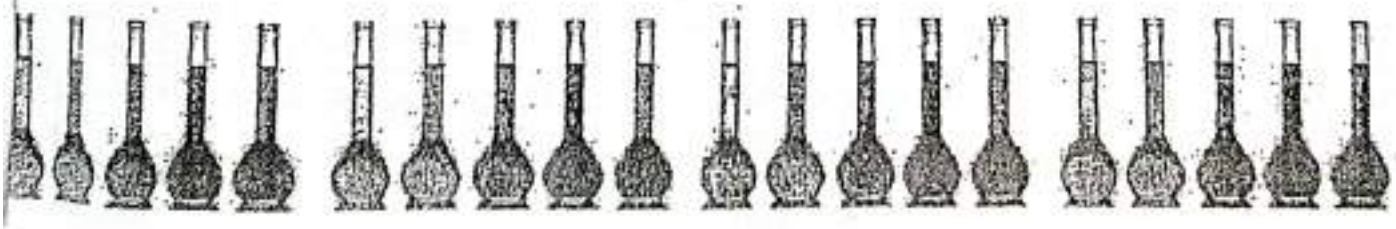
السلامة المختبرية

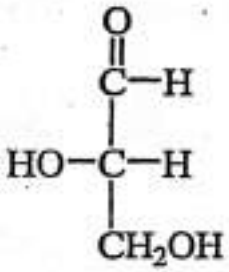
- يجب ارتداء الصدرية البيضاء الخاصة بالمختبر للحفاظ على ملابسك من تأثير المواد الكيمياوية بمجرد دخول المختبر.
- تعلّم أسماء كافة الأجهزة والأدوات والمواد التي تستعملها في التجارب وحافظ عليها من التلف والكسر ونظفها بعد انتهاء التجربة.
- ضع جميع حاجياتك والكتب داخل الدولاب أمامك ورتب الأدوات التي تحتاجها للتجربة على الدولاب بحيث تترك مجالاً كافياً للعمل.
- كن مرتباً في عملك وحافظ على مكانك نظيفاً وامسح الطاولة التي تشتغل عليها بأسفنجه رطبة كلما انسكب عليها سائل ما.
- لا تضع أي شيء في فمك ولا تدخن.
- يجب عدم الإسراف في استعمال المواد الكيمياوية والماء المقطر والتقيد بالكميات المذكورة في طريقة أجراء التجربة.
- بعد استخدام زجاجيات المواد الكيمياوية يجب إعادتها إلى مكانها مع أحكام إغلاقها.
- يجب فتح صنبور المياه لفترة عند إلقاء الحوامض المركزة في الأحواض لكي تصبح مخففة منعاً لتآكل أنابيب تصريف المياه.
- عند الانتهاء من العمل أغلق التيار الكهربائي لجميع الأجهزة المستخدمة.
- مطالعة التجربة قبل القدوم إلى المختبر وفهمها جيداً والتقيد بخطوات العمل في كل تجربة.
- يجب تدوين ملاحظات عن كافة المشاهدات والاستنتاجات التي تحصل عليها أثناء إجراء التجارب.



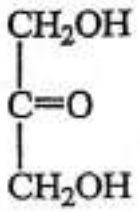
Carbohydrates

Carbohydrates are one of a major type of big biomolecules, also are a class of organic compounds. It is about 15% organic compounds of cell structure.

Carbohydrates were considered to be hydrates of carbon because they contain hydrogen and oxygen in the ratio of 2:1 just as in water. The general formula of carbohydrates is $C_n(H_2O)_n$, where $n \geq 3$ carbohydrates are now defined as aldehydes or ketones of polyhydroxy alcohol.



Aldosugar



Ketosugar

In most organisms, carbohydrates material, largely in the form of simple sugar glucose, is the primary foodstuff, providing most of the energy and carbon required in the biosynthesis of proteins, nucleic acids, lipids and other carbohydrates.

Some carbohydrates have a structural role such as cellulose in plants and glycogen in animals and bacteria.

Carbohydrates are classified into three major categories according to their structure or hydrolysis to:

Monosaccharides	السكريات الأحادية	1
Disaccharides Oligosaccharides	السكريات الثنائية السكريات قليلة الوحدات	2
Polysaccharides	السكريات المتعددة	3

- **Monosaccharides:** are simple sugars that cannot be changed into simple sugar upon hydrolysis (reaction with H_2O) . It can be classified according to the number of carbon atoms in the chain. Those found most commonly in humans include the following:
  - **a- Trioses:** composed of three carbon atoms [e.g. glyceraldehydes].
  - **b- Tetroses:** composed of four carbon atoms [e.g. erythrose].
  - **c- Pentoses:** composed of five atoms [e.g.: ribose, xylose, arabinose].
  - **d- Hexoses:** composed of six carbon atoms :
    - Aldohexoses: contain aldehyde group [e.g glucose in blood, galactose in milk].
    - Ketohexoses: contain ketone group [e.g fructose in honey].

They are easily soluble, colorless, sweet taste, readily diffusible and highly reactive pentoses and hexoses.

- **Disaccharides** or **Oligosaccharides:** composed of (2-10) molecules of simple sugar linked together by glycosidic linkages [e.g. lactose, maltose and sucrose]. It can also be classified according to reaction to:

A. Reducing sugar: they contain free aldehyde or ketone groups that are capable of reducing an oxidizing agent. Examples are maltose, that is composed of two molecules of glucose, and lactose, that is composed of glucose and galactose.

$$\text{Maltose} + H_2O \xrightarrow[\text{Maltase}]{\Delta} 2\ \text{Glucose}$$

B. Non-reducing sugars: they lack the free aldehyde or ketone group. An example is sucrose (sugarcane). It is found in red beet, pineapple and root carrot which is composed one molecule of (glucose) and one molecule of fructose linked together by (1-2) glucosidic linkage.

$$\text{Sucrose} + H_2O \xrightarrow[\text{or } H^+, \Delta]{\text{Sucrase}} \text{Glucose} + \text{Fructose}$$

- **Polysaccharides:** they are polymer of monosaccharide units linked together by glycosidic linkages which hydrolysis by acid and heat or specific enzymes. So are complex saccharides on hydrolysis yield many simple sugars. All polysaccharides are non-reducing. The large size of these polysaccharide molecules is responsible for their relatively low solubility and diffusion. For the same reason their solution are colloidal in nature and opalescent in appearance.
- Structural polysaccharides such as cellulose and chitin.
- Storage polysaccharides such as starch and glycogen.

Quantitative test for carbohydrates

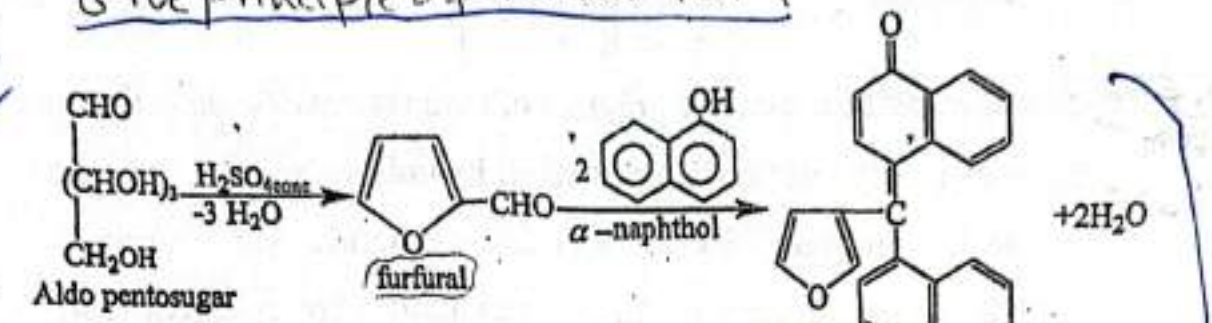
1. Molish's test

Principle

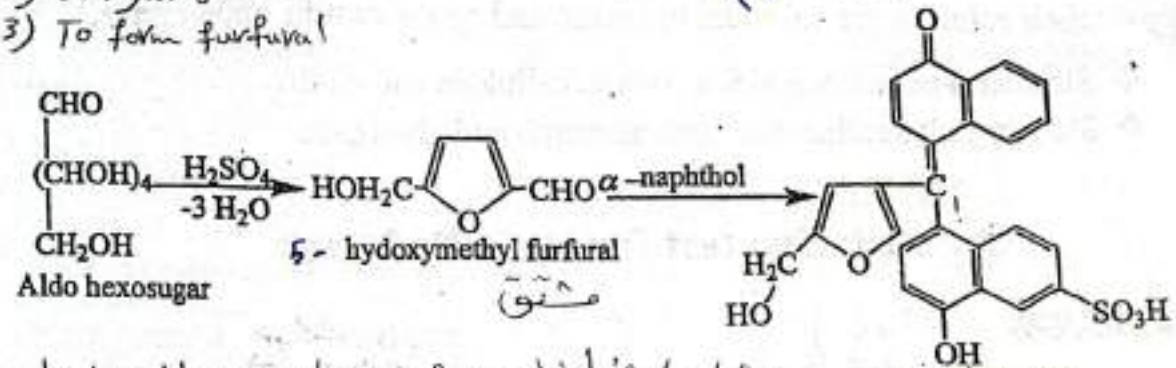
Carbohydrates on treatment with strong concentrated sulphuric acid undergo dehydration to give furfural or furfural derivative which on condensation with α-naphthol yields violet or purple ring complex. If oligo saccaride or polysaccharides are present they are first hydrolyzed to the constituent monosaccharides which are then dehydrated. Pentose yield furfural and hexoses yield-5-hydroxy methyl furfural.

$$\left[\begin{matrix}\text{Polysaccharide} \\ \text{Disaccharide}\end{matrix} \xrightarrow[\text{Conc.}]{H^+} \text{Monosaccharide}\right]$$

Give principle of molish test?



molish test في H2SO4 لماذا نستخدم
1) hydrolysis of Poly & Di to mono sugar
2) dehydration.
3) To form furfural



what is the content of molish's test?
1) H2SO4 2) α-naphthol 3) sugar simple

Materials

1. 5% alcoholic α-naphthol fresh prepare in 95% ethanol (Molish's reagent).
2. Conc. H_2SO_4.
3. Water.
4. 1% monosaccarides, disaccharides, polysaccharides.

*** Precaution**

Test tube for this test should be completely dry.

1) 1ml test (Sugar)
2) 3-4 drops molish's test

Procedure

1. In a clean and dry test tube, take 0.5 ml of the carbohydrate solution (sugar).
2. Add (3-4) drops of molish reagent, mix well.

3. Carefully add about 0.5 ml of conc. H_2SO_4 by the side of the tube. Without mixing so that two layers will be formed, the upper layer for carbohydrate solution and the lower layer for conc. H_2SO_4.
4. Observe the purple ring at the junction of the two layers indicate the presence of carbohydrates.

Notes:

1. You may observe the formation of a green ring beside the purple ring this is due to the reaction between α-naphthol and sulphuric acid and not to the presence of carbohydrate.
2. Black ring indicate to sugar charring due to adding conc. H_2SO_4 quickly.

2. Bendict's test

This test is positive for reducing sugars only some carbohydrate with a free aldo or keto group process a reducing power. So they are known as reducing sugars. Benedict's test is a test used to distinguish between reducing sugar from non-reducing sugars.

Principle:

The reducing sugars (mono or disaccharides) will reduce the cupric ions (Cu^{+2}) present in the reagent to the cuprous ion and precipitated as cuprous oxide Cu_2O in alkaline medium at high temperature. The test can be used for the detection of glucose in urine.

Principle:

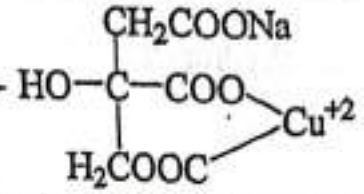
$$\text{Reducing Sugar} + CuSO_4 \xrightarrow{OH^-} Cu_2O\downarrow + V$$

$$(HC\text{-}OH)_4 + CH \xrightarrow{OH^-} CH^{+1} + (H\text{-}C\text{-}OH)_n$$

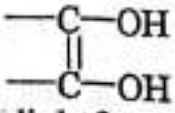
CH_2OH — $COOH$

$CuSO_4 \longrightarrow Cu^{++} + SO_4^{=}$

غير مطلوب

$Cu^{++} + Na_3C_6H_5O_7 \longrightarrow$ 

cupric: sodium citrate complex

Reducing Sugar $\xrightarrow{Na_2CO_3}$ 

Enediols forms of reducing sugar

Enediols + Cupric : Sodium citrate complex $\xrightarrow{B.W.B}$ Cu^{+1} + Mixture of sugar acids

$Cu^{+1} + OH^- \longrightarrow CuOH$

$2CuOH \xrightarrow{Heat} Cu_2O\downarrow + H_2O$

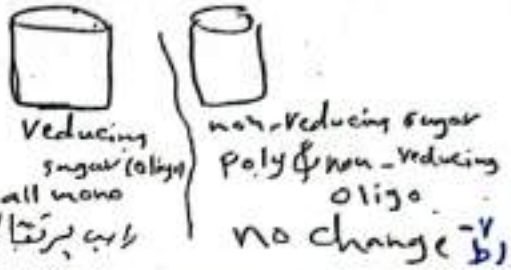
Red ppt.

Materials

1. Benedict's reagent is prepared by dissolving 173 g of sodium citrate ($Na_3C_6H_5O_7$) 100 g of anhydrous Na_2CO_3 in 800 ml of distilled water. Slightly heat the contents to dissolve.
   Dissolve 17.3 g of $CuSO_4.5H_2O$ in 100 ml of water. Add this solution slowly and with stirring to the above solution complete to 1 L, the mixed solution is ready for use.
2. 1% monosaccharides (glucose) and disaccharides (lactose).

Procedure:

1. Add 1ml of Benedict's reagent in the test tube and then add 4 drops of the test solution, mix well.
2. Put the reaction test tube in boiling water bath for (2-3) minutes.
3. Then take it out of the water bath and let it cool.
4. Observe and record the change in color (yellow, orange, green or red and brown ppt.)



Notes:

1. $CuSO_4.5H_2O$ is the source of Cu^{+2}.
2. Na_2CO_3 to make alkaline medium.
3. Sucrose may be hydrolysis in a long time of heating so may give positive result.
4. Degree of color change depends on the conc. of reducing sugar.

3. Barfoed's test

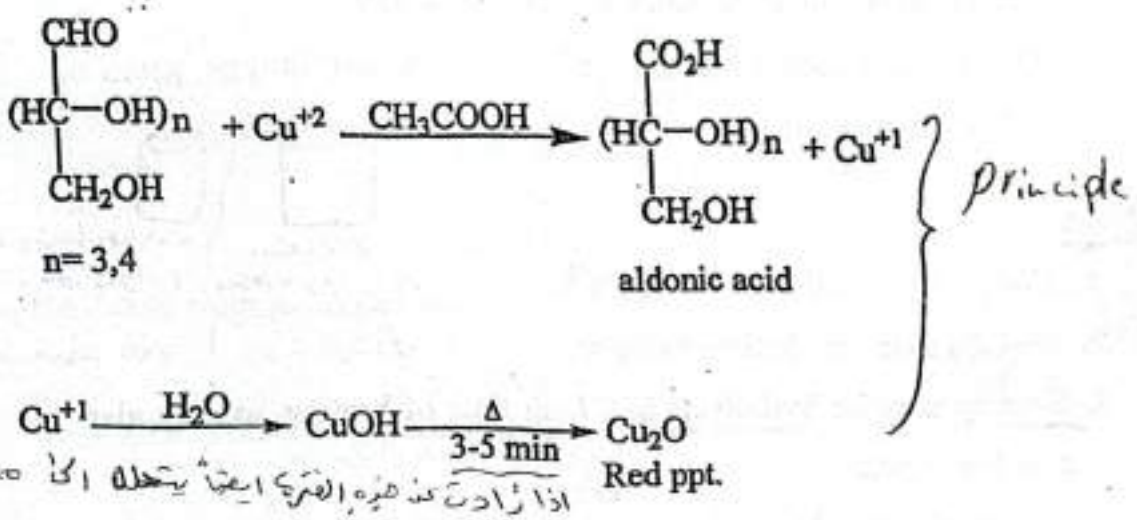
This test is used to distinguish monosaccharide from reducing disaccharide.

Principle:

Only monosaccharides are capable of reducing cupric ions in weakly acidic solution to the corresponding coprous ions (ppted as Cu_2O). whereas disaccharide give negative result (solution remain blue) unless they present in high concentration or hydrolyzed to their main constituents of monosaccharide due to prolonged heating of the reaction mixture. The test cannot be used for the detection of glucose in urine due to the presence of chloride ions in the urine, which interfere with the test.

Barfoed's test is a reduction test carried out in an acidic medium. The acidity makes it a weaker oxidizing reagent. Therefore, only

monosaccarides, will reduce cupric ions. However, if heating is prolong, disaccarides may be hydrlyzed by the acid and the resulting monosaccaride will give a positive test.



Materials

1. Barfoed's reagent is prepared by dissolving 13.3 gm of copper (II) acetate in 200 ml distilled water, filter then add 1.8 ml glacial acetic acid.
2. 1 % monosaccharide and disaccharide.

Procedure:

1. To 1 ml of Barfoed's reagent add 5 drops of test solution, mix.
2. Place the test tube in boiling water bath for exactly 3 minutes.
3. Then remove the test tube and allow cooling gradually a red precipitate of Cu_2O is formed in case of the presence of monosaccharide.

Notes:

1. Time factor is very important since prolonged heating of di-or polysaccharide may hydrolyze it and give positive test.
2. The rate of reaction depends upon the concentration of cupric ions (Cu^{+2}). It also depends on the time of heating.

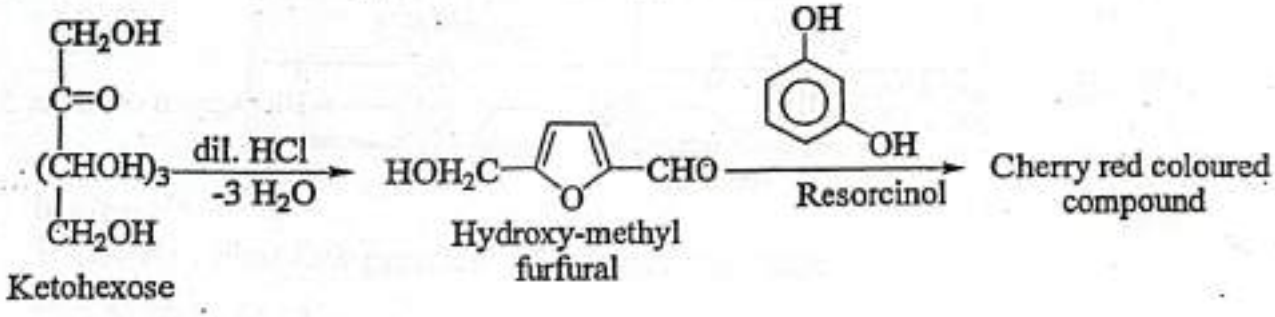
4. Selivanoff's test

This test is positive for ketohexoses only and hence is used in the detection of fructose.

Selivanoff's test distinguishes between fructose and glucose.

Principles

Ketohexoses are dehydrated more easily than aldohexoses and aldopentoses to give hydroxyl methyl furfural which condenses with resorcinol to form a cherry red colored complex.



Materials:

1. 0.05 gm resorcinol in 200 ml dil. HCl (2:1) .
2. 1% aldo and keto sugars.

Procedure:

1. Place I ml of Selivanoff's reagent in a test tube.
2. Add 3 drops of carbohydrate solution, mix.
3. Place in boiling water bath for (2) minutes exactly or heat over a flame for 30 seconds only. Cool the solution.
4. An appearance f cherry red color indicates the presence of fructose.

Notes:

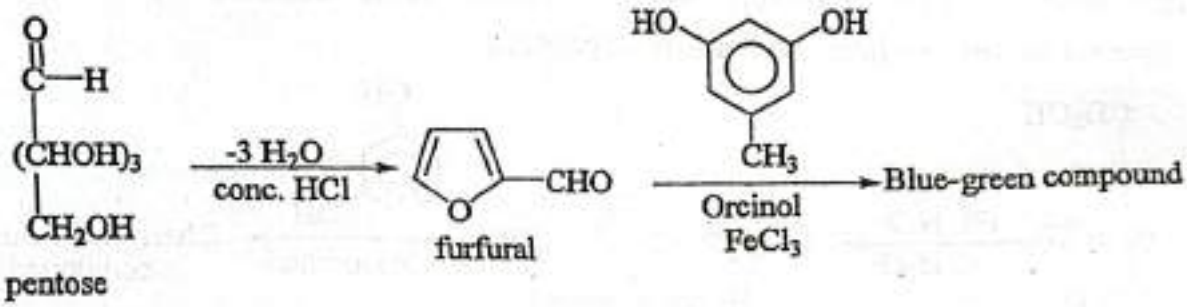
1. The time factor Selivanoff's test is very important.
2. Over heating of the solution is avoided because on continuous boiling, aldose will also give this test positive because of their conversion to ketoses by hydrochloric acid.
3. Sucrose also gives a positive test because it is readily hydrolyzed during the course of the test yielding fructose as one of the products.

