Estimation of protein using standard curve & Biochemistry analyzer

Experiment 1 & 2

BBT 314

ACh

Introduction

- Proteins are an abundant component in all cells, and almost all except storage proteins are important for biological functions and cell structure.
- Proteins vary in molecular mass, ranging from approximately 5000 to more than a million Daltons.

- Plasma proteins are synthesized predominantly in the liver plasma cells, lymph nodes, spleen and in bone marrow.
- In the course of disease the total protein concentration and also the percentage represented by individual fractions can significantly deviate from the normal values.
- **Hypoproteinemia** can be caused by diseases and disorders such as loss of blood, nephritic syndrome, severe burns, salt retention syndrome and kwashiorkor (acute protein deficiency).
- **Hyperproteinemia** can be observed in case of severe dehydration and illness such as multiple myeloma changes in the relative percentages of one plasma protein fraction.
- The total protein measurements are used in the diagnosis and treatment of a variety of diseases involving the liver, kidney or bone marrow as well as other metabolic or nutritional disorders.

Aim and significant

Aim:

- To estimate the amount of total proteins in plasma.
- To make standard curve.
- To use Bioanalyzer

Significant:

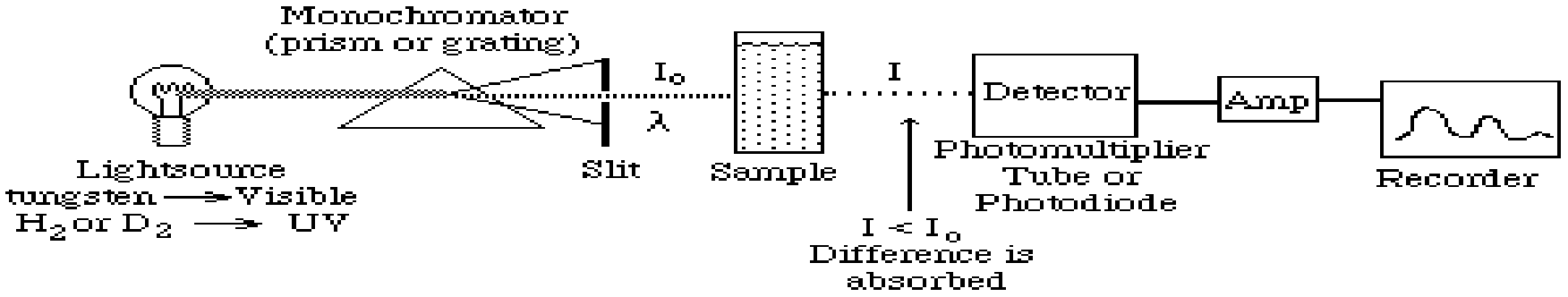
- The quantization of protein content is important and has many applications in clinical laboratory practices and in research especially in the field of biochemistry. The accurate quantization of protein content is a critical step in protein analysis.

Colorimetric assays : **Biuret test**

- Colorimetric assays allow for indirect determination of specific substrate concentrations, such as proteins or carbohydrates as well as determination of enzyme activity, via a color change. These reactions can be performed directly inside the spectrophotometer.
- In principle, all measurements occur in the visible range of light (approx. 380 nm - 780 nm).

Spectrophotometer

- A **spectrophotometer** is employed to measure the amount of light that a sample absorbs. The instrument operates by passing a beam of light through a sample and measuring the intensity of light reaching a detector is an optical instrument that measures the light energy transmitted throughout the continuous band of wavelength in spectromagnetic spectrum.
- A beam of light is focused by a lens onto an entrance slit, where it is collected by a second lens and refocused on the exit slit after being reflected and dispersed by a diffraction grating (used to select λ).
- After passing the slit , the light goes through the sample being measured and picked up by a phototube. The amount of light absorbed by the sample is read on the dial.
- Colorimetry: white light passed through a solution containing colored compounds .
- Standard solutions: known concentrations of samples


