Experiment No. 9: Estimation of Haemolymph Proteins.

Aim: To estimate the haemolymph proteins using Lowry's method.

Principle: The –CO-NH- bond (peptide) in polypeptide chain reacts with copper sulphate in an alkaline medium to give a blue colored complex. In addition, tyrosine and tryptophan residues of protein cause reduction of the phosphomolybdate and phosphotungstate components of the Folin-Ciocalteau reagent to give bluish products which contribute towards enhancing the sensitivity of this method.

Reagents Required:

1. **Reagent A:** 2% sodium carbonate in 0.1 N sodium hydroxide.
2. **Reagent B:** 0.5% copper sulphate ($CuSO_4.5H_2O$) in 1% potassium sodium tartarate. Prepare fresh by mixing stock solutions.
3. **Reagent C (Alkaline copper solution):** Mix 50 ml of reagent A and 1 ml of reagent B prior to use.
4. **Reagent D (Diluted Folin's reagent):** Dilute Folin-Ciocalteau reagent with an equal volume of 0.1 N NaOH
5. *Standard:* Dissolve 50 mg BSA in 50 ml of 0.1 N NaOH in a volumetric flask. Take 10ml of this stock standard and dilute to 50 ml in another flask for working standard solution. One ml of this solution contains 200 µg protein.
6. **Haemolymph Sample:** Dilute the haemolymph 250 times with distilled water containing 1mM thiourea.

Apparatus and Glass wares required: Test tubes, Pipettes, Colorimeter, *etc*.,

Procedure:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to the series of labeled test tubes.
2. Pipette out 1 ml of the sample in another test tube.
3. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of distilled water serves as the blank.
4. Now add 5 ml of reagent C to all the test tubes including the test tubes labeled 'blank' and 'sample' (diluted haemolymph).
5. Mix the contents of the tubes by vortexing / shaking the tubes and allow to stand for 10 min.

6. Then add 0.5 ml of reagent D rapidly with immediate mixing and incubate at room temperature in the dark for 30 min.
7. Now record the absorbance at 660 nm against blank.
8. Then plot the standard curve by taking concentration of protein along X-axis and absorbance at 660 nm along Y-axis. *For drawing the standard graph please refer last page figure 1.*
9. Now from this standard curve calculate the concentration of protein in the given sample.

Observations and Calculations:

Volume of standard BSA (ml)	Volume of distilled Water (ml)	Concentration of protein (µg)	Volume of reagent C (ml)	Incubate at room temperature for 10 min	Volume of reagent D (ml)	Incubate at dark room temperature for 30 min	A_{660}
0.0	1.0	00	5		0.5		0.00
0.2	0.8	40	5		0.5		
0.4	0.6	80	5		0.5		
0.6	0.4	120	5		0.5		
0.8	0.2	160	5		0.5		
1.0	0.0	200	5		0.5		
1.0 ml of sample	0.0	To be estimated			0.5		

Result: The given haemolymph sample contains ----µg protein/ml.
