

VARIOUS METHOD OF GLUCOSE ESTIMATION , GTT AND PRINCIPAL OF CARBOHYDRATES CHEMISTRY TEST

Subject : Biochemistry

Student name :

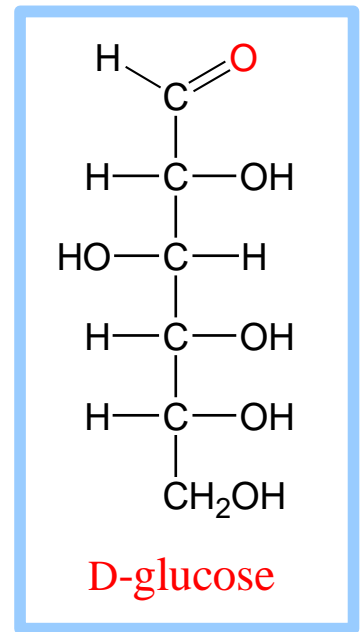
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Content :

- Introduction
- Entry of glucose into cell
- Blood collection
- Method for glucose estimation
 - 1) Enzymatic method
 - 2) Chemical method
- Estimation of glucose in urine sample
- Estimation of glucose in CSF sample
- Normal values
- GTT
- Principle of carbohydrate chemistry

INTRODUCTION

- Glucose is a monosaccharide.
- It is central molecule in carbohydrate metabolism.
- Stored as glycogen in liver and skeletal muscle.



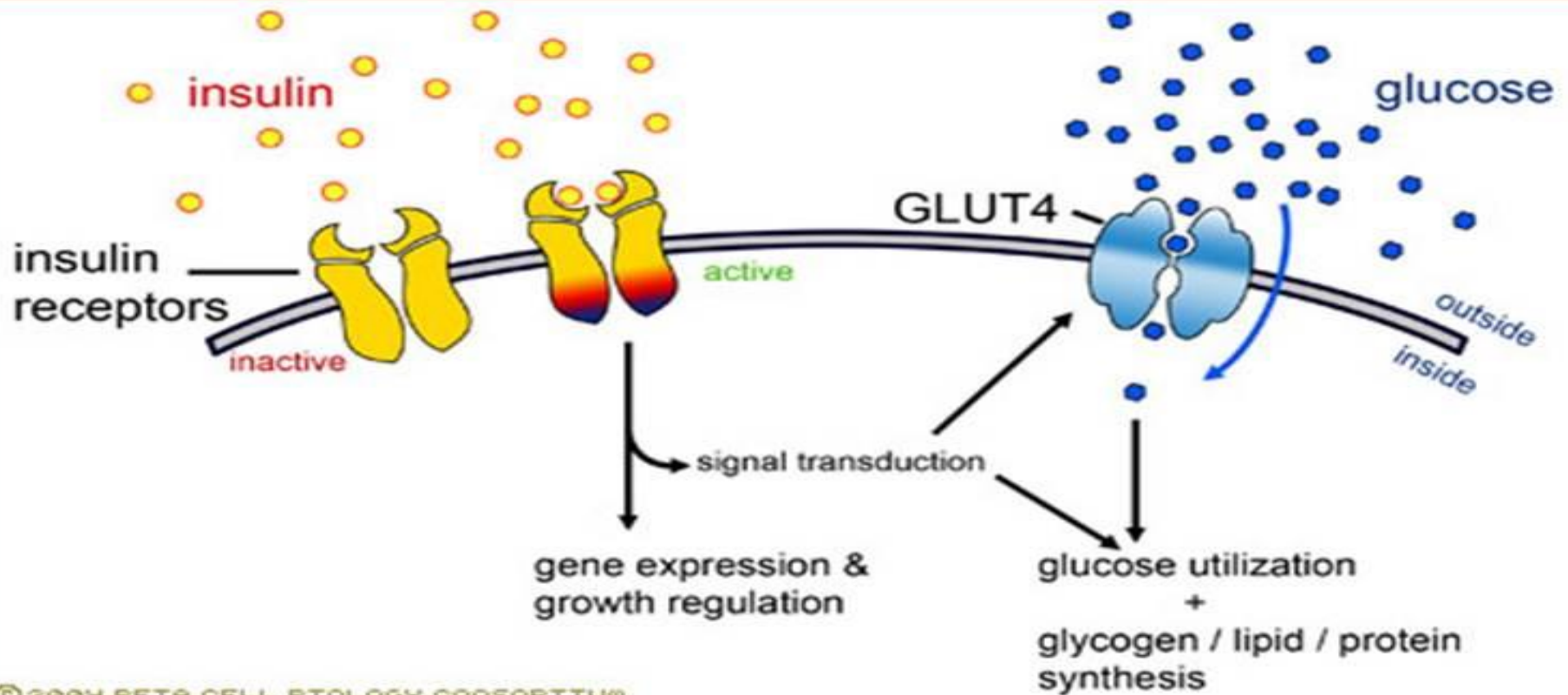
Entry of glucose into the cell

Two specific transport system are used :

- Insulin –independent transport system:
- Carrier mediated uptake of glucose
- Not dependent on insulin.
- Present in hepatocytes, erythrocytes & brain.
- Insulin dependent transport system :
- Present in Skeletal muscle.

ENTRY OF GLUCOSE INTO CELL :

Insulin - dependent GLUT 4 –mediated



- Insulin/GLUT4 is not only pathway.
- cellular uptake of glucose into muscle and adipose tissue (40%).

Insulin – independent glucose disposal (60%)

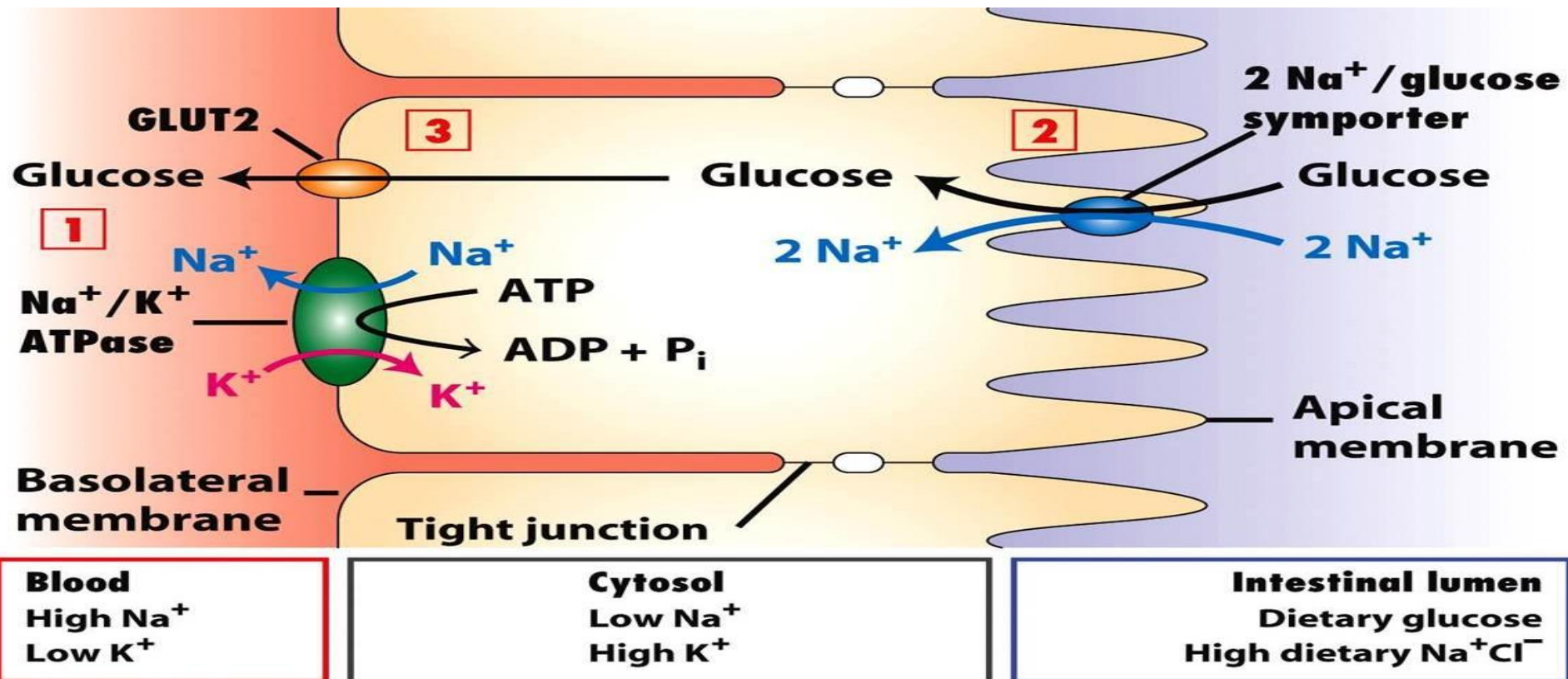
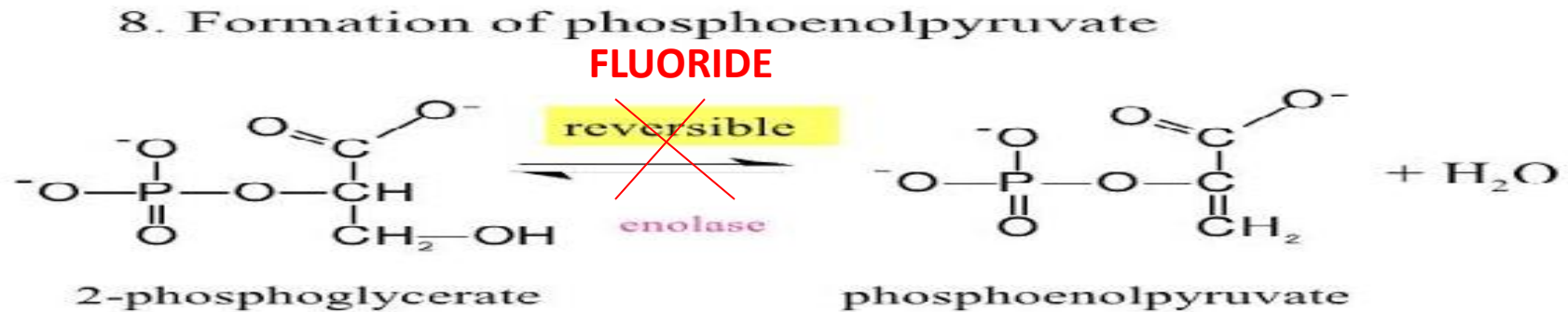