Monochromator
(prism or grating)
I_o
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Detector
Amp
Photomultiplier
Tube or
Photodiode
Recorder
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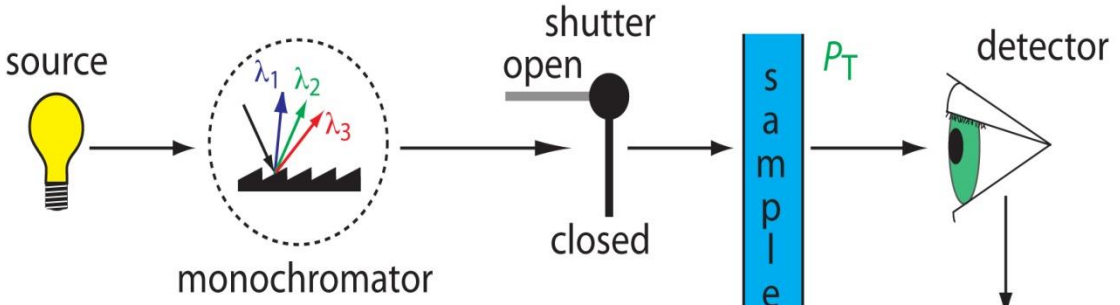
tungsten → Visible
H_2 or D_2 → UV
Slit
Sample
I < I_o
Difference is
absorbed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monochromator
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detector
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signal
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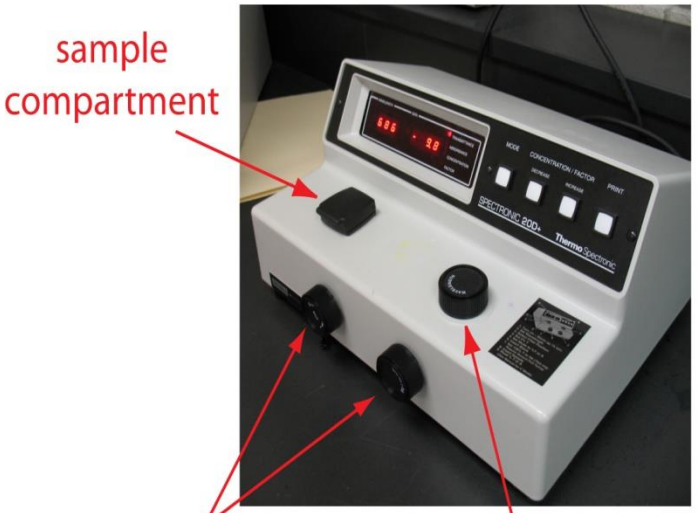

sample
compartment
0% T and 100% T
adjustment
wavelength
dial


A&E Lab

Biuret test

- For routine use, the biuret procedure is simple to perform, producing a stable color that obeys Beer's Law.
- UV-Vis Spectroscopy is primarily used for quantitative analysis in chemistry and one of its many applications is in protein assays.

