment, the test is treated not exactly the same as the standard (dilution, units … etc) the equation should be multiplied by a factor considering the variation.

Wavelength and Choice of Light Colour for Colorimetry

'White' light (coming from the sun or tungsten lamp) is a mixture of lights of various colors and wavelengths. A red solution absorbs the green component and reflects red so it appears red to the eye. Hence, for the quantitation of a colored compound in a solution, the sample has to be illuminated by a colored light supplied by the proper wavelength to get maximum absorbance of that light.

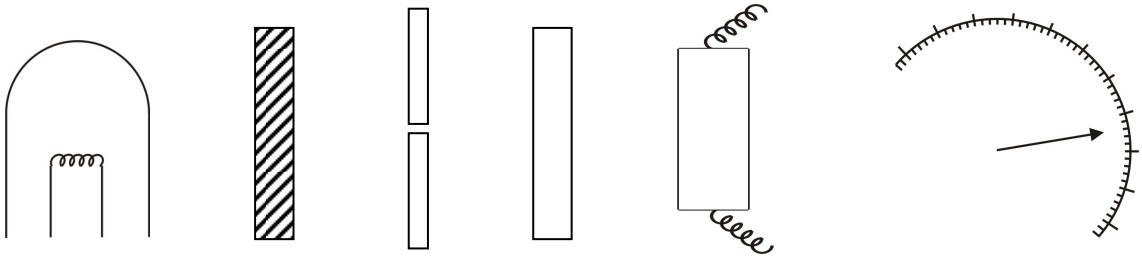
Measurement of absorbance 'A' (which is directly proportional to concentration 'C') is usually achieved using colorimeter or spectrophotometer. A diagram of the basic arrangement of colorimeter is given in fig. (3).

The major components of a simple spectrophotometer consist of

1- Light source supplied by a lamp (tungsten, deterium, or U.V.).
2- Monochromator or filter to provide selection of the desired light colour or wavelength for maximum absorbance.
3- Slit for isolation of a narrow beam and improvement of chromatic purity made of special kind of glass.
4- Absorption cell or (cuvette) to contain the sample. Here, light is absorbed depending on the concentration and nature of the solution. The remaining light is transmitted to the photocell.

5- Photocell or phototube which converts transmitted light energy to electric energy.

6- Meter or recorder to register the electric energy according to the concentration of the compound in the solution.



lamp filter slit cuvette photocell meter

Solutions Required for Photometric Measurements

In general, it is necessary to prepare three solutions:

1- Test solution that is made from serum, plasma or blood or other specimen being analysed.

2- Standard solution that is made from a known quantity of the substance to be measured.

3- Blank solution containing all the reagents used except of the substance to be measured. The blank solution compensates for non-specific colour already present such as reagent colour. Its absorbance is usually deducted from that of the test and standard respectively.

It is important to avoid cloudiness, turbidity or bubbles which absorb light and introduce error. The solutions should be optically clear.

CALIBRATION CURVE

For accurate work, standard solutions should be included with each determination. Variations in chemical reactions of new batches of reagents and in instrument behavior combine to cause variability in the absorbance 'A' of samples. It is useful to document the successive absorbance 'A' readings of the standard for quality control purposes. Furthermore, when Beer-Lambert law is obeyed, calibration curve is prepared for the range of concentrations intended to be covered in practice. This curve is prepared by taking several dilutions of the substance to be measured, running the experiment as described, then plotting absorbance against concentration on a graph paper. When running the experiment, the concentration of the unknown is found from this curve.

Preparation of calibration curve

An alternative procedure to find the concentration of 'test' of unknown protein sample is to prepare a calibration curve, then read the concentration of the unknown.

Procedure

Three known concentrations of protein standard will be provided. Place 3 ml of each concentration in a glass tube and label (St_1, St_2 & St_3). Place 3 ml of distilled water in another tube and label B. Add 5 ml of Biuret colour reagent to all tubes, mix, incubate for 30 min. at room temp. or for 10 min. at 37 °C water bath. Read the absorbance of the standard and blank by the spectrophotometer at 540 nm against distilled water. Subtract 'B' reading from each reading of the standards, then construct a calibration or standard curve with absorbance on Y-axis and concentrations in g/L ml on X-axis as in fig. (1). Find the concentration of the unknown solution 'T' from the curve.