Figure 11-29
Molecular Cell Biology, Sixth Edition

- GLUT 1 -3 in the Brain, Placenta, Kidney
- SGLT 1 and 2 (sodium glucose symporter)
- intestinal epithelium, kidney.

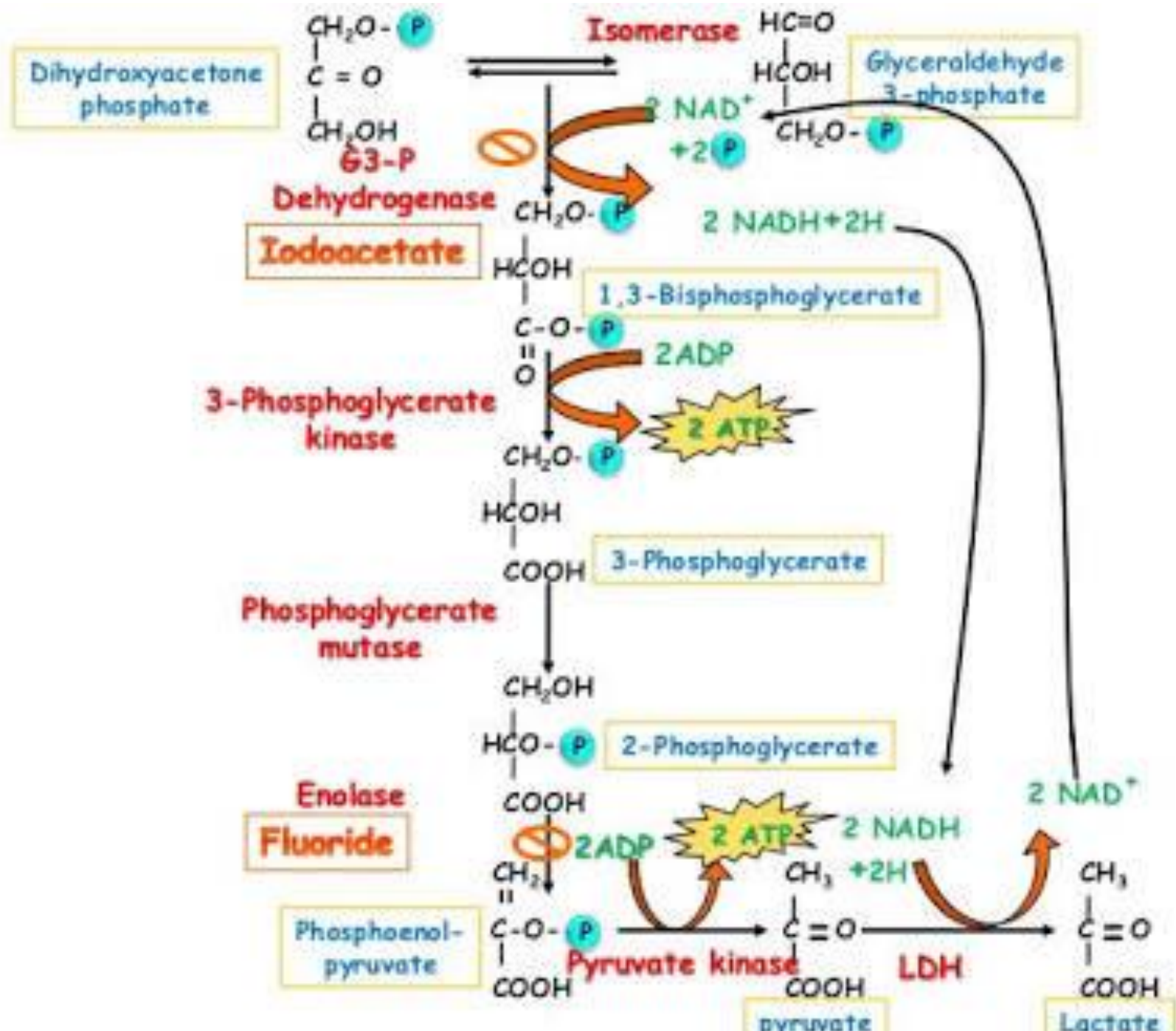
Blood collection for glucose estimation :

- Fluoride containing vials are used.
- Fluoride inhibit glycolysis by inhibiting enolase enzyme.
- 2-phosphoglycerate is converted into phosphoenol pyruvate by enzyme enolase by removing one water molecule.



- Fluoride irreversibly inhibit enolase there by stop the whole glycolysis.
- Therefor, fluoride is added to blood during estimation of blood sugar.

- 5
- 6
- 7
- 8
- 9
- 10



Normal ranges :

[Reference: American Diabetes Association(ADA)]

Random blood glucose test :

- It is a blood sugar test taken from a non-fasting subject.
- Normal range is 79-160 mg/dl.

Fasting blood glucose :

- A determination of blood glucose level after an 8 hour period of fasting.
- Normal range is 70 – 100 mg/dl.
- Levels between 100 – 126 mg/dl are referred to as impaired fasting glucose or pre-diabetes.
- Diabetes is typically diagnosed when fasting blood glucose levels are 126 mg/dl or higher.

Postprandial blood glucose test :

- It is determination of the glucose in the blood after a meal.
- Normal range is under 140mg/dl.
- High value indicate diabetes.

Glycosylated hemoglobin :

- hemoglobin to which glucose is bound.
- Also known as glycohemoglobin or as hemoglobin A1C.
- Tested for to monitor long-term control of diabetes mellitus.
- Its level increase in of person with poorly controlled diabetes mellitus.
- Since glucose stay attached to hemoglobin up to life of RBC (120 days).
- It reflect the average blood glucose level over past 3 month.

Ranges of glycosylated hemoglobin :

- Normal range is between 4 to 5.7%.
- Hemoglobin A1C between 5.7 to 6.4 % mean you have a greater change of getting of diabetes.
- Level of 6.5% or higher means you have diabetes.

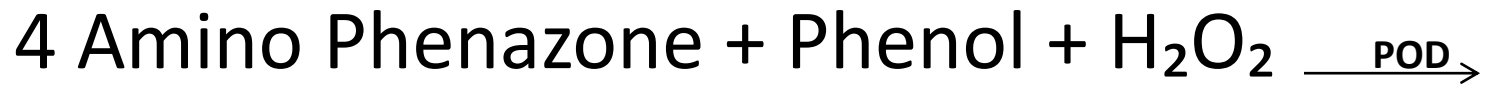
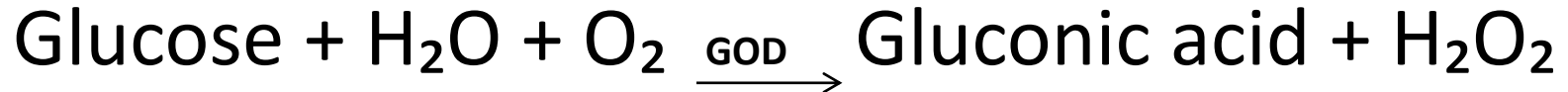
Enzymatic determination of glucose in blood

- 1) Glucose oxidase – peroxidase method (GOD
POD method)
- 2) hexokinase method (HKS method)
- 3) Glucose dehydrogenase method (GDH
method)

GOD POD METHOD

(glucose oxidase – peroxidase method)

PRINCIPLE:



Quinonimine – Pink colour compound

- Intensity is determined at on 505 nm filter.

