QUANTITATIVE EXPERIMENTS

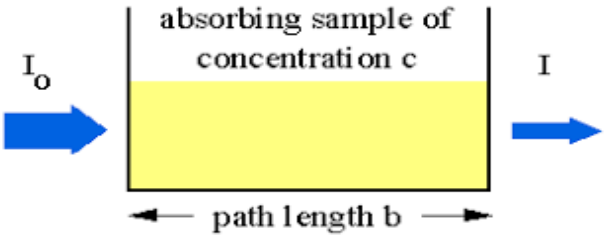
COLORIMETRY

- Colorimetry means measurement of colour. This technique is used in Biochemistry to measure the concentration of substances that are coloured or that can be converted to coloured compounds by suitable reactions. This technique is very sensitive and requires small quantity of sample.

- When ordinary light passes through a coloured solution, a portion of the light is absorbed by the coloured substances (chromogen) and the rest is transmitted.
- monochromatic light of definite wavelength, which is maximally absorbed by the chromogen, is used in colorimetry.
- The absorption is directly proportional to the number of chromogen particles in the path traveled by light

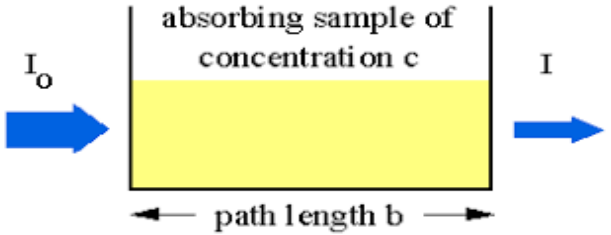
Beer's Law

- Beer's Law States that the log of the ratio of intensities of incident light (I_{in}) and emergent light (I_{em}) is directly proportional to the concentration of the chromogen in the solution provided.
- The length of the light path is constant.
- Log I_{in} / I_{em} is directly proportional to C



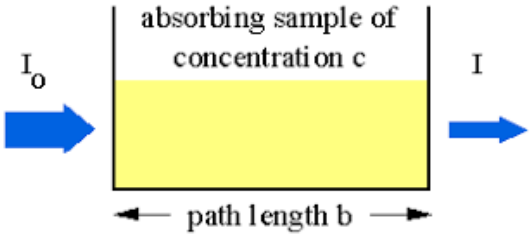
Lambert's Law

- Lambert's Law States that the log of the ratio of intensities of incident light (I_{in}) and emergent light (I_{em}) is directly proportional to the length of the light path (L) provided that the concentration of the chromogen in the solution is constant.
- Log I_{in} / I_{em} is directly proportional to L



