قسم الكيمياء

الكيمياء الحياتية العملي

المرحلة الرابعة

2021-2020

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الكورس الأول والكورس الثاني

Experiments in Biochemistry for 4th Year Chemistry Students

2020-2021

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الكورس الأول والكورس الثاني

Collection and handling of blood samples

LAB EXPERIMENT 1

Blood and the circulatory system

The circulatory system is made up of the heart, blood and blood vessels.

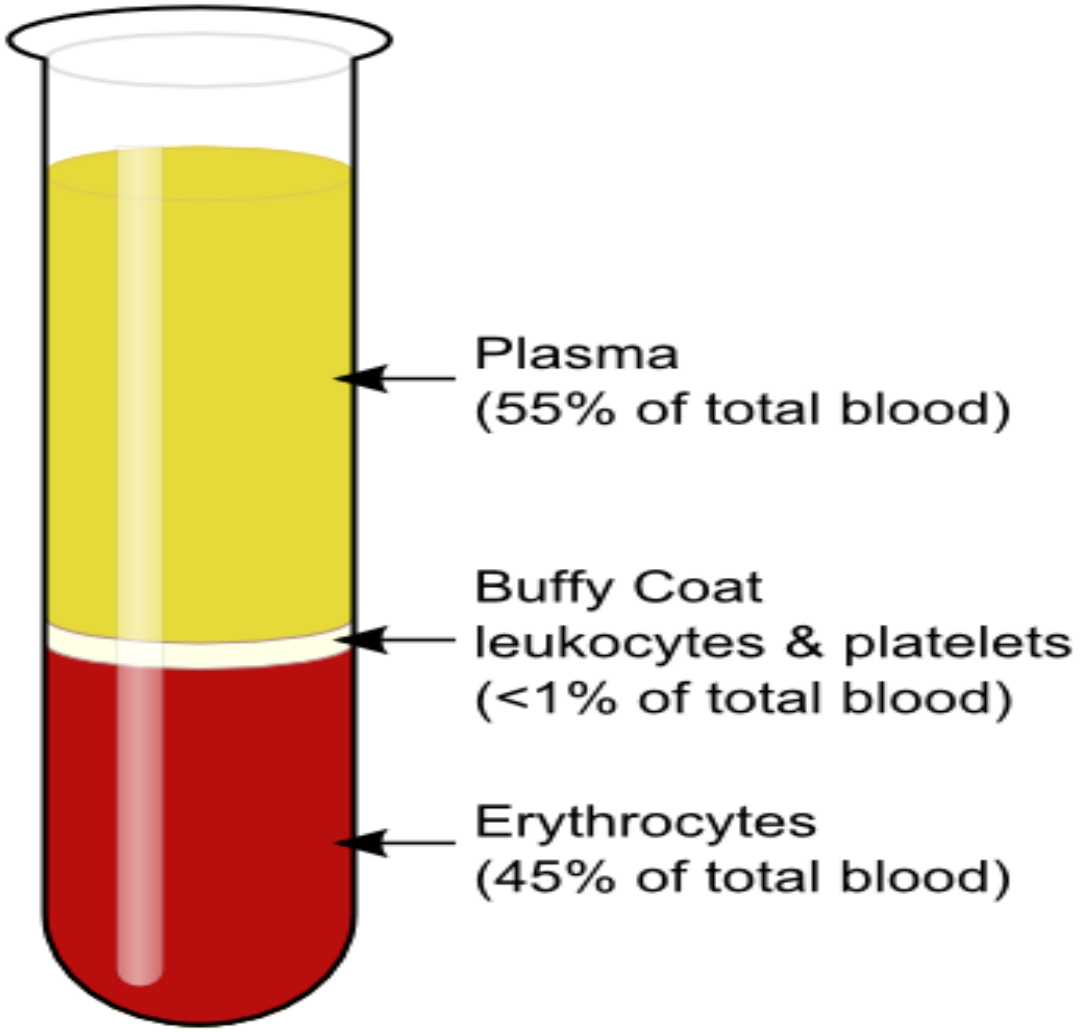
What exactly is blood and what does it do?

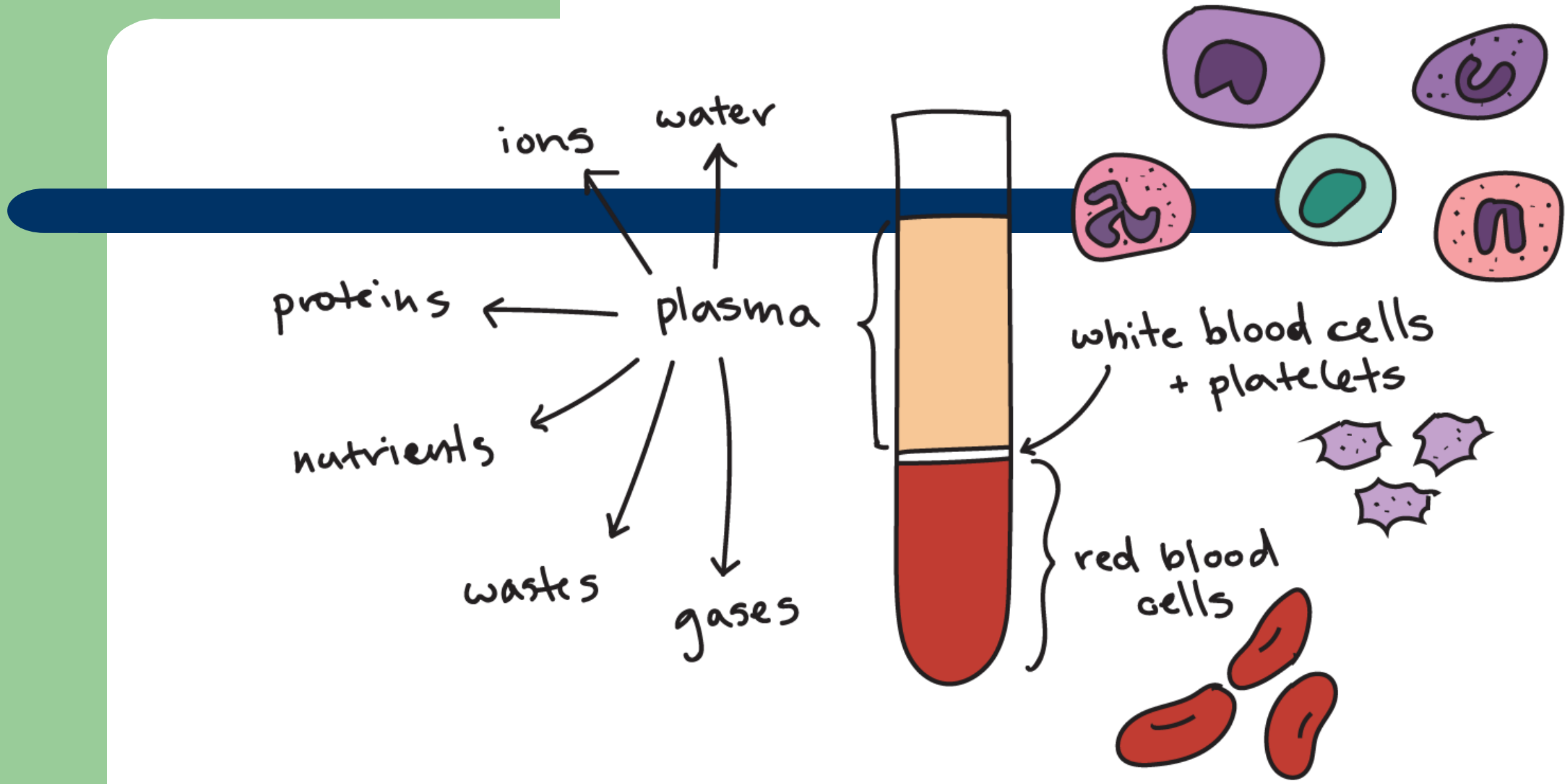
Blood is a tissue that circulates in a closed system of blood vessels.

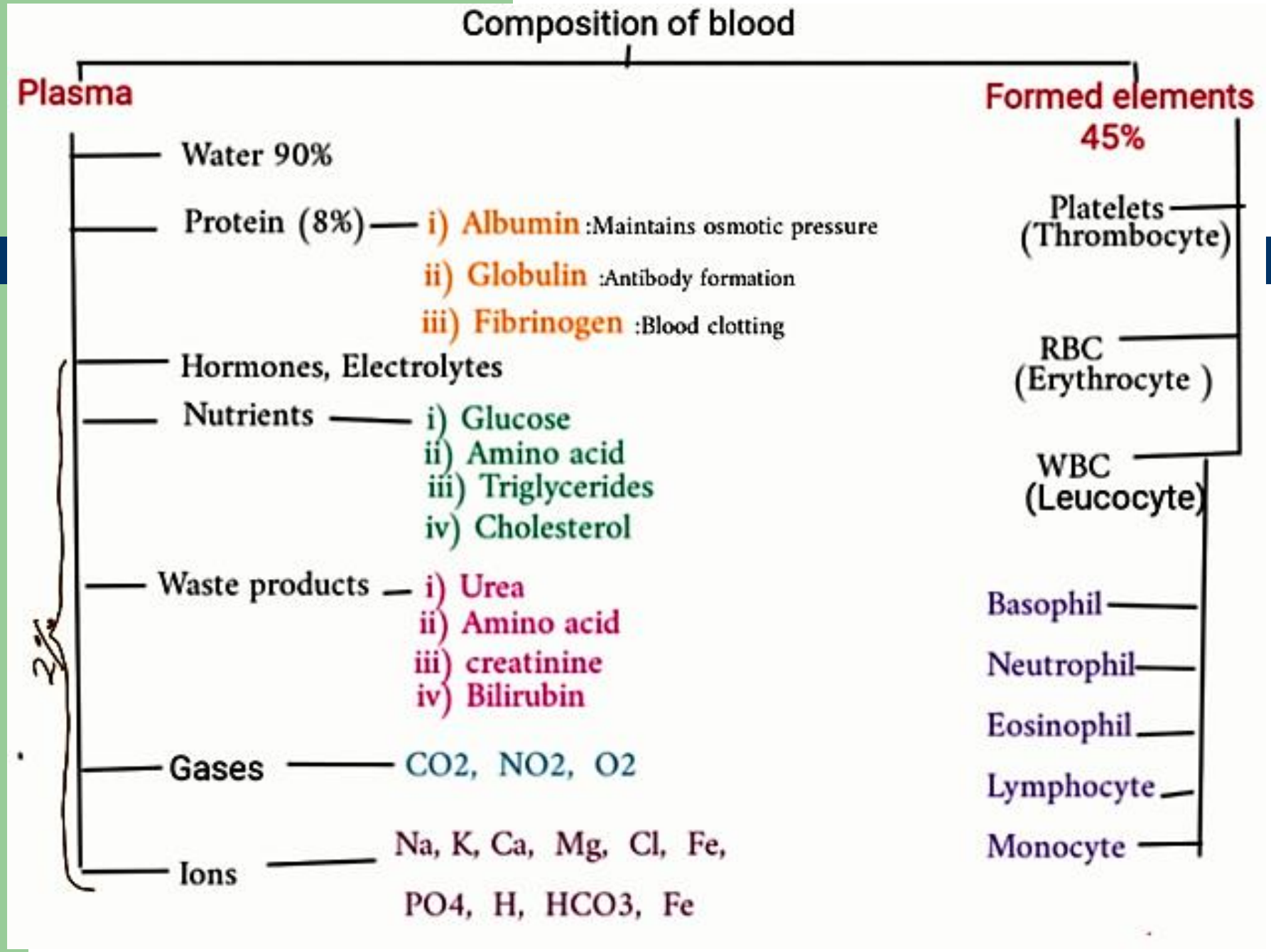
PROPERTIES OF BLOOD

Colour	Bright red in arteries & dark red in veins
Mass	8 % of the body mass
pH	Slightly alkaline (pH = 7.35 – 7.45)
Taste	Salty
Temperature	38° C (100.4° F)
Viscosity	3 – 4 times more viscous than water
Volume	5 – 6 litre

Blood fractionation




ions
water
proteins
plasma
nutrients
wastes
gases
white blood cells
+ platelets
red blood
cells


Composition of blood
Plasma
Water 90%
Protein (8%)
i) Albumin :Maintains osmotic pressure
ii) Globulin :Antibody formation
iii) Fibrinogen :Blood clotting
Hormones, Electrolytes
Nutrients
i) Glucose
ii) Amino acid
iii) Triglycerides
iv) Cholesterol
Waste products
i) Urea
ii) Amino acid
iii) creatinine
iv) Bilirubin
Gases
CO2, NO2, O2
Ions
Na, K, Ca, Mg, Cl, Fe,
PO4, H, HCO3, Fe
Formed elements
45%
Platelets
(Thrombocyte)
RBC
(Erythrocyte)
WBC
(Leucocyte)
Basophil
Neutrophil
Eosinophil
Lymphocyte
Monocyte

Steps of safe blood sampling

Step 1: select equipment, prepare area
Step 2: prepare patient, collect blood sample
Step 3: transfer blood sample
Step 4: waste management

BLOOD COLLECTION TOOLS

Material:

- • Tourniquet.
- • Vacutainer or syringe.
- • Alcohol swab.
- • Bandage/ medi-plast

Blood can be collected from 3 different sources:

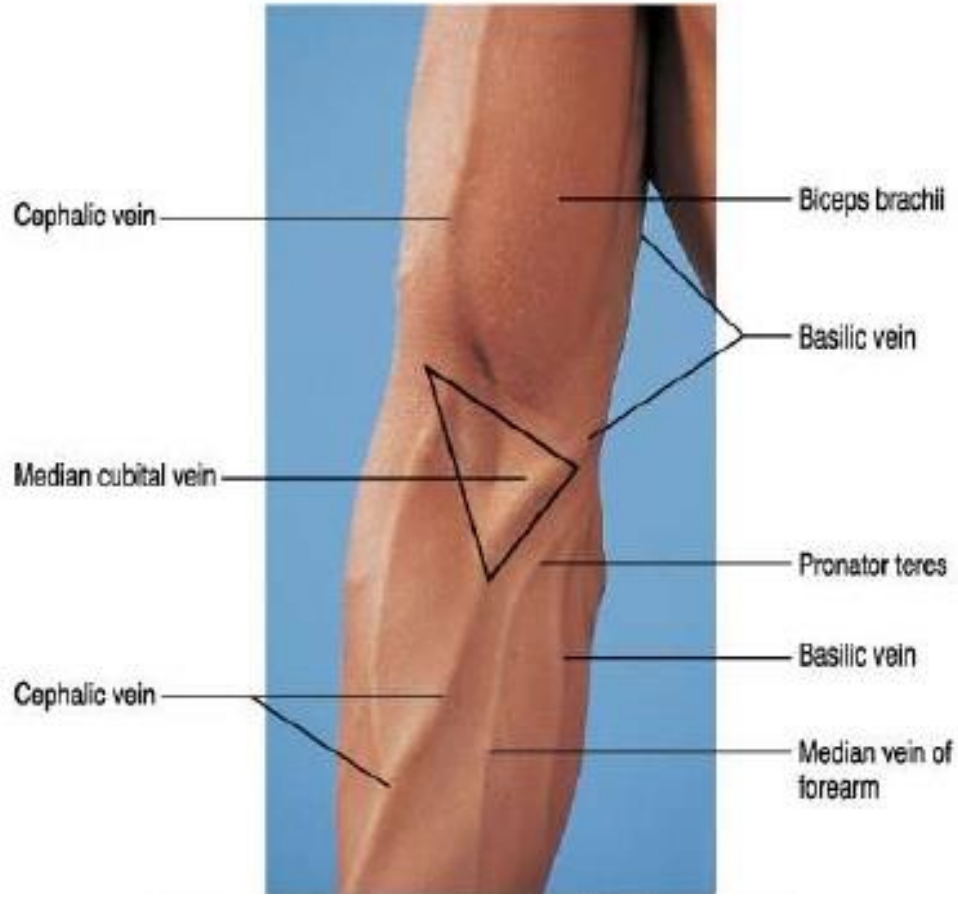
- I. Venous blood.
- II. Arterial blood.
- III. Capillary blood.

VENOUS BLOOD COLLECTION

Blood is collected from the veins of the patient. it is require when the large amount of blood is required

SITES FOR VENOUS BLOOD

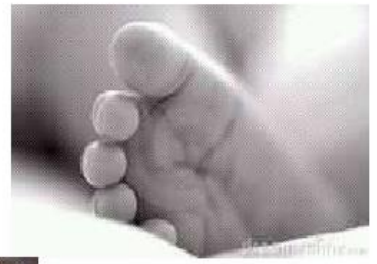
- Anticubital veins
- Radial vein
- Dorsal vein

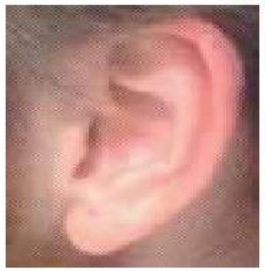


Arterial blood

- Specially required for estimation of blood gases (ABG):
- PH, CO2 and O2
- Collect quickly, fill completely and seal both ends immediately
- No air bubbles

SITES FOR CAPILLARY BLOOD COLLECTION





CAPILLARY BLOOD COLLECTION

- Blood from capillary network can be used to perform test for which only few drops of blood is required.
- Capillary blood is not recommended for those tests which require large amount of blood.
- It is not used for platelet count.

SUITABLE FOR

- Hb , TLC, DLC, RBC count by microdilution method.
- For preparation of thin blood film to determine blood picture.

- Most blood collection tubes contain an additive that either accelerates clotting of the blood (clot activator) or prevents the blood from clotting (anticoagulant).
- A tube that contains a clot activator will produce a serum sample when the blood is separated by centrifugation and a tube that contains an anticoagulant will produce a plasma sample after centrifugation.
- Some tests require the use of serum, some require plasma, and other tests require anticoagulated whole blood.

The table below lists the most commonly used blood collection tubes.

Tube cap color	Additive	Function of Additive	Common laboratory tests
Light-blue 	3.2% Sodium citrate	Prevents blood from clotting by binding calcium	Coagulation
Red or gold (mottled or "tiger" top used with some tubes is not shown) 	Serum tube with or without clot activator or gel	Clot activator promotes blood clotting with glass or silica particles. Gel separates serum from cells.	Chemistry, serology, immunology
Green 	Sodium or lithium heparin with or without gel	Prevents clotting by inhibiting thrombin and thromboplastin	Stat and routine chemistry
Lavender or pink 	Potassium EDTA	Prevents clotting by binding calcium	Hematology and blood bank
Gray 	Sodium fluoride, and sodium or potassium oxalate	Fluoride inhibits glycolysis, and oxalate prevents clotting by precipitating calcium.	Glucose (especially when testing will be delayed), blood alcohol, lactic acid

ANTICOAGULANT TUBE

- **EDTA** (Ethylene Diamine Tetra-Acetate) liquid:
- Types: Na and K2 EDTA (1.5-0.25mg /ml)
- Functions by forming Ca salts to remove Ca.
- Uses: most hematology studies. such as:

CBC, PCR and HbA1c.

Sodium citrate (1:9 ratio).

- Anticoagulant: 32g/l.
- Action: Remove Calcium.
- Uses: Coagulation studies and platelet function.

Sodium Heparin or Lithium Heparin anticoagulant.

- Action: inactivate thrombin and thromboplastin.
- Uses:
- For Lithium level use Na Heparin anticoagulant
- &for Ammonia level use Na or Lithium Heparin

Thank you

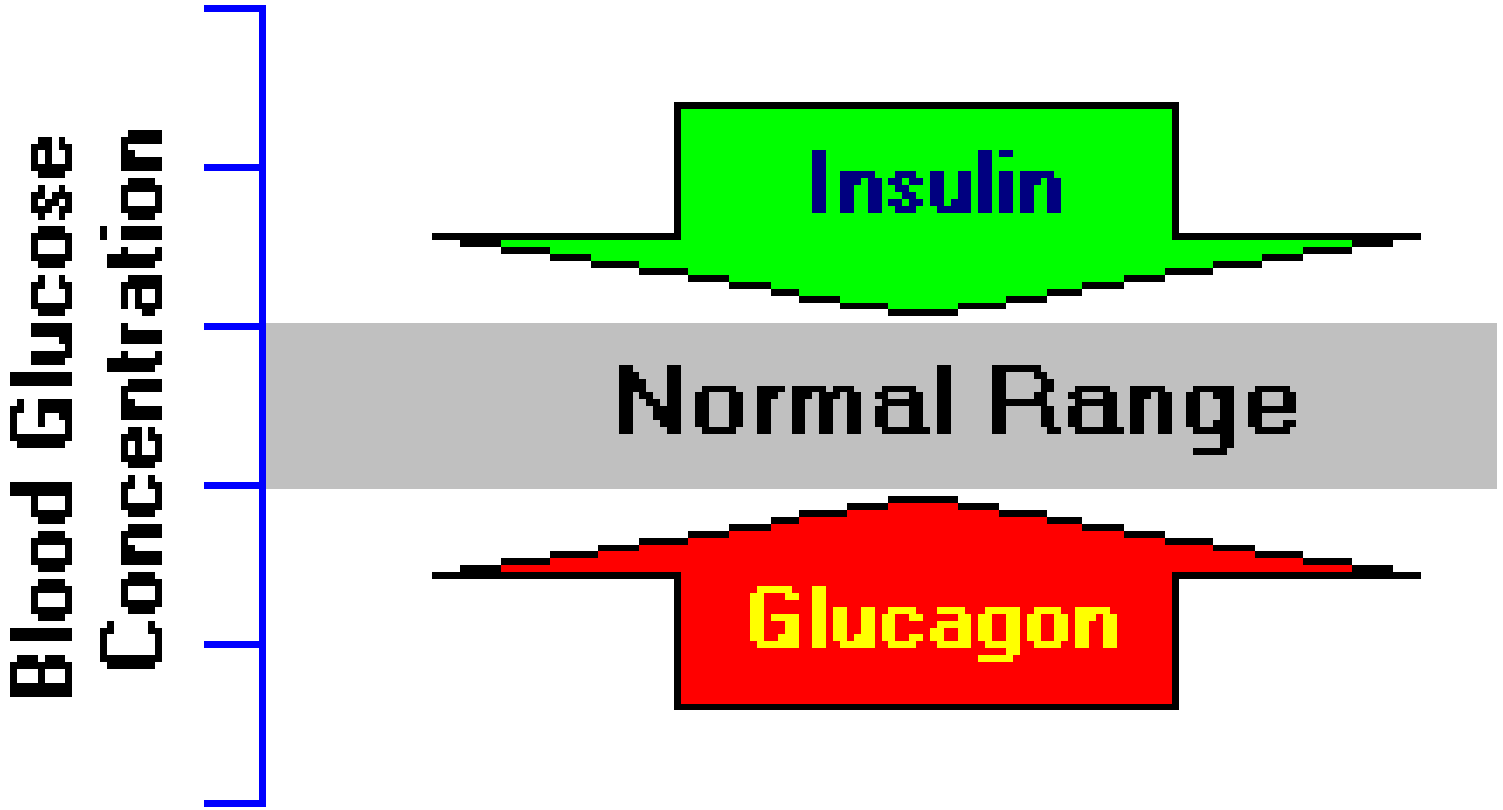
Blood glucose

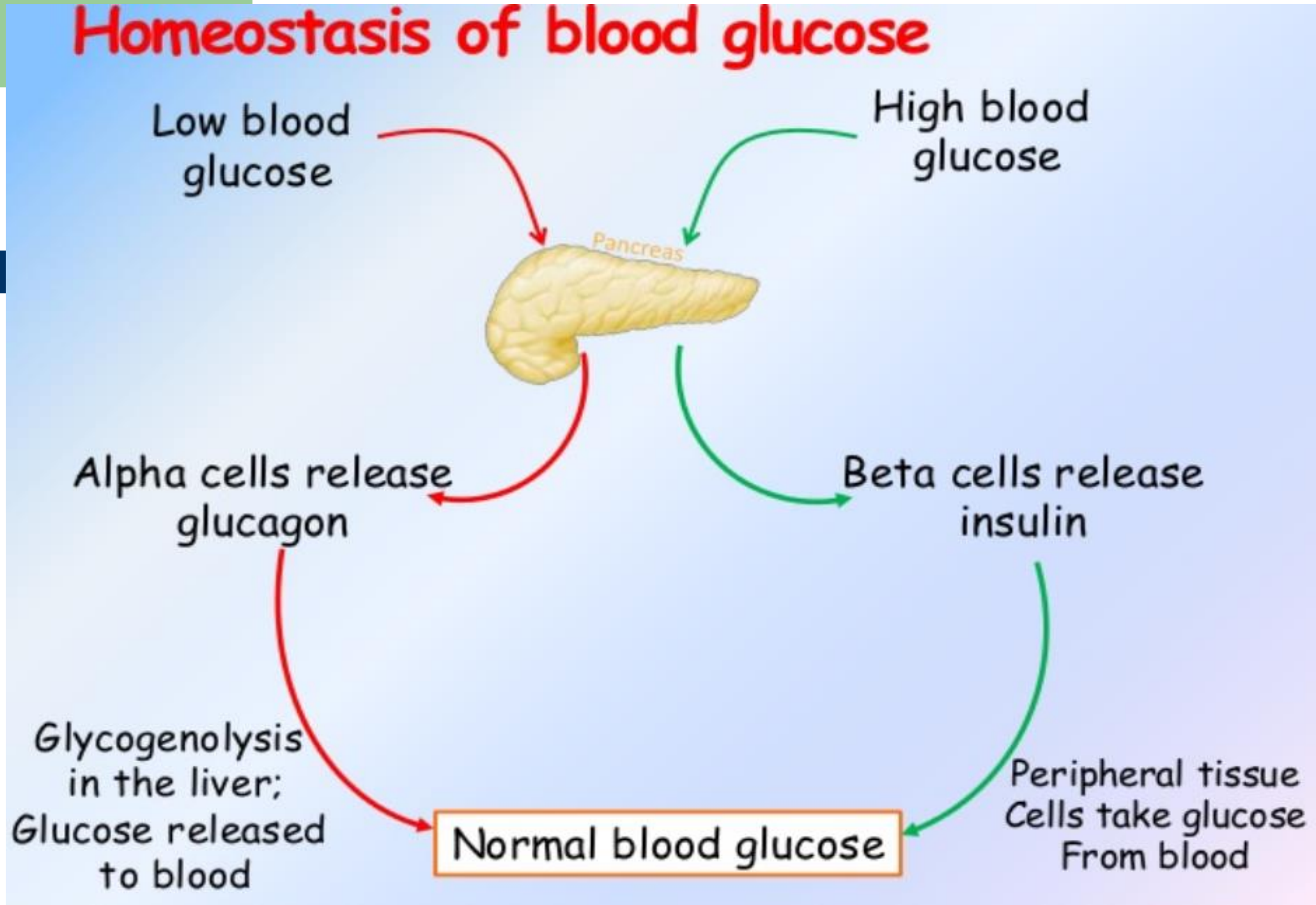
LAB EXPERIMENT 2

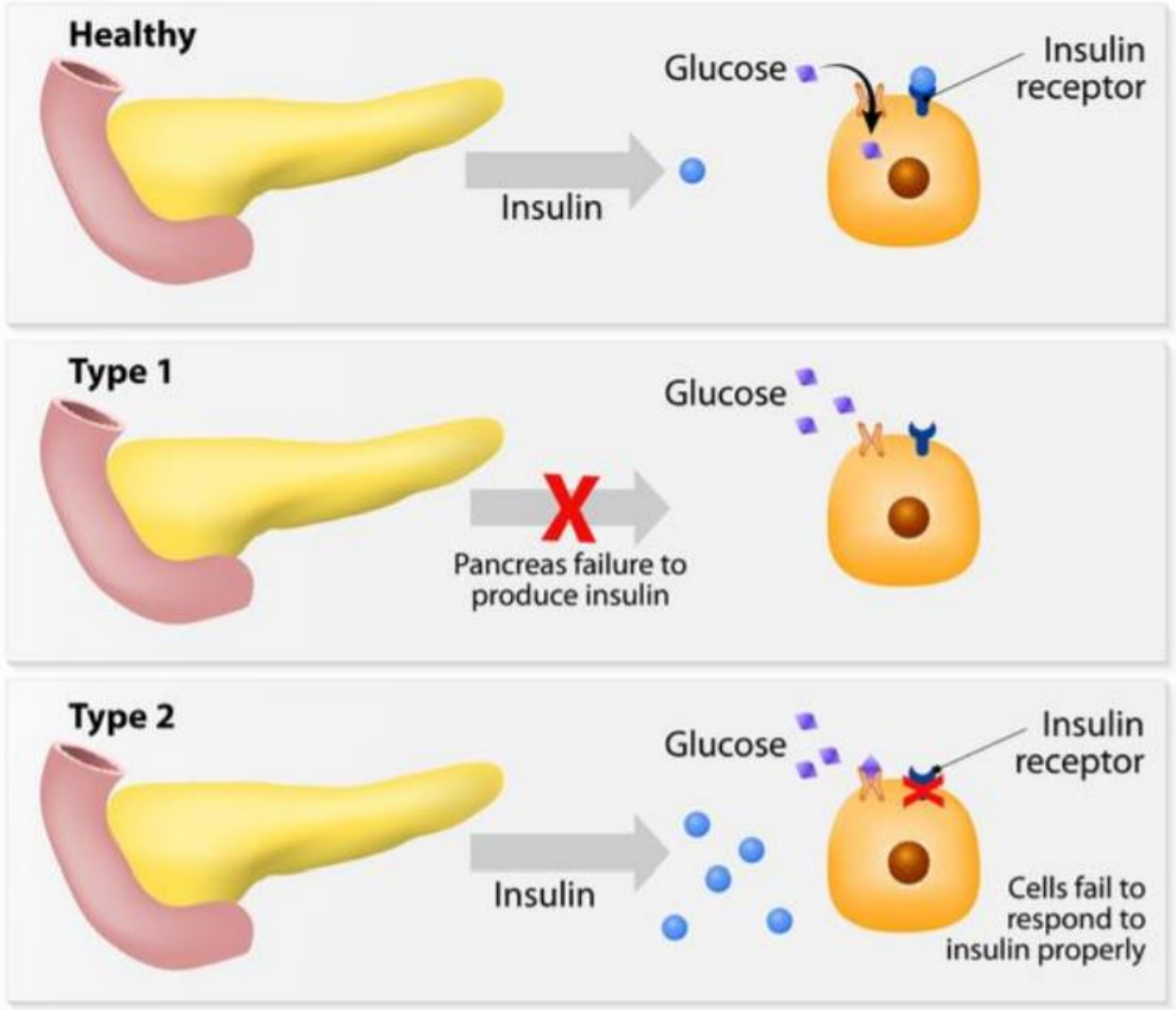
Dr. Nuha Nihad

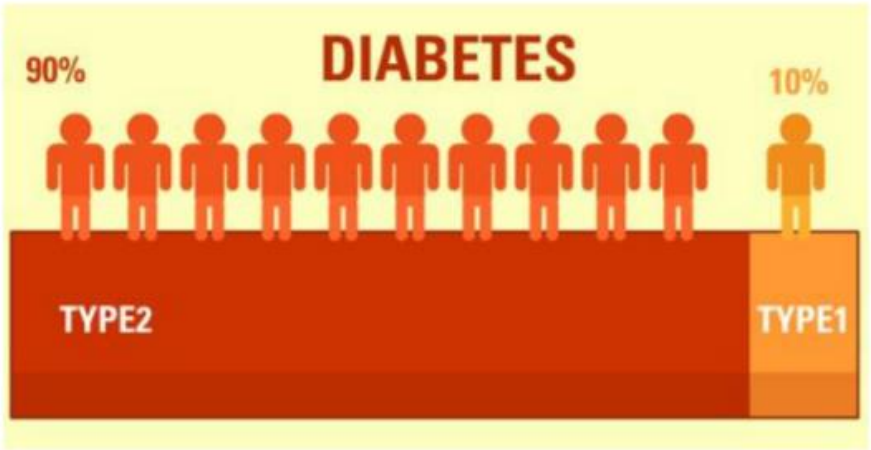
LETS LEARN SOME GREEK!!!!