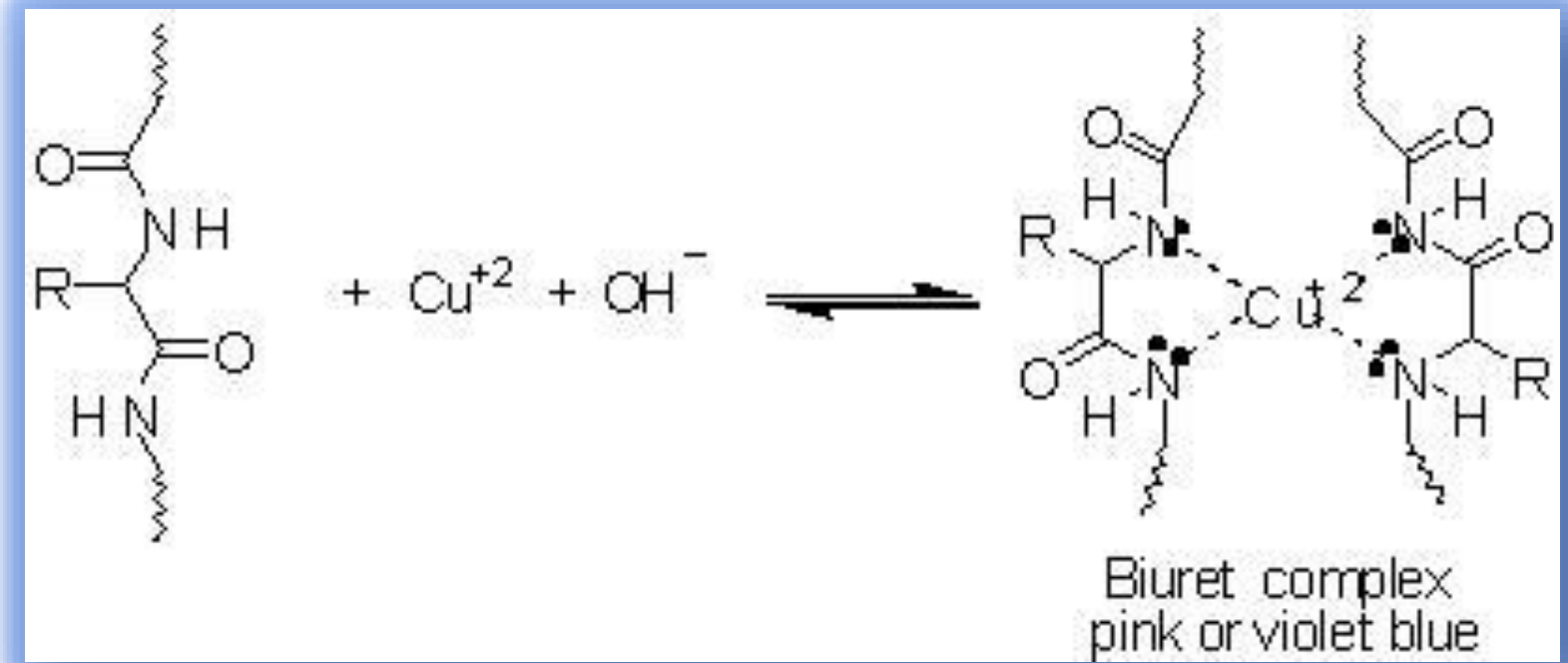
Biuret reagent.

- Hydrated copper sulphate: this provides the cu(II) ions which form the chelate complex.

  Cu(II) ions give the reagent its characteristic blue color.
- Potassium hydroxide does not participate in the reaction but provides the alkaline medium.
- Potassium sodium tartarate: (KNAC4H4O6.4H2O) stabilizes the chelate complex, prevent precipitation of copper hydroxide and potassium prevent auto reduction of copper.

Principle

- One commonly used method for determining the total protein in a sample is the Biuret method.
- The Biuret method is based on the complexation of Cu^{2+} to functional groups in the protein's peptide bonds.
- The formation of a Cu^{2+} protein complex requires two peptide bonds and produces a violet-colored chelate product which is measured by absorption spectroscopy at 540 nm.
- Over a given concentration range, the measured absorption at 540 nm is linear with respect to the concentration of total protein.
- The intensity of the color and hence the absorption at 540nm, is directly proportional to the protein concentration, according to the beer lamber law.
- Molecules containing 2 or more peptide bonds associate with the cupric ions to form a coordination complex that imparts a purple color to the solution with Amax = 540 nm.
- The purple color of the complex can be measured independently of the blue color of the reagent itself with a spectrophotometer or colorimeter.


+ Cu+2 + OH−
Biuret complex
pink or violet blue

Standard curve

- A standard curve is a type of graph used as a quantitative research technique.
- Standard curve for protein concentration is often created using known concentrations of bovine serum.
- The protein we will analyze is bovine serum albumin (BSA).
- Albumin is a serum protein that transports fatty acids and is important in maintaining plasma pH.
- In protein quantization assays, BSA serves as a reference protein that is used to construct protein standard curves. Other proteins can be used depending on the physical/chemical properties of your protein of interest.
- The preparation of a standard curve is necessary to check whether the method of assaying a particular substances increases in a linear way with its concentration.
- The general formula for obtaining different concentrations of a solution by dilution with diluent is:
- $C1V1=C2V2$

