
بسم الله الرحمن الرحيم

2013

Kingdom of Saudi Arabia
Ministry of Higher Education
King Saud University
College of Science
Biochemistry Department

قسم الكيمياء الحيوية
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Practical Note
General Biochemistry
(BCH 302)

Prepared By

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1. General Laboratory Guidelines

1.1 Safety

Safe practices in the biochemistry laboratory are of great significance. Students must adopt safe and skillful methods while working in the lab. To achieve this goal the student must obtain the prerequisite knowledge of properties of materials present in the lab and must be acquainted with different hazards and harmful effects associated with their improper handling.

1.1.1 Regulations

1. Throughout your stay in the laboratory, you must wear safety goggles.
2. Immediately inform your instructor in case of any accident.
3. Do not eat, drink, chew, or smoke in the laboratory.
4. Do not depart from the lab leaving an experiment unattended. If you need to leave the lab, you must inform your instructor before leaving the lab.
5. After finishing the experiment turn off all the equipments, clean your workbench and reshelf all the equipments or chemicals.
6. Not sticking to these rules will result in instant removal from the lab.

1.1.2 Precautions

1. You must come to the lab with a serious awareness of personal liability and utmost consideration for others in the lab.
2. You must acquaint yourself with safety equipment location, acid-base neutralizing agents, eyewash, fire extinguisher, emergency shower, broom & dustpan and broken glass container.
3. You must listen carefully to all the instructions given by your instructor. If you are unsure of anything, always ask your instructor.
4. You must immediately clean all chemical spills.
5. While handling the chemicals you must wear gloves, otherwise some chemicals may result in skin irritation.
6. While handling all electrical and heating equipments extra precautions must be taken to prevent shocks and burns.
7. Do not handle broken glassware with your bare hands.
8. You must wash your hands with soap after finishing the experiment**.**

1.1.3 Personal clothing

Selection of clothing for the laboratory is generally left to the discretion of the student. However, due to the harmful nature of some chemicals, it is in the best interest of the student to wear proper and suitable clothing. You must wear a lab coat to help keep clothes protected. Open toed shoes must not be worn because they cannot protect you against chemical spills. Long hair should be tied back to avoid interference with motion or observation.

1.1.4 Equipments

Equipment must be placed in a safe and secure manner. Hot plate must be placed in safe location and kept away from the edge of the bench to reduce chances of body contact.

1.1.5 Glassware Handling

Glassware in the lab is generally delicate and fragile, and if not handled properly, may cause serious injuries. Do not use any chipped or broken glassware. After finishing the experiment, all glassware must be cleaned and kept back at the proper place.

1.1.6 Acids and Bases

In metabolism lab experiments, you will be using different acids and bases. Hence, care must be taken to avoid skin contact. While handling these chemicals, avoid eye and face contact. In case of acid or base contact with your skin, wash it with large amount of clean, cold water and inform your instructor immediately. For your own protection, neutralize acid or base spills before cleaning them up.

1.1.7 Laboratory Notebooks

For all laboratory experiments, use a bound notebook to keep record of all primary data and observations. You must organize your notebook every week before coming to the lab by writing the title of the experiment on a new page, with important equations or formulae from the lab manual, and all necessary calculations involving solution preparations, molar masses, etc.

Try to understand theoretical concepts and particular instructions given by your instructor before the experiment. The lab notebook must have a record of every experiment. The lab notes should be written in a manner that other people could understand them. Excellent note taking in the lab is an important skill that can be learned with little effort and practice.

1.1.8 Guidelines to be followed

1. Carry your notebook to the lab for each experiment.
2. Number all the pages in sequential order.
3. Use your notebook to record values directly and do not use loose scraps of paper.
4. Mention each measured quantity by its name and indicate the units.
5. Simply strike through the sentence and write the new sentence next to it, if you make a mistake in your notebook.
6. Tables are very useful to simplify the data entry; they should be prepared in advance before starting the experiment.
7. Do not depend on your memory and write down all observations, for example color and phase changes, etc.
8. Last but not the least, you must write a brief conclusion of your experiment. It should address the objectives of conducting the experiment.

1.2 Guidelines for preparing Laboratory reports

The laboratory reports are major written assignments and should be written in the form of a scientific paper. The laboratory reports should contain the following sections:

- Title Page
- Brief Introduction
- Materials and Methods
- Results/Discussion
- References

All laboratory reports are expected to be well written, typed in English. Follow the following guidelines for each section to write a lab report.

1.2.1 Abstract section:

The abstract is a short and yet thorough summary of the report so that one can get an idea about the experiment without reading your whole report. It should include the purpose of your experiment, the procedures you used to carry out the experiment, results you obtained from the experiments, and your conclusions. The abstract should be no longer than a small paragraph (10-12 lines).

1.2.2 Introduction section:

This part should consist of any theoretical background information pertinent to understand your report. This section should be around 30-40 lines.

1.2.3 Materials and Methods section:

In this section you will write the material and methods that you used, you must also mention exact volumes, amounts, incubation times, and any modifications from the procedure mentioned in the manual.

1.2.4 Results section:

In this section of your lab report, you should report all your results that you get from your experiment such as calculations, exact volumes, amounts, incubation times, etc.). You should present them in a tabulated form so it will be easy for quick reference. You must number and label all the tables and figures (graphs, diagrams). This way it will be easy for you to refer to them in your discussion section. You should also include your sample calculations (if any) in the result section.

1.2.5 Discussion section:

In this section you are required to give a thorough description of what happened in the experiment. The discussion section is also where you interpret your results and make conclusions. You should refer to your tables and diagrams while explaining your results. You should compare your results to expected values (calculated or from the literature). Even if you obtained unexpected results, the discussion section is the section to justify or explain the reasons why you have obtained such results. Please remember how you interpret your results carries more weight than the results themselves.

1.2.6 Conclusion section:

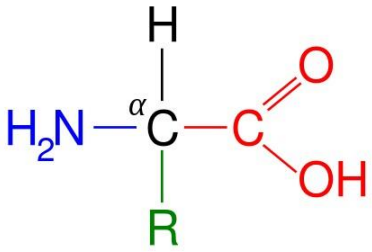
The conclusion section is just a quick overview of what was done and how. However, more importance is given to the results.

1.2.7 References section:

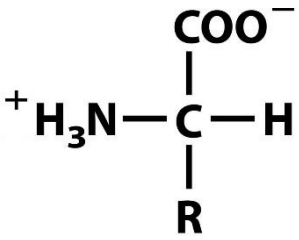
In this section, you will provide an alphabetical listing (by first author's last name) of the references that you actually cited in the body of your report.

2. Amino Acids

Amino acids plays central roles both as building blocks of proteins and as intermediates in metabolism. There are 20 natural amino acids found within proteins convey a vast array of chemicals versatility. All of them are L-α amino acids.



All amino acids found in proteins have this basic structure, differing only in the structure of the R-group or the side chain.
The simplest, and smallest, amino acid found in proteins is glycine for which the R-group is hydrogen (H).
All these amino acids found in solutions in their ionized form (Zwitter ion), i.e. they are polarized and their ionization depends on the pH of the medium where they are located.



According to their ionization (polarity) in water, they are classified into 4 categories:

1. Non-polar.
2. Uncharged polar.
3. Basic polar (positively charged).
4. Acidic polar (negatively charged).

- Polar amino acids are more soluble in water than non-polar, due to presence of amino and carboxyl group which enables amino acids to accept and donate protons to aqueous solution, and therefore, to act as acids and bases. A molecule that functions as such is known as an amphoteric.
- The pH value at which concentration of anionic and cationic groups are equal (i.e the net charge of this molecule equals zero) is known as isoelectric point (pI), a point at which the molecule

does not move to either cathode or anode if it is put in electric field and its solubility is minimum.

- Amino acids are able to rotate polarized light either to the left (livo) L. or to the right (dextro) D, since they have an asymmetric C atom (a carbon atom linked to 4 different groups), glycine which lacks asymmetric C atom (has 2 H^+ on α-C) is an exception.

2.1. Qualitative tests of amino acids:

2.1.1 Solubility test:

The physical proprieties of amino acid are mainly result of their structure, both the solid state and in various solutions .In this part of experiment you will investigate the solubility of selected amino acid in various solutions

Objective: to test the solubility of amino acids in different solvent.

Principle:

Polar amino acids are more soluble in water than non-polar, due to presence of amino and carboxyl group which enables amino acids to accept and donate protons to aqueous solution

Materials:

- Different amino acid solutions: glycine, lysine, glutamine.
- Solvents: H_2O - HCl - NaOH - Chloroform
- Test tubes
- Water bath

Method:

Test solubility of each amino acid sample (0.5ml) by mix with different solvents (2ml).