5. Bial's test

This sensitive test for the detection of pentoses.

Principles

When pentoses are heated with conc. HCl, furfural is formed which condenses with orcinol in the presence of ferric ions to give a blue green color.



Materials

1. 1.5 gm orcinol in 500 ml concentrated HCl. Add (20-30) drops of 10% of ferric chloride (Bial's reagent).
2. 1% pentose and hexose sugars.

Procedure:

1. To 1 ml of Bial's reagent add 5 drops of pentose solution , mix.
2. Heat in a boiling water bath for (3) minutes to get a blue-green color indicates a positive result.

6. Iodine test

A test to distinguish between simple sugars and polysaccharides.

Principles

Iodine can form an adsorption complex with the helical structure (coil-like) of the polysaccharide. So it give a blue color with starch, red with dextrin and brow-red with glycogen depending on coil size and molecular weight. In order to achieve a good result. The test solution

should be either neutral or slightly acidic but never alkaline, since alkaline medium react with iodine molecule and dissociate it.

$$3I_2 + 6NaOH \longrightarrow 5NaI + NaIO_3 + 3H_2O$$

oligosaccharides	Color with I_2
Starch	Blue
Glycogen	Red-brown
Dextrin	Red-violet
Cellulose	Yellow
Inulin	Yellow

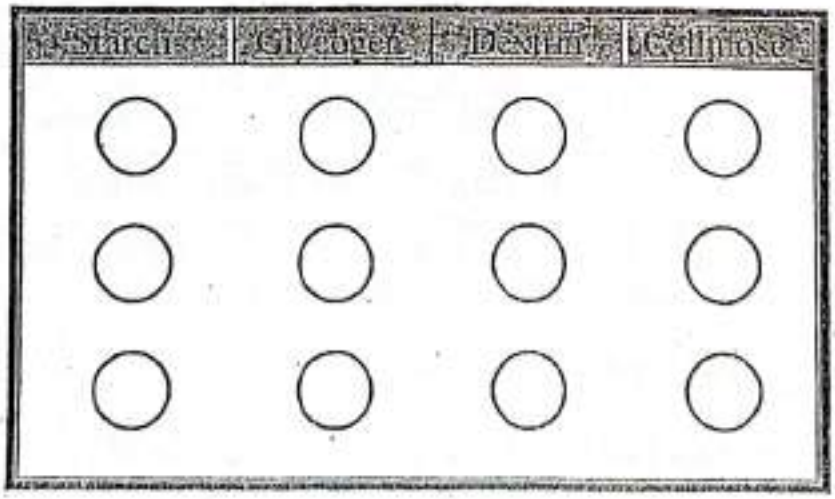
H.w) Cellulose & I_2 test

Materials

1. 2% KI . Add few granules of I_2 to get red color.
2. 3 N HCl solution.
3. 1% monosaccharide and polysaccharides.

Procedure:

1. Place 1 ml of test solution in dry porcelain test tile. 1 ml
2. Add (1-2) drops of 6N HCl. 2 drop Hcl 6N
3. Mix by glass rod, add (1-2) drops of I_2. 2drop iodine test
4. Record your observations.



Notes:

1. On heating, the coil shape of starch expand and the adsorption complex dissociated into iodine and starch on cooling, the original coil shape return back and the adsorption complex formed again.
2. Treatment of the adsorption complex with abase e.g NaOH solution also disappear the color complex due to the reaction of iodine with base to give sodium hypoiodide. Reacidification return the color complex.

$$\underset{\text{Sodium iodate}}{NaIO_3} + HCl \longrightarrow HIO_3 + NaCl$$

$$\underset{\text{Sodium iodide}}{NaI} + HCl \longrightarrow HI + NaCl$$

$$HIO_3 + HI \longrightarrow I_2 + 3H_2O$$

3. The polysaccharides are believed to have coil-like structurs in solution. Iodine molecules enter into the coils and make adsorption complexes which appear in different colors depending on the size and orientation of the molecules.

Disaccharides

The important disaccharides used in the laboratory are maltose, lactose and sucrose. They are colorless, crystalline, water-soluble, sweet and optically active compounds. They can be readily hydrolyzed by digestive enzymes or by acids into their constituent monosaccharide units.

a- Reducing sugar:

These sugar contain free functional group (-C-) not form glycosidic linkage for example (Maltose).

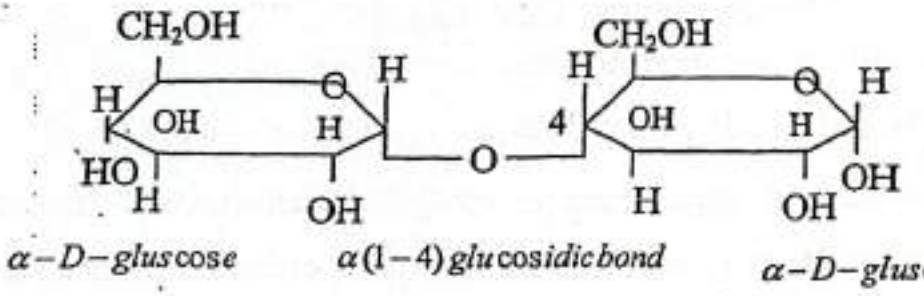
b- Non reducing sugars:

These sugar cannot contain free carbonyl group (in glycosidic bond) e.g (sucrose = glu. +fru.).

The reducing power of carbohydrates decrease as the number of their sugar components increases.

Maltose:

It is reducing disaccharides which gives a positive bencdict's test but negative Barfoed's test. On hydrolysis it is converted in to their constituent monosaccharide molecules and therefore show a greatly increased reducing power. Maltose form osazones with very characteristic crystalline forms. Maltose is composed of 2 glucose monomers in an α-(1-4)glucosidic bond.



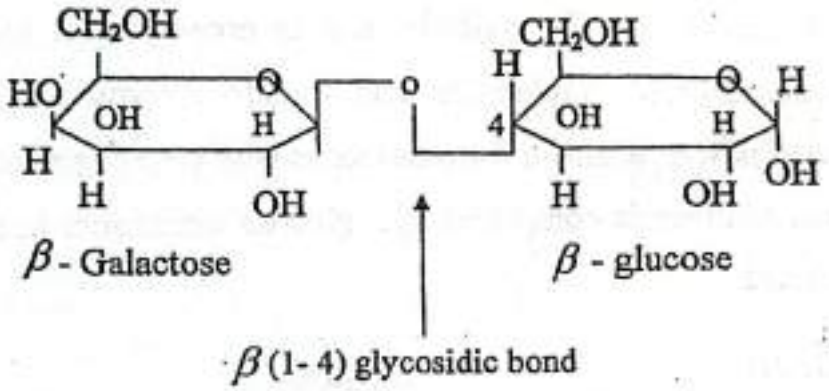
α-D-gluscose α(1-4)glucosidic bond α-D-gluscose

Lactose

It is found exclusively in the milk of mammals. Some time in pregnant urine specially in pregnancy and period of lactation. This sugar posses sufficient reducing power to give a positive bendedict's test , so it form osazone with very characteristic crystalline form (puffs shape). Lactose is not fermented by ordinary yeast which serve to distinguish maltose and lactose by using conc. nitric acid to give mucic acid that is less soluble due to containing galactose molecules. In acidity of milk by microorganisims like *streptococcus lacticus* and *lactobacillus acids hilus* which ferment lactose to lactic acid:

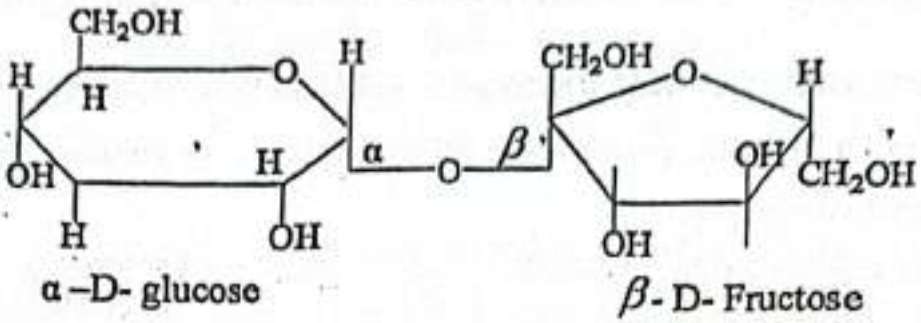
Lactose $\xrightarrow[S.\ Lact]{L.aadio}$ Lactic acid

Lactose is composed of 2 glucose molecule and galactose in an α-(1-4) glycosidic bond.



Sucrose:

Prevalent in sugar cane and sugar beets, sucrose shows no reducing power. It forms no osazone. These properties are unusual sugar can therefore, be used for distinguish sucrose from other sugar. Sucrose gives a positive Selivanoff's test, it is easily fermented by ordinary yeast, sucrose is readily hydrolyzed under the influence of the appropriate enzyme or of an acid into its constituent monosaccharide units, glucose and fructose. Which show marked reducing properties. A non-reducing sugar which gives a positive Selivanoff's test is identified as sucrose further confirmation may be obtained by hydrolyzing this sugar and showing that the hydrolysate prossesses a marked reducing power. Sucrose is composed of glucose and fructose through an α-(1-2) glycosidic bond.



α –D- glucose β- D- Fructose

Sucrose

Test of disaccharides:

Perform the same test of monosaccharide, then compare with all the results in table.

Hydrolysis of sucrose:

Sucrose is a disaccharides composed of glucose and fructose joined by α-(1-2) β-glycosidic bond, thus lack the presence of free carbonyl group. The glycosidic bond can be hydrolyzed by the action of acid and heat to give the two monosaccharides.

Materials :

1. 3M HCl.
2. 1% sucrose.

Procedure:

1. Take a clean two test tubes.
2. **The first tube :** before hydrolysis. Place 0.5 ml of sucrose.
3. Add 1 ml of Benedict's reagent, mix well. Put it in boiling water bath (2-3) minutes. Cool under the tap observe the color.
4. **The second tube:** after hydrolysis, place 1 ml of sucrose, add (1-2) drops of 6N HCl, put it in boiling water bath (2-3) minutes.

(3-4) drop

5. Cool and neutralize with 10% NaOH solution (why?), using litmus paper as an indicator perform the following tests for solution before and after hydrolysis.

Test	Sucrose solution	
	Before hydrolysis	After hydrolysis
Benedict's		
Barfoed's		

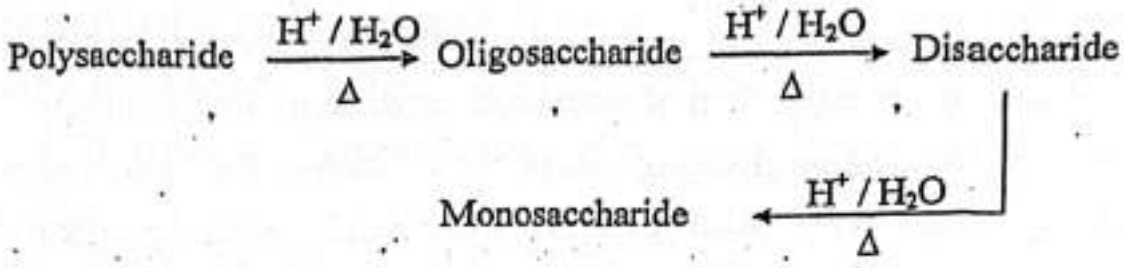
why we addition NaoH 10% with bendict test?

Notes:

1. Neutralization is a very important step since bendict's reagent work only in neutral or slightly alkaline solution.
2. Hydrochloric acid is preferred not to be used in the hydrolysis of sucrose due to the interferences of chloride ions with the subsequent barfoed's reagent.

Polysaccharides:

Most of the carbohydrate found in nature occur in the form of high molecular weight polymers called polysaccharides, starch , glycogen serves the same purpose in animals. This type of sugar contain only one reducing group for several hundred or more residue so that they are effectively non-reducing. The large size of these polysaccharide molecules is responsible for their relatively low solubility and diffusion. For the same reason their solution are colloidal in nature and opalescent in appearance. Under enzymatic digestion starch, dextrin, and glycogen are broken up. Under the influence of amylase into maltase unit, which are then acted upon by the enzyme maltase to form 2 glucose molecules. The acid hydrolysis of starch, dextrin and glycogen, however yield glucose directly.



When polysaccharides are composed of single monopolysaccharides building block, they are termed.

1. **Homo polysaccharides:** it contain the same kind of monosaccharides unit as starch and glycogen which bonded large number of (α-D-glucose), inulin, (fructose). That found in artichoke and vine.

Dextrose may be looked upon as an intermediate product of a polysaccharide nature, formed in the early stage of the hydrolysis of starch.

Polysaccharides composed of more than one type of monosaccharide are termed.

2. **Hetero polysaccharides:** it contain different kinds of mono sacch. Units as vegetable gums like gum Arabic, agar-agar, and pectins, are examples of hemicelluoses which yield on hydrolysis not only simple sugar but also certain other sugar derivatives, such as uronic acid (glycuronic and galacturonic acids). Hylaronic acid and heparin.

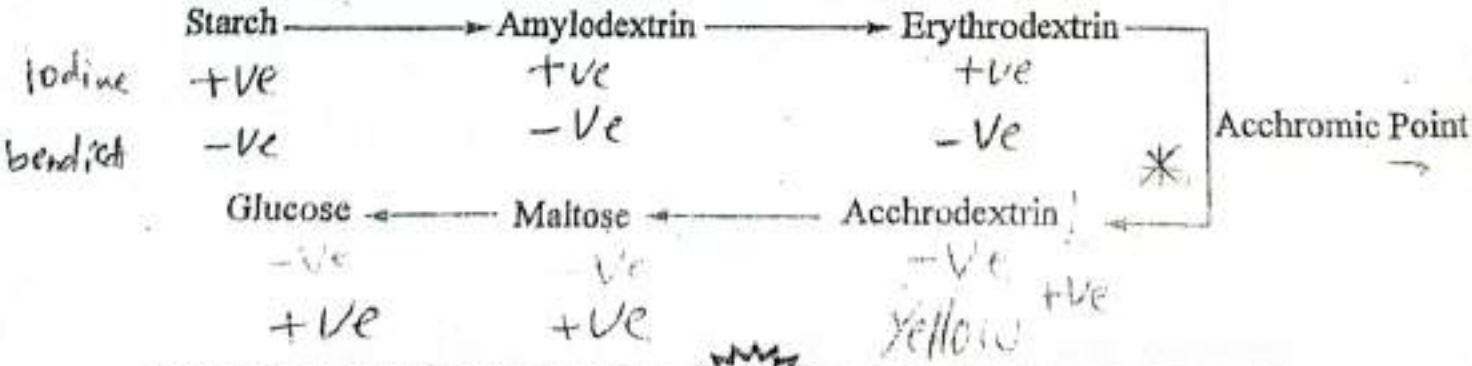
Write structure?

Starch

Starch is the major form of stored carbohydrate in plant cells. Its structure is identical to glycogen, except for a much lower degree of branching (about every 20-30 residues). Unbranched starch is called amylase, branched starch is called amylopectine which usually exist in the ratio 1:3. The amylase molecule appears to consist of about 300 units in an unbranched chain. Amylose forms a clear solution with water. It gives a deep blue color with iodine and it is completlely hydrolysed to maltose by beta-amylase. Amylopectin on the other band consists of about 6000 glucose units arranged in a branched chain pattern, each chain containing about 25 glucose unit. Amylopectin further from amylose in forming an opalescent solution with water, in producing a purple color with iodine and in being only partly hydrolyzed to maltose by beta-amylase. the colour Yellow ⇒ iodine & Sugar

Acid hydrolysis of starch:

Starch is a polysaccharide contain only one reducing group for several hundred or more of monosaccharide residues, so that they are effectively non reducing acid hydrolysis of starch gives the constituent monosaccharide (glucose) which have a reducing property(Therefore the reducing power of starch increase gradually with the progress of hydrolysis, while the ability of starch to react with iodine and form adsorption complex disappear gradually. Such process is done within second by amylase enzyme present in saliva or experimentally by acid and heat as below.



Materials

1. 3N HCl, starch solution.
2. 10% NaOH.
3. Benedict's reagent and iodine reagent.

Procedure:

1. Start heating a water bath to get it boiling.
2. Arrange in a rack six test tubes numbered from 1 to 6.
3. Measure 2 ml Benedict's reagent into each of these tubes.
4. Now take 10 ml of 5% soluble starch solution in a big test tube and add 1 ml of HCl to it.
5. Transfer one drop of this solution to porcelain test tile, do the iodine test by adding one drop of diluted iodine solution. This is zero time.
6. Start heating in boiling water bath, mix, after (3) minutes.
7. Transfer one drop of the solution to fresh depression of the test tile and add 1 ml to the second test tube containing Benedict's reagent.
8. Perform the iodine test with the solution on the tile and note the result.
9. Withdraw similar samples of the boiling solution of intervals of 5, 8, 12, 20 minutes after the commencement of the boiling for the iodine test and for transference to the series of test tubes containing Benedict's reagent.
10. Now place all the test tubes containing the Bendicts reagent and the added solution in the boiling water bath for just 5 minutes.

At the end of the period of heating, remove the test tubes to the rack and allow them to cool spontancously. Note the degree of reduction in each case and correlate it with the hydrolysis of starch as judged by the progressive iodine test.

11. Record your result and put it in table.

1	Blue	Blue	No reduction	Starch
5	Violet	Green	Reduction starts (+)	Amylodextrins
8	Reddish-violet	Red	Initiation of reduction (++)	Amylo and erythrodextrine
12	Red	Red	Partial reduction (+++)	Achrodextrin
20	No color	Red	Completely reduced (++++)	Glucose

Notes:

1. The acidified starch takes about 20 minutes for complete hydrolysis.
2. Achromic point is that point at which no color is obtained with iodine.
3. Polysaccarides have a high M.wt. that is responsible for their relatively low solubility in water, diffusion and colloidal in nature.
4. Cellulose, inulin, disaccharides or monosaccharides gives negative result with iodine.

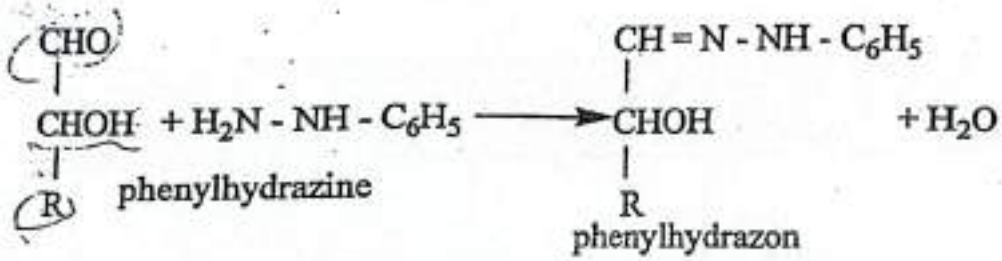
Osazone test:

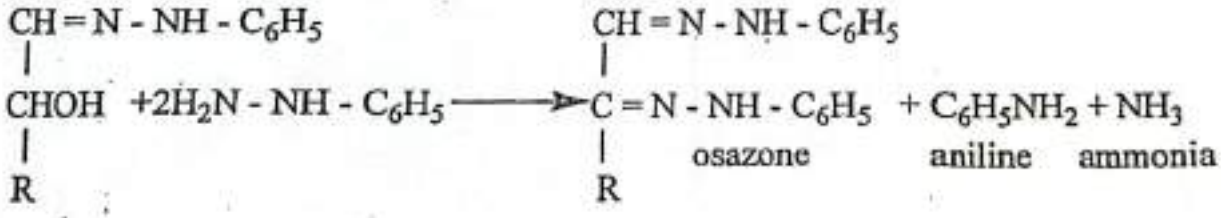
Reducing sugar can be distinguished by phenylhydrazine test when characteristic osazone crystals are formed. These osazones have definite crystal structure, precipitation time and melting point and hence help in the identification of reducing sugars.

