Practical Biochemistry 1
الكيمياء الحيوية العملية 1

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Chemistry Department

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<table>
<thead>
<tr>
<th>Chemical subs.</th>
<th>Reacts with</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>With chromic acid, hydroxy-containing compounds, ethylene glycol, perchloric acid peroxides and permanganates.</td>
</tr>
<tr>
<td>Acetylene</td>
<td>With copper (tubing). Fluoring, bromine, chloride, iodine, silver, mercury, carbon</td>
</tr>
<tr>
<td>Alkali metal</td>
<td>Such as calcium potassium and sodium-with water, carbon dioxide, carbon tetraoxide and other chlorinated hydrocarbons.</td>
</tr>
<tr>
<td>Ammonium-anhydrous</td>
<td>With mercury, halogens, calcium hypochlorite hydrogen fluoride.</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>With acids, metal powders, Flammable liquids, chlorates nitrates, sulfur and finely divided organics or combustible.</td>
</tr>
<tr>
<td>Aniline</td>
<td>With nitric acid, hydrogen peroxide</td>
</tr>
<tr>
<td>Bromine</td>
<td>With ammonia, acetylene, butadiene, butane, hydrogen, sodium carbide. Turpentine and finely divided metals.</td>
</tr>
<tr>
<td>Carbon acetate</td>
<td>With calcium hypochlorite – with all oxidizing agents.</td>
</tr>
<tr>
<td>Chlorates</td>
<td>With ammonium salts, acids, metal powders. Sulfur finely divided organics or combustibles carbon.</td>
</tr>
<tr>
<td>Chromic acid</td>
<td>With acetic acid, naphthalene caniper, alcohol, glycerol turpentine and other flammable liquids.</td>
</tr>
<tr>
<td>Chlorine dioxide</td>
<td>With ammonia, methane, phosphate, hydrogen sulfide.</td>
</tr>
<tr>
<td>Chlorine</td>
<td>With ammonia, acetylene, butadiene, benzene and other petroleum fractions, hydrogen, sodium carbide, turpentine and finely divided powdered metals.</td>
</tr>
<tr>
<td>Copper</td>
<td>With acetylene, hydrogen peroxide.</td>
</tr>
<tr>
<td>Cyanides</td>
<td>With acids and alkali.</td>
</tr>
<tr>
<td>Liquids</td>
<td>With ammonium nitrate, chromic acid, hydrogen peroxide, nitric acid, sodium peroxide and halogens.</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>With copper, chromium, iron, most metal or their respective salts, flammable fluids, and other combustible materials, aniline and nitromethane</td>
</tr>
<tr>
<td>Hydrogen sulfide</td>
<td>With fuming nitric acid, oxidizing gases.</td>
</tr>
<tr>
<td>Hydrocarbons</td>
<td>general With fluorine, chlorine, formine, chromic acid and sodium peroxide.</td>
</tr>
<tr>
<td>Iodine</td>
<td>With acetylene, ammonia</td>
</tr>
</tbody>
</table>
### Chemicals and Reported Effects

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Reported effects</th>
</tr>
</thead>
</table>
| Trichloroethylene (ethylene trichloride) | Acute: Narcotic effects  
                          Chronic: Liver damage, nonspecific neurological impairment |
| m-Xylene (1,3-dimethylbenzene) | Acute: Narcotic effects, headache, dizziness, fatigue, nausea  
                          Chronic: Nonspecific neurological impairment |
| o-Xylene (1,2-dimethylbenzene) | Acute: Narcotic effects, headache, dizziness, fatigue, nausea  
                          Chronic: Nonspecific neurological impairment |
| p-Xylene (1,4-dimethylbenzene) | Acute: Narcotic effects, headache, dizziness, fatigue, nausea  
                          Chronic: Nonspecific neurological impairment |