Then leave the solution for about one minute,

Record your result

- **Results:**

Amino acid	Solvent	Degree of solubility

2.1.2 Ninhydrin test

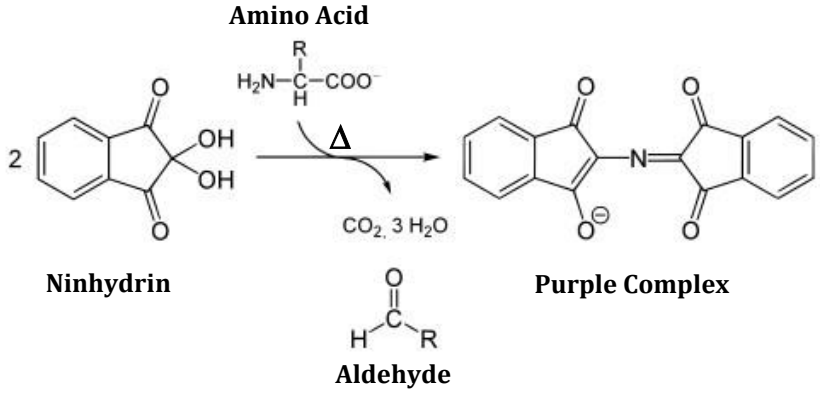
Objective: a general test to detect α-L-amino acids.

Caution:

Ninhydrin is a strong oxidizing agent, it should be handled with care, and applied apart from contact with skin or eyes, gloves and mask is a must, using hood is required, if accidently get in touch with the skin, the resulting stains is a temporarily one, that will be eliminated within 24 hours.

Principle:

- Ninhydrin (triketohydrindene hydrate) degrades amino acids into aldehydes (on pH range 4-8), ammonia and CO_2 through a series of reactions. The net result is ninhydrin in a partially reduced from hydrindantin.
- Ninhydrinthen condenses with ammonia and hydrindantin to produce an intensely blue or purple pigment, sometimes called ruhemann's purple:
- The color varies slightly from acid to acid. Proline and hydroxyproline (amino acids) give yellow color.
- Many substances other than amino acids, such as amines will yield a blue color with ninhydrin, particularly if reaction is carried out on filter paper.



Materials:

- Amino acids (0.1% solution of Glycine, Tyrosine, Tryptophan and Proline).
- Distilled water.
- Ninhydrin (0.2% prepared fresh)
- Test tube.

Method:

- You are provided with 5 solutions (1-5). Place 1 ml of each of the solutions in a test tube and add 5 drops of ninhydrin solution.
- Boil the mixture over a water bath for 2 min.
- Allow to cool and observe the color formed
- Complete the below table.

Results:

Tube	Result	Conclusion
1		
2		
3		
4		
5		

Table1: Summarize the result after adding ninhydrin reagent to the sample (A-F).

Questions:

1. Depending on its sensitivity to amino acids, when could ninhydrin be used or applied?

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2. Why proline gives a yellow color?

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2.1.3. Xanthoproteic test

Objective:

- 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.

Material:

- Amino acids solutions (0.1% w/v solution of glycine, 0.2% of tyrosine, tryptophan and phenyl alanine).
- Concentrated nitric acid 10% (HNO_3).
- Sodium hydroxide solution 10 M (NaOH)

Method:

- Label five tubes (1 - 5), then add 0.5 ml of each amino acid solution.
- Add 0.5 ml of conc. HNO_3 to all tubes.
- Mix well and write your observations in following table.

Results:

Tube	Result	Conclusion
1		
2		
3		
4		
5		

Question:

Discuss the reasons of which aromatic amino acids give positive result but not aliphatic ones in Xanthoproteic test.

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2.1.4. Millon's test

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

Objective:

- To detect the presence of tyrosine in the sample.

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.

Material:

- Amino acids solutions (0.1% (w/v) solution of glycine, tyrosine and phenylalanine).
- Millon's reagent (prepared by dissolving 100g $HgNO_3$ then diluted with distilled water (1:2) freshly prepared.
- Phenol 100% (w/v).
- Boiling water bath.
- Test tubes.

Method:

- Label 4 test tubes (1 - 4).
- Add 1 ml of test solutions in separate tubes and the phenol solution in one tube.
- Add to each tube 0.5 ml Millon's reagent and shake it well.
- Place the test tubes in the boiling bath with care, for 10 min.
- Write your observation in the following table.

Results:

Tube	Result	Conclusion
1		
2		
3		
4		

Table of results after adding Millon's reagent to the tested samples

Questions:

1. Would phenol give positive results, explain your answer?

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2. Discuss the results of tyrosine comparing it with that of tryptophan and phenyl alanine?

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2.1.5. Sakaguchi Test

Objective:
Sakaguchi test is a specific qualitative test for the detection of amino acid containing gauanidium group [R-NH-C= $(NH2)^{2+}$-NH2]. In other words, it is a test for guanidines, i.e arginine.

Principle:
In alkaline solution, arginine react with α-naphthol and sodium hypobromite /chlorite as an oxidize agent, to form red complexes as a positive result.

Material:

- Amino acids samples (glycine, tryptophan, arginine) (1gm in 500ml)
- 10%NaOH
- α-naphthol in 10% ethanol
- 5%sodium hypobromate (or

Method:

- Label 3 test tube and put in each one 1 ml of the amino acid solution.
- Add to each tube 0.5 ml of NaOH solution. Mix well
- Add to each tube 5 drops of α-naphthol solution. Mix well
- Add to each tube 0.5 ml of sodium hypobromite solution **and avoid mixing**.
- Record your result

Results:

Tube	Result	Conclusion
1		
2		
3		

2.1.6. Detection of amino acids containing sulfhydryl group (- SH)- Lead Sulfite Test

This test is specific for –SH containing amino acid (Cysteine).

Principle:

- Some of sulfur in cysteine converted to sodium sulfide by boiling with 40% NaOH.
- The Na_2S can be detected by the precipitation of PbS from an alkaline solution.
- The amino acids containing sulfhydryl group when heated with base, the sulfhydryl group (cysteine) and disulfhydryl (cystine) directly converted to inorganic sulfur. Which is confirmed by the black precipitate of PbS (lead sulfide) when adding lead acetate $Pb(CH_3COO)_2$.

Materials:

- Amino acids solutions (1% (w/v) of cysteine, methionine and glycine).
- 10% (w/v) sodium hydroxide (NaOH).
- $Pb(CH_3COO)_2$ 5 % (w/v). poisonous chemical
- Boiling water bath.
- Test tubes.

Method:

- Label three test tubes (1 - 3).
- Add 2 ml of amino acids solutions in each tube.
- Add to each test tube 1 ml of NaOH solution.
- Add 0.5 ml of $Pb(CH_3COO)_2$ to each tube and mix by vortex.
- Place the test tube carefully in the boiling bath for 3 min.
- Finally write your observation in the following table.

Results:

Tube	Result	Conclusion
1		
2		
3		

Questions:

Which of the amino acids contain (-SH) group?

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What is the difference between cysteine and cystine ?

……

Give an example of a functional protein with a disulfide bridge?

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3. Proteins

3.1. Qualitative chemical reactions of amino acid protein functional groups:

Certain functional groups in proteins can react to produce characteristically colored products. The color intensity of the product formed by a particular group varies among proteins in proportion to the number of reacting functional or free groups present and their accessibility to the reagent. In this part of the experiment, we will use various color producing reagents (dyes) to detect the presence of certain functional groups in proteins qualitatively.

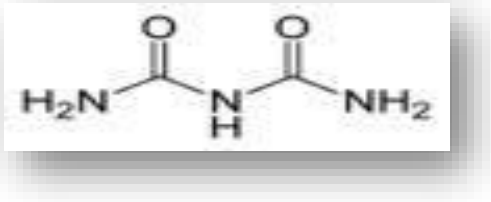
3.1.1. Biuret test:

This test is specific for the peptide bond. Substances containing not less than two peptide linkages give this test. In this reaction, proteins form a pink-purple colored complex with $CuSO_4$ in a strongly alkaline solution.

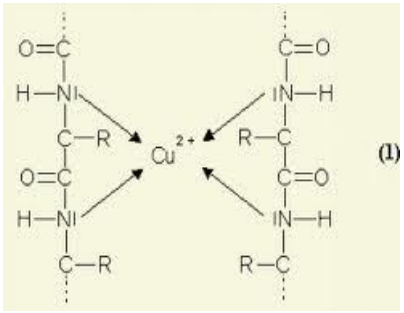
Objective: to detect the presence of peptides or proteins in a sample.

Principle:

- This test used to detect the presence of proteins and peptides (i.e. peptide bonds) by treating them with an alkaline solution of dilute copper sulfate. A positive test indicated by the formation of a pink-violet color. The name of the test derived from a specific compound, biuret that gives a positive test with this reagent.



Biuret compound



Purple complex

Materials:

- Proteins solutions [2% gelatin , 2%BSA, or 2% raw egg albumin dissolved in 0.1 NaCl and 1% casein] (casein is to be dissolved in diluted NaOH) .
- NaOH (3M).
- Copper sulfate (1% $CuSO_4 .5H_2O$, Fehling's solution A diluted 1/10 with water).
- Test tubes.

Method:

- in 3 different test tubes put 2ml of each protein solution.
- to each tube add 1ml 3M NaOH.
- Add 0.5 ml of $CuSO_4 .5H_2O$, and mix well.
- Observe the colors produced and write your observation in the table.