Procedure

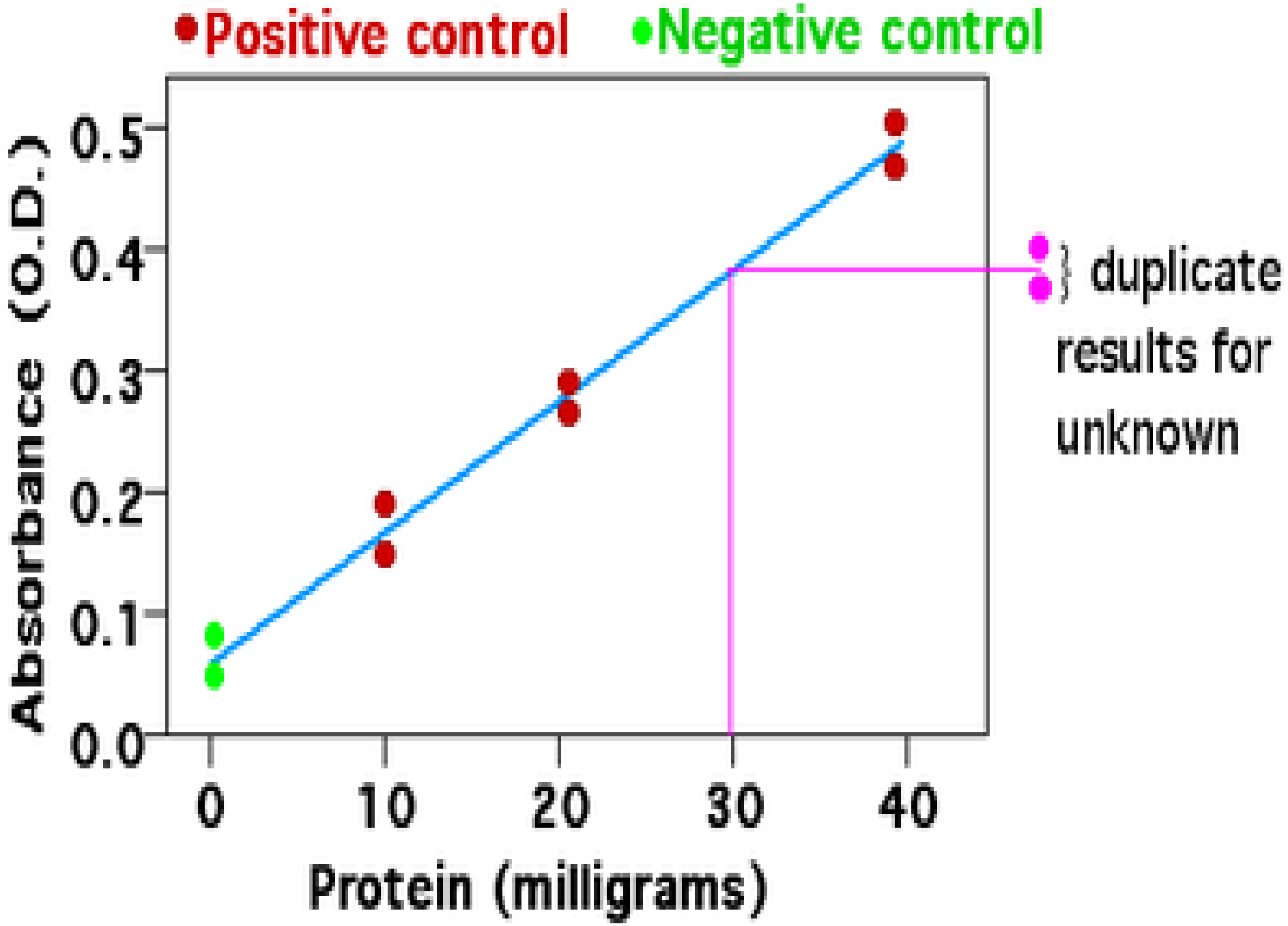
Tubes	D.W (ml)	Protein standard (ml)	Unknown (ml)	Conc. mg/ml
1	0.5	0		0
2	0.4	0.1		0.4
3	0.3	0.2		0.8
4	0.2	0.3		1.2
5	0.1	0.4		1.6
6	0	0.5		2
unknown	0	0	0.5	

After this, add 1 ml of biuret reagent to each tube and mix.
Incubate the tubes for 30 mints at room temperture.
Read at 540 nm on spectrophotometer.
Make the standard curve and measure the concentration of unknown.

