University of Mysore

YUVARAJA'S COLLEGE

(Autonomous)

Mysuru - 570005

Graduate Course - Semester Scheme

SERICULTURE

A

E – BOOK

FOR

PAPER - DSC 4 LAB

PHYSIOLOGY OF MULBERRY AND SILKWORM

Editor

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Editor acknowledges all scientists who have pioneered and developed methods described in this e-book.

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CONTENTS

PART-A: PHYSIOLOGY OF MULBERRY

Experiment No. 1: Determination of Stomatal Index.

Introduction: Stomatal index is the measurement of the surface density of stomata. This parameter has been found useful in comparing leaves of different mulberry varieties as it plays very important role in photosynthesis and transpiration.

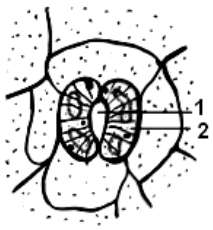
Aim: To determine stomatal index of the given mulberry variety.

Procedure: Select a matured mulberry leaf. Apply a thin layer of either gum or nail polish to the lower surface of the leaf and leave it for 10 min for drying. After complete drying peel the gum or nail polish layer carefully with the help of forceps without damaging the layer. Mount the peeled layer with a drop of diluted safranin and a drop of glycerin on a clean glass slide using cover glass and observe under a microscope.

Stomatal Index: Number of stomata present in a unit area of the leaf (may be as seen under microscope field or may be calculated using micrometers) in percentage. Count the number of stomata as well as number of epidermal cells in a given unit area and calculate the stomatal index using the following formula.

$$\text{Stomatal Index (\%)} = \frac{\text{Stomatal density} \times 100}{\text{Stomatal density + epidermal cell density}}$$

Epidermal Cells and Stomata Under Microscope



Stomata – Enlarged

1: Stomatal Pore; 2: Guard Cell

Report: The stomatal index of the given mulberry variety is ------ %.

Experiment No. 2: Kranz Anatomy.

Introduction: Kranz means wreath in German (A wreath is an assortment of flowers, leaves, fruits, twigs, cells or various materials that is constructed to resemble a ring). Kranz anatomy refers to an arrangement of bundle sheath cells surrounded by mesophyll cells in C_4 plants. This arrangement ensures that mesophyll cells are no more than 2-4 cell layers away from the bundle sheath cells. Hence, the transport of C_4 cycle metabolites is facilitated via plasmodesmata through these two cell types.

Plants are categorized as C_3 and C_4 plants based on the nature of carbon compounds formed during carbon dioxide fixation. Generally in plants like dicots *eg.*, in mulberry a three carbon compound phosphoglyceric acid (PGA) is formed as a first stable compound during CO_2 fixation and therefore such plants are called as C_3 plants. However, in some monocots *eg.*, sugarcane, a four carbon compounds like oxaloacetate, malate and aspartate are formed during CO_2 fixation. Therefore, these plants are called as C_4 plants.

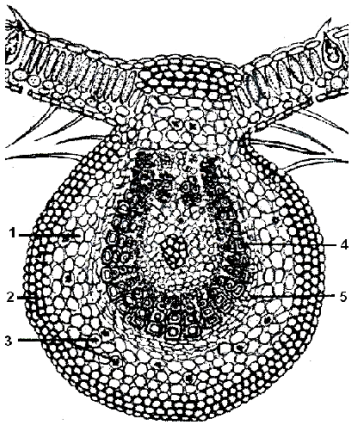
Aim: To study the Kranz anatomy.

Procedure: Prepare a thin transverse section of mulberry as well as sugar cane leaf and mount on a clean glass slide with a drop of glycerin and diluted Safranin. Observe under a microscope and list out the differences.

Differences between C_3 and C_4 plants

Sl. No.	C_3 Plants	C_4 Plants
1	Non Kranz type anatomy.	Kranz type anatomy *i.e.*, has a concentric arrangement of the bundle sheath and mesophyll layer, the bundle sheath is also thicker.
2	Bundle sheath cells are absent. Carbon fixation and Calvin cycle reactions occur in mesophyll cells only.	Bundle sheath cells are present and contain chloroplasts. Carbon is fixed in mesophyll cells and then transported to bundle sheath cells where Calvin cycle reactions occur.
3	A 3 carbon compound phosphoglyceric acid (PGA) is	Four carbon compounds like oxaloacetate, malate and aspartate are formed during CO_2

	formed as a first stable compound.	fixation as first stable compound
4	Ribulose 1,5 bisphosphate carboxylase (RuBisCo) is the CO_2 accepter.	Phosphoenolpyruvate (PEP) carboxylase is the CO_2 accepter, and is more efficient than RuBisCo.
5	Only C_3 pathway is present and involves in CO_2 fixation.	Both C_3 and C_4 pathways present and involves in CO_2 fixation. Hence they are called as "Efficient Plants"



Cross Section of C_3 Plant Leaf

1. Parenchyma
2. Collenchyma
3. Druses
4. Xylem
5. Phloem

Courtesy: Krishnaswamy *et al*. 1973. *Sericulture Manual 1*, FAO ASB, Rome.

Cross Section of C_4 Plant Leaf

1. Bundle Sheath
2. Bundle Sheath Cell
3. Mesophyll Cell

Experiment No. 3: Estimation of Mulberry Leaf Proteins.

Aim: To estimate the mulberry leaf protein by Biuret method.

Principle: The –CO-NH- bond (peptide) in polypeptide chain reacts with copper sulphate in an alkaline medium to give a purple colour which can be measured at 540 nm.

Reagents Required:

1. **Biuret Reagent:** Dissolve 3 g of copper sulphate ($CuSO_4.5H_2O$) and 9 g of sodium potassium tartarate in 500 ml of 0.2 mol/liter sodium hydroxide; add 5 g of potassium iodide and make up to 1 liter with 0.2 mol/liter sodium hydroxide.
2. **Protein Standard:** Dissolve 250 mg Bovine Serum Albumin (BSA) in 50 ml of 0.1 N NaOH in a volumetric flask. One ml of this solution contains 5 mg BSA.
3. **Mulberry Leaf Extract:** Prepare 0.5% homogenate in 0.1 N NaOH / Distilled water using mortar and pestle, centrifuge at 3000 rpm for 10 min, collect the clear supernatant and use as sample.