Procedure :

	TEST	STAN.	BLANK
1)Glucose reagent (ml)	1.0	1.0	1.0
2)Serum(ml)	0.01	---	----
3)Glucose standard(ml)	---	0.01	----
4)Distilled water(ml)	---	----	0.01

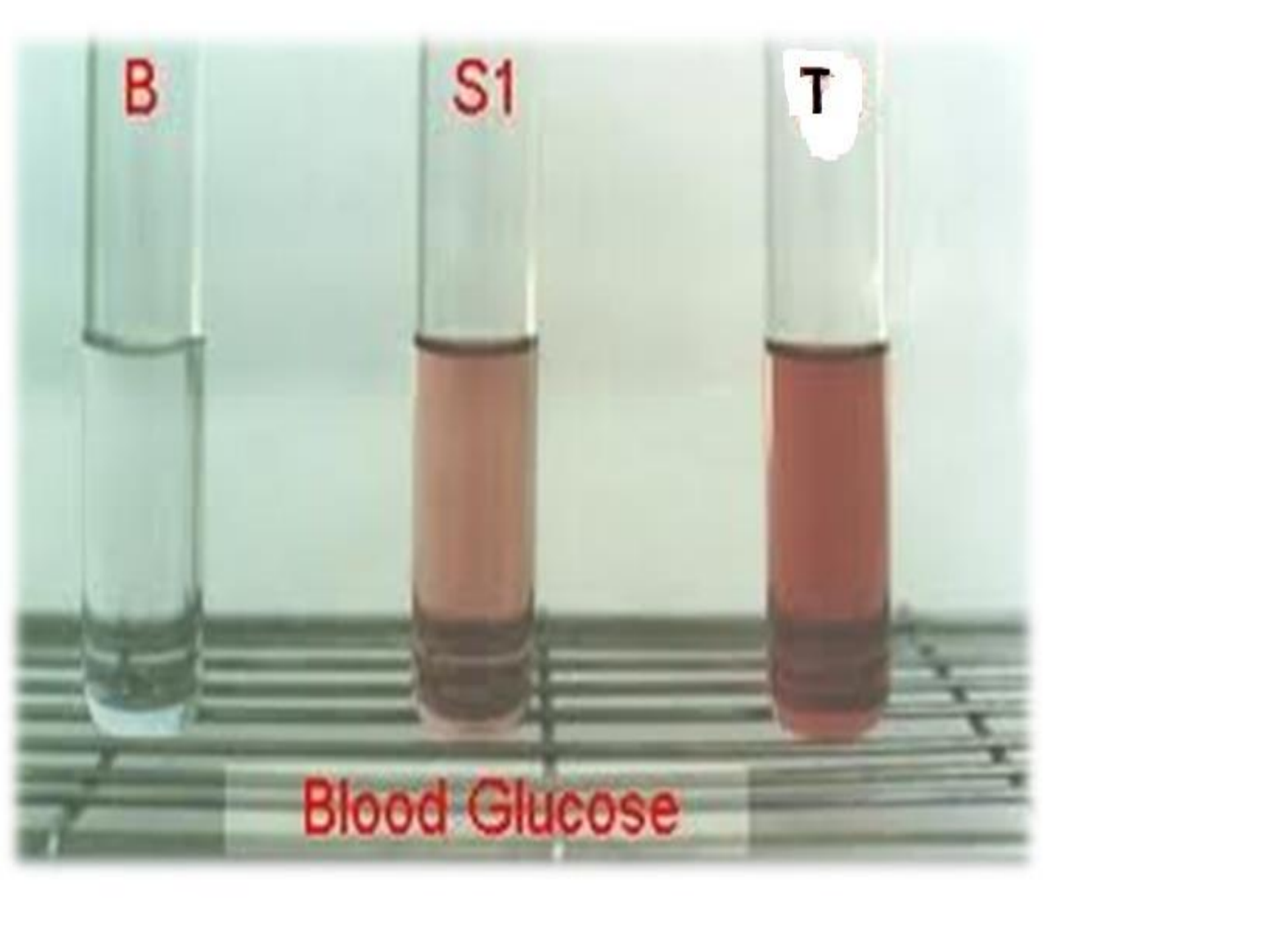
- Mix & keep it for incubation at 37°C for 15 min or at room temperature for 30 min.
- Measure the intensity of colour at 505 nm filter (Green filter).

B

S1

T

Blood Glucose



Calculation:

Concentration of Substance =

$$\frac{\text{O.D. of Test} - \text{O.D. of Blank}}{\text{O.D. of Std.} - \text{O.D. of Blank}} \times \text{Concentration of Std.}$$

General Parameter:

- Reaction type : End point
- Standard Concentration : 100 mg/dl
- Linearity is up to 500 mg/dl
- If sample value is 500mg/dl ,dilute the sample 1:2 with distilled water & repeat assay.

ADVANTAGES :

- It is specific for glucose estimation.
- This method is sensitive, simple.
- It is cheap method.

DISADVANTAGE:

- This method show linearity only up to 500 mg/dl of glucose concentration.

Hexokinase method

PRINCIPLE:

- Glucose + ATP \leftrightarrow Glucose 6 phosphate + ADP
- Glucose 6 Phosphate + NAD \leftrightarrow 6-Phosphogluconate + NADH + H⁺
- Conversion of NADH from NAD at 340nm increase in O.D. is measured at fix interval
- Increase O.D. /min is directly conc. of glucose in the specimen = Δ O.D.

PROCEDURE:

Pipette 1.0 ml Of Glucose Reagent in Cuvette

incubate ↓ water bath at 37° for 1 minute

add 10 µl of sample

↓ mix well

read O.D./minute up to 3 minute

- Repeat each steps by using Standard.

CALCULATION:

- Plasma glucose =
$$\frac{\text{Delta O.D./min(test)}}{\text{Delta O.D./min(Std.)}} \times 100$$

ADVANTAGES :

- It is most sensitive method.

Disadvantages :

- This method show linearity only up to 500 mg/dl of glucose concentration.

GLUCOSE DEHYDROGENASE METHOD (GDH Method)

PRINCIPLE:



- The appearance of NADH is measured at 340 nm.

Chemical method for determination of blood glucose

- Ortho- toluidine method
- Folin – vui method
- Glucometer

Ortho-toluidine method

PRINCIPLE:

- Glucose react with ortho-toluidin in hot acidic medium to form a Green color complex

- Color intensity \propto Conc. Of Glucose

Measured in photometer at 620 nm to 660 nm.

- It can measured other monosaccharide also.

PROCEDURE:

Tube no.	Tube 1 (Sample 1)	Tube 2 (Sample 2)	Tube 3 (Standard)	Tube 4 (Blank)
O – toluidine	5 ml	5 ml	5 ml	5 ml
Test 1	0.1 ml	-	-	-
Test 2	-	0.1 ml	-	-
Standard (100mg/dl)	-	-	0.1 ml	-
Water	-	-	-	0.1 ml
	Shake, then incubate in boiling water bath for 10 min, then cool under tap water			

Calculation :

- The concentration of glucose in the standard solution is 100mg/100ml.

The concentration of glucose in urine is given by:

$$\frac{\text{O.D. TEST}}{\text{O.D. STANDARD}} \times 100 = \text{mg Glucose /100ml blood}$$

Disadvantages :

- It is Non-Specific Method.
- Time consuming method.
- Ortho-toluidine is carcinogenic, so not utilized nowadays.

Folin - Vui Method

Principle :

- When glucose or other reducing agent are treated with alkaline copper solution they reduce the copper with the result insoluble cuprous oxide is formed.
- The cuprous oxide form is allowed to react with phospharomolybdate to form molybdenum blue colour complex which can be read at 420nm.

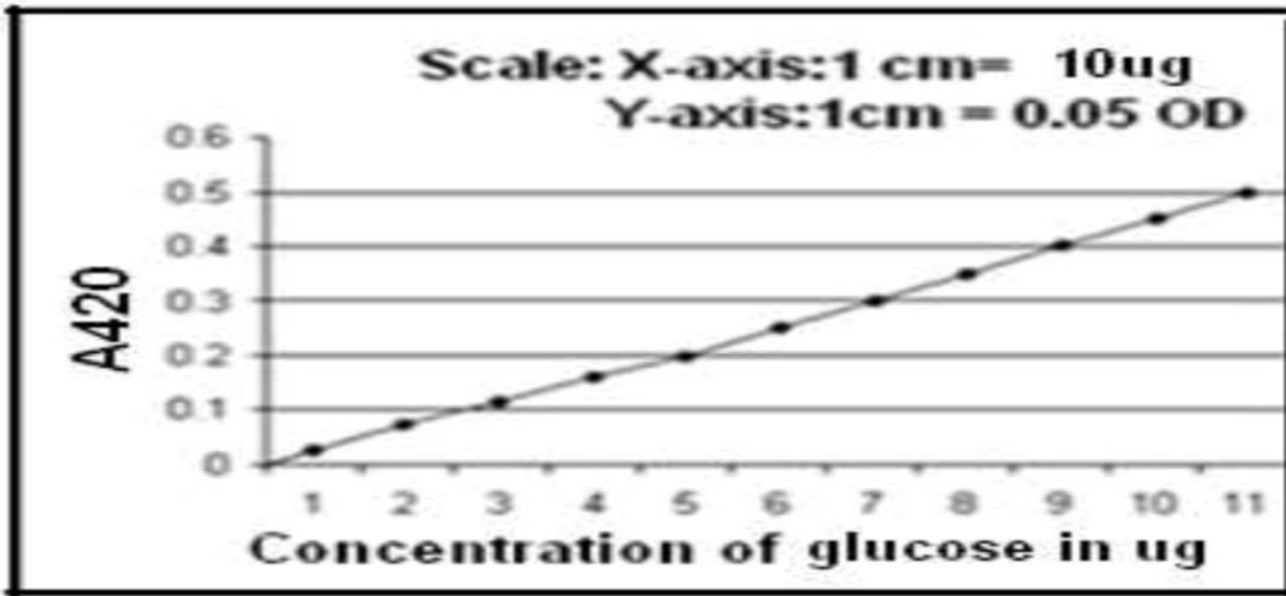
Procedure :

- 1 ml blood added in boiling tube contain 7 ml water+ 1 ml 10% sodium tungstate mix it + 1 ml of 2/3N H₂SO₄ with shaking.
- Allowed to stand for 10 min.
- Filtered it(This filtered is tungastic acid blood filtrate and it taken as a test sample).

