COURSE BOOK OF

CHEMISTRY 2 (BIOCHEMISTRY)

Department of Biochemistry
Benha University, Agriculture College

PRACTICAL

PRACTICAL BIOCHEMISTRY

Course name	Practical Biochemistry
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Course link at the University	
Course overview: In two or three constructive paragraphs mention the importance and the necessity of this course	

Biochemistry can be defined as the science concerned with the chemical basis of life. The cell is the structural unit of living system, thus biochemistry can also be described as the science concerned with the chemical constituents of living cells and with the reactions and processes they undergo. By this definition, biochemistry encompasses large areas of cell biology, of molecular biology, and of molecular genetics.

Course objective: in two or three paragraphs mention the aims of the course and the main points students should have learned by the end of the course.

We learn the students the general and specific tests for determine the normal subjects of biochemistry also the abnormal one. That related with diseases.

	The Topics :	
I	**CARBOHYDRATES**	
	1-	General tests: Molisch, Benedict, Fehling and Barfoid.
	2-	Specific tests: Seliwanoff.
	3-	Tests for polysaccharide: (Iodine, and Hydrolysis of polysaccharide).
II	**LIPIDS**	
	1-	General tests: (Solubility test of lipids, Grease spot, Emulsification of lipids).
	2-	Specific test: (Copper acetate, iodine, Iodine number determination, Acid value, Saponification of oils and fats, Saponification value.)
III	**PROTEINS**	
	1-	General tests:- a- Protein composition test. b- Precipitation of proteins; By salts of heavy metals, By alkaloidal reagents. By neutral salts: Half saturation and Complete saturation, and By alcohol. c- Coagulation of proteins by heat. d- Biuret's test, Ninhydrin's test.
	2-	Specific tests for Amino acids: (Milon's test, Xanthoproteic's test, Lead acetate test, and sakaguchi's test.
IV	**Enzyme**	
	1-	General information.

Practical of Chemistry 2
BIOCHEMISTRY

AC 0102

Introduction:
The good practical worker will therefore seek to obtain accurate and precise measurements at the bench. Errors may be random, or caused by carelessness or inaccurate instruments. To reduce such random errors which are individually unpredictable, take a large number of measurements and calculating the average value.

Accuracy:
Accuracy is defined as the degree of conformity to the truth and expressed as absolute error.
Absolute error = experimentally measured value – true value

Precision:
Precision is defined as the degree of agreement between replicate experiments and expressed as standard deviation.
Precision does not mean accuracy, since measurements may be highly precise but inaccurate due to a faulty instrument or technique.

Biological variation
An additional factor to be considered when working with material derived from living matter is biological variation. A physical quantity such as the refractive index of a liquid, for example, may be measured and the value obtained compared with the correct figure, but for biochemical measurements there is rarely a single value which can be considered as correct, but a range of
so-called normal values. This means that if an animal is healthy and free from stress then the value of say a serum constituent should be within the normal range.

Standards and blanks:
To obtain a value as accurate as possible from an estimation, errors must be reduced to a minimum. This can be done by careful working and the use of standard solutions.
Standard solutions of the substance to be estimated should be

included with any test even when a calibrated instrument and standard reagents are used. This provides a useful check on the accuracy of a method since the measured figures should fall within the acceptable limits of the true values. Ideally the standard solution should be treated in an identical manner to the fluid under investigation.

A standard curve can then be constructed showing the variation of the quantity measured with concentration. Values obtained for the test solution should fall within the range of the standard curve and the value of the test can then be read. Control solutions of body fluids are now commercially available and are used in clinical laboratories as a check on methods.

Blank solutions should be included in any measurements. The same volume of distilled water replaces the substance to be estimated and the blank is then treated in exactly the same way as the test and standard. Any value obtained for the blank is, of course, subtracted from the value for the test and standard in the final calculation, since the blank value is due to the reagents used and not the substance under investigation.

Standard pH solutions:

The pH meter is calibrated before use by means of a standard solution. The meter should be calibrated with a solution whose pH is close to that under test and several convenient standards are given below.

Buffer Solutions:

A buffer solution is one that resists pH change on the addition of acid or alkali.
Buffer consisted of weak acid + its salt (acetic acid + sodium acetate) or weak base +
its salt (ammonium hydroxide + ammonium chloride).
Such solutions are used in many biochemical experiments where the pH needs to be accurately controlled.

Qualitative assay of carbohydrates

Objective:

To characterize carbohydrates present in the unknown solution on the basis of various chemical assays.

Theory:

Carbohydrates are polyhydroxy aldehydes and ketones or substances that hydrolyze to yield polyhydroxy aldehydes and ketones. Aldehydes (– CHO) and ketones (=CO) constitute the major groups in carbohydrates.
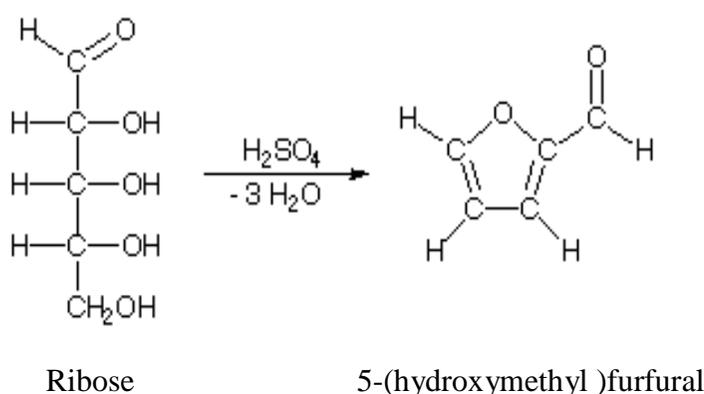
Carbohydrates are mainly divided into monosaccharides, disaccharides and Polysaccharides. The commonly occurring monosaccharides includes glucose, fructose, galactose, ribose etc. The two monosaccharides combined together to form disaccharides which include sucrose, lactose and maltose. Starch and cellulose fall into the category of polysaccharides which consists of many monosaccharide residues.

1. Molisch's Test:

This test is specific for all carbohydrates, Monosaccharide gives a rapid positive test, Disaccharides and polysaccharides react slower.

Principle:

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

Ribose   5-(hydroxymethyl)furfural

Method:
- Add 2 drops of the α-naphthol solution (5% in ethanol, prepare fresh) to 2 ml of test solution in a test tube.
- Carefully, pour about 1 ml of conc. H_2SO_4 down the side of the tube so as to form two layers.
- Carefully observe any colour change at the junction of the two liquids.
- Repeat the test, using water instead of the carbohydrate solution.