Principles

Compounds containing the H-C(=O)-C(=O)-H group form crystalline

osazone with phenyl hydrazine. The osazone crystals have characteristic shapes and melting points which assist in the identification of the reducing sugar. Further evidence for identification is obtained by noting the time of formation of the crystals and whether the osazone is precipitated from the hot solution or only on cooling this method is instead of Benedict's and Barfoed's tests. Phenyl hydrazine reacts with the carbonyl group of the sugar to give the phenyl hydrazone, which then reacts with a further two molecules of phenyl hydrazine to form the osazone. The formation of an osazone from an aldose is outlined below, although the mechanism of the reaction is more complex than that illustrated. The reaction of ketones is very similar.





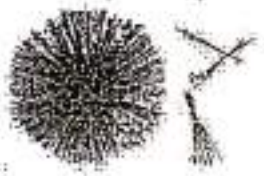
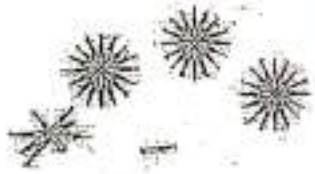
Materials

1. 1% glucose, maltose, lactose solution.
2. Mixture of phenyl hydrazine hydrochloride and anhydrous sodium acetate (2:3). Then closed to protect of hydrated.
3. Glacial acetic acid.
4. Microscope and ice.

Procedure:

1. In a clear and dry three test tube, add 5 ml of glucose, maltose and lactose successively.
2. Add 1 ml of glacial acetic acid.
3. Take approximately 0.5 gm of solid mixture to each tube, mix.
4. Place the test tubes in boiling bath for 5 minutes with occasional shaking.
5. Tubes for maltose and lactose stay in boiling water bath for (20-30) minutes.
6. Cool very slowly , examine and draw the osazone.

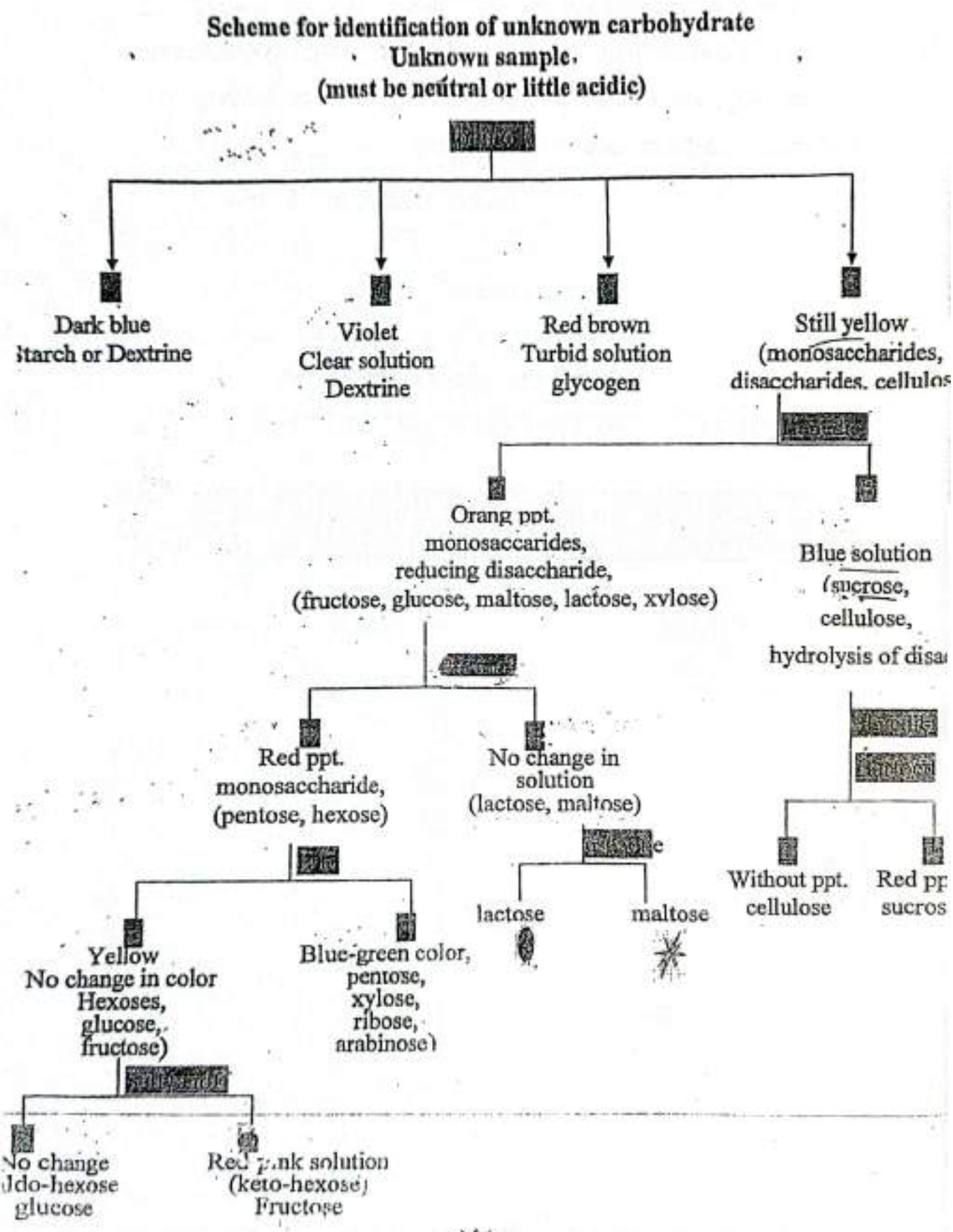
Crystals under the microscope: compare their shapes with those drawn in table

[illegible]	[illegible] color	[illegible] time/min	Crystal form
glucose	yellow	5	
lactose	yellow	20–25	
maltose	yellow	20–25	
sucrose	No form		

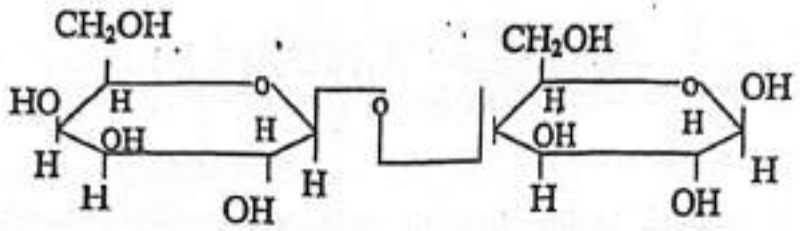
Notes:

1. Carefully note the time when the osazone is precipitated and also whether it is formed in hot or cold solution.
2. Mannose actually forms white crystals of the phenyl hydrazone at room temperature, but the yellow osazone separates on heating.
3. Sucrose. It will give osazone test negative.

Scheme for identification of unknown carbohydrate



Determination Lactose in milk



β- (1,4) glucosidic bond

(1-4) D - glucose + D-galactose

Lactose

Lactose is a disaccharides that is found in milk and is formed glucose and galactose. It has formula of $C_2H_{22}O_4$. It gets positive result with Molish, Benedict and osazone tests. It is present in milk and sometimes note in urine of women pregnant especially in pregnancy and period of lactation. It constitutes 4.4-5.2 % of cow's milk and 7% of human milk, it makes up about 2-8% of milk by weight, the intestinal enzyme called lactose (β-D-glactosidase) cleaves the lactose molecule into two subunits, the simple sugars glucose and galactose which can be observed. Since lactose occurs mostly in milk, in most mammals the production of lactose gradually decrease with maturity due to a lack of constant consumption. Many people with ancestry in west Asia , India and part of east Africa have lack for this enzyme. These who are lactose intolerant, lactose is not broken down and provides food for gas-producing gut flora which can lead to diarrhea, bloating, flatulence and other gastroin testinal symptoms. Lactose is responsible for drink milk as liquid.

$$\text{Lactose} + H_2O \xrightarrow{\text{lactase}} \text{galactose} + \text{glucose}$$

$$\text{Gal} + \text{ATP} \xrightarrow{\text{galactokinase}} \text{gal 1-P} + \text{ADP}$$

$$\text{Gal 1-P} + \text{UDP-6} \xrightarrow{\text{[redacted]}} \text{UDP-gal} + \text{glu-1-P}$$

Principle: in this method protein-free milk is prepared by Folin-method. Then the lactose concentration is determined by Nelson's colorimetric method of sugars. Cupric ions are reduced by sugars (lactose) to cuprous ions, which quantitatively reduce phosphomolybdic acid to a blue color complex.

$$\text{Lactose} + Cu^{+2} \xrightarrow{OH^-} Cu^{+1} \xrightarrow[\text{ybdic acid}]{\text{phosphomol}} \text{Blue color complex}$$

Beer's law . This law states that absorption is directly proportional to the lactose concentration

$\mathbf{A = \varepsilon bc}$ .....(Beer's law)

A = Absorbance.

ε = molar extinction coefficient.

b = cell length.

c = concentration in mol / L.

Materials:

1. Fat-free milk, distilled water.
2. Standard lactose: dissolve 50 mg lactose in 100 ml of distilled water, add 0.2 gm benzoic acid as preservative.
3. Alkaline copper reagent: dissolve 40 mg of pure anhydrous sodium carbonate in about 100 ml of distilled water , add 7.5 gm of tartaric acid with stirring until dissolved. Then add 4.5 gm of copper sulphate ($CuSO_4.5H_2O$) to the carbonate solution. Mixed well and

dilute to (1 litter) , if a sediment of cuprous oxide forms , filter the solution.

4. Phosphomolybdic acid reagent : dissolve (10 g) of sodium tungestate and (70 gm) of molybdic acid in (700 ml) of (5%) sodium hydroxide solution. Boil to remove the ammonia in the molybdic acid. Cool and add (250 ml) of (85%) phosphoric acid.
5. (0.3 M) H_2SO_4 : add (16.6 ml) of H_2SO_4 conc. Then complete to (100 ml) distilled water.

Procedure:

1. Place 1 ml of fat-free milk (to prevent form collidol precipitates) in (100 ml) volumetric flask.
2. Add (2 ml) of (10 %) sodium tungestate solution.
3. Add slowly and with constant shaking (2 ml) of (0.3 M) H_2SO_4, make the mixture up to the mark and allow it to stand for 5 minutes, then filter the mixture through a whatman No. 42 filter paper.
4. Complete as this table:

Reagents	Test/ml	Standard/ml	Blank/ml
filtrate	1 + 1ml D.W	-	-
Distilled water	-	-	2
Standard lactose	-	2	-
Alkaline copper solution	2	2	2
Mix and plug with a piece of cotton)this prevents reoxidation of Cu^{+2} ion). Heat the tubes in a boiling water bath for exactly 8 min. cool and add			
Phosphomolybdic acid	4	4	4

5. Mix well, allow the tube to stand for 1-3 min.
6. Then dilute the mixture with 25 ml of distilled water the blue color is formed.
7. Read absorbance at 630 nm.
8. Record the results.

Calculation:

$$\text{Lactose concentration} = \frac{\text{O.D of unk.} - \text{O.D of blank}}{\text{O.D of st.} - \text{O.D of blank}} \times \frac{0.6}{1000} \times \frac{100}{0.01}$$

$$= \quad \text{mg/ 100 ml}$$

Notes :

1. The volume is (0.01 ml) due to take 1 ml of milk diluted to 100 ml

Maillard reaction:

Also known as the browning reaction, is the phenomenon responsible for turning meat brown and converting bread to toast, along with hundreds of other example.

The Maillard reaction is named for Louis-camille Maillard (1878-1936) French-chemist and physician, who first described and studied it in the analysis of food chemistry and the science of browning as a part of his ph.D thesis which was published in 1913 for this work he received several a wards including the French academy of medicine award in 1914, Lately, it has been found to play a key role in many health-related issues. It is now associated with diabetes, ageing and cancer.

The Maillard reaction can be a complicated bit of biochemistry, but what's most important to know is the effect it has on foods and other protein-based technologies.

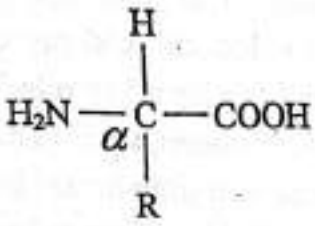
There are two major categories of browning that occur in foods:

- Enzymatic browning is chemical process, involving polyphenyloxidase, catechol oxidase and other enzymes that creat melanins and benzoquinon form natural phenol, resulting in a brown color, also requires exposure to oxygen, thus the browning that occurs when an apple, for example, is cut.
- Non-enzymatic is a chemical reaction between an amino acid and reducing sugar. The sugar interacts with the amino acid producing a variety of odors and flavors. Which it is responsible for heating or store along time of milk, due to damage of amino acid and harmful effect of milk products, Also toxic impact of Maillard reactions not only damage and loss amino acids but also appear intermediate compounds, that is responsible for many colors and flavors in foods:

- The browning of various meats like steak.
- Toasted bread.
- Biscuits.
- Dried or condensed milk.
- French fries.
- Roasted coffee.
- The burnished surface (crust) of bioche, cake, yest and quick breads.

Proteins

Proteins are polyamides and have high molecular weight above 5000 Dalton, also they are complex nitrogenous composed essentially of 20 α-amino acid residues. All microorganisms, plants and animals contain the same 20 α-amino acid.

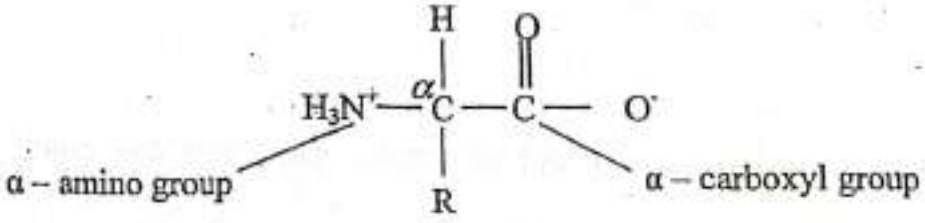


They constitute a major part of the living cytoplasm of cells. Although plants and many bacteria are capable of synthesizing proteins from simple organic and inorganic nitrogenous compounds, the higher animals have, for the most part, lost this power and therefore they depend, for the continuance of life, upon the proteins or the amino acids synthesized by plants.

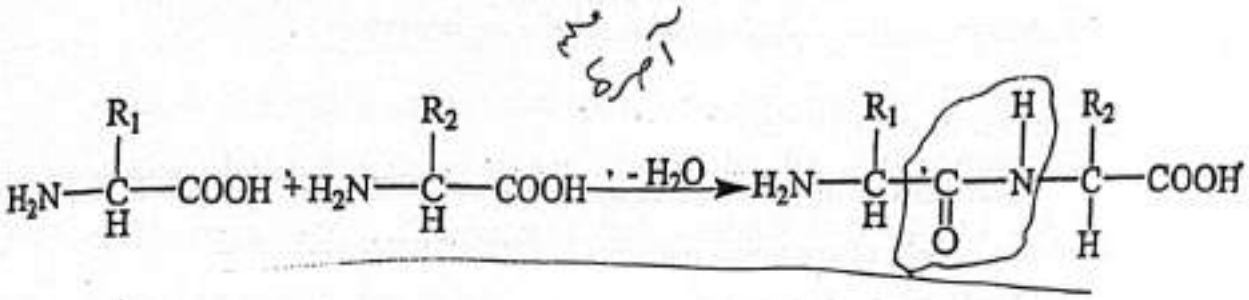
Proteins composing 50-70% of the cell's dry, provide an organism with H, C, N and S.

All protein contain nitrogen. This fact distinguishes them from most carbohydrates and fats. Proteins also contain carbon, oxygen and hydrogen and a smaller quantity of sulphur, iodine and phosphate.

Proteins as linear polymers of α-amino acids in which the carboxyl group (COO^-) of one amino acid linked to the amino group ($-NH3^+$) of another amino acid by peptide bond ($-\overset{O}{\overset{\|}{C}}-\overset{H}{N}-$).



α – amino acid



The almost infinitive number of proteins which occur in nature however, show certain common characters which help to distinguish them from other substances:

1. All proteins form colloidal solutions of the emulsion type, which are usually opalescent, and from which the proteins can be precipitated by a variety of reagents;
2. Proteins give a number of color reactions due to either the existence of certain amino acids in the protein molecule or to special chemical groups as associated with molecular structure of proteins.
3. Gives absorption on U.V spectrum so, they have aromatic amino acid at wavelength = 280 nm.

Protein may act as an enzymes, hormones, muscles (action and myosin), structural function of cell wall ..... etc.

Types of proteins:

1. Simple proteins: as albumin, globulin, gelatin, and prolamin.
2. Conjugated proteins compounds proteins which are bonded with non proteinous compound as lipoprotein, glycoprotein and nucleoprotein.
3. Derivative proteins: this type result of some nature and chemical factors so can changes the nature structure but still keep the specific characters as pepton.

Separation of proteins:

Proteins can be precipitated by a different source using the physical methods instead of chemical methods to prevent denaturation like :

1. High conc. of protein.
2. Temperature.
3. pH.

The proteins are usually characteristic of the species and often, of the specific organ in which they are found, therefore, protein as a class exhibit much greater variety in chemical composition and properties than do the carbohydrates or than lipids. The almost infinite number of proteins which occur in nature, however, show certain common characteristics which help to distinguish them from other substances.

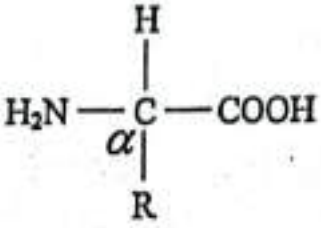
Some important definitions:

1. **Denaturation:** it is a process in which proteins lose the tertiary and secondary structure which is present in their native state, by application of some external stress or compound such as strong acid or base, a concentrated inorganic salt, an organic solvent or heat. In denatured proteins can exhibit a wide range of characteristics, from loss of solubility to communal aggregation. The most common observation in the denaturation process is the precipitation or coagulation of the protein

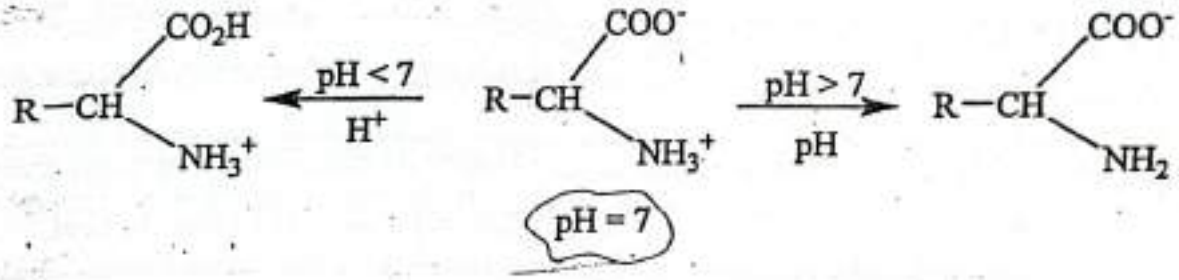
Some of process denaturate proteins is:

1. Physical: such as temperature, pressure, freeze, x-ray, ultraviolet and ultra sound.
2. Biochemical : some enzymes hydrolyzed proteins.
3. Chemical: the degree of pH , organic solvent and some chemical substances.

2. **Amino acids:** it is a smaller building unit of peptide and protein structure. The alpha amino acids consist of a carboxylic (-COOH) and amino (-NH_2) functional group attached to the same tetrahedral carbon atom. some of characters colorless soluble in water and less in alcohole and insoluble in ether.



3. **Zwitter ion:** the amino acids exist in neutral solution or inside body as doubly charged ions known as zwitter ions, and not as unionized molecules.



So this ion in acid solution positively charged and in alkaline solution negative charge.

ppt increase
Solub. decrease

4. **Iso electric point (IP):** iso electric pH is defined as the pH at which the protein molecule does not migrate to the cathode or to the anode in an electric field. At this pH, the protein molecule exists as zwitter ion. At the isoelectric point, the precipitation of protein is maximum i.e., in other words the solubility of proteins is minimum. At the isoelectric pH, the electrostatic repulsive force which normally prevents the protein molecules coming together is minimum as a result of no net charge on the protein molecule and hence give rise to

يحصل له ترسيب لتيجة عدم قدرة البروتين على الفقدان او الاكتساب ع

maximum precipitation. IEP casein =5, Gelatine=7, Keratin =6 , phosphoproteins =4.

5. **Albumins and globulins:** albumins and globulins often occur together, as in blood serum, in egg white and in milk. They give all the ordinary precipitation and color tests of protein but their most characteristic property is that they are heat coagulabel.