Observations and Calculations:

Volume of standard glucose (ml)	Volume of distilled water (ml)	Concentration of glucose (μg)	Volume of copper sulfate solution (ml)	Incubate In boiling water bath for 8 min and cool to R T	Volume of phospho molybdic acid (ml)	Dilute the solution up to 25 ml mark on the Folin-Wu tube.	A_{420}
0.0	2.0	00	1.5		2		0.00
0.4	1.6	40	1.5		2		
0.8	1.2	80	1.5		2		
1.2	0.8	120	1.5		2		
1.6	0.4	160	1.5		2		
2.0	0.0	200	1.5		2		
2.0 Sample	0.00	To be Estimated	1.5		2		

Standard curve



- Plot the standard curve by taking concentration of glucose along X-axis and absorbance at 420 nm along Y-axis.

From the standard curve calculate the concentration of glucose in the given sample.

Calculation :

$$\text{Mg of glucose/100 ml of blood} = \text{mg of glucose in standard} \times (\text{OD of test/OD of standard}) \times 100/0.2$$

Disadvantages:

- . Time consuming method.
- . Non-specific method, also measure fructose.

Glucometer



- Blood is placed onto a test strip & insert into the glucometer to measure blood sugar level.
- On each strip, there is about 10 layers, including a stiff plastic base plate and other layers containing chemicals or acting as spacer.
- For instance there is a layer containing two electrodes (silver and other similar metals).
- There also is a layer of a immobilized enzyme, glucose oxidase and another layer containing microcrystalline potassium ferricyanide.
- The reaction interest between glucose and enzyme.

Principle:

Glucose $\xrightarrow{\text{GOD}}$ Glucuronic acid

Glucuronic acid + ferricyanide \longrightarrow

ferrocyanide $\longrightarrow \bar{e} \longrightarrow$ Current

- The principle behind blood glucose meter is based on reactions that are analyzed by electrochemical sensor.
- The glucose in the blood sample react with the glucose oxidase to form glucuronic acid which then reacts with ferricyanide to form ferrocyanide.
- The electrode oxidizes the ferrocyanide, and this generate a current directly proportional to the glucose concentration.

- Glucometer is only type of dry chemistry.
- Currently many type of glucometer are available that give result as
 - plasma equivalent
 - whole blood glucose
- Glucose level in plasma is generally 10- 15% higher than whole blood.
- So it is important for patients to know whether it give result in “whole blood equivalent ” or “plasma equivalent ”.

- **Advantages:**

- Can do from capillary blood collection method.

- **Disadvantages:**

- It is coastly.
- It gave slightly higher result than actual.

MEASUREMENT OF GLUCOSE IN URINE

- For glucose estimation from urine is collected.
- **METHOD:**

1. Qualitative

2. Quantitative

3. Semi- quantitative

1) **QUALITATIVE METHOD:**

- It is determination by Benedict test

2) QUANTITATIVE METHOD:

- It is Determination By Hexokinase & Glucose Dehydrogenase

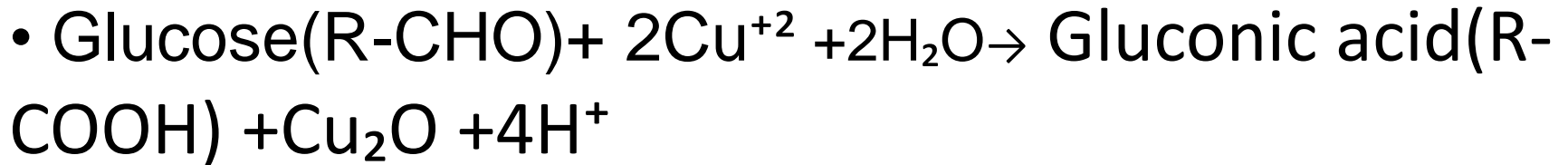
3) SEMI QUANTITATIVE METHOD:

- It is determination by Glucose Oxidase strip test
- E.g. Urine strip

Benedict's Test

This is a very simple and effective method of the amount of glucose in the urine

❖ Principle:



Procedure:

5 ml of Benedict's reagent



8 to 10 drops of urine

↓ Boiling the mixture & cool down it

observe changes colour

Result & Interpretation on Benedict Test

- Blue - sugar absent;
- Green - 0.5 gm% sugar = +1
- Yellow – 1.0 gm% sugar = +2
- Orange - 1.5 gm% sugar = +3
- Brick red – 2.0 % or more sugar = +4



colour :	blue	green	orange	brick red
gm% sugar :	absent	0.5%	1.5%	2.0%
grade :	-	+1	+3	+4

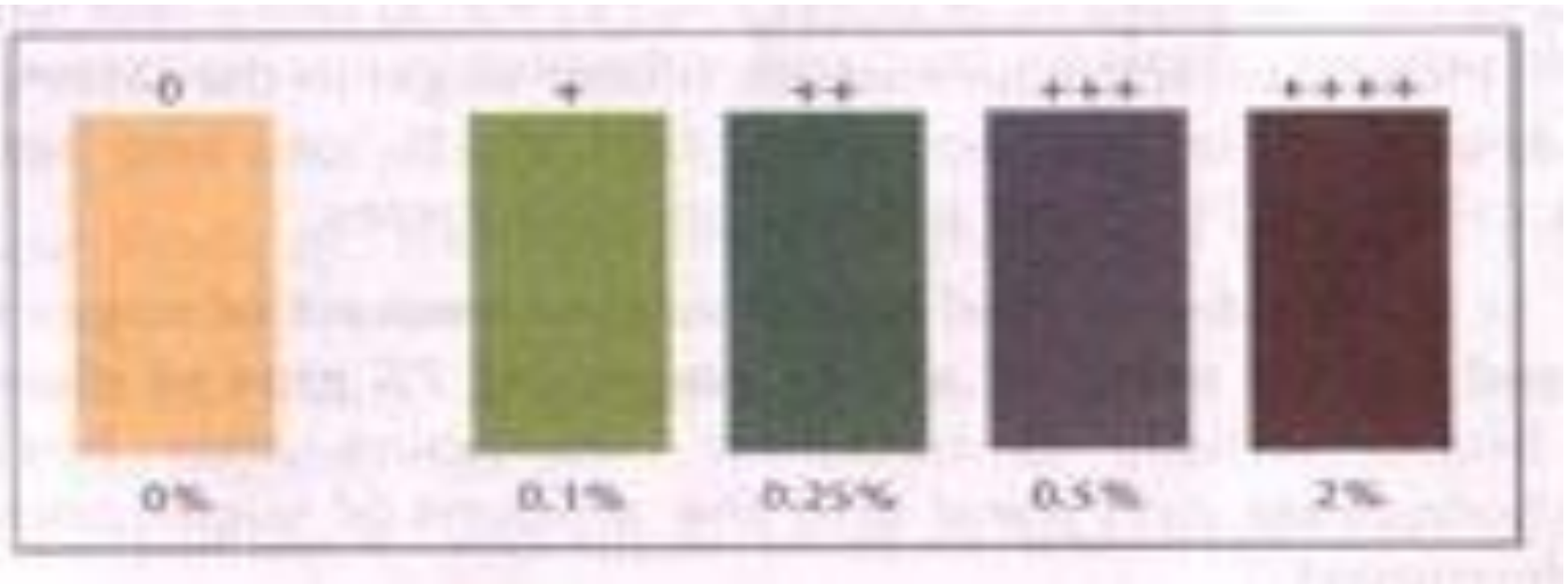
Significant of Benedict Test

- Each reducing substance gives positive test
- So Following substance can gives false positive test E.g. Vitamin – C, B-Complex vitamin, Salicylic acid

Glucose Oxidase Test or Diastix

- Paper or plastic strips, called diastix .
- A color-chart is provided with the strips.
- Strip contain dye are O-toluidine, tetramethylbenzidine , potassium iodide, 4-amino phynazome, phenol.
- The dye changes colour on coming in contact with the urine.
- After 30 to 60 seconds the colour of the strip matched with the colours of the provided chart.

Oxidase Strip



• **GLUCOSE ESTIMATION IN CSF**

- CSF is a fluid that flows through and protects the subarachnoid space of the brain and spinal cord.
- CSF fluid is mostly collected into plain vial and put it into a ice-pack & transport to laboratory for to analyze it immediately.
- In CSF, bacteria & other cells are also present so analyzed immediately
- It's obtained by lumbar puncture, L 3-L 4
- In CSF, Glucose is estimation by GOD - POD method.
- In CSF Contain $\frac{2}{3}$ part of plasma glucose.