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 protein solution in to the series of labeled test tubes.
2. Pipette out 1 ml of the given sample (mulberry leaf extract) in another test tube.
3. Make up the volume to 1 ml in all the test tubes with distilled water. A tube with 1 ml of distilled water serves as the blank.
4. Now add 3 ml of Biuret reagent to all the test tubes including the test tubes labeled 'blank' and 'sample' (mulberry leaf homogenate).
5. Mix the contents of the tubes by vortexing / shaking the tubes and warm at 37 ºC for 10 min.
6. Now cool the contents and record the absorbance at 540 nm against blank.
7. Then plot the standard curve by taking concentration of protein along X-axis and absorbance at 540 nm along Y-axis.

   For drawing Standard Graph please refer last page figure 1.
8. Then from this standard curve calculate the concentration of protein in the given sample.

Observations and Calculation:

Volume of BSA solution (ml)	Volume of distilled water (ml)	Concentration of standard protein (mg)	Volume of biuret reagent (ml)	Incubate at 37ºC for 10 min & cool to room temperature	A_{540}
0.0	1.0	0.0	3		0.00
0.2	0.8	1	3		
0.4	0.6	2	3		
0.6	0.4	3	3		
0.8	0.2	4	3		
1.0	0.0	5	3		
1.0 ml of mulberry leaf extract	0.0	To be estimated	3		

Result: The given unknown sample contains ------ mg protein/ml.

Experiment No. 4: Separation of Photosynthetic Pigments by Paper Chromatography.

Aim: To separate photosynthetic pigments by paper chromatography.

Principle: The separation of photosynthetic pigments is based on partition takes place between the static phase adsorbed to the cellulose matter of the paper and mobile phase.

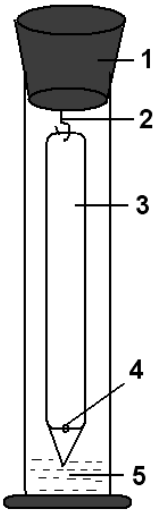
Materials Required: Mulberry leaves, pestle and mortar, 80% acetone, calcium carbonate, Buchner filter, beaker, measuring cylinder, glass jar/test tube with a tight cork, Whatman No.1 filter paper, petroleum ether, acetone, hook, micro pipette.

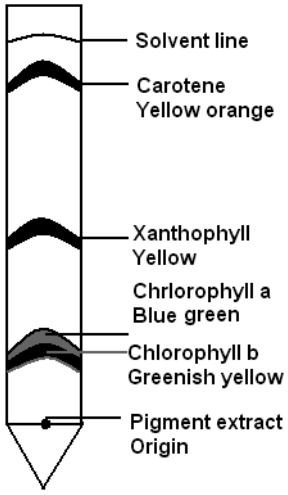
Procedure:

1. Take 50 g of fresh leaves in a pestle and mortar. Crush them with 20 ml of 80% acetone. Add a pinch of calcium carbonate and again crush.
2. Filter the extract on a Buchner filter or with double layered muslin cloth. The deep green coloured filtrate containing chlorophylls and carotenoids is obtained. Evaporate the extract to

concentrate.

3. Take a glass jar (about 45cm high) with a tight cork fitted in it. The cork should have a hole in the centre to fix the hook.
4. Now prepare the solvent by mixing 25 ml petroleum ether and 3 ml acetone. Pour the solvent into the jar and allow the jar to become saturated.
5. Cut a strip of filter paper of the size which can easily be hung on the hook. Apply a circular spot of pigment extract about 3cm from the base of strip with the help of a micropipette. Now hang the strip inside the jar to the hook of cork and close the cork. Care should be taken that the spot is not dipped in the solvent. Make the apparatus air tight and observe.





Experimental set up during running **Chromatogram**

1. Cork to minimize evaporation of solvent
2. Paper clip hook to hold the paper
3. Filter paper
4. Spot of pigment
5. Solvent

Observation:

The solvent will run on the filter paper. After few hours, the chloroplast pigments will be separated in the form of different spots on the paper. Take out the paper when the solvent reaches up to the upper level. After drying the paper, identify the different pigments with the help of their specific colours. Carotene is yellow, Xanthophyll is yellow-brown, Chlorophyll-a is blue green and Chlorophyll-b is olive green in colour.

Experiment No. 5: Extraction of Photosynthetic Pigments by Solvent Wash Method.

Aim: To separate photosynthetic pigments by solvent wash method.