### Chemicals and Reactions

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercury</td>
<td>With acetylene, fulminic acid hydrogen.</td>
</tr>
<tr>
<td>Nitric acid</td>
<td>With acetic, chromic and hydrocyanic acids, aniline, carbon, hydrogen sulfide, fluids or gases and substances that are readily nitrated.</td>
</tr>
<tr>
<td>Oxygen</td>
<td>With oils, grease, hydrogen, flammable liquids, solids and gases.</td>
</tr>
<tr>
<td>Perchloric acid</td>
<td>With acetic anhydride, bismuth and its alloys, alcohol paper, wood and other organic materials.</td>
</tr>
<tr>
<td>Phosphorus pentoxide</td>
<td>With water.</td>
</tr>
<tr>
<td>Potassium permanganate</td>
<td>With glycerol, ethylene, glycol, benzaldehyde, sulfuric acid.</td>
</tr>
<tr>
<td>Silver</td>
<td>With tartaric acid, ammonium compounds.</td>
</tr>
<tr>
<td>Sodium peroxide</td>
<td>With any substance, for instance, methanol, acetic acid, acetic anhydride, benzaldehyde, carbon disulfide, glycerol, ethylene, glycol, ethyl acetate, furfural.</td>
</tr>
<tr>
<td>Sodium</td>
<td>With carbon tetrachloride, carbon dioxide, water.</td>
</tr>
<tr>
<td>Sulfuric acid</td>
<td>With chlorates, perchlorates, permanganates and water.</td>
</tr>
</tbody>
</table>
How to protect yourself in the laboratory

Safety Rules

Every experiment is designed to minimize hazards, but the following rules are a necessary adjust to that design.

1- Wear safety glasses all times when you are in the laboratory. Those who wear prescription glasses have considerable protection already. At the very least, others should wear inexpensive plastic nonprescription glasses. By "safety glasses" we mean industrial quality eye protective devices meeting the standards of the American standard safety code for head, eye, and respiratory protection.

Those who wear contact Lenses are warned of a special problem. If a chemical splashes onto the eyes, it may seep under the edge of the contact lenses. The lens must be removed as soon as practicable, so that both lens and eye can be thoroughly washed.

2- Learn the exact locations of eyewash fountains, fire extinguishers, fire alarms, fire blankets, and other safety features in your laboratory, as well as how to use these devices. sketch the laboratory and indicate their location.

3- Work only during the scheduled laboratory periods and perform only authorized experiments. Your instructor will advise you about local regulations. An important safety rule, however is never work alone in laboratory. If an accident occurs, the other person may be able to aid you.

4- If you feel faint, sit down right away.

5- If you burned and require the attention of a doctor, has someone accompany you to the doctor's office. Do not apply salves or ointments on the burned areas, let the doctor decide the treatment. Prompt cooling of a burned area with cold water markedly reduces subsequent pain and facilitates heating of the area.

6- Some accidents happen when labels are not read carefully. Get in the habit of reading out loud (but softly) the label of bottle you intend to use.

7- To avoid contamination;
   (a)Discard unused chemicals : do not return them to reagent bottles ;clean up anything you spilled..
   (b)Never put a medicine cropper or a pipette from your desk into a reagent bottle but, instead pour a very small amount of the reagent quantities.
(c) Try to keep inner walls of bottle stoppers or corks from touching tops of desks or shelves where they might pick up dust or other chemicals.

8- Discard all waste solids-water-insoluble chemicals, litmus paper, used matches, broken glass, paper towels-into crocks at the end of your laboratory bench. When sinks are used as wastebaskets they may over flow.

9- Your shoes should cover your feet to protect them from spilled chemicals or dropped objects.

10- If your hair is long, fluffed with chemicals, it is quite flammable. At least pin or tie it back in some way while you work around benzene burner flames.

11- Food and beverage are not allowed in the laboratory.

12- Every time you select a flask, cylinder, or test tube for some experiment, examine it for cracks and broken edges. Sometimes a broken edge can be tolerated, but under no circumstances use a cracked container.

13- Never taste chemical. Check odors only very cautiously.

14- Always pour concentrated acid into water (never water into acid).

15. Mobile phones should be turned off during all lab sessions!
Preparing the lab report..

The following should be included in a given lab report:

- Title of lab session e.g., "Carbohydrates"
- Title and number of the experiment
- The Aim of the experiment
- The principle of the method's used in achieving the experiment
- Results (including pic., calculations if any!)
- Comments
<table>
<thead>
<tr>
<th>List of Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>7</td>
</tr>
<tr>
<td>Lipids</td>
<td>26</td>
</tr>
<tr>
<td>Amino acids</td>
<td>33</td>
</tr>
<tr>
<td>Proteins</td>
<td>41</td>
</tr>
<tr>
<td>Enzymes</td>
<td>50</td>
</tr>
<tr>
<td>Metabolism</td>
<td>54</td>
</tr>
<tr>
<td>Vitamins</td>
<td>57</td>
</tr>
</tbody>
</table>
Carbohydrates

Background

Carbohydrates, or saccharides, are sugars and starches, which provide energy for both humans and animals. Cellulose which makes up many plant structures is also considered carbohydrate. “Carbs,” as they are now commonly referred to, have become both a blessing and a curse, as the process of modern food production has changed the way we consume them.