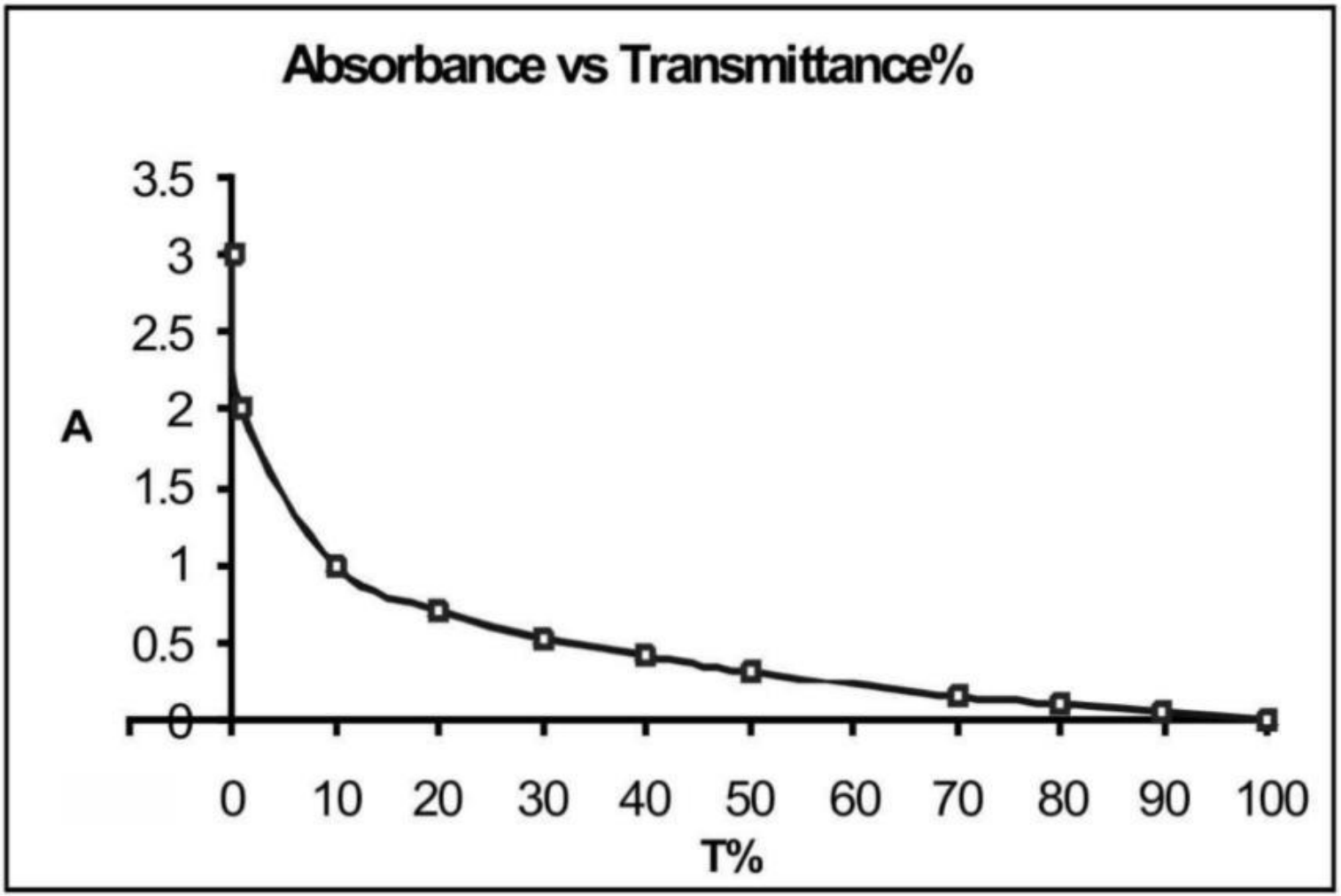
Beer – Lambert Law

- Log I_{in} / I_{em} is directly proportional to CL
- The ratio of the intensities of emergence and incident light (I_{em}/ I_{in}) is known as transmittance (T), a measure of the ability of a solution to transmit light.
- Log 1/T is directly proportional to CL
- Log 1/T is known as the optical density (OD) or the absorbance (A). this is a measure of the ability of the solution to absorb light.
- SO, A is directly proportional to CL



- As A has a direct relationship with C and L, A is used in calculations in all colorimetric estimations.
- Calculations are based on comparison of two solutions, one standard whose concentration is known and the other unknown whose concentration is to be determined.

Following graph describe relationship between T% and A.