Result:

Sample	Observation	Conclusion
1		
2		
3		

Questions:

1. Write the chemical formula of this reaction and the resulting copper complex.

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2. Do you think free amino acids will give a positive result with this reaction? Why?

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3. What is the least number of amino acids bonded together by peptide bonds that will respond positively to this test?

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3.1.2. Effect of salt concentration on the protein solubility:

This experiment is used to separate different proteins using salting-out theory. Each protein can precipitated at specific salt concentration.

Objective: to investigate the effect of different salt concentration on protein solubility.

Principle:

The low salt concentration solutions makes protein solubility easier using the attraction of salt ions to the functional groups of the protein. On contrast, high salt concentration or solids dissolved in the reaction medium until saturation, causes the protein to precipitate since salt ions, in this case, compete with the protein molecules in binding water molecules.

Materials:

- Egg albumin and casein.
- Sodium chloride NaCl 0.1M.
- Ammonium sulfate $(NH_4)_2SO_4$ solid.
- 50% $(NH_4)_2SO_4$ solution (29.1gm in 100 ml)

Method:

- Separate globulin by centrifugation. from the egg white, the supernatant is albumin and the undissolved part is globulin.
- After separation of globulin add 4 ml of NaCl , shake and label the tube as T1
- Take 2 ml of T1, slightly add 50% $(NH_4)_2SO_4$ solution, observe the precipitation of globulin and label the tube as T2
- Check the ability of precipitated globulin to dissolve in NaCl 0.1 M.
- Label a tube as T3, take 2 ml of albumin sample, add 50% $(NH_4)_2SO_4$ solution, and note what happened.
- To the tube T3 Add solid add $(NH_4)_2SO_4$,and note what happened.
- Compare T_2 with T_3 and give reason.
- Compare T_3 with T_4 and give reason.

Results:

Step	Observation	Comment
T_1- Globulin ppt.+ NaCl		
T_2- (Globulin + NaCl) +50%saturated (NH4)2SO4		
T_3-Albumin+ 50% saturated (NH4)2SO4		
T_4-Albumin+ 50% saturated (NH4)2SO4) +100%saturated (NH4)2SO4		

Question:

Can we use this method in fractionating mixture of proteins? Explain you result with example.

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3.1.3. Precipitation of proteins by acids:

This experiment used to precipitate different proteins using strong acid solution. Each protein can precipitated at specific acid concentration.

Objective: to investigate the effect of concentrated acids on protein solubility.

Principle:

- Strong acids cause proteins to precipitate by affecting different bonds of the molecule; there are many applications of this test in laboratories, i.e. in the detection of small amounts of protein in urea sample, also in the separation and purification of proteins or to stop the enzymatic action of an enzyme.
- This test depend on affecting solubility of the protein as a function of changes in pH in highly acidic media, the protein will be positively changed, which is attracted to the acid anions that cause them to precipitate.

Materials:

- Concentrated nitric acid.
- Trichloroacetic acid (TCA).
- BSA (bovine serum albumin) 0.5%, or 2% egg albumin dissolve in 0.1 NaCl solution

Method:

- In a test tube, put 3ml of conc. nitric acid.
- Using a dropper add to the tube the protein solutions you have (albumin, casein, gelatin) drop-wise on the inner wall of the tube to form a layer up the acid.
- Note the white precipitate at the inner face of the protein in contact with the conc. acid.
- To another tube add 3 ml of the protein solution add T.C.A drop-wise till a precipitate forms.
- Observe what happen to the precipitate if a few drops more will added? Dose it dissolve in excess acid?

Brief your notifications in the following table:

Tube	**Observation**			**Comments**
1-H_2NO_3	albumin	casein	gelatin	
2-T.C.A				

Questions:

1. Why albumin dissolved in 0.1 NaCl?

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3.1.4. Protein denaturation:

Denaturation is destruction of the usual nature of a substance, as by the addition of methanol or acetone. Most globular proteins exhibit complicated three-dimensional folding described as secondary, tertiary, and quaternary structures. These conformations of the protein molecule are rather fragile, and any factor that alters the precise geometry said to cause denaturation. Extensive unfolding sometimes causes precipitation of the protein from solution. Denaturation defined as a major change from the original native state without alteration of the molecule's primary structure, i.e., without cleavage of any of the primary chemical bonds that link one amino acid to another.

Objective: to investigate the effect of high temperature, strong acids or bases, high concentrations of inorganic salts or organic solvents (e.g., alcohol or chloroform), or irradiation on protein structure.

Principle:

- Treatment of proteins with strong acids or bases, high concentrations of inorganic salts or organic solvents (e.g., alcohol or chloroform), heat, or irradiation all produce denaturation to a variable degree. Loss of three-dimensional structure usually produces a loss of biological activity.
- This test illustrates the importance of week bonds in globular protein's tertiary structure (the functional structure). Acid and heat disrupt ionic bonds and hydrogen bonds, respectively causing loss of the quaternary structure. This leads to denaturation and loss of biological function.

Materials:

- Albumin (2% raw egg albumin in 0.1 NaCl), 1% gelatin ,1% casein,1% globulin in 0.1 NaCl.
- Diluted acetic acid.

Method:

- Add 10 ml of protein solutions in different test tubes
- Add 3 drops of acetic acid to each tube.
- Place them in a boiling water bath for 10 minutes.
- Remove aside to cool at room temperature.
- Note the change in each tube.

Brief your results in the following table:

Results:

Tube	Observation	Conclusion
Globulin		
Albumin		
gelatin		
casein		

Questions:

Are Albumin and gelatin compact, globular proteins?

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By the end of this test did casein and gelatin coagulated, are they still biological active? Why?

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3.1.5. Precipitation of protein by salts of heavy metals:

Heavy metal salts usually contain Hg^{+2}, Pb^{+2}, Ag^{+1} Tl^{+1}, Cd^{+2} and other metals with high atomic weights. Since these salts are ionic, they disrupt salt bridges in proteins. The reaction of a heavy metal salt with a protein usually leads to an insoluble metal protein salt.

Objective: to investigate the effect of heavy metals on protein structure.

Principle:

- At pH≤7 the protein is normally negatively charged. Addition of the heavy metal cation will neutralize those negative charges and will cause the protein to precipitate, but any elevation of pH of the medium to higher than 7 (to basic) will precipitate protein as hydroxides, whereas more of metal cations will dissolve this precipitate.
- The family's application of this technique is to eliminate poisoning by lead (Pb^{++}) and mercury salts (Hg^{++}).

Materials:

- Albumin (2% dissolved in 0.1 NaOH) ,1% gelatin ,1% casein (dissolved in 0.1 NaOH)
- Pb acetate (CH_3-COO Pb) or 2% silver nitrate (2g $AgNO_3$) dissolved in 100ml distilled water)
- Mercury chloride 5% $HgCl_2$.

Method:

- In different test tubes, take 1ml of protein solutions.
- Add to each tube 0.5ml of $AgNO_3$ (be careful).
- Repeat the process using $HgCl_2$ instead of $AgNO_3$, compare the results.

Brief them in the following table:

Results:

Tube	Observation	Conclusion
Albumin		
gelatin		
casein		

Questions:

How can this technique help eliminating poisoning by Pb^{++} from water pipes or accidental poisoning of Hg^{++}?

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3.2. Quantitative Proteins Estimation by Lowry method:

3.2.2. Introduction:

Biochemical research often requires the quantitative measurement of protein concentrations in solutions. Several techniques have been developed, since the amino acid content varies from protein to protein, no single assay will be suitable for all protein.

In four of the five methods, chemical reagents added to the protein solutions to develop a color whose intensity will measured via a spectrophotometer.

Comparison of various methods in the following table:

Method	Sensitivity	Time	Principle	Interferences	Comments
Biuret	**Low 1-20 mg**	**Moderate 20-30min**	**Peptide bonds + alkaline Cu^{2+} → Purple complex**	**Zwitterionic buffers, Some amino acids**	**Similar color with all proteins. Destructive to protein samples.**
Lowry	**High ~ 5 µg**	**Slow 40-60min**	**1) Biuert reaction 2) Reduction of phosphomolybdate-phosphotungstate by Tyr and Trp**	**Ammonium sulfate ,glycine, Zwitterionic ,buffers, Mercaptans**	**Time-consuming. Color varies with proteins. Critical timing of procedure. Destructive to protein samples.**
Bradford	**High ~ 1 µg**	**Rapid 15 min**	**λ_{max} of Coomassie day shift from 465 nm to 595 nm when protein-bound**	**Strongly basic Buffers, detergents TritonX-100 ,SDS**	**Stable color, which varies with proteins. Reagent commercially available. Destruction to protein samples Discoloration of glassware.**
BCA	**High ~ 1 µg**	**Slow 60 min**	**1) Burit reaction 2) Copper complex with BCA; λ_{max} =562 nm**	**EDTA,DTT , Ammonium sulfate**	**Compatible with detergents. Reagents commercially available. Destructive to Protein samples.**
Spectrophoto-metric (A280)	**Moderate 50-100 µg**	**Rapid 5-10 min**	**Absorption of 280-nm light by Aromatic residues**	**Purines , pyrimidines, Nucleic acids**	**Useful for monitoring column eluents. Nucleic acid absorption can be corrected. Nondestructive to protein samples. Varies with proteins.**

3.2.2. Objective:

To determine the concentration of protein by Lowry method

3.2.3. Materials and Equipment:

1. Albumin standard (200µg/ml): Dissolve 0.02g albumin in water and make up to 100 ml with water.
2. **Reagent A**: Dissolve; 2 g Na_2CO_3 (2%), 0.4 g NaOH (0.4%), 0.16 g sodium, potassium tartarate (0.16%), 1g SDS (1%) in water and make up to 100 ml. Store at room temperature.
3. **Reagent B**: 4% $CuSO_4.5H_2O$, Dissolve 0.4g $CuSO_4.5H_2O$ in a little volume of water and make up to 10 ml. Store at room temperature.
4. **Reagent C**: 100 parts of reagent A + 1 part reagent B. Take 100 ml reagent A and add 1ml reagent B.
5. **Folin-Ciocalteu reagent**: Dilute commercial reagent by 1: with water. Freshly prepared.
6. Spectrophotometer.
7. Test tubes.
8. Cuvettes.