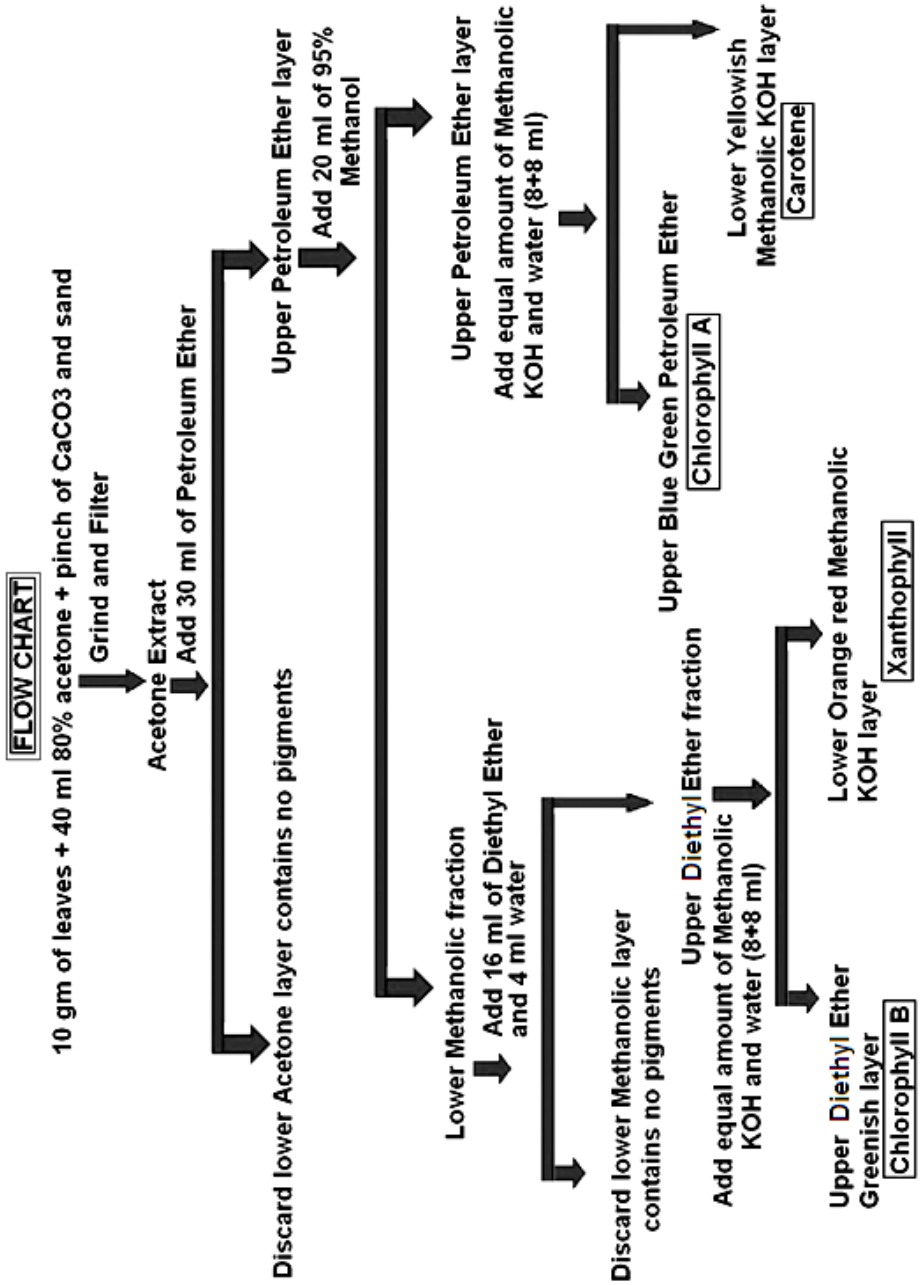
Principle: The separation of photosynthetic pigments is based on solubility of different pigments with specific solvents.

Materials Required: Mulberry leaves, pestle and mortar, 80% acetone, calcium carbonate, separating funnel, beaker, measuring cylinder, petroleum ether, acetone, diethyl ether, 30% methanolic KOH, 95% methanol, distilled water *etc*.,.

Procedure:

1. Take 10 g of fresh leaves in a pestle and mortar. Crush them with 40 ml of 80% acetone. Add a pinch of calcium carbonate, crush again and filter the extract on a Buchner filter or double layered muslin cloth.
2. Fill the homogenate in to separating funnel, add equal amount of petroleum ether, shake the contents gently and leave it for separation of acetone and petroleum ether layers. The upper petroleum ether layer contains all the pigments. Care should be taken to release the pressure built up in the separating funnel by opening the top lid.
3. Discard the lower acetone layer by opening the tap of separating funnel. Wash the petroleum ether layer with 20-30 ml of distilled water, discard lower water layer.
4. Now to the petroleum ether layer add 20 ml of 95% methanol mix the contents by shaking and leave it for separation of upper petroleum ether and lower methanol layers.
5. Collect the lower methanol layer and store it separately.
6. To the upper petroleum ether layer add 16 ml of 30 % methanolic KOH and 4 ml of distilled water shake gently; leave the contents for the separation of two layers. Collect the upper blue green petroleum ether layer and lower yellowish methanolic KOH layers separately as chlorophyll a and carotenes.
7. Now take methanol fraction (collected and stored at step 5) in separating funnel and add equal amount of diethyl ether (16 ml) as well as distilled water (4 ml). Mix the contents and discard lower methanol layer as it contains no pigments.

8. To the upper diethyl ether fraction add equal amount of methanolic KOH and distilled water (8+8 ml), mix gently and collect upper diethyl ether layer contains greenish chlorophyll b and lower methanolic KOH layer contains orange red xanthophylls.



Observation: After isolation identify the different pigments with the help of their specific colours as shown in the previous experiment.

Experiment No. 6: Determination of Water Potential of Potato Tubers.

Aim: To determine the water potential of potato tubers.

Principle: Water potential is the potential energy unit volume relative to pure water in reference conditions. The measure of the relative tendency of water to move from one area to another, and is commonly represented by the Greek letter Ψ (Psi).

Water potential (Ψ) is a measure of the driving force that governs the movement of water from the soil into plants and finally into the atmosphere. Water potential is the amount of energy per unit volume (or pressure) contained in a system (like a plant cell, tissue, or soil) and is expressed in units of megapascals (Mpa). For reference, pure water in a free standing solution has a water potential of zero, while most plant cells have a negative water potential. Water potential of a plant cell is made up of two important components, and the relationship among these components is expressed mathematically as:

$\Psi = \Psi s + \Psi p$

Ψ is the overall water potential of a cell.

Ψs is the solute or osmotic potential and represents the contribution made by dissolved solutes to Ψ.

Ψp is the pressure potential and represents the contribution made by pressure to Ψ.