The name glucose comes from the Greek word *glykys* (γλυκύς), meaning "sweet", plus the suffix "-ose" which denotes a sugar

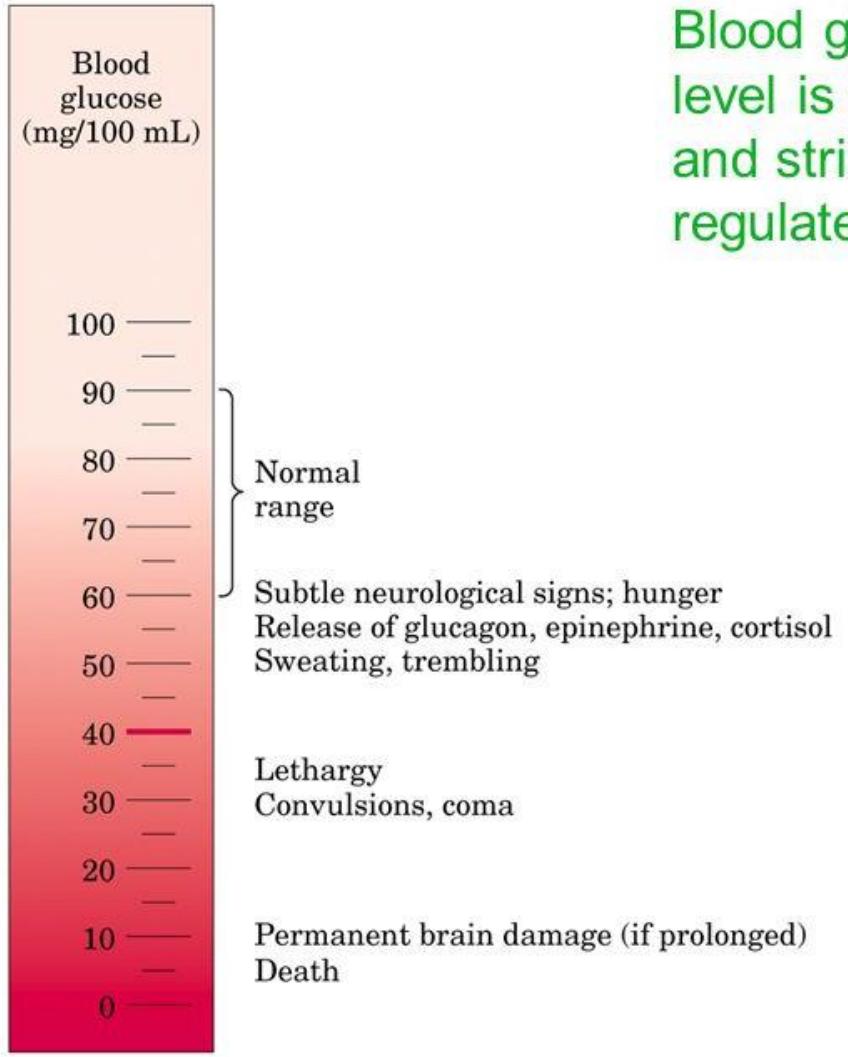

Blood Glucose Concentration
Insulin
Normal Range
Glucagon

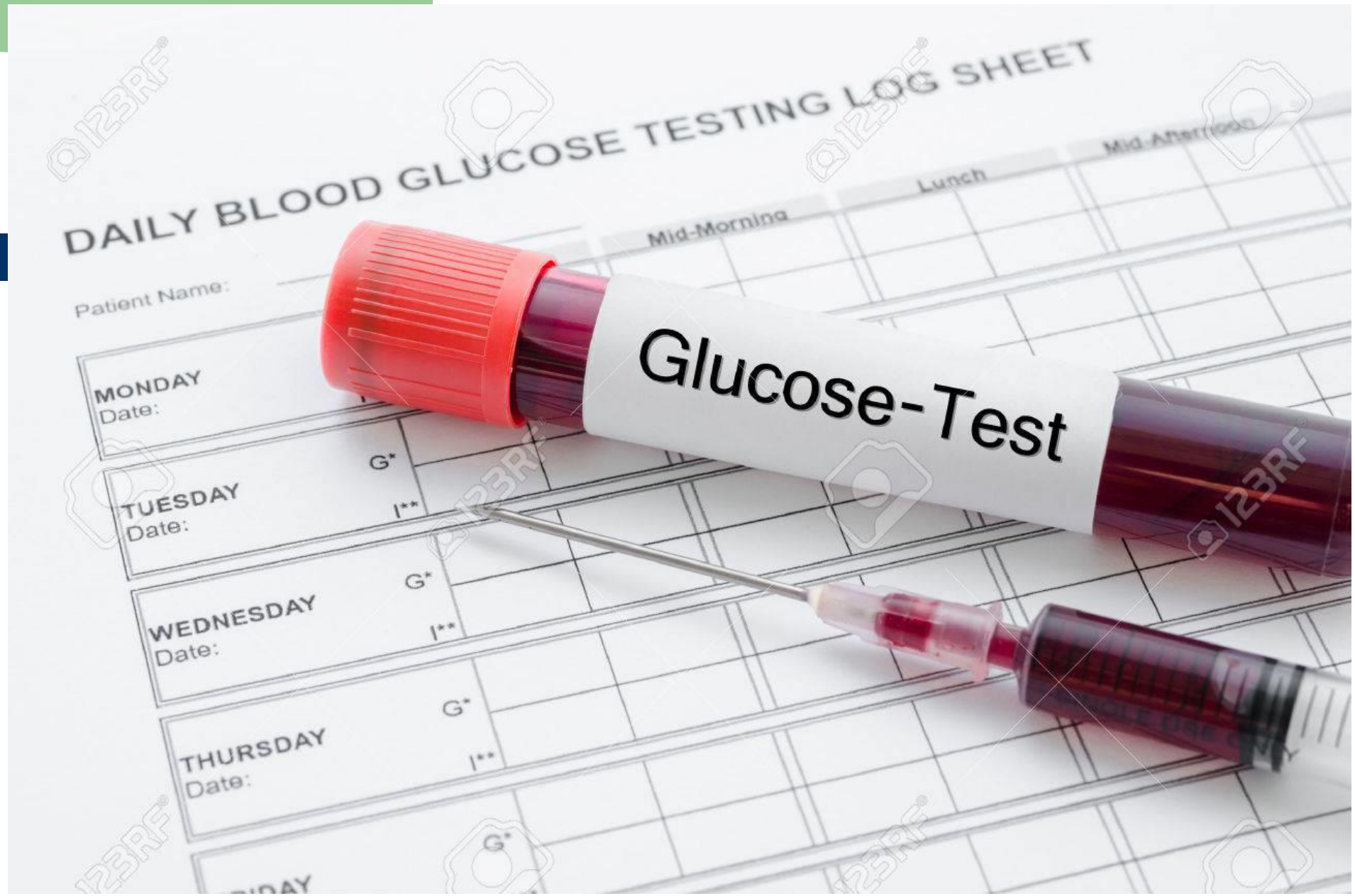

Homeostasis of blood glucose
Low blood glucose
High blood glucose
Pancreas
Alpha cells release glucagon
Beta cells release insulin
Glycogenolysis in the liver; Glucose released to blood
Normal blood glucose
Peripheral tissue Cells take glucose From blood


Healthy
Glucose
Insulin receptor
Insulin
Type 1
Glucose
X
Pancreas failure to produce insulin
Type 2
Glucose
Insulin receptor
Insulin
Cells fail to respond to insulin properly


DIABETES
90%
10%
TYPE2
TYPE1

Blood glucose level is critical and stringently regulated




DAILY BLOOD GLUCOSE TESTING LOG SHEET
Patient Name:
Mid-Morning
Lunch
MONDAY
Date:
TUESDAY
Date:
WEDNESDAY
Date:
THURSDAY
Date:
Glucose-Test

Whole blood left at room temperature

Glycolysis @ 7mg/dl/hr

(-)

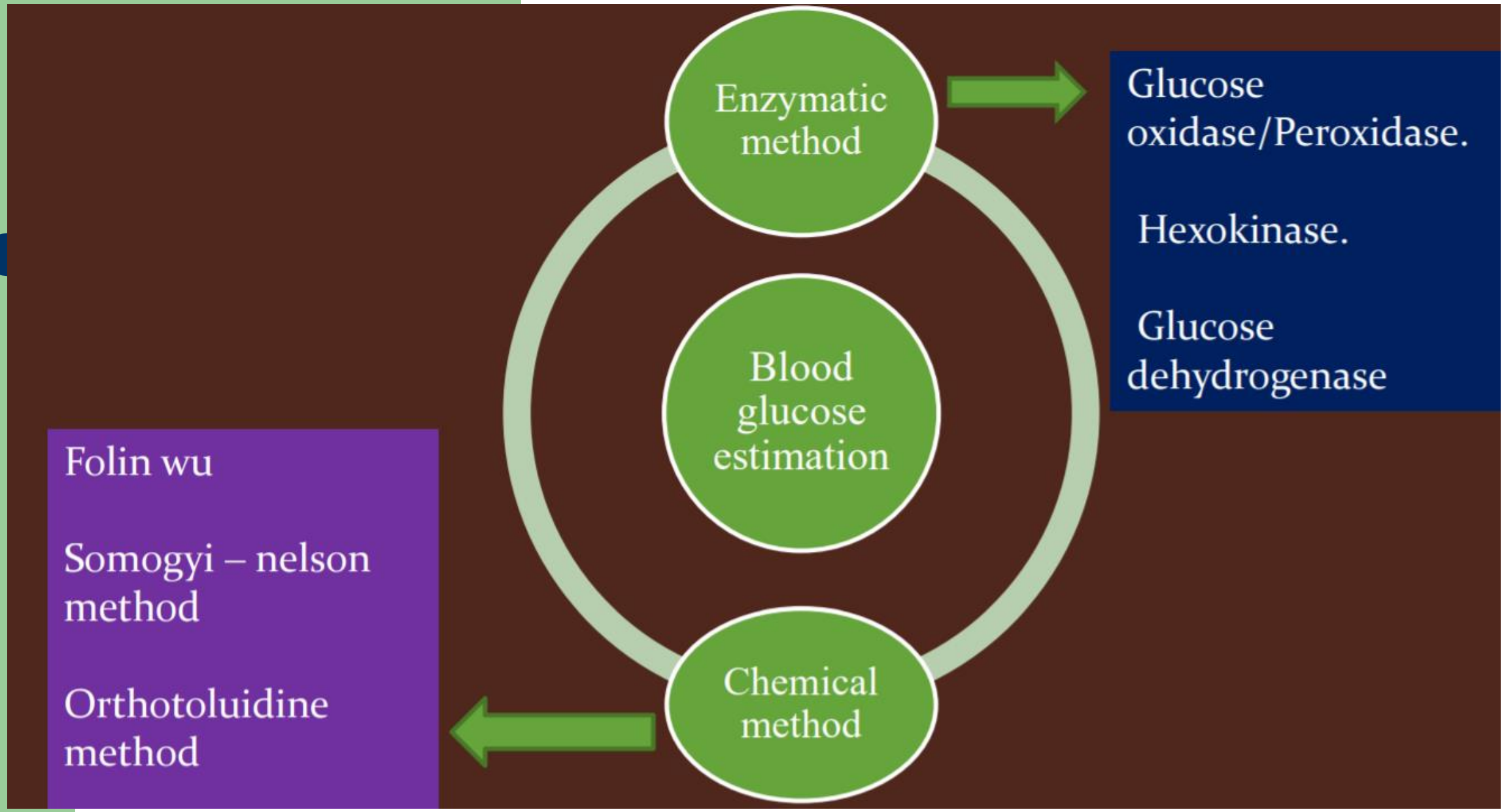
Sodium fluoride

(+)

Leucocytosis and bacterial contamination

BLOOD GLUCOSE ESTIMATION

- The blood is collected using an anticoagulant (potassium oxalate) and an inhibitor of glycolysis (sodium fluoride).
- Fluoride inhibits the enzyme, enolase, and so glycolysis on the whole is inhibited
- The **glucose oxidase** (GOD) method is the one most widely used.


Enzymatic method
Glucose oxidase/Peroxidase.
Hexokinase.
Glucose dehydrogenase
Blood glucose estimation
Chemical method
Folin wu
Somogyi – nelson method
Orthotoluidine method

GLUCOSE

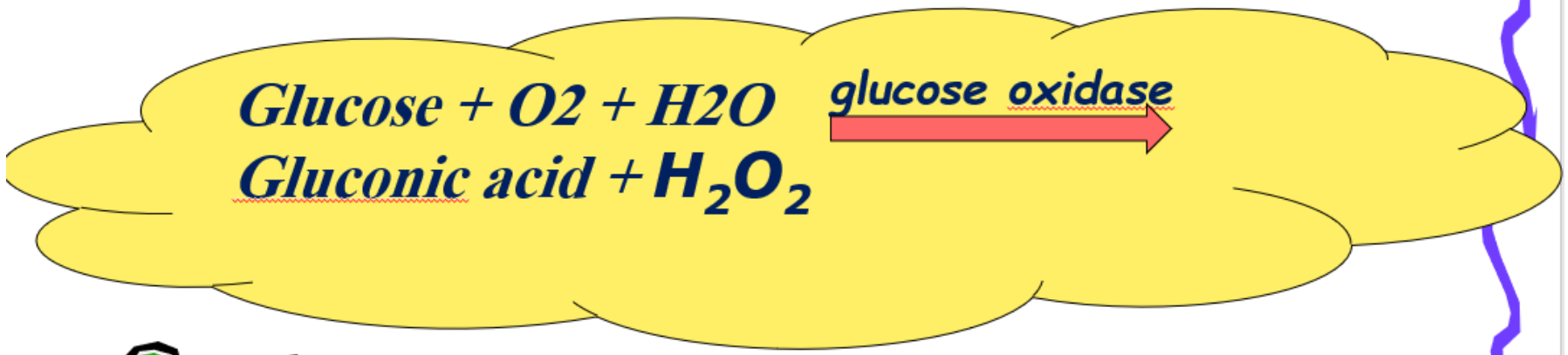
GOD-PAP

GLUCOSE GOD-PAP

Cat n°	Presentation	Main features	Principle
REF 80009	**R1** : 1 x 500 mL **R2** : 1 x 7.5 mL **R3** : 1 x 5 mL Insert	Excellent stability. Dry powder. Rapid and easy reconstitution (2 minutes). Standard enclosed in the kit. Linearity up to	TRINDER method (GOD-POD). Reagent for quantitative determination of glucose in human plasma, serum, cerebrospinal fluid (CSF) or urines. Reaction in 10 minutes at 37°C. Reading: End Point at 500 nm (460-560).

Principle of the glucose oxidase reaction

- Glucose is oxidized by glucose oxidase to form gluconic acid with liberation of hydrogen peroxide.



Principle of the glucose oxidase reaction

- Hydrogen peroxide is dissociated to water and oxygen atom by a peroxidase enzyme.
- The liberated oxygen is captured by a chromogen (a mixture of 4-amino antipyrine & phenol) which is converted to a red violet complex.

H_2O_2 + *Phenol + 4-amino antipyrine* $\xrightarrow{\text{peroxidase}}$ ***red colored product***

1. Reduction method, which is based on the ability of glucose to **reduce** Cu^{++} to Cu^{+} →less sensitive, →substances that could reduce Cu^{++} : fructose, galactose,vitamin C, creatinin, uric acid, glutathion, etc.
2. Enzymatic method (more specific and precise result) : Glucose is **oxidized** by glucose oxidase →gluconic acid + H_2O_2 → **red dye**.

Somogyi - Nelson

Glucose + Cu^{2+} >>>
Gloconate + Cu^{+}

pH > 7 , heat

Cu^{+} + arsenomolybdate >>>
blue molybdenium complex

λ = 660 nm

Folin - Wu

Glucose + Cu^{2+} >>>Gloconate + Cu^{+}

pH > 7 , heat

Cu^{+} + phosphomolybdate
Blue molybdenium complex

λ = 660 nm

TWO METHODS OF MEASURING BLOOD GLUCOSE LEVEL

1. Reduction method, which is based on the ability of glucose to **reduce** Cu^{++} to Cu^{+} →less sensitive, →substances that could reduce Cu^{++} : fructose, galactose,vitamin C, creatinin, uric acid, glutathion, etc.
2. Enzymatic method (more specific and precise result) : Glucose is **oxidized** by glucose oxidase →gluconic acid + H_2O_2 → **red dye**.

Folin - Wu

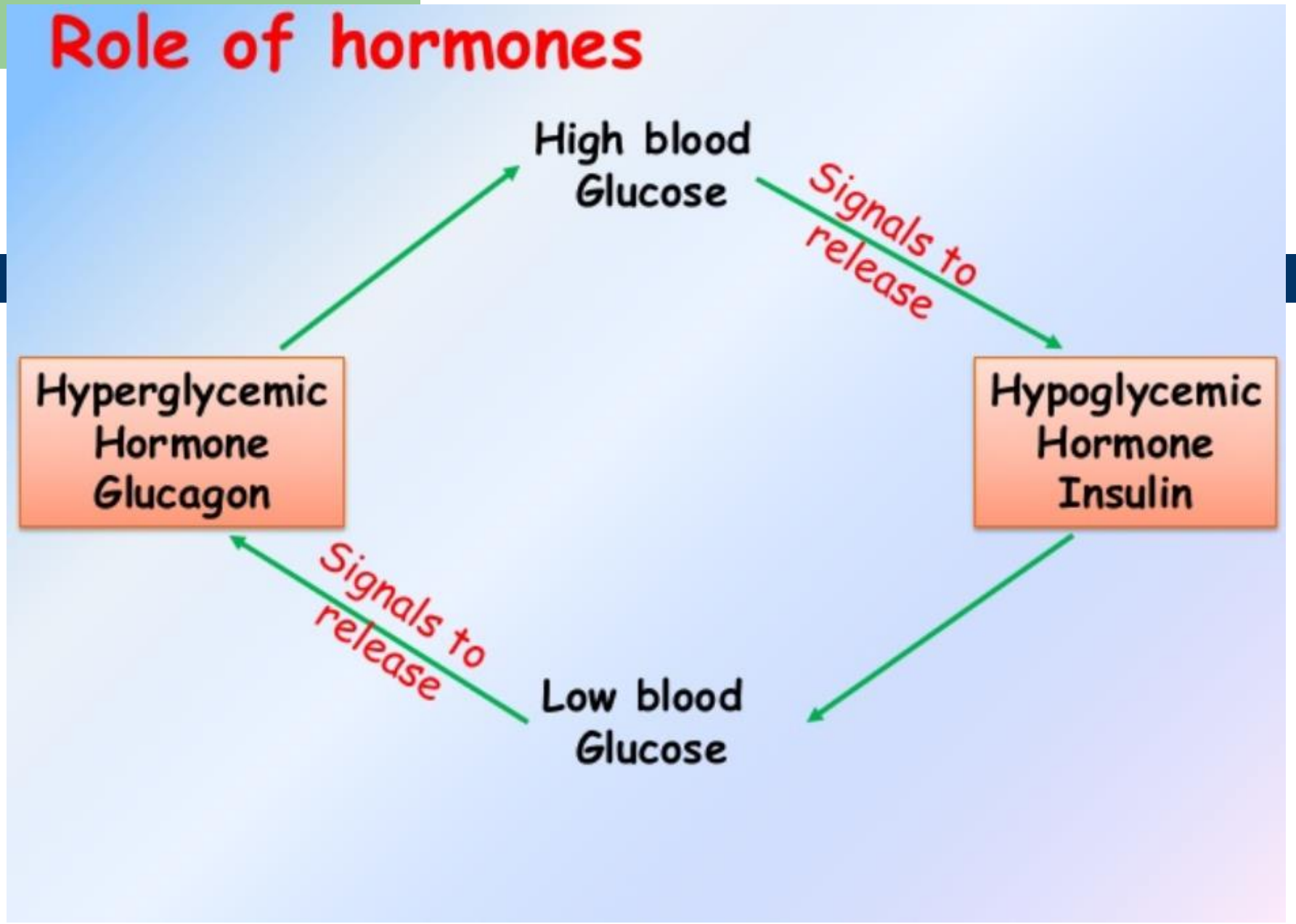
Glucose + Cu^{2+} >>>Gloconate + Cu^{+}

pH > 7 , heat

Cu^{+} + phosphomolybdate

Blue molybdenium complex

λ = 660 nm


Role of hormones
High blood Glucose
Signals to release
Hypoglycemic Hormone Insulin
Low blood Glucose
Signals to release
Hyperglycemic Hormone Glucagon

Hypoglycaemia Symptoms

low blood sugar

Hyperglycaemia Symptoms

high blood sugar

NORMAL VALUES- common terms

FASTING PLASMA GLUCOSE [FPG/FBS]	• A FBS is taken after at least eight hours of fasting
RANDOM PLASMA GLUCOSE [RBS/RPG]	• Test done any time of day without regard to time since last meal.
POST-PRANDIAL BLOOD GLUCOS [PPBS]	• The test done about 2 hr after a good meal

NORMAL VALUES

FASTING FBS	RANDOM RBS	POST-PRANDIAL PPBS
• 70-110mg/dL • 4-6.1mmol/L	• < 140 mg/dl • <7.8 mmol/l	• 140 mg/dL • 7.8 mmol/L

Blood Sugar Classification	Fasting Blood Sugar Levels	Post Meal Blood Sugar Levels
Normal	70-100 mg/dL	70-140 mg/dL
Prediabetes	101-125 mg/dL	141-200 mg/dL
Diabetes	125 mg/dL and above	200 mg/dL and above

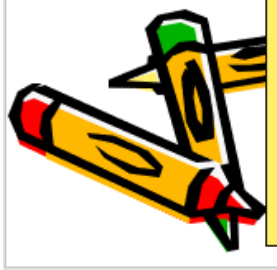
Oral Glucose Tolerence Test
OGTT

Definition :

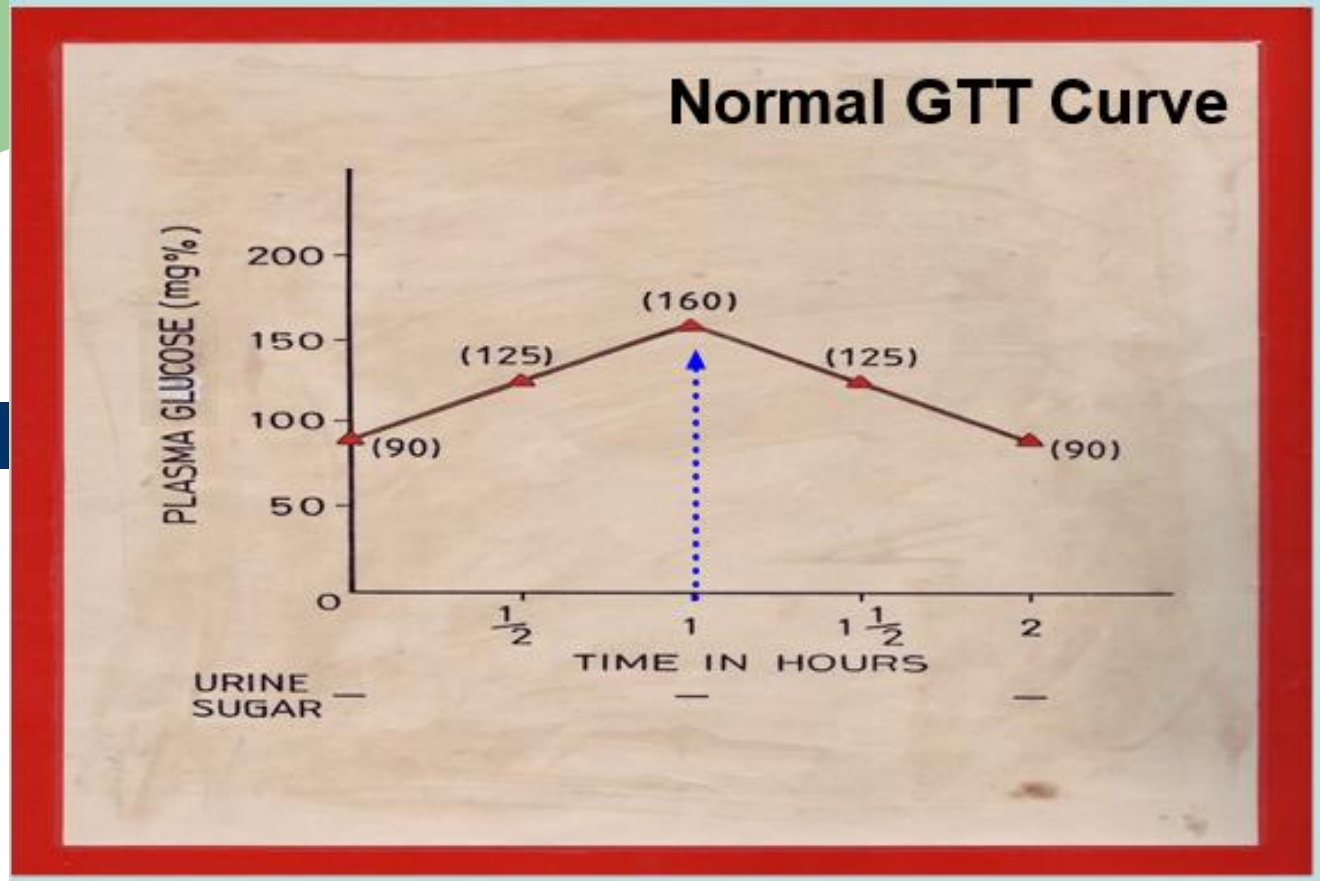
The administration of glucose to determine how quickly it is cleared from the blood

This patient was subjected to an OGTT and the following results were obtained:

	Fasting	1/2hour	1hour	1 1/2hour	2hour
Plasma Glucose %	100	140	170	165	160
Urine Glucose	-------	--------	-------	-------	-------



Draw the curve and comment on the results

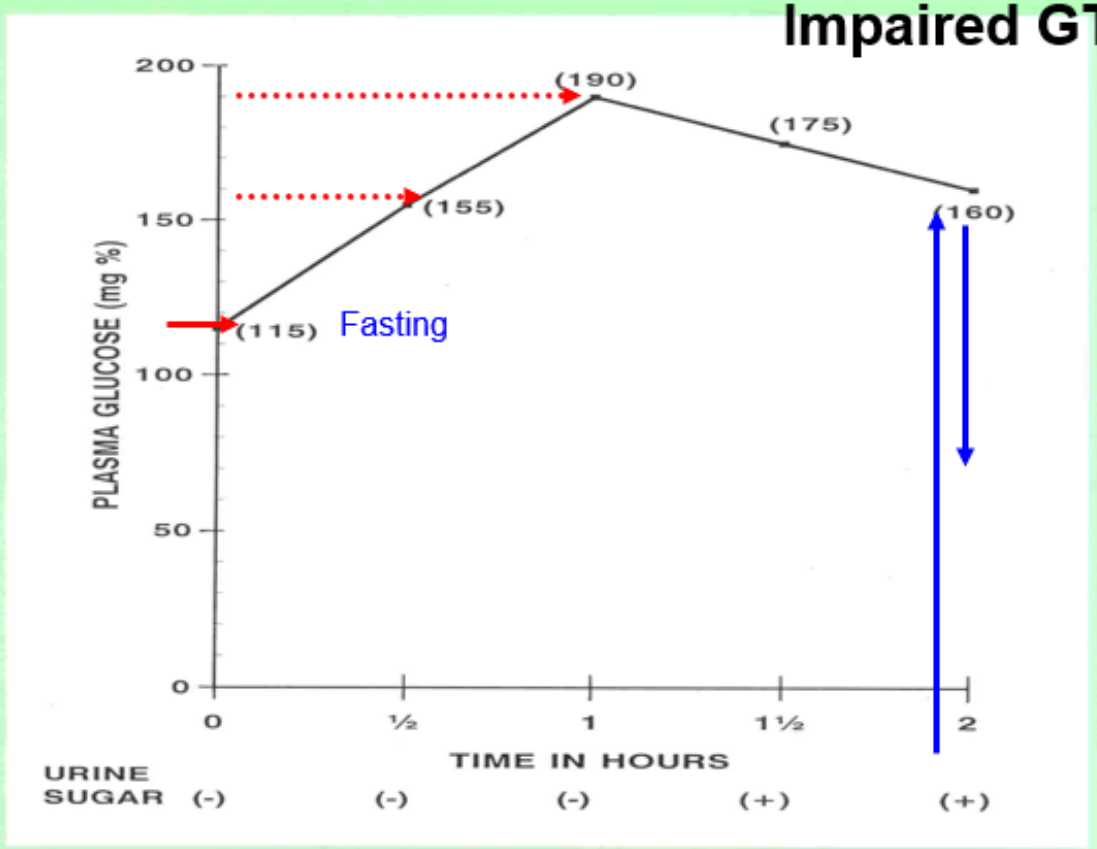


Benedict's Test

Features of a normal GTT curve:

1. Fasting level within normal (70-110mg/dL).
2. Peak value at 1 hour less than **"Renal threshold value"** (180mg/dL).
3. After 2 hours comes back to fasting value.
4. No sugar in the urine at any time.

Impaired GTT Curve

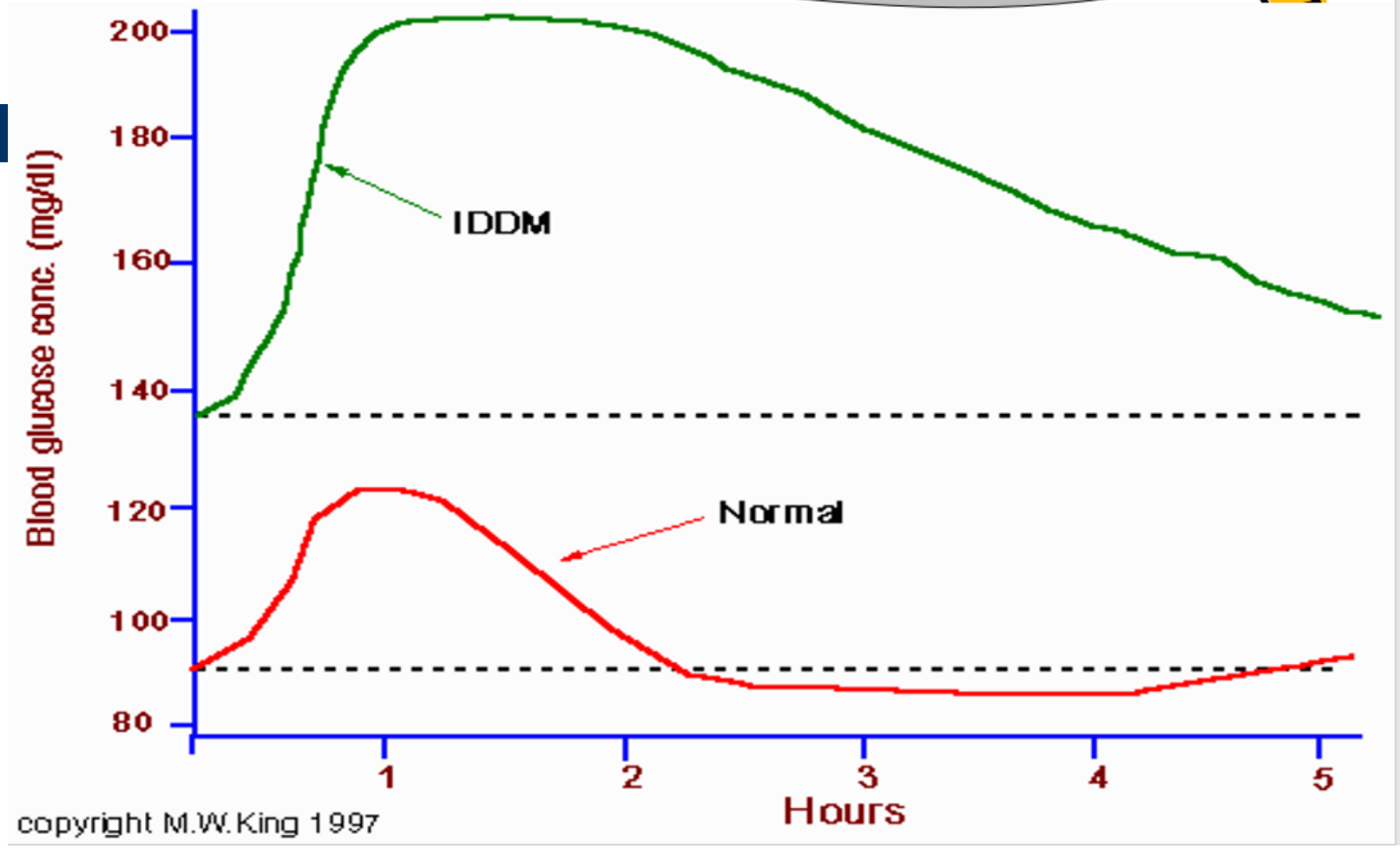


Benedict's Test

Features of Impaired Glucose Tolerance:

1. Fasting level above normal (115mg/dL).
2. Peak value at 1 hour more than renal thresh hold 190mg/dL.
3. After 2 hours does not come back to fasting value.
4. Sugar in the urine seen after the plasma value cross the renal thresh hold value.
5. Such patients may become diabetic after few years.

Interpretation of OGTT curve in normal & diabetic patients

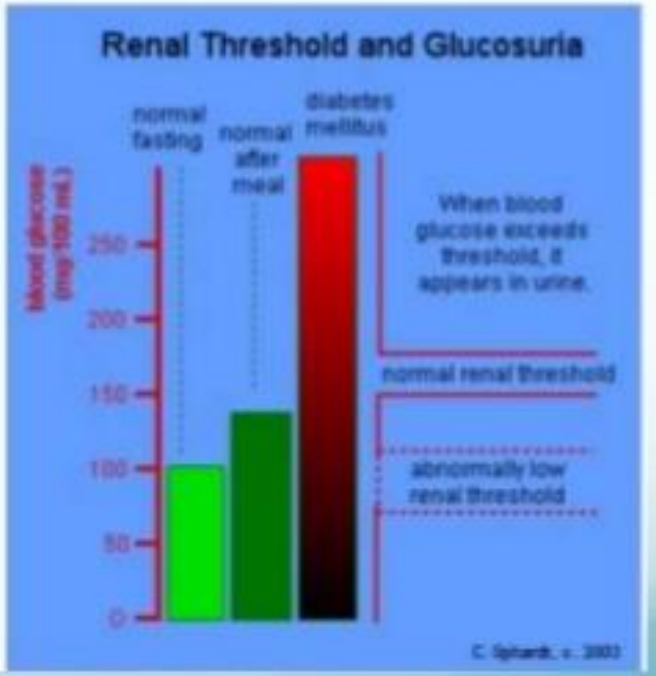


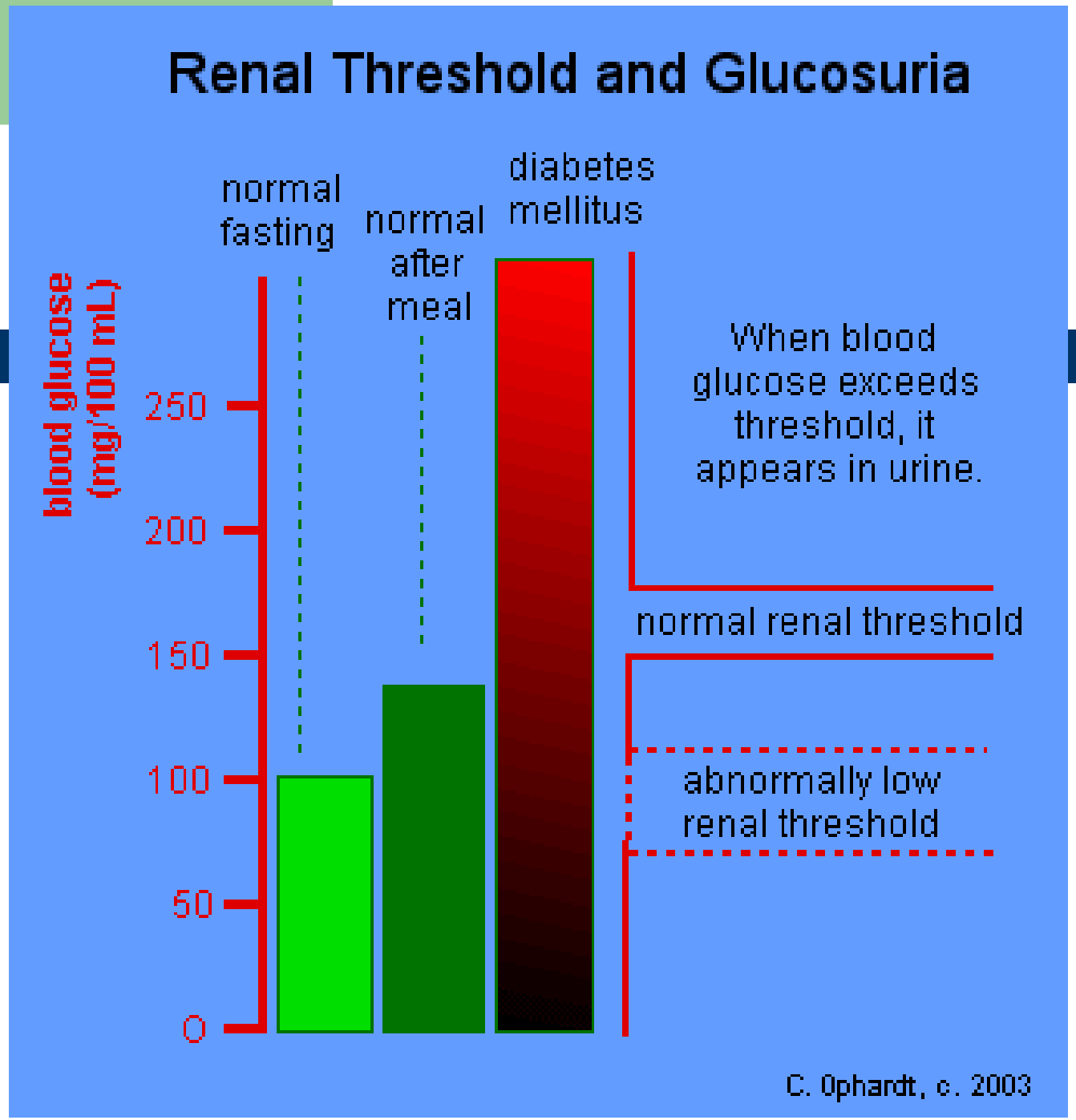
Glycosuria

What is Glycosuria?