3.2.4. Method

1. Set up 8 tubes as follows:

Tube	**Water (ml)**	**albumin standard (ml)**	**unknown (ml)**
A(blank)	1.0	-	-
B	0.8	0.2	-
C	0.6	0.4	-
D	0.4	0.6	-
E	0.2	0.8	-
F	-	1.0	-
G	-	-	1.0
H	-	-	1.0

2. Add 3ml reagent C to all tubes. Mix and let stand at room temperature for 15 min.

3. Add 0.3 ml of Folin-Ciocalteu reagent. (Add this reagent to one tube at a time and immediately after adding it mix well).
4. Let the tubes stand at room temperature for 45 min.
5. Read absorbance at 750nm against the blank.

3.2.5. Results and Calculations:

Tube	**Albumin concentration (µg/ml)**	**A_{750}**
A	0	
B	40	
C	80	
D	120	
E	160	
F	200	
G		
H		

Plot a standard curve for absorbance at 750nm against Albumin concentration (µg/ml). From the standard curve, obtain the concentration of unknown samples.

Enzymes

4.1. Some factors affecting polyphenol oxidase activity

1.1.1. Introduction:

When we consider enzyme catalyzed reactions in the living cell, the reacting substances, upon which an enzyme acts, are termed the substrates. The substances produced as a result of the reaction are the products. Enzyme catalyzed reactions are mostly reversible and involve the formation of an intermediate enzyme-substrate complex.

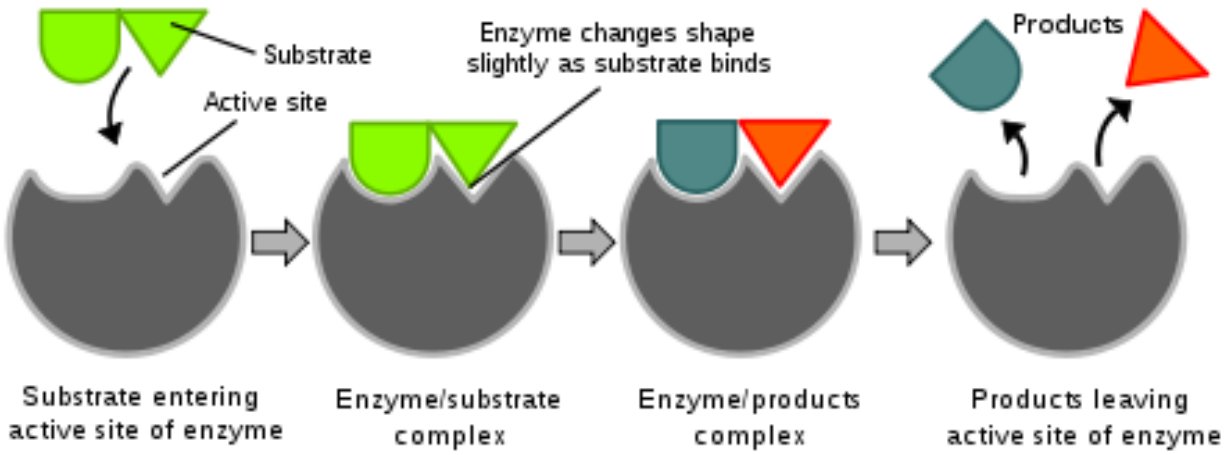
Enzyme Substrate Enzyme -substrate complex Enzyme Product

The formation of an enzyme-substrate complex increases the possibility for chemical reaction by:

1- Lowering the energy of activation, and
2- Reducing the element of chance in the collisions of molecules or ions.

The rate of reaction is accelerated through the catalytic action of the enzyme. A single enzyme molecule, even though it can react over and over, is only capable of combining with a given total number of substrate molecules per minute. This number known as the turnover number varies from enzyme to enzyme. Many enzymes have a high turnover number. For example, catalase has a turnover number of 5 million per minute. Thus enzymes are generally effective in relatively minute concentrations in the living cell.

The formation of enzyme-substrate complex is confined to relatively small areas of the enzyme molecule, known as active sites. The structure of a particular substrate may induce the enzyme to "mold" itself over the substrate. This may be illustrated schematically in the following way:

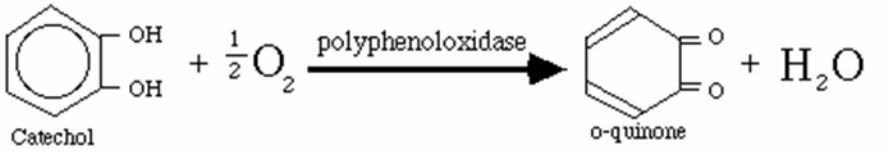


http://biochemistryquestions.wordpress.com/2008/07/15/induced-fit-model-of-enzyme-substrate

The "induced fit" hypothesis suggests that differences in the surface configuration (three-dimensional shape) of the active site are essential to specificity. In other words, only certain types of substrate molecule would be able to establish a close fit with a given type of enzyme molecule.

Because hundreds of reactions are simultaneously carried out in the living cell, it becomes difficult to study a single reaction in an intact living cell. However, it is possible to extract enzymes from cells and thus study enzyme catalyzed reactions in a test tube. In this experiment, a crude extract of the enzyme polyphenol oxidase will be prepared from the potato.

Polyphenol oxidase is a copper-containing enzyme with an optimum pH of 6.7. It catalyses the oxidation of di- and tri- hydroxyl phenol to the corresponding quinine.



This oxidation-reduction reaction is accompanied by a color change (quinines absorb light in the visible region of the spectrum). This reaction commonly occurs in nature and accounts for the "browning" of peeled potatoes and bruised fruits. You will familiarize yourself with the reaction catalyzed by the enzyme polyphenol oxidase, as it occurs removed from the intact living cell, i.e. in a test tube. This experiment is in four parts, corresponding to the four objectives listed on the first page.

4.1.2. Objectives:

- To demonstrate activity of the enzyme polyphenol oxidase in crude extract prepared from potato.
- To demonstrate the chemical nature of the enzyme.
- To investigate the substrate specificity of the enzyme.

To investigate the effects of various temperatures on the activity of the enzyme

4.1.3. Materials:

0.01M catechol

Dissolve 1.1g of catechol in 1 liter of distilled water. Adjust the pH to 6.0 with dilute NaOH to prevent auto oxidation. Keep this solution in a brown bottle in a refrigerator. (If the solution turns brown it is unfit for use.)

0.1M NaF

Dissolve 4.2g of sodium fluoride in 1 liter of distilled water.

0.01M hydroquinone

Dissolve 0.11g of hydroquinone in 100ml of distilled water.

0.01M phenol

Dissolve 94mg of phenol in 100ml of distilled water.

5% trichloroacetic acid

Dissolve 5g of trichloroacetic acid in 100ml of distilled water.

5% trypsin

Dissolve 5g of trypsin in 100ml of distilled water.

Phenylthiourea

A few grams of crystalline phenylthiourea should be available for the experiment.

Potatoes.

Homogenizer.

Cheesecloth.

Water baths at 37 and 70 ºC.

Container of crushed ice.

4.1.4. Method:

To detect and follow the progress of the reaction in this experiment a simple, qualitative method will be used. More sophisticated, quantitative methods of following enzyme catalyzed reactions will be introduced later in the course. In this experiment, record your observations according to the following scheme:

Degree of color intensity in test tube	**symbol**
No color change (colorless)	–
Faint color (just detectable)	+
Definite color	++
Dark (deep) color	+++

The color should be brown, i.e. the color of the product (quinone). The intensity of this color will be proportional to the enzym's activity in the tube under observation.

Preparation of enzyme extract

1- Peel a small potato.
2- Cut a cube (2-4cms square) from the potato. Then cut this cube into small pieces and place them in a homogenizer.
3- Add 50ml of sodium fluoride solution (this inhibits other potato enzymes).
4- Grind the potato pieces for 1-2min in the homogenizer.
5- Pour the homogenate, through several layers of cheesecloth, into a 100ml beaker.

This is your crude extract containing the enzyme polyphenol oxidase.