Procedure:

1) Prepare the following sucrose solutions *i.e.*, 0.0 (distilled water), 0.2, 0.4, 0.6, 0.8 and 1 M sucrose.
2) Cut 6 cylinders from a potato and trim each cylinder to 4 cm in length with a knife.
3) Quickly blot the cylinders on paper towels to remove any excess moisture and weigh the cylinders. Record the weights in following table. After weighing, quickly transfer one cylinder to each beaker and ensure that they are fully immersed in the solution.
4) After 45 minutes, remove the cylinders, blot excess moisture with paper towels, and reweigh them, exactly as in step 3. Record the weights in following table.

Observations and Calculations:

Record the observations and calculate as shown in the following table. Then plot graph using the per cent change in weight at Y-axis and different concentration of sucrose on X-axis. Using this graph determine the exact concentration of sucrose that would cause no change in weight in the potato tubers. The water potential of this solution will equal the water potential of the potato tissue. *For drawing the graph please refer last page figure 2.*

Table: Weight change in potato tissues in sucrose solutions of different concentration.

	Sucrose					
	0 M	0.2M	0.4M	0.6M	0.8M	1.0M
Initial weight						
Final weight						
Difference in weight						
Difference in %						

In an open solution where there is no turgor pressure, the Ψp is equal to zero. Thus, the Ψ of such a solution is equal to the Ψs of a solution. Calculate the Ψs of the solution causing no change in weight of the potato tissues using the following formula:

Ψs = -miRT
m = molarity

i = ionization constant *i.e.*, 1 for sucrose

R = gas constant *i.e.*, 8.31 J K^{-1} mol^{-1}

T = room temperature in K (ºC + 273 = K)

(For example [-0.4 x 1 x 8.31 x {25+273}] = -990.55)

Report: Water potential of the given potato tuber cells is ____ Mpa

Experiment No. 7: Estimation of Moisture Per Cent Age and Moisture Retention Capacity of Mulberry Leaf.

Aim: To determine the moisture per cent age and moisture retention capacity of the given mulberry varieties.

Principle: Water is one of the important constituents of mulberry leaves and it plays an important role in silkworm crop production. Moisture content varies with variety, season, age of the plant and preservation techniques followed. It is usually expressed in terms of per cent age.

Procedure: Collect the mulberry leaf samples from garden by plucking the lateral branches as a whole. Separate the individual types of leaves as tender (for chawki worms) and matured (for late age worms) leaves. Record the initial weight of the leaves (W_1) and preserve them under standard conditions for 12 or 24 hours. After this preservation, record the second weight of leaves (W_2). After 12 or 24 hours of preservation, keep the same mulberry leaves in an oven at 100 ^oC for 2-3 hours or until complete drying of mulberry leaves. After drying, record the weight of dried mulberry leaves (W_3) as final weight. From this observations calculate the per cent age of water in mulberry leaves, per cent age of water loss and moisture retention capacity of the different mulberry varieties using the following formulae.

Observations and Calculations

Mulberry variety	Fresh weight (W_1)	Second weight (W_2)	Final weight (W_3)	Moisture %	Moisture loss %	Moisture retention capacity %
M_5 Tender						
M_5 Matured						
Mysore Local Tender						
Mysore Local Matured						

$$\text{Moisture \%} = \frac{W_1 - W_3 \text{ X } 100}{W_1}$$

$$\text{Moisture loss \%} = \frac{W_1 - W_2 \times 100}{W_1}$$

W_1 – Fresh weight of leaves

W_2 – Second weight of leaves

W_3 – Dried weight of leaves

Moisture Retention Capacity = 100 – Moisture loss %

**Fresh weight is considered as 100%*

Experiment No. 8: Hill Reaction.

Aim: To study the Hill reaction.

Principle: One of the first steps of photosynthesis is the photolysis of water to donate two electrons to the reaction center, P680. This reaction is known as the Hill reaction. The electrons donated to P680 move through an electron transport chain to the reaction center P700, and eventually, to reduce NADP to NADPH. The complete reaction can be summarized as follows:

$$H_2O + NADP \xrightarrow[\text{Chloroplast}]{\text{Light}} NADPH + 1/2\,O_2 + H^+$$

Other electron acceptors can be substituted for NADP, which allow the Hill reaction to be measured, and some of its components studied. The most commonly used electron acceptor is the dye 2,6 dichlorophenol indophenol (DCIP), which can accept electrons instead of P700. When DCIP is added to a chloroplast or thylakoid suspension, the following reaction occurs:

$$H_2O + DCIP \xrightarrow[\text{Photosystem II}]{\text{Light}} DCIP\ (\text{reduced}) + 1/2\,O_2$$

DCIP is a blue color in its oxidized form. When reduced, it is colorless. This property of DCIP allows the measurement of the rate of the Hill reaction.

Reagents Required:

1. **Potassium phosphate buffer**: 125 mM pH 7.5 with 0.3 M sucrose.
2. **DCIP**: 30 µg/ml DCIP in distilled water.

Procedure: Prepare 25% homogenate of fresh Spinach leaves in potassium phosphate buffer with 0.3M sucrose, filter and centrifuge the filtrate at 3000 rpm for 5 min. Re-suspend the chloroplast pellet in 10 ml of buffer and store on ice. In a test tube take 1.9 ml water, 3 ml buffer, 0.5 ml chloroplast suspension and 0. 1 ml DCIP, mix and incubate in sunlight for 1 hour. A suitable control should also be prepared and incubate in dark. Prepare a standard curve using different concentrations of DCIP on X-axis against absorption at 550 nm on Y-axis.

For drawing Standard Graph please refer last page figure 1.

Then from this standard curve calculate the amount of DCIP reduced.

Standard Curve:

Sl. No.	Water (ml)	DCIP (ml)	Concentration of DCIP (µg)	A_{550}
1	5.5	0.0	0	0.00
2	5.3	0.2	6	
3	5.1	0.4	12	
4	4.9	0.6	18	
5	4.7	0.8	24	
6	4.5	1.0	30	

Test:

Experiment	Water (ml)	Buffer (ml)	Chloroplasts (ml)	DCIP (ml)	A_{550} against water
T-1 Incubated in dark	1.9	3	0.5	0.1	
T-2 Incubated in sunlight	1.9	3	0.5	0.1	

Calculation:

Amount of DCIP reduced = O D of T-1 – O D of T-2 = ……..