Definition

Carbohydrates may be defined as polyhydroxy aldehydes or ketones or compounds which produce them on hydrolysis.

Classification

Carbohydrates of physiological importance are classified as

1. Monosaccharides
2. Oligosaccharides
3. Polysaccharides

Monosaccharides

Monosaccharides are the simplest of carbohydrates and they cannot be further hydrolyzed into smaller units. They are found in fruits and dairy products, are more easily digested by the body. They are also often found in processed, refined foods such as white sugar, pastas, and white bread.

The most common monosaccharides of biological importance are glucose, fructose, galactose, mannose, and ribose. The monosaccharides are classified into different categories, based on the functional group and the number of carbon atoms.
Based on Functional Group

**Aldoses:** Possessing aldehyde as functional group,  
* e.g. glyceraldehydes, glucose  
**Ketoses:** Possessing keto as functional group,  
* e.g. dihydroxyacetone, fructose

Based on Number of Carbon Atoms

<table>
<thead>
<tr>
<th></th>
<th>Aldoses</th>
<th>Ketoses</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Triose</strong></td>
<td>Glyceraldehyde</td>
<td>Dihydroxyacetone</td>
</tr>
<tr>
<td><strong>Tetrose</strong></td>
<td>Erythrose</td>
<td>Erythulose</td>
</tr>
<tr>
<td><strong>Pentose</strong></td>
<td>Ribose</td>
<td>Ribulose</td>
</tr>
<tr>
<td></td>
<td>Xylose</td>
<td>Xylulose</td>
</tr>
<tr>
<td><strong>Hexose</strong></td>
<td>Glucose</td>
<td>Fructose</td>
</tr>
<tr>
<td></td>
<td>Galactose</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mannose</td>
<td></td>
</tr>
<tr>
<td><strong>Heptose</strong></td>
<td>Glucoheptose</td>
<td>Sedoheptulose</td>
</tr>
</tbody>
</table>

Oligosaccharides

Oligosaccharides contain 2 to 10 monosaccharide units. Based on the number of monosaccharide units present, the oligosaccharides are further classified into:

- Disaccharides
- Trisaccharides
• Tetrasaccharides

Disaccharides

Disaccharides are formed by the union of two monosaccharide units (glycosidic linkage) with the elimination of one molecule of water. Disaccharides may be reducing or non-reducing depending upon the availability of free (potential) aldehyde/keto group in their molecular structure. The most common disaccharides of biological importance are maltose, lactose, and sucrose.

**Maltose:** Maltose or malt sugar occurs in germinating seeds and also formed during digestion of starch by enzymes or by dilute acids. It is formed by the union of two glucose units by α-1,4 glycosidic linkage. Maltose is a reducing disaccharide, since it contains one free aldehyde group in its molecular structure. It can be hydrolyzed either by acid or by the enzyme maltase of the intestinal juice.

**Lactose:** lactose is present in the milk (milk sugar). Lactose is formed by one molecule of galactose and one molecule of glucose linked β-1,4 glycosidic linkage. It is a reducing disaccharide, since it contains one free aldehyde group in its molecular structure. It can be hydrolyzed either by acid or by the enzyme lactase of the intestinal juice.

**Sucrose:** It is the sweetening agent. Present in the sugar cane. Sucrose is formed by one molecule of glucose and one molecule of fructose linked by α β, 1-2 glycosidic linkage. Sucrose is a nonreducing disaccharide, since the reducing groups of glucose (aldehyde group) and fructose (keto group) are involved in the formation of glycosidic bond. It can be hydrolyzed by acid or by the enzyme sucrose (invertase) of the intestinal juice.
Polysaccharides

They are the macromolecular polymers of monosaccharide units linked by glycosidic linkage with high molecular weight. It takes longer for the body to digest polysaccharides. Examples of polysaccharides such as starch, cellulose, glycogen.