Do not dispose of this enzyme extract until you have completed all parts of this experiment. Dispose of all waste (potato peel, cheesecloth, potato mash etc.) in the laboratory's waste container. It is absolutely essential to keep your glassware clean. Wash all your glassware thoroughly before and after each of the following experiments.

4.1.4.1 .Test tube enzymatic activity:

a) Label three clean test tubes A, B and C.

b) Prepare each tube as follows:

Tube A: 15 drops of enzyme extract.

15 drops of 0.01M catechol solution.

Tube B: 15 drops of enzyme extract.

15 drops of distilled water.

Tube C: 15 drops of 0.0M catechol solution.

15 drops of distilled water.

c) Place all three tubes in a water bath at 37 ºC.

d) Shake each tube every 5 minutes to aerate, thereby adding oxygen to the solution.

e) Every 5 minutes, after shaking, hold the tubes up to the light and examine. Record the color in each tube, according to the scheme described in this page, in the table in the "Results" section. Continue for 25 minutes.

4.1.4.2.Chemical nature of polyphenol oxidase:

a) Label four clean test tubes A, B, C and D.

b) Prepare, and treat, each tube as follows:

Tube A: Add 15 drops of enzyme extract.

Add 15 drops of 0.01M catechol solution.

Shake tube and place in water bath at 37 ºC for 10 minutes.

Set tube aside as <u>control</u> with which to compare results of tubes B, C and D.

Tube B: Add 10 drops of enzyme extract.

Add 10 drops of 5% trypsin solution.

Shake tube thoroughly.

Place tube in a water bath at 37 ºC for 10 minutes.

Add 10 drops of 0.01M catechol solution.

Replace in the same water bath for 10 minutes.

Examine and compare with tube A.

Record your observations in the table in the "Results" section.

Tube C: Add 10 drops of enzyme extract.

Add 10 drops of 5% trichloroacetic acid.

Shake tube thoroughly and wait 5 minutes.

Add 10 drops of 0.01M catechol solution.

Place tube in water bath at 37 ºC for 10 minutes.

Examine and compare with tube A.

Record your observations in the table in the "Results" section.

Tube D: Add 15 drops of enzyme extract.

Add a few crystals of phenylthiourea.

Shake tube thoroughly and continue shaking it frequently during a period of 5 minutes.

Then add 15 drops of 0.01M catechol solution.

Place tube in water bath at 37 ºC for 10 minutes.

Examine and compare with tube A.

Record your observations in the table in the "Results" section.

Trypsin is a proteolytic enzyme in other words it hydrolyses the peptide bonds which link the amino acid residues, to denature and precipitate proteins, including enzymes. Phenylthiourea has a very strong chemical affinity for the element copper. It is able to bind with copper, even when the copper is attached to other chemical substances, as in the active site of polyphenol oxidase.

4.1.4.3. Substrate specificity:

a) Label three clean test tubes A, B and C.

b) Add 15 drops of enzyme extract to each tube.

c) Prepare each tube as follows:

Tube A: Add 15 drops of 0.01M catechol solution.

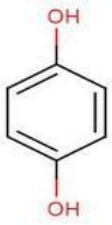
Tube B: Add 15 drops of 0.01M phenol solution.

Tube C: Add 15 drops of 0.01M hydroquinone solution.

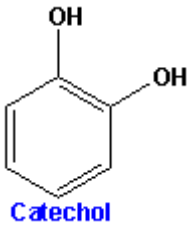
d) Shake the tubes gently and place them in a water bath at 37 ºC.

e) Examine the tubes after 5 minutes and after 10 minutes. Record the color in each tube, according to the scheme described on the page 3, in the table in the "Results" section.

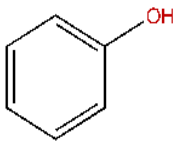
The three compounds used as substrates in this part of the experiment are structurally related, as shown in the figure below. Each is capable of reacting with oxygen to form various colored products.



Hydroquinone



Catechol



Phenol

4.1.4.4. Temperature and enzymatic activity:

a) Label three clean test tubes A, B and C.

b) Add 15 drops of enzyme extract to each tube.

c) Place each tube containing the enzyme extract, in a water bath for 10 minutes at the following temperatures:

Tube A: 0 ºC (in a container of crushed ice).

Tube B: 37 ºC.

Tube C: 70 ºC.

d) Add 15 drops of 0.01M catechol solution to each tube.

e) Shake each tube gently and quickly return it to its proper temperature condition.

f) Wait for 15 minutes. After this time, examine each tube, without removing it from its temperature condition, and record the color in each tube, according to the scheme described in the table of "Results" section.

4.1.5. Results:

1) Enzymatic activity

Incubation time (minutes)	Degree of color intensity (Symbol: −, +, ++ or +++)		
	Tube A	Tube B	Tube C
0			
5			
10			
15			
20			
25			

2) Chemical nature of polyphenol oxidase:

Tube	Treatment	Degree of color intensity (Symbol: −, +, ++ or +++)
A	Control	
B	Trypsin	
C	TCA	
D	Phenylthiourea	

3) Substrate specificity:

Substrate	Degree of color intensity (Symbol: −, +, ++ or +++)	
	5 minutes	10 minutes
Catechol		
Phenol		
Hydroquinone		

4) Temperature and enzymatic activity:

Temperature (ºC)	Degree of color intensity (Symbol: −, +, ++ or +++)
0	
37	
70	

In each of the above tables, the degree of color intensity may be considered proportional to enzymic activity.

4.1.6. Discussion and conclusions:

4.1.7. Questions:

1- In part 1 of the experiment, did you detect any reaction in tube B (enzyme extract and distilled water)? Give reasons why some reaction may be possible in this tube.
2- Can the enzyme be restored to an active state after TCA treatment (part 2)? Explain your answer.
3- What can you deduce about the specificity of polyphenol oxidase from part 3 of the experiment?
4- Explain the effects of temperature on the activity of polyphenol oxidase (part 4).
5- Suppose that two solutions of equal concentration were prepared, one of purified polyphenol oxidase and one of purified trypsin. Which solution, would you expect, would lose its enzymic activity first and why?

5. Carbohydrates (1)

Qualitative analysis of Carbohydrates

5.1. Introduction:

The term carbohydrates applied generally to the group of Polyhydroxy Aldehydes or Ketones commonly known as Sugars. Carbohydrates are produced from carbon dioxide and water by plants through the process of photosynthesis. Carbohydrates are the major food supply and energy source for the people of the world. Depending on the dietary habits, 50-90% of the carbohydrates consumed comes from grain, starchy vegetables and legumes. Despite the major utilization of carbohydrates for energy only a small amount is stored in the body. The average adult reserve is about 370 g stored mainly as liver and muscle glycogen.

Carbohydrates can be classified as:

1) **Simple carbohydrates**, often called monosaccharides or simple sugars, contain one saccharide unit.
2) **Complex carbohydrates** are those containing more than one saccharide group.

Also it can be classified as:

- **Monosaccharides** contain one monosaccharide unit.
- **Disaccharides** contains two monosaccharide units.
- **Oligosaccharides** contains 3-6 monosaccharide units.
- **Polysaccharides** can contain over 7 or more monosaccharide units. Complex carbohydrates can be broken down into smaller sugar units through a process known as hydrolysis.

Monosaccharides can be classified in a number of ways:

1. They can be classified by the number of carbon atoms they contain; pentoses (5 carbons) and hexoses (6 carbons) are the most common.
2. Monosaccharides can also be classified as ketoses or aldoses. A ketose contains a carbonyl group attached to two R groups having one or more hydroxyl groups. An aldose contains terminal aldehyde group in addition to R group containing -OH.

5.2. Physical properties:

5.2.1. Solubility:

Monosaccharide and Disaccharide can be dissolved freely in water because water is a polar substance, while polysaccharide cannot be dissolved easily in water, because, it has high molecular weight , which give colloidal solutions in water soluble.

5.3, Chemical properties:

5.3.1. Molisch Test:

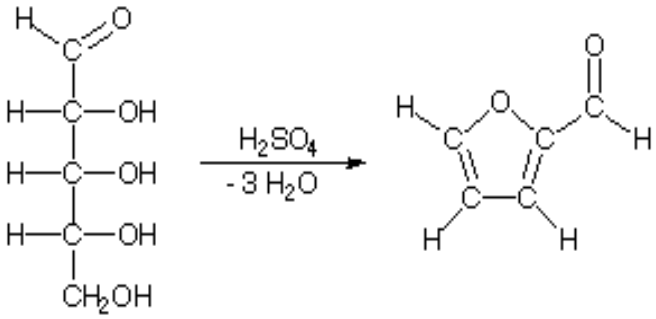
This test is specific for all carbohydrates, Monosaccharide gives a rapid positive test, Disaccharides and Polysaccharides react slower.

Objective:

To identify the carbohydrate from other macromolecules Lipids and Proteins.

Principle:

The Conc. sulfuric acid 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.