Glycosuria

- Definition: abnormal excretion of glucose in the urine
- Causes:
  - Elevated blood glucose levels
    - Diabetes mellitus
  - Renal glycosuria
    - Problem with reabsorption of glucose in kidney tubules




Renal Threshold and Glucosuria
normal fasting
normal after meal
diabetes mellitus
blood glucose (mg/100 mL)
250
200
150
100
50
0
When blood glucose exceeds threshold, it appears in urine.
normal renal threshold
abnormally low renal threshold
C. Ophardt, c. 2003

Glycosuria

- Normally the urine contains about 0.05gm/dl of sugar.
- Which cannot be detected by Benedict's test.
- But under certain circumstance considerable amount of glucose or other sugar may be excreted in the urine.
- Excretion of detectable amount of sugar in the urine is known as **glycosuria**.
- Generally is called as "sugar in the urine".
- Glucosuria may different type....
  - Alimentary (lag storage) glucosuria
  - Renal glucosuria
  - Diabetic glucosuria.

Thank you

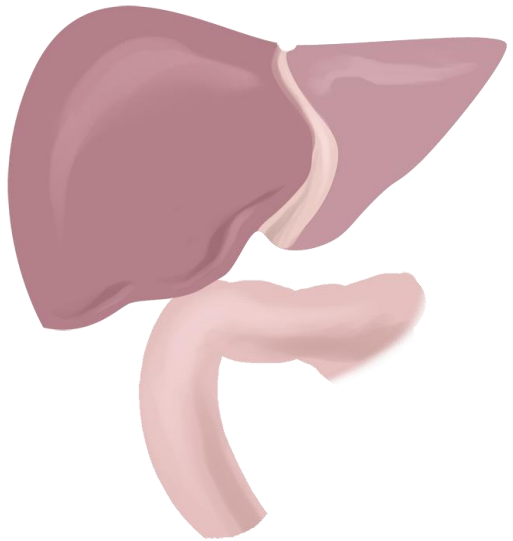
Renal function test
Blood urea.

LAB EXPERIMENT 3

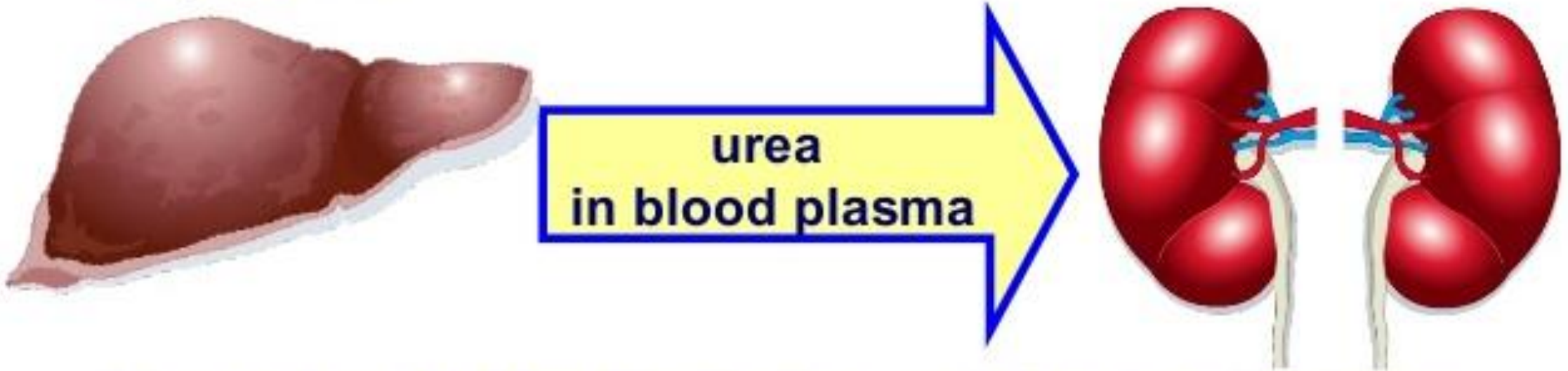
Dr. Nuha Nihad

What is urea?

Urea is the main end product of **protein** metabolism.
Removal of amino groups from amino acids, from which urea is formed takes place in the **liver**.
Urea represents about 50% of non-protein nitrogen (**NPN**) of normal blood.



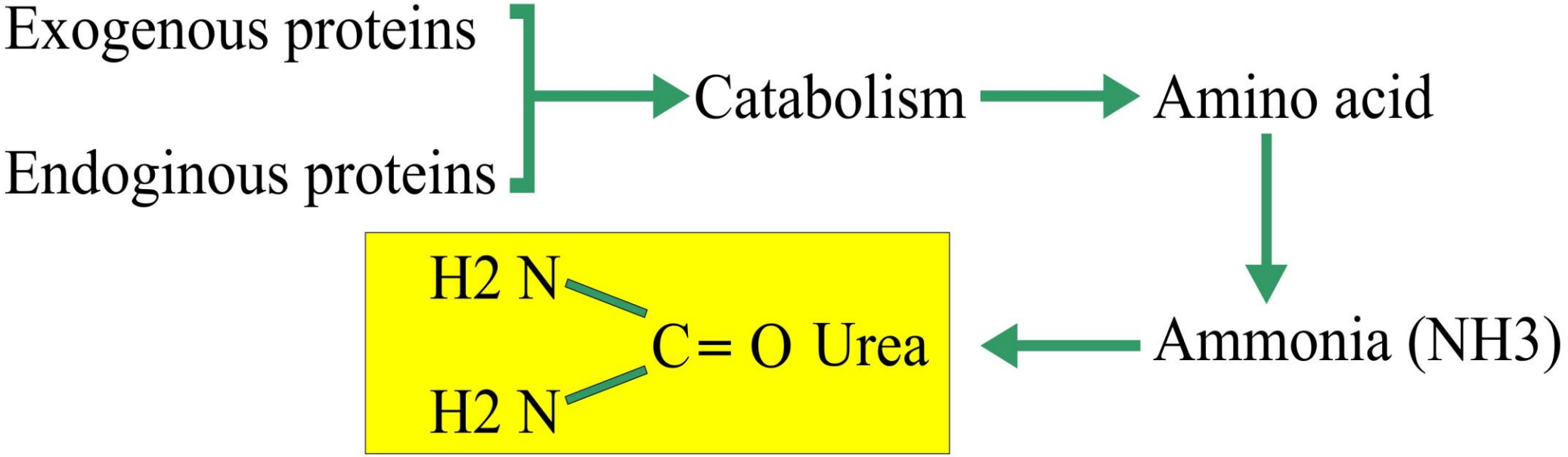
Urea is **toxic** to the body and must be removed as quickly as possible.

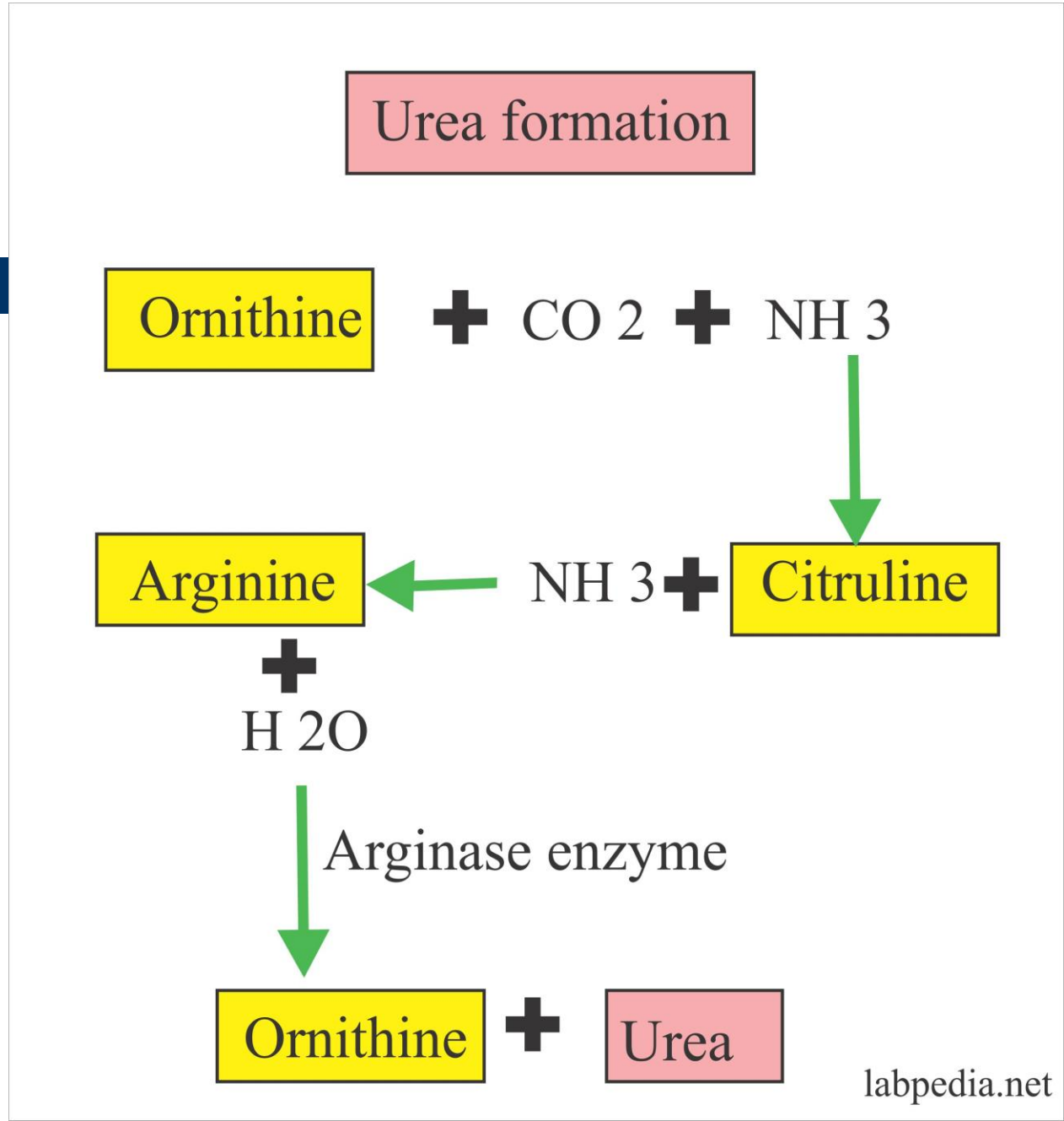


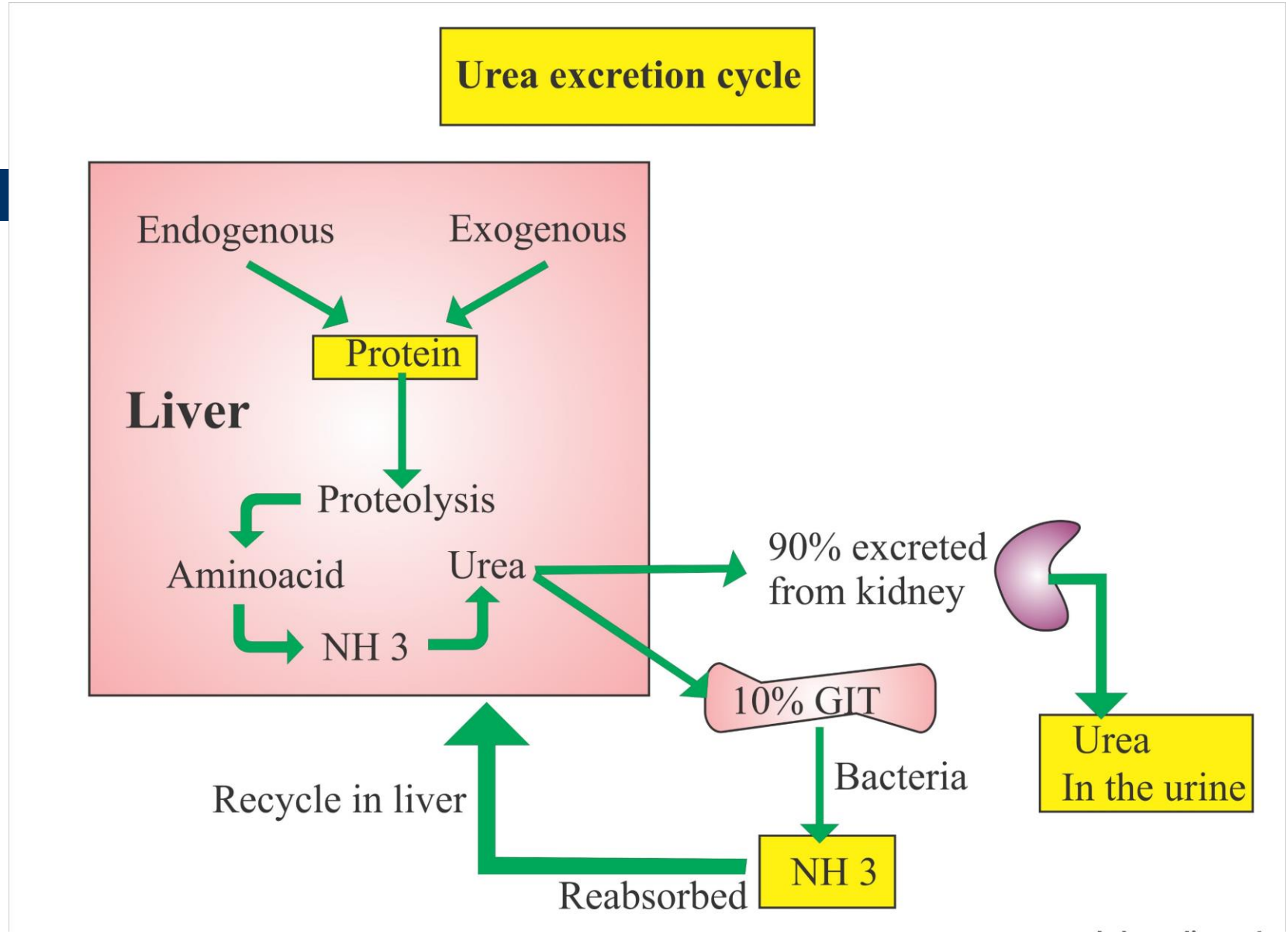
Urea enters the blood in the liver and dissolves in plasma. It is then carried from the **liver** to the **kidneys**.

The kidneys filter blood and remove urea from plasma. This waste product is then removed from the body in **urine**.

Formation of Urea

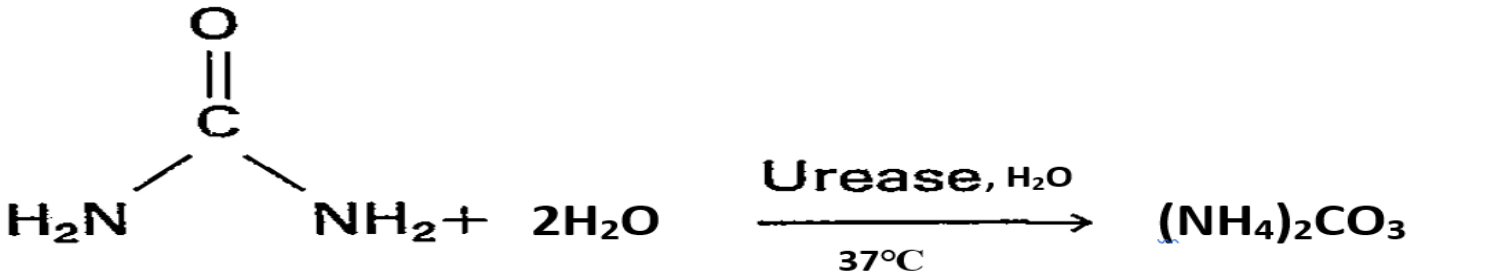



Urea formation
Ornithine
CO 2
NH 3
Citruline
NH 3
Arginine
H 2O
Arginase enzyme
Ornithine
Urea
labpedia.net


Urea excretion cycle
Endogenous
Exogenous
Protein
Liver
Proteolysis
Aminoacid
Urea
NH 3
90% excreted
from kidney
10% GIT
Urea
In the urine
Bacteria
Recycle in liver
NH 3
Reabsorbed

Method for quantitative measuring of urea are based on colorimetric and spectrophotometric analyses

(**urease** catalyzes the hydrolysis of urea to ammonium carbonate by the reaction)



$$(NH_4)_2CO_3 \xrightarrow{OH^-} 2NH_4OH + CO_3^-$$

Estimation of ammonia by Nessler’s method

- Principle:

The Nessler’s reagent is an alkaline solution of potassium mercuric iodide.

- The reaction between **Nessler’s reagent** and **ammonia** may be represented as:

$$2(KI)2HgI_2 + NH_4OH + KOH \longrightarrow NH_2Hg_2I_3 + 5KI + NH_4I$$

Amm.Dimeric iodide

(Yellow orange)

A known amount of sample was treated with Nessler’s reagent which produces a yellowish brown color.

The intensity of the color is directly proportional to the amount of ammonia originally present.

The main difficulty with Nessler method is the **turbidity**. **Zinc hydroxide** is the preferred deproteinizing agent, since it eliminates the small amounts of turbidity.

Normal blood contains 15-40 mg urea/100ml. Up to 500mg/100ml or even **higher values** are found in conditions associated with **impaired renal function**.

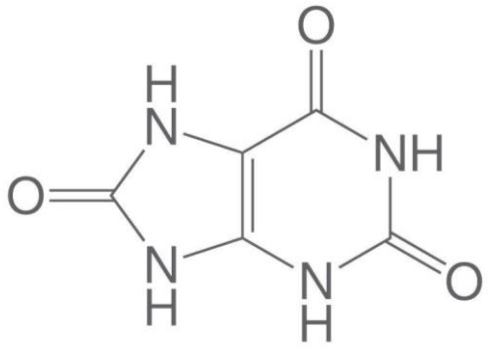
Thank you

Uric acid

LAB EXPERIMENT 4

Dr. Nuha Nihad

Uric Acid



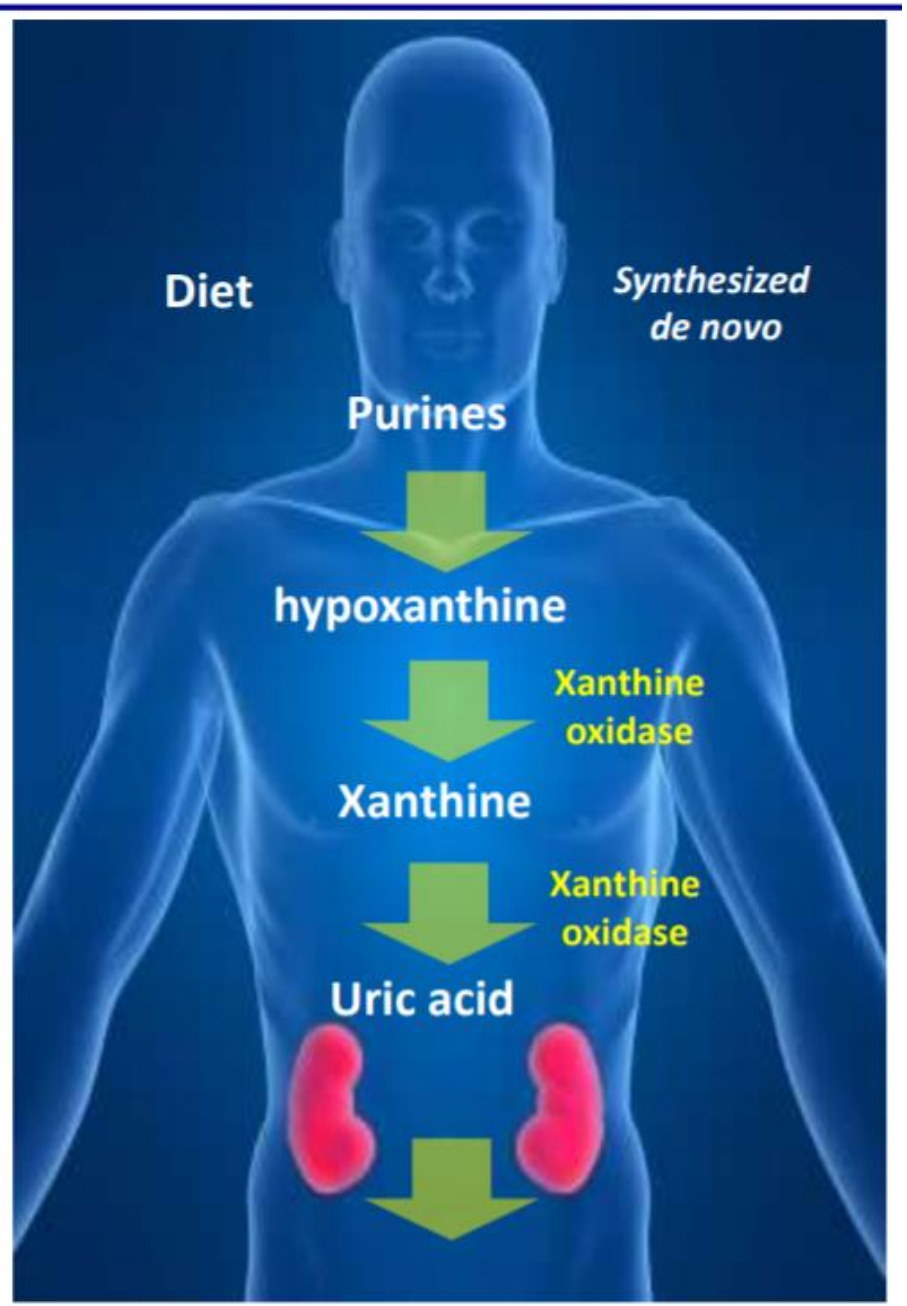
- Uric acid is formed from the breakdown of **nucleic acids** and is an end product of **purine** metabolism.
- Uric acid is transported by the plasma from the liver to the kidney, where it is filtered and where about 70% is excreted in Urine.
- The remainder of uric acid is excreted into the GI tract.

Uric acid

Uric acid is the final breakdown product of purine degradation in humans .

Uric acid is synthesized from compounds containing purines, and it is a waste product derived from purines of the diet such as liver, thymus, and organ meat.

Uric acid is mainly excreted in urine by glomerular filtration. A part of it is reabsorbed by the renal tubules



Formation of uric acid

- **Endogenously** → **nucleoproteins metabolism**
- **Exogenously** → **purines metabolism**

Normal Rang

- 2-7mg/dL

Interpretation of the result

If the result was at Normal value, the interpretation will be :

This patient has Normal Level of uric acid.

If the result was Higher than normal value, the interpretation will be :

This patient has Hyperuricemia

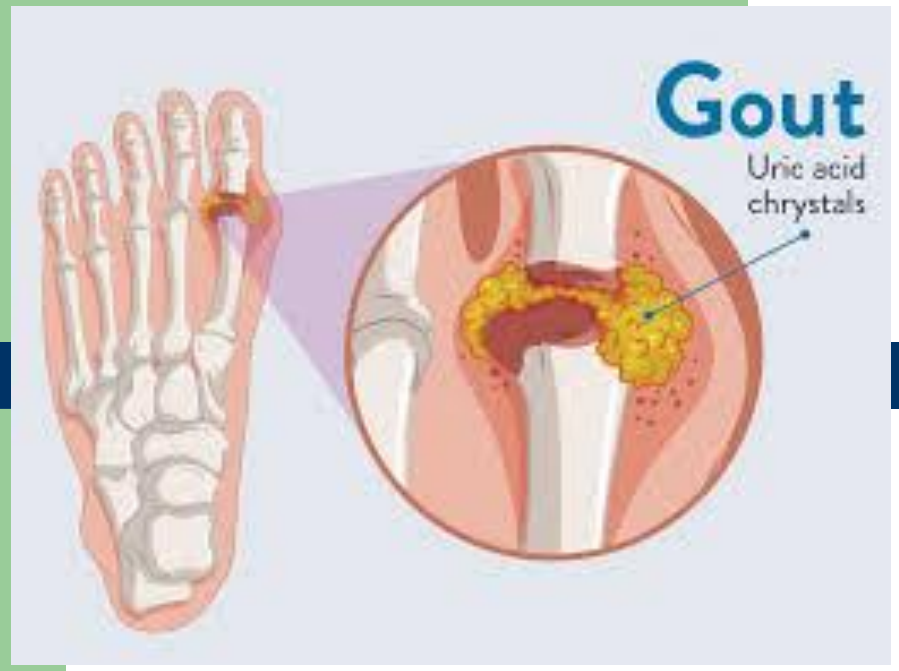
If the result was Lower than normal value, the interpretation will be :

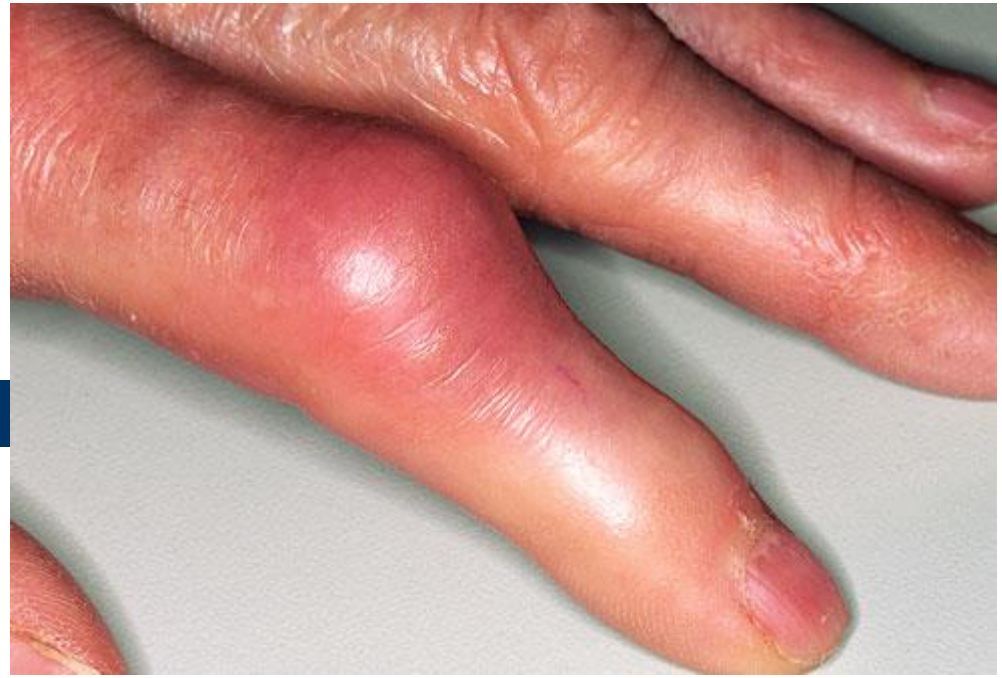
This patient has Hypouricemia

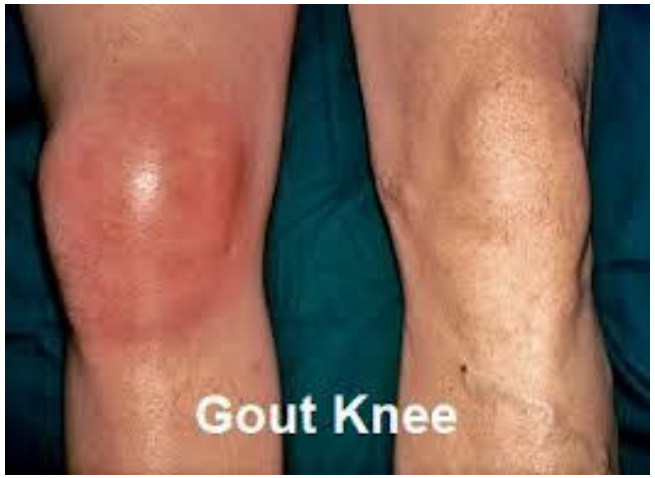
GOUT

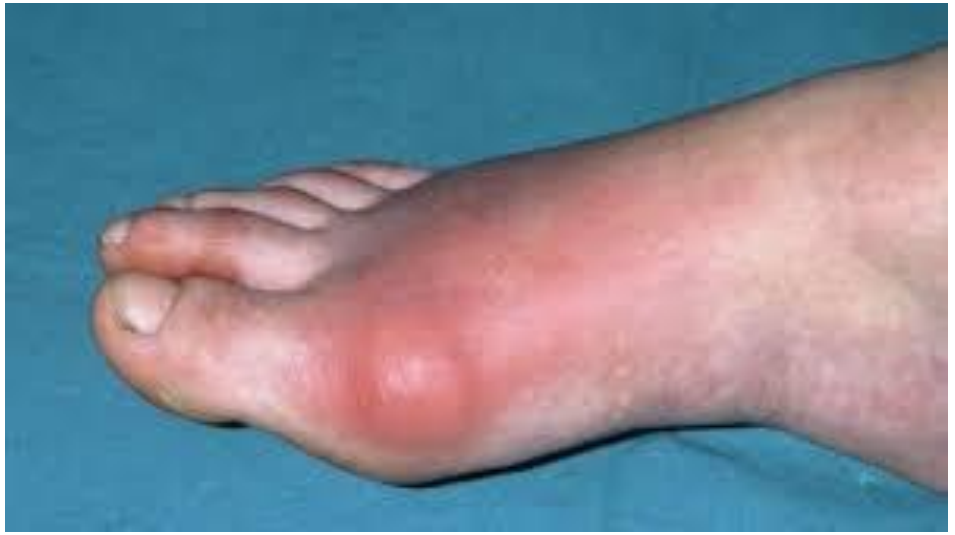
"The disease of kings" or "Rich man's disease".

In gout the blood levels of uric acid are increased and abnormal deposition of uric acid crystals occur in joints, tendons bone leading to painful condition of these structures.


Gout
Uric acid
chrystals




Gout Knee



Specimen

- Serum or plasma may be used
- Stability in serum / plasma:
  - 6 months at -20°C
  - 7 days at 4-8°C
  - 3 days at 20-25°C

ANALYTIC METHODS—URIC ACID

- **Chemical Method (old) :**
  - **Phosphotungstic Acid, read the absorbance(Ab) at 700nm (UV). blue Colored product**
- Enzymatic Method: is More specific
  - By using Couple reaction of uricase and Peroxidase. Pink solution (Ab at 500nm spectrophotometric) Pink color solution.

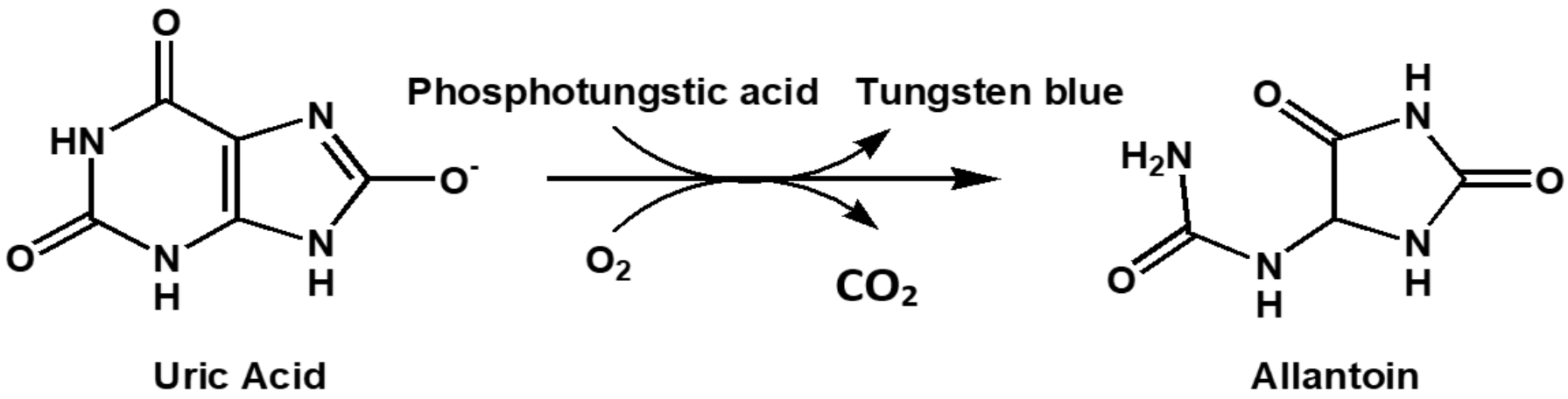
Chemical Method

Henry-Caraway – Phosphotungstic acid method

- Principle : Uric acid in the protein free filtrate reacts with **phosphotungstic reagent** ($H_3PW_{12}O_{40}$) in the presence of sodium carbonate (alkaline medium) to form a blue colored complex. The intensity of the color formed is directly proportional to the concentration of uric acid present in the sample.

Measuring uric acid

- Uric acid is oxidized to **allantion** and carbon dioxide by a phosphotungstic acid regent in alkaline solution
- Phosphotungstic acid is reduced in this reaction to **tungsten blue** which is measured at 700 nm.
- Protein have been removed by precipitation with tungstic acid.



Uric acid is a reducing agent in strong alkaline condition. It reduces colorless phosphotungstic acid to tungsten blue.