2. Fehling's Test:

This forms the reduction test of carbohydrates. Fehling's solution contains blue alkaline cupric hydroxide solution, heated with reducing sugars gets reduced to yellow or red cuprous oxide and is precipitated. Hence, formation of the yellow or brownish-red colored precipitate helps in the detection of reducing sugars in the test solution.

Preparation of Fehling's solution A:
Dissolve 35g of $Cu_2SO_4.7H_2O$ in water and make up to 500ml
Preparation of Fehling's solution B:
Dissolve 120 g of KOH and 173 g of Sod. Pot. Tartarate (Rochelle salt) in water and make up to 500 ml
Fehling's reagent: Equal volumes of Fehling A and Feling B are mixed to form a deep blue solution.
Note: If you do not have sodium potassium tartarate, it can prepared using tartaric acid as described below.
Method:
- Mix equal volumes of Fehling's solution A and B.
- Add 5 drops of the test solution (glucose, fructose, and sucrose solution) to the mixed Fehling's solution and boil.

Results

Glucose solution Orange-brown color is appeared. Fructose solution Orange-brown color is appeared. Sucrose solution No change.

Discussion:

Fehling's tests for aldehydes are used extensively in carbohydrate chemistry. A positive result is indicated by the formation of a brick red precipitate. Like other aldehydes, aldoses are easily oxidized to yield carboxylic acids. Cupric ion complexed with

tartrate ion is reduced to cuprous oxide.

The cupric ion (Cu^{++}) is complexed with the tartarate ion. Contact with an aldehyde group reduces it to a cuprous ion, which the precipitated as orange-brown Cu_2O.
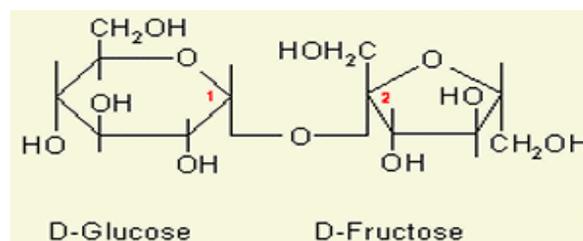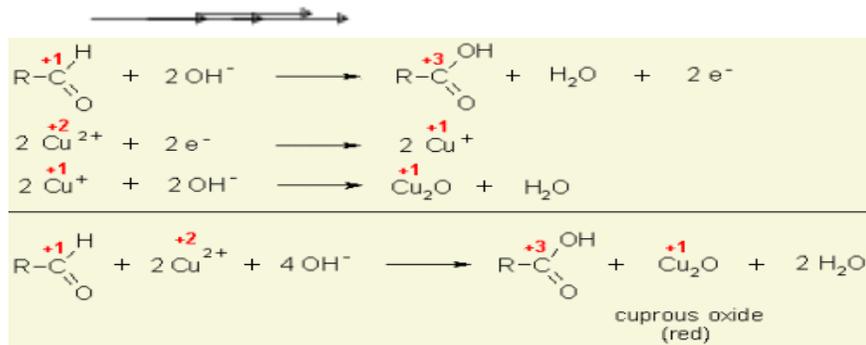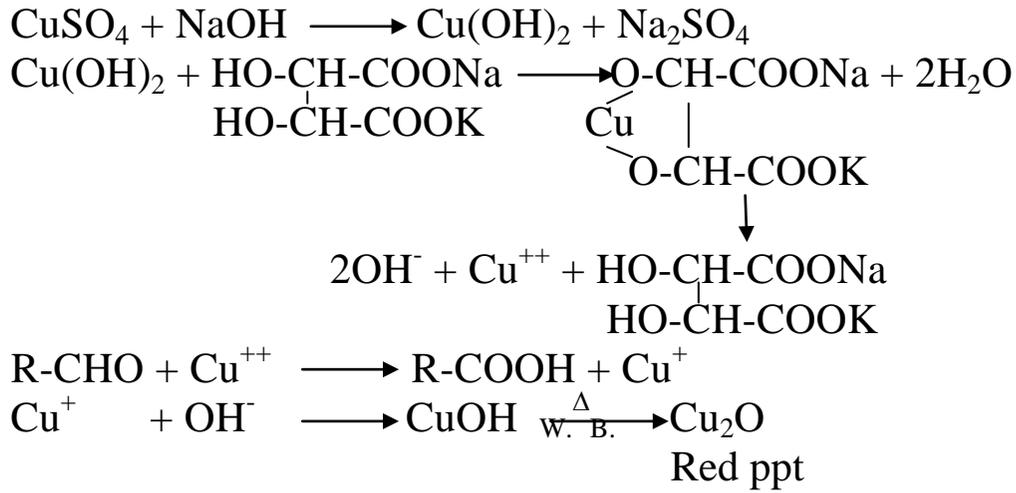
The sucrose does not react with Fehling's reagent. Sucrose is a disaccharide of glucose and fructose. Most disaccharides are reducing sugars, sucrose is a notable exception, for it is a non-reducing sugar. The anomeric carbon of glucose is involved in the glucose- fructose bond and hence is not free to form the aldehyde in solution.

On the other hand, glucose, a reducing sugar, reacts with Fehling's reagent to form an orange to red precipitate. Fehling's reagent is commonly used for reducing sugars but is known to be not specific for aldehydes. For example, fructose gives a positive test with Fehling's solution too, because fructose is converted to glucose and mannose under alkaline conditions. The conversion can be explained by the keto-enol tautomerism.

The reduction of Fehling solution using fructose is not only to be attributed to the fact that the ketose is isomerized into an aldose. The treatment of fructose with alkali - e.g. Fehling solution - causes even decompostion of the carbon chain. More products with reducing capability are formed.

Note:

Fehling's test takes advantage of the ready reactivity of aldehydes by using the weak oxidizing agent cupric ion (Cu^{2+}) in alkaline solution. In addition to the copper ion, Fehling's solution contains tartrate ion as a complexing agent to keep the copper ion in solution. Without the tartrate ions, cupric hydroxide would precipitate from the basic solution. The tartrate ion is unable to complex cuprous ion Cu^+, so the reduction of Cu^{2+} to Cu^+ by reducing sugars results in the formation of an orange to red precipitate of Cu_2O. Copper-tartrate-complex

$$CuSO_4 + NaOH \longrightarrow Cu(OH)_2 + Na_2SO_4$$

$$Cu(OH)_2 + \begin{matrix} HO\text{-}CH\text{-}COONa \\ | \\ HO\text{-}CH\text{-}COOK \end{matrix} \longrightarrow \begin{matrix} O\text{-}CH\text{-}COONa \\ | \\ Cu \\ | \\ O\text{-}CH\text{-}COOK \end{matrix} + 2H_2O$$

$$2OH^- + Cu^{++} + \begin{matrix} HO\text{-}CH\text{-}COONa \\ | \\ HO\text{-}CH\text{-}COOK \end{matrix}$$

$$R\text{-}CHO + Cu^{++} \longrightarrow R\text{-}COOH + Cu^+$$

$$Cu^+ + OH^- \longrightarrow CuOH \xrightarrow[W.\ B.]{\Delta} Cu_2O$$

Red ppt

D-Glucose   D-Fructose

3. Benedict's test:

Benedict modified the Fehling's test to produce a single solution which is more convenient for tests as well as being more stable than Fehling's reagent.
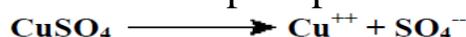
<u>Preparation of Benedict's reagent:</u>