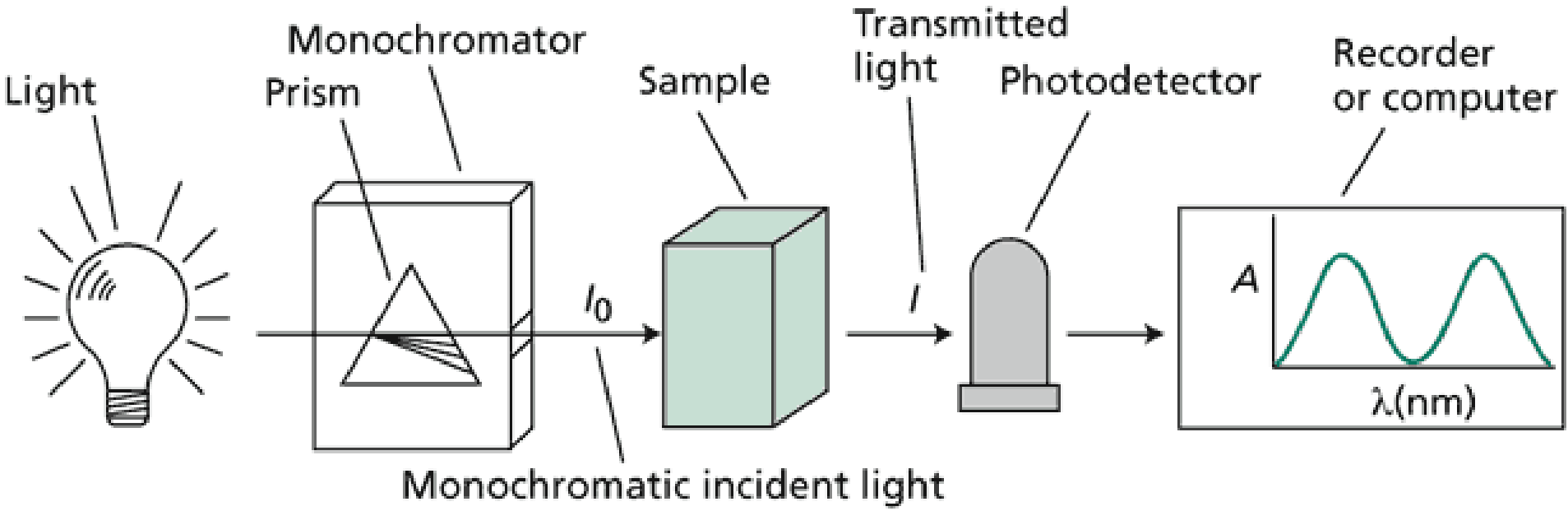
Photoelectric Colorimeter

- The basic components of a photoelectric colorimeter are :
- Light source : A tungsten lamp is, is used as a light source.
- Filters : The filters are usually coloured glass or dyed gelatin. When the light passes through a filter a light of very narrow range of wave length is allowed to pass through, the remaining wave length being absorbed.

- Cuvettes : Cuvettes are made of glass of fine quality with even surface and definite width to hole solution. The width of the cuvette varies with instrument.
- Photocell of Phototube : They are devises to measure the intensity of light by converting light energy in to electric energy.

- Galvanometer : Galvanometer measures the potential difference in the form of flow of current. The galvanometer is calibrated to read directly transmittance or absorbance or both.

The arrangement of various components is a single cell photoelectric colorimeter is as under.



- Optical Density Range (OD): As OD is a logarithmic scale, the accuracy of the reading is decreased if it is more than 0.9 and less than 0.1.

Filter Choice: Commonly the most suitable colour of filter used to measure the concentration of a particular chromogen is complimentary to its colour as shown in table below:

	Colour of solution	Colour of filter	λ-Range
1	Bluish – Green	Red	700-650
2	Green Blue	Orange	650-600
3	Blue	Yellow	600-575
4	Violet	Yellow-Green	575-555
5	Purple	Green	555-505
6	Red	Blue-Green	505-495
7	Orange	Green-Blue	495-475
8	Yellow	Blue	475-430
9	Yellow-Blue	Violet	430-350

Use of Blank

- **To eliminate errors, two types of blanks are used.**
- 1. Water blank: it is used to adjust the O.D. to zero and % T to 100
- 2. Reagent blank: the preparation of blank is essential in colorimetric analysis because of the fact, that some amount of light is absorbed by the coloured reagent itself. Therefore, the blank is prepared by adding all reagents except the substance to be estimated.

Use of the standard solution

- It is the solution of known concentration of the substance in pure form to be estimated. Both the concentration and OD of the standard solution are known, therefore, the concentration of unknown can be calculated.

Application

- Colorimetry is used for the estimation of various biochemical compounds in various biological sample like blood, plasma, serum, CSF, urine and other body fluids. Colorimetric technique is routinely used for biochemical estimations, such as glucose, urea, creatinine, uric acid, bilirubin, lipids, total proteins and albumin and enzymes like ALP, SGOT, SGPT, minerals like calcium, phosphorus etc.

A. ESTIMATION OF TOTAL PROTEIN IN SERUM BY BIURET METHOD

1. Principle

- When serum is treated with biuret reagent, the peptide bonds of proteins react with cupric ions in alkaline medium to form a violet coloured copper co-ordinate complex. The absorbance of this complex is measured colorimetrically at 540 nm using green filter. A standard protein solution is also treated similarly and the colour intensities are compared.