Thank you

Plasma creatine and creatinine

LAB EXPERIMENT 5

Dr. Nuha Nihad

Creatine & Creatinine

Creatine :

Creatine is a nitrogenous organic acid, synthesized primarily in the liver,

then transported to the muscle and converted to creatine phosphate, where it serves as high-energy source

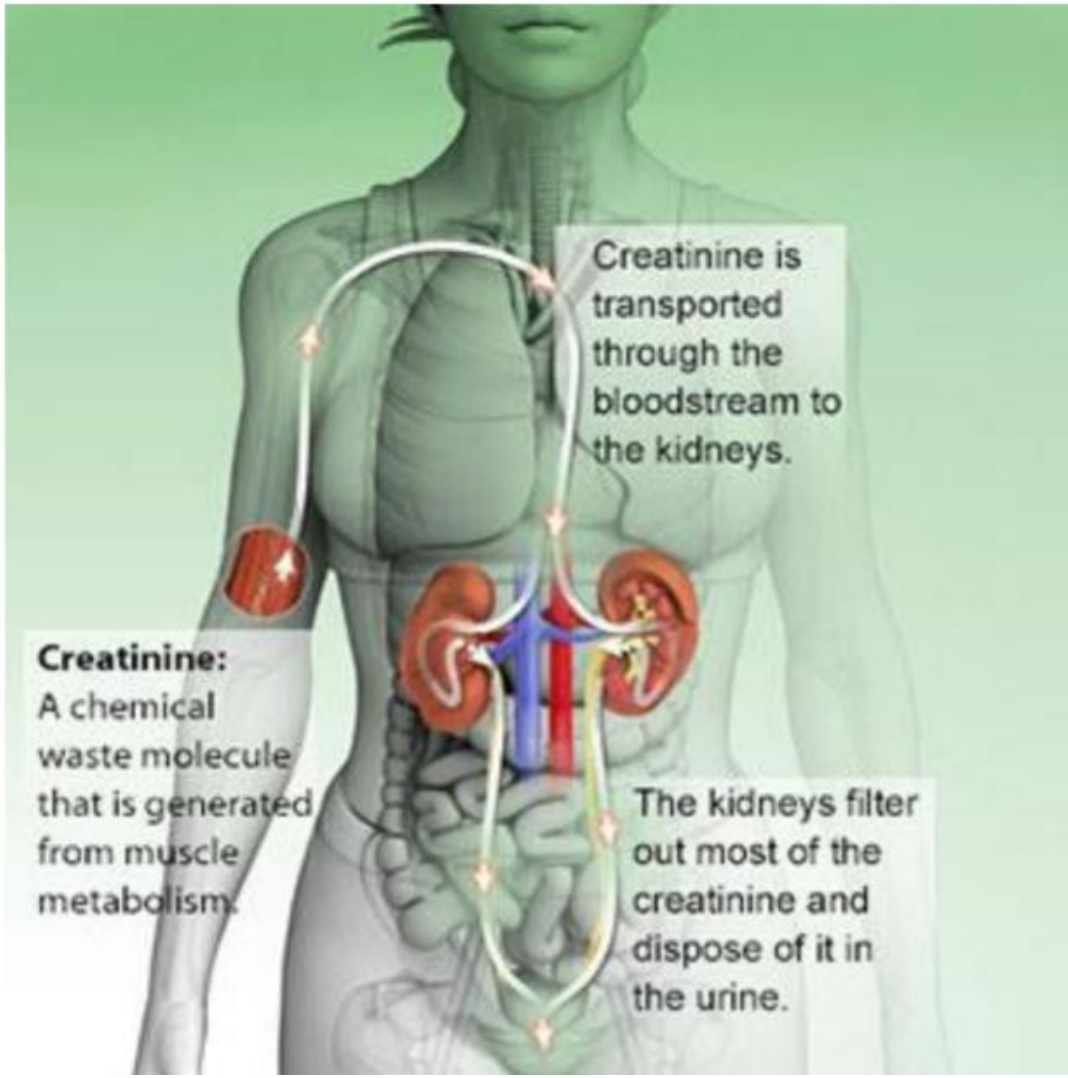
Creatinine :

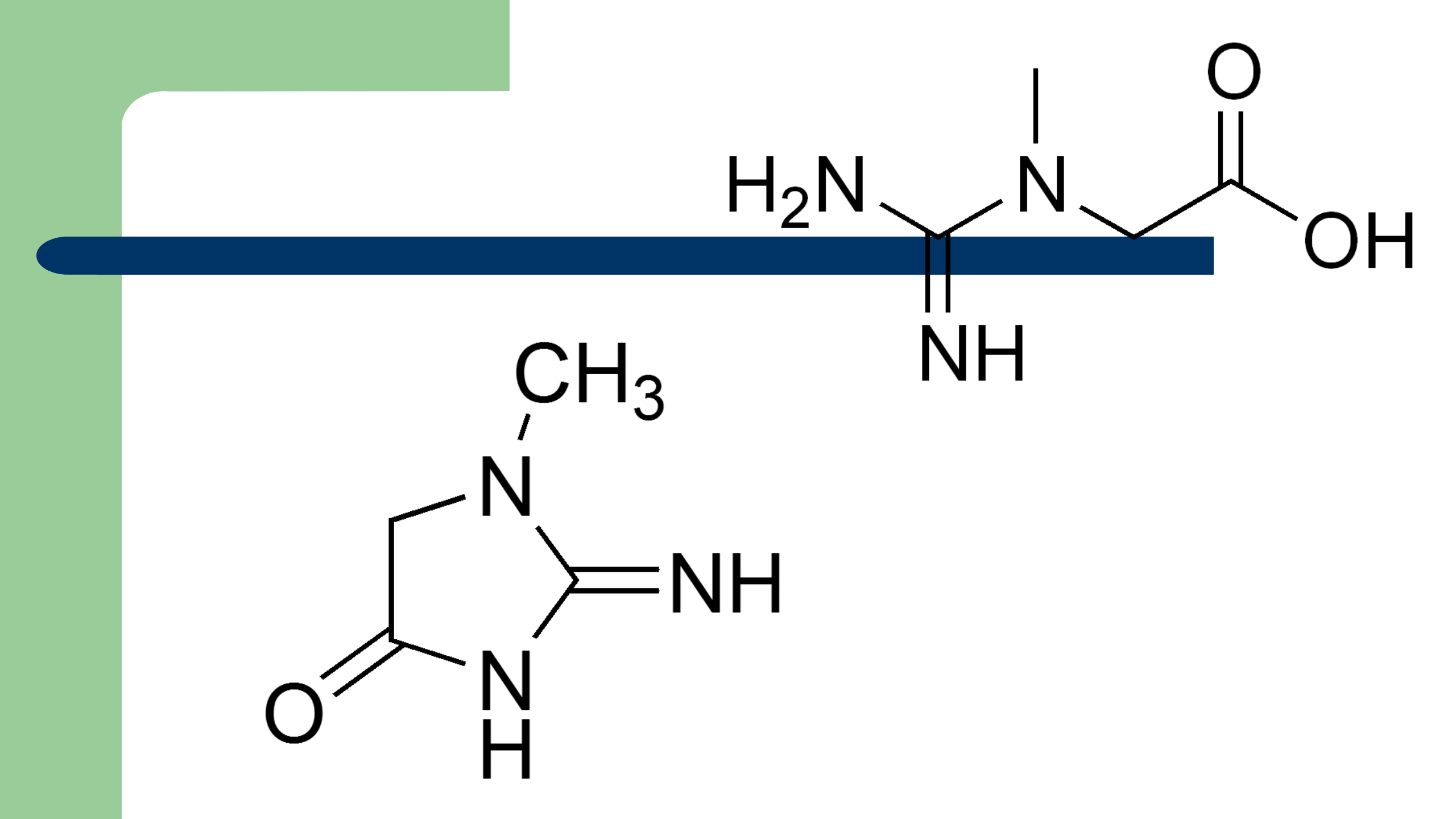
Creatinine is a chemical waste molecule that is generated from muscle metabolism, and it is produced from creatine.

Reference ranges :

Male : 0.9 – 1.3 mg/dl

Female : 0.6 – 1.1 mg/dl




H2N
N
O
OH
NH
CH3
N
NH
O
N
H

Significance

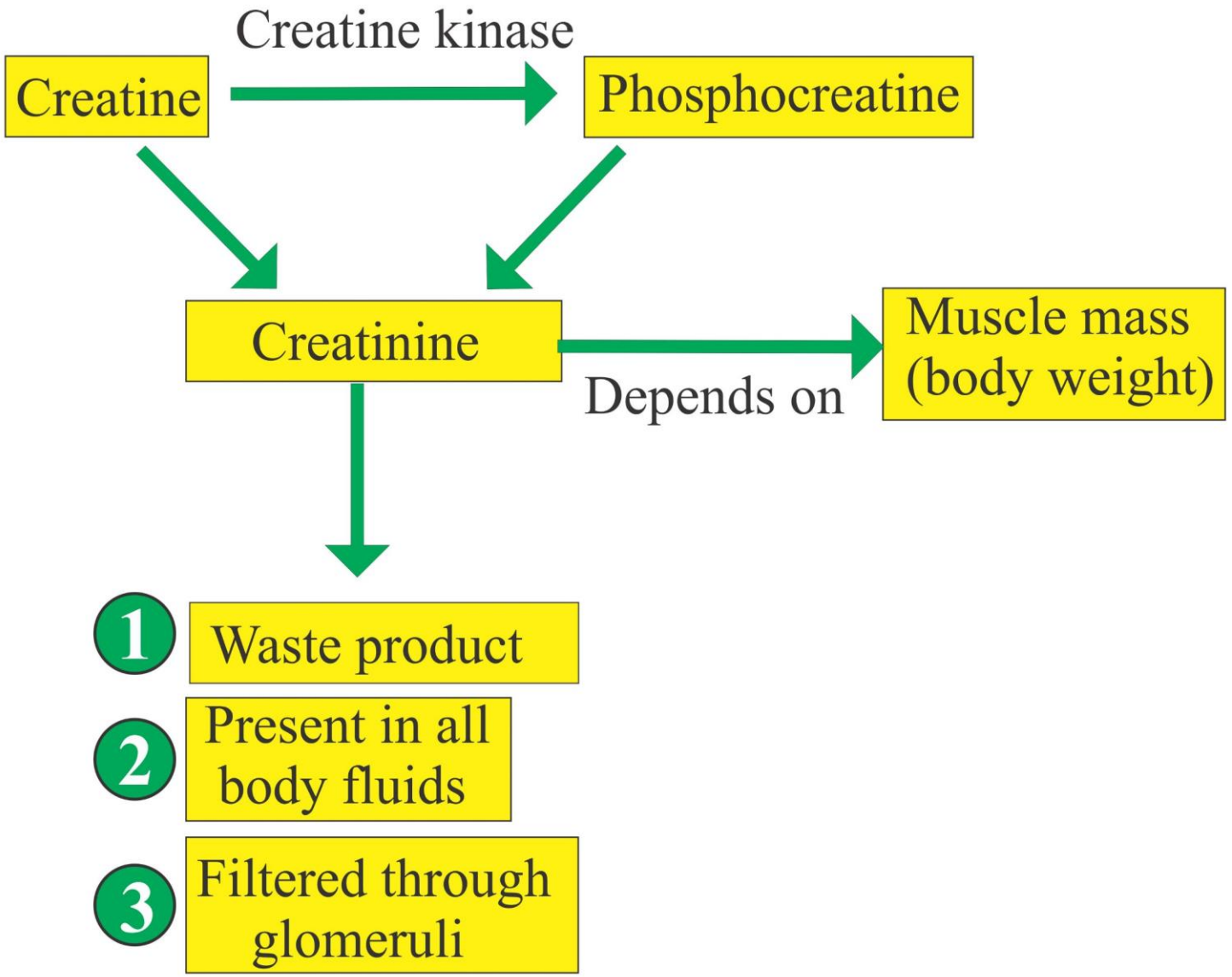
- **Creatine**

Creatine is very important for the body. It is a natural compound created in the body and is obtained from the diet through meat products.

- **Creatinine**

Creatinine does not perform any vital function in the body..
However, Creatinine levels in the blood can help your doctor assess how well your kidneys are working.

Creatinine Formation



Specimen

- One can analyze serum, plasma, or diluted urine.
- The common anticoagulants (fluoride and heparin) do not cause interference, though heparin, which can be formulated as the ammonium salt, must be avoided in enzymatic methods that measure ammo production.
- Storage
  - 7 days at 4-25^{o}C
  - At least 3 months at -20^{o}C

METHODS

- Colorimetric: **Jaffe Reaction**

NaOH

creatinine + picric acid → (reddish-orange complex)

alkaline picrate

  - Noncreatinine chromagens (color-producing substances) such as glucose, ketones and vitamin C may interfere with this reaction
  - Jaffe KINETIC method modification was designed to monitor the rate of the reaction at selected time intervals to avoid picking up noncreatinine chromagens. (Typically 25 – 60 seconds)
- Enzymatic: Creatinine aminohydrolase
  - A variety of coupled enzymatic methods are available.

Jaffe reaction Method

Sodium picrate in alkaline solution is added to plasma filtrate.

1- Plasma creatinine:

Creatinine levels are measured by a reaction between creatinine and sodium picrate to form creatinine picrate, which is red in color.

Creatinine + alkaline picrate → Creatinine-picrate complex
(red -orange)

2- Plasma creatine:

Creatine levels are usually measured by difference in the creatinine before and after conversion to creatinine by acid and heat.

CLINICAL INTERPRETATION

INCREASES

- Any renal factors
- Creatinine is NOT affected by diet
- Creatinine is NOT typically reabsorbed by the tubules
- Creatinine levels reflect the glomerular filtration rate (GFR)

DECREASES

- No medical significance

CLINICAL INTERPRETATION

- Creatinine is more specific than BUN since it is not affected by nonrenal factors.

- **Remember:** Creatinine serum levels are not above normal until ½ to ¾ of kidney function is lost.

Explain why this test provides more sensitive measure of renal damage than blood urea?

Because renal damage is the only cause of creatinine increasing levels.

Clinical correlation of Creatinine

Creatinine concentration Measurement :

Is used to determine the sufficiency of kidney function & severity of kidney damages.

Creatinine clearance test :

Is a test that compares the creatinine level in urine with its level in blood,

and it is used to assess the renal function.

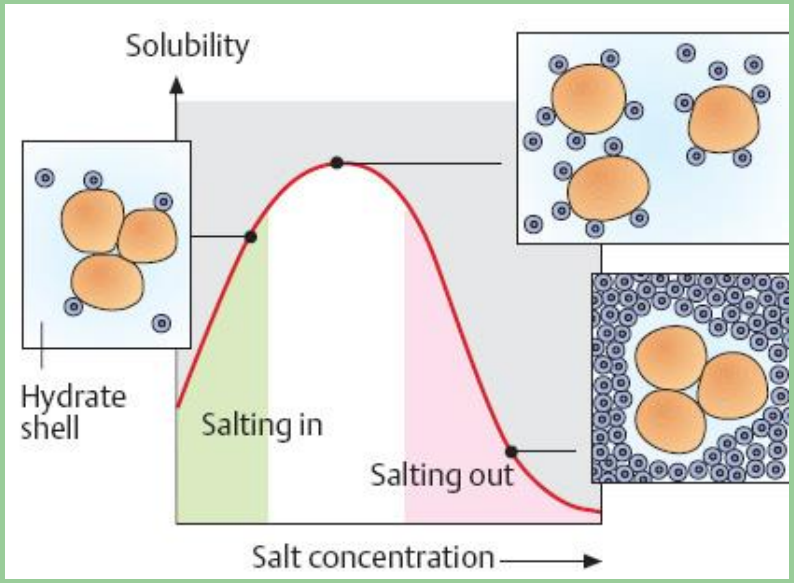
Thank you

University of Baghdad

College of Science

Department of Chemistry

Scheme for salt fractionation of serum proteins



LAB EXPERIMENT 6

Dr. Nuha Nihad

Aims

Using salt fractionation and **biuret method** to determine the followings in the serum:

- Total proteins
- Albumin + α-globulin
- Albumin
- γ-globulin

Serum Total Protein

- Serum total protein, also called plasma total protein or total protein, is a biochemical test for measuring the total amount of protein in blood plasma or serum.
- **Protein** in the serum is made up of **albumin** and **globulins**.
- ***Note:*** the **globulins** in turn is made up of α1, α2, β, and γ globulins.

γ-globulins: IgG, IgM, IgA, IgD, IgE

Exp. (5) :- Scheme for salt fractionation of serum proteins

Principle :-

Total protein (TP) is determined by the usual biuret method.

1- Albumin (A) is determined in the solution remaining after precipitation of all globulins with Na_2SO_3 at a final conc. of 28%. the total globulins (G) are calculated as follows:-

$$TP - A = G$$

And the ratio A/G can be calculated.

2- γ-globulin is determined by applying the biuret reaction to the precipitate obtained at a final concentration of 1.4% $(NH_4)_2SO_4$ in 3% NaCl salt solution.

3- α-globulins and β-globulins can be estimated when the following additional determination ((Albumin + α-globulin)) is done in the solution remaining after precipitation of β and γ globulins with a final conc. of 23% Na_2SO_4. α and β globulins are then calculated by the difference as follows :-

α-globulin = (Albumin + α-globulin) - Albumin

β-globulin = (Globulin – γ-globulin) - α-globulin

Note:- If fibrinogen is to be determined, plasma species must be used instead of serum. Fibrinogen normal values are about 0.02-0.06 gm/100ml.

Salt fractionation

- Without → Total proteins
- Using 23% Na_2SO_4 (sodium sulfate) → (Albumin + α-globulin) (supernatant)
- Using 28% Na_2SO_3 (sodium sulfite) → Albumin (supernatant)
- Using 1.4% $(NH_4)_2SO_4$ → γ-globulin (ppt)

Albumin

- L. *albus* = white
- Most abundant plasma protein (~50%)
- Synthesized by hepatocytes
- Single polypeptide - 66 kDa

Globulins

- Heterogeneous family of proteins
- Mol weight: 12-900 kDa
- Less water soluble than albumin
- Types
  - Alpha (α) :
    - ✓α1 (α1-antitrypsin, α1-acid glycoprotein, HDL)
    - ✓α2 (Haptoglobin, α2-macroglobulin, Ceruloplasmin)
  - Beta (β) :
    - ✓β1 (Transferrin,C4,LDL)
    - ✓β2 (C3, β2-microglobulin)
  - Gamma (γ) : Immunoglobulins, C-reactive protein

What is protein fractionation?

It depends on the principle that most **proteins** **are** less soluble in solutions of **high salt concentrations** because the addition of **salt** ions shield **proteins** with multi-ion charges. Those charges help **protein** molecules interact, aggregate, and **precipitate**.

Salting In

- Low concentrations → of salt the solubility increases.

This could be explained by the following:

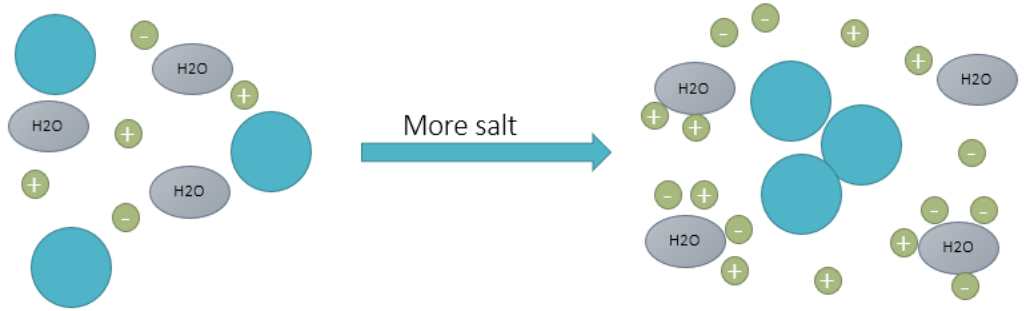
- Salt molecules stabilize protein molecules by :
- Decreasing the electrostatic energy between the protein molecules which increase the solubility of proteins.

Salting out

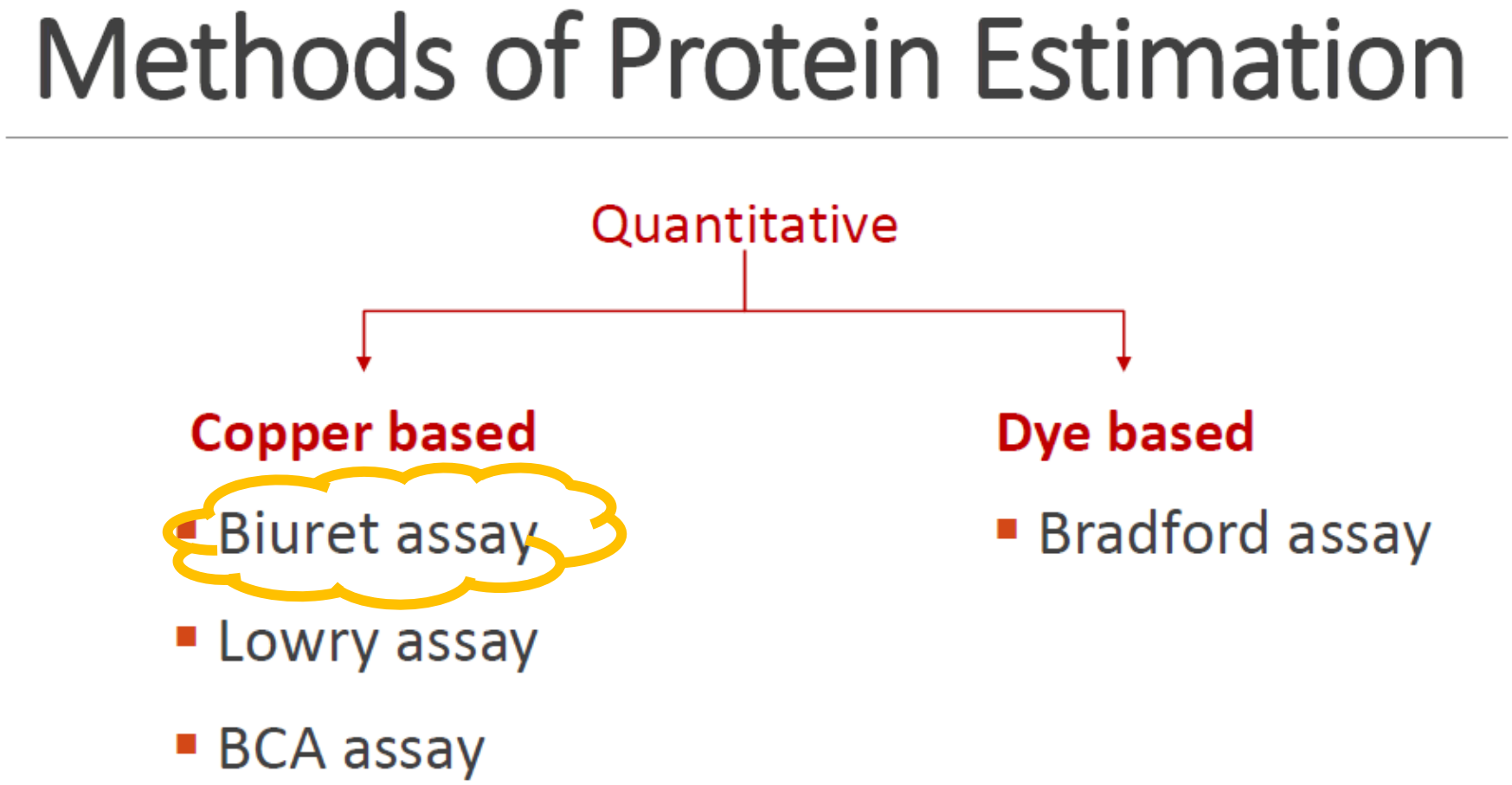
- High concentration of salts ⟶ the solubility decreases, and protein precipitates.

This could be explained by the following:

- because the excess ions (not bound to the protein) compete with proteins for the solvent.
- The decrease in solvation allows the proteins to aggregate and precipitate .

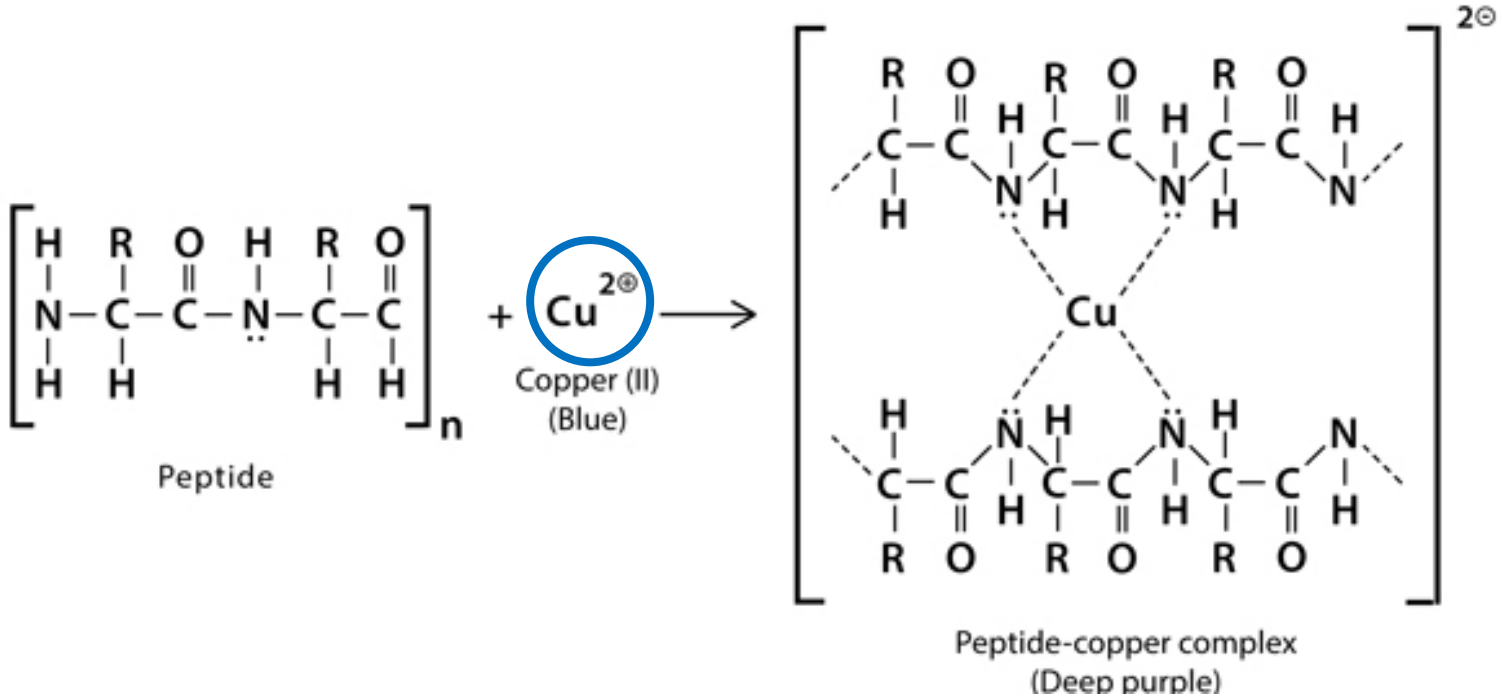


The traditional method for measuring total protein uses the biuret reagent, but other chemical methods are also available



Biuret Reaction

- Compounds with 2 or more peptide bonds, react with $CuSO_4$ in alkaline conditions to produce a complex which is **violet colored**.



at 540nm

Biuret Reagent Composition

- Sodium hydroxide — provide an alkaline medium
- Copper sulphate
- Sodium potassium tartrate
- Potassium iodide

Specimen

- Serum and plasma may be used, and all usually yield comparable results, though, because of the presence of fibrinogen, plasma levels for total protein higher than serum levels.

Total protein is stable in serum and plasma for

- 1 week at room temperature,
- and for at least 2 months at –20° C

Calculations

$$\text{Serum Total Protein (gm/100 ml)} = \frac{A_{Test} - A_{Blank}}{A_{Standard} - A_{Blank}} \times \text{Conc. of Standard } x \frac{100}{0.1}$$

$$\text{Serum albumin (gm/100 ml)} = \frac{A_{Test} - A_{Blank}}{A_{Standard} - A_{Blank}} \times \text{Conc. of Standard } x \frac{100}{0.1}$$

$$\text{Serum globulin (gm/100 ml)} = \text{Serum total protein} - \text{Serum albumin}$$

Normal Values in Serum

- Total protein : 6.7 – 8.6 g/dL
- Albumin : 3.5 - 5.5 g/dL
- Globulins : 2.0 - 3.5 g/dL
- A:G ratio : 1.5 to 2.5

Thank you

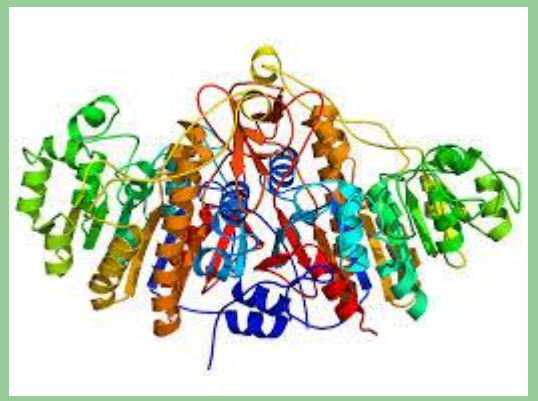
University of Baghdad

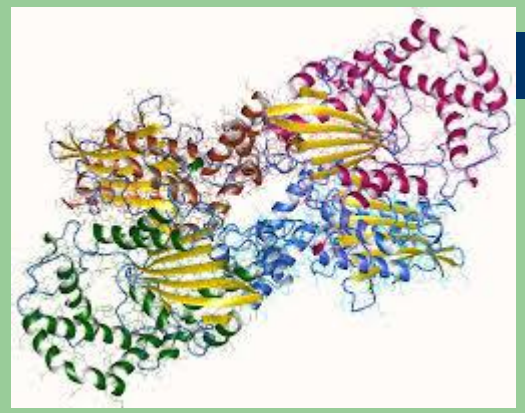
College of Science

Department of Chemistry

Serum Phosphatases
Alkaline Phosphatase and Acid Phosphatase

LAB EXPERIMENT 7





Dr. Nuha Nihad

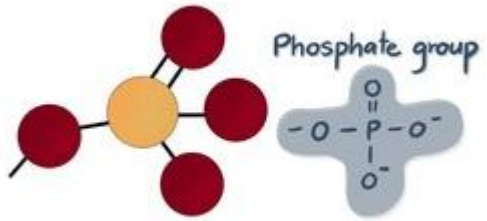
Aims

Using King-Armstrong method to determine the levels of:

- Serum Alkaline Phosphatase
- Serum Acid Phosphatase

Phosphatases

Phosphatases are enzymes which catalyze the splitting the terminal phosphate group from monophosphate esters.



The Phosphatases belong to a class of enzymes called hydrolases.

Phosphatases

EC (3.1.3.X)

Exhibits optimum activity

In alkaline solution pH=9-10

In acid solution pH=5-6

Alkaline Phosphatase

Acid Phosphatase

EC (3.1.3.1)

EC (3.1.3.2)

EC (3.1.3.1)

1- **Bone**
2- **Biliary system**

ALP

- **Alkaline Phosphatase**

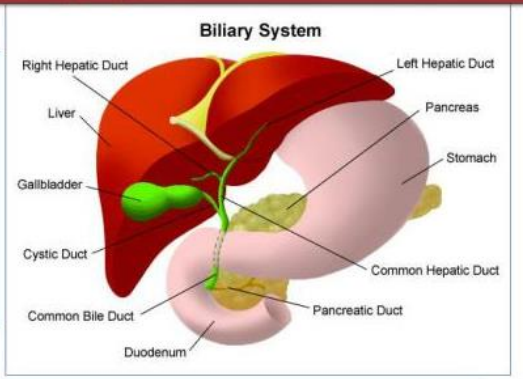
Alkaline medium

Removes phosphate groups from molecules

Enzyme

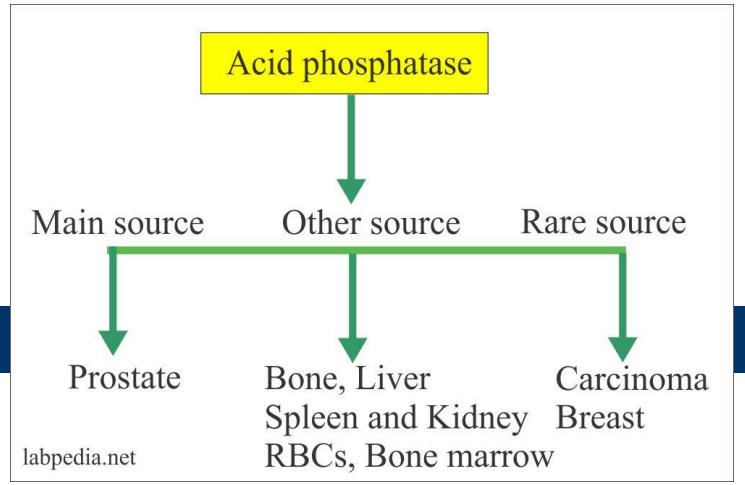
Biliary System

The biliary system consists of the **liver**, **gallbladder** & **bile ducts** that are involved in the **production**, **storage** & **transportation of bile.**



Bile is secreted by the liver cells at a constant rate of about 40 ml per hour. When digestion is not taking place, the bile is **stored** and **concentrated** in the gallbladder; later, it is delivered to the duodenum.

EC (3.1.3.2)



Acid Phosphatase

ACP

Acid medium

Removes phosphate groups from molecules

Enzyme

EC (3.1.3.x)

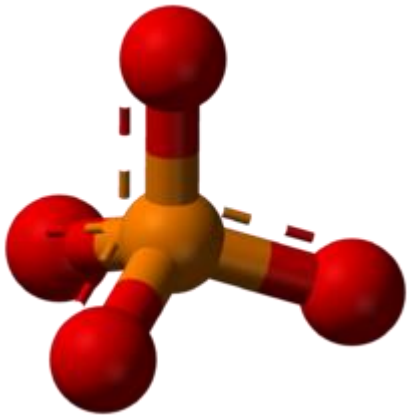
Enzyme Commission number

Hydrolases

Ester bond

phosphatases

removing phosphate group

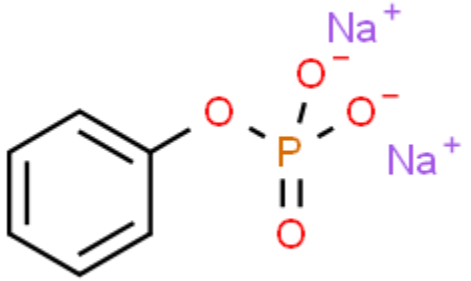


King Armstrong Method

Principle:

- Disodium phenylphosphate is cleaved by the enzyme phosphatase and the released phenol is detected (by converted to red quinon complex under alkaline conditions and with the addition of 4-amino antipyrine).

- Substrate → Disodium phenylphosphate



- Temp. → 37℃

- **Alkaline Phosphatase** pH=10, time 30min.
- **Acid Phosphatase** pH=4.9, time 1hr.

King-Armstrong units was using to estimate activities of ALP and ACP.

King-Armstrong units, one unit being milligrams of phenol liberated by 100ml of serum under the following condition:

- pH 10, 37℃ and 30 min. for ALP.
- pH 4.9, 37℃ and 1hr. For ACP.

Calculation

Conc. of st.

1mg 100ml
X 1ml

Serum alkaline phosphatase units (K.A.U) for each 100 mL serum

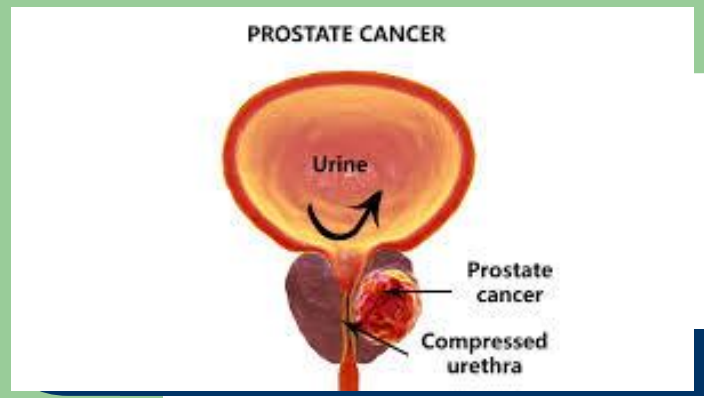
$$= \frac{As - Ac}{Ast - A\,B} \times \frac{0.01}{0.1} \times 100$$

Vol. of test

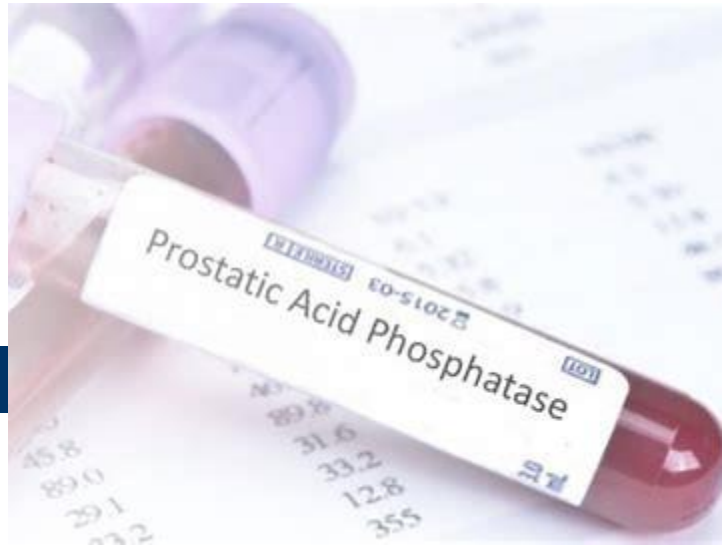
$$K.A.U/100ml = \frac{A\ sample - A\ control}{A\ standard - A\ Blank} \times 10$$

The King-Armstrong unit (K.A.U) of alkaline phosphatase is defined as the amount of phosphatase that releases 1 mg of phenol from substrate (pH 10) at 37°C for 30min.