CLINICAL SIGNIFICANCE

Increased glucose : (hyper glycemia)

- Diabetes mellitus
- Adrenocortical hyper activity

Decreased glucose: (hypo glycemia)

- Bacterial infection
- Hypo thyroidism
- Hypo adrenalism

GTT

(GLUCOSE TOLERANCE TEST)

INTRODUCTION

- **Glucose tolerance** means ability of the body to utilize (tolerate) glucose in blood circulation.
- The effect of ingested carbohydrate can be studied under reasonably standard condition by means of the **Glucose Tolerance Test**.
- It is indicated by the nature of blood glucose curve following the administration of glucose.

- Temporary rise of blood sugar after food intake for few hours.
- Extent and duration of rise depends on type of food (**Glycemic index**).
- Glucose level returns to normal within 2 hrs.
- If it take >2 hours = Decrease glucose tolerance.

Types of GTT

1) Oral GTT (OGTT)

2) INTRAVENOUS GTT (IVGTT)

- OGTT is mostly preferred & explain in detail here.
- IVGTT use for patient who are unable to absorb an oral dose of glucose (malabsorption syndrome).

PRINCIPLE :

a glucose tolerance test is the administration of glucose in a controlled and defined environment to determine how quickly it is cleared from the blood. The test is usually used to test for diabetes, insulin resistance and sometimes reactive hypoglycemia. The glucose is most often given orally.

INDICATIONS FOR GTT

- Having symptoms like diabetes mellitus, but fasting blood sugar value is inconclusive (between 100-126mg/dl)
- During pregnancy and past history of miscarriage.
- To rule out benign renal glucosuria.

CONTRA-INDICATION

- Person with confirmed Diabetic patients.
- Mal-absorption disease(OGTT).
- Test should not done in acutely ill patients.

PRE-CAUTION

- Normal diet intake in last 3 days.
- Avoid heavy exercise.
- Report to lab after fasting for 12-16 hrs.
- Avoid drug that change glucose level.
e.g. steroid , Insulin ,Oral Hypo Glycemic Drug.
- Addiction : Alcohol & smoking.

METHOD OF THE TEST

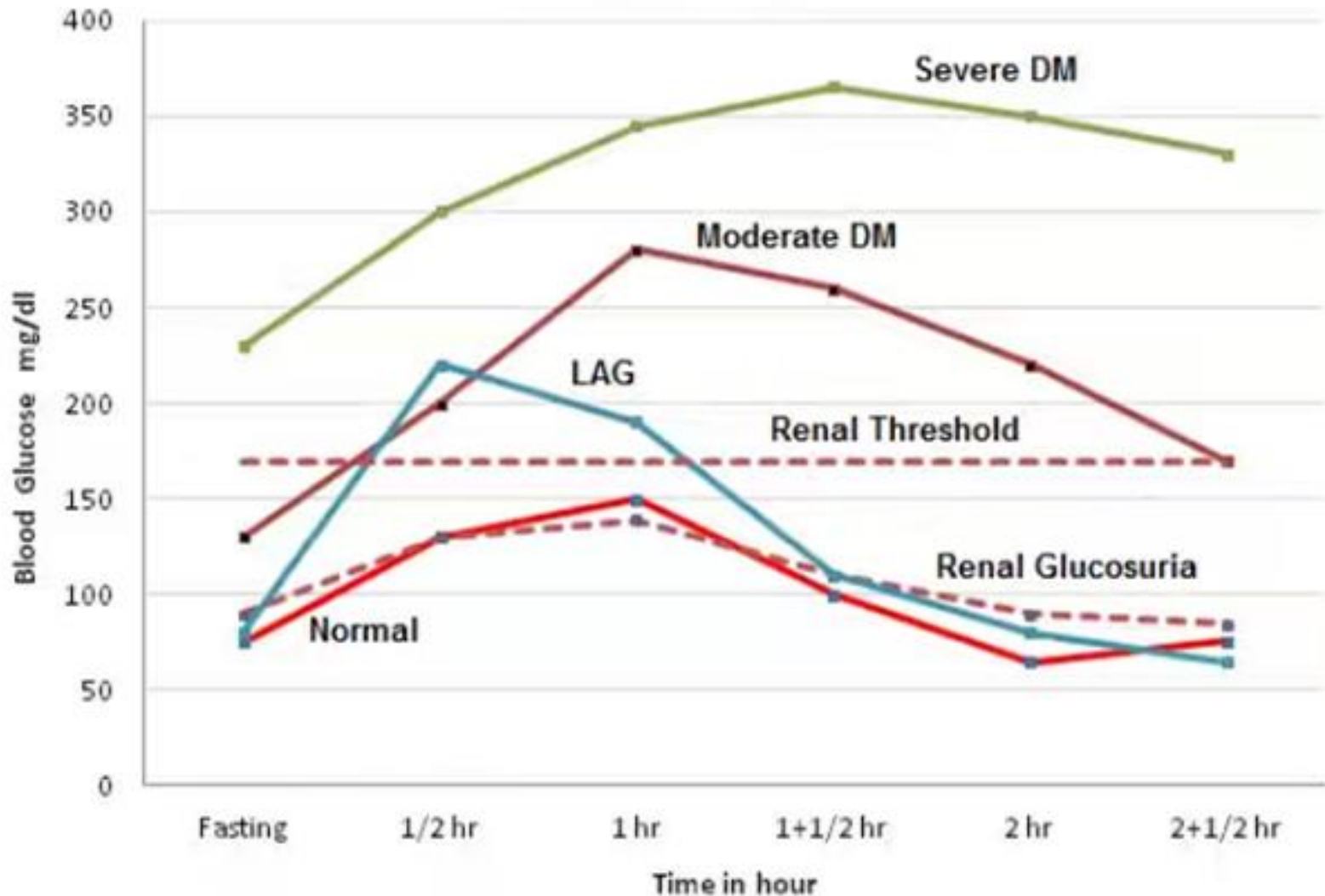
- Collection of fasting urine & blood (in fluoride) sample.
- Give 75gm or 100gm of glucose dissolved in lemon water to the patient.
- Note the time of oral glucose administration.
- In pediatric patient 1.5 - 1.75 gm/kg glucose/dextrose powder.
- Collect five sample of venous blood and urine are collected at the half hourly intervals.
- Determine blood glucose by the specific method. e.g. GOD-POD method.
- Urine glucose = Semi-Quantitative Method – Benedict's Test
- Prepare a **glucose tolerance curve**(plasma glucose level - time).