It is obtained by full saturation of $(NH_4)_2SO_4$.	It is obtained by half saturation of ammonium sulfate.
It is soluble in water.	It is in soluble in water but soluble in dilute mineral acids and salt solution.
It is a smaller molecule having more charge.	It is bigger molecule with less charge.
In isoelectric point field it travel faster than globulins.	Due to its big molecule and less charge it travel slowly in isoelectric field.

6. **Phosphoproteins:** they are conjugated proteins formed by the organic union of the protein molecule with phosphoric acid. Casein of milk and vitellin of egg yolk are example of known phosphoproteins. Casein occurs in milk as colloidal complex of its calcium salt. It is precipitated from milk by acidification with acetic acid. Maximum precipitation of casein occurs at its isoelectric point at pH = 4.55. casein is rich in tyrosine, arginine and tryptophane but loss cystine.
7. **Scleroproteins :** they are simple proteins which form the chief organic constituents of skeletal tissues such as bones, cartilage tendon, skin, hair and nails. Scleroproteins are insoluble in neutral solvents, water or dilute acid and alkali. These proteins can be classified into two subdivisions:

a- Collagen: present in cartilage, tendon, bone and teeth. It is insoluble and indigestible. On boiling with water, it produces glue like substance.

b- Keratin: present in hair and nails. It is insoluble in all neutral solvent and it is indigestible. Keratin is rich in sulfur containing amino acid cystein, phenylalanine, and tyrosine. Boiling keratine with 40% NaOH converts the organically bound sulfur into inorganic form.

8. **Gelatin:** uncomplete animal protein it is digestible, it lack many essential amino acids such as tyrosine and tryptophane. Gelatin is rich in glycine , prolin and hydroxy proline but few in cysteine. It form in connection tissues, bones and skin.

9. **Glycoproteins:** they are conjugated proteins composed of strongly bound carbohydrate groups other than nucleic acids into the protein molecule. Mucin is a well-known member of this group. It occurs in the mucous secretions of the digestive, respiratory and the genital tracts. The carbohydrate units present in the mucin molecule have no free reducing groups and therefore mucin does not give positive Benedict's test. When heating mucin sample with mineral acids, hydrolysis of mucin occurs and reducing groups of carbohydrate units are released and therefore Benedict is (+ ve).

Some specific properties of proteins:

Heat coagulation:

The Heat coagulation property of albumin and globulin is most marked near the isoelectric point of protein (pH =5.4). This fact is the basis for the detection of albumin in urine and also for removal of protein from solution.

Materials:

1. 1% Albumin.
2. 1% Gelatine.

Procedure:

1. Place 1 ml of albumin in a test tube.
2. Place 1 ml of gelatin in another test tube.
3. Heat the upper layer until boiling.
4. Record your observation.

   a- Coagulated albumin which indicate denaturate it.

   b- Gelatin is soluble.

Some color tests for amino acids and proteins:

1. Elementary composition of protein: all proteins contain nitrogen, carbon, oxygen, hydrogen and a smaller quantity of sulphur, phosphate and iodine.

Materials:

1. Powder albumin.
2. Red litmus paper.
3. 40% NaOH.
4. Lead acetate.

Procedure:

1. Select a narrow pyrex test tube, which is clean and perfectly dry, place a very small quantity of powdered protein in it.
2. Place a piece of moistened red litmus paper on the upper part of the test tube.
3. Heat until you get a smell of burning hair.

Sulfur

4. The red litmus paper turns blue. The characteristic smell and the change of the color of litmus paper due to the evaluation of ammonia from the heated protein. This indicates the presences of nitrogen in the protein molecule.
5. The moisture on the upper part in test tube is due to the condensation of water, which indicates the presence of hydrogen and oxygen in protein.
6. The charring of the heated protein demonstrate the presence of carbon in protein.

2. Test of sulfur:

1. Place a small quantity of powdered albumin.
2. Add (1 ml) of 40 % NaOH solution boil gently about 20 min.
3. Add a drop of lead acetate solution. A black or brown ppt. indicates the presence of sulfur in the protein molecule.

Note:

On heating the protein with NaOH some of the organically combined sulfur present in the protein is converted into inorganic sodium sulfide, this reacts with lead acetate producing black precipitate of lead sulfide.

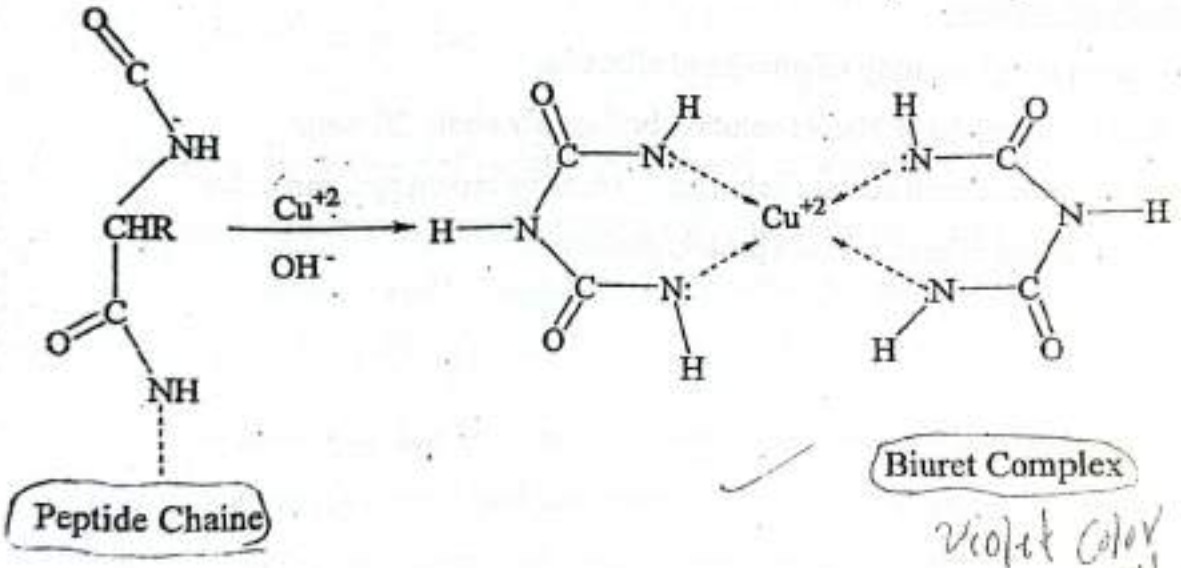
$$Na_2S + Pb(OCOCH_3)_2 \longrightarrow PbS\downarrow + 2NaO_2CCH_3$$

3. Biuret test:

Biuret test is given by all compounds that contains three or more peptide bonds. Since proteins are polypeptide, hence it is a general test for proteins. All proteins contain large number of peptide bond when a protein solution is treated with cupric ion

Cu^{+2} in a moderately alkaline medium (Biuret solution) a colored coordinate complex is formed between the Cu^{+2} ions and the peptide bond present in the protein. A positive test indicated by the formation of a pink-violet to purple-violet color. The intensity of the color produced is proportional to the number of peptide bond availabel for reaction.

Amino acids and dipeptides cannot give the reaction (still blue color). The name of the reaction is derived from the organic compound, Biuret , which gives a positive test with this reagent.

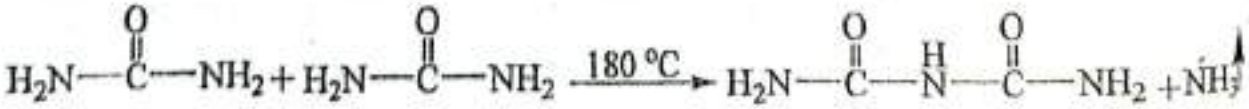


Violet color
depends with Peptide bond

a- Urea :

Formation of Biuret and Biuret reaction.

Place (0.2 g) of urea in a dry test tube, heat very gently just above the m.p and note the production of ammonia. After 1-2 minutes the liquid suddenly solidifies with the formation of biuret.



Dissolve the solid residue in few ml of warm (10%) NaOH solution, cool and add (1 drop) of very dilute copper sulphate solution. A purple coloration is obtained due to the formation of complex between copper and two –CONH- grops.

b- Proteins:

Materials

1. Biuret solution (0.5 % $CuSO_4$ + 10 % NaOH).
2. 1% white egg albumin.
3. 1% gelatin.
4. 1% amino acid.

Procedure:

1. Prepare and label four test tubes.
2. Place 1ml of test solution in each test tube.
3. Add 1ml of 10% NaOH, mix.
4. Add drop by drop of 0.5% $CuSO_4$, mix.
5. Note the colors produced.

Notes:

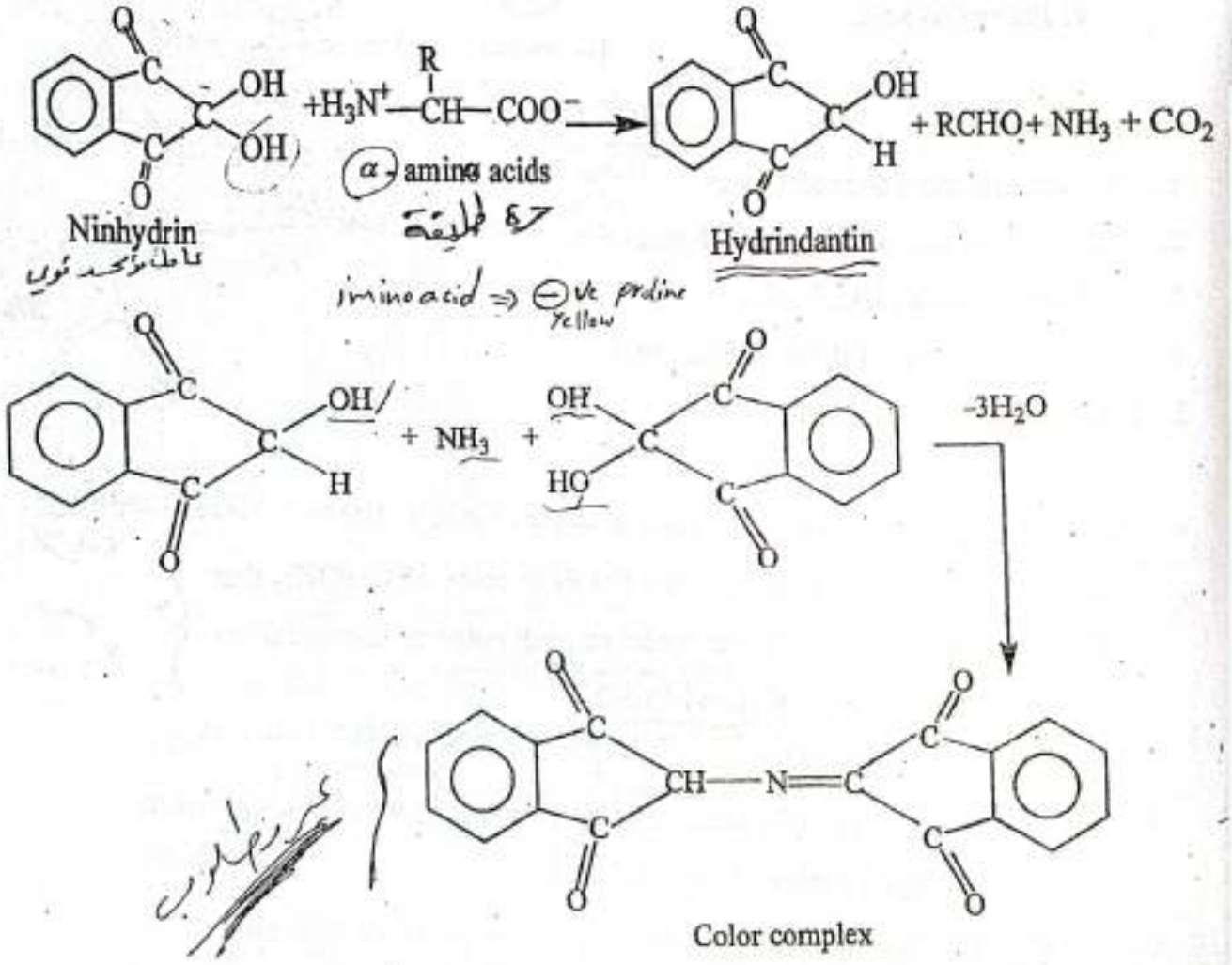
$CuSO_4 + NaOH \rightarrow Cu(OH)_2 + Na_2SO_4$ blue

1. Avoid the excess of $CuSO_4$, since the blue color of $Cu(OH)_2$ that formed will interfere with the violet or pink color of Biuret due to the reaction between NaOH and $CuSO_4$.
2. Peptones give a pink color.
3. Peptides give a very light pink color and gelatin gives violet-blue color (due to high number of peptide bonds).
4. Amino acids and dipeptides cannot give the reaction still blue color.

This observation indicate that the color produce depend on the number of peptide bonds.

4. Ninhydrin test:

Ninhydrin (triketohydrindene hydrate) is a general test for proteins and for all products of protein hydrolysis including α-amino acids. Ninhydrin is a powerful oxidizing agent which oxidizes amino acids producing CO_2, NH_3 and aldehyde. Then ninhydrin and hydrindentin react with NH_3 forming a blue color complex.



This reaction is essential for quantitative determination of amino acids by using colorimetric method.

Materials:

1. 0.1 % amino acids and 0.1% tryptophane.
2. 0.1% proline.
3. Albumin solution (egg white).
4. Freshly prepared 0.2% ninhydrin solution and put in brown bottle reagent.

Procedure:

1. Prepare and label four test tubes.
   - a- 1 ml protein solution.
   - b- 1 ml amino acid solution.
   - c- 1 ml proline.
   - d- 1 ml distilled water.
2. Add 2-5 drops of freshly prepared 0.2% ninhydrin solution.
3. Heat in boiling water bath about 2-5 minutes or boil for 1 minute directly on flame.
4. Allow to cool, a blue color is produced.

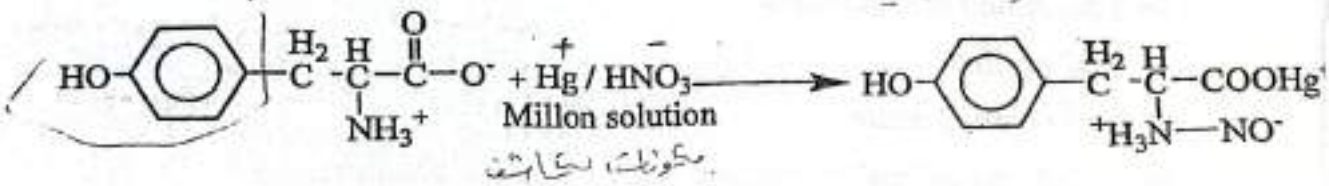
Notes:

1. The solution to be tested must be between pH = 5-7.
2. Ninhydrin is readily soluble in water but its solution is not stable for more than two days therefore must put in dark place.
3. This test is positive for proteins and for all products of protein hydrolysis including amino acid.
4. Most amino acids give the same color, except proline and hydroxy proline makes a pale – yellow product with ninhydrin. This is due to the fact that proline is an imino acid instead of having traditional α-amino acid structure.

5. Millon's test:

This test is specific for tyrosine and is an indication of the presence of tyrosine in the protein because tyrosine is the only amino acid containing hydroxy phenyl group. (+ve)

Millon's reagent contains Hg ion which forms red color complex with tyrosine. If the unknown is protein solution, a red precipitate will be formed because heavy metal (Hg) are precipitating agents, while if the unknown is tyrosine a red color solution will be formed.



Material:

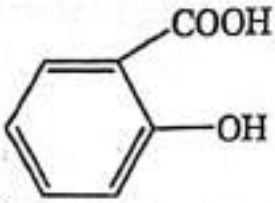
1. Millon solution (I volume Hg + 2 volume HNO_3 + 2 volume H_2O).
2. 0.2 % tyrosine.
3. 0.1% alanine.
4. Egg white (albumin).

Procedure:

1. Prepare and label four test tubes.
   - a- 1 ml tyrosine.
   - b- 1 ml alanine.
   - c- 1 ml albumin.
   - d- 1 ml H_2O.
2. Add(3-5) drops of millon's reagent to each test tube, mix well.
3. Heat gently in boiling water bath for 5 minutes.
4. Record your observation.

Notes:

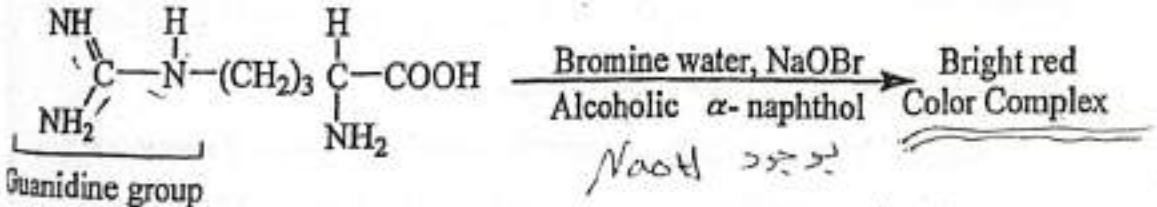
1. Excess of chlorides interfere with this test by combining with the mercury of the reagent, and rendering is inert. This test is therefore never used for the detection of proteins in urine.
2. Millon's test will be conducted with one compound which is not an amino acid nor a protein. This compound is salicylic acid which is simply a 2-hydroxy benzoic acid. This compound is tested with millon's reagent.



3. Gelatin gets a negative result with millon's test because it has little tyrosine.
4. This test can be easily performed on solid proteins also, suspend the solid protein in a little water and then carry out the test as usual. The solid particles will turn red.

6.Sakaguchi test:

This test is used to detect arginine amino acid. Arginine is the only amino acid containing the guanidine group, which react with α-naphthol in alkaline medium. The product of condensation is then oxidized by the oxidizing agent such as bromine water to give a red color complex, which indicates, presence of arginine.



Materials:

1. 0.1 % amino acids (arginine and glycine).
2. 10% sodium hydroxide.
3. 1% alcoholic α-naphthol.
4. Bromine water (5-9 drops liquid bromine to 100 ml H_2O).

Procedure:

1. Prepare and label three test tubes:

a- 1 ml of arginine solution.

b- 1 ml of glycine.

c- 1 ml of D.W.

2. Add (8) drops of 10% NaOH to all tubes, mix.
3. Add (3) drops of alcoholic α-naphthol, mix.
4. Add drop by drop of bromine solution.
5. Bright red complex indicates the presence of arginine.
6. Record your observation.

Notes :

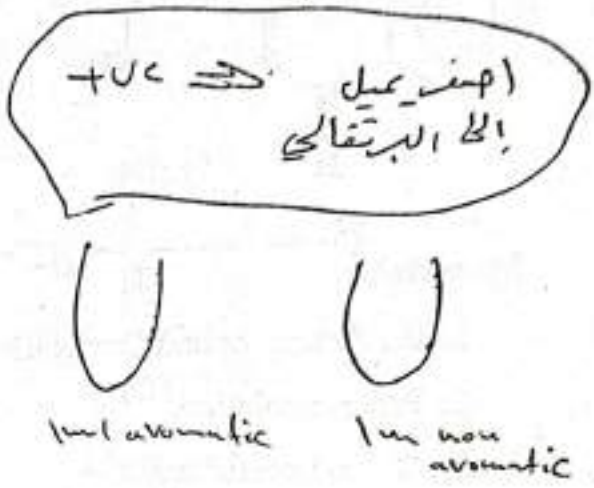
1. Avoid adding excess bromine because the red color might fade away.
2. Bromine give very nasty burn if spilt on the skin.
3. This is a very sensitive test for free or combined arginine since all known proteins contain sufficient amount of arginine to give a positive Sakaguchi's reaction this test may well be used as a general test for proteins.

7. Xanthoproteic test:

Amino acid containing an aromatic nucleus form a yellow nitro derivative on heating with conc. nitric acid. The salts of these derivatives are orange.

Materials:

1. 1 % egg albumin.
2. 0.1 % alanine, phenylalanine.
3. 0.1 % tyrosine.
4. Conc. HNO_3.
5. 10 % NaOH.