Standard value for King-Armstrong method: 3 -13 K.A.U/100ml



Calculation



Acid phosphatase units(K.A.U) for each 100 mL serum

$$= \frac{As - Ac}{A\,st - A\,B} \times \frac{0.01}{0.2} \times 100$$

$$K.A.U/100ml = \frac{A\,sample - A\,control}{A\,standard - A\,Blank} \times 5$$

Vol. of test

The King-Armstrong unit (K.A.U) of acid phosphatase is defined as the amount of phosphatase that releases 1 mg of phenol from substrate (pH 4.9) at 37°C for 1 hour.

Standard value for King-Armstrong method: 1-3.5 K.A.U/100ml

Thank you

الكورس الثاني

Experiments in Clinical Biochemistry for 4th Year Chemistry Students

2020-2021

University of Baghdad

College of Science

Department of Chemistry

Quantitative determination of carbohydrates

LAB EXPERIMENT 1
2020-2021

Dr. Nuha Nihad

Aim: To Estimate reducing sugars by:

- The Somogyi-Nelson method.
- Sumner’s method.
- Ferricyanide method (a volumetric method).

1-The Somogyi-Nelson method for estimation of sugars

Principle

- In the Nelson method Cu^{+2} is reduced to Cu^{+1} by the reducing activity (free aldehyde or ketone groups) of the sugar (e.g. glucose)(step 1)

$$H_2O + RCHO + 2Cu^{+2} \longrightarrow RCOOH + 2Cu^{+1} + 2H^{+}$$

Orange/ red ppt

- Cu^{+1} is oxidized to Cu^{+2} by addition of phospho- or arsenomolybdate solution (colorless) (step 2).

$$Cu^{+1} + Na_3PO_4.12Mo^{+6}O_2 \longrightarrow Cu^{+2} + 12Mo^{<+6}$$ (molybdenum blue)

- The blue color developed is compared with a set of standards in a colorimeter at 700 n

colorless

The Somogyi-Nelson method

1-Measures the total reducing capacity of a sample (blood or any other biological fluid) which normally contains small amounts of other reducing substances beside glucose:

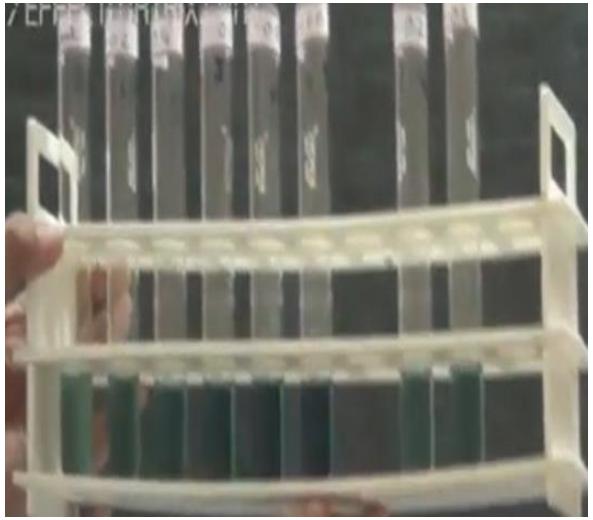
- Salts
- Proteins
- Glutathione in RBC

2-The proteins must be precipitated completely without changing the pH of the solution, then glucose or the reducing sugar is determined by Somogyi-Nelson method.

Materials

- Somogyi-Nelson copper reagent (Alkaline Copper tartrate).
- Phospho- or arsenomolybdate Reagent (color reagent).
- Stock glucose solution : (0.1mg/ml).

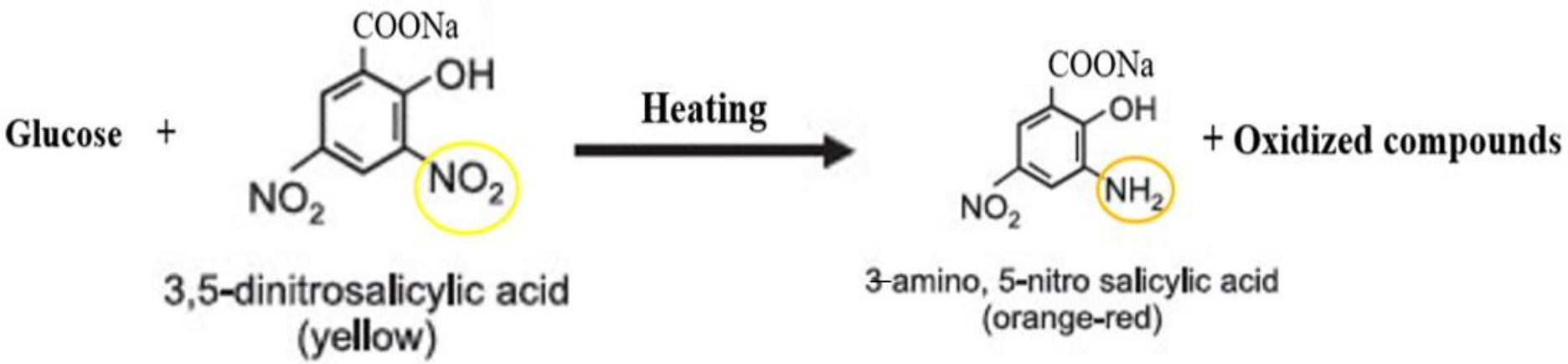
Somogyi-Nelson method



Sumner's method for estimation of sugars

Principle

In alkaline solution reducing sugar reduces the 3,5-dinitrosalicylic acid  (DNS) to 3-amino-5-nitro salicylic acid. 

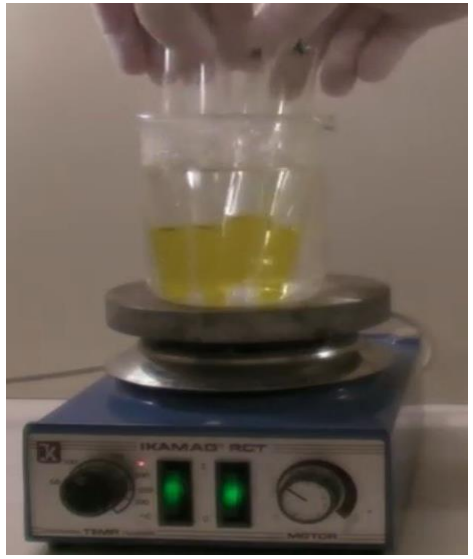


Amount of absorbance directly related to amount of reducing sugar (at 540 nm).

Materials

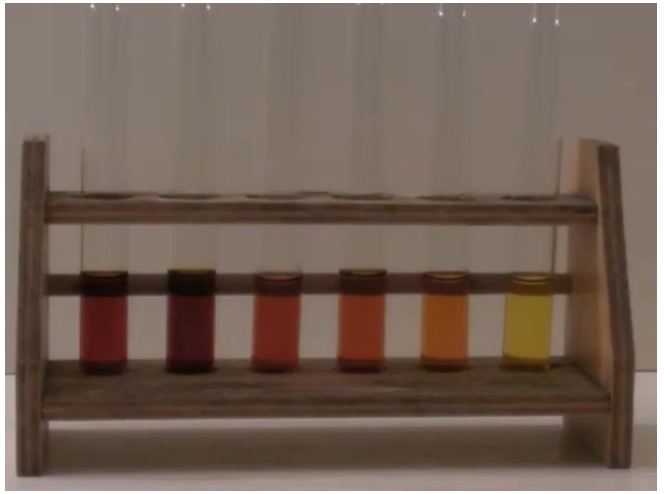
- DNS reagent (3,5-dinitrosalicylic acid) (color reagent).
- NaOH.
- Stock glucose solution : (10mM).

Sumner’s method for estimation of sugars



1

2



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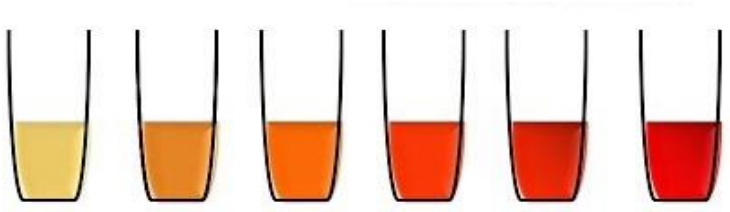


3-Determination of soluble reducing sugars by ferricyanide method (a volumetric method).

Principle:

A part of potassium ferricyanide is reduced to potassium ferrocynide when heated with alkaline solution by reducing sugars (glucose).

$$2K_3[Fe(CN)_6] + 2KOH \rightarrow 2K_4[Fe(CN)_6] + H_2O + 1/2O_2$$

(potassium ferricyanide) (potassium ferrocynide)

$$C_6H_{12}O_6 + 1/2O_2 \rightarrow C_6H_{12}O_7$$

(Glucose) (Gluconic acid)

3-Determination of soluble reducing sugars by ferricyanide method (volumetric method).

The ferrocynide formed a stable complex of potassium zinc ferrocynide. The remaining ferricyanide is determined from the amount of iodine liberated.

$$2K_3[Fe(CN)_6] + 2KI \rightarrow 2K_4[Fe(CN)_6] + I_2$$

$$K_4[Fe(CN)_6] + ZnSO_4 \rightarrow K_2Zn_3[Fe(CN)_6]_2 + K_2SO_4$$

The liberated iodine is estimated by titrating against sodium thiosulfate using starch as an indicator.

$$I_2 + 2Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2NaI$$

Materials

Potassium ferricyanide (K_3 [$Fe(CN)_6$]) solution.
Sodium thiosulfate ($Na_2S_2O_3$) solution.
Starch indicator.
Potassium iodide.
Unknown concentration of glucose.

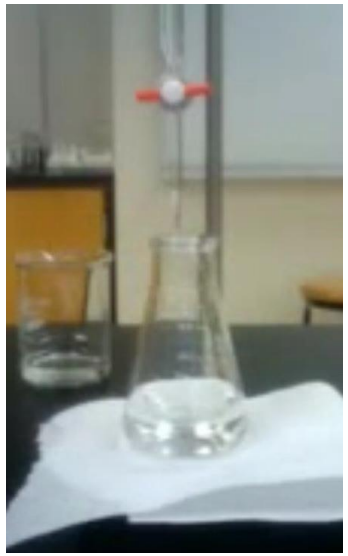
Steps of titration using starch solution as an indicator.

1

2

3

4



5

Determining the Concentration of a Solution

- Beer's Law
- Standard Curve

Beer's Law

$Abs = \varepsilon b C$

where Abs = absorbance (no units)

ε = molar absorptivity ($M^{-1}cm^{-1}$)

b = path length (cm)

C = concentration (M)

These measurements all take place at the wavelength at which our absorbing species absorb!

- $A_{unknown}$
- Cst
- Ast

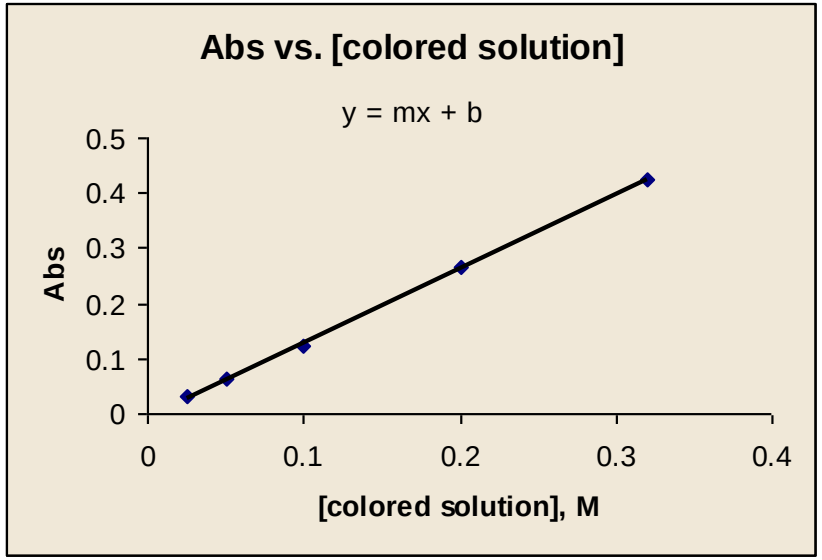
$$C_{unknown} = \frac{A_{unknown} \times Cst}{Ast}$$

To obtain the standard curve:

- Prepare a series of colored solutions of known concentration (“standards”).
- The absorbance of each solution is measured.
- Absorbance versus concentration is plotted.
- Using the calibration curve equation, the concentration of the unknown solution can be calculated, given the absorbance of the unknown solution:

Standard Curve

Plotting Abs vs. [colored solution] gives:



This is called a “calibration curve.”
For y = m x + b

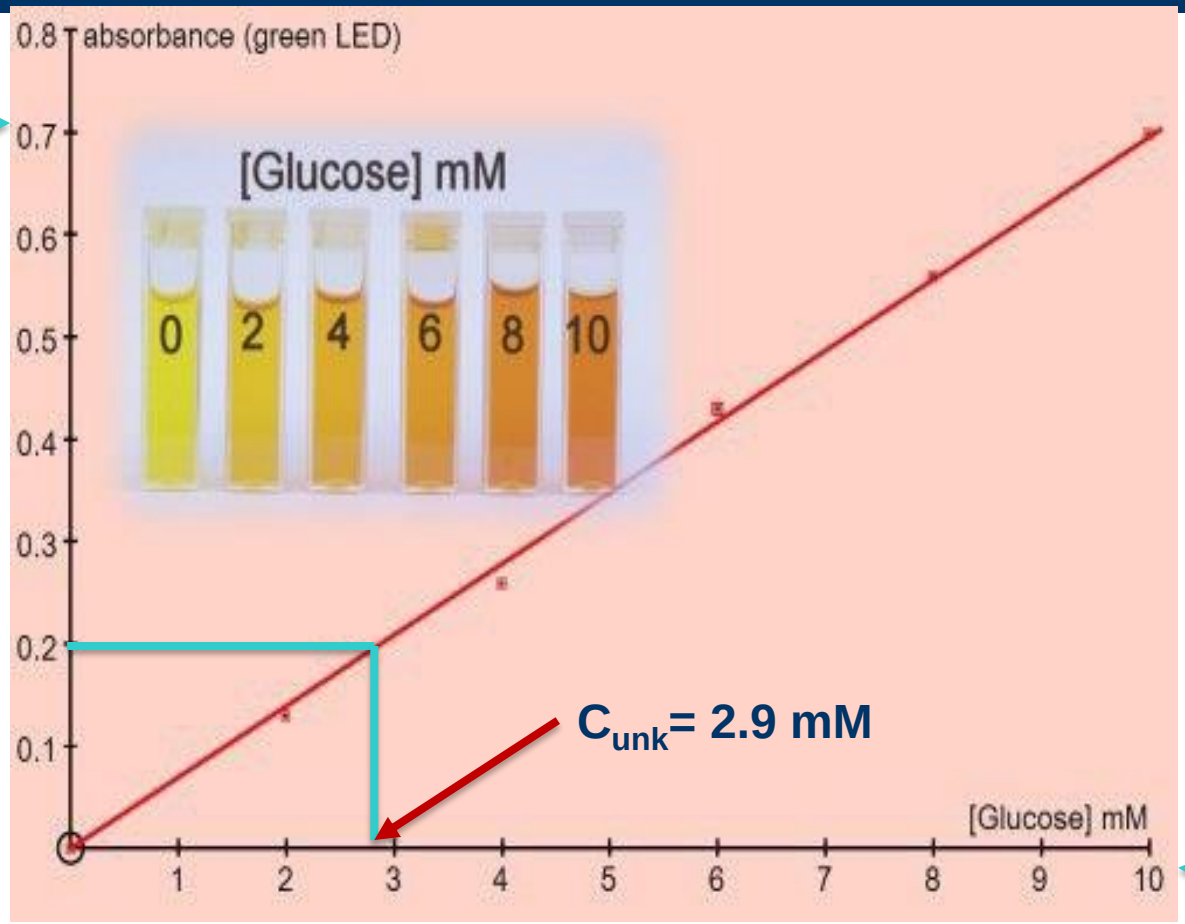
Abs = m [colored solution] + b

Abs = ε b c

After the calibration curve is established with your standard solutions, the equation is used to calculate the concentration of the unknown solution, given the absorbance of the unknown solution.

Standard curve

A-AB



Conc. (mM)

Unit of concentration

- mg/ml
- mg/100ml
- M
- mM

1mg/ml=100mg/100ml

$$\text{Molarity} = \frac{\text{Weight of solute(g)}}{\text{Molecular weight of solute(g/mol)}} \times \frac{1000}{\text{Volume of solution(ml)}}$$

$$mM = M \times 1000$$

- 0.06 mg/ml
- 6 mg/100ml
- $6 \div 1000 = 0.006$ g/100ml
- $\frac{0.006}{180} \times \frac{1000}{100} = 0.0003\ M$
- $0.0003\ M \times 1000 = 0.3$ mM

Exp(1):Quantitative determination of Carbohydrates

Exp. (1.1) :- The Somogyi-Nelson method for estimation of sugars

The Somogyi-Nelson method is an adaptation of the original method of Somogyi, which depends upon the ability of the sugar (e.g. glucose) to reduce the cupric ion (Cu^{+2}) to the cuprous ion (Cu^{+1}) by the aldehyde or the carbonyl group of the simple sugars.

$$H_2O + RCHO + 2Cu^{+2} \longrightarrow RCOOH + 2Cu^{+} + 2H^{+}$$

The cuprous ion is precipitated as cuprous oxide (Cu_2O) .The cuprous ion, in turn is used to reduce an arseno-molybdate solution or phospho-molybdate solution giving a blue coloration, which is estimated photometrically. Thus the amount of blue coloration produced is dependent upon the initial reducing ability of the sample solution.

$$Cu^{+2} + Na_3PO_4.12Mo^{+6}O_2 \longrightarrow Cu^{+2} + 12Mo^{<+6} \quad \text{(blue)}$$

The Somogyi-Nelson method measures the total reducing capacity of a sample (blood or any other biological fluid) which normally contains small amounts of other reducing substances beside glucose (e.g.. urea, urates, ammonia and its salts, proteins, glutathione in RBC, etc.).The proteins must be precipitated completely without changing the pH of the solution, then glucose –(or the reducing sugar)-is determined by heating with alkaline copper solution reagent, and the cuprous oxide formed is treated with arseno- or phospho-molybdate reagent, which is oxidized to a colored compound whose intensity is proportional to the cuprous ions and thus to glucose –(or the reducing sugars)-in the sample.

Materials:

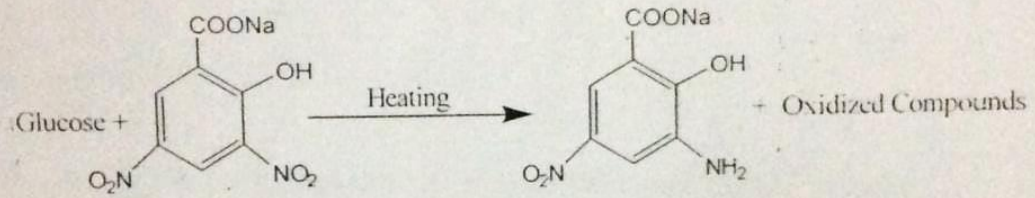
1- Stock glucose solutions:- (0.1mg/ml) and (1mg/ml)

2-Somogyi-Nelson copper reagent:- (Equal volumes of solution A +solution B)
Solution A :- A solution of 0.013%$CuSO_4.5H_2O$.
Solution B :- Dissolve 50 gm $NaHCO_3$ and 40 gm anhydrous Na_2CO_3 in 700 ml dist.water then add 27 gm pot.oxalate. Dissolve 24 gm Na K tartrate (Rochelle salt) in 100 ml dist. water .Mix the two solutions and make up the volume to 1L.(if Na K tartrate is not available use 87 gm Na_2SO_4).

3- Arsenomolybdate reagent:-Dissolve 25 gm of ammonium molybdate in 450 ml dist. water, then add 21 ml conc.H_2SO_4 . Dissolve 3 gm sodium arsenate in 30ml dist. water .Mix the two solutions with continuous stirring . Transfer the mixed solution into a dark reagent bottle and keep at 37° C for 24-48 hr

Exp. (1.2) :- Sumner's method for estimation of sugars

When an alkaline solution of 3,5-dinitrosalycilic acid (DNS) is heated with a reducing sugar, the DNS reagent which is yellow in color is reduced to an orange to red colored solution of 3-amino-5-nitrosalycilic acid . The resultant color is compared with standards. This is a rapid method of sugar determination in aqueous solutions; also applicable as well to glycosuric urine .The reaction is followed by measuring the absorbances at 540 nm.



Materials:

1-Stock glucose solution (10 mM).

2-Sodium hydroxide (2N).

3-DNS reagent : Dissolve 1gm of 3,5-dinitrosalycilic acid, 18.7 gm Na K tartrate , 0.2 gm crystalline pure phenol and 0.05 gm sodium sulphite in 100 ml of 1% NaOH.

4-Unknown concentrations of sugar samples (glucose or any suitable reducing sugar).

Method:

Using the stock glucose solution prepare the following concentrations (0.0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, 4.0 and 6.0 mM glucose) in 2 ml solutions using dist. water for preparing the dilutions. Add 0.5 ml of 2N NaOH and 0.5 ml of DNS reagent to each test tube. Mix well and heat in a boiling water bath for 2- 5 min. Cool and record the absorbances at 540 nm after the addition of 10 ml of dist. water to each test tube .The unknown solution of a sugar sample is provided in 2 ml patches which has to be treated as the standard solutions.

For the determination of the unknown concentration of the sugar sample provided earlier, a standard curve is prepared by placing the absorbances on perpendicular axis- (Y-axis)-against the known glucose concentrations as mM or mg/ml or mg /100 ml or mmol/reaction mixture on the abscissa-(X –axis).

Exp. (1.3):- Determination of soluble reducing sugars by ferricyanide method (a volumetric method)

Soluble reducing sugars are oxidized by potassium ferricyanide in alkaline solutions and the excess of potassium ferricyanide is reduced by potassium iodide solution. The unreacted excess of potassium iodide is volumetrically determined as I_2 by titration with sodium thiosulfate ($Na_2S_2O_3$).

$$2K_3[Fe(CN)_6] + 2KOH \longleftrightarrow 2K_4[Fe(CN)_6] + H_2O + 1/2\ O_2$$

$$C_6H_{12}O_6 + 1/2\ O_2 \longrightarrow C_6H_{12}O_7$$

(Glucose) (Gluconic acid)

The excess of pot.ferricyanide is reduced with pot iodide solution to pot.ferrocyanide and iodine is quantitatively librated representing the excess pot.ferricyanide

$$2K_3[Fe(CN)_6] + 2\ KI \longleftrightarrow 2K_4[Fe(CN)_6] + I_2$$

And pot.ferrocyanide reacts with zinc sulfate present in the pot.iodide solution to form a stable complex of pot.zinc ferrocyanide

$$ZnSO_4 + K_4[Fe(CN)_6] \longrightarrow K_2Zn_3[Fe(CN)_6]_2 + K_2SO_4$$

Then iodine is titrated against sod.thiosulfate

$$I_2 + 2Na_2S_2O_3 \longrightarrow Na_2S_4O_6 + 2NaI$$

The amount of pot.ferricyanide consumed by the reducing sugar is determined by running a blank in which the sugar solution is replaced by dist. water.

Materials:

1-A solution of unknown concentration of glucose - or any other reducing sugar - of concentration between (1mM – 4mM).

2-Potassium ferricyanide solution : Dissolve 8.25 gm pot.ferricyanide and 10.6 gm anhydrous sod.carbonate in 500 ml dist. water and make up the volume to 1L.

3-Potassium iodide solution : Dissolve 12.6 gm pot.iodide , 25 gm zinc sulfate and 125 gm sod. chloride in 500 ml dist. water. Filter and store in a dark reagent bottle .

4-Sodium thiosulfate (0.01N) : Dissolve 2.507 gm sod.thiosulfate in 1L dist. water.


Thank
you

University of Baghdad

College of Science

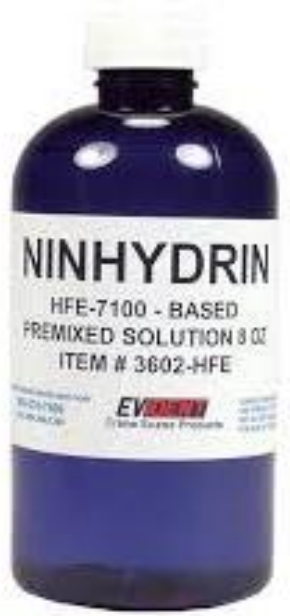
Department of Chemistry

Quantitative determination of amino acids
Quantitative determination of proteins

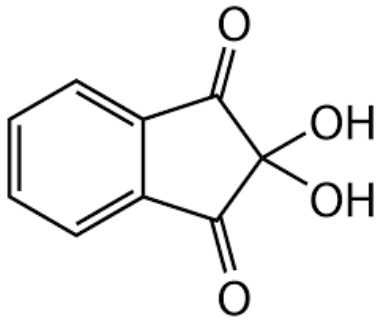
LAB EXPERIMENT 2

Dr. Nuha Nihad

1- Quantitative determination of amino acids



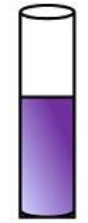
Ninhydrin reaction.



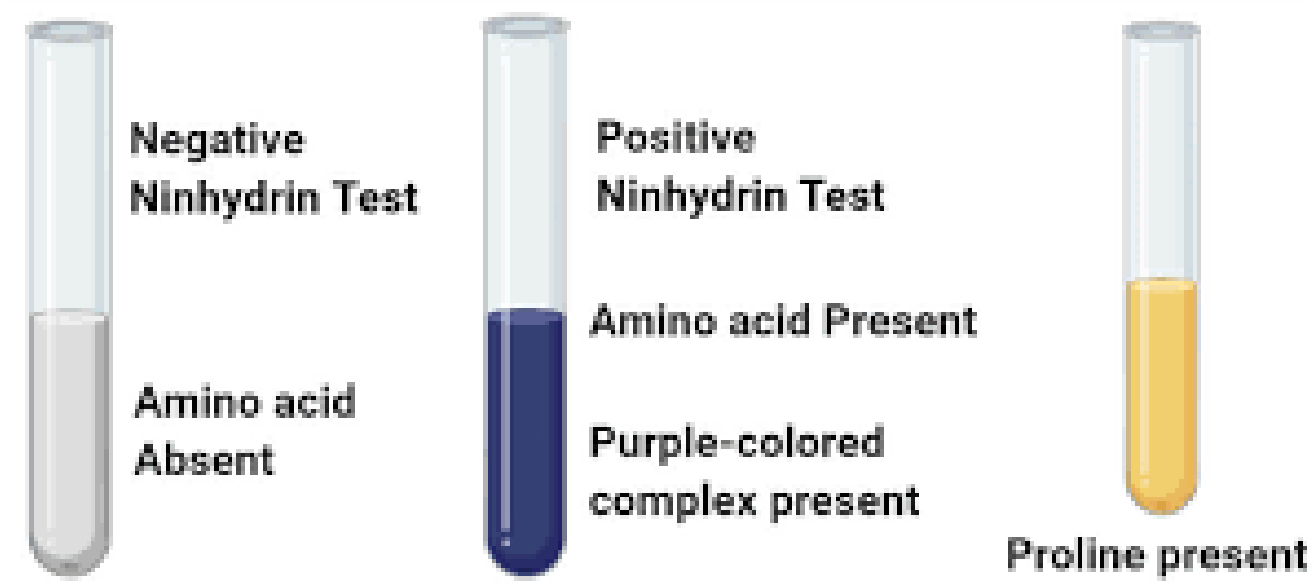
Ninhydrin (triketohydrindene hydrate) is used to assess amino acid qualitatively and quantitatively in ninhydrin reaction.

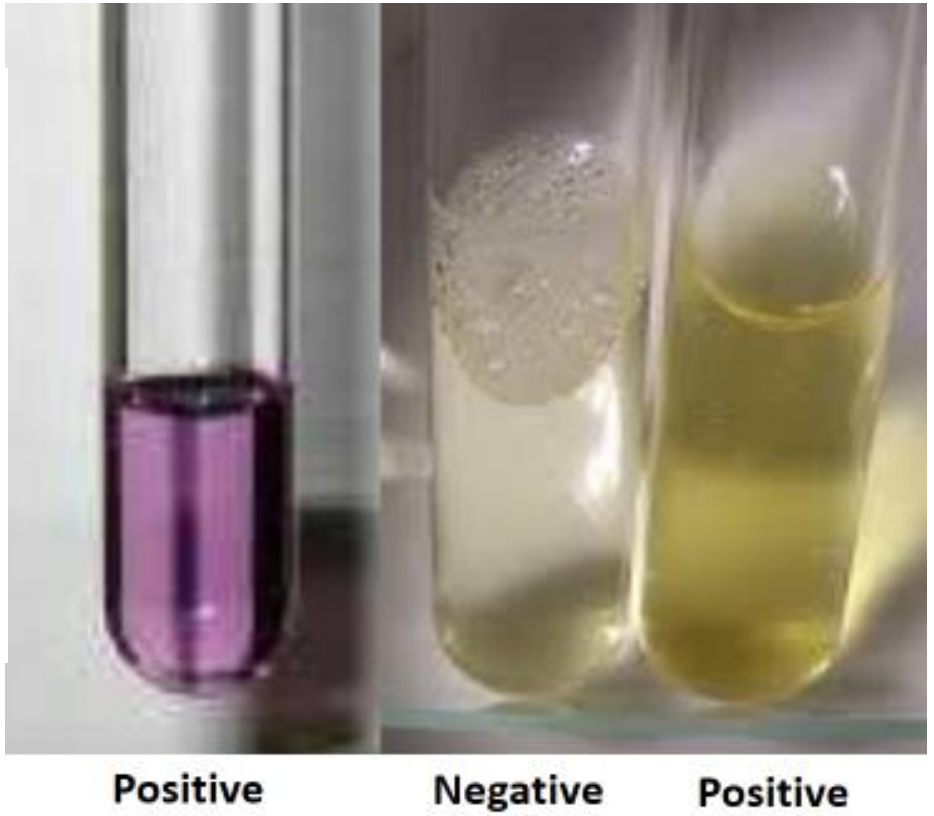
1- Quantitative determination of amino acids

Ninhydrin (Triketohydrindene hydrate) oxidizes α-amino acids (containing a free NH2 & free COOH) between pH 4-8 to give purple-colored diketohydrin .



But proline and hydroxyproline (an imino acid, a secondary amino group) gives yellow color with ninhydrin.


Negative
Ninhydrin Test
Amino acid
Absent
Positive
Ninhydrin Test
Amino acid Present
Purple-colored
complex present
Proline present


Positive
Negative
Positive

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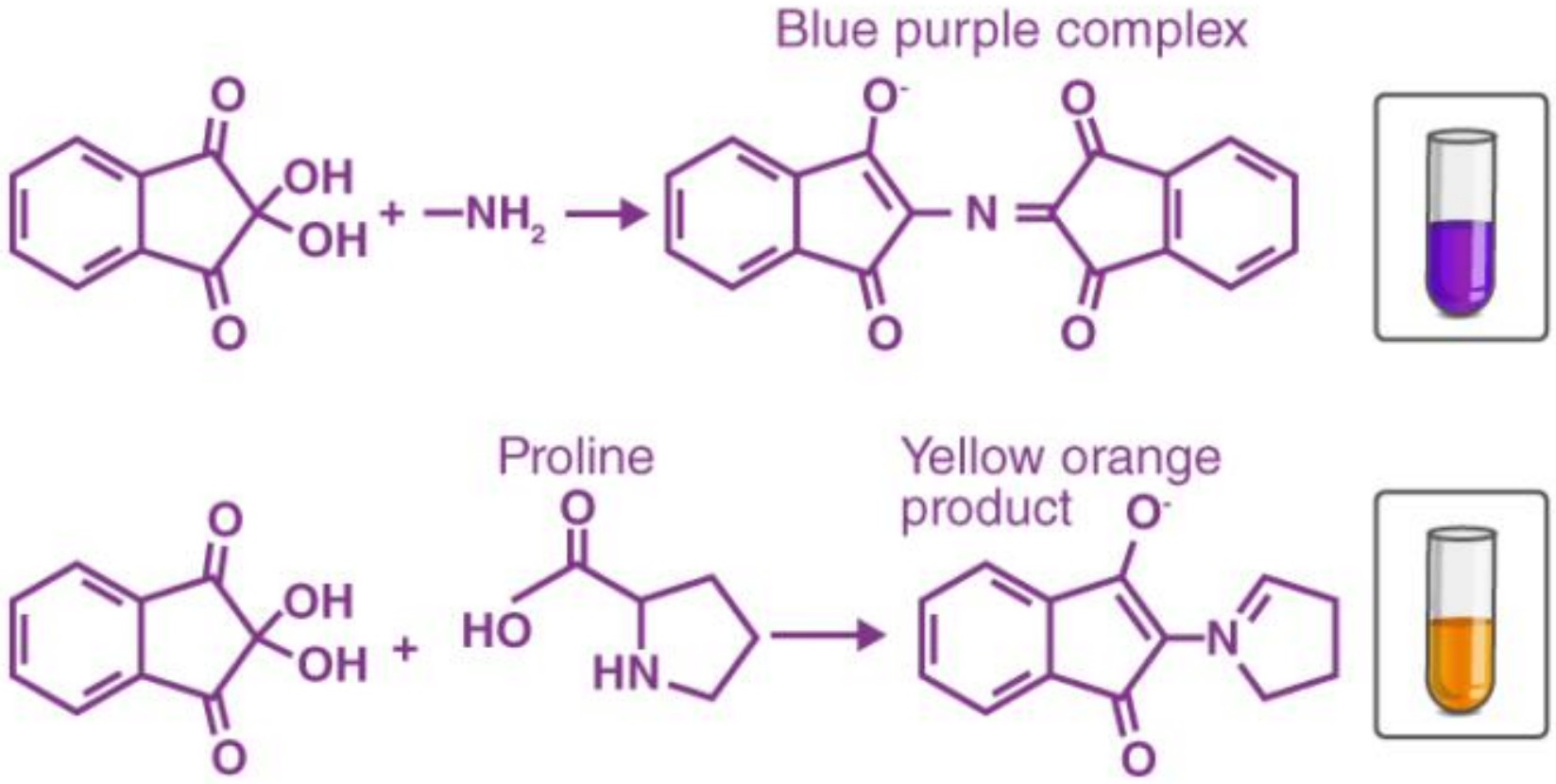
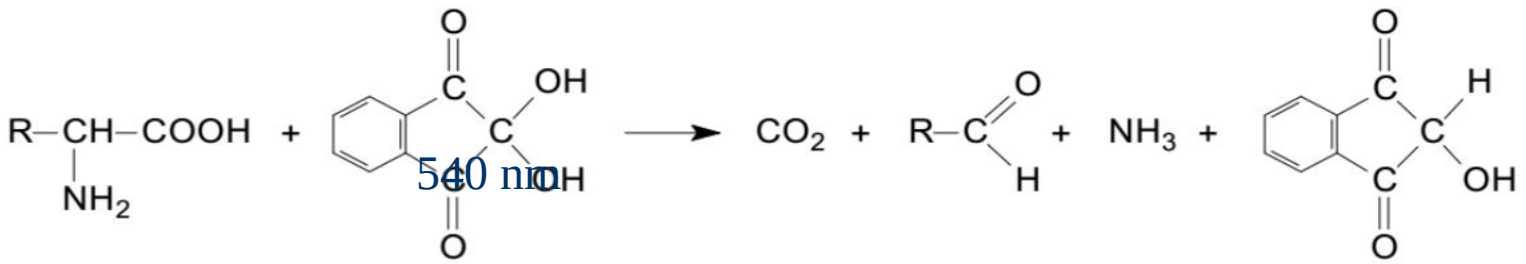

Blue purple complex
OH
OH
O
O
+ —NH2
O-
N
O
O
Proline
HO
HN
O
Yellow orange
product
O-
N
O

1- Quantitative determination of amino acids



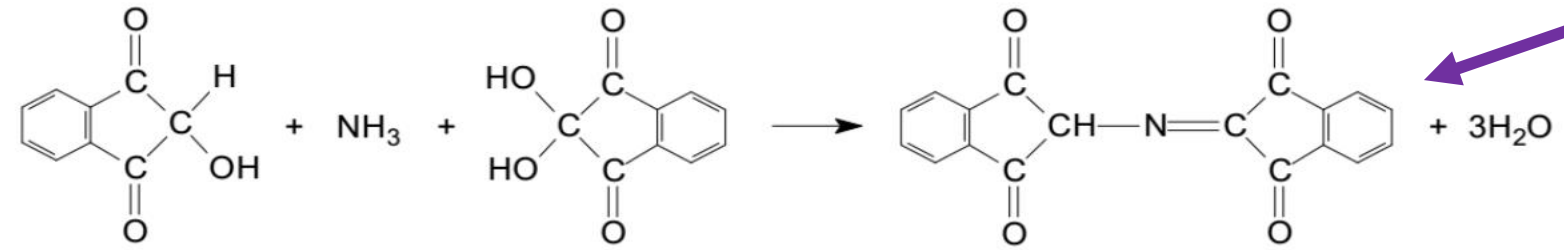
540 nm

Ninydrin

Reduced ninydrin

Ruhemann's purple

570 nm



Reduced ninhydrin

Ninhydrin

Violet-blue condensation product

2- Quantitative determination of proteins.