NORMAL GLUCOSE TOLERANCE CURVE

TIME (MIN.)	FASTING	30	60	90	120	150
BLOOD SUGAR (mg/dl)	75	125	145	100	70	75

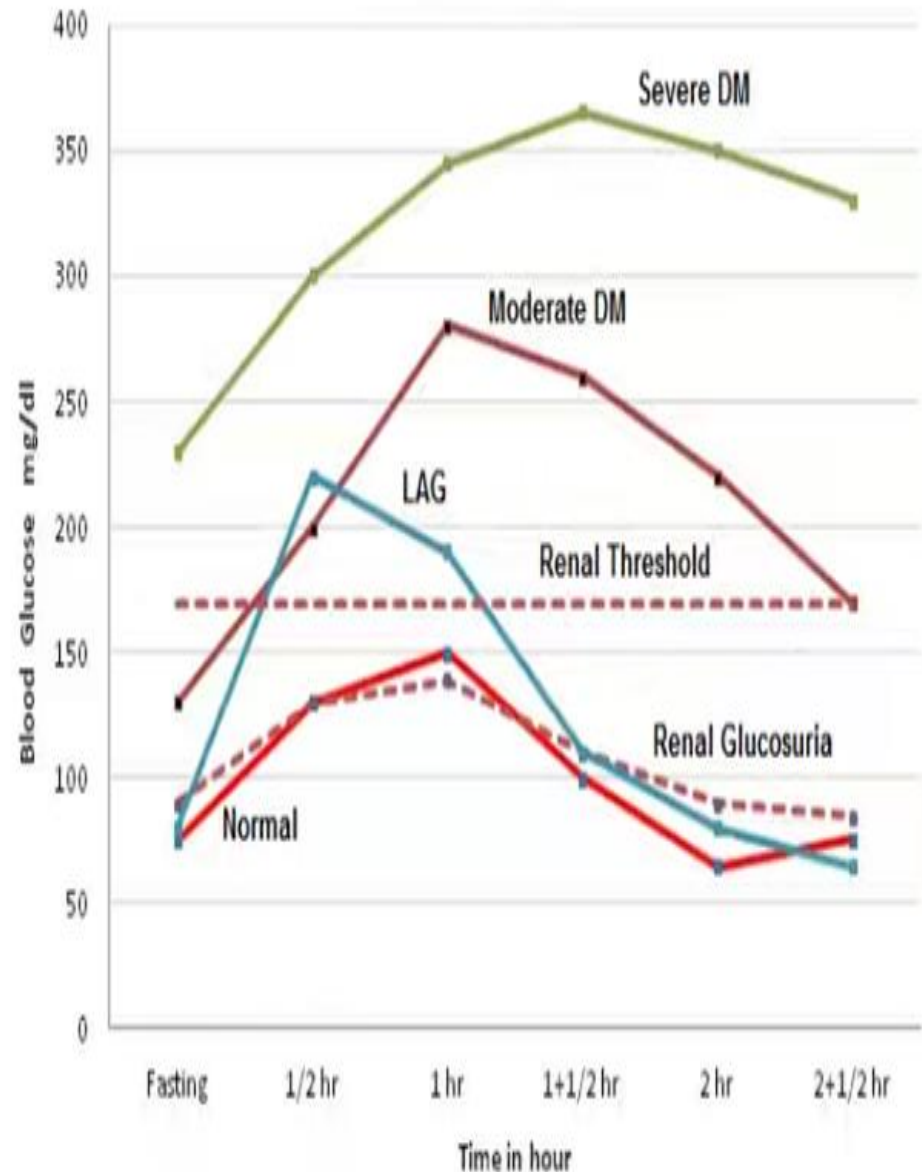
← Urine Sugar absent throughout →

ORAL GLUCOSE TOLERANCE TEST (OGTT): glucose tolerance curve



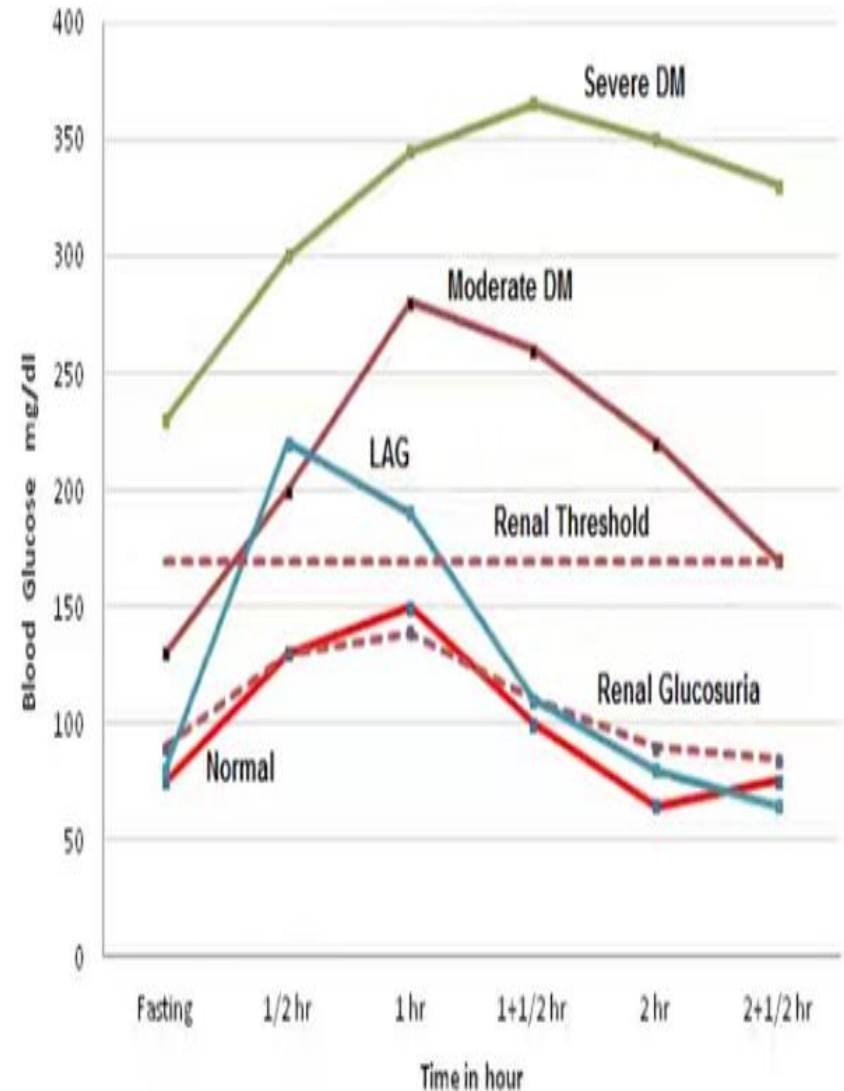
NORMAL RESPONSE

- Initial fasting glucose within normal limits.
- The highest peak value is reached within 1 hour.
- The highest value does not exceed the renal threshold (160-180mg/dl).
- The fasting level is again reached by 2-2.5 hours.
- No glucose or ketone bodies are detected in any specimen of urine.



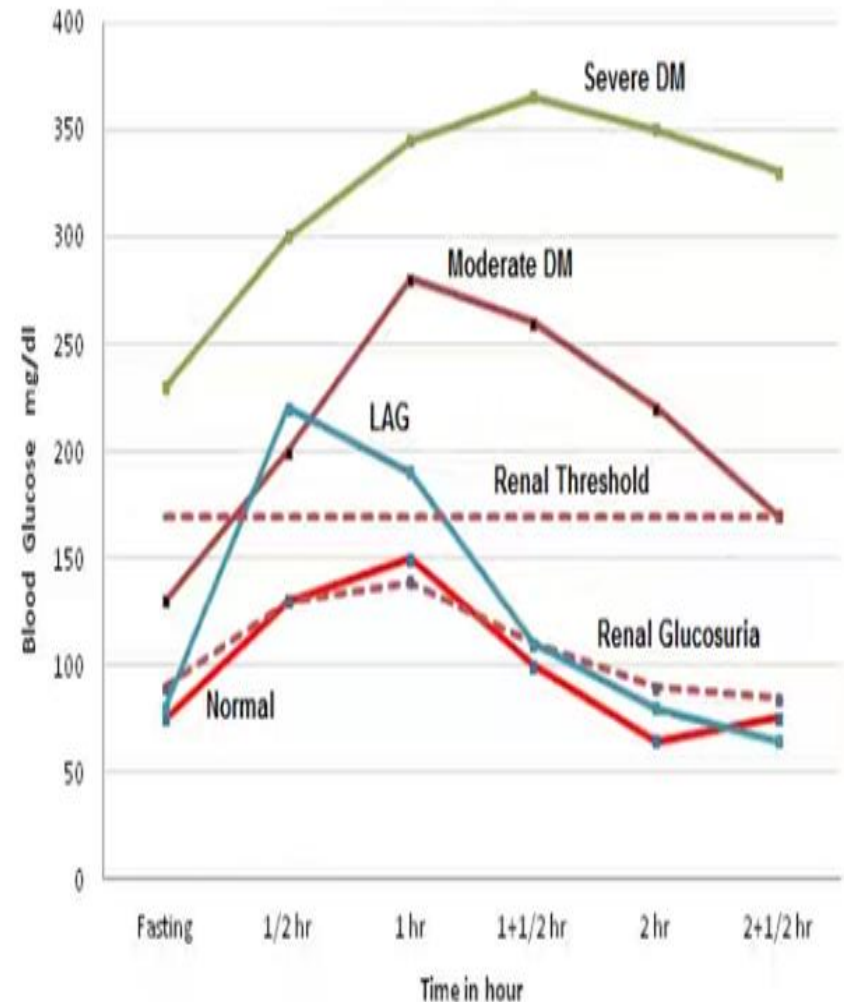
RESPONSE OF DIABETIC PATIENTS

- Fasting blood glucose is definitely raised above 110 mg/dl.
- The highest value exceed the renal threshold.
- The blood glucose level dose not return to fasting level within 2.5hours. This is the most characteristic feature of DM.
- Urine sample always contains glucose except in some chronic diabetes or nephritis who may have raised renal threshold.
- **According to severity , GTC may be :**
 - Mildely diabetic curve
 - Moderately severe diabetic curve
 - Severe diabetic curve



LAG CURVE FOR OXYHYPERGLYCEMIA

- Fasting glucose level is normal.
- Raises rapidly in the $\frac{1}{2}$ to 1 hour and exceeds the renal threshold so that the corresponding urine specimens show glucose.
- The return to normal value is rapid and complete.
- **The curve is obtained in :**
- Hyperthyroidism
- After gastroenteroscopy
- During pregnancy
- Also in early diabetes