Dissolve 173 g of sodium citrate and 100 g sodium carbonate in about 800 ml of warm water. Filter through a fluted filter paper into a 100 ml measuring cylinder and make up to 850 ml with water. Meanwhile dissolve 17.3 g of copper sulfate in about 100 ml of water and make up to 150 ml. Pour the first solution into a 2-liter beaker and slowly add the copper sulfate solution with stirring.
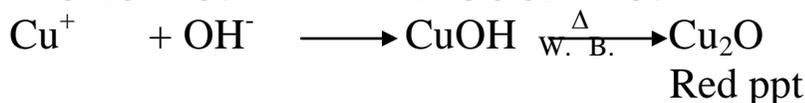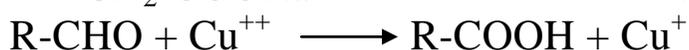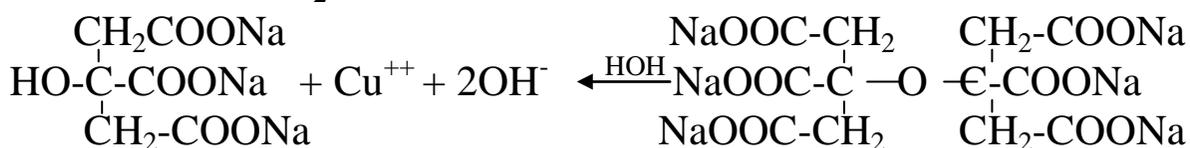
<u>Principle:</u>

The copper sulfate ($CuSO_4$) present in Benedict's solution reacts with electrons from the aldehyde or ketone group of the reducing sugar. Reducing sugars are oxidized by the copper ion in solution to form a carboxylic acid and a reddish precipitate of copper (I) oxide.

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

$$2\ Cu^{++} + \underset{\text{(electron donor)}}{\text{Reducing Sugar}} \longrightarrow Cu+$$

$$Cu^+ \longrightarrow Cu_2O \text{ (precipitate)}$$

<u>Method:</u>

1. Add 5 drops of the test solution to 2 ml of Benedict's reagent and place in a boiling water bath for 5 min. Orange-brown color is appeared.
2. Compare the sensitivity of Benedict's and Fehling's test, using increasing dilutions of 1% glucose.
3. Both fehling's and benedict's test are used as a test for the presence of reducing sugars such as glucose, fructose, galactose, lactose and maltose, or more generally for the presence of aldehydes (except aromatic ones). It is often used in place of Fehling's solution.

$$CuSO_4 + Na_2CO_3 \longrightarrow CuCO_3 + Na_2SO_4$$

$$CuCO_3 + \underset{\underset{CH_2\text{-COONa}}{|}}{\overset{\overset{CH_2\text{-COONa}}{|}}{HO\text{-}C\text{-COONa}}} \qquad H_2CO_3 +$$

$$\underset{\underset{CH_2\text{-COONa}}{|}}{\overset{\overset{CH_2COONa}{|}}{HO\text{-}C\text{-COONa}}} + Cu^{++} + 2OH^- \xleftarrow{HOH} \underset{\underset{NaOOC\text{-}CH_2}{}}{\overset{\overset{NaOOC\text{-}CH_2}{}}{NaOOC\text{-}C}} \!-\!O\!-\! \underset{\underset{CH_2\text{-COONa}}{}}{\overset{\overset{CH_2\text{-COONa}}{}}{C\text{-COONa}}}$$

$$R\text{-CHO} + Cu^{++} \longrightarrow R\text{-COOH} + Cu^+$$

$$Cu^+ + OH^- \longrightarrow CuOH \xrightarrow[\text{W. B.}]{\Delta} \underset{\text{Red ppt}}{Cu_2O}$$

4. Barfoed's Test:

Barfoed's test is used to detect the presence of monosaccharide (reducing) sugars in solution. Barfoed's reagent, a mixture of ethanoic (acetic) acid and copper (II) acetate, is combined with the test solution and boiled. A red copper (II) oxide precipitate is formed will indicates the presence of reducing sugar. The reaction will be negative in the presence of disaccharide sugars because they are weaker reducing agents. This test is specific for monosaccharides. Due to the weakly acidic nature of Barfoed's reagent, it is reduced only by monosaccharides.

Preparation of Barfoed's reagent:

Dissolve 13.3 g of copper acetate in about 200 ml of water and add 1.8 ml of glacial acetic acid.

Method:

• Add 1 ml of the test solution to 2 ml of Barfoed's reagent.

• Boil for 1 min and allow to stand.

5. Iodine Test:

This test is used for the detection of starch in the solution. The blue black colour is due to the formation of starch-iodine complex. Starch contain polymer of α-amylose and amylopectin which forms a complex with iodine to give the blue black colour.

Iodine forms colored adsorption complexes with polysaccharides, starch gives a blue color with iodine, while glycogen and partially hydrolyzed starch react to form red- brown colors.

Method:

Acidify the test solution (1% starch, glycogen or cellulose) with dilute HCl, then add two drops of iodine (0.005 N in 3% KI) and compare the colors obtained with that of water and iodine.
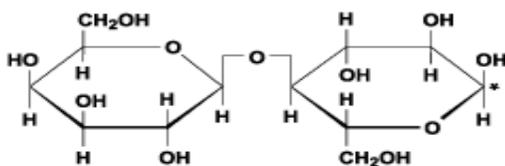
6. Seliwanoff's test:

It is a color reaction specific for ketoses. When conce: HCl is added. ketoses undergo dehydration to yield furfural derivatives more rapidly than aldoses. These derivatives form complexes with resorcinol to yield deep red color. The test reagent causes the dehydration of ketohexoses to form 5-hydroxymethylfurfural. 5-hydroxymethylfurfural reacts with resorcinol present in the test reagent to produce a red product within two minutes. Aldohexoses reacts so more slowly to form the same product.