Procedure:

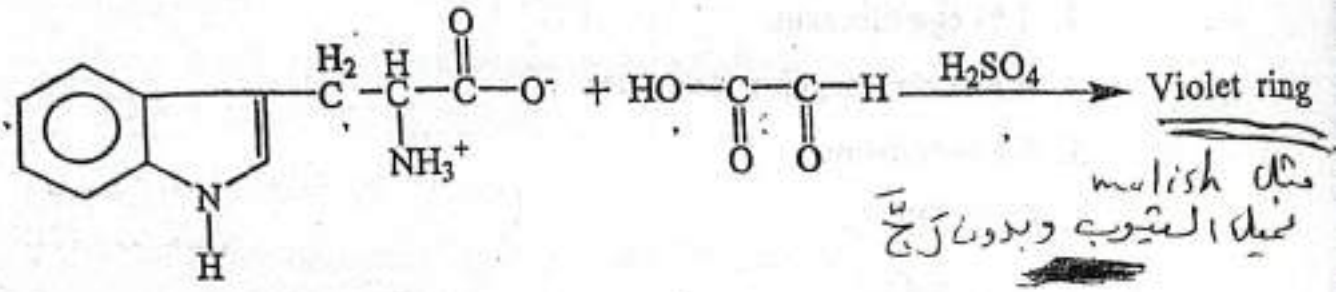
1. Prepare and label three test tubes:
   a- 1 ml of alanine.
   b- 1 ml of tyrosine .
   c- 0.5 ml of egg albumin.
2. Add 10 drops of conc. HNO_3.
3. Heat the test tubes 3-5 minutes in boiling water bath and observe the color change.
4. Add NaOH to make the solution strongly alkaline.
5. A yellow color in acid solution turn to bright orange with alkaline because a nitrogen added to aromatic ring.

Notes:

1. Proteins that contain significant level of tyrosine or tryptophane produce a yellow color in this test.
2. The aromatic ring of phenylalanine does not react with nitric acid under condition of the test.

6. Hopkin cole test:

This test is used to detect the presence of tryptophane which contains an indole ring, which it reacts with glyoxylic acid in the presence of conc. H_2SO_4 to give a purple ring.



Materials:

1. 0.1 % tryptophane.
2. Protein solution.
3. Glacial acetic acid.
4. Pure conc. H_2SO_4.

Procedure:

1. Add 1 ml of test solution on test tube.
2. Add 1 ml of glacial acetic acid.
3. Add drop by drop of conc. H_2SO_4 carefully down the sides of a sloping test tube without mix.
4. A purple ring formed at the junction between layers of fluid, on shaking, the whole solution may become red.

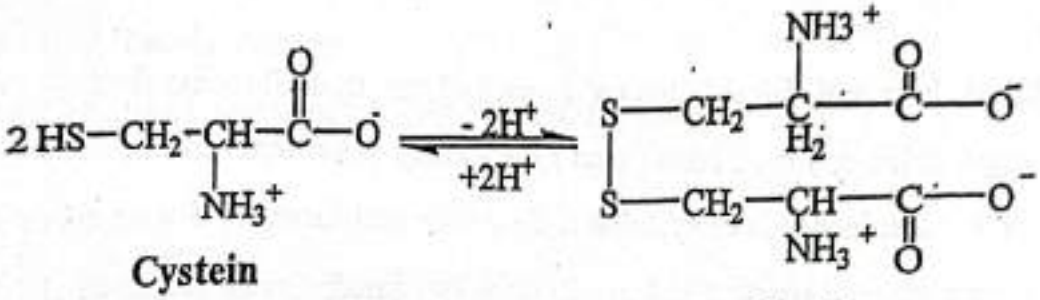
Notes:

1. Glacial acidic acid usually contains some glyoxilic acid as an impurity, therefore it is a suitable source of glyoxilic acid in this test.
2. H_2SO_4 used should be pure, otherwise no color will develop.
3. This test is due to the presence of indole ring in tryptophan.

9. Cystine, cysteine test:

This test is done to detect the presence of sulfur containing amino acids such as cysteine and cystine.

By boiling the sample with 40% NaOH, the organically combined sulfur in the amino acid or protein molecule (which contain cysteine or cystine) is converted to sodium sulfide. Brown or black precipitate (PbS), is formed by the addition of lead acetate solution.



Cystein

Cystine

Cystein or Cystine + 2NaOH ⟶ Na_2S + ROH + H_2O

40%

Na_2S + $(CH_3COO)_2$ Pb ⟶ PbS↓ + 2 CH_3 COONa

Lead sulphide

Materials:

1. 0.1 % cysteine or cystine solution.
2. 0.1 % glycine solution.
3. 40 % NaOH.
4. 0.1 N lead acetate.

0.5ml/NaOH 40% 5min ∆ 1ml lead acetate.

Procedure:

1. Prepare and label three test tube:

a- 1 ml of amino acid containing sulfa hydryl group (-SH) solution.

b- 1 ml of glycine solution.

c- 1 ml D.W.

2. Add 5 drops of 40% NaOH for all tubes.

3. Boil for 3 minutes then cool.
4. Add 1 ml of lead acetate solution.
5. Report your observation.

Notes:

1. Wool, hair and fingernails give a marked reaction albumins from eggs, milk or blood serum also give afairly good test. Casein of milk containes less of cysteine and cystine and therefore does not give a marked reaction.
2. The sulfur of methionine is not affected by this reaction, why?

Precipitation of proteins

When proteins are dissolved in a medium such as water or dilute salt solution, their colloids particles will carry on effective negative electrical charge. Neutralization of this charge brings proteins to the isoelectric point (IEP). The type of precipitation is :

1. **Precipitation by heavy metals:** at (IEP) maximum precipitation of proteins takes place and the protein particles bear zero net charge. Salts of heavy metals like iron, copper, zinc, mercury, silver, ...etc. are very suitable for this purpose. The precipitation of proteins by salts of heavy metals is also due to the formation of insoluble compounds of proteins.

Note:

Addition excess amounts of salts of heavy metals solutions to protein solutions may lead to positively charged protein particles and hence, these particles may become dissolved again.

Proteins are precipitated from solutions by salts of heavy metals probably by combination of the metal ions with the anionic form of the protein. On the alkaline side of the isoelectric point proteins exits as negative ions.

Materials:

1. Albumin solution.
2. Salts of heavy metals (Hg_2Cl_2, $CuSO_4$ and $FeCl_3$).

Procedure:

1. Place (2) ml of albumin solution in a test tubes.
2. Add drop wise solutions of heavy metals as lead acetate ($(CH_3COO)_2Pb$), mercuric chloride (Hg_2Cl_2), cupper sulphate ($CuSO_4$) and ferric chloride ($FeCl_3$) until maximum precipitation occurs in it.
3. See the turbidity of solution and white colloid precipitate.

Note:

We can make use of this phenomenon in treatment of lead, mercury or any heavy poisoning by using egg albumin or milk to precipitate with heavy metal and then reduce the absorption form intestine.

2. Precipitation by alcohol:

Alcohol are dehydrating agents and precipitate proteins from their solution, so increase the interpenetrate between protein molecules with each other. This ppt. dissolve again with distill water.

Materials:

1. Albumin solution.
2. 95 % ethanol.

Procedure :

1. Place 1 ml of protein solution in a test tube.
2. Add 1ml of 95% ethanol drop by drop.
3. White gleaming precipitate of protein will be formed .
4. Add 2 ml of D.W, note the precipitate is dissolve.

3. Precipitation by neutral salts:

When high concentration of inorganic salts are present in protein solution, the solubility of the protein is reduced leading to its precipitation. This happens due to the ability of the ion of salts to become hydrated (to bind water and therefore to compete with the protein molecules for water.

Concentration solution of neutral salts like ammonium sulfate, sodium sulfate and sodium chloride are used to precipitate proteins from solutions.

When a solution of two substances which differ in their solubility in water are mixed with each other, then an excess addition of one of them causes removal of water (dehydration), and this will cause precipitation of the other substance.

For example, if albumin and ammonium sulphate come in contact together on addition of excess of ammonium sulfate will cause removal of water from the protein solution because the affinity of ammonium sulfate to water is more than the protein, so the protein will be precipitated and the process called salting out .

Salting out : It is the process in which the slightly soluble substance is precipitated from its solution when more easily soluble salt is added to the solution.

Materials:

1. Albumin solution.
2. Solid ammonium sulphate.

Procedure:

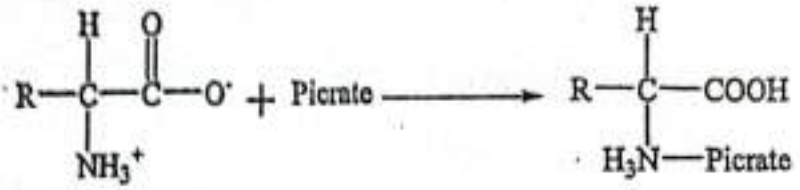
1. Place 1 ml of protein solution in a test tube.
2. Add powdered ammonium sulfate $(NH_4)_2SO_4$ and mix .
3. Keep adding this salt until the solution is saturated with ammonium sulfate, notice a white precipitate of protein is formed.
4. If you add water, the precipitate will disappear due to decrease of concentration of the dehydrating agent (the ammonium sulfate).

4. precipitation by alkaloidal reagents: The addition of an acid to a protein solution causes the protein particles to acquire a positive charge. Proteins are precipitated from the solution by combination between the acid anions and the positively charged protein molecule by forming all insoluble complex. The alkaloidal reagents precipitate proteins by combination of the acidic radical of the former with the cationic form of the protein, which predominates when the solution is on the acidic radicals of the former with the cationic form of the protein , which predominates when the solution is on the acidic side of the isoelectric point.

Alkaloidal reagents include [picric acid is 2,4,6 trinitrophenyl, tannic acid has a formula $C_{76}O_{52}O_{46}$. Trichloroacetric acid Cl_3CCOOH, sulfosalicylic acid (S.S.A) has a formula $C_7H_6O_6S$ phosphotungestic acid $H_3PW_{12}O_{40}$.

Tannic acid and picric acid are used in solution for burns treatment because they are safe. They prevent the growth of microorganisms by precipitating bacterial protein as shown below.

When pH of a protein is below isoelectric point, the net charge of the protein is positive. The protein will now act as a base and can salts with added acids.



Materials :

1. Dil. Albumin solution.
2. TCA trichloroacetic acid.

Procedure:

1. Place I ml of dilution albumin in a test tube.
2. Add drop by drop of TCA. A white precipitate will be formed.
3. Add excess of TCA will dissolving of the precipitate .

Notes:

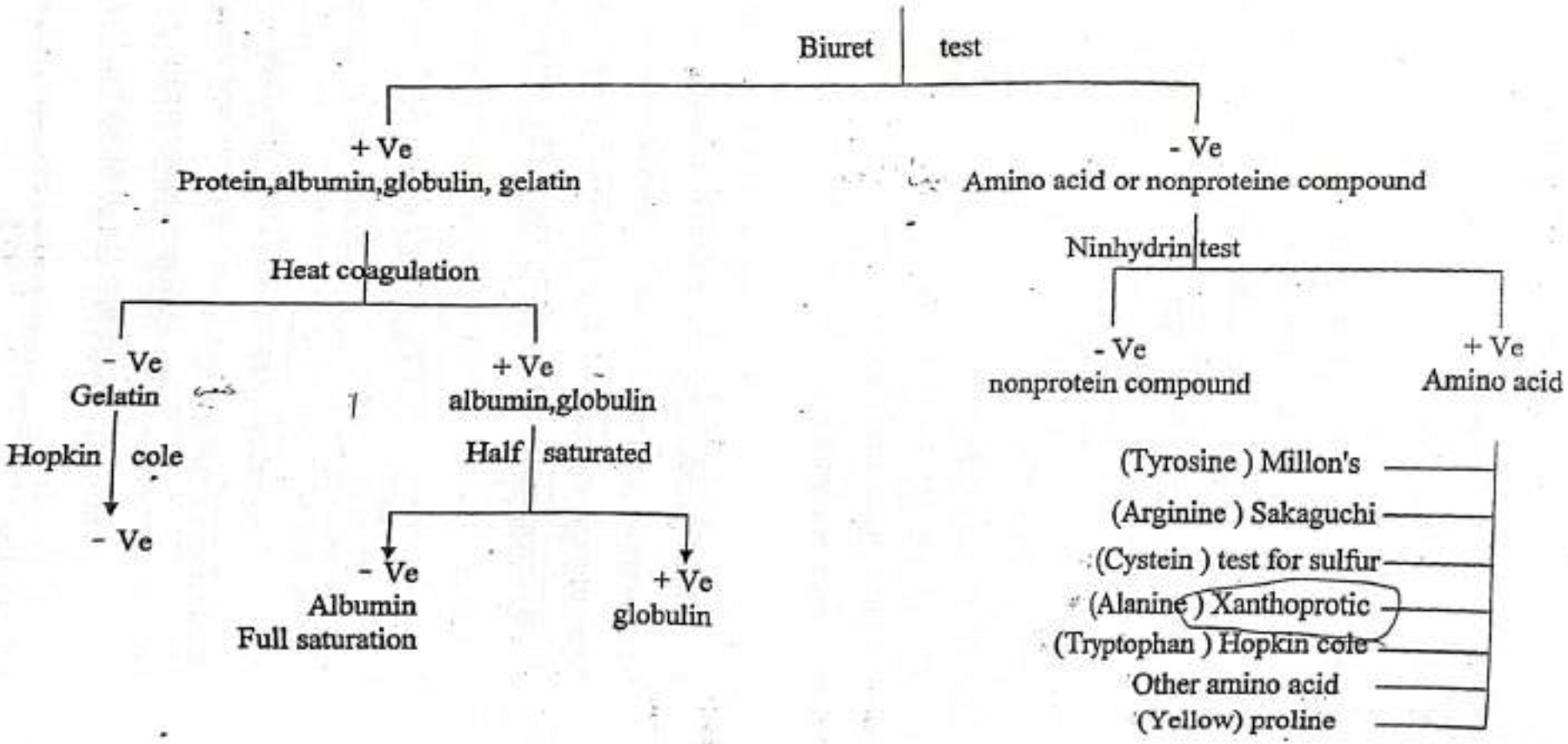
1. This method is used to detect proteins in urine.
2. Excess reagent should be avoided to prevent redissolving of the precipitate.

5. precipitation by acidic reagents: We can use conc.HNO_3 to precipitate protein due to denaturations so, this method is used to detect protein in urine.

Schedule of amino acids and protein tests result

	Biuret	Ninhydrin	Millons	Hopkins & Cole	Sakaguchi	Test of sulfur
	+	+	+	+	+	+
	+	+	-	-	+	+
	-	+	+	-	-	-
	-	+	-	+	-	-
	-	+	-	-	+	-
	-	+	-	-	-	+
	-	+	-	-	-	-

General scheme for identification for unknown amino acids and proteins



Determination of protein concentration

Methods of quantitative measurements of protein

1. **Folin ciocalteau method:** It has sensitively about (25 – 500) µg for low concentration which is apply for tissues or plants

2. **Biuret method:** It has sensitivity about (1 – 20) mg for high concentration , which is used for determination protein in urine and blood

3 **Ninhydrin method:** This method usage for nitrogen in amino group. Also for quantitative determination of amino acid. Not all amino acids give exactly the same intensity or color and this must be allowed for in any calculation . the imino acids proline and hydroxyl proline give a yellow color, so these are read at 440 nm .

4. **Spectrophotometric method:** Tyrosine and Tryptophan absorb at 275 nm and 280 nm and so proteins containing these amino acids will also absorb in this region. The specific extinction coefficient $\varepsilon^{1\%}_{1cm}$ g/l varies according to how much of these amino acids are present in the particular protein. The values found in practice range from 5 to 60 , although many proteins have a value close to 10, that is 1 mg/ml of protein gives an extinction of 280 nm of about 1 when viewed through 1 cm light path. The disadvantage of this method is that many other compounds absorb in this region, particularly nucleic acids which have a peak at 260 nm. Pure proteins have a ratio of absorption at (280 nm / 260 nm) of about 1.8, while nucleic acids have a ratio of 0.5.

Kieldal's method:

All protein contain nitrogen, the average content being 16% nitrogen determinations are therefore often carried out to estimate proteins quantitatively.

Kieldal's method determines total nitrogen Protein is digested with concentrated sulphuric acid in the presence of catalyst such as selenium dioxide, copper sulphate or potassium sulphate, to yield inorganic ammonium sulphate which treated with sodium hydroxide to yield ammonia, which also treated with conc. HCl of known concentration from this information we can determine the nitrogen in the sample.

$$\text{Organic-N} \xrightarrow[\text{Se}]{H_2SO_4} CO_2 + H_2O + NH_4HSO_4$$

Folin – Ciocaltue Method

Protein reacts with the Folin – Ciocaltue reagent to give a colored complex. The color so formed is due to the reaction of the alkaline copper with the protein and the reduction of phosphomolybdic-phosphotungstic reagent by tyrosine and tryptophan present in the protein under examination. The intensity of color depends on the amount of these aromatic amino acids present and will thus vary for different protein. This method is rapid and sensitive (100 times more sensitive than the Biuret method)

Materials:

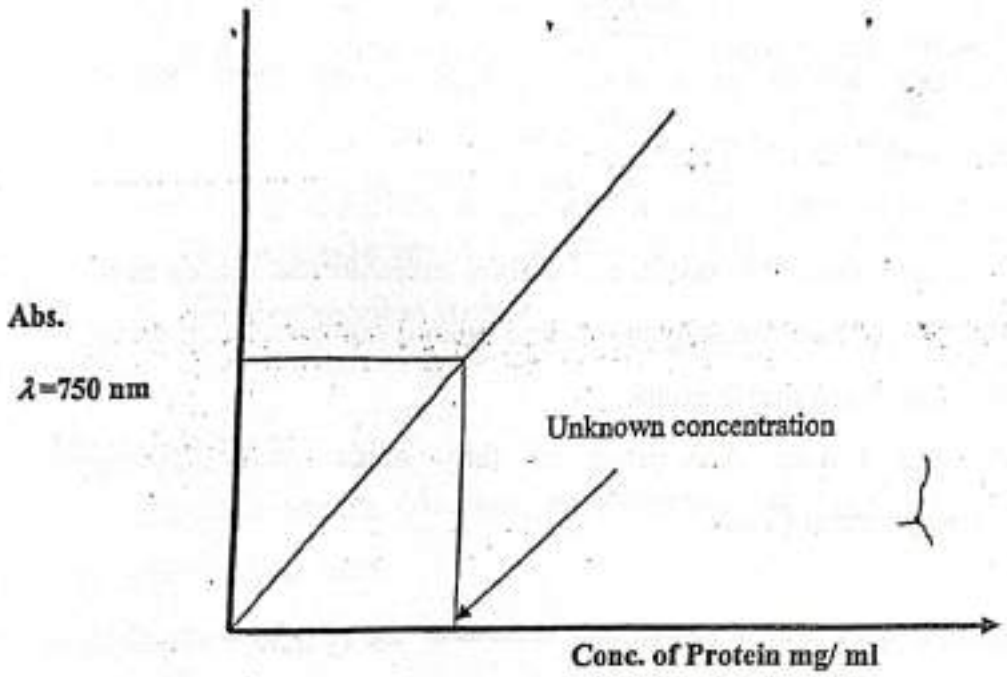
1. Protein solution 1 mg / L.
2. Reagent A: 0.2% Na_2CO_3(anhydrous) in 0.1 NaOH.
3. Reagent B: 0.5% $CuSO_4.5H_2O$ in 1% sodium or potassium tartarate.

4. Reagent C: 50 ml solution A + 1ml of solution B.
5. Folin – Ciocaltue reagent: sodium molybdate+ sodium tungestate solution in perchloric acid and hydrochloric acid . The commercial reagent is diluted with equivalent volume of H_2O.

Procedure:

[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
0	5	1	0	(Blank) 1
5	5	0.8	0.2	2
10	5	0.6	0.4	3
15	5	0.4	0.6	4
20	5	0.2	0.8	5
25	5	0	1	6
؟	5	0.5	0.5	Unknown

Mix well and allow to stand for 10 min. at room temperature. Add (0.5) ml of folin reagent to each tube with immediate mixing. After (30) min., read at 750 nm plot a standard curve and determine the protein concentration of the unknown solution in gm %.


Abs.
λ=750 nm
Unknown concentration
Conc. of Protein mg/ ml

Lipids

Lipids are defined as a group of fatty nature which are a heterogenous group of compounds which share the property of being relative insoluble in water and soluble in organic solvent such as alcohol, acetone, benzene, ether, chloroform and carbon tetrachloride. Lipids thus include fats, oils. Waxes prostaglandins and related compounds . Lipids are classified into three major groun

1. **Simple lipids:** Are esters of fatty acids with glycerol (Triglyceride) (T.G).

Simple lipids + H_2O ⟶ hydrolysis ⟶ Fatty acid + alcohol

If the alcohol produced is glycerol, then the simple lipids is called fat. Simple lipids e.g. fat, oils, waxes.