2. Reagents

1. Biuret reagent: Sodium -potassium tartarate. Copper sulphate, Potassium iodide and NaOH.
2. Normal saline : 0.9%
3. Protein standard : 6.00mg/ml

3. PROCEDURE

- Pipette into clean, dry test tube labelled as Blank (B), Standard (S) and Test (T).
- Then add the solution in each of test tubes separately as shown in table below.

	BLANK	STANDARD	TEST
Biuret Reagent	1000 µl	1000 µl	1000 µl
Distilled Water	20 µl	---	---
Standard	---	20 µl	---
Sample	---	---	20 µl

Mix and read absorbance (A) after 10 minutes of incubation at 540 nm.

4. OBESERVATION TABLE

1. O.D. of Blank (B): _____
2. O.D. of Standard (S):_____
3. O.D. of Test (T): _____

5. DATA

- Total Protein standard concentration is 6 gm/dl

6. CALCULATION

$$\frac{\text{O.D. of test} - \text{O.D. of blank}}{\text{O.D. of standard} - \text{O.D. of blank}} \times \text{Concentration of standard}$$

7. **RESULT:** Concentration of total protein in given serum sample is _____ gm/dl.

8. **REFERENCE RANGE:** 6.0-8.0 gm/dl

9. INTERPRETATION

- **A low total protein level** can suggest a liver disorder, a kidney disorder, or a disorder in which protein is not digested or absorbed properly. Low levels may be seen in severe malnutrition and with conditions that cause malabsorption, such as celiac disease or inflammatory bowel disease (IBD).
- **A high total protein level** may be seen with chronic inflammation or infections such as viral hepatitis or HIV. It also may be associated with bone marrow disorders such as multiple myeloma.

B. Estimation of serum albumin by Bromocresol Green (BCG) Binding Method

1. Principle

- Albumin in serum has an affinity for anionic forms of many indicators at pH below 5.
- Bromocresol green (BCG), an anionic dye binds tightly to albumin when added to serum and the complex absorbs light much more at pH = 4.1 and 620 nm than does the unbound dye.
- The increase in absorption of light is directly proportional to the albumin concentration.

2. Reagents

- 1. Bromocresol Green dye
- 2. Normal saline
- 3. Albumin standard 4.0 gm/ml

3. PROCEDURE

- Pipette into clean, dry test tube labelled as Blank (B), Standard (S) and Test (T).
- Then add the solution in each of test tubes separately as shown in table below.

	BLANK	**STANDARD**	**TEST**
Working Reagent	1000 µl	1000 µl	1000 µl
Distilled Water	20 µl	---	---
Standard	---	20 µl	---
Sample	---	---	20 µl

Mix and read absorbance (A) after 10 minutes of incubation at 620 nm.

4. OBESERVATION TABLE

1. O.D. of Blank (B): _____
2. O.D. of Standard (S):_____
3. O.D. of Test (T): _____

5. DATA

- Albumin standard concentration is 4 gm/dl

6. Calculations

$$\frac{\text{O.D. of test} - \text{O.D. of blank}}{\text{O.D. of standard} - \text{O.D. of blank}} \times \text{Concentration of standard}$$

7. Result

- Concentration of albumin in given serum sample= …… gm/dl

8. REFERENCE RANGE: 3.5-5.5 gm/dl

9. INTERPRITATION

- **Low albumin levels** can also be seen in inflammation, shock, and malnutrition.
- **Higher albumin levels** may be caused by acute infections, burns, and stress from surgery or a heart attack.

III. Determination of Globulin

- Total protein = Albumin + Globulin(gm/dl)
- Globulin = Total protein – albumin
- Globulin= ……. gm/dl.

Normal Range

- Serum total Protein : 6.0gm/dl - 8.0 gm/ dl
  Serum total Albumin: 3.5gm/dl - 5.5 gm/ dl
  Serum total Globulin: 1.5gm/dl - 3.0 gm/ dl.
  Serum A/G ratio= 1.2- 2.5

INTERPRETATION

- **A low A/G ratio** may reflect overproduction of globulins, such as seen in multiple myeloma or autoimmune diseases, or underproduction of albumin, such as may occur with cirrhosis, or selective loss of albumin from the circulation, as may occur with kidney disease (nephrotic syndrome).
- **A high level of albumin/globulin ratio indicates**: Conditions causing underproduction of globulins, like leukemia, hypothyroidism, and some genetic disorders

THANK YOU