- Determination of proteins by the biuret method
- Determination of proteins by the Folin-Lowry method
- The Bradford assay method
- The ultraviolet absorption method

Colorimetric methods

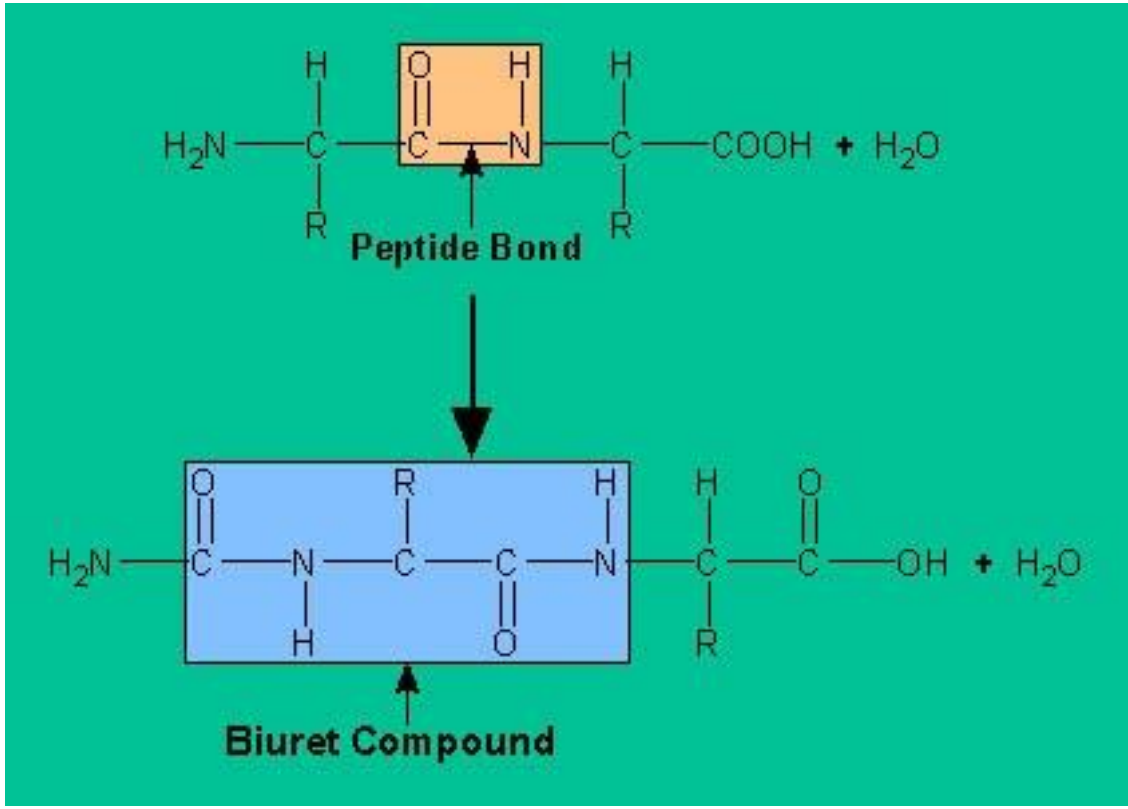
UV absorption

Biuret method.

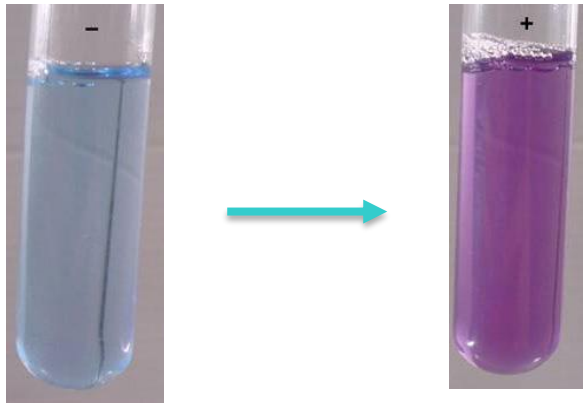
This method requires relatively large quantities of protein (1-20 mg protein/ml) for detection.

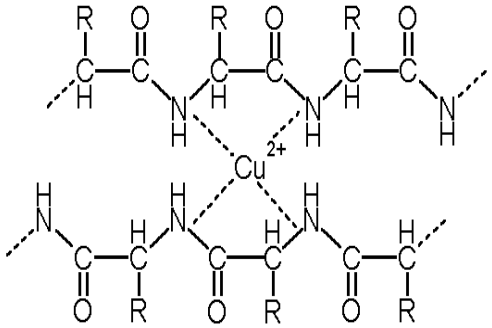
In alkaline solutions, Cu^{+2} complexes with compounds containing two or more peptide bonds (C-N bonds).

The reagent does not in fact contain biuret ($NH_2CONHCONH_2$). The test is so named because the substance biuret gives a positive reaction under the same conditions.



Peptide (N atom) bonds + alkaline Cu^{+2} → purple coordination complex of copper and measure Amax = 540 nm.



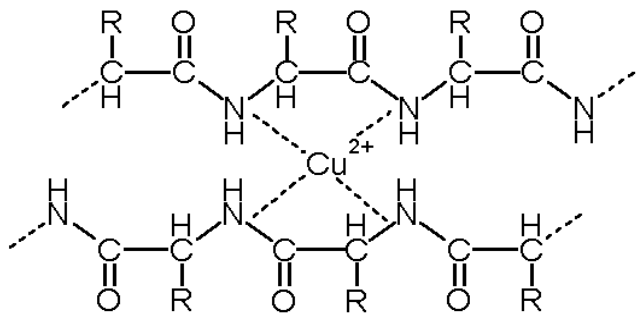


Biuret method.

The **biuret reagent** is made of $CuSO_4.5H_2O$ and NaOH, together with sodium potassium tartrate. The reagent turns from blue to purple in the presence of proteins.

Protein + Biuret Reagent

NaOH, $CuSO_4$ & Na/K-Tartrate



(Tetra-dentate Complex)

Folin-Lowry method (Lowry *et al*, 1951)

This method is about 10 times more sensitive than the Biuret method (can detect lower concentrations of protein)

Two reactions make the blue color develop:

1- The reaction of alkaline copper with protein as in the biuret test

Cu^{+2} + peptide bonds → Cu^{+1}-peptide bond complex, produces purple-blue color

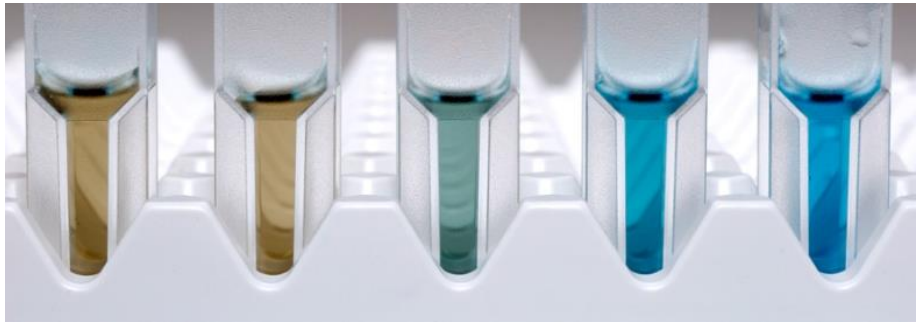
2- The reduction of Folin-Ciocalteu reagent（Phosphomolybdic and Phosphotungstic acid）by aromatic amino acids (e.g. tyrosine and tryptophan) present in the protein molecule which subsequently causes a color change of the solution into **blue** molybdenum or tungsten complex with an absorption at 700 nm.

Folin reagent + Cu^{+1}-complex → reduced Folin reagent, produces **dark blue**
(by aromatic amino acids)

Bradford Method

Principle:

- λ_{max} of Coomassie Brilliant Blue dye G-250 changes from 465 nm to 595nm upon binding to protein (a greenish-blue color complex)
- Measure A_{595}



Sensitivity: High (~lower than 100μg/ml)

Interferences: detergents, Triton X-100, & SDS

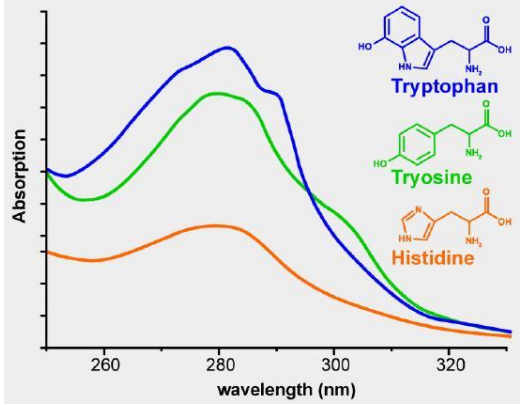
Time: rapid

Test tubes containing:
Bradford reagent alone.

(λmax) of the dye 465nm

Test tubes containing:
Bradford reagent with protein added.
(λmax) is shifted to 595nm.

The ultraviolet absorption method



Warburg-Christian Method (A280/ A260 Method)

Principle

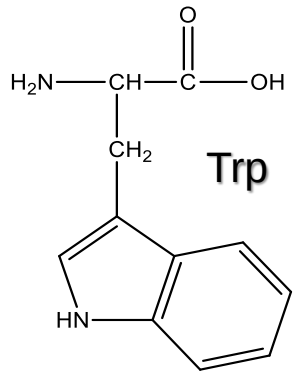
Proteins show strong absorption at UV 280 nm, primarily due to aromatic amino acids of tryptophan and tyrosine residues in proteins.

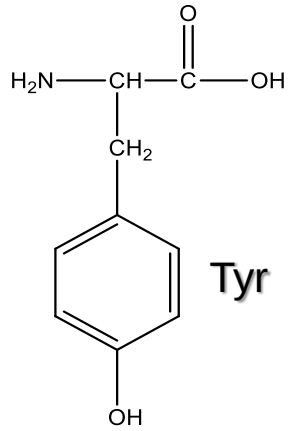
Advantages

Rapid

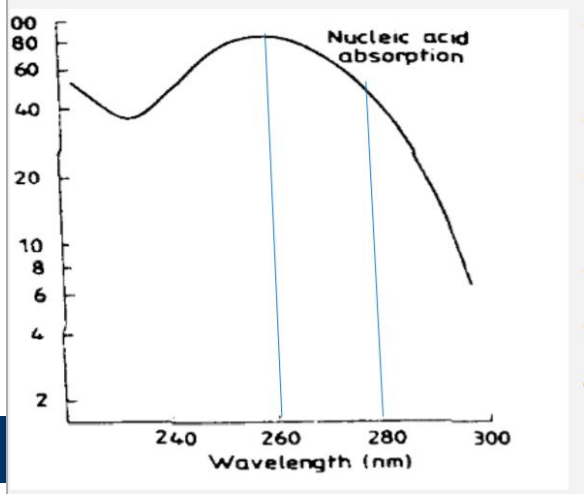
Disadvantages

- Contaminants (especially nucleic acids) also absorb at 260 nm.
- Variable amounts of aromatic amino acids in various proteins.





Protein concentration [mg/ml] = $[1.55 \times A_{280}] - [0.76 \times A_{260}]$



-A protein solution that has a high A280/A260 ratio: Less contaminated by DNA.

[It shows a lower absorbance at 260nm comparing to absorbance at 280nm].

-A protein solution that has a low A280/A260 ratio: Highly contaminated by DNA.

[It shows a higher absorbance at 260nm comparing to absorbance at 280nm].

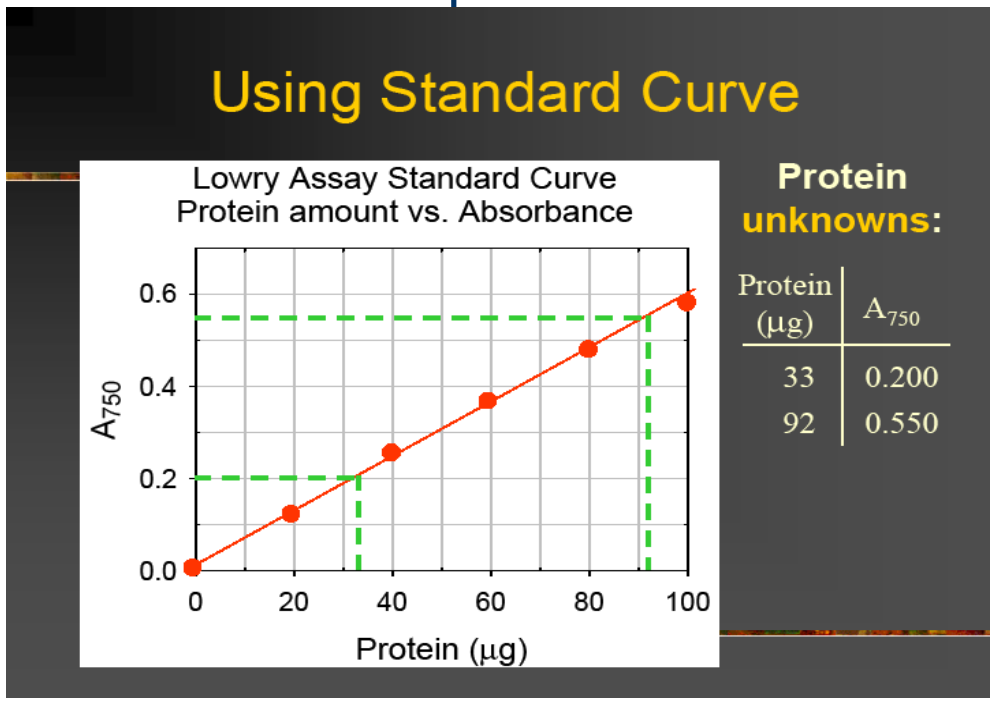
Quantitative determination of proteins. للاطلاع

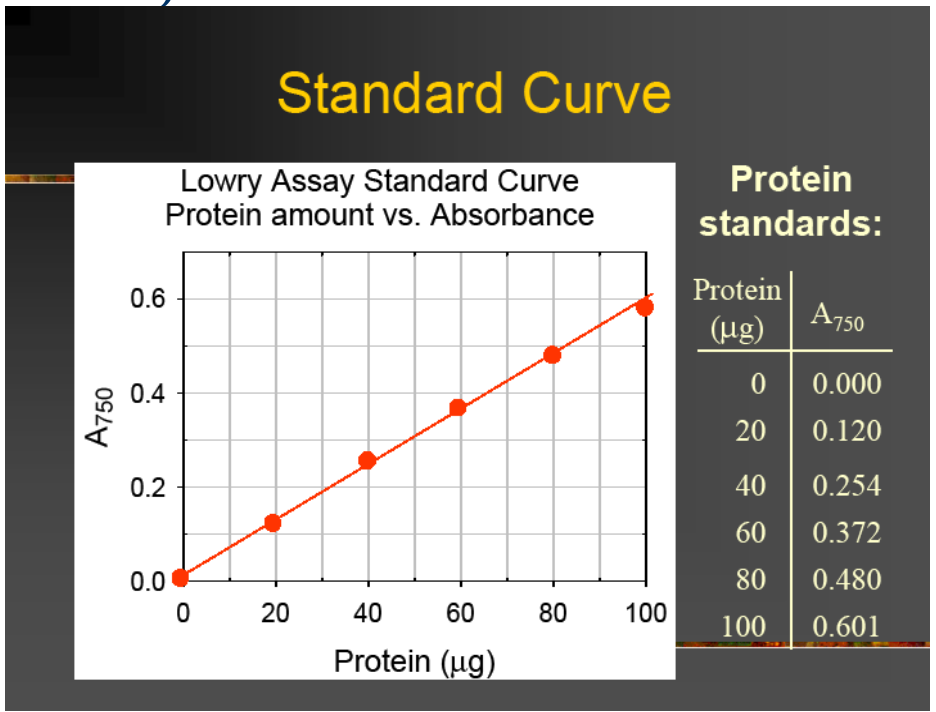
Method	sensitivity	Time	Reagent
Biuret	Low (0-1mg)	20-30 min	Alkaline copper-sulphate
Lowry	High (0-0.1mg)	40-60min	Cu+ Folin-Ciocalteu reagent
Bradford	High (0-0.01mg)	15min	Coomassie brilliant blue G-250
Warburg christian (A_{280}/A_{260})	Moderate about 0.05- 2.0 mg protein/ml	-----	No reagent

Determination the concentration of the unknown protein sample

1- Standard curve

- There is a linear relationship between absorbance and concentration.
- The amount of protein in the sample can be estimated using a standard curve of a selected standard protein solution such as (albumin).





Determination the concentration of the unknown protein sample

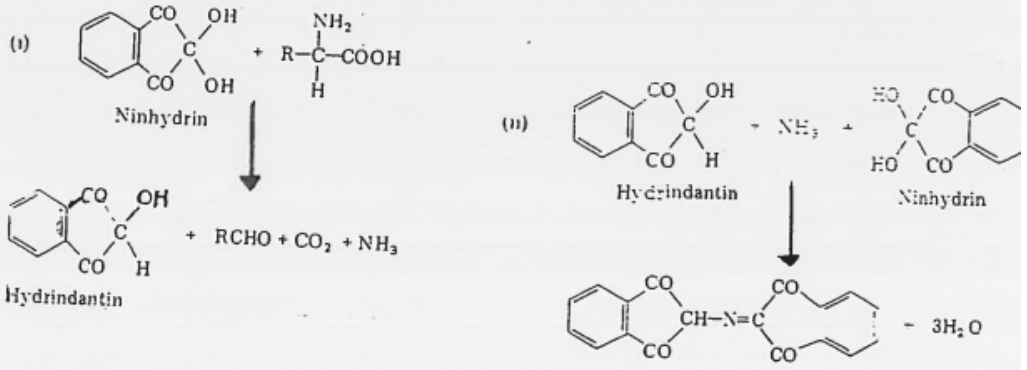
2- Beer's Law

$$\text{Conc. of Unknown} = \frac{\text{Ab of Unknown} \times \text{Conc. of Std}}{\text{Ab of Std}}$$

Exp(2):Quantitative determination of Amino Acids

Exp.(2.1):- The quantitative determination of amino acids using the ninhydrin reaction

The ninhydrin reaction which is used as a qualitative test for amino acids can be modified to a quantitative determination method as ninhydrin (triketohydridene hydrate) oxidizes α -amino acids between pH 4-8 to give purple-colored soluble diketohydrin (Ruhemann's purple) which can be determined colorimetrically at 570 nm. The imino acids proline and hydroxyproline also react with ninhydrin to give a yellow colored compound with maximum absorbance at 440 nm.



Materials:

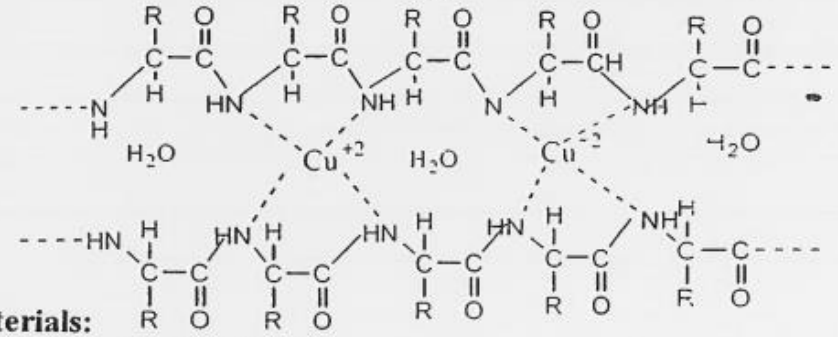
1- Amino acids (0.1 Mm)
2- Acetate buffer 4M,pH 5.5
3- Methyl cellosolve
4- Ninhydrin reagent: Dissolve 2 gm ninhydrin and 0.3 gm hydrindantin in 68 ml methyl cellosolve and make up the volume to 100 ml with acetate buffer 4M, pH 5.5. Prepare freshly and keep in a dark reagent bottle
5- Ethanol 50%

Method:

Pipette 2 ml of amino acid solution into a test tube, add 2 ml of ninhydrin reagent and heat in a boiling water bath for 15 min. Cool ,add 3 ml of 50% ethanol and read the absorbance at 570 nm(or 440 nm) after 10 min. against the appropriate blank. Make a standard curve of known concentrations (0.0—0.1mM) with the same amino acid in study (the unknown).

Exp.(3.1):- Determination of proteins by the biuret method

Compounds containing two or more peptide bonds form a characteristic purple color when treated with dilute alkaline copper sulfate solution. The color is due to coordination complex of copper with four nitrogen atoms and is related to the familiar blue color obtained with copper sulfate and ammonia



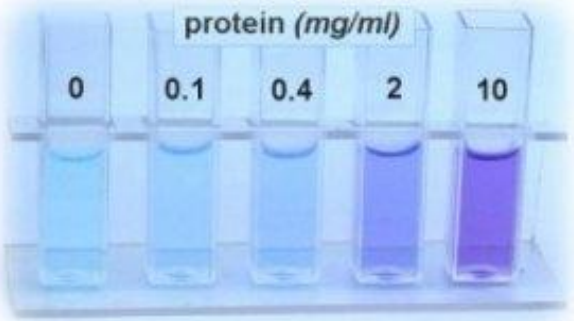
Materials:

1-Sodium chloride (0.9%)

2-Stock protein solution (10 mg albumin /ml in 0.9%NaCl)

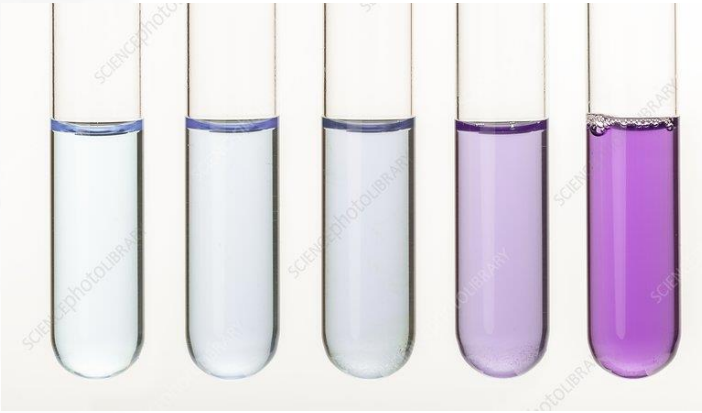
3-Biuret reagent : Dissolve 3gm $CuSO_4$ .5 H_2O and 9gm Na K tartrate in 700 ml of 0.2N NaOH. Add 5gm pot.iodide and make up the volume 1L with 0.2N NaOH

4-Unknown concentrations of albumin as unknown samples. Also blood plasma or blood serum might be used as unknown samples.



Method:

In eight dry and clean test tubes prepare 1 ml of the following protein concentrations (0.0, 0.5, 1, 2, 4, 6, 8 and 10 mg/ml) using the stock protein solution and 0.9%NaCl to prepare the protein standard concentrations. Add 3 ml of biuret reagent to each test tube and mix well. Treat 1ml of the unknown protein sample also with 3ml biuret reagent. Place all the tubes in a water bath of 37° C for 15min. Cool and record the absorbances at 540nm. Plot the biuret standard curve (absorbance vs. protein concentration as mg/ml or mg/100 ml) to determine the concentration of the unknown protein sample.



Notes

1. For best results 5ml biuret reagent is used .
2. To determine total protein concentration of blood plasma or serum 0.1ml of plasma or serum is diluted to 1ml with 0.9%NaCl and used as an unknown

Exp. (3.2):- Determination of proteins by the Folin-Lowry method

Proteins react with the Folin-Ciocalteau reagent to give a colored complex. The color complex so formed is due to the reaction of the alkaline copper with protein molecules -as in the biuret method- and the reduction of phosphomolybdate and phosphotungstate present in the Folin-Ciocalteau reagent by the aromatic amino acids (e.g..tyrosine and tryptophan) present in the protein molecule.

Materials:

1-Alkaline solution: Mix freshly (50 ml solution A + 1ml solution B)
Solution A: Prepare (2%Na_2CO_3 in 0.1N NaOH)
Solution B: Prepare freshly (0.5%$CuSO_4$ in 1% Na K tartrate).

2-Folin –Ciocalteau reagent: Available commercially and contains sodium molybdate ,phosphoric acid and hydrochloric acid. Dilute freshly 1:3

3- Stock protein solution: Prepare (1 mg albumin / ml)

4-Unknown concentration of albumin as unknown sample. Serum might be used as an unknown sample by diluting 0.01ml serum to 1 ml.

Method:

Prepare 1 ml of the following standard protein concentrations using the stock protein solution (0.1, 0.2, 0.4, 0.6. 0.8 and I mg /ml) in six clean and dry test tubes and transfer 1 ml of the unknown sample into the seventh test tube. Add 3 ml of "Alkaline solution" to each tube and mix well thoroughly. Allow standing at 37°C for 10 min then add 0.5 ml of the diluted Folin –Lowry reagent, mix rapidly and thoroughly- (use vortex if possible)- and let stand at room temperature for 30 min Prepare the appropriate blank. Record the absorbances at 700 nm and prepare the Folin-Lowry protein standard curve to calculate the concentration of the unknown sample.

Notes:

- For best results 5 ml of "Alkaline solution" is used.
- For preparation of the standard protein concentrations, dist. water or 0.9% NaCl is used.

Exp.(3.3):- The Bradford assay method

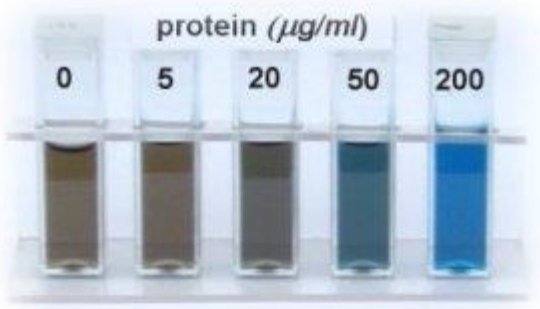
The protein dye Coomassie-Brilliant Blue G-250 interacts with proteins giving a greenish-blue color complex, which develops in less than 3 min., with λ max at 595 nm. The complex is stable for at least one hour, thus making the assay method simple and rapid. The Bradford assay method is sensitive to protein concentrations lower than 100µg/ml, and unlike biuret and Folin-Lowry assay methods, materials such as NH_4^+, K^+, Na^+, phenols, carbohydrates do not interfere. However detergents like SDS and Triton X-100 hamper the assay method

Materials:

1. Phosphate buffer (0.1M, pH 7.5).
2. Stock standard protein (0.1mg / ml in 0.1 M phosphate buffer pH 7.5). Bovine serum albumin is usually used as stock standard protein.
3. Bradford reagent: Dissolve 0.1 gm Coomassie-Brilliant Blue G-250 in 50 ml ethanol, add 100 ml of 85% phosphoric acid and make up the volume to 1L with dist. water.
4. Unknown concentration of protein sample.

Method:

Prepare 1 ml of the following protein concentration using the stock standard protein and the phosphate buffer to prepare the dilutions (10, 20, 40, 80 and 100µg/ml) and the appropriate blank. Deliver 1 ml of the unknown concentration of the protein sample into a clean and dry test tube. Add 5 ml of Bradford reagent to each test tube; mix thoroughly –(use vortex if possible)- for two minutes. Record the absorbances at 595nm. Plot the protein standard curve to determine the concentration of the unknown protein sample.



Exp.(3.4):-The ultraviolet absorption method

Tyrosine and tryptophan -(the aromatic amino acids)- absorb light at 280nm of the UV region and so proteins containing these amino acids will also absorb in this region of light. The disadvantage of this method is that many compounds absorb light in this region particularly nucleic acids, which have a peak at 260nm. Pure proteins have a ratio of absorption at 280nm / absorption at 260nm of about 1.8. While nucleic acids have a ratio of 0.5

Method:

Using a spectrophotometer, record the absorbance of a protein and of its blank solutions at 260nm & 280nm. To calculate the protein concentration as follows:

$$\text{mg protein / ml} = (1.55A_{280}) - (0.76A_{260})$$

THANK YOU.....

DO YOU HAVE ANY QUESTIONS ?

University of Baghdad

College of Science

Department of Chemistry

Nucleic Acids part one (RNA)

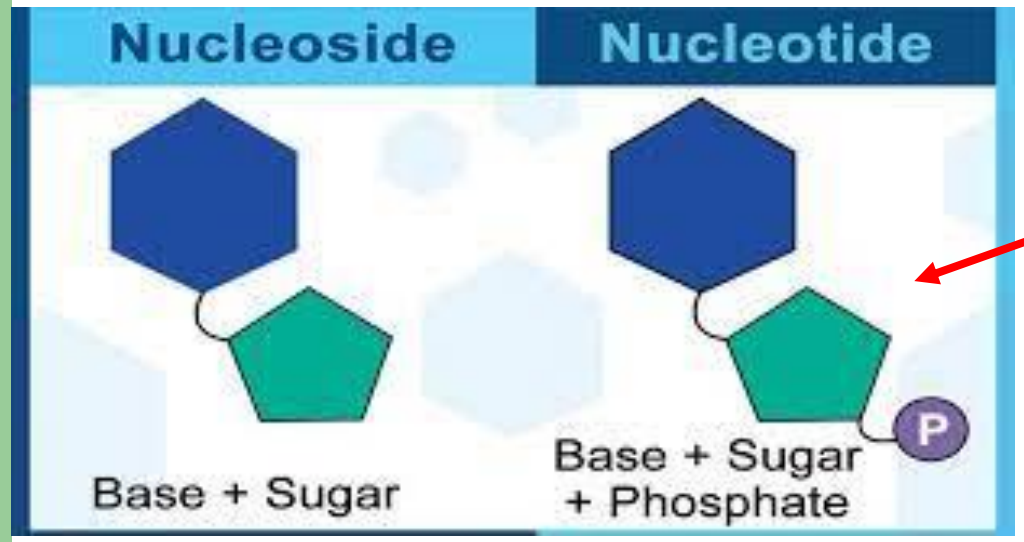
LAB EXPERIMENT 3

Dr. Nuha Nihad

Aims

- Isolation RNA from Yeast.
- Estimation of RNA by orcinol method.

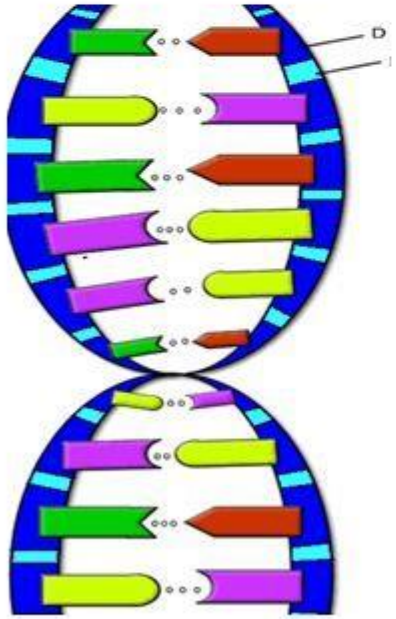
Nucleic acids



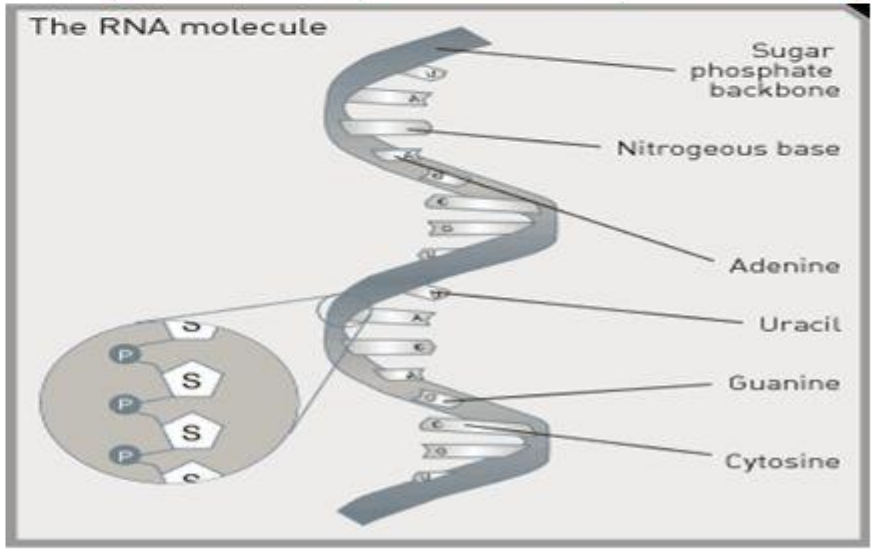
Nucleic acids are biopolymers, or small biomolecules, essential to all known forms of life. They are composed of nucleotides, which are monomers made of three components: a 5-carbon sugar, a phosphate group and a nitrogenous base. If the sugar is a compound ribose, the polymer is RNA (ribonucleic acid); if the sugar is derived from ribose as deoxyribose, the polymer is DNA(deoxyribonucleic acid).