Report: The given sample showed ------- µmole of DCIP reduced per hour.

PART-B: PHYSIOLOGY OF SILKWORM

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.

Experiment No. 10: Estimation of Haemolymph Glucose Level.

Aim: To estimate haemolymph glucose level by DNS method.

Principle: Several reagents have been employed which assay sugars by using their reducing properties. One such compound is 3, 5- dinitrosalicylic acid (DNS) which in alkaline solution is reduced to 3-amino 5-nitrosalicylic acid.

Reagents Required:

1. **Sodium potassium tartarate:** Dissolve 300 gm of this salt in about 500 ml of water.
2. **3, 5-dinitrosalicylic acid:** dissolve 10 gm of this powder in 200 ml of 2 mol/lt sodium hydroxide.
3. **Dinitrosalicylic acid reagent:** Prepare this fresh by mixing solutions 1 and 2 and make up to 1 liter with water.
4. **Sodium hydroxide:** 2 mol/liter
5. **Stock glucose standard:** 1gm/liter solution in saturated benzoic acid.
6. **Working sugar standard:** Dilute stock standard solution in 1:1 ratio with distilled water to get 500 µg/ml glucose.

Preparation of protein free filtrate: To 1 ml haemolymph sample, add 8 ml distilled water, 0.5 ml of 2/3 N sulfuric acid and 0.5 ml of 10% sodium tungstate solution in a stoppered centrifuge tube and mix the contents. Then centrifuge at 3000 rpm for 10 min and collect the supernatant as sample.

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 given haemolymph sample (protein free filtrate) 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 0.5 ml of DNS to all the test tubes including the test tubes labeled 'blank' and 'sample'.
5. Mix the contents of the tubes by vortexing / shaking the tubes and incubate for 10 min in a boiling water bath and cool to room temperature.
6. Then to the cooled test tubes add 2.5 ml of distilled water mix the contents and record the absorbance at 540 nm against blank.
7. Plot the standard curve by taking concentration of glucose along X-axis and absorbance at 540 nm along Y-axis. *For drawing the standard graph please refer last page figure 1.*

Now from this standard curve calculate the concentration of glucose in the given sample.

Observations and Calculations

Volume of standard glucose (500 µg/ml)	Volume of distilled water (ml)	Concentration of glucose (µg)	Volume of DNS reagent (ml)	Incubate for 10 min in a boiling water bath and cool	Volume of distilled water (ml)	A_{540}
0.0	1.0	000	0.5		2.5	0.00
0.2	0.8	100	0.5		2.5	
0.4	0.6	200	0.5		2.5	
0.6	0.4	300	0.5		2.5	
0.8	0.2	400	0.5		2.5	
1.0	0.0	500	0.5		2.5	
1.0 sample	0.0	To be estimated	0.5		2.5	

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

Experiment No. 11: Preparation of Silkworm Haemocytes.

Aim: To prepare the silkworm haemocytes.

Reagents Required:

1. **Phosphate-buffered saline with 10% formalin:** 10mM Na_2HPO_4, 138mM NaCl, and 2.7mM KCl, pH 7.4 containing 10% formalin and 1mM thiourea.
2. **Lishman's Stain:** Dissolve 0.6 g Leishman's stain powder in 400 ml Methanol. Or ready solution may be used.

Procedure:

1. Collect the haemolymph from 5^{th} instar silkworm larvae in a clean pre cooled micro centrifuge tube by puncturing the caudal horn.
2. Then, immediately mix 200 µl of haemolymph with one ml of phosphate buffered saline and leave it for 5 min at room temperature.
3. Now, centrifuge the above preparation at 1000 rpm for 3 min.
4. After centrifugation remove the supernatant with a micro pipette carefully, re-suspend the palette with 50 µl of phosphate buffered saline, drop on a clean glass slide.
5. Air dry the preparation, stain with Lishman's stain for 2 min, wash excess stain in running distilled water, air dry and observe under a microscope with a magnification of 450 X.

Observation:

Observe the haemocytes and record the results as number of different types with reference to the following photograph.

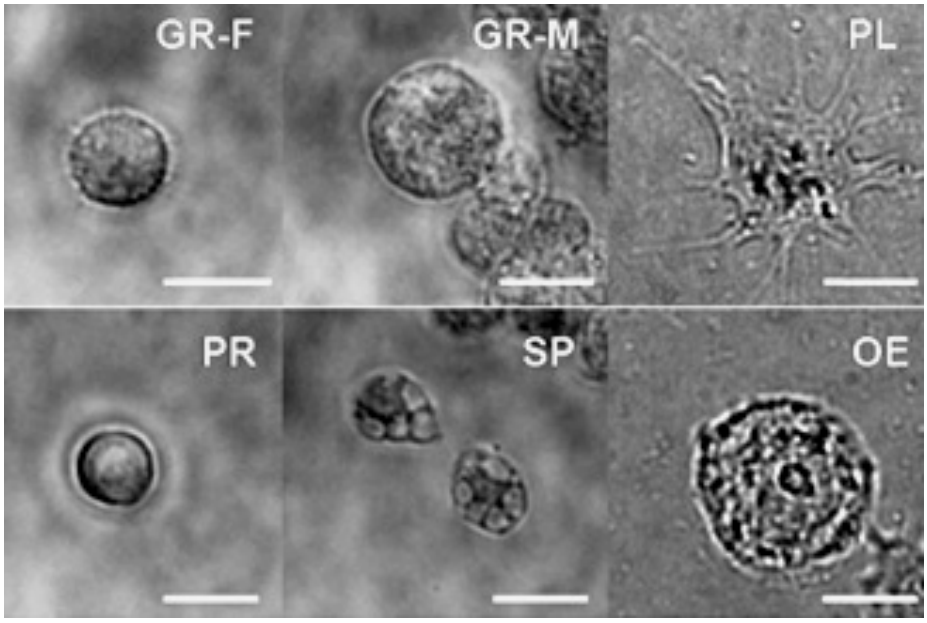


Figure:

GR-F: Granulocyte in the feeding phase.
GR-M: Granulocyte in the molting phase.
PL: Plasmatocyte.
PR: Prohemocyte.
SP: Spherulocyte.
OE: Oenocytoid.