Results

Test	Monosaccharide		Disaccharide		Polysaccharide	
	Glucose	Fructose	Sucrose	Lactose	Starch	Dextrin
1. Molisch						
2. Fehling						
3. Benedict						
4. Barfoed						
5. Iodine						
6.Seliwanoff						

Lactose

Milk sugar or 4-O-β-D-galactopyranosyl-D-glucose.

This reducing disaccharide is obtained as the α-D anomer (see formula, where the asterisk indicates a reducing group); the melting point is 202°C (396°F). Lactose is found in the milk of mammals to the extent of approximately 2–8%. It is usually prepared from whey, which is obtained by a by-product in the manufacture of cheese.

Hydrolysis Test:

This test is used to convert sucrose (non-reducing disaccharide) to glucose and fructose (reducing mono saccharides).
Principle:
Sucrose is the only non-reducing disaccharide so it does not reduce the Cu^{++} solution (Bendict's and Fehling's test) because the glycosidic bond is formed between the two hemiacetal bonds. So there is no free aldehydic or ketonic group to give potitive reducing properties. This bond can be hydrolysed and the individual components of sucrose (glucose + fructose) are then able to give positive reducing test.
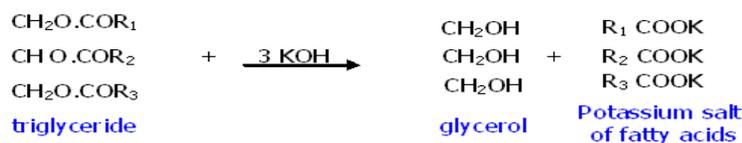

Sucrose
Glc(α1↔2β)Fru

Method:
- 6ml of a sucrose solution is placed in two test tubes.
- Add two drops of concentrated hydrochloric acid (HCl) to only one tube.
- Heat the tubes in boiling water bath for 15 minutes.

LIPIDS

Determination of triglycerides

Esters of glycerol and fatty acids are known as glycerides. The trihydric alcohol glycerol can be esterified to give mono-, di-, and triglycerides. The fatty acids may be the same or different. On saponification, free glycerol and fatty acids are obtained:

$$
\begin{array}{llll}
CH_2O.COR_1 & & CH_2OH & R_1\ COOK \\
CH\ O.COR_2 & +\ \underline{3\ KOH} \longrightarrow & CH_2OH\ + & R_2\ COOK \\
CH_2O.COR_3 & & CH_2OH & R_3\ COOK \\
\text{triglyceride} & & \text{glycerol} & \text{Potassium salt of fatty acids}
\end{array}
$$

Naturally occuring glycerides are called fats or oils depending on whether they are solid or liquid at room temp.

Animal fat is made up largely of triglycerides containing fully saturated fatty acids with straight chains and an even number of carbon atoms.

Methods for the quantitation of plasma triglycerides include chemical and enzymatic methods. The chemical methods require solvent extraction of the plasma to solublize triglycerides and to denature and remove protein. The extract is treated with an adsorbent material to remove phospholipids and interfering substances; isopropanol extracts are treated with a zeolite mixture or with alumina, and chloroform extracts are treated with silicic acid. Once isolated and purified, triglycerides are quantitated by either chemical or enzymatic reactions directed against their glycerol component.

In the chemical methods: glycerol is released from triglycerides in the purified extracts by saponification with alcoholic potassium hydroxide. The glycerol is then oxidized to formaldehyde by sodium periodate. The formaldehyde is reacted with a chromotropic-sulfuric acid mixture to form a product that absorbs at 570 nm.

Qualitative tests of lipids:

1- Solubility test:

Principle:

Fats are not dissolved in water due to their nature, non-polar (hydrophobic), but it is soluble in organic solvents such as chloroform, benzene, and boiling alcohol. Different lipids have ability to dissolve in different organic solvent. This property enable us to separate a mixture of fat from each other for example, undissolve phosphatide lipid in acetone; undissolve of cerebroside,

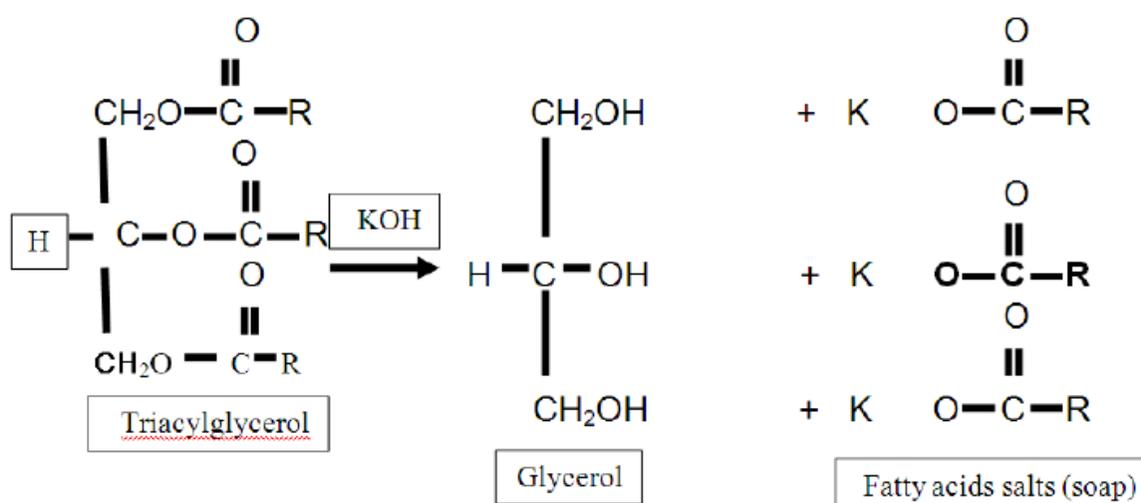as well as sphingomyline in the ether.

Method:

- Place 0.5ml of oil in 6 test tubes clean, dry containing 4ml of different solvents (acetone, chloroform and ether and ethanol, cold ethanol and hot water),
- Shake the tubes thoroughly, then leave the solution for about one minute,
- Note if it separated into two layers , the oil are not dissolve; but if one layer homogeneous transparent formed , oil be dissolved in the solvent.

2- Saponification test:

Triacyl glycerol can be hydrolyzed into their component fatty acids and alcohols. This reaction can also be carried out in the laboratory by a process called **saponification** where the hydrolysis is carried out in the presence of a strong base (such as NaOH or KOH).

Principle:

Saponification is a process of hydrolysis of oils or fat with alkaline and result in glycerol and salts of fatty acids (soap) and can be used the process of saponification in the separation of saponifiable materials from unsaponified (which are soluble in lipid). The process of saponification as follows:

Soap can be defined as mineral salts of fatty acids. The soap is soluble in water but insoluble in ether. Soap works on emulsification of oils and fats in the water as it works to reduce the attraction surface of the solution.