2. **Compound lipids:** Esters of fatty acids with glycerol in addition of other compounds e.g. phospholipids, glycolipids lipoproteins.
3. **Derivative lipids:** There are compounds derived from hydrolysis of simple and compound lipids, such as fatty acids , glycerol, steroids, alcohols, ketone bodies, hydrocarbons and vitamins.

Lipids that are solid at room temperature are called fats. Lipids that are liquid at room temperature are called oils. lipids serve as the main energy reserve for living system form part of cell membranes, and regulate the activities of cells and tissues.

Test for lipids

1 **Solubility test:** The provided oils is insoluble in water, hence it floats on the surface of water, forming a separate layer. The oil is freely soluble in ethyl being heavier than alcohol. some of the oil (undissolves) settle down at the bottom as minute droplets, whereas it is extremely soluble in chloroform. The resulting solution is clear.

Materials :

1. Organic solvents (Acetone, chloroform, ethanol) distilled water, sodium carbonate.
2. Oil.

Procedure :

1. Take five perfectly dry test tube.
   - a- Add 1 ml of distilled water.
   - b- Add 1 ml of acetone.
   - c- Add 1 ml of chloroform.
   - d- Add 1 ml of 2% of sodium carbonate.
   - e- Add 1 ml of cool (ethanol then 1 ml of oil shake gently and observe, heat on water, shake gently and observe again.
2. Add for all tubes 1 ml of oil.
3. Shake gently and record your results.

2. Emulsification test

Most lipids are soluble in 95% ethanol but form an emulsion of fine droplets on addition of water. This gives the solution a characteristic milky appearance and is a very sensitive test for fats.

Emulsion: Amilky liquid prepare by mixing oil and water, there are two types of emulsion:

1. Temporary emulsion (in this case the oil particles separate, and carried above the surface of water).
2. Permanent emulsion (in this case the oil particles mix water and don't separate).

Materials:

1. Oil.
2. Albumin.

Procedure:

1. Take two perfectly clean test tubes.
2. To the first add 2 ml of distilled water.
3. To the second add 2 ml of albumin.
4. To each of two tubes, add 2 drops of oil.
5. Close the mouth of test tube with your thumb.
6. Shake both vigorously and observe.
7. In the first tube a temporary emulsion of oil in water is formed. Let it to stand for 10 minutes. It remains turbid.
8. In the second tube a permanent emulsion of oil in albumin is formed, which is separate into two distinct layers.

Conclusion: Albumin which is a protein act as emulsifying agent that convert oil to emulsion to accelerate the absorption from the intestines, and then transport in blood by a transport from protein (albumin).

3. Rancidity test :

Qualitative test for fry oil specially oil restaurant by adding a few drops 0.5% NaOH then add a few drops ph.ph. indicator. Disappearance of the pink color of indicator indicate the rancidity of oil.

Materials :

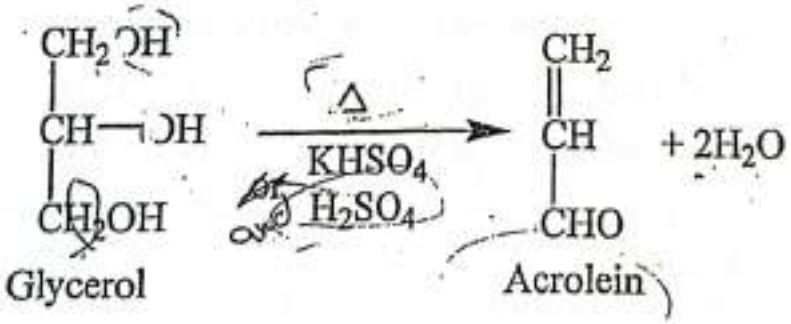
1. Rancidity oil.
2. 0.5 % NaOH.
3. Ph.ph. indicator.

Notes:

1. Ph.ph. color in alkaline medium is pink.
2. Due to the hydrolysis of rancidity fat or oil to fatty acid which cause the medium acidic.

4. Acroline test:

When glycerol is heated with potassium bisulphate ($KHSO_4$) and conc. H_2SO_4. This treatment results in dehydration occurs and aldehyde acrolein is formed which has a characteristic odor. The test is given by glycerol free or combined as an ester.



Materials :

1. Test compound (oil or fat, oleic acid).
2. Potassium bisulfate or conc.H_2SO_4.

Procedure :

1. Place 1 g of $KHSO_4$ in a dry test tube, then add 2 drops of conc. H_2SO_4.
2. Add 2 drons of oil.
3. Heat the test tube directly.
4. Observe the pungent smelling fumes arise from a test tube.

Notes:

1. Use dry test tube because H_2O react with double bonds.
2. Acrolein is evolved which has a pungent smell.
3. All the triglycerides give this test.
4. Any fat or oil give this smell since they all have glycerol as their alcohol component.

5. Unsaturated test :

The fatty acids present in animal fat, are usually fully saturated , where as those found in vegetable oils contain one or more double bond. Halogens readily add a cross the double bonds and the decolorization of a solution of bromine or iodine by a lipid, indicates the presence of double bonds. This test is used to distinguish between saturated and unsaturated fatty acids, as well as between oils and fats.

$CH_3(CH_2)_7-CH=CH(CH_2)_7COOH + I_2 \longrightarrow CH_3(CH_2)_7CH(I)-CH(I)-(CH_2)_7COOH$

Oleic acid

Di-Iodo Oleic acid

Materials:

1. Oil, fat or oleic and stearic acids.
2. Organic solvents (chloroform or ethanol).
3. Bromine water or iodine.

Procedure:

1. Place 1 ml of oil.
2. Slowly add bromine water to the oil drop by drop, shaking after each addition.
3. Count the number of bromine drops needed to produce a permanent color.

Notes:

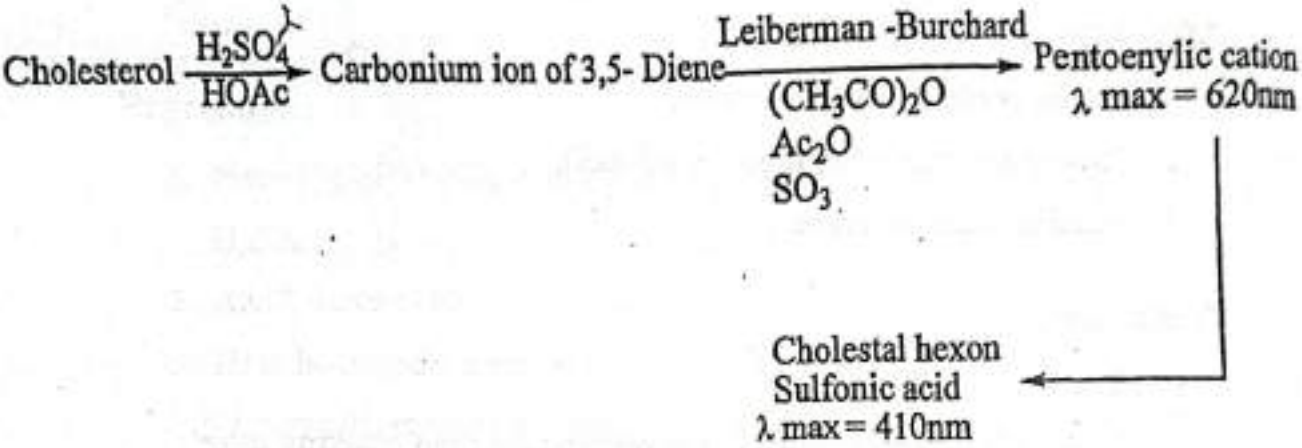
1. Unsaturated fatty acids consume a large number of bromine drops than saturated fatty acids do.
2. This test is used to determine the degree of unsaturation.
3. Hydrogenation of double bonds change the liquid fat to solid.
4. Oil contains a higher percent of unsaturated fatty acids than solid fats. So oils can react with more bromine.

Cholesterol :

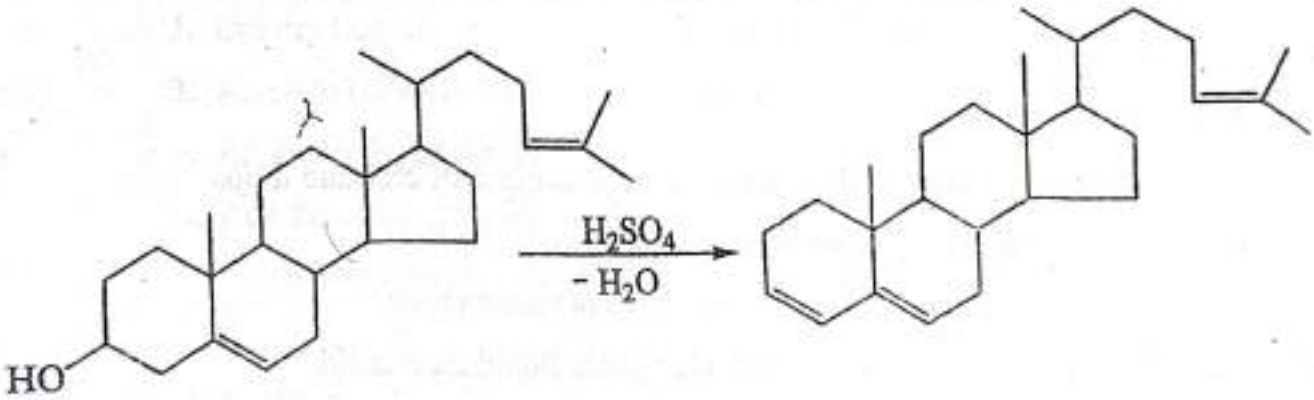
Is one important precursor of steroid hormones and vitamin D for human being and is supplied by diet. However higher concentration of cholesterol in blood is related to a number of diseases, e.g. cardiac infraction and atherosclerosis. The following are simple qualitative test

for cholesterol which form the basis of quantitative determination of cholesterol in blood.

a- Salkowski test



The reaction is not clear but dehydration is probably in the first step:



When sulfuric acid is added to a chloroform solution of cholesterol, characteristic colors develop in the two layers. The chloroform layer exhibits a red to blue color and the acid layer shows a green fluorescence. The nature of these color is not known at present time.

Materials:

1. 0.5% cholesterol in chloroform.
2. Conc. H_2SO_4.

Procedure :

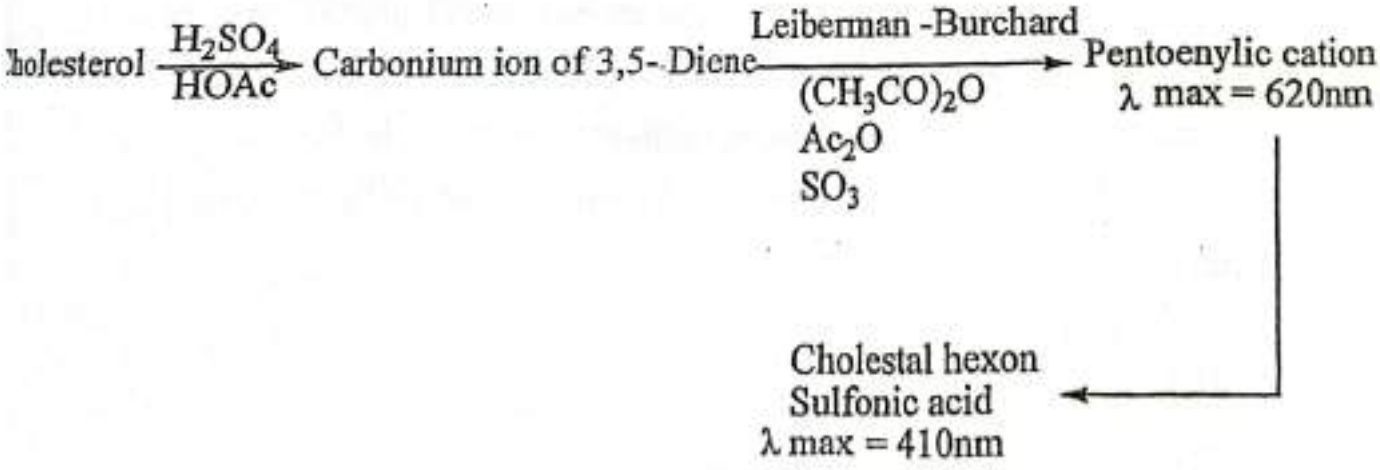
1. Place 1 ml of 0.5% cholesterol in chloroform in a dry test tube.
2. Add 1 ml of con. H_2SO_4.
3. Mix carefully allow to stand. Note the red color of the upper (chloroform) and green flourescence in the lower layer.

Notes:

1. Cholesterol is a constitute of all animal cells. It is a colorless substance.
2. Absence of water is absolutely necessary for the success of this experiment.

b- Leiberman –Burchard test:

Acetic anhydride reacts with cholesterol in a chloroform solution to produce a characteristic blue-green color. The exact nature of the chromophore is not known, but the reaction probably includes esterification of the hydroxyl group at the three position as well as other rearrangements in the molecule.



Materials:

1. 0.5% cholesterol in chloroform.
2. Acetic anhydride.
3. Conc. H_2SO_4.

Procedure :

1.Place 1 ml of 0.5% cholesterol in chloroform in dry test tube.

2.Add 10 drops of acetic anhydride.

3.Add 2 drops of conc. H_2SO_4, mix.

4.Observe the appearance of deep blue color which gradually turns in to green.

Notes:

1. The test is used in the estimation of blood cholesterol.
2. Absence of water is absolutely necessary for the success of this experiment.

6. Acid value:

During storage fats may become rancid due to peroxide formation at the double bonds by atmospheric oxygen and hydrolysis by microorganisms with the liberation of free acid. The amount of free acid present therefore gives an indication of the age quality of the fat.

The acid value is the number of milligrams of KOH required to neutralize the free acid present in 1 g of fat.

Materials:

1. Fat or oil.
2. 0.1 N KOH.
3. 25 ml of each (95% alcohol +ether).
4. Dissolve (0.1) g of ph.ph. in 10 ml of 95% ethanol.

Procedure :

1. Place 10 g of fat or oil in a conical flask.
2. Add 50 ml of equal volume of alcohol and ether.
3. Add 1 ml of phenolphthaline as indicator.
4. Heat with shaking in water bath (65° C) for (10) minutes then cool.
5. Titrate the solution with 0.1 N KOH until pink color appear 10 sec. after end point.
6. Record the volume of KOH.
7. Calculate the acid value of the fat using the following law:

$$\text{Acid value} = \frac{\text{Volume of KOH} \times 0.1 \times 5.6}{\text{Weight of fat or oil}}$$

Acid value = mg KOH / 1g oil

7. Iodine number:

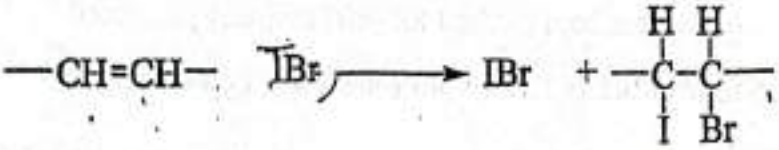
Halogens add across the double bonds of unsaturated fatty acids or triglycerides to form additions compounds.

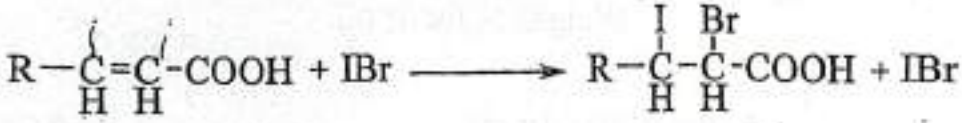
Halogens e.g. iodine or bromine are take up by the fat because of the presence of double bonds in the fatty acid part of the fat.

Iodine number is a measure of the degree of unsaturation of a fat. The higher the iodine number, the more is the unsaturation present in the fats. Iodine number is useful characteristic for assessment of both purity and nutritive of the fat.

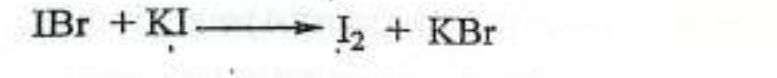
Bromine is often used instead of iodine because it is more reactive. The value is influenced by the percentage of each unsaturated fatty acid, the degree of unsaturation of each acid and the mean moleculear weight of the fat.

Iodine number is defined as the number of grams of iodine absorbed by 100 gm of the fat. The given amount of fat treated with a measured excess of hanus solution (IBr):

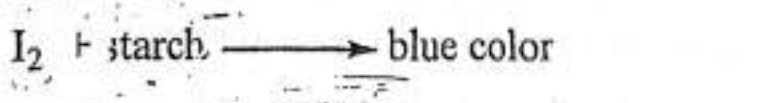




Iodine bromide is allowed to react with the fat in the dark, and the amount of iodine consumed is then determined by titrating the iodine released (after adding KI) with standard thiosulphate and comparing with a blank in which the fat is omitted. The reaction mixture is kept in the dark and the titration carried out as quickly as possible since the halogens are oxidized in the light .



At the end point:



Iodine number	Oil or fat
119–106	Cotton seed
88–79	Olive
204–175	Linseed
9	Coconut
90–80	Castor-oil plan
128–104	Corn
180–144	Whale liver
53	Pig
153–120	Sea fish
142–153	Caw
40–25	Cream
65	Human under skin
135	Liver

Materials :

1. oil or fat.
2. Chloroform.
3. Hanus iodine solution: Dissolved 13.2 g for pure iodine in 1L of acetic acid, add 3 ml of bromide, mix and kept in dark flask.
4. 0.1 N sodium thiosulphate: Place 24.13 g of ($Na_2S_2O_3.5H_2O$) in 1 L H_2O, freshly prepared.
5. 15% KI.
6. 1% starch solution or solid.

Procedure :

1. In a 250 ml conical flask , place 0.25 g of oil, add 10 ml of chloroform, mix this is "T" test.
2. Add 15 ml of Hanus solution (IBr), mix well.
3. Cover the mouth of the flask with a watch glass and keep it for 30 minutes for reaction to take place.
4. After 30 minutes, add 5 ml of KI solution into it.
5. Mix well, followed by 50 ml of distilled water.
6. Titrate the contents of the flask with 0.1 N/$Na_2S_2O_3$, till the yellow color is disappear , add starch as indicator.
7. Complete the titration till the blue color is disappear .
8. Calculate the volume from burette.
9. Calculate the equivalent volume of hanus alone usage 10 ml of chloroform without oil this flask "B" blank.

Calculation:

Titrate volume obtained for test solution = T

Titrate volume obtained for black titration = B

According to normality equation

1 M of $Na_2S_2O_3 \equiv$ 1 M of I_2

1 M of $Na_2S_2O_3 \equiv$ 127 g of I_2 equivalent of iodine = 127

I ml of 0.1 N $Na_2S_2O_3 \equiv 0.127 \times 0.1$ g of I_2

(B-T) × 0.1 = (B-T) × 0.127 × 0.1 of I_2 absorbed by 0.25 g of oil

Iodine number: Number of grams of iodine absorbed by 100 g of the oil

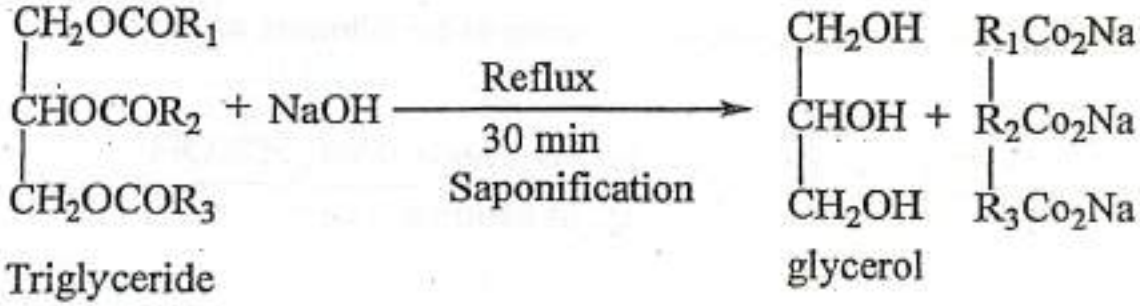
$$\text{The iodine number} = \frac{(B-T) \times 0.127 \times 0.1 \times 100}{0.25}$$

$$= \text{g } I_2 / 100 \text{ g oil}$$

Saponification number:

Saponification number is defined as the number of milligrams of potassium hydroxide (KOH) required to saponify completely 1 g of fat since fats are mixture of triglycerides, most of which are of mixed type, so saponification number is a measure of average molecular weight of the fatty acids comprising the fats (i.e. the measure of the average chain length of the fatty acid).