Courtesy: Takashi *et al.* 2008. *J. Insect Physiology* (54): 454–461.

Experiment No 12. Estimation of Amylase Activity in Haemolymph of Bivoltine and Multivoltine Races.

Aim: To estimate the amylase activity in haemolymph of bivoltine and multivoltine silkworm strains.

Principle: When amylase acts on starch, it is converted in to glucose units. The resultant glucose units react with 3, 5-dinitrosalicylic acid (DNS) in alkaline solution to give rise to orange coloured complex, which can be measured at 540 nm.

Reagents Required:

1. **Phosphate buffer 0.1 M pH 7.8:**
   a. 0.1 M KH_2PO_4: Dissolve 1.360 g of KH_2PO_4 in 100 ml of distilled water.
   b. 0.1 M K_2HPO_4: Dissolve 1.7418 g of K_2HPO_4 in 100 ml of distilled water.

Mix the solutions a and b at 1:1 ratio and adjust the pH 7.8.

2. **Haemolymph Sample:** Dilute 100 µl of silkworm haemolymph with 1900 µl phosphate buffer containing 1mM thiourea.
3. **Substrate (1% starch):** Dissolve 1 g soluble starch in 90 ml of distilled water and boil to get clear solution. Make up to 100 ml with water.
4. **DNS reagent:** Please refer experiment number 11.

Procedure for Standard Curve: For preparation of standard curve please refer experiment number 11 or same standard curve may used here for the estimation of amylase activity.

Procedure for Experiment:

1. **Blank:** Take 2 ml of phosphate buffer, 0.5 ml of substrate and 0.5 ml of inactivated haemolymph sample or distilled water in a clean dry test tube.
2. **Test 1:** Take 2 ml of phosphate buffer, 0.5 ml of substrate and 0.5 ml of multivoltine haemolymph samples in a clean dry test tube.
3. **Test 2:** Take 2 ml of phosphate buffer, 0.5 substrate and 0.5 ml of bivoltine haemolymph samples in a clean dry test tube.
4. Mix the contents of the tubes by vortexing / shaking the tubes and incubate for 30 min at 37°C.
5. Now add 0.5 ml of DNS to all the test tubes, mix the contents of the tubes by vortexing / shaking the tubes and incubate for 10 min in a boiling water bath and cool to room temperature.
6. Then to the cooled test tubes add 0.5 ml of distilled water, mix the contents and record the absorbance at 540 nm against blank.

Observations and Calculations

	Buffer (ml)	Substrate (ml)	Enzyme sample (ml)	Mix, incubate at 37°C for 30’	DNS (ml)	Incubate for 10 min in a boiling water bath and cool	D W (ml)	A_{540}
Blank	2	0.5	0.5 IE*		0.5		0.5	0.00
Test 1 (MV)	2	0.5	0.5		0.5		0.5	
Test 2 (BV)	2	0.5	0.5		0.5		0.5	

*IE - Inactivated Enzyme

Amylase activity = Standard Curve Value X 60 X Dilution Factor (20)
Sample taken (ml) X Time of incubation (min)

= -----µg of glucose released/ml of haemolymph sample/hour at 37°C

Result: The given multivoltine and bivoltine haemolymph samples shown ----- and ----- µg /ml / hour at 37°C respectively.

Experiment No 13. Estimation of Succinate Dehydrogenase Activity in the Eggs/ Tissue.

Aim: To estimate succinate dehydrogenase activity in silkworm eggs.

Principle: Succinate dehydrogenase is one of the mitochondrial enzymes, which catalyzes the conversion of succinate to fumerate. In this reaction FAD reduces to $FADH_2$. In *in vitro*, the lemon yellow colored INT accepts electrons and becomes red coloured farmazan which can be measured at 495 nm.

Reagents Required:

1. **Sodium phosphate buffer (0.1M, pH 7.4):** Mix 16 ml (0.2 M) of monobasic and 84 ml (0.2 M) of dibasic and makeup to 200 ml with distilled water.
2. **INT** [2(4-iodophenyl)-3(4-nitrophenyl)-5-Phenyltetrazolium chloride]: 1mg/ml in distilled water.
3. **Sodium succinate:** 15mM
4. **Glacial acetic acid, Toluene *etc.*,**
5. **Sample:** Prepare 0.2% egg (5 days old or more) homogenate in cold phosphate buffer/ distilled water using mortar and pestle. Centrifuge the homogenate at 3000 rpm for 10 min, collect the clear supernatant and use as sample.

Procedure for Standard Curve:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of INT in to the series of labeled test tubes.
2. Make up the volume to 1 ml in all the test tubes. A tube with 1 ml of distilled water serves as the blank.
3. Now add 1 ml of buffer, 1 ml of sodium succinate and 1 ml of sample (egg homogenate) to all the test tubes including the test tubes labeled 'blank' and 'test'.
4. Mix the contents of the tubes by vortexing / shaking the tubes and incubate at 37°C for 24 h or until complete reduction of INT.
5. Now add 6 ml of glacial acetic acid to stop the reaction.
6. Then add 6 ml of toluene, mix and keep them in a refrigerator for separation of toluene layer.
7. Now collect the upper red coloured toluene layer containing farmazan in to a tube, cool to room temperature and record the absorbance at 495 nm against blank.
8. Then plot the standard curve by taking concentration of farmazan (equivalent to INT) along X-axis and absorbance at 495 nm along Y-axis.

   For drawing the standard graph please refer last page figure 1.