DNA vs. RNA

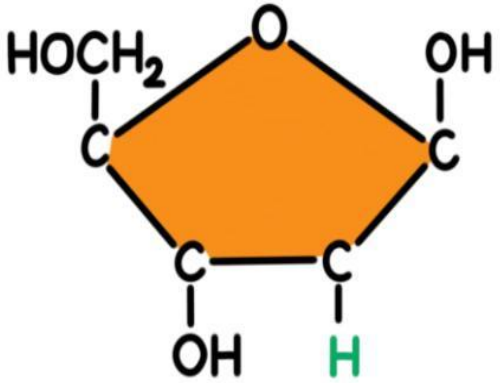
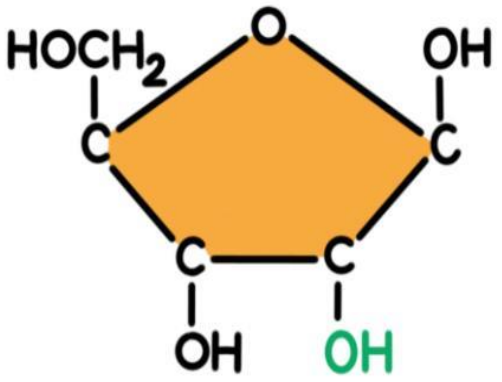
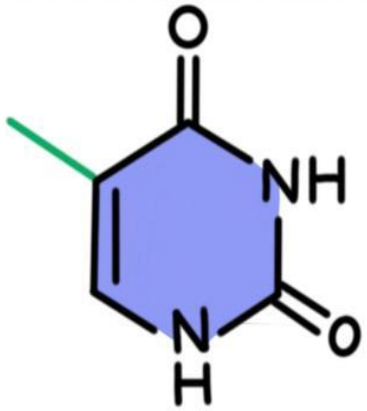
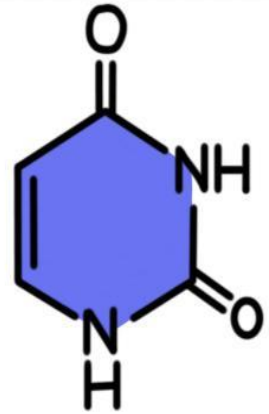
- Double stranded
- Deoxyribose sugar
- Bases: C,G A,T
- Self replicate

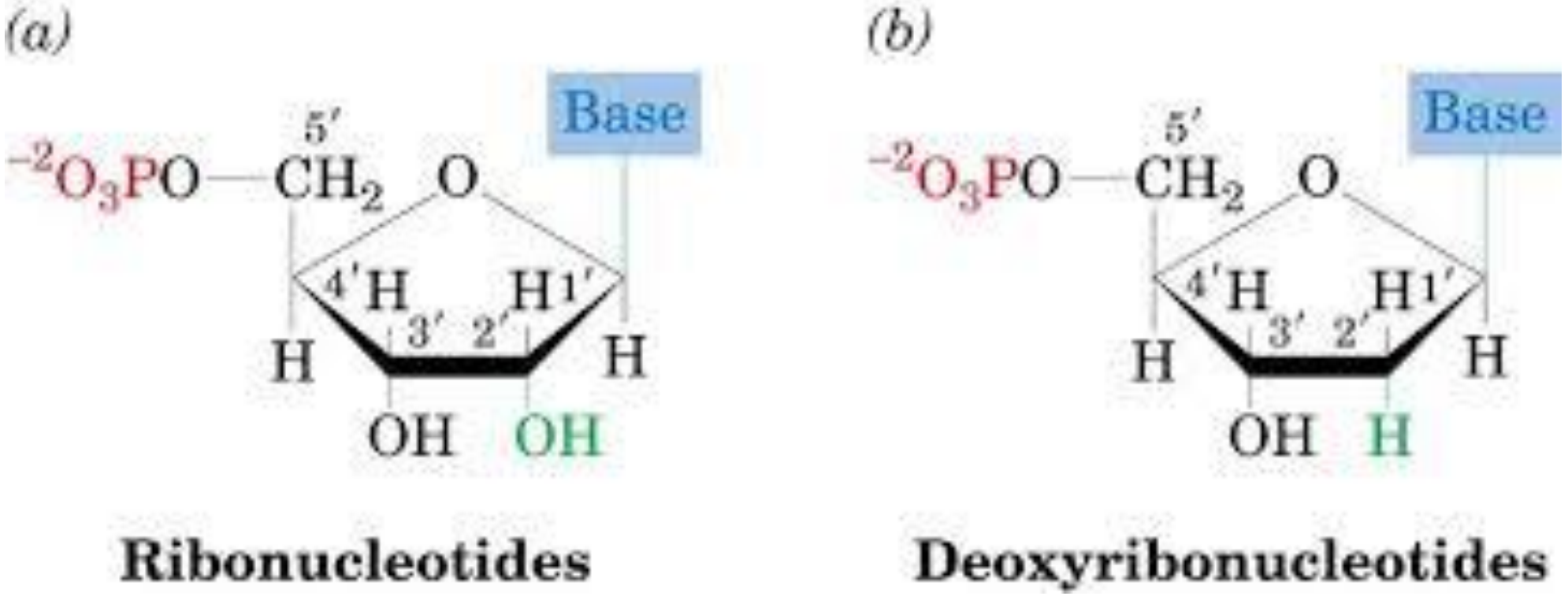


- Single stranded
- Ribose sugar
- Bases: C,G,A,U
- Can't self replicate
- mRNA, tRNA, rRNA



Both contain a sugar, phosphate, and base.

	DNA	RNA
SUGAR	 DEOXYRIBOSE	 RIBOSE
BASE	 THYMINE	 URACIL

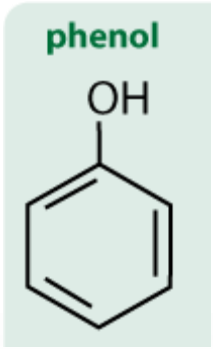


Ribonucleotides **Deoxyribonucleotides**

1- The isolation of RNA from Yeast.

Principle

Total yeast **RNA** is obtained by extraction a whole cell homogenate with **phenol** which disrupts hydrogen bonding causing denaturation of the protein.



1- The isolation of RNA from Yeast.

After centrifuging, the homogenate is separated into 2 phases: an aqueous phase (contains RNA, carbohydrate and some denatured proteins) and an organic (phenol) phase (contains DNA and some denatured proteins). **RNA** is precipitated by using ice-cold **95%** ethanol then RNA pellet is washed using ice-cold **70%** ethanol.

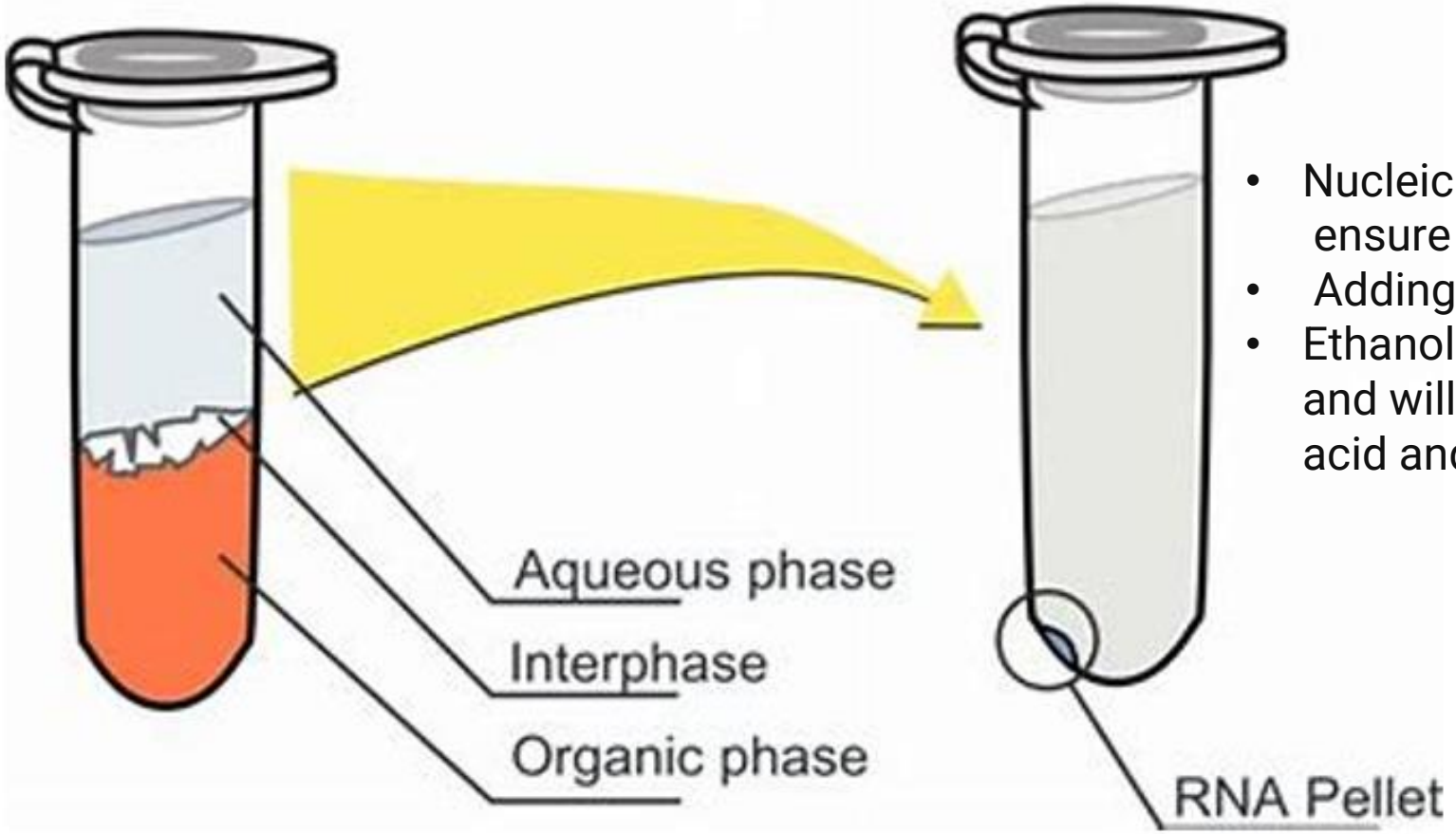
Why are two concentrations of ethanol used in the RNA extraction method?

- **95%** ethanol
- **70%** ethanol

Ethanol helps to promote RNA aggregation

Extraction using phenol

95% ethanol precipitation



للاطلاع

- Nucleic acids are insoluble in ethanol, so this will ensure that they precipitate out.
- Adding salts will aid in the precipitation.
- Ethanol has higher dielectric constant than water and will thus "soak-out" the water from nucleic acid and leave it dehydrated.

70% ethanol

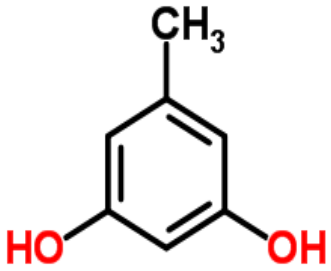
70 % percent of ethanol solution is used during the RNA washing steps. This allows the salts to dissolve while minimizing RNA solubility.

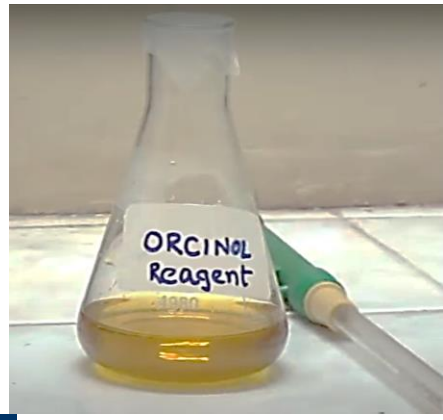
The Role of Salt (potassium acetate)

للاطلاع

The role of salt in the method is to neutralize the charges on the sugar-phosphate backbone. In solution, potassium acetate breaks up into K^+ and $[CH_3COO]^-$. The positively charged potassium ions neutralize the negative charge on the PO_3^- groups on the nucleic acids, making the molecule far less hydrophilic and, therefore, much less soluble in water.

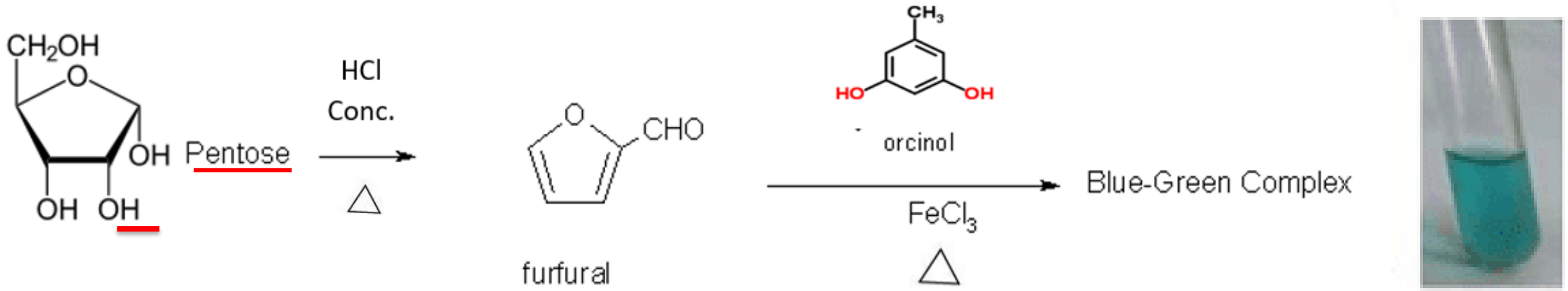
2- The orcinol method for RNA estimation.

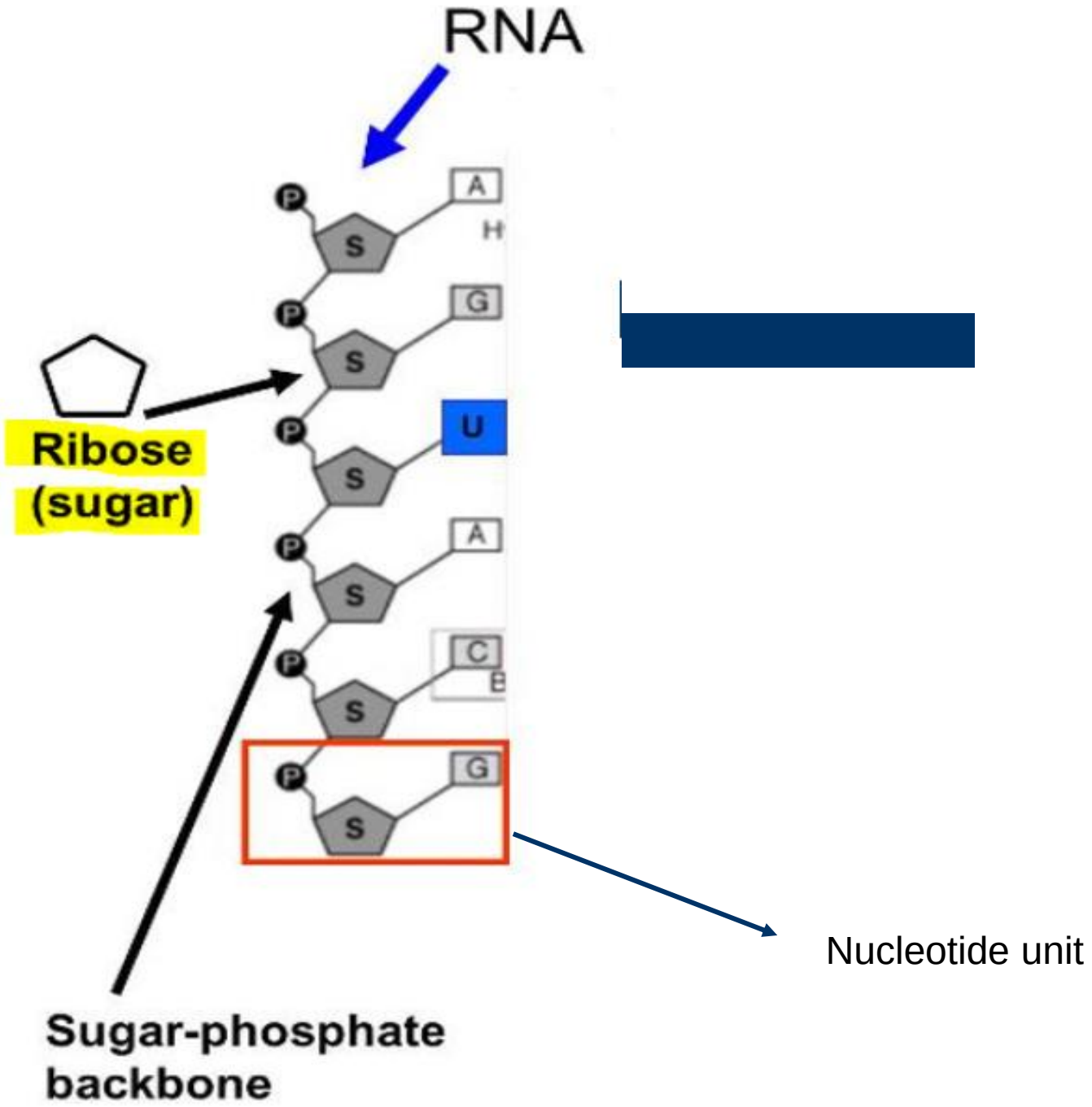




Principle

The orcinol method depends upon the formation of furfural when pentoses (in the backbone of the RNA molecule) are heated with HCl conc. Orcinol reacts with furfural in the presence of Fe^{+3} as a catalyst to give a soluble bluish-green colored complex




RNA
A
G
U
A
C
G
Ribose
(sugar)
Sugar-phosphate
backbone
Nucleotide unit

Bial’s test

للاطلاع

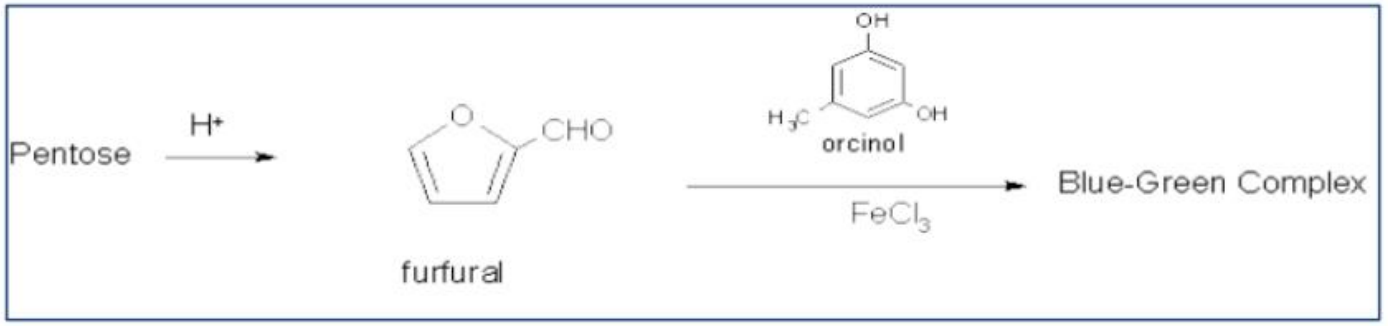
To distinguish between pentose monosaccharide and hexose monosaccharide (to detect pentoses).

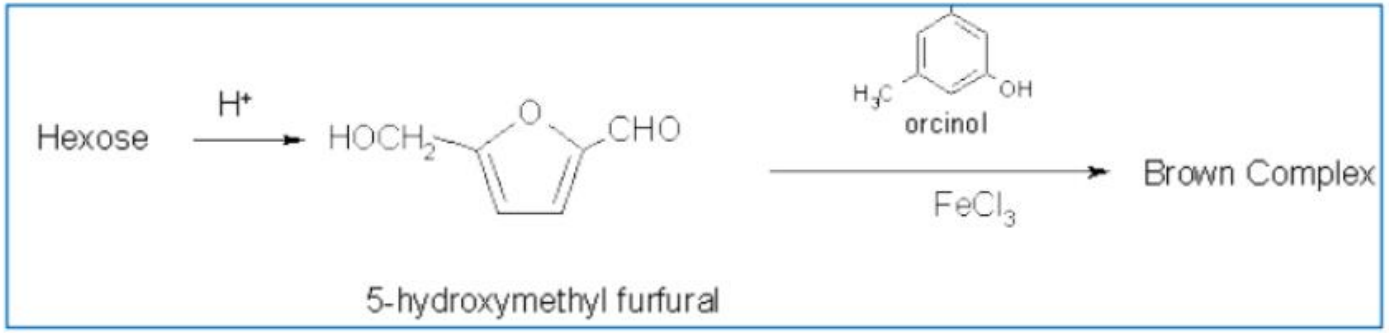
Principle:

- Bial's reagent (a solution of orcinol, HCl and ferric chloride).
- Bial’s test uses concentrated HCl as a dehydrating acid and orcinol + traces of ferric chloride [FeCl3] as condensation reagent.
- The test reagent dehydrates **pentoses** to form furfural. Furfural further reacts with orcinol and the iron ion present in the test reagent to produce a bluish or green product, while **hexoses** yield muddy-brown to grey condensation product.

Bial's test

للاطلاع





Ribose
(+)

Glucose
(-)

Exp (4.2):- The isolation of RNA

Total yeast RNA is obtained by extracting a whole cell homogenate with phenol. The concentrated solution of phenol disrupts hydrogen bonding in the macromolecules, causing denaturation of the protein. The turbid suspension is centrifuged and two phases appear; the lower phenol phase contains DNA and denatured proteins and the upper aqueous phase contains RNA, soluble carbohydrates and some denatured proteins. The denatured proteins existing in both phases are removed by subsequent centrifugation and RNA is then precipitated with alcohol at pH 5. The obtained RNA is free of DNA but usually contaminated with carbohydrates; treating with amylase preparations can make further purification. RNA can also be extracted with sodium hydroxide to dissolve membrane lipids and to dissociate the nucleoprotein complexes.

RNA EXTRACTION FROM YEAST

Materials:

1. Dried yeast.
2. Phenol solution (90%).
3. Potassium acetate (20%, pH 5).
4. Sodium hydroxide (0.1N).
5. Acetic acid (1M).
6. Ethanol (95%) & (70%).
7. Diethyl ether.
8. Litmus paper.

Method (a): Extraction using phenol.

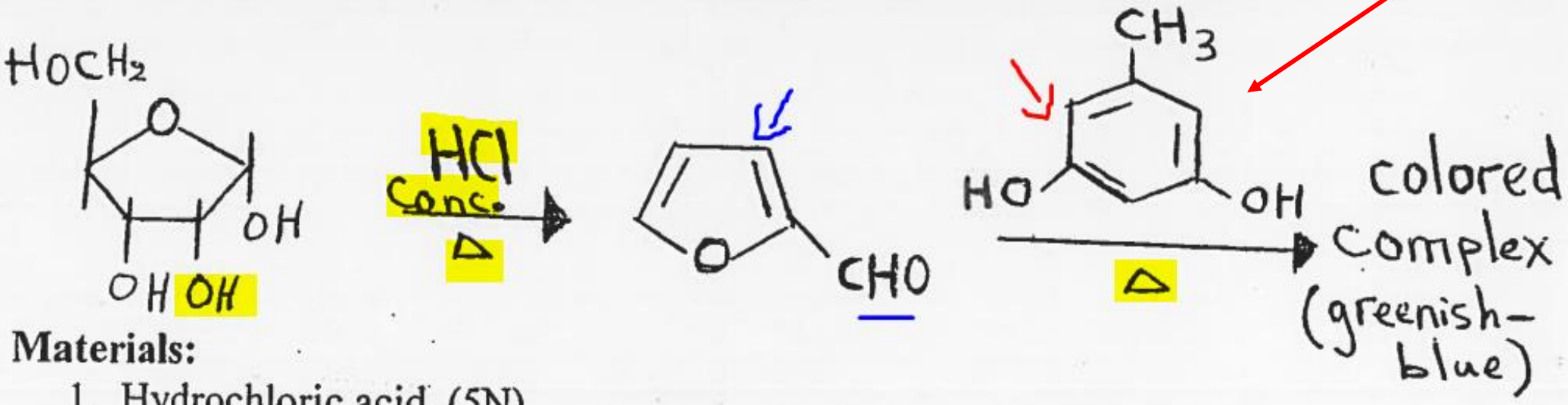
(a-1) Extraction of yeast RNA:

Procedure:

1. Suspend 0.3gm yeast in 2ml dist. water in a glass centrifuge tube and incubate at 37°C for 15 min.
2. Add 2ml of 90%phenol with care mix and incubate at room temperature for 30 min. with occasional stirring.
3. Cool the suspension in an ice bath for 5 min, then centrifuge at 3000rpm for 15 min. remove the aqueous upper phase carefully with Pasteur pipette into a previously weighed centrifuge tube. Make a subsequent centrifugation for the aqueous phase to clear the solution.
4. Add one-tenth the volume 20%pot.acetate (0.5—1ml), mix well then add two volumes of ice-cold 95%ethanol (≈ 5ml). Cool the solution on ice for 15 min. to precipitate the RNA.
5. Centrifuge at 3000rpm to collect the RNA. Wash the precipitate once with 3ml of 70%ethanol and once with 3ml of diethyl ether, centrifuge at each washing step.
6. Air-dry the RNA and weigh the pellet.
7. Calculate the amount of yeast RNA as mg RNA/ gm yeast & as %RNA in yeast. Compare the amount of RNA using the orcinol method (**4.3**).

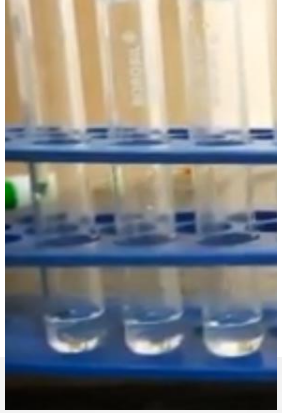
Exp (4.3):- The orcinol method for RNA estimation

The orcinol method depends upon the formation of furfural when pentoses are heated with concentrated hydrochloric acid. Orcinol reacts with furfural in the presence of Fe^{+3} as a catalyst to give a soluble bluish-green colored complex. On estimating RNA, only the purine nucleotides give any significant reaction.



Materials:

1. Hydrochloric acid (5N).
2. Stock standard RNA (50 mg/ml in 5N HCl).
3. Sample RNA (prepared in exp.**4.2**).
4. Orcinol reagent: Mix freshly (100 ml solution A + 3.5 ml solution B).
   Solution A: Dissolve 0.1 gm of $FeCl_3.6H_2O$ in 100ml conc HCl..
   Solution B: dissolve 0.6 gm orcinol in 10 ml ethanol.

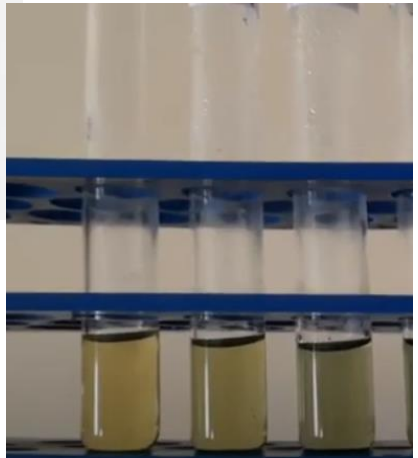


3ml of Orcinol reagent

Method:

1. Dissolve 0.1 gm of dry sample RNA in 5 ml of 5N HCl.
2. In three test tubes prepare the following:
   a)-Test (T): 1ml of sample RNA.
   b)-Standard (S): 1ml of standard RNA (5—50 mg/ml).
   c)-Blank (B): 1 ml of 5N HCl.
3. To all the tubes add 3 ml orcinol reagent, heat in a boiling water bath for 2 min. Cool and record the absorbencies at 670 nm.
4. Calculate the amount of sample RNA as mg RNA /ml, mg RNA / gm yeast & %RNA in yeast.
   (Note : for best results 5 ml orcinol reagent may be used)

Δ
2min.



Read A670 nm

- mg RNA/ml
- mg RNA/gm yeast
- % RNA in yeast

Calculations

Standard

1- Stock standard (50mg/ml RNA)

50mg/ml RNA in 5N HCl

Dissolve 0.05gm of RNA in 1ml HCl (5N) or dissolve 5g of RNA in 100ml HCl (5N)

2- **Standard** (25mg/ml RNA)

$50mg/ml \times V = 25mg/ml \times 1$

V= **0.5** ml of **Stock standard + 0.5ml HCl (5N)**

Calculations

ارقام افتراضية

- $A_T = 0.12$
- $Ast = 0.72$
- $A_B = 0.02$
- **Con. of standard** (25mg/ml RNA)

$$C_T = \frac{AT - AB}{Ast - AB} \times Cst$$

$$C_T = \frac{0.12 - 0.02}{0.72 - 0.02} \times 25mg/ml = 0.14 \times 25mg/ml$$

C_T = 3.5 mg RNA /ml

ملاحظة: قد يتم استخدام وحدة µg/ml لتحضير المحلول القياسي وبالتالي النتيجة تكون ب µg RNA/ml

1- RNA

Amount of RNA	Vol. of sample
3.5 mg	1ml
X	2ml

X = 7 mg/2ml

X = 0.007 gm/2ml

2- Yeast

Amount of yeast	Vol. of sample
0.3gm	**2ml**

0.3 gm/2ml

3- g%(w/w) = (gRNA/100gm yeast)

Amount of RNA

0.007 gm/2ml

X

X = 2.3 g% (w/w)

Amount of yeast

0.3 gm/2ml

100gm

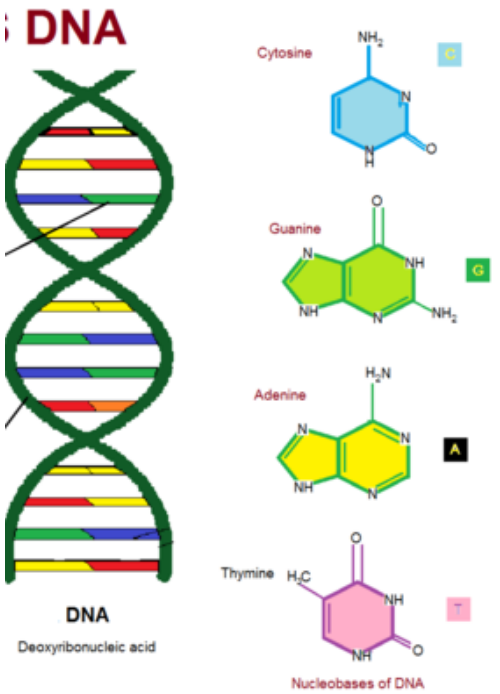

Thank
you

University of Baghdad

College of Science

Department of Chemistry

Nucleic acids part 2 (DNA)

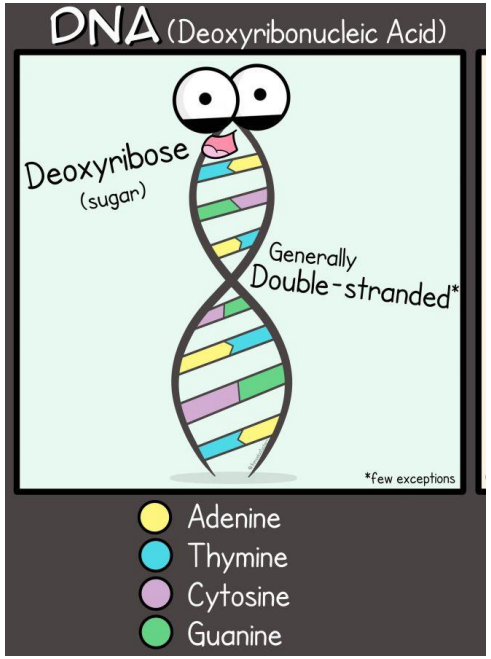


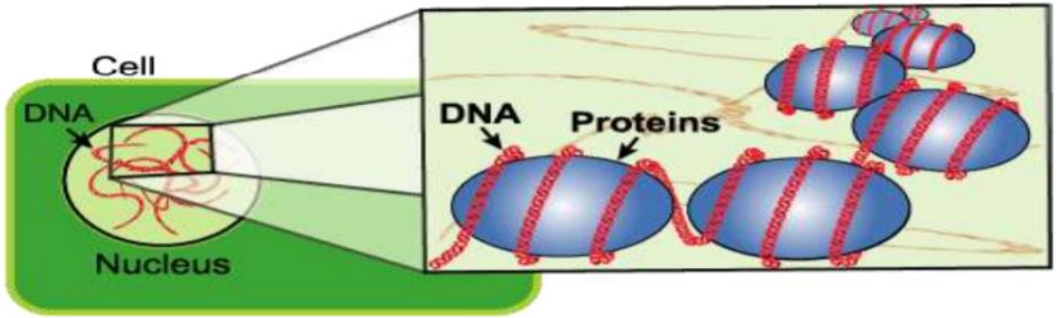
LAB EXPERIMENT 4

Dr. Nuha Nihad

Aim

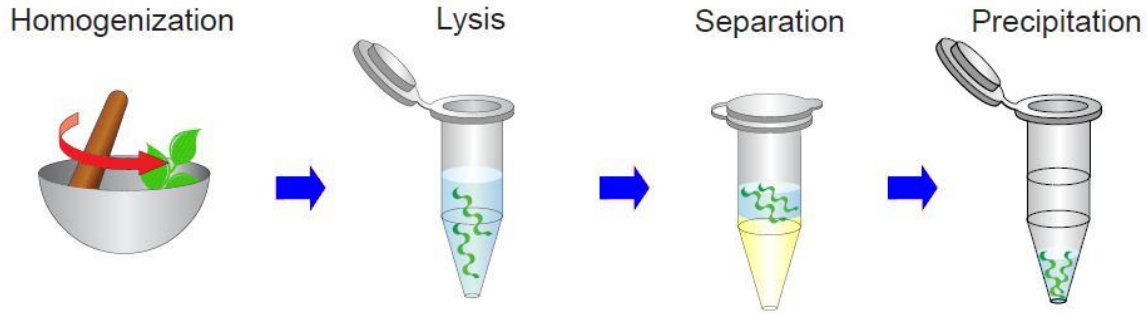
- The isolation of DNA.
- The diphenylamine method for DNA estimation.





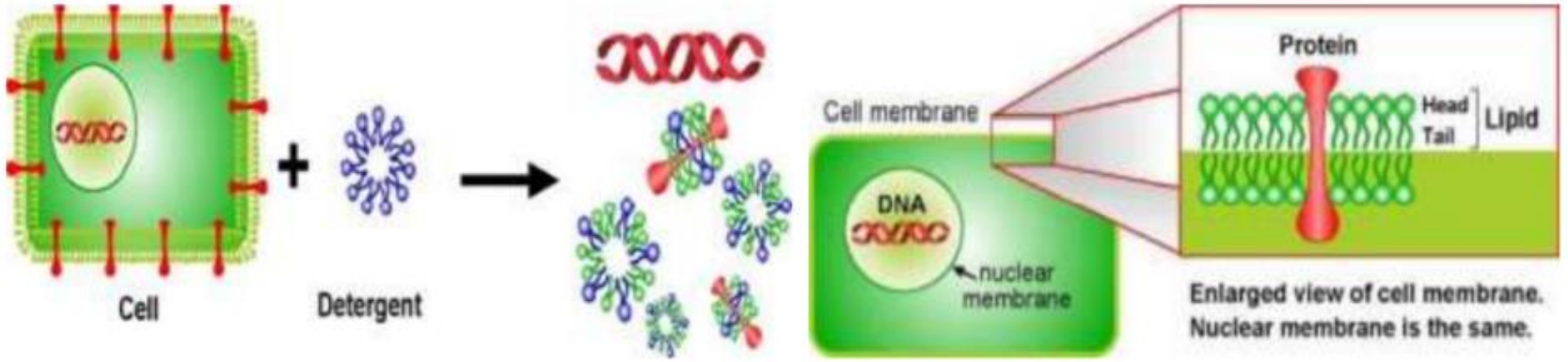
The isolation of DNA.

- The DNA is extracted from an animal or a plant tissue by using cold isotonic saline solution buffered with sodium citrate at pH=7.4 and a detergent solution containing the compound SDS (sodium dodecyl sulfate).
- DNA is precipitated as a fibrous white by using ice-cold 95% ethanol then precipitated DNA is washed using ice-cold 70% ethanol.