Glucose 5-(hydroxymethyl)furfura

Materials:

- α–Naphthol in 95% ethanol(50gm of α–Naphthol in 1000 ml ethanol)
- Different sugar solutions.1%w/v
- Concentrated sulfuric acid

Method:

- Place 2ml of a sample solution in a test tube.
- Add 2 drops of the Molisch reagent (a solution of α-napthol in 95% ethanol).
- Add 2 ml of concentrated sulfuric acid with care on tube wall so that two layers form, producing violet ring appear as liaison between the surface separations.

Results:

Sample	**Observation**	**Result**
1		
2		
3		
4		

Question:

Name the complex formed by the addition of concentrated sulfuric acid to sugar solution and explain the reaction?

.....
.....
.....

5.3.2 Benedict's test:

Benedict's reagent is used as a test for the presence of reducing sugars. All monosaccharides are reducing sugars; they all have a free reactive carbonyl group. Some disaccharides have exposed carbonyl groups and are also reducing sugars. Other disaccharides such as sucrose are non-reducing sugars and will not react with Benedict's solution. Starches are also non-reducing sugars.

Objective:

To distinguish between the reducing and non-reducing sugars.

Principle:

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

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

$$2\ Cu^{++} + \text{Reducing Sugar} \longrightarrow Cu^{+}$$

(electron donor)

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

Materials:

- Solution of different Carbohydrate (1%)
- Benedict's reagent (a solution of sodium citrate and sodium carbonate mixed with a solution of copper sulfate)
- Boiling water bath

Method:

- Place 1 ml of a sample solution in a test tube.
- Add 2 ml of Benedict's reagent
- The solution then heated in a boiling water bath for 3 minutes.

A positive test is indicated by: The formation of a reddish or orange precipitate within 3 minutes.

Results:

Tubes	Observation	Discussion
1		
2		
3		
4		
5		

Questions:

Why sucrose gives negative Benedict test?

.....
.....
.....

Explain, although starch has free hemiacetal bond it gives negative Benedict test?

.....
.....
.....

Why glucose (monosaccharide) and maltose (disaccharide) give positive Benedict test?

.....
.....
.....
.....

5.3.3. Barfoed's Test:

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

Objective:

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

Principle:

Barfoed's test used copper (II) ions in a slightly acidic medium reducing monosaccharides are oxidized by the copper ion in solution to form a carboxylic acid and a reddish precipitate of copper (I) oxide within three minutes. Reducing disaccharides undergo the same reaction, but do so at a slower rate. The non-reducing sugars give negative result.

Materials:

- Barfoed's reagent (a solution of cupric acetate and acetic acid)
  (13.3 gm. of cupric acetate in 100ml dis. H_2O + 1.8 ml conc. acetic acid)
- Glucose, Sucrose
- Boiling water bath.

Method:

- Place 1 ml of a sample solution in a test tube.
- Add 3 ml of Barfoed's reagent (a solution of cupric acetate and acetic acid).
- Heat the solution in a boiling water bath for 3 minutes.
- Note your observation

Results:

Tubes	Observation	Discussion
1		
2		
3		
4		

Questions:

What is the difference between Benedict and Barfoed's reaction?

.....
.....

Why should be avoid boiling more than 5 minutes?

.....
.....

5.3.4. Bial's Test:

Objective:

To distinguish between pentose monosaccharide and hexose monosaccharide

Principle:

Bial's test uses concentrated HCl as a dehydrating acid and Orcinol + traces of Ferric Chloride as condensation reagent. The test reagent dehydrates Pentoses to form furfural. Furfural further reacts with Orcinol and the iron ion present in the test reagent to produce a greenish blue product, while hexoses yield muddy-brown to grey condensation product.

Material

- Ribose, Fructose
- Bial's reagent (a solution of Orcinol, HCl and Ferric Chloride)
  1.5 gm. of Orcinol in 500 ml of Conc. HCl + 30 drops of 10% Ferric Chloride
- Water bath.

Method:

- Put 2 ml of a sample solution in a test tube.
- Add 2 ml of Bial's reagent to each tube.
- Heat the tubes gently in boiling water bath.

A positive test indicated by the formation of a greenish blue product. All other colors indicate a negative result for pentoses.

Note that hexoses generally react to form red, or brown products.

Results:

Tubes	Observation	Discussion
1		
2		
3		
4		

Questions:

What is the principle of Bial's reaction?

.....
.....
.....

5.3.5. Seliwanoff's Test:

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

Objective:

To distinguish between aldose and ketone sugars.

Principle:

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

Material:

- Seliwanoff's reagent (a solution of resorcinol and HCl)
  5.5 gm. Resorcinol in 269 ml of 6M HCl then add dis.H2O up to 1000 ml
- Glucose, Fructose.
- Boiling water bath

Method:

- Put 0.5 ml of a sample solution in a test tube.
- Add 2 ml of Seliwanoff's reagent (a solution of Resorcinol and HCl).
- The solution then heated in a boiling water bath for 2 minutes.

Results:

Tubes	Observation	Discussion
1		
2		

Questions:

What are the carbohydrates' that give positive result with Seliwanoff? why?

.....
.....

6. Carbohydrate: Qualitative analysis of Carbohydrates (2)

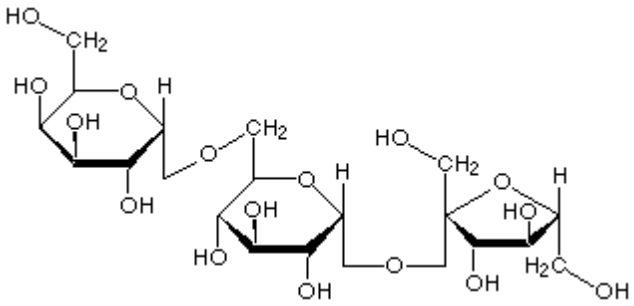
6.1. Introduction:

Structures of common complex carbohydrates

The most abundant carbohydrate molecules found in nature are actually large complex structures consisting of mixtures of monosaccharide derivatives. Carbohydrates consisting of several monosaccharides are called **oligosaccharides**, a designation that could also include disaccharides, whereas, carbohydrates with ~10 or more monosaccharide units are called **polysaccharides**. Polysaccharides can either be homopolymeric (same repeating monosaccharide unit) or heteropolymeric (mixture of monosaccharides).

Disaccharides can be broken into two monosaccharide units by hydrolysis and lost one molecule of water, examples of disaccharides Sucrose, Lactose and Maltose.

Oligosaccharides can be broken into 3-6 monosaccharide units by hydrolysis and lost one or more molecules of water, See below example of oligosaccharides.



Glucose Glucose fructose

Raffinose

Polysaccharides can be broken into 7 or more monosaccharide units by hydrolysis and lost one or more molecules of water examples Starch.

Plants and animals store glucose in the form of very large polysaccharide glucose homopolymers that contain both α1-4 and α1-6 glycosidic bonds. The glucose homopolymer produced in plants is called **starch**, while the glucose homopolymer produced in animal cells is called **glycogen**. Plants synthesize two forms of starch, **amylose**, a linear polysaccharide containing about ~100 glucose units linked by α1-4 glycosidic bonds, and **amylopectin**, a branched polysaccharide containing ~100,000 glucose units connected by α1-4 and α1-6 glycosidic bonds.

6.2. Practical experiments

6.2.1. Sucrose Hydrolysis Test:

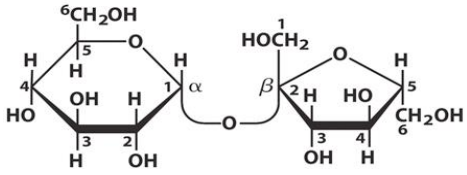
This test is used to convert sucrose (non-reducing disaccharide) to glucose and fructose (reducing mono saccharides).

Objective:

To identify the products of hydrolysis of disaccharides.

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.



Materials:

- Sucrose
- Concentrated hydrochloric acid (HCl)
- Concentrated NaOH
- Benedict's reagent
- Seliwanoff's reagent

Method:

1. Set up 2 tubes add to each one 4 ml of a sucrose solution. Label the tubes (Sucrose with HCl, Sucrose without HCl)
2. Add 4 drops of concentrated hydrochloric acid (HCl) to the 1st tube.
3. Heat both in boiling water bath for 10 minutes.
4. Add 4 drops of concentrated NaOH to each tube (why?)
5. Set up 2 new tubes and from the 1st tube take 2 ml in each to do Benedict's test and Seliwanoff's test, label the tubes (Benedict+HCl) and (Seliwanoff's+HCl). Add 2 ml of Benedict's reagent and 3 ml of Seliwanoff's reagent to the corresponding tube. WHAT do you expect?
6. From the tube which contain only sucrose (without HCl) take 2 ml to do Benedict's test only (add 2 ml of Benedict's reagent) WHAT do you expect?

Results:

Tube	**Sucrose with HCl**		**Sucrose without HCl**
Test	Benedict's test	Seliwanoff's test	Benedict's test
Result			

Questions:

How you can convert non-reducing sugar to reducing?

.....

.....

.....

6.2.2. The Iodine/Potassium Iodide Test:
This test used to distinguish between polysaccharides and mono or oligo saccharides.