Procedure for Test:

7. **Blank:** Take 1 ml of phosphate buffer, 1 ml of sodium succinate, 1 ml of INT and 1 ml of inactivated enzyme sample (distilled water may be used) in a clean dry test tube, and incubate at 37°C for one hour.
8. **Test:** Take 1 ml of phosphate buffer, 1 ml of sodium succinate, 1 ml of INT and 1 ml of enzyme sample in a clean dry test tube, and incubate at 37°C for one hour.

After incubation add 6 ml of glacial acetic acid to both blank as well as test to stop the reaction. Then add 6 ml of toluene to each tube, mix and keep them in a refrigerator to separate the red farmazan. After separation, collect the upper red coloured toluene layer containing farmazan in to a cuvette and record the absorbance at 495 nm against blank.

Observations and Calculations:

Standard Curve:

INT (ml)	H_2O (ml)	Buffer (ml)	Succinate (ml)	Sample (ml)	Mix, incubate at 37ºC for 24 h.	Acetic acid (ml)	Toluene (ml)	Mix & keep in a fridge for 24 h. Then collect the upper layer	A_{495}
0.0	1.0	1	1	1		6	6		
0.2	0.8	1	1	1		6	6		
0.4	0.6	1	1	1		6	6		
0.6	0.4	1	1	1		6	6		
0.8	0.2	1	1	1		6	6		
1.0	0.0	1	1	1		6	6		

Test:

Ex.	INT (ml)	Buffer (ml)	Succinate (ml)	Sample (ml)	Mix & incubate at 37ºC for 1hr	Acetic acid (ml)	Toluene (ml)	Collect the farmazan as above	A_{495}
B	1	1	1	1		6	6		0.00
T	1	1	1	1		6	6		

B - Blank

T - Test

Optical density of the test: ---

SDH activity level = $\frac{\text{Standard Curve Value X 60}}{\text{Tissue taken (mg) X Incubation time (min)}}$

= ----- µg of farmazan formed per hour per mg at 37°C.

Report: The SDH activity in the given sample is ----- µg of farmazan formed per hour per mg tissue at 37°C.

Experiment No 14. Estimation of Glycogen in Silkworm Fat Body/Tissue.

Aim: To estimate the amount of glycogen.

Principle: Glycogen is released from the tissue by heating with strong alkali and precipitated by the addition of ethanol. Sodium sulphate is added as co-precipitant to give a quantitative yield of glycogen.

Simple sugars, oligosaccharides, polysaccharides, and their derivatives, give an orange yellow color when treated with phenol and concentrated sulfuric acid, which can be read at 490 nm.

Requirements:

1. **30% KOH.**
2. **Ethyl alcohol:** 95% ethanol
3. **2 N H_2SO_4**
4. **Glycogen Standard:** 100µg/ml in double distilled water.
5. **Phenol:** 5% solution.
6. **H_2SO_4:** 96-98%.

Procedure:

Isolation of Glycogen:

1. Place 1 g of freshly collected silkworm tissue into a calibrated stoppered centrifuge tube containing 2 ml of 30% KOH and heat in a boiling water bath for 20 min with occasional shaking.
2. Cool the tubes in ice, add 0.2 ml of saturated Na_2SO_4, and mix thoroughly.
3. Precipitate the glycogen by adding 5 ml of 95% ethanol, stand on ice for 5 min, and centrifuge.
4. Discard the supernatant and dissolve the precipitated glycogen in about 5 ml of water with gentle warming, then dilute with distilled water to the 10 ml calibration mark and mix thoroughly. One ml of this sample is equivalent to 100 mg of tissue.

Estimation of glycogen:

1. Pipette out 0.0, 0.2, 0.4, 0.6, 0.8 and 1 ml of glycogen standard in to the series of labeled test tubes.
2. Pipette out 1 ml of isolated glycogen 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 1 ml of phenol. Then rapidly pipette out 5 ml of H_2SO_4 to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
5. Mix the contents and incubate on ice bath for 30 min.
6. Then record the absorbance at 490 nm against blank.
7. Then plot the standard curve by taking concentration of glycogen standard along X-axis and absorbance at 490 nm along Y-axis.
8. Then from this standard curve calculate the concentration of glycogen in the given test sample.

Observations and Calculations:

Volume of standard glycogen (ml)	Volume of distilled water (ml)	Concentration of glycogen (ml)	Volume of phenol (ml)	Volume of H_2SO_4	Incubate on Ice bath for 30 min	A_{490}
0.0	1.0	00	1	5		0.00
0.2	0.8	20	1	5		
0.4	0.6	40	1	5		
0.6	0.4	60	1	5		
0.8	0.2	80	1	5		
1.0	0.0	100	1	5		
1.0 ml sample	0.0	To be estimated	1	5		

Amount of glycogen silkworm litter = $\frac{\text{Standard Curve Value}}{\text{Silkworm Tissue taken (mg)}}$

= -------µg of glycogen/mg tissue

Result: The given unknown sample contains ----µg glycogen /mg tissue.

Experiment No 15. Estimation of Uric Acid in Silkworm Litter.

Aim: To estimate the amount of uric acid.

Principle: The uric acid reduces phosphotungstic acid in the presence of sodium carbonate to blue coloured complex. The concentration of uric acid is directly proportional to intensity of colour, which can be read at 700 nm.

Requirements:

1. **Tungstic acid:** Mix 25 ml of 10% sodium tungstate, 25 ml of 2/3 N H_2SO_4 and drop of phosphoric acid, make up to 400 ml with distilled water. Store in a brown bottle.
2. **Phosphotungstic acid (stock) solution:** Dissolve 50 g of sodium tungstate in about 400 ml of water. Add 40 ml of 85% phosphoric acid and reflux gently for 2 hours. Cool, transfer to 500 ml flask and make up to the mark with water. Keep this in a brown bottle
3. **Dilute solution for use:** Dilute 10 ml of the stock solution to 100 ml with water and store in a brown bottle.
4. **Sodium carbonate solution (10%):** Dissolve 10 grams sodium carbonate in 100 ml of distilled water.
5. **Uric acid standard solution:** Dissolve 60 mg of lithium carbonate in 15-20 ml of water in a test tube. Heat the solution to 60 °C and pour on to 100 mg of uric acid taken in a small beaker. Stir until dissolved, heat further if necessary. Add 2 ml of 40% formalin and then slowly with shaking add 1 ml of 50% acetic acid. Make up to volume 100 ml and store in brown bottle.
6. **Uric acid working standard:** Dilute 1 ml of the stock to 200 ml with water. Store in a brown bottle. This contains 0.05 mg uric acid per ml.