15

Method:
- Place 2 ml of oil in a large
  test tube (or flask).
- Add 4 ml of alcoholic potassium hydroxide (preferably add
  little small pieces of porcelain to regulate the boiling point).
- Boil the solution for 3 minutes. After this period, make sure
  it is perfectly saponification process, by taking a drop of the
  solution and mix with the water if oil separated indicates
  that the non-completion of the saponification. In this case,
  continued to boil until all the alcohol evaporates.
- Take the remaining solid material (soap) and add about 30
  ml of water and keep it for the following tests.
- Shake the solution after it cools and
  noted to be thick foam.

3- Copper acetate test:

This test is used to distinguish between oil or neutral fat
and fatty acid saturated and unsaturated.

Principle:

The copper acetate solution does not react with the oils (or fats),
while saturated and unsaturated fatty acids react with copper acetate
to form copper salt.

Copper salt formed in the case of unsaturated fatty acids can only be
extracted by petroleum ether.

Method:
- Take three test tubes put 1 / 2 g of each sample and then
  added 3 ml of petroleum ether and an equal volume of a
  solution of copper acetate.
- Shake the tube and leave it for some time.
- In the case of olive oil notice that petroleum ether upper layer
  containing the dissolved oil and appears colorless, aqueous
  solution remains blue in the bottom.
- In the case of oleic acid the upper layer of petroleum ether
  becomes green as a result of copper oleate. The lower layer
  becomes less in blue.
- In the case of stearic acid notice that the petroleum ether
  upper layer remains colorless, while consists of pale green
  precipitate of copper stearate at the bottom.

Proteins

The aim of this practical session is to:

1. Obtain a simplified knowledge about protein structure.
2. Practically apply this knowledge by performing some protein color and precipitation reactions.

Practical

Using the provided solutions of albumin (egg white) and gelatin (animal collagenous material), **perform the following**:
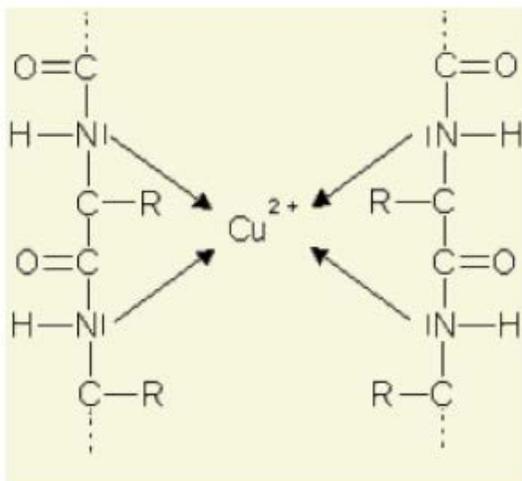A. General tests
B. Color reactions
C. Precipitation reactions

A. General tests for proteins

1- Biuret test:

Principle:

The biuret reagent (copper sulfate in a strong base) reacts with peptide bonds in proteins to form a blue to violet complex known as the "biuret complex". N.B. Two peptide bonds at least are required for the formation of this complex.

Biuret complex

Biuret assay
Method:

(a) Add 2 ml of protein solution to 1 ml of NaOH.
(b) Add one drop of Fehling's A.
(c) Shake this solution carefully and then you see purple color.

B. Color reactions of proteins

Color Reactions of Proteins

- A color test for a protein tests for a particular functional group or structural component present in a protein or in a particular amino acid found in proteins.

(a) Biuret reaction.

(b) Xanthoproteic reaction

The color is produced as the result of the formation of nitro-derivatives of the compounds which contain a benzene ring, for example, tyrosine.

(c) Million's reaction.

The reaction serves as a test for the presence of tyrosine.

(d) Sulphur reaction

The precipitate, which is lead sulphide, is formed as the result of the decomposition of the cysteine by the alkali.

(e) Hopskins Cole Reaction

The color produced is due to the formation of a compound from the glyoxylic acid in the reagent and the tryptophan in the protein

1- Reduced sulfur test:

Principle:

Proteins containing sulfur (in cysteine and cystine) give a black deposit of lead sulfide (PbS) when heated with lead acetate in alkaline medium.

Procedure & observation:

1- To 1 ml of protein solution in a test tube, add 2 drops of 10% sodium hydroxide solution and 2 drops of lead acetate.
2- Mix well and put in a boiling water bath for few minutes; a black deposit is formed with albumin and gelatin gives negative result.

2- Xanthoproteic acid test:

This test is used to differentiate between aromatic amino acids which give positive results and other amino acids.

Amino acids containing an aromatic nucleus form yellow nitro derivatives on heating with concentrated HNO_3. The salts of these derivatives are orange in color.
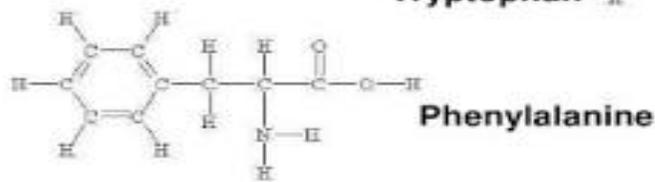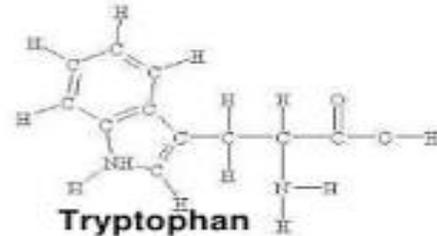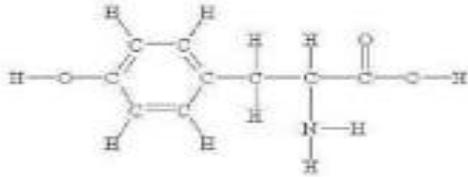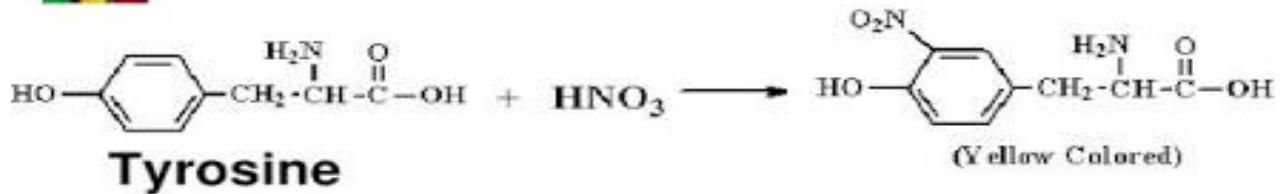
Principle:

Concentrated nitric acid reacts with the aromatic rings that are derivatives of benzene giving the characteristic nitration reaction. Amino acids tyrosine and tryptophan contain activated benzene rings which are easily nitrated to yellow colored compounds. The aromatic ring of phenyl alanine dose not react with nitric acid despite it contains a benzene ring, but it is not activated, therefore it will not react.