Saponification number is an important constant particularly in distinguishing or identifying certain oils. The saponification value gives an indication of the nature of the fatty acids in the fat since, the longer the carbon chain, the less acid is liberated per gram of fat hydrolyzed. When triglycerides are heated with alkaline potassium hydroxide the ester bonds may be hydrolyzed to give glycerol and salt of fatty acid (soaps).



Materials:

1. Fat.
2. 0.5 N alcoholic potassium hydroxide.
3. 0.5 N HCl.
4. Phenonaphthalein.

Procedure:

1. Take a clean and dry 100 ml conical flask.
2. Transfer accurate weight 5g of oil or fat in flask.
3. Add 50 ml of 0.5 N alcoholic potassium hydroxide.
4. Then connect the flask with condenser and reflux on a boiling water bath for 30 minutes till the oil globules disappear and a yellow cake is formed by potassium salts of fatty acids.
5. Cool it at room temperature and titrate with 0.5 N hydrochloric acid solution using 2 drops of phenonaphthalien as an indicator until the pink color disappears and then record the volume as (T).
6. Now take 50 ml of alcoholic KOH and titrate with 0.5 N HCl solution. This is blank solution (B).

Calculate the saponification number according to the following equation:

$$\text{Sap.No.} = \frac{\text{normality} \times \text{volume} \times \text{equivalent wt. of KOH}}{\text{wt. of sample}}$$

$$= \frac{0.5 \times (B-T) \times 56}{5}$$

Properties of some lipids

Oil	Acidity (%)	Iodine number	Saponification number
Butter	0.5-30	25-40	210-230
Castor oil	0.2-4	80-90	175-187
Coconut oil	2.5-6	7-10	254-262
Corn oil	1-2	104-128	187-195
Halibut liver oil	1	120-135	170-180
Linseed oil	1-4	170-200	188-195
Olive oil	0.2-3	79-90	190-195

Enzymes

Enzymes are proteins and biocatalyst responsible for acceleration and activate metabolic reaction that maintain animal homeostasis. Because of their role in maintaining life processes, the assay and pharmacological regulation of enzymes have become necessary in clinical diagnosis and therapeutics.

Enzymes are found in all tissues and fluids of the body. Intracellular enzymes catalyze the reactions of metabolic pathways, enzymes of the circulatory system are responsible for regulating the clotting of blood, digest and oxidation diet, amino acid metabolism and construction of hemoglobin. Many enzymes were purified and crystallized.

Characteristic :

1. Enzymes require substrate.
2. Enter in reaction as a small amount without structure change.

$$S + E \rightleftharpoons ES \longrightarrow P + E$$

3. The activity of enzymes are influenced markedly by many environmental variables such as temperature, pH, concentration of the substrate, enzyme and inhibitor, activator.
4. Enzymes have high M.wt. about 10^4-10^6 dalton.
5. Reactions with enzyme, all substrate turnover to product at high rate and capability.
6. Many of enzymes require nonprotein organic compound in order to function such as:

1. Metal ions which serve as activators Mg^{+2} is a common one, Ca^{+2}, Fe^{+2}, Zn^{+2}, Mn^{+2} and called cofactor.
2. Coenzyme such as ascorbic acid, vitamin B complex. If these groups is a strongly bonded enzyme called prosthetic group.

7. All enzymes have active side, which is amino acid units as hole or encircle to share in catalysis e.g. acetyl cholinesterase has active side (histidine, serine, tyrosine).

Some specific definition

a- Units of enzyme or activity: The amount of enzyme which will catalyze the transformation of 1μmole (10^{-6}) mole of substrate per minute (v): rate of enzymatic reaction.

b- Specific activity:

This describes the purity of an enzyme, and is usually the number of units per milligram of protein or nitrogen. Increase through purification.

$$\text{Specific activity} = \frac{\text{Activity of enzyme}}{\text{protein (mg)}}$$

3. Molecular activity:

If the molecular weight of the enzyme is known which is defined as the units per micro mole of enzyme.

4. Turnover Number:

It is the number of moles of substrate transformed per minute per moles of enzyme under specified conditions. For example one molecule of catalase (M.wt = 225,000) will decompose 2,600,000 molecules of H_2O_2 in 1 minute at 0°C. The turnover number is 2,600,000. This is extremely active enzyme.

5. **Substrate:** Material or chemicals which enzyme react with it to get product.

6. **Michaels – Menten constant (km):** The substrate concentration when maximum velocity equal to half, when the observed reaction rate is half of the maximum possible reaction rate, the substrate concentration is numerically equal to the Michaelis –Menten constant.

$$Km = [S] \approx \frac{Vmax}{2}$$

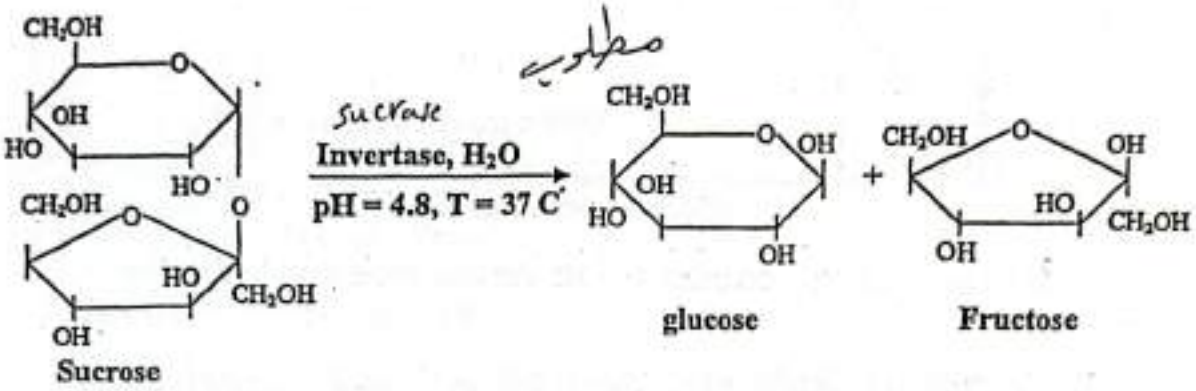
Factors effect on velocity of an enzyme reaction

Since the activity of enzymes is influenced by many environmental variables including:

1. Concentration of substrate.
2. Concentration of enzyme.
3. pH.
4. Temperature.
5. Time of reaction.
6. Inhibitors and activitors.

First :Effect of substrate concentration on reaction velocity in an enzymatic reaction:

Usage invertase or suctase [EC.3.2.1.1], properties of yeast invertase, among the earliest known and widely studied enzyme are those that hydrolyze sucrose.

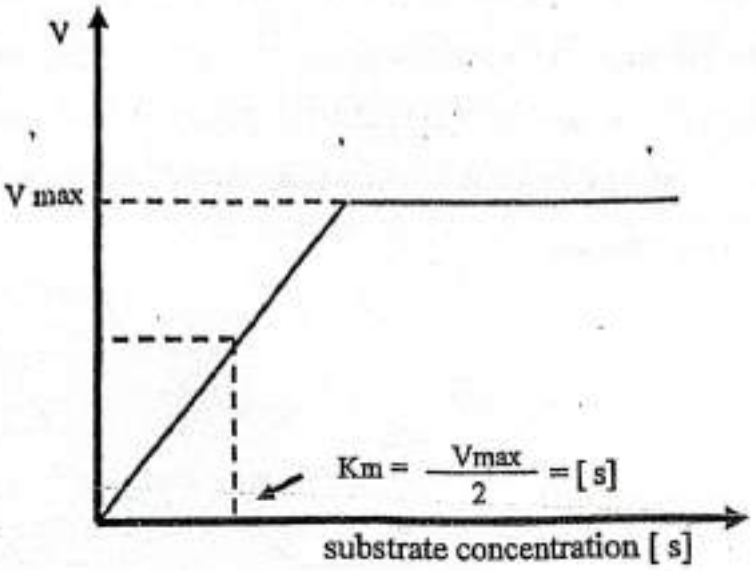


The name invertase sacharase more recently, β-fructofuran-asidase have been used with reference to the enzymetic hydrolysis sucrose. Invertase activity has been demonstrated in yeast, many bacteria, plants and higher animals.

Concentration of substrate with a given quantity of enzyme. In this reaction velocity increases with increasing substrate concentration until a limiting value is reached (enzyme saturated with substrate) .

The catalytic event that covert substrate to product involves the formation of a transition state, and it occurs most easily at specific binding site on the enzyme. This site called the catalytic site of the enzyme. The complex is called the enzyme substrate ES.

$$S + E \rightleftharpoons ES \longrightarrow P + E$$

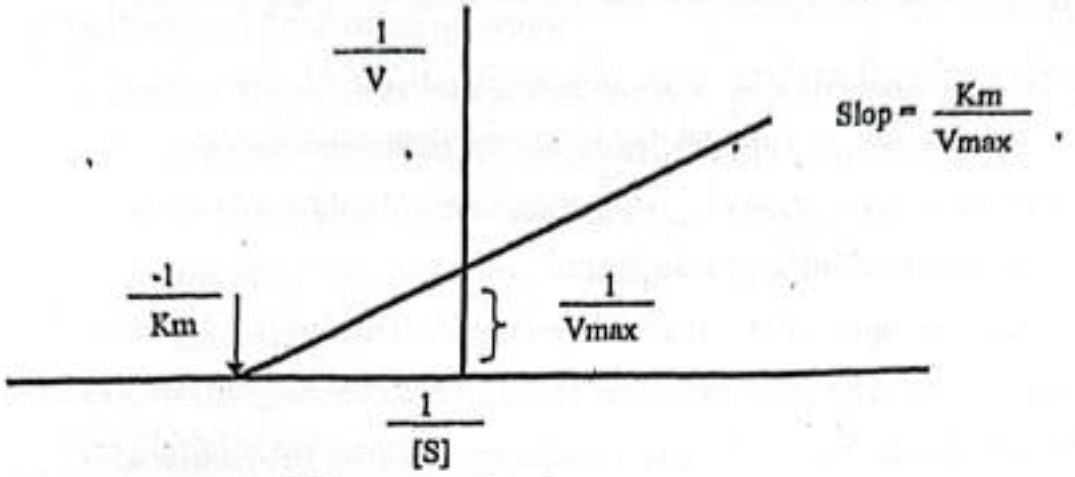


Plot of substrate concentration versus reaction velocity

The Michaelis –Menten equation is a quantitative description of the relationship among the rate of an enzyme catalyzed reaction.

The Michaelis –Menten equation can be used to demonstrate that the substrate concentration produces exactly half of the maximum reaction rate i.e. 1/2 Vmax the substrate concentration is numerically equal to km. The opposite and rearrangement of the Michaelis Menten equation, which it a lineweaver-burk equation.

$$\frac{1}{V} = \frac{Km}{Vmax\,[S]} \times \frac{1}{Vmax}$$



Lineweaver-burk

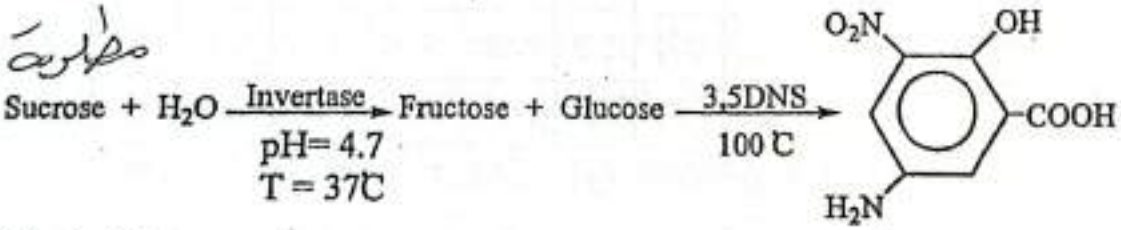
$$\frac{1}{V} = \frac{Km}{Vmax\,[S]} \times \frac{1}{Vmax}$$

This equation usage for:

1. Study to determine the kind of inhibitors (compitative, noncompitative and uncompitative).
2. Exact values of km as well as Vmax

Principle:

This experiment uses the reducing power assay procedure which consist in stopping the reaction with alkaline and subsequently measuring the reducing power by means of the 3,5-dinitrosalicylate method, which reducing to 3-amino,5-nitrosalicylitic acid. It absorbance at 540 nm.



Materials :

1. Prepare a crude enzyme:

Place 10 gm of dry yeast in (30 ml) (0.1 N) of $NaHCO_3$, in (250 ml) conical flask, mix well until get homogeneous solution covered by cotton and incubate at 37°C for 24 hours, then centrifuge it on 15000g for 15 minutes.

2. Take the filter and keep it in volumetric flask at 2 °C.
3. Enzyme solution: Dilute the filter on 10% of distilled water.
4. 0.03 M sucrose, place (10.2) gm of sucrose in 1L distilled water.
5. 3,5-dinitrosalciylate dye reagent:

Place (5)gm of 3,5-dinitrosaliyselic acid in (100) ml of 2N NaOH. place (150 gm) potassium sodium tartarate (Rachall salt) in (250 ml) distilled water. Mix two solutions and complete it to 500 ml of D.W.

6. A solution: 74.6 gm sodium acetate in (1L) D.W.

B solution : 12.5 ml acetic acid in 1L D.W.,

Acetate buffer solution =(750 ml of A+ 500 ml of B).

Procedure:

Prepare and label at least eight test tubes, which content as the table below:

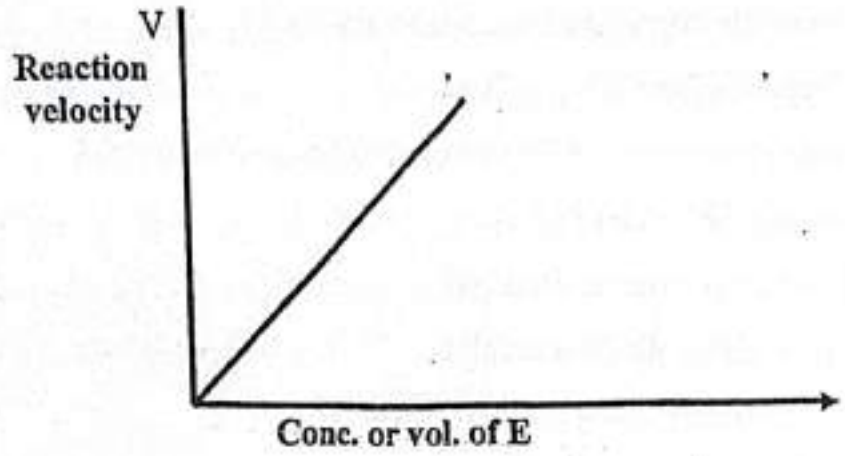
No. of tube	1	2	3	4	5	6	7	8
0.03M sucrose substrate	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7
Buffer solution pH = 4.7 sodium acetate +acetic acid	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Distilled water	1.5	1.4	1.3	1.2	1.1	1.0	0.9	0.8
Enzyme solution	1	1	1	1	1	1	1	1
Mix, and incubate for exactly 5 min at 37 °C on water bath								
3,5-DNS solution	2	2	2	2	2	2	2	2
Place all tubes in boiling water bath for 5 min								
Dilute all tubes to 20 ml of D.W								

1. Read the optical density at (540) nm versus blank (tube No.1).
2. Plot results substrate concentration [S] against velocity (υ).

3. Answer the following questions:
   a- Discuss the type of curve you have obtained by plotting the substrate concentration against velocity.
   b- Prepare a Line Weaver-Burk plot.
4. Determine the value of Vmax from Michaels –Menten plot.
5. Determine the value of km.
   a- From Line Weaver-Burk plot.
   b- From Michaelis-Menten plot.
   c- Theoretically using the above data.
   d- From the plot versus [S].
6. Discuss what is practical significance of km when
   a- Km value is large.
   b- Km value is small.
7. know the type of inhibition .

Second: effect of enzyme conc.

In the presence of excess substrate the velocity of the reaction is proportional to the concentration of enzyme. Some enzymes are inhibited by the products of the reaction. As the reaction proceeds. Various states of relative enzyme and substrate concentration are possible. One can have a high [S] and so, little enzyme that velocity cannot be accurately determined. Similar too much enzyme makes it difficult to determine an extremely high velocity. Also-called kinetic assay in which the reaction rate is followed continuously is advantageous.



Effect of [E] with velocity of reaction

Procedure:

8	7	6	5	4	3	2	1	Tube no.
1	1	1	1	1	1	1	1	Sucrose solution (ml)
0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	Buffer solution (ml)
0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	D.w (ml)
0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.0	Enzyme solution (ml)
Put all tubes in water bath at 37° C								
2	2	2	2	2	2	2	2	3,5-DNS solution
Place all tubes in boiling water bath for 5 min Dilute all tubes to 20 ml of D.W								

1. Read the optical density at (540 nm) for all test tubes.
2. Draw a plot between enzyme and velocity.
   (direct relationship)

Third: effect of temperature

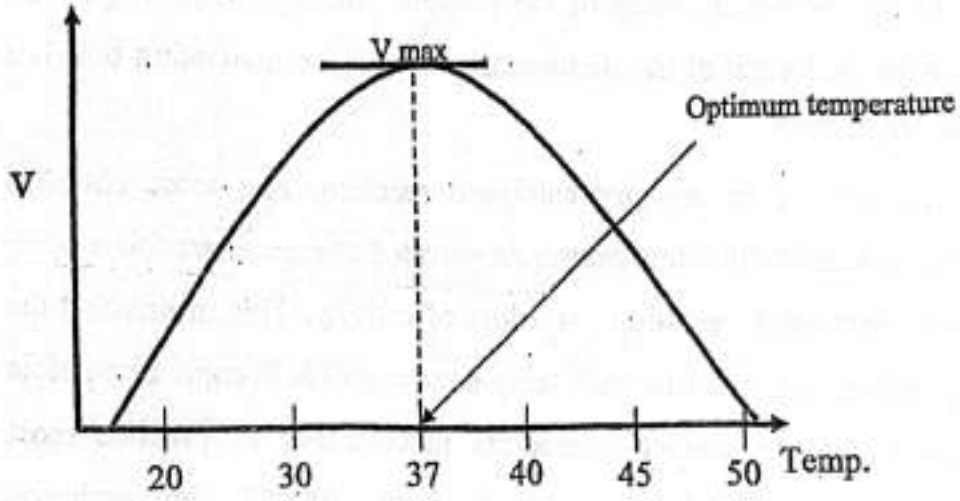
Enzyme reactions are influenced by temperature changes in much the same way as chemical reactions in general are influenced, the effects i.e. The point at which the acceleration effect of temperature increase is balanced by the denaturating of the enzyme. The optimum temperature varies with the length of the incubation period if the incubation time is a high as 60°C-70°C.

The rate of an enzyme-catalyzed reaction, like most chemical reaction, increase with temperature. At elevated t temperature the enzyme protein is denatured, resulting in a loss of activity. This means that the initial reaction rate will rise with temperature until it become impossible to measure due to almost immediate inactivation. In practice most enzymes are completely inactivated above 70 °C. the optimum temperature is the result of the balance between the rate of increase of activity and the rate of destruction of the enzyme. The optimum temperature is not a constant value for a given enzyme, but depends upon the time during which measurements of activity are made. The shorter the time of measurement, the higher will be the apparent optimum temperature.

Procedure:

6	5	4	3	2	1	Tube no.
0.5	0.5	0.5	0.5	0.5	0.5	Sucrose solution (ml)
0.5	0.5	0.5	0.5	0.5	0.5	Buffer solution (ml)
0.5	0.5	0.5	0.5	0.5	0.5	Enzyme solution (ml)
1	1	1	1	1	1	D.w (ml)
Put all tubes in water bath for 5 minutes as bellow						
50	45	40	37	30	20	Temperature °C
2	2	2	2	2	2	3,5-DNS solution

1. Dilute the test tube to 20 ml by distilled water.
2. Read optical density at 540 nm for all test tubes.
3. Draw a plot between temperature and velocity.



Effect of temperature on the enzyme velocity

4. Effect of pH :

The activity of an enzyme is very much dependent upon $[H^+]$. Generally, an enzyme will be active over relatively narrow pH range. The maximum value is referred to as the optimum pH although this value will vary with temperature, buffer used, presence of activators or inhibitors, etc. Enzymes are active over a limited pH range only and a plot of activity against usually gives a bell-shaped curve. The pH value of maximum activity is known as the optimum pH and this is a characteristic of the enzyme, provided that the enzyme is stable under the conditions studied. The variation of activity with pH is due to the change in the state of ionization of the enzyme protein and other components of the reaction mixture. Michaelis and Davidsohn suggested in 1911 that only one of the large number of ionized forms of the protein is active, so that a change in pH either side of the optimum procedures and decrease of this form and hence a fall in the activity.