Preparation of protein free filtrate: Take 100 mg of silkworm litter, wash with 10 ml petroleum ether followed by 10 ml of acetone to remove green pigments and to the residue, add 10 ml of tungstic acid reagent in a stoppered centrifuge tube and homogenize the contents. Then centrifuge at 3000 rpm for 10 min and collect the supernatant as sample. 5 ml of supernatant is equivalent to 10 mg of litter.

Procedure:

9. Pipette out 0.0, 1, 2, 3, 4 and 5 ml of working uric acid standard in to the series of labeled test tubes.
10. Pipette out 5 ml of the given sample/ protein free filtrate in another test tube.
11. Make up the volume to 5 ml in all the test tubes. A tube with 5 ml of distilled water serves as the blank.
12. Now add 1 ml of sodium carbonate solution and 1 ml of dilute phosphotungstic acid solution to all the test tubes including the test tubes labeled 'blank' and 'unknown'.
13. Mix the contents of the tubes by vortexing / shaking the tubes and incubate for 3 min at 25°C in a water bath.
14. Then record the absorbance at 700 nm against blank.
15. Then plot the standard curve by taking concentration of uric acid along X-axis and absorbance at 700 nm along Y-axis.
16. Then from this standard curve calculate the concentration of uric acid in the given sample.

Observations and Calculations:

Volume of standard uric acid (ml)	Volume of distilled water (ml)	Concentration of uric acid (µg)	Volume of sodium carbonate solution (ml)	Volume of phosphotungstic acid solution (ml)	A_{700}
0	5	0	1	1	0.00
1	4	5	1	1	
2	3	10	1	1	
3	2	15	1	1	
4	1	20	1	1	
5	0	25	1	1	
5 ml of sample	0	To be estimated	1	1	

Amount of uric acid in silkworm litter = $\frac{\text{Standard Curve Value}}{\text{Silkworm Litter taken (mg)}}$

= -------µg of uric acid/mg litter

Result: The given unknown sample contains ----µg uric acid/mg litter.

Figure 1: Model Standard Graph

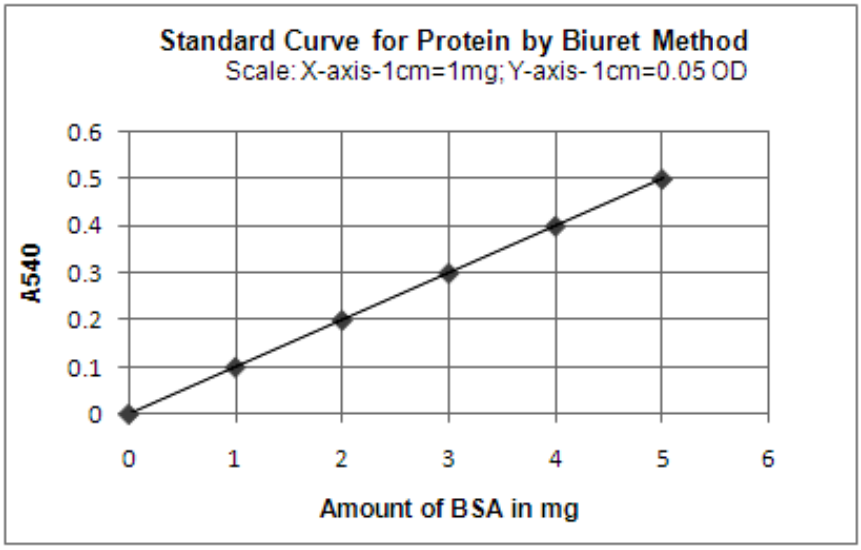
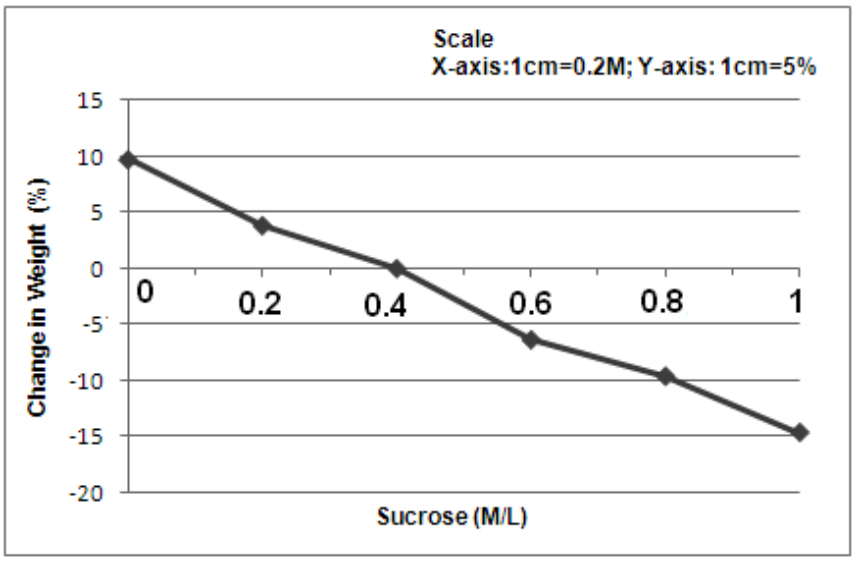


Figure 2: Model Graph Showing Water Potential of Tissue



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