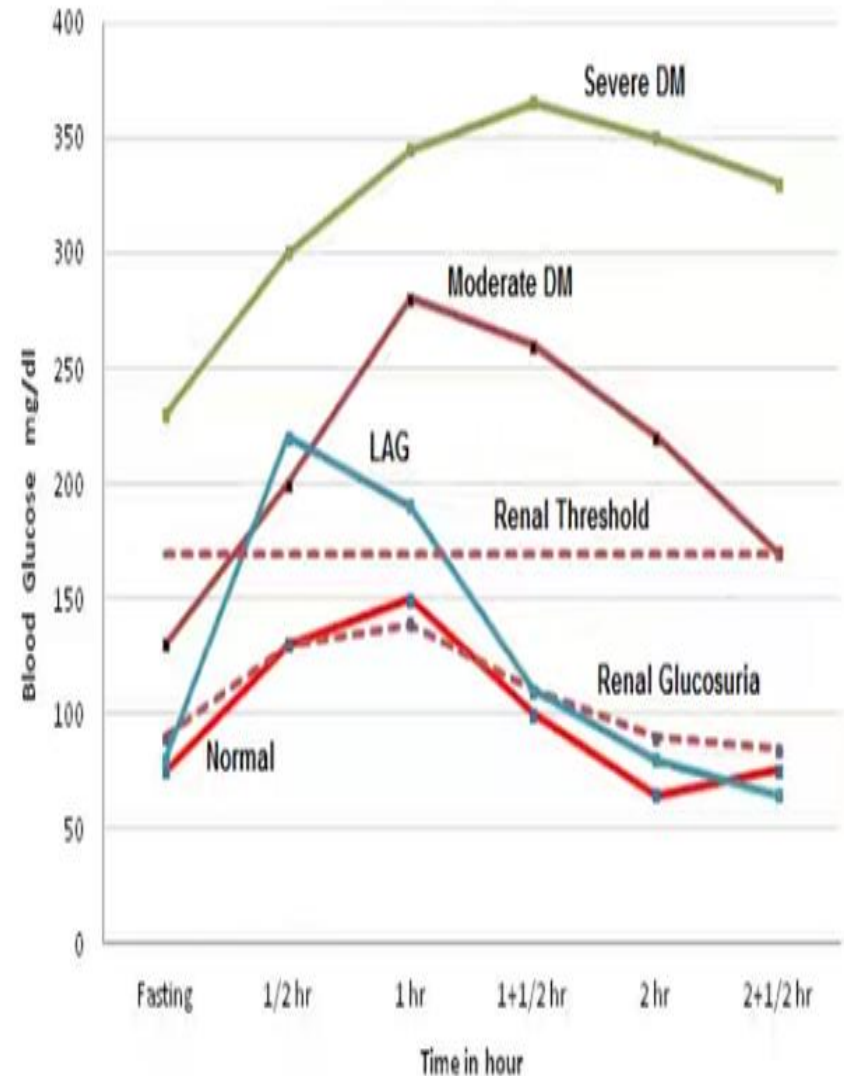
CURVE FOR RENAL GLUCOSURIA

- Glucose appears in the urine at level of blood glucose much below renal threshold.

- Patients who show no glucosuria when fasting may have glucosuria when blood glucose is raised.

It may be seen renal in :

- Renal disease and pregnancy
- early diabetes



ADVANTAGES

- It is useful in recognizing of border line cases of diabetes.
- GTT is useful in early diagnosis diabetes melitus.
- It is useful in diagnosis of gestational diabetes.

DISADVANTAGES

- GTT is not necessary in known cases of hyperglycemic patient.
- Oral GTT is also not necessary in known cases of mal-absorption.
- This time I-V glucose tolerance test is required.

**PRINCIPAL OF
CARBOHYDRATE
CHEMISTRY TEST**

Tests:

- 1) Molisch's test
- 2) Benedict's test
- 3) Barfoed's test
- 4) Seliwanhoff's test
- 5) Inversion test
- 6) Iodine test

Molisch test

This test is specific for all carbohydrates.

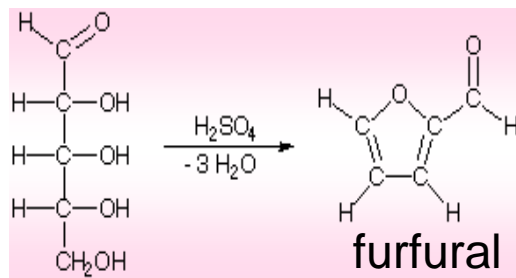
Monosaccharide gives a **rapid** positive test, Disaccharides and polysaccharides react **slower**.

Objective: To identify the carbohydrate from other macromolecules lipids and proteins.

Principle:

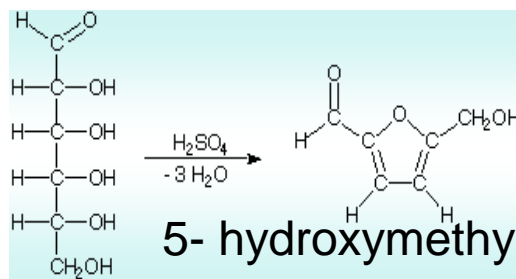
The test reagent (H_2SO_4) dehydrates pentose to form furfural and dehydrates hexoses to form 5-hydroxymethyl furfural.

The furfural and 5-hydroxymethyl furfural further react with α -naphthol present in the test reagent to produce a purple product.



α -naphthol

Purple color



α -naphthol

Purple color



Procedure :

2 ml of a sample solution

↓ add

2 drops of the Molisch reagent (α -naphthol in 95% ethanol)

↓ add

2 ml of concentrated sulfuric acid

↓

violet ring appear

Tube	observation
1-glucose	+
2-ribose	+
3-sucrose	+
4-starch	+

Benedict's test

Benedict's reagent is used as a test for the presence of reducing sugars.

All monosachharides are reducing sugars; they all have a free reactive carbonyl group.

Some disaccharides have exposed carbonyl groups and are also reducing sugars. Other disaccharides such as sucrose are non-reducing sugars and will not react with Benedict's solution.

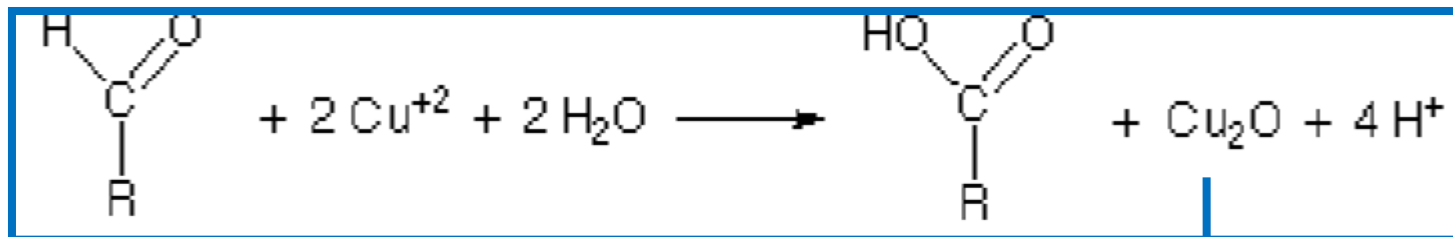
Large polymers of glucose, such as starch, are not reducing sugars

Objective: To distinguish between the reducing and non-reducing sugars.

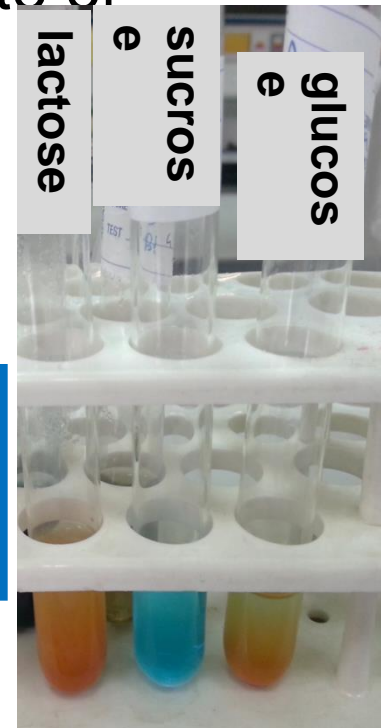
Principle:

The copper sulfate (CuSO_4) present in Benedict's solution reacts with electrons from the aldehyde or ketone group of the reducing sugar in **alkaline medium**.

Reducing sugars are oxidized by the copper ion in solution to form a carboxylic acid and a reddish precipitate of copper oxide.



reddish precipitate of copper



Colour of precipitate give the idea about quantity of sugar present in the solution hence the test is semi-quantitative.

- It can give false positive result because of non –glucose reducing substance.
- Grade Color of Reaction Mixture
Approximate Glucose concentration

Grade	Color of Reaction Mixture	Approximate Glucose concentration
+	Green	0.5-1 gm%
++	Yellow	1-1.5 gm%
+++	Orange	1.5-2 gm%
++++	Red	>2 gm%

Procedure :

1 ml of a sample solution

↓add

2 ml of Benedict's reagent

↓ heated in a boiling water bath for 5 minutes

formation of a reddish precipitate.

Tube	observation
1-glucose	+
2-sucrose	-
3-lactose	+

Barfoed's Test

This test is performed to distinguish between reducing monosaccharides, reducing disaccharides and non reducing disaccharides.

Objective: To distinguish between mono- , di- and poly saccharides.

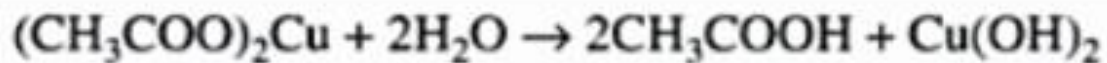
Principle:

Barfoed's test used copper (II) ions in a **slightly acidic medium**

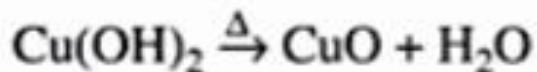
Reducing monosaccharides are oxidized by the copper ion in solution to form a carboxylic acid and a reddish precipitate of copper (I) oxide within three minutes. Reducing disaccharides undergo the same reaction, but do so at a slower rate.