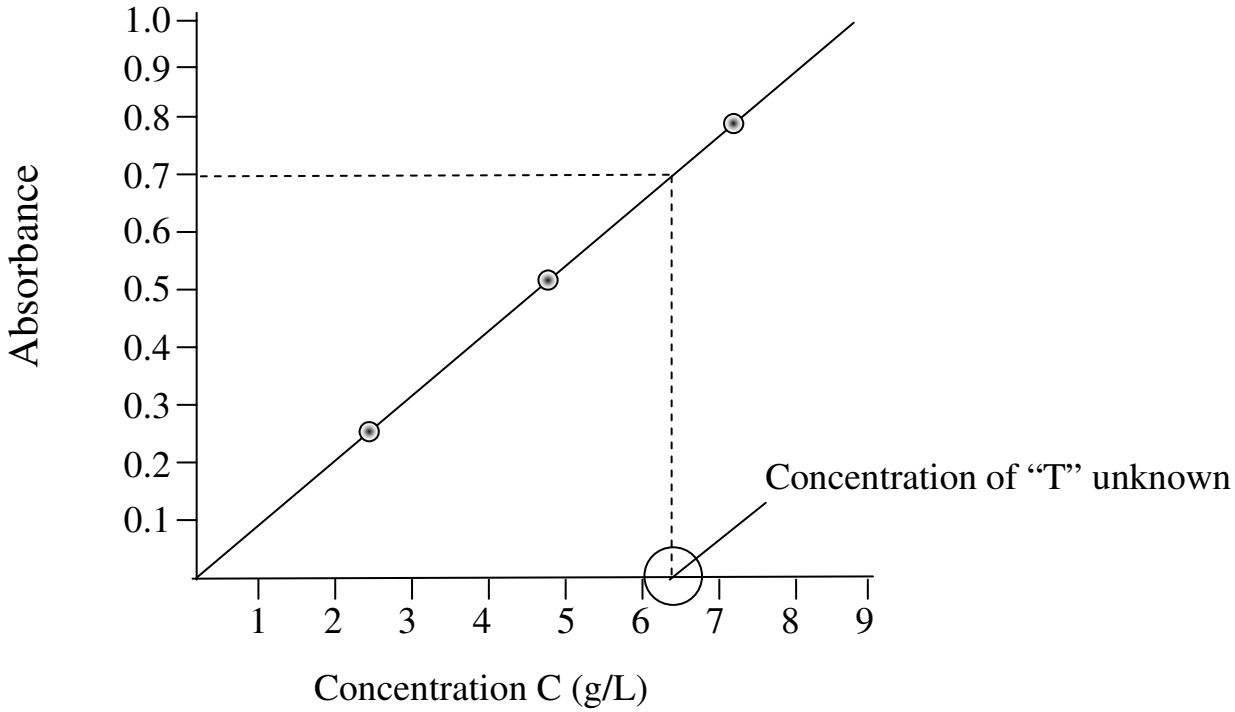


Fig. 1

$$C_T = \frac{A_T}{A_{St}} \times C_{St}$$

Reagents

1- Biurt reagent (as for total protein).

2- Protein standards : 2.5, 5.0, 7.5 g/L. They are equivalent to: 37.5, 75.0, 102.5 g/L in serum.

Prepare stock std: 5gm bovine albumin in 100 ml H_2O or (50 g/L).

a. Take 12.5 ml of stock and dilute to 250 ml with DW. This will give 2.5 g/L.

b. Take 25 ml of stock and dilute to 250 ml with DW. This will give 5.0 g/L.

c. Take 37.5 ml of stock and dilute to 250 ml with DW. This will give7.5 g/L.

3- Unknowns: the above standards can be given as unknowns or any other dilution could be made.