Caution:

Concentrated HNO_3 is a toxic, corrosive substance that can cause severe burns and discolor your skin. Prevent eye, skin and cloth contact. Avoid inhaling vapors and ingesting the compound. Gloves and safety glasses are a must; the test is to be performed in a fume hood.

Procedure & observation:

1- To 2 ml of protein solution in a test tube, add 2 drops of concentrated nitric acid.
2- A white precipitate is formed and upon heating in a boiling water bath, it turns yellow with "tyrosine" and orange with the essential amino acid "tryptophan" indicating a high nutritive value.

$$HO-\text{C}_6\text{H}_4-CH_2\cdot CH(NH_2)-C(=O)-OH + HNO_3 \longrightarrow O_2N\text{-}HO\text{-}C_6H_3\text{-}CH_2\text{-}CH(NH_2)\text{-}C(=O)\text{-}OH$$

Tyrosine (Yellow Colored)

Tryptophan

Phenylalanine

3- Millon's test:

This test is specific for tyrosine, the only amino acid containing a phenol group, a hydroxyl group attached to benzene ring.

Principle:

In Milon's test, the phenol group of tyrosine is first nitrated by nitric acid in the test solution. Then the nitrated tyrosine complexes mercury ions in the solution to form a brick-red solution or precipitate of nitrated tyrosine, in all cases, appearance of red color is positive test.

Note: all phenols (compound having benzene ring and OH attached to it) give positive results
in Millon's test.

Procedure & observation:
1- To 2 ml of protein solution in a test tube, add 3 drops of Millon's reagent.
2- Mix well and heat directly on a small flame.
3- A white ppt is formed with albumin and casein (but not gelatin); the ppt gradually turns into brick red.
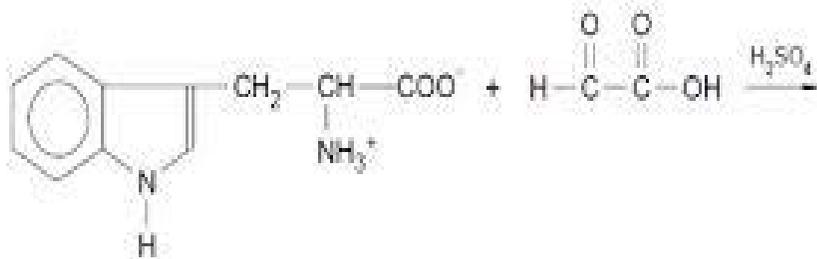
4- Hopkins-Colé test:

Principle:

Hopkins-Colé reagent (magnesium salt of oxalic acid) gives positive results with proteins containing the essential amino acid "tryptophan" indicating a high nutritive value.

Procedure & observation:

1- To 1 ml of protein solution in a test tube, add 1 ml of Hopkins-Colé reagent and mix well.
2- Incline the test tube and slowly add 1 ml of concentrated H2SO4 on the inner wall of the test tube to form 2 layers.
3- Put the test tube in a boiling water bath for 2 minutes.
4- A reddish violet ring is formed at the junction between the 2 layers with albumin and casein; gelatin gives negative results.

Principle:

Tryptophan + Hopkins–Cole $\xrightarrow{H_2SO_4}$ purple ring

Name of the Test	Reagent Used	Positive results	Remarks
Biuret Test	NaOH, dilute $CuSO_4$	violet	+ results with polypeptides and proteins
Xanthroproteic Test	Conc. H_2SO_4	AA with benzene ring (yellow)	Proteins with trp, tyr, phe
Million's Test	$Hg(NO_3)$ and $Hg(NO_2)_2$	Tyrosine (red)	+ phenolic compounts
Sulphur Test	Lead acetate, dissolved with NaOH	Gray or black precipitate	+ lead sulphide, formed as the result of the decomposition of the cysteine by the alkali.
Hopkins Cole Test	Glyoxylic acid, sulfuric acid	Tryptophan (violet ring)	+ with any compound with indole ring
Ninhydrin Test	ninhydrin	Free-NH_2 group (blue)	+ results given by NH_3, primary amines, amino acids, peptides, and proteins

Colour reactions of amino acids

S.No.	Test	Significance
1)	Ninhydrin reaction	Given by all Alpha amino acids
2)	Xanthoproteic test	Given by aromatic amino acids
3)	Millon's test	Confirmatory test for Tyrosine
4)	Biuret test	Not given by free amino acids
5)	Sakaguchi test	Given by Arginine
6)	Hopkins Cole reaction	Confirmatory test for Tryptophan
7)	Lead acetate test	Given by cysteine and cystine but not given by Methionine
8)	Nitroprusside reaction	Given by SH group containing amino acids

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C. Precipitation reactions of proteins

1. Precipitation by heavy metals:

Principle:

Heavy metals (e.g. **Hg2+, Pb2+, Cu2+**) are high molecular weight cations.

The positive charge of these cations counteracts the negative charge of the carboxylate group in proteins giving a precipitate.

Procedure & observation:

1- To 1 ml of protein solution in a test tube, add 1 drop of lead acetate; a white ppt is obtained.
2- To 1 ml of protein solution in a test tube, add 1 drop of 10% copper sulfate; a blue ppt is obtained.

2. Precipitation by alkaloidal reagents:

Principle:

Alkaloidal reagents (e.g. tannate & trichloroacetate) are high molecular weight anions. The negative charge of these anions counteracts the positive charge of the amino group in proteins giving a precipitate.

Procedure & observation:

1- To 1 ml of protein solution in a test tube, add tannic acid drop wise until a buff ppt is obtained.
2- To 1 ml of protein solution in a test tube, add 1 ml of trichloroacetic acid (TCA); a white ppt is obtained.

N.B. Precipitation of proteins by heavy metals and alkaloidal reagents indicates the presence of both negative and positive charges and hence the **amphoteric** nature of proteins.

3. Precipitation by denaturation:
a. Denaturation by heat (heat coagulation test):

Principle:

Heat disrupts hydrogen bonds of secondary and tertiary protein structure while the primary structure remains unaffected. The protein increases in size due to denaturation and coagulation occurs.

Procedure & observation:

1- Put 2 ml of protein solution in a test tube, incline it and heat to boiling.
2- A permanent clotting and coagulation is obtained with albumin only.

b. Denaturation by acids (Heller's test):

Principle:

      Nitric acid causes denaturation of proteins with the formation of a white ppt (this differs from the nitration reaction in "xanthoproteic acid test").