The nonreducing sugars give negative result.

Barfoed's reagent, cupric acetate in acetic acid , so in acidic medium , disacchride is a weaker reducing agent than monosacchride, so mono sacchride (1 to 2 minute) will reduce the copper in less time & disaccharide (7 to 12 minute)take time.



Cupric hydroxide



Cuprous oxide
(Red ppt.)



Procedure :

1 ml of a sample solution

↓ add

3 ml of Barfoed's reagent (a solution of cupric acetate and acetic acid)

↓

Heat mixture in a boiling water bath for 6 min.(after the 3 min check the tubes)

↓

Reddish precipitate formed

Tube	observation
1-glucose	+
2-sucrose	-
3-lactose	-

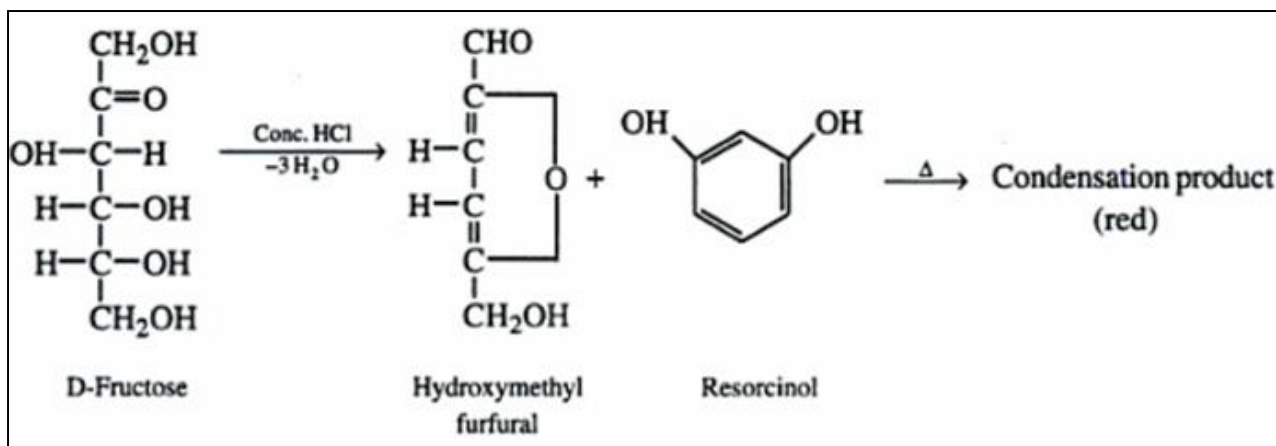
Seliwanoff's Test

This test is used to distinguish between aldoses (like glucose) and ketoses (like fructose).

Objective: To distinguish between aldose and ketone sucrose.

Principle:

Seliwanoff's Test uses 6M HCl as dehydrating agent and resorcinol as condensation reagent. The test reagent dehydrates ketohexoses to form 5-hydroxymethylfurfural. 5-hydroxymethylfurfural further condenses with resorcinol present in the test reagent to produce a cherry red product within two minutes. Aldohexoses react to form the same product, but do so more slowly giving yellow to faint pink color.



Procedure :

1/2 ml of a sample solution

↓add

2 ml of Seliwanoff's reagent

↓

heat mixture in a boiling water bath for 2ml

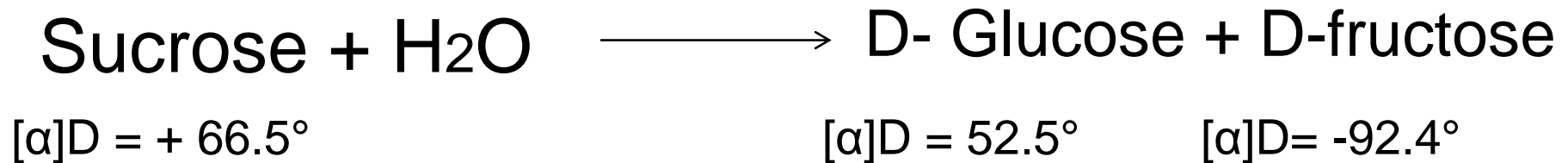
↓

colour develope

Tube	observation
1-glucose	-
2-fructose	+

Inversion test

Principle:



- Sucrose is dextrorotatory.
- The optical rotation change from dextrorotatory to laevorotatory on hydrolysis, since fructose causes a much greater laevorotation than the dextrorotation caused by glucose.
- This is known as inversion.
- The resultant hydrolysate is called invert sugar, which is more sweeter than sucrose.

Procedure :

5ml of OS



2 drop of conc. HCl

↓ boil for 2 min. & cool down

5 drop of 40% NAOH(alkaline)



perform benedict & seliwanoff test



observe colour reaction

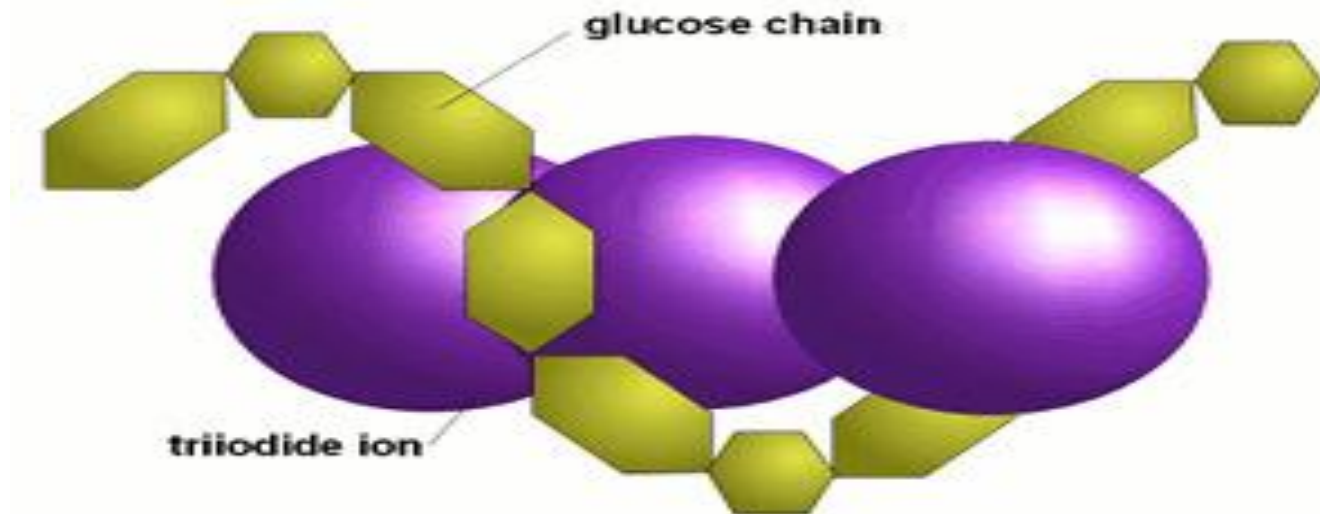
Iodine test:

Principle:

Iodine binds starch to give blue colored complex.

Shorter chain gives a red color.

Longer chain give more intense color reaction.



Procedure :

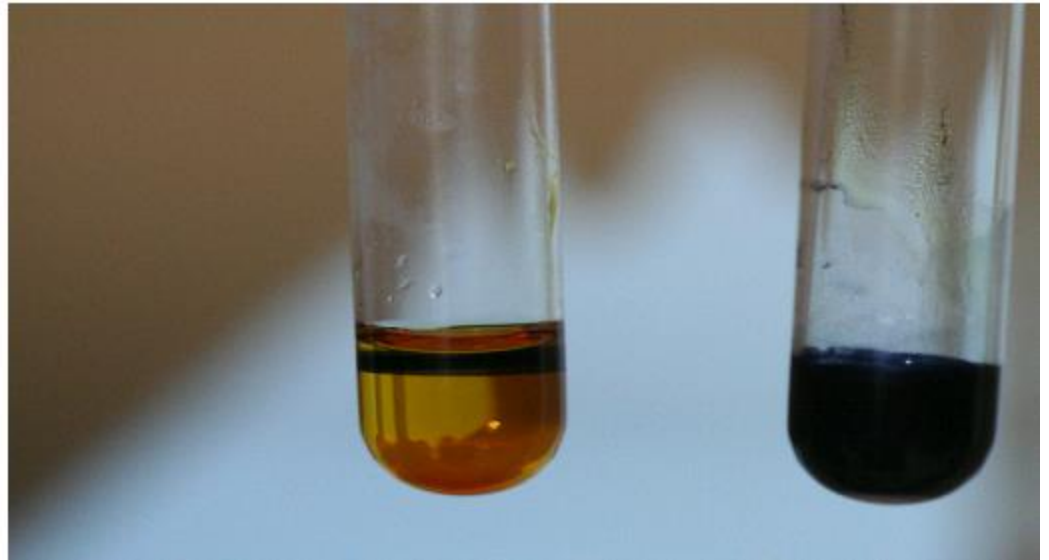
1 ml of OS



2 drop of iodine solution

↓mix

colour develop
(blue ,violet)



THANK YOU