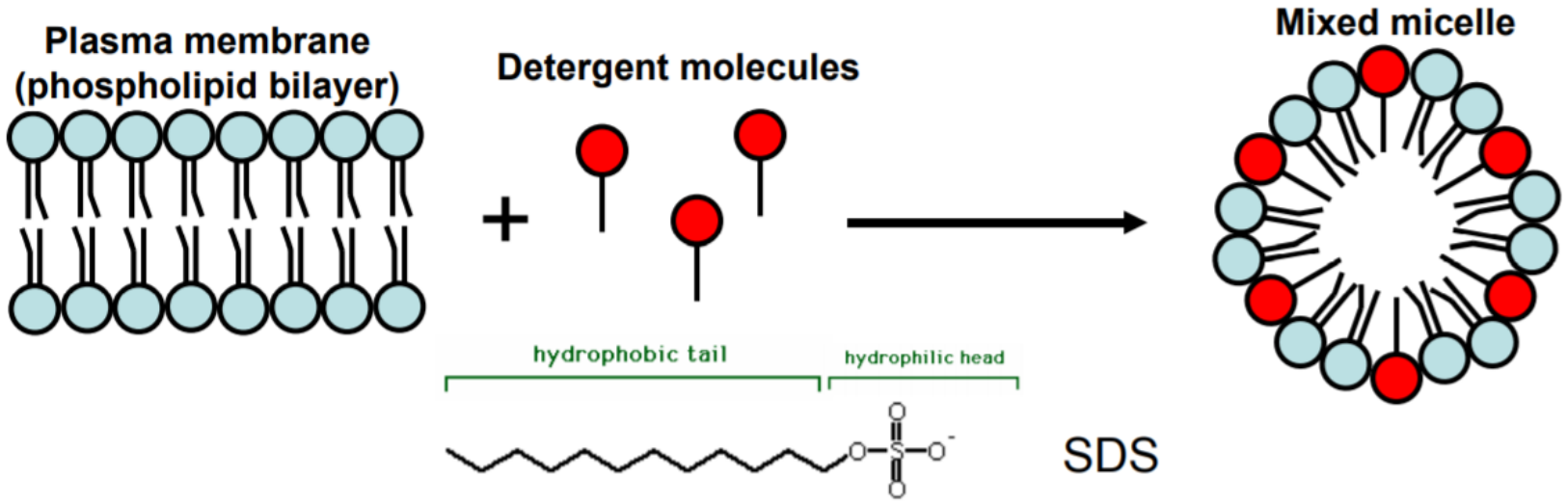


- At this ionic strength, the deoxyribonucleoprotein are insoluble and separate well from other proteins.
- The citrate ions **inhibit** the activity of the enzyme DNAase by binding Ca^{+2} and Mg^{+2} which are co-factors for this enzyme.

Use Detergent to solubilize the membrane lipid.

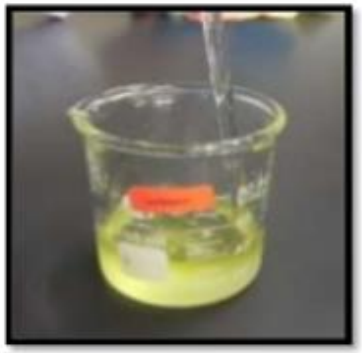


Use of Detergents to Lyse Cells:



Preparation of DNA extraction of onion cell

Chopped the onion

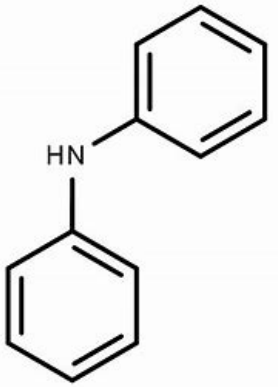


Poured water, salt and detergent

Poured the solution into the blender

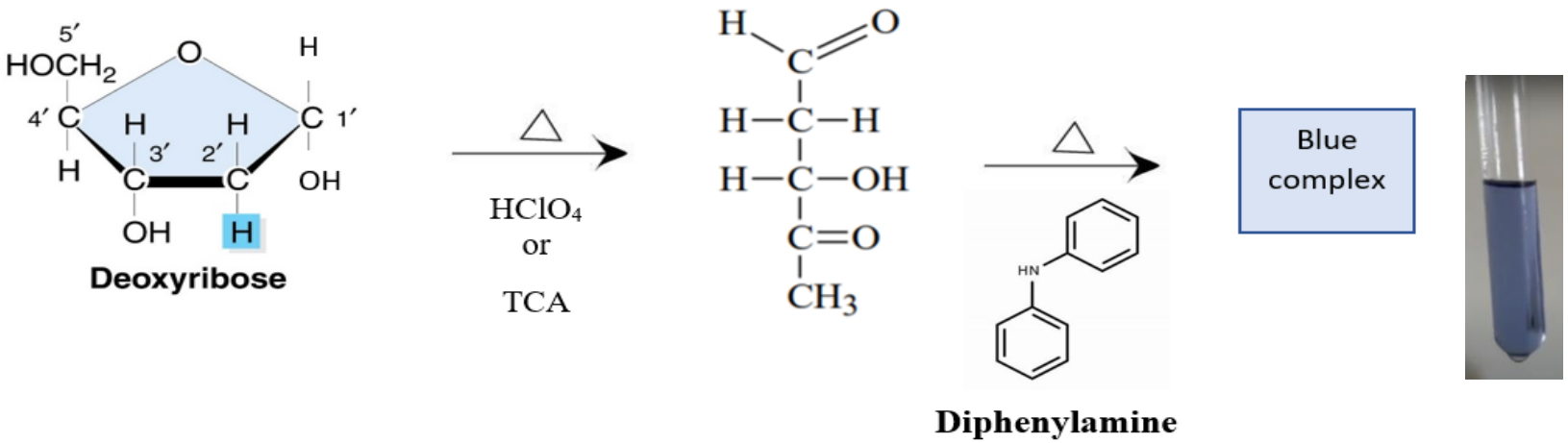
Filtered the homogenate

Precipitated by using ice-cold 95% ethanol



The diphenylamine method for DNA estimation.

- When DNA is treated with diphenylamine under acidic conditions, a blue compound is formed with the sharp λ_{max} at 595 nm.
- In acid solution, the straight chain of the 2-deoxyribose is converted to the highly reactive β-hydroxylevulinaldehyde, which reacts with diphenylamine to produce a blue complex.



Exp(4.4): The isolation of DNA

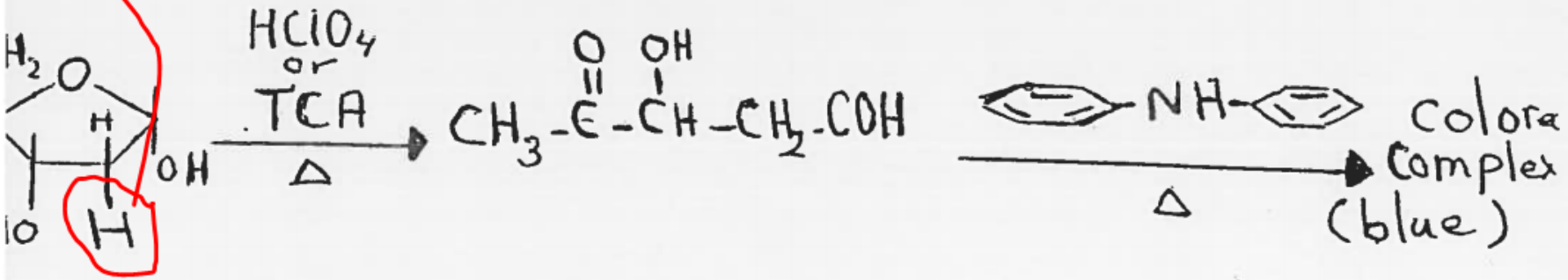
To extract the DNA from an animal or a plant tissue, the tissue is homogenized in isotonic saline solution buffered with sodium citrate at pH 7.4. At this ionic strength the deoxynucleoproteins are insoluble and separate well from other proteins. The citrate ions inhibit the activity of the enzyme DNA-ase by binding Ca^{+2} & Mg^{+2} which are co-factors for this enzyme. The extraction procedure must be carried out in cold so that any residual DNA-ase activity is minimal. Glass or plastic vessels are used throughout the experiment to avoid DNA degradation and direct contact with fingers must be avoided.

The DNA is precipitated as a fibrous white mass by the addition of ethanol. The material is best stored frozen and does not undergo any damaging changes for several months, while drying exposes the DNA strands to denaturation.

For further purification of the nucleic acids (RNA & DNA). urea, SDS, lysozymes, RNA-ase or DNA-ase and their inhibitors may be used.

Exp (4.5): The diphenylamine method for DNA estimation

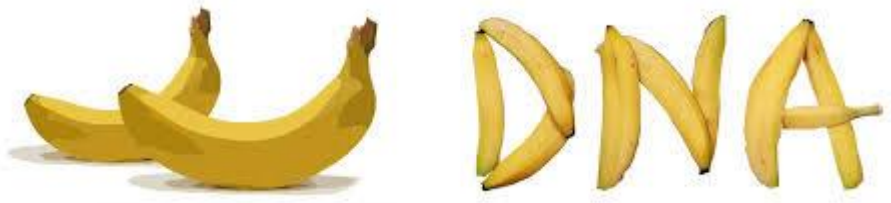
The diphenylamine method for quantitative estimation of DNA requires heating the DNA sample with diphenylamine in acidic conditions to produce a blue compound with a sharp λ_{max} at 595nm.which is a general reaction given by the 2-deoxypentoses (not specific for DNA). In acidic solution, the straight chain of the 2-deoxyribose is converted to β-hydroxylevulinaldehyde, which reacts with diphenylamine to give a blue complex. In DNA only the deoxyribose of the purine nucleotides react, so the value obtained represents half the value of the deoxyribose present in DNA sample.



Materials:

1. Trichloroacetic acid (10%TCA).
2. Stock standard DNA (10mg/ml in 10%TCA): Dissolve DNA (commercial sample) in 10 ml of 10%TCA and heat in a boiling water bath for 30 min, cool and make up the volume to 10 ml.
3. Sample DNA (prepared in exp4.4), dissolved in 10 ml of 10% TCA.
4. Diphenylamine reagent (DPA reagent): Prepare freshly by dissolving 1 gm


Thank
you

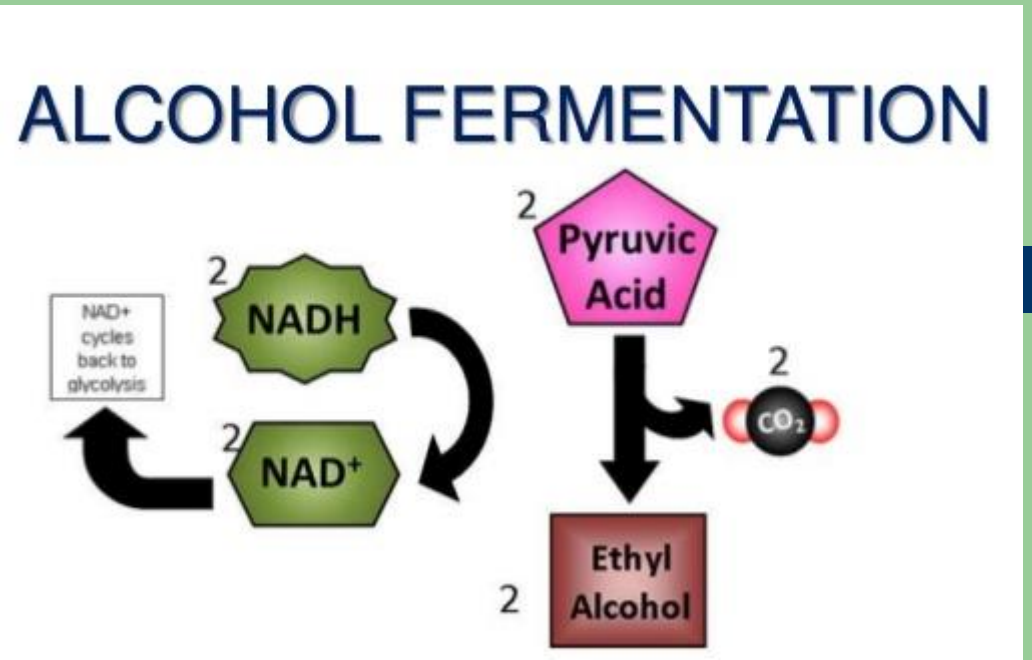

DNA

University of Baghdad

College of Science

Department of Chemistry

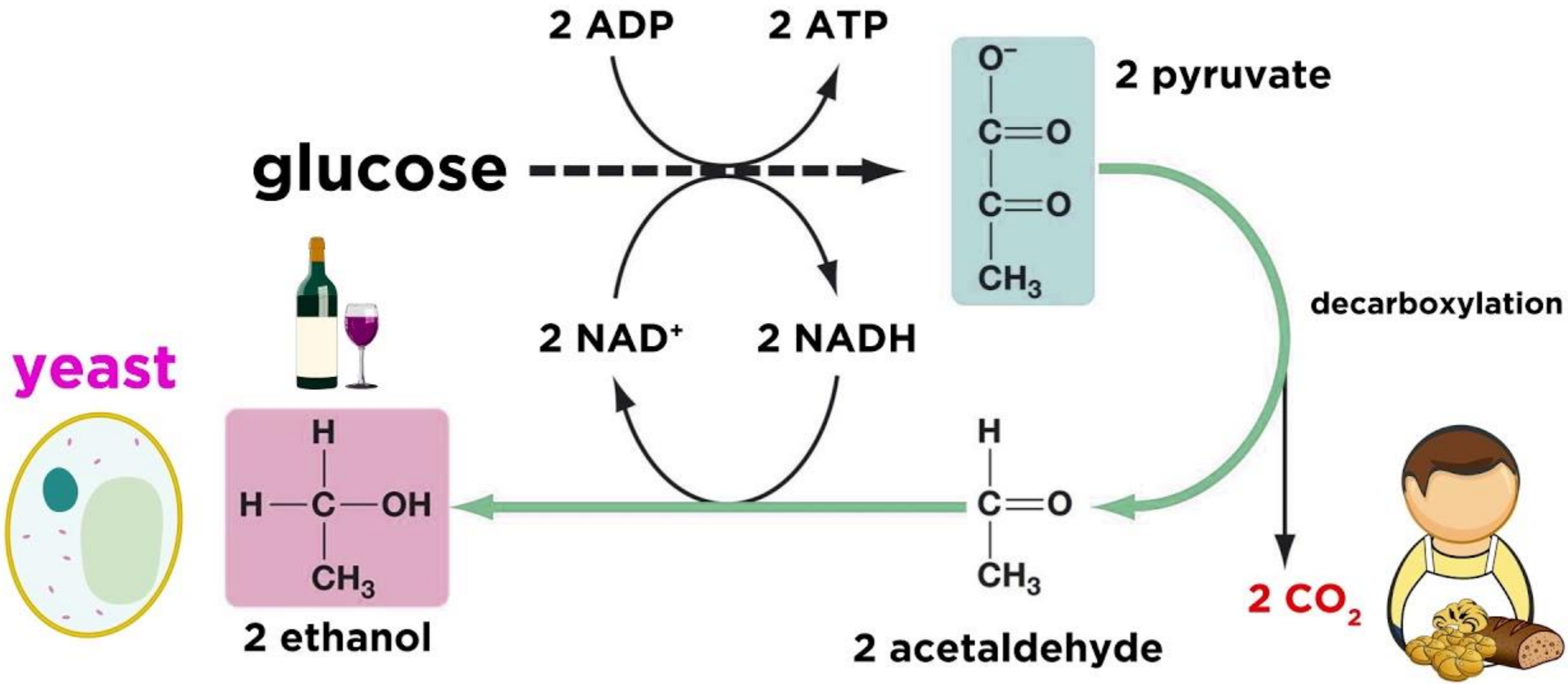
The production of pyruvate and acetaldehyde during the fermentation of glucose



LAB EXPERIMENT 5

Dr. Nuha Nihad

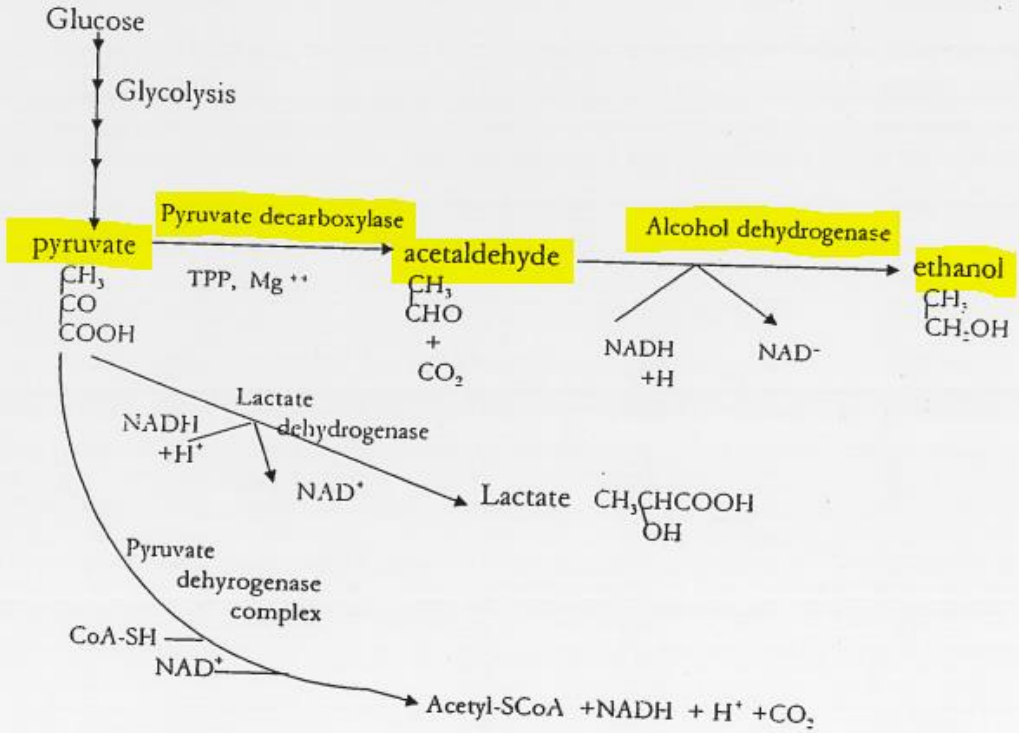
Alcohol Fermentation



Exp(7) The production of pyruvate and acetaldehyde during the fermentation of glucose.

The metabolic breakdown of glucose is basically the same in yeast and animals up to the production of pyruvate. Under anaerobic conditions, pyruvate is further metabolized to lactate in animals while ethanol (alcohol) is the end product in yeasts.

Alcohol is formed by the oxidative-decarboxylation of pyruvate with the intermediate formation of acetaldehyde.



In yeasts and other microorganisms, the conversion of glucose into ethanol is called alcohol fermentation and the net result of glycolysis in yeasts is:

$$\text{Glucose} + 2\text{ADP} + 2\text{Pi} + 2\text{H}^+ \longrightarrow 2\text{ethanol} + 2\text{CO}_2 + 2\text{ATP} + 2\text{H}_2\text{O}$$

It is important that NAD^+ and NADH do not appear in this equation . Acetaldehyde is reduced to ethanol so that NAD^+ is regenerated for use in the reaction catalyzed by glyceraldehydes -3-phosphate dehyrogenase in the glycolytic pathway.

As Pyruvate decarboxylase (2-oxo-acid carboxy-lyase ,4.1.1.1) is inactive in slightly alkaline solutions , pyruvate will accumulate at this pH and its presence is detected.

The metabolic intermediates such as pyruvate and acetaldehyde are normally present at very low concentrations to be detected by the conventional chemical methods .So in order to prove their existence as intermediates in the pathway some means has to be found to prevent the metabolic reactions to proceed any further either by adding an enzyme inhibitor or blocking the enzyme activity or changing the physiological conditions or by adding trapping agents that react with one of the intermediates to form a compound that is not metabolized any further.

Sodium sulphite salt, when added into one of the incubation tubes as a trapping agent, reacts with acetaldehyde and prevents the metabolic reaction to move any further, so acetaldehyde can be detected. Ethanol is detected in the tubes in which the physiological conditions are right and no trapping agents are present.

Method:

In four glass centrifuge tubes add the following solutions :-

The glucose solution as the primary substrate for the glycolytic pathway,

The yeast suspensions in slightly alkaline and slightly acidic solutions as different physiological conditions for enzymatic activity.

The yeast suspensions in dist. H_2O with or without sodium sulphite salt to show the effect of the trapping agent on the pathway .

Test tube contents	The glass centrifuge tubes A	B	C	D
Glucose 10%	5 ml	5 ml	5 ml	5 ml
Yeast suspension in Na_2HPO_4	5ml	_____	_____	_____
Yeast suspension in KH_2PO_4	_____	5ml	_____	_____
Yeast suspension in dist. H_2O	_____	_____	5ml	_____
Yeast suspension in dist. H_2O with 10% Na_2SO_3	_____	_____	_____	5ml
Mix well and incubate the four centrifuge tubes at 37°C for 60 minutes (for best results the incubation time should be 4 hours). Then add 10% TCA to stop the enzymatic reactions.				
TCA 10%	1ml	1ml	1ml	1ml
Mix well and centrifuge at 3000 rpm for 5—10 minutes and transfer the clear supernatant into four clean test tubes. Perform the chemical tests using the clear supernatant of A , B , C , D .				

Tests for pyruvate :- Use supernatants A & B

1)- Sodium nirtoprusside test :- Add 2ml of boiled supernatant to about 1 gm of solid amm.sulphate ,add 2 drops of 5% sod. nitroprusside ,mix thoroughly then add without mixing conc. ammonium hydroxide solution to form two layers. If pyruvate is present, a green or blue ring forms at the junction of the tow layers. Sometimes a transient pink ring forms due to the presence of thiol groups before the characteristic blue or green color of pyruvate.

2)- The 2,4-di-Nitrophenyl hydrazine (DNPH) test :- Add 0.5 ml of the DNPH solution to 2 ml of the supernatant ,mix thoroughly then add I ml of 10% NaOH solution and dilute to 5ml with dist. water. A red color forms if pyruvate is present.

Tests for acetaldehyde:- Use supernatants C & D

1)- Nitroprusside test:- add 0.5 ml of freshly prepared 5% sod. nitroprusside to 2 ml of supernatant. A dark green color is formed if acetaldehyde is present which turns blue if 3% piperidine is added.

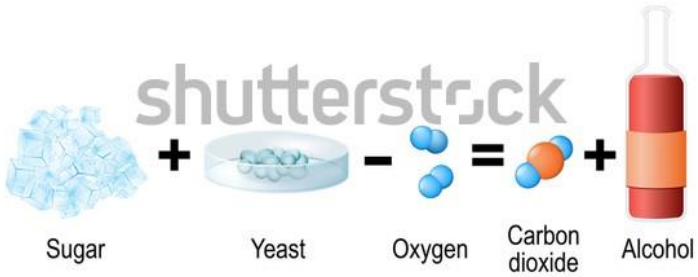
2)- Potassium chromate test:- Add 1 ml of 10% pot. chromate to 2 ml of supernatant ,a green color is formed if acetaldehyde present.

Tests for ethanol:- Use supernatants C & D

1)- Cerric amm. nitrate test :- Add 0.5 ml of 10% cerric amm. nitrate to 1 ml supernatant .A dark red color appears if ethanol is present (heat in a boiling water bath for 3—5 min if needed) .

2)- The N-Bromosuccinamide test:- Add 1 ml of the reagent to 1 ml of supernatant then add a few drops of Br_2/CCl_4 . Mix thoroughly and notice the color formed if primary alchohol is present.

Thank you


FERMENTATION
Sugar
Yeast
Oxygen
Carbon dioxide
Alcohol
shutterstock
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University of Baghdad

College of Science

Department of Chemistry

Sugar analysis

LAB EXPERIMENT 6

Dr. Nuha Nihad

Aim

Estimation of glucose, fructose, and sucrose in honey (foodstuff).

Quantitative Analysis of carbohydrate (sugar).

- Volumetric Methods.
- Gravimetrical Methods.
- Chromatographic Methods.

Reducing property of sugar used in :

- Qualitative analysis→ to identify carbohydrates.
- Quantitative analysis → to determine the amounts of carbohydrates in the sample.

Volumetric Method (Luff method)

The sugars content will be determined in honey through the volumetric method which is based on the reducing properties of sugars, such as glucose and fructose using LUFF reagent.

Luff Reagent

Contains

- $CuSO_4.5H_2O$
- Na_2CO_3
- Na-K tartrate (prevent $CuSO_4$ precipitated in reagents)
- $NaHCO_3$
- KI
- KIO_3

LUFF reagent

$R - COH + Cu^{+2} \rightarrow Cu_2O + R - COOH$

- LUFF reagent contains cupric ions that are reduced by reducing sugars and the **excess** is used to reduce KI solution to form I_2 which is determined by titration against $Na_2S_2O_3$ solution.

KI and acid adding

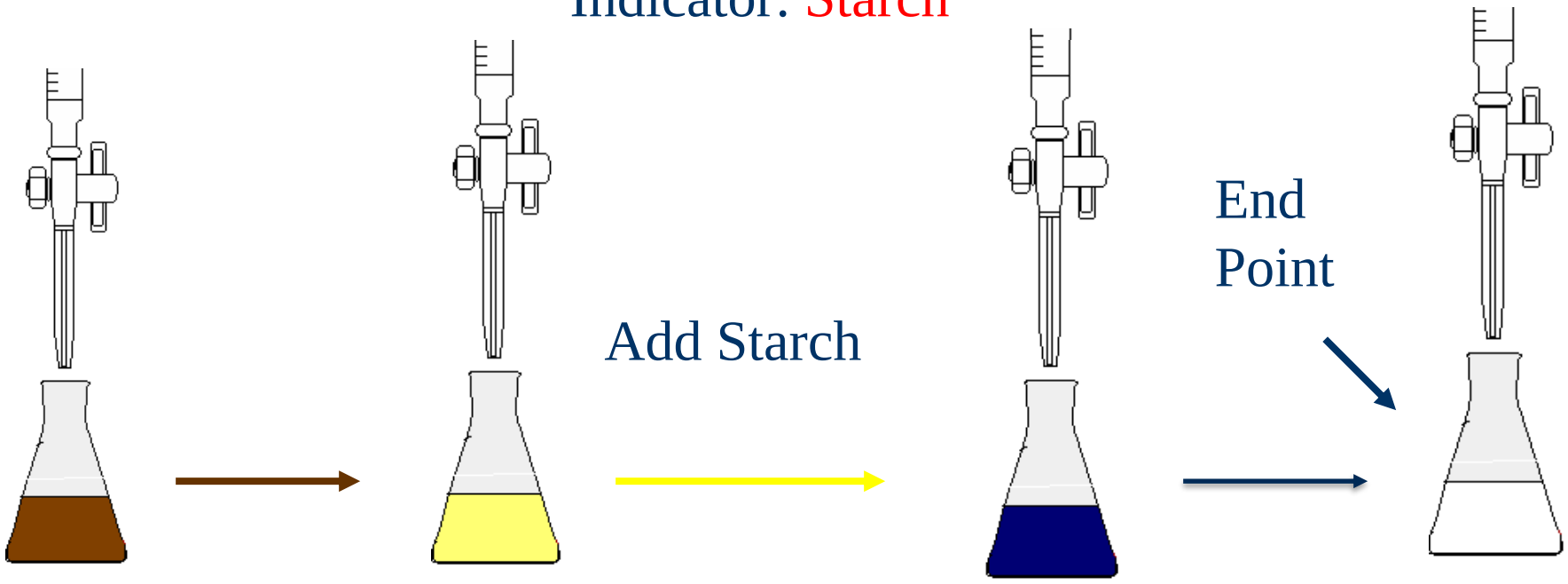
Titration with $Na_2S_2O_3$

Iodine x Thiosulphate

$I_2 + Na_2S_2O_3 \rightarrow Na_2S_4O_6 + NaI$

Colorless

Indicator: Starch



I_2 Soln. Brown

I_2 Soln. Pale Yellow

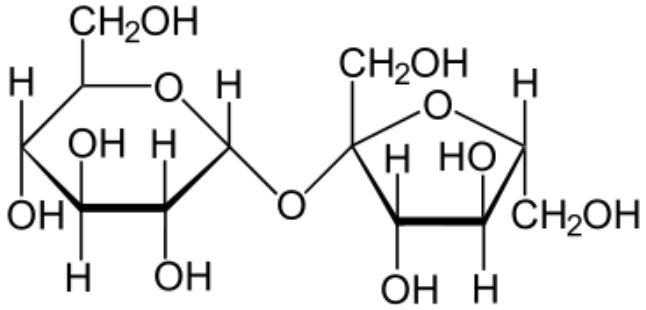
Blue Black

Colorless

I_2 + starch : blue

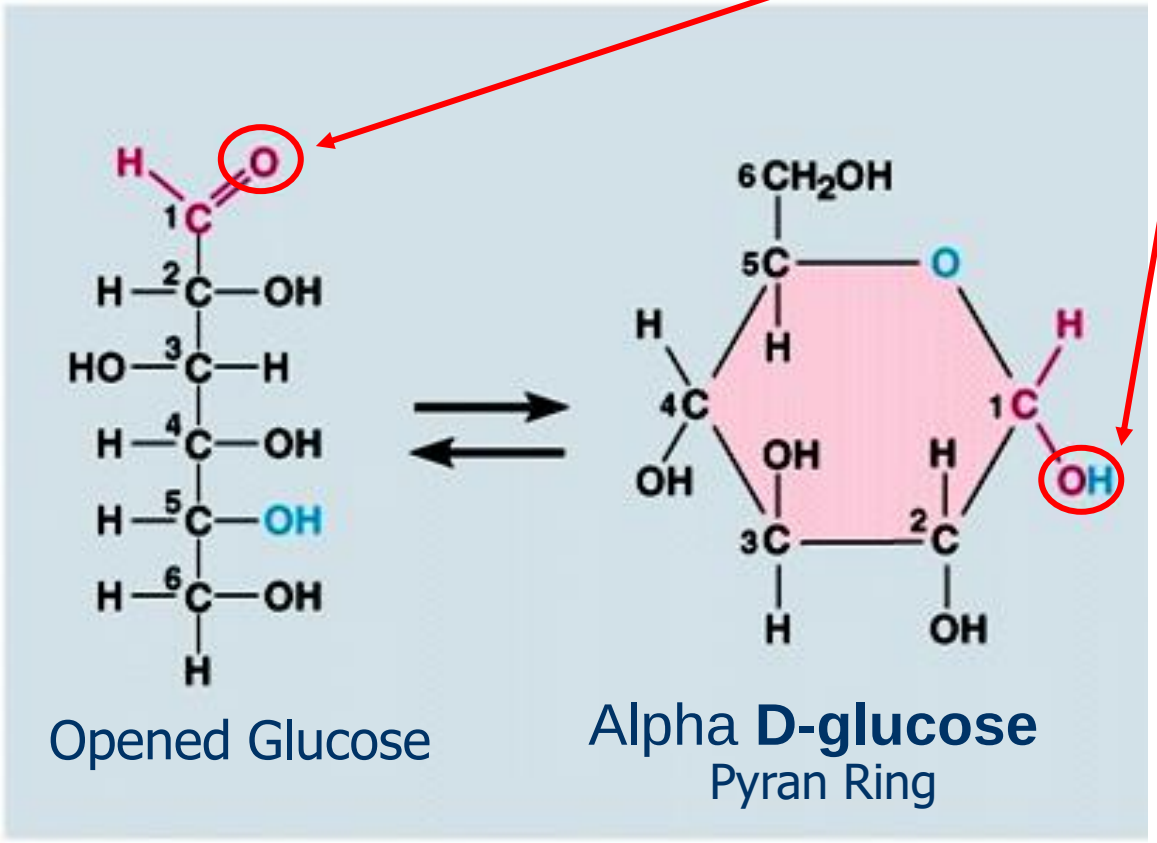
- Sucrose does not react with complex copper (II) ions, but glucose and fructose can react with this reagent due to the presence of aldehyde groups in glucose and alpha hydroxy ketones in fructose.
- To be analyzed by this method, sucrose is hydrolyzed

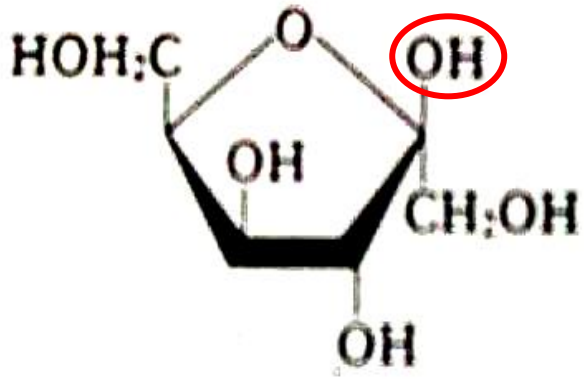
into glucose and fructose.



Reducing sugars

Reductive group

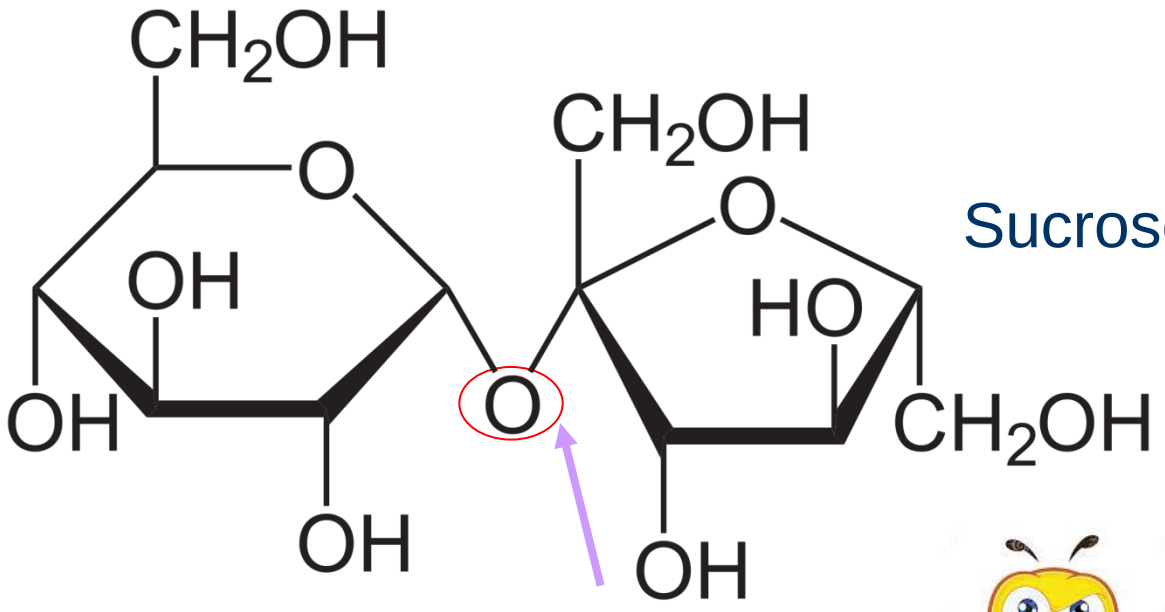




Beta- **D-fructose**

Furan Ring

Non-reducing sugars



Sucrose = α Glucose + β Fructose

Reductive group close each other (non-reductive)