Procedure & observation:

1- Put 2 ml of concentrated nitric acid in a test tube.
2- Incline the tube and slowly add 1 ml protein solution drop wise to form a layer above the nitric acid layer.
3- A white ring is formed at the interface between the 2 layers.

4. Fractional precipitation by ammonium sulfate (salting out):

Principle:

      Protein molecules contain both hydrophilic and hydrophobic amino acids.

In aqueous medium, hydrophobic amino acids form protected areas while hydrophilic amino acids form hydrogen bonds with surrounding water molecules (**solvation** layer).

When proteins are present in **salt** solutions (e.g. ammonium sulfate), some of the water molecules in the solvation layer are attracted by salt ions. When salt concentration gradually increases, the number of water molecules in the solvation layer gradually decreases until protein molecules coagulate forming a precipitate; this is known as "**salting out**".
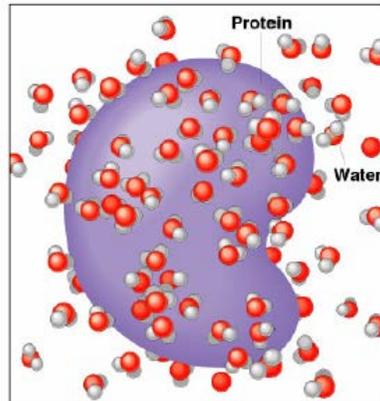
As different proteins have different compositions of amino acids, different proteins precipitate at different concentrations of salt solution.

Procedure & observation:

1- To 2 ml of egg-white solution (containing both albumin & globulin), add an equal volume of saturated ammonium sulfate solution; globulin is precipitated in the resulting half saturated solution of ammonium sulfate.
2- Separate globulin by centrifugation and recover the clear supernatant.
3- Add ammonium sulfate crystals gradually to the clear supernatant until full saturation occurs; another precipitate (albumin) is obtained.
4- Separate albumin by centrifugation.

N.B. The reason for the precipitation of globulin and albumin at different ammonium sulfate concentration could be that the solvation

layer around globulin is looser and thinner than that around albumin. Therefore, globulin needs only half-saturated ammonium sulfate to loose its solvation layer while albumin looses its solvation layer in a fully saturated ammonium sulfate solution.

Laboratory exercise:

Using the provided solutions of **albumin** and **gelatin** perform the tests in the table below and write down your observations.

	Albumin	Casein	Gelatin
Biuret test			
Reduced sulfur test			
Xanthoproteic acid test			
Heavy metal pptn			
Alkaloidal reagent pptn			
Heat coagulation test			
Heller's test			

Enzyme assay

Enzyme assays are <u>laboratory</u> methods for measuring <u>enzymatic</u> activity. They are vital for the study of <u>enzyme kinetics</u> and <u>enzyme inhibition</u>.

Enzyme units:

Amounts of enzymes can either be expressed as <u>molar</u> amounts, as with any other chemical, or measured in terms of activity, in <u>enzyme units</u>.

Enzyme activity = moles of substrate converted per unit time, mol/min or mol/sec. Enzyme activity is a measure of the quantity of active enzyme present and is thus dependent on conditions, *which should be specified*.

The SI unit is the <u>katal</u>, 1 katal = 1 mol s^{-1}, but this is an excessively large unit.

UV/VIS Spectrophotometer.

Types of assay:

All enzyme assays measure either the consumption of substrate or production of product over time.

A large number of different methods of measuring the concentrations of substrates and products exist and many enzymes can be assayed in several different ways. Biochemists usually study enzyme-catalyzed reactions using four types of experiments:

1. Initial rate experiments:

When an enzyme is mixed with a large excess of the substrate, the enzyme-substrate intermediate builds up in a fast initial transient. Then the reaction achieves a steady- state kinetics in which enzyme substrate intermediates remains approximately constant

over time and the reaction rate changes relatively slowly. Rates are measured for a short period after the attainment of the quasi-steady state, typically by monitoring the accumulation of product with time. Because the measurements are carried out for a very short period and because of the large excess of substrate, the approximation free substrate is approximately equal to the initial substrate can be made. The initial rate experiment is the simplest to perform and analyze, being relatively free from complications such as back-reaction and enzyme degradation. It is therefore by far the most commonly used type of experiment in enzyme kinetics.

2. Progress curve experiments:

In these experiments, the kinetic parameters are determined from expressions for the species concentrations as a function of time. The concentration of the substrate or product is recorded in time after the initial fast transient and for a sufficiently long period to allow the reaction to approach equilibrium. We note in passing that, while they are less common now, progress curve experiments were widely used in the early period of enzyme kinetics.

3. Transient kinetics experiments:

In these experiments, reaction behavior is tracked during the initial fast transient as the intermediate reaches the steady-state kinetics period. These experiments are more difficult to perform than either of the above two classes because they require rapid mixing and observation techniques.

4. Relaxation experiments:

In these experiments, an equilibrium mixture of enzyme, substrate and product is perturbed, for instance by a temperature, pressure or pH jump, and the return to equilibrium is monitored. The analysis of these experiments requires consideration of the fully reversible reaction. Moreover, relaxation experiments are relatively insensitive to mechanistic details and are thus not typically used for mechanism

identification, although they can be under appropriate conditions.

Enzyme assays can be split into two groups according to their sampling method:

1. Continuous assays, where the assay gives a continuous reading of activity.

2. Discontinuous assays, where samples are taken, the reaction stopped and then the concentration of substrates/products determined.

Temperature-controlled cuvette holder in a spectrophotometer.

Continuous assays are most convenient, with one assay giving the rate of reaction with no further work necessary. There are many different types of continuous assays. Spectrophotometric assay:

In spectrophotometric assays, you follow the course of the reaction by measuring a change in how much light the assay solution absorbs. If this light is in the visible region you can actually see a change in the color of the assay, these are called colorimetric assays.

UV light is often used, since the common coenzymes NADH and NADPH absorb UV light in their reduced forms, but do not in their oxidized forms. An oxido-reductase using NADH as a substrate could therefore be assayed by following the decrease in UV absorbance at a wavelength of 340 nm as it consumes the coenzyme.

Direct versus coupled assays

Coupled assay for hexokinase using glucose-6-phosphate dehydrogenase.

Even when the enzyme reaction does not result in a change in the absorbance of light, it can still be possible to use a spectrophotometric assay for the enzyme by using a coupled assay. Here, the product of one reaction is used as the substrate of another, easily-detectable reaction.

Fluorometric assay:

Fluorescence is when a molecule emits light of one wavelength after absorbing light of a different wavelength. Fluorometric assays use a difference in the fluorescence of substrate from product to measure the enzyme reaction. These assays are in general much more

sensitive than spectrophotometric assays, but can suffer from interference caused by impurities and the instability of many fluorescent compounds when exposed to light.