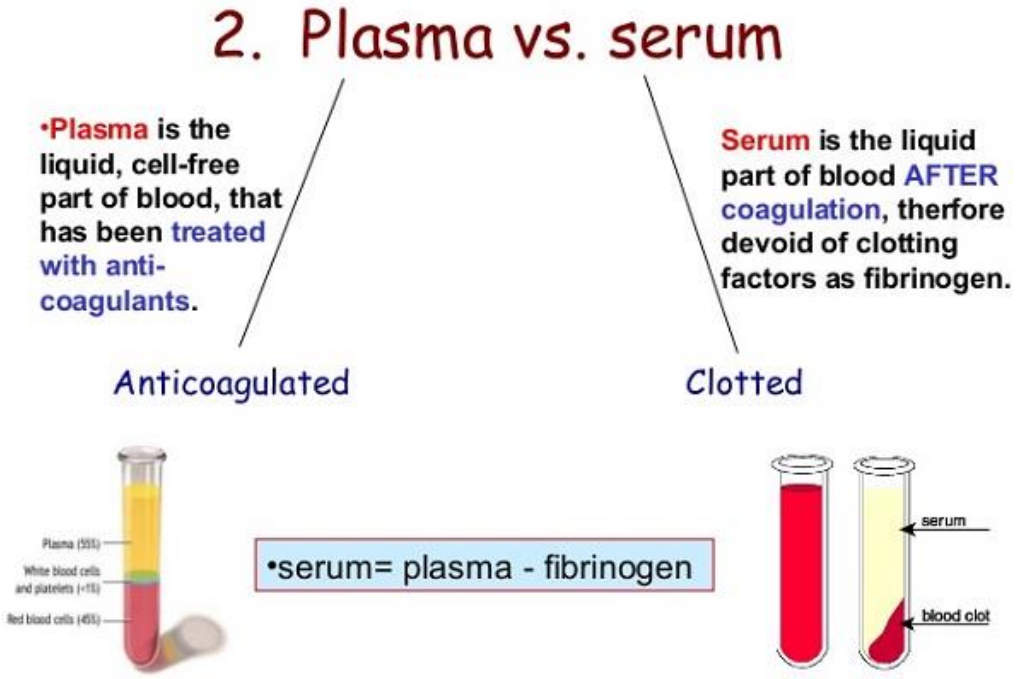
Blank solution: A blank solution is a solution containing little to no analyte of interest, usually used to calibrate instruments such as a colorimeter.

HOW TO MAKE STANDARD CURVE

- Multiple samples with known properities are measured and graphed, which then allows the same properties to be determined for unknown samples by interpolation on the graph.
- The samples with known properties are the standards, and the graph is the standard curve.
- Draw the points with protein concentrations as x values and the average absorbance as y values on a grid or graph paper
- Draw a straight line through the points
- Lookup the unknown protein concentration from the plot using the absorbant value of the unknown protein.


Positive control
Negative control
Absorbance (O.D.)
0.0
0.1
0.2
0.3
0.4
0.5
0
10
20
30
40
Protein (milligrams)
duplicate results for unknown

Biochemistry Analyzer



Calculation: Calculate the total Protein concentration by using the following formula:

Total Protein concentration =
(Absorbance of sample/Absorbance of standard) x [Standard]
(Unit conversion: mg/dl x 1.45 = ___ mmol/L)

Expected value:

Adults	6.3-8.3 g/dL
Children	
> 1 year	6.0-8.0 g/dL
< 1 year	4.6-7.6 g/dL

Home Work

- **Graph preparation: Produce two graphs**
  - ❑ Plot an abs vs. concentration graph using the values using a graph paper. (after drawing take a picture of the graph and paste it . *In exam you have to draw one by hand. So practice this.*
  - ❑ Plot an abs vs. concentration graph using the values using excel sheet (paste the excel generated graph)
- **Calculation:**
- Calculate the concentration of SAMPLE A from the standard curve, (can use Beer-Lambart law & y= mx+C)
- How to prepare dilution of the BSA?
- How would you interpret the result of SA?
- Any limitations you faced? How could you improve them?

- **Experiment 2**
- **Estimation of total protein in serum by enzymatic method using semi-automated Biochemistry Analyzer**
- **Calculation:**
- Calculate the total Protein concentration using the formula. Show your calculation here in details (Both in mg/dl & mmol/L).
- Does your result seem ok? If not, why? How could you have improved your result?

The End