Objective: to detect the presence of starch in a sample

Principle:
Starch forms deeply blue color complex with iodine. Starch contains α-amylase, a helical saccharide polymer and amylopectin, a branched form of starch. Iodine forms a large complex with α-amylose helix. This complex absorbs light and reflects the blue light only. Simple oligosaccharides and mono saccharides do not form this complex.
Note that other polysaccharides like glycogen may give other colors (red or brown).

Materials:
- Iodine/potassium iodide solution
- Starch- Glycogen- Dextrin, glucose

Method:
1. Take 2 ml of a sample solution in a test tube.
2. Add 3 drops of iodine/potassium iodide solution.
3. A positive test indicated by the formation of a colored complex between Iodine and polysaccharide.
4. Heat the tubes in a water bath and write your observations.

Result:

Tubes	Observation	Discussion
1		
2		
3		
4		

Questions:
Why glucose does not give positive result with iodine test but not starch?
……

……

……

Explain why the blue color disappears upon heating?
……

……

6.2.3. Hydrolysis of Starch:

This experiment illustrates the conversion of starch (non-reducing sugar) to a reducing sugar by the action of hydrochloric acid (HCl) at boiling point. The longer the starch is exposed to the acid the further hydrolysis proceeds.

Objective: to establish the effect of concentrated HCl on a glycosidic bond in starch**.**

Principle:

Although starch has free hemiacetal in the terminal glucose residue, it has no reducing properties, because the percentage between the free residues is very low in comparison to the whole molecule. Heating starch solution in acid medium hydrolyses the glycosidic bonds giving many free glucose residues. These glucose molecules give reducing properties to the hydrolysis product.

Materials:

- Starch
- Benedict's reagent
- Iodine reagent (10 ml of concentrated iodine in 100 ml of dis.H2O)
- Water bath
- concentrated NaOH

Method:

1. Take 2 ml of starch in large tube
2. Add 5 drops of HCl, heated in boiling water bath for 10 mints. then cool the solution.
3. Add sufficient amount of NaOH to convert the medium to basic.
4. Divide the whole amount in 2 tubes (A and B)
5. In tube (A) add 3 drops of iodine solution and note the result.
6. In tube (B) add 1 ml of Benedict reagent, mix well and heated for 3 mint then record result.

Result:

Tube	**Starch with HCl**	
Test	**(A) Iodine test**	**(B) Benedict's test**
Result		

Questions:

Although starch has free hemiacetal bonds it is non reducing sugar, explain?

……

……

……

6.3. Quantitative estimation of glucose by enzymatic method:

6.3.1. Introduction :

There are three main methods of estimation the reducing sugar content in solution :

a. reduction of cupric to cuprous salts ;
b. reduction of ferricyanide to ferrocyanide ; and
c. enzymatic methods

Of the above three methods, method "c" is the most commonly used in clinical laboratories for glucose estimation.

a) Reducing sugars contains an aldehyde or keto groups reduced alkaline copper to cuprous oxide. Cuprous oxide allowed reacting with phosphomolybdate solution, which reduced and forms blue color. The intensity of color measured on colorimeter against standard.
b) Reduction of ferricyanide to ferrocyanide by reducing sugars in alkaline solution. In presence of zinc ions, the ferrocyanide formed precipitated as a zinc complex.

c) Enzymatic methods

Glucose commonly measured using an enzyme to covert the glucose to a product that can be easily detected, common enzymes used are glucose oxidase, glucose dehydrogenase and hexokinase.

(1) Glucose oxidase:

Converts glucose, in the presence of oxygen, to gluconolactone and hydrogen peroxide:

$$\text{Glucose} + O_2 \xrightarrow{Glucose\ oxidase} \text{Gluconolactone} + H_2O_2$$

Hydrogen peroxide can converted to water and oxygen by the action of the enzyme peroxidase. A chromogenic oxygen acceptor (the reduced form) captures the released oxygen (now the oxidized form), and this allows quantitation of the glucose. The oxygen acceptor is usually a non-colored substance that becomes colored when oxidized.

$$H_2O_2 + \text{o-Dianisidine} \xrightarrow[\text{(colorless)}]{Peroxidase} \text{Oxidized o-dianisidine} + H_2O \quad \text{(colored)}$$

(2) Glucose dehydrogenase

$$\text{Glucose} + NAD^+ \xrightarrow{Glucose\ dehydrogenase} \text{Glucono-}_{\delta}\text{-lactone} + NADH + H^+$$

The amount of reduced nicotinamide adenine dinucleotide (NADH) produced is proportional to the concentration of glucose in the sample. The production of NADH is monitored by an increasing absorbance at 340 nm.

(3) Methods using hexokinase produce glucose-6-phosphate, which is used as a substrate with NAD^+ in a second enzyme reaction using glucose-6-phosphate dehydrogenase. NADH is produced in proportion to the glucose concentration in the sample.

$$\text{Glucose + ATP} \xrightarrow{hexokinase} \text{Glucose-6-phosphate + ADP}$$

$$\text{Glucose-6-phosphate + } NAD^+ \xrightarrow{G-6-P\ dehydrogenase} \text{6-Phosphogluconate + NADH + } H^+$$

6.3.2. Objective:

Measurement of glucose concentration by enzymatic method .

6.3.3 .Material and method:

As shown in the provided kit.

6.3.4. Result

6.3.5.DISCUSSION:

7. Lipids

7.1. Introduction

Lipids found naturally in all living organisms. It has a structural function in the cell, since it presents in cell membranes, and also it is an essential source of energy in the body. It gives more energy than carbohydrate and proteins.

It can be defined as nonpolar organic compound insoluble in polar water, but soluble in organic solvents such as benzene, ether, chloroform and boiling alcohol.

Fatty acids:

The building blocks of lipid. Fatty acid have a long hydrocarbon chain containing a carboxyl group at the end. They are divided into: **saturated** fatty acids and **unsaturated** fatty acids (unsaturated contain double bonds). The general formula for fatty acids:

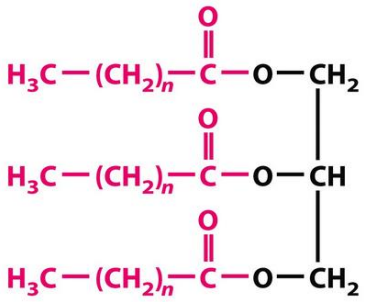
$CH_3 (CH_2)_n COOH$

Fats can be divided according to their chemical composition to:

A- Simple lipids:

Triglycerides are esters of fatty acids with glycerol. It is found in fats and oils. The triacyglycerol is the simplest and most common fat. It is the form in which lipids are stored in the cell.

The general formula of fats and oils



Triacylglycerol

B - Compound (conjugated) lipids:

Lipids are linking with other compounds, such as phospholipids and glycolipids.

C - Derived lipids:

They are substances that are soluble in lipid or derived from the above groups of lipids by hydrolysis; for examples, cholesterol and fat-soluble vitamins.

7.2. Qualitative tests of lipids:

7.2.1. Solubility test:

Objective: to test the solubility of oils in different solvent.

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, undissolved phosphatide lipid in acetone; undissolved cerebroside, as well as sphingomyline in the ether.

Materials :

- Olive oil (or cotton seed oil) - butter - corn oil.
- Solvents: diluted acid - dilute alkaline - ethanol - ether - chloroform - acetone
- Test tubes
- Water bath

Method:

1. 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),
2. Shake the tubes thoroughly, then leave the solution for about one minute,
3. 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.

Results:

Tube	Solvent	Degree of solubility

Questions:

Which solvent is the best for lipid?

.....
.....
.....

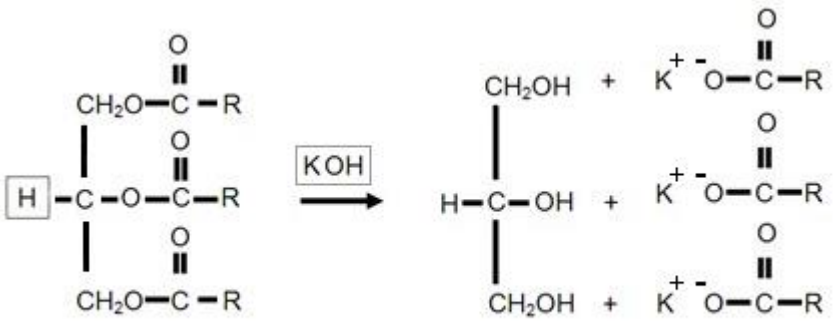
7.2.2. Saponification test:

Triacylglycerol can be hydrolyzed into their component of 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).

Objective: to form the soap.

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:



Triacylglycerol **Glycerol** **Salts of Fatty Acids (SOAP)**

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.

Materials:

- Types of oils like corn oil, butter, olive oil.
- KOH solution in alcohol (2O% KOH)
- Water bath (boiling)

Method:

1. Place 2 ml of oil in a large test tube (or flask).
2. Add 4 ml of alcoholic potassium hydroxide (preferably add little small pieces of porcelain to regulate the boiling point).
3. 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.
4. Take the remaining solid material (soap) and add about 10 ml of water and keep it for the following tests.
5. Shake the solution after it cools and noted to be thick foam.

Results:

Tube	**Observation**	**Conclusion**

Questions:

What is the chemical composition of soap?