An example of these assays is again the use of the nucleotide coenzymes NADH and NADPH. Here, the reduced forms are fluorescent and the oxidised forms non- fluorescent. Oxidation reactions can therefore be followed by a decrease in fluorescence and reduction reactions by an increase. Synthetic substrates that release a fluorescent dye in an enzyme-catalyzed reaction are also available, such as 4- methylumbelliferyl-β-D-galactoside for assaying β-galactosidase.

Calorimetric assay:

Chemiluminescence of Luminol

Calorimetry is the measurement of the heat released or absorbed by chemical reactions. These assays are very general, since many reactions involve some change

in heat and with use of a microcalorimeter, not much enzyme or substrate is required. These assays can be used to measure reactions that are impossible to assay in any other way.

Chemiluminescent assay:

Chemiluminescence is the emission of light by a chemical reaction. Some enzyme reactions produce light and this can be measured to detect product formation. These types of assay can be extremely sensitive, since the light produced can be captured by photographic film over days or weeks, but can be hard to quantify, because not all the light released by a reaction will be detected.

The detection of horseradish peroxidase by enzymatic chemiluminescence (ECL) is a common method of detecting antibodies in western blotting. Another example is the enzyme

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luciferase, this is found in fireflies and naturally produces light from its substrate luciferin.

Light Scattering assay:

Static Light Scattering measures the product of weight-averaged molar mass and concentration of macromolecules in solution. Given a fixed total concentration of one or more species over the measurement time, the scattering signal is a direct measure of the weight-averaged molar mass of the solution, which will vary as complexes form or dissociate. Hence the measurement quantifies the stoichiometry of the complexes as well as kinetics. Light scattering assays of protein kinetics is a very general technique that does not require an enzyme.

Discontinuous assay:

Discontinuous assays are when samples are taken from an enzyme reaction at intervals and the amount of product production or substrate consumption is measured in these samples.

Radiometric assay:

Radiometric assays measure the incorporation of radioactivity into substrates or its release from substrates. The radioactive isotopes most frequently used in these assays are ^{14}C, ^{32}P, ^{35}S and ^{125}I. Since radioactive isotopes can allow the specific labelling of a single atom of a substrate, these assays are both extremely sensitive and specific. They are frequently used in biochemistry and are often the only way of measuring a specific reaction in crude extracts (the complex mixtures of enzymes produced when you lyse cells).

Radioactivity is usually measured in these procedures using a scintillation counter.

Chromatographic assay:

Chromatographic assays measure product formation by separating the reaction mixture into its components by chromatography. This is usually done by high- performance liquid chromatography (HPLC), but can also use the simpler technique of thin layer chromatography. Although this approach can need a lot of material, its sensitivity can be increased by labelling the substrates/products with a radioactive or fluorescent tag. Assay sensitivity has also been increased by switching protocols to improved chromatographic instruments (e.g. ultra-high pressure liquid chromatography) that operate at pump pressure a few-fold higher than

HPLC instruments (see HPLC#Pump_pressure).
Factors to control in assays

- **Salt Concentration**: Most enzymes cannot tolerate extremely high salt concentrations. The ions interfere with the weak ionic bonds of proteins. Typical enzymes are active in salt concentrations of 1-500 mM. As usual there are exceptions such as the halophilic (salt loving) algae and bacteria.

- **Effects of Temperature**: All enzymes work within a range of temperature specific to the organism. Increases in temperature generally lead to increases in reaction rates. There is a limit to the increase because higher temperatures lead to a sharp decrease in reaction rates. This is due to the denaturing (alteration) of protein structure resulting from the breakdown of the weak ionic and hydrogen bonding that stabilize the three dimensional structure of the enzyme. The "optimum" temperature for human enzymes is usually between 35 and
40 °C. The average temperature for humans is 37 °C. Human enzymes start to denature quickly at temperatures above 40 °C. Enzymes from thermophilic archaea found in the hot springs are stable up to 100 °C. However, the idea of an "optimum" rate of an enzyme reaction is misleading, as the rate observed at any temperature is the product of two rates, the reaction rate and the denaturation rate. If you were to use an assay measuring activity for one second, it would give high activity at high temperatures, however if you were to use an assay measuring product formation over an hour, it would give you low activity

   at these temperatures.

- **Effects of pH**: Most enzymes are sensitive to p H and have specific ranges of activity. All have an optimum pH. The pH can stop enzyme activity by denaturing (altering) the three dimensional shape of the enzyme by breaking ionic, and hydrogen bonds. Most enzymes function between a pH of 6 and
8; however pepsin in the stomach works best at a pH of 2 and trypsin at a pH of 8.

- **Substrate Saturation**: Increasing the substrate concentration increases the rate of reaction (enzyme activity). However, enzyme saturation limits reaction rates. An enzyme is saturated when the active sites of all the molecules are occupied most of the time. At the

saturation point, the reaction will not speed up, no matter how much additional substrate is added. The graph of the reaction rate will plateau.

- **Level of crowding**, large amounts of <u>macromolecules</u> in a solution will alter the <u>rates</u> and <u>equilibrium constants</u> of enzyme reactions, through an effect called <u>macromolecular crowding.</u>

Protocol for Enzyme Assay

- Isolation of enzymes
- Different enzymes can be isolated from different sources. It depends on the type of enzyme and the rich source of the required enzyme.
- Adenosine triphosphatase (ATPase) can be isolated from cells and nervous tissues.
- Acetyl cholinesterase (AChE) can be isolated from red blood cells.
- Butyrile choline esterase (BuChE) can be isolated from plasma.
- Hepatic soluble enzymes (GPT, GOT, AlP) can be isolated from blood.

Tissues should be chopped, homogenized in buffer solution and then centrifuged at certain round per minute (rpm).

Pellets or supernatant are taken as a source of enzyme. Pellets may be suspended in buffer while supernatant is diluted with buffer.

In case of blood, sample should be centrifuged at 5000 rpm to separate plasma and red blood cells (RBC's)

References:

- Abousalah, K. and Alnaser, A., 1996, Principles of practical biochemistry.
- Farid Shokry Ataya, 2007, Practical Biochemistry. AlRoshd Publisher, Riyadh, Saudi Arabia.
- Milio, F. R. and Loffredo, W. M., 1995, Qualitative Testing for Amino Acids and Proteins, modular laboratory program in chemistry, REAC 448.
- Collection from internet researches.
- Dr. AHMED KHAMIS MOHAMED SALAMA (2013): Practical BIOCHEMISTRY. Medical Laboratories Dept., Colleges compound at Zulf