Procedure:

1	1	1	1	1	1	Sucrose solution (ml)
5.6	4.8	4.4	4	3.7	2.7	Buffer solution (ml)
Put all tubes in water bath for 5 minutes						
1	1	1	1	1	1	Enzyme solution (ml)
The time between each addition must be 1 minute						
2	2	2	2	2	2	3,5-DNS solution(ml)
Put all tubes in boiling water bath for 5 minutes						
Dilute all tubes to 20 ml						
Read the absorbance at 540 nm.						

1. (G+F) conc. From standard curve previously.
2. To calculate the velocity from this equation.

$$V = \frac{G+F}{2t}$$

3. Draw a plot between υ and pH.

Standard curve of sucrose hydrolysis by invertase:

To determine glucose and fructose from hydrolysis of sucrose by invertase at limited time. Draw a standard curve. React know amount of (G & F) with inhibition (3,5-dinitrosalycelic acid), then record the absorption at (540 nm). Plot a standard curve between (G + F) conc. And absorption.

Procedure:

1. Prepare 6 label test tubes, each one content as .

						Tube no.
1	0.8	0.6	0.4	0.2	0	(G + F) (ml)
0	0.2	0.4	0.6	0.8	1	D.w (ml)
1	1	1	1	1	1	Buffer solution (ml)
1	1	1	1	1	1	Sucrose solution(ml)
2	2	2	2	2	2	3,5-DNS solution(ml)
Put all tubes in water bath for 5 minutes then cold it						

2. Dilute all test tube to (20 ml) with distilled water.
3. Record the optical density at (540 nm) except tube No. 1 as blank.
4. Draw standard curve between (G+F) concentration and optical density.

(G+F) solution : it is a mixture prepare (0.03) mole/L .

Calculations :

Concentration of (G+F) of each test tube is:

V ml	(G + F) conc.
1000	0.03
0.2	X

$$X = \frac{0.03 \times 0.2}{1000} \times \frac{0.006}{1000} = 6 \times 10^{-6} \text{ mol / L} \quad \text{No.1}$$

Also the same method use to calculate the conc. in each tube .

5. Effect of inhibitors: are chemical compounds decrease the activity of enzyme by decrease the velocity of enzymatic reaction.

Accumulation of the product of reaction may retard (inhibitor) enzymatic activity in certain instances. Many compounds react with enzymes and reduce the measured activity. This property of enzymes is used in designing drugs and insecticides which selectivity inhibit enzymes in the infective bacteria or insert, but don't affect the animal or plant. Two classical types of inhibition are recognized:

1. **Competitive:** In this case, the inhibitor reacts with the enzyme by competing with the substrate for the active site.
2. **Non-competitive:** In the non-competitive type of inhibition, the inhibitor combines with the enzyme but not at the active site, so that the enzyme can bind both substrate and inhibitor at the same time. The binding site of the inhibitor is usually sufficiently far removed from the active center so that the binding of the substrate is unaffected. Example the reaction of cyanide with the iron-prophyrin enzymes.

Vitamins

Vitamins are organic molecules that function a wide variety of capacities within the body which needs it few amount. The most prominent function is as coenzymes for enzymatic reactions as well as for growth, synthesis and regulate the metabolic reactions. The distinguishing feature of the vitamins is that they generally cannot be synthesized by mammalian cell and therefore, must be supplied in the diet. The vitamins are of two distinct types :

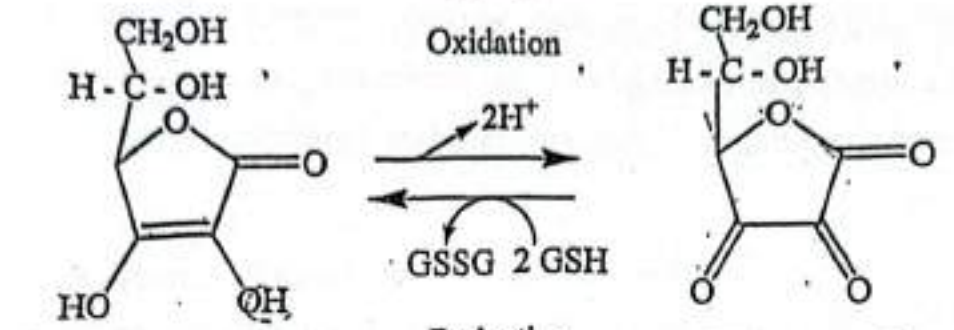
1. Fat soluble vitamins [A, D, E, K]
2. Water soluble vitamins [Thiamine (B_1), riboflavin (B_2), niacin (B_3), pantothenic acid (B_5), pyridoxal, pyridoxamine, pyridoxine (B_6), biotin, cobalamin (B_{12}), folic acid , ascorbic acid.

The excretion of water soluble vitamins with urine because body cannot storage it, while the fat soluble vitamins storage in liver so in a large amount of it cause hypervitaminosis.

Ascorbic acid:

Ascorbic acid is more commonly known as vitamin C, it was first isolated in pure crystalline from lemon juice by the American biochemists. It is one of the simplest vitamins in structure, being a lactone of a sugar acid.

Ascorbic acid is required in the diet of only a few vertebrates man, monkeys, the guinea pig but most other higher animals and plants can synthesize ascorbic acid from glucose of other simple precursors. Ascorbic acid is a strong reducing agent it is belong to water soluble antioxidant. It is affect of light, temperature , base and enzymes especially in Fe and Cu present. So readily losing hydrogen atoms to become dehydroascorbic acid, which also has vitamin C activity.



Ascorbic acid $AscH_2$ Dehydroascorbic acid Asc.

Ascorbic acid in food is largely destroyed by cooking and a long store.

1. Ascorbic acid is an important vitamin which participates in a great variety of biological events concerning electron-transport reaction.
2. Vitamin C functions as an antioxidant by reacting directly with ROS (Reactive Oxygen Species or regenerating vitamine E from α-tocophenoxyl radical. Thus, it protects cell membranes from external oxidants and has a glutathione-sparing effect.

Determination of vitamin C

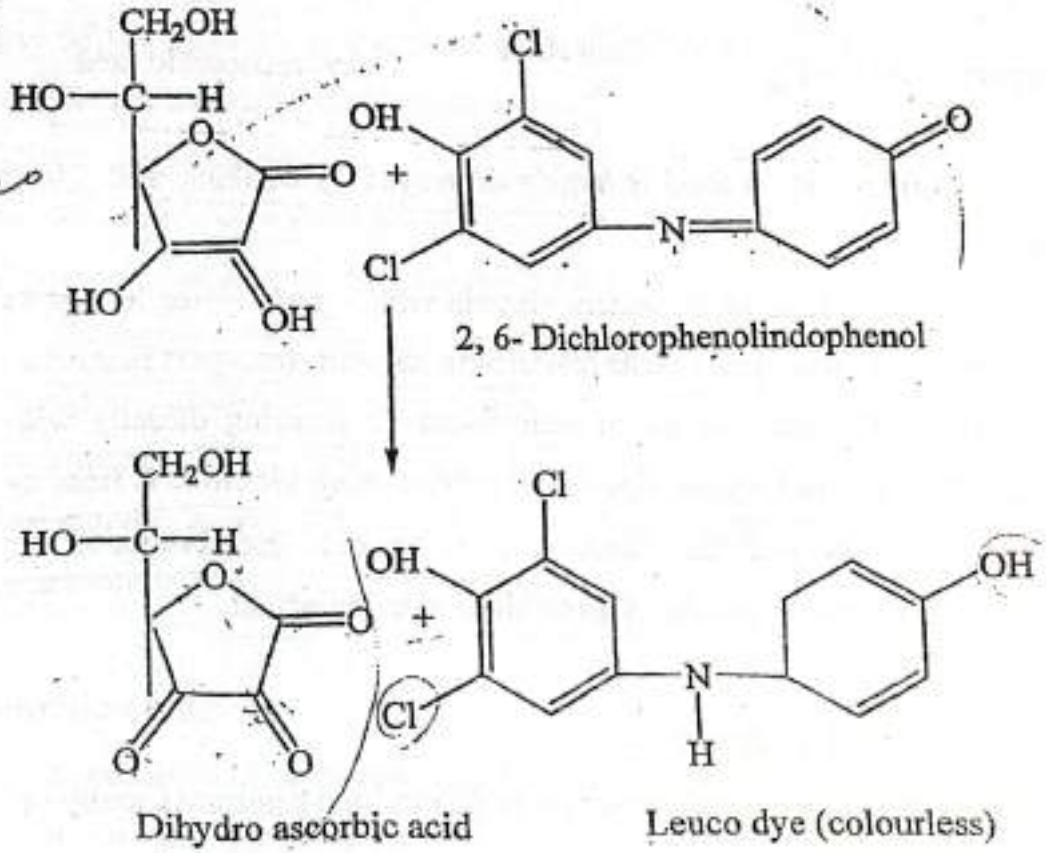
vitamin C in serum and plant can be determined photometrically or titration methods.

1. Titration method :

Principle:

Ascorbic acid is oxidized by the colored dye 2,6-dichlorophenolindophenol to dehydroascorbic acid. At the same time the dye is reduced to a colorless compound so that the end point of the reaction can be easily determined. The dye is decolorized by other compounds as well as ascorbic acid, but the specificity can be increased

to some extent by using acidic mediu so other substance will react so slowly. 2,6-dichlorophenolindophenol is obtainable commercially in convenient tablet form, such that one tablet is equivalent to 1 g of ascorbic acid.



Materials :

1. Ascorbic acid standard solution (2 mg) in (100) ml distilled water (prepare just before use).
2. 2,6-dichlorophenolindophenol solution (1 mg complete to 100 ml distilled water.
3. 6 N HCl or 5% CH_3COOH.
4. Fruit juices (orange, lemonjuice, green pepper).

Procedure:

1. When the sample is orange, press and filter the juice then record the total volume.
2. Place (1 ml) of juice, complete to (50 ml) of distilled water (mixture).
3. Place 5 ml of mixture and add 1 ml of 6N HCl.
4. Titrate with dye (in burette) till the pink color is fix, record the dye volume from burette.
5. Place 5 ml of standard vitamin C add 1 ml of 6N HCl.
6. Titrate with the dye till the pink color fix then record the dye volume.

Calculations:

Conc.	Vol. ml
2	100
X	5

X = 0.1 mg/ 5ml

From biurret →

Vol. (ml)	Conc. (mg)
10	0.1
8	y

y = 0.08 mg/ 5ml

Vol. (ml)	Conc. (mg)
0.08	5
Z	50

$0.08 \times 17 = 13.6$ mg/ 17ml To complet Vol. for example is 17 ml

Vol. (ml)	Conc. (mg)
17	13.6
100	C

$$C = \frac{100 \times 13.6}{17} = 80 \text{ mg/ } 100\text{ml}$$ To calculate the conc. for 100 ml

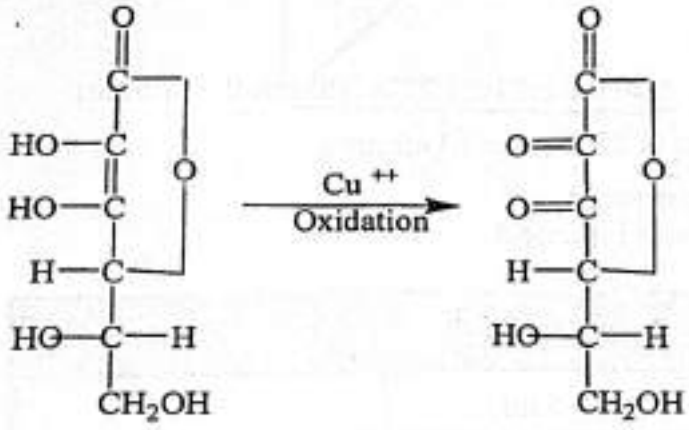
Notes :

1. This type of titration called selfindicator.
2. This is select reaction, so to avoid the secondary reaction.
3. Addition of acid to make the medium of reaction acidic so prevent the intervarance of other reducing substances.

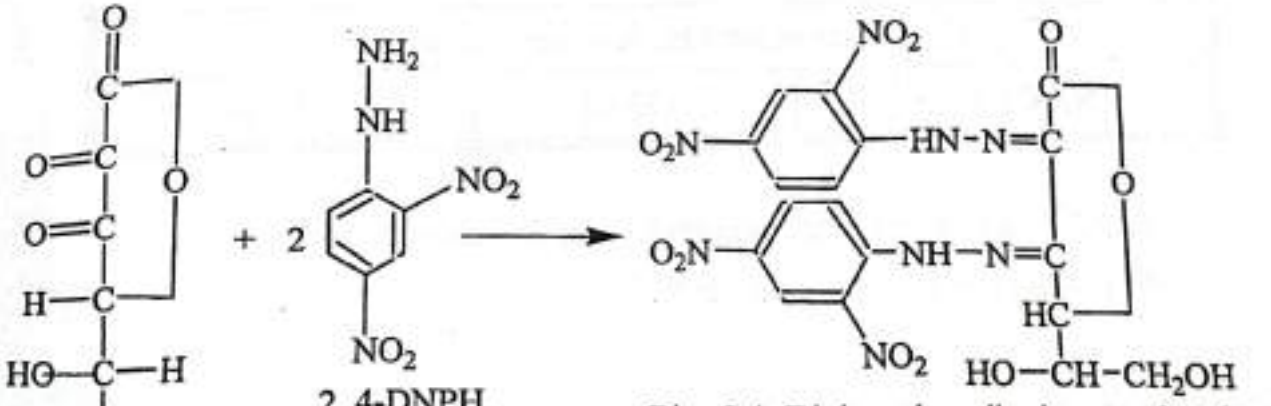
Colorimetric method

Principle :

One can obtain a protein-free sample by treating sample with metaphosphoric acid. Ascorbic acid in the protein-free supernatant is oxidized by Cu (II) ions to dehydro ascorbic acid (DHAA) and diketogluconic acid. These ketones react with 2,4-dinitrophenylhydrazine (2,4-DNPH) in sulfuric acid to form the phenyl hydrazone product, which absorbed at 520nm:



L. Ascorbic acid DHAA



DHAA 2, 4-DNPH

Bis - 2,4- Dinitro phenylhydrazone derative

λ (520 nm)

Materials :

1. 10% TCA in H_2O.
2. 2,4-dinitrophenylhydrazine solution/ thiourea/ Cu(DTC). Place 3 g of 2,4-dinitrophenylhydrazine, 0.05 g $CuSO_4.5H_2O$ and 0.4 g thiourea. All compounds dissolved in 100 ml of 9N H_2SO_4, keep it in cool place.
3. 65% H_2SO_4. This solution was stable at room temp. for 2 years.

Procedure :

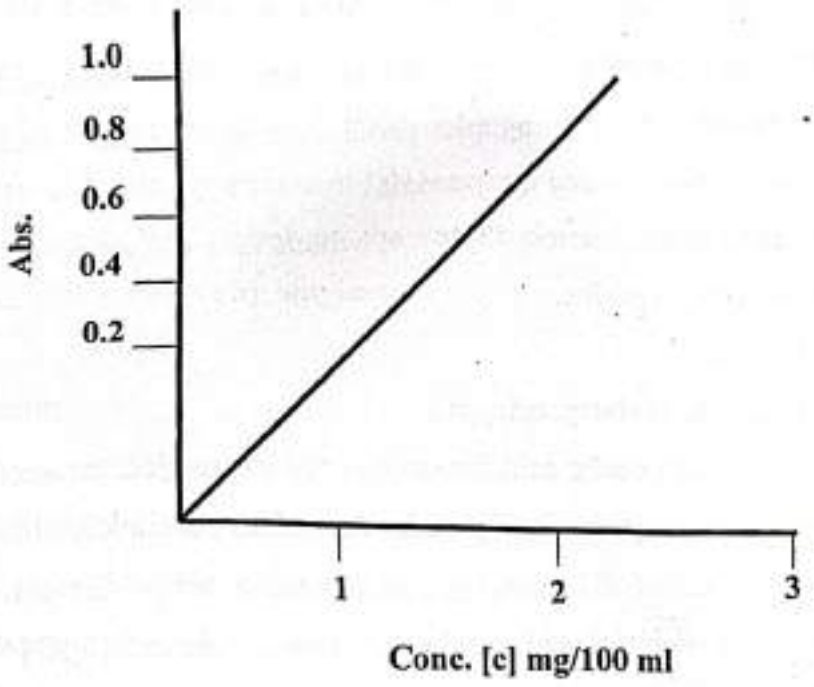
1. Mix 0.5 ml of sample with 0.5 ml 10% TCA. (to ppt.the protein).
2. Put them in centerfuge at 3500 g for 20 minutes.
3. Filter and take the supernatant.
4. Complete as follows.

Materials	Test	Blank
Supernatant	0.5 ml	-
Distilled water	-	0.5 ml
DTC	0.1 ml	0.1 ml
Mix and stand for 3 hr. in water bath at 37°C.		
H_2SO_4 %65	0.75 ml	0.75 ml

5. Mix well, left at room temperature for 30 minutes.
6. Record the absorbance at λ= 520 nm.

Calculation :

Determine vit. C concentration by using standard curve. So preparing different concentration of vitamin C solution (0-2) mg / 100 ml by usage 5% TCA solution.



Standard curve for vit. C determination

Nucleic acid

Organic compounds which have a high molecular weight. It is form many nucleotides bonded by phosphodiesters bond. Each nucleotide contains three characteristic components :

1. A nitrogenous heterocyclic base, which is a derivative of either pyrimidine or purine.
2. A pentose.
3. A molecule of phosphoric acid .

The usage of nucleic acid for storage and expression for hereditary information, also in synthesis of protein. As a class , the nucleotides may be considered one of the most important metabolites of the cell. Two main groups of nucleic acid are known ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) .

❖ RNA

Consist of a long chain of polynucleotide. The three types of ribonucleic acid in cells are called messenger RNA (m RNA) , ribosomal RNA (r RNA) and transfer RNA (t RNA), which is found in cytoplasm rather than in nucleus or mitochondria. RNA is a single-band.

❖ DNA

DNA would exit as a helix of two complementary antiparallel strands, wound around each other in a rightward direction and stabilized by H-bonding between bases in adjacent strands. It is exist in the neculus. Ahigh temperature and pH separate the DNA bond.

Extraction the RNA from the yeast:

This method depended on homoextrate by phenol which broken hydrogen bonds for a big molecules due to denaturation of protein, which separate by using centerfuge for suspension, separate two layer (phases). The down phenolic layer content DNA and up water layer content RNA. RNA precipitate by alcohol, so the end product is free of DNA, only polysaccharides remove by amylase.

Materials:

1. Dry yeast 30 gm .
2. Phenol solution.
3. 200 gm / L . pH =5 . potassium acetate.
4. Absolute ethanol.
5. Diethyl ether.

Procedure:

1. Dry yeast of (30 gm), add (120 ml) of distilled water at (37° C) , stand about (15 min).
2. Add carefully (160 ml) phenol solution, mechanical stirring of mixture about 30 min. at room temperature.
3. Cooling centrifuge (3000 xg) about (30 min.).
4. Raise the up layer by dropper, then return to centerfuge again (1000×g) for (15 min).
5. Add amount of potassium acetate to supernatant fluid until reach 2% concentration.
6. Add two volume of ethanol to precipitate RNA, cool, stand for (15 min.).
7. Place centerfuge (2000×g) for (5 min.).

8. Wash RNA subsequence by mixture of ethanol-water (1-3), ethanol then ether.
9. Leave it to dry, then weight it.

Determination of nucleotide concentration:

Using Bail's method to form furfural, when the pentose from RNA react with HCl conc. Orcinol interaction furfural in $FeCl_3$ as a catalysis to get a green complex color.

Materials:

1. Standard RNA solution (5-40) µg / ml.
2. Orcinol reagent: Dissolve (100 mg) of ($FeCl_3$. $6H_2O$) in (100) ml HCl conc. and (3 ml) orcinol dissolve in 6% alcohol .

Procedure:

1. Prepare six concentration of RNA solution : 5µg, 10µg, 15µg, 20µg, 30µg, 40µg.
2. Place (2 ml) of unknown nucleotide (content pentose).
3. Add (3 ml) of orcinol reagent, heat in boiling water bath about 10 min.
4. Cool and record the absorption at (670 nm), repeat for all concentrations.
5. Blank : (5ml) of orcinol reagent , heat about (10 min.), cool and read absorption at 670 nm.
6. Draw standard curve, then obtained the concentration of unknown nucleotide.

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