.....
.....
.....

Why potassium hydroxide is used in this test?

.....
.....
.....

If you use cocoa butter, what kind of soap that will get it?

……
……
……

If you use fatty acids instead of oil do you expect to get the soap?

……
……
……

7.2.3. Testing the separation of soap from the solution by salting out:

Objective: to investigate the effect of NaCl on soap solubility.

Principle:

To get the soap out of solution by salting out when added solid sodium chloride to the solution until saturation; separated soap in the form of insoluble and floats above the surface.

Materials:

- Soap (which was prepared in the previous experiment)
- Solid sodium chloride NaCl
- A small beaker.

Method:

Place about 3 ml of soap in a 25ml beaker, then add small amounts of sodium chloride in batches, stirring until saturated solution.

Tube	Observation	Conclusion

Questions:

Q/Why is a separation layer on the surface of soap when you add salt?

.....
.....
.....

7.2.4. Test formation insoluble fatty acids salt (insoluble soaps):

Working calcium, magnesium, lead or iron ions to the deposition of soap and make it insoluble in water, where solve these ions replace the sodium or potassium ions are present in soap. Due to the hard water to contain significant quantities of $Ca^{+2,}$ Mg^{+2} and some Fe^{+3} are difficult to foam.

Potassium soap + calcium sulfate ===> calcium soap + potassium sulfate.

(Consisting of a white precipitate from calcium stearate or oleate).

Objective: to investigate the effect of different cations on soap solubility.

Materials:

- Soap (which was prepared in the previous experiment)
- Calcium chloride($CaCl_2$) 5%
- Magnesium chloride or sulfate 5%
- Lead acetate .
- Test tubes.

Method:

1. Add about 2 ml of soap in three test tubes
2. Add to the first tube a few drops of calcium chloride, to second tube MgCl, and third tube lead acetate.

Results:

Tube	Observation	Conclusion

Questions:

Q1/ Write the equation of reacting calcium chloride with soap?

.....
.....
.....

Q2 / What happens to the soap when washing with water hardness?

.....
.....
.....

7.2.5. Copper acetate test:

Objective: 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.

Materials:

Olive oil - oleic acid (polyunsaturated fatty acid) - stearic acid (saturated fatty acid) - petroleum ether - copper acetate solution (5%)

Method:

- Take three test tubes put 0.5 gm 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.

Results:

Tube	Observation	Conclusion

Questions:

Why olive oil does not form green color?

.....
.....
.....

What do you expect if you used palmitic or linoleic acid?

.....
.....
.....

7.2.6. Qualitative estimation of Cholesterol by Liebermann - Burchard Test

Objective:

To detect the presence of cholesterol

Principle:

Liebermann - Burchard Test , is a chemical estimation of cholesterol, the cholesterol is react as a typical alcohol with a strong ,concentrated acids; the product are colored substances.

Acetic anhydride are used as solvent and dehydrating agents, and the sulfuric acid is used as dehydrating and oxidizing agent .A positive result is observed when the solution becomes red , then blue, and finally bluish –green color.

Material:

- cholesterol
- Acetic anhydride
- Concentrated sulfuric acid
- Chloroform

Method:

- Dissolve a few crystals of cholesterol in 2 ml of chloroform in a dry test tube
- Now add 10 drops of acetic anhydride
- Add 2 to 3 drops of conc. sulfuric acid
- Record your result

Result:

Tube	**Observation**	**Conclusion**
Cholesterol		

7.2.7. Unsaturation Test:

Principle:

All neutral lipid contain glycerides of some unsaturated fatty acids. These unsaturated fatty acids become saturated by taking up iodine. If the fat contains more unsaturated fatty acids, it will take up more iodine.

Material:

- Hub's iodine reagent (alcoholic solution of iodine containing mercuric chloride)
- Chloroform
- Mustard oil, coconut oil, olive oil, and groundnut oil

Method:

- Add 10 drops of Hub's iodine reagent to 10 ml of Chloroform. The chloroform shows pink color due to presence of iodine.
- Divide solution equally into 4 tubes:
  (i) To one test tube add Mustard oil drop by drop shaking the tube vigorously for about 30 seconds after addition of each until the pink color is discharged and count the number of drops. The pink color is discharged owing to the taking up of iodine by the unsaturated fatty acids of the oil.
  (ii) Repeat above experiment with the remaining three test tubes taking fats (coconut oil, olive oil, and groundnut oil).
  (iii) Compare unsaturation , it should be remembered that more the number of drops required to discharge the pink color, the less is the saturation.

Result:

Tube	Observation	Conclusion

……

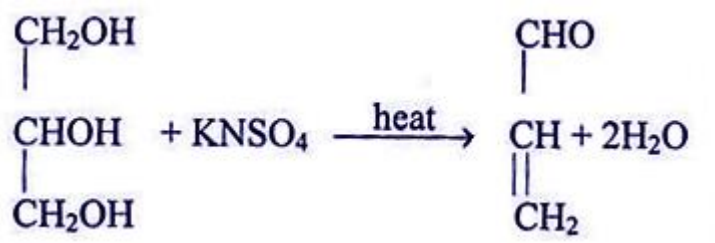
7.2.8. Acrolein test

Most lipids are found in the form of triglycerides, an ester formed from glycerol and fatty acids. When a fat is heated strongly in the presence of a dehydrating agent such as $KHSO_4$, the glycerol portion of the molecule is dehydrated to form the unsaturated aldehyde, acrolein

Principle:

When a fat is heated strongly in the presence of a dehydrating agent such as $KHSO_4$, the glycerol portion of the molecule is dehydrated to form the unsaturated aldehyde, acrolein

CH_2=CH-CHO , which can be distinguished by its irritating acrid smell and as burnt grease.



Glycerol **Acrolein**

Lipid can detect by dye Sudan IV (general dye for lipid), which produce red color with lipid.

Materials:

- different types of vegetable oils (such as corn oil, olive oil, butter)
- glycerol
- Solid potassium hydrogen sulfate $KHSO_4$
- test tubes
- a water bath (boiling)

Method:

1. Place an amount of solid potassium hydrogen sulfate $KHSO_4$ to about 0.5ml of glycerol in a test tube, then carefully heat the tube in boiling water bath and observed the emergence of the smell of acrolein (irritating to the membranes).
2. Repeat the previous step using oil from different sources instead of glycerol

Results:

Tube	Observation	Conclusion
Glycerol		
Palm oil		
Sun Flower oil		

Questions:

Why acrolein test is used as a general test for oils and fats?

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.....
.....

Do you expect to get a positive result if you use free fatty acid like oleic acid or palmitic acid and why?

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.....
.....

Do you expect to get a positive result if you use beeswax and why?

.....
.....
.....

8. Spectral Characterization of DNA

Objective:

1- To establish the effect of temperature on the absorbance of DNA
2- To determine the optimum wave length for DNA
3- To determine the concentration of DNA in the sample.

Principle:

Nucleic acids are characterized by a strong absorption at wave length 260 nm.
Denaturation of a double stranded nucleic acids (DNA), produce an increase in absorption (this is called hyperchromic effect)

Material:

- DNA concentrated sample(extracted from yeast)
- 1X saline solution (NaCl with Tri Sodium Citrate)
- Quartz Cuvette
- UV Spectrophotometer

Method:

1- Pipette 0.5 ml of isolated DNA solution and add to it 4.5 ml of 1X saline-citrate as blank.

2- Read the absorbance of the solution at 260 nm using 1X saline -citrate as blank.

Note: (if the absorbance is greater than 1.0, dilute the solution until you obtain A260 of 1.0 or slightly less.)

3- When the absorbance of the solution ≈ 1.0 is obtained, read the absorbance of the solution at the following wave lengths:

(240,245,250,255,260,265,270,275,280), using a blank of 1X saline citrate.

Note: (for each wave lengths, zero the spectrophotometer against a blank of 1X saline citrate)

4-Using the same isolated DNA solution (solution of A≈ 1.0), heat the DNA solution in a boiling water bath for 15 min.

7- Rapidly determine the absorbance of heated sample at different wave lengths:

(240,245,250,255,260,265,270,275,280), using a blank of 1X saline citrate.

Note: (for each wave lengths, zero the spectrophotometer against a blank of 1X saline citrate)

8- Plot the absorption spectra of the native DNA solution and the denatured DNA heated 15 min at 100 °C.

Result and calculation:

Part 1:

Wave length (nm)	Absorbance of isolated DNA	Absorbance of heated DNA
240		
245		
250		
255		
260		
265		
270		
275		
280		

Plot the absorption spectra of the native DNA solution and the denatured DNA heated 15 min at 100 °C.

Part 2:

For calculation of DNA concentration of samples free of RNA, the following conversion factor is used: (OD260) 1 = 50 ug of DNA/ml.

You can calculate the concentration of the DNA in your sample as follows:
DNA concentration (µg/ml) = (OD260 of sample) x (dilution factor) x (50 ug of DNA/ml) / (OD260 unit)

Part3:
Purity of DNA :

OD260/OD280 =1.7 -1.8
If a value out of this range, it may indicate the DNA sample are contaminated (i.e., protein)

Discussion

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