Reducing And Nonreducing Sugars

Sugars exist in solution as an equilibrium mixture of open-chain and closed-ring (or cyclic) structures. In the open-chain form, the carbon atom that contains the C=O bond is called the carbonyl carbon. In the closed-ring (cyclic) structure, the carbonyl carbon is the one which is attached to the O of the ring and an OH group. Sugars that can be oxidized by mild oxidizing agents are called reducing sugars because the oxidizing agent is reduced in the reaction. A non-reducing sugar is not oxidized by mild oxidizing agents. All common monosaccharides are reducing sugars. The disaccharides maltose and lactose are reducing sugars. The disaccharide sucrose is a nonreducing sugar.

Common oxidizing agents used to test for the presence of a reducing sugar are:
- Benedict's reagent
- Fehling's reagent
- Tollen's reagent

Structure of Monosaccharides

When the closed-ring (cyclic) structure of a monosaccharide opens to form a chain, the result may be either

- an aldehyde (alkanal)
- a ketone (alkanone)

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{or} & \\
\text{||} & \quad \text{||} \\
\text{R - C - H} & \quad \text{R - C - R'}
\end{align*}
\]
Sugars that contain an aldehyde group are aldoses, and the ones containing keto group are ketones. A monosaccharide containing 5 carbon atoms is known as a pentose. The open-chain form is therefore either: an aldopentose if it is an aldehyde (alkanal) or a ketopentose if it is a ketone (alkanone).

A monosaccharide containing 6 carbon atoms is known as a hexose. The open-chain form is therefore either: an aldohexose if it is an aldehyde (alkanal) or a ketohexose if it is a ketone (alkanone).

<table>
<thead>
<tr>
<th>Monosaccharide</th>
<th>Glucose</th>
<th>Fructose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Closed-ring (cyclic) Structure</td>
<td><img src="image1" alt="Glucose Structure" /></td>
<td><img src="image2" alt="Fructose Structure" /></td>
</tr>
<tr>
<td>Open-chain Structure</td>
<td><img src="image1" alt="Glucose Structure" /></td>
<td><img src="image2" alt="Fructose Structure" /></td>
</tr>
<tr>
<td>Classification</td>
<td>aldohexose</td>
<td>ketohexose</td>
</tr>
</tbody>
</table>
Testing for the Presence of a Reducing Sugar

The common oxidizing agents used to test for the presence of a reducing sugar:

<table>
<thead>
<tr>
<th>Oxidizing Reagent</th>
<th>Benedict's Solution</th>
<th>Fehling's Solution</th>
<th>Tollen's Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Composition</strong></td>
<td>copper sulfate in alkaline citrate</td>
<td>copper sulfate in alkaline tartrate</td>
<td>silver nitrate in aqueous ammonia</td>
</tr>
<tr>
<td><strong>Color of Solution</strong></td>
<td>deep blue</td>
<td>deep blue</td>
<td>colorless</td>
</tr>
<tr>
<td><strong>Color After Reaction with a Reducing Sugar</strong></td>
<td>brick red precipitate</td>
<td>brick red precipitate</td>
<td>silver mirror forms</td>
</tr>
<tr>
<td></td>
<td>$\text{Cu}_2\text{O}_x$</td>
<td>$\text{Cu}_2\text{O}_x$</td>
<td>$\text{Ag}_x$</td>
</tr>
<tr>
<td><strong>Species Being Reduced</strong></td>
<td>$\text{Cu}^{2+}$</td>
<td>$\text{Cu}^{2+}$</td>
<td>$\text{Ag}^+$</td>
</tr>
<tr>
<td>(the oxidant)</td>
<td>$\text{Cu}^{2+} + e \rightarrow \text{Cu}^+$</td>
<td>$\text{Cu}^{2+} + e \rightarrow \text{Cu}^+$</td>
<td>$\text{Ag}^+ + e \rightarrow \text{Ag}_x$</td>
</tr>
<tr>
<td><strong>Species Being Oxidized</strong></td>
<td>reducing sugar</td>
<td>reducing sugar</td>
<td>reducing sugar</td>
</tr>
<tr>
<td>(the reductant)</td>
<td>oxidized to carboxylate</td>
<td>oxidized to carboxylate</td>
<td>oxidized to carboxylate</td>
</tr>
</tbody>
</table>

Oxidation of Monosaccharides

**Oxidation of Aldoses**

Glucose and galactose are both examples of aldoses.

Oxidation Using Benedict's or Fehling's Solution:

\[
\begin{align*}
\text{O} & \\
\text{||} & \\
\text{R - C - H} & + 2\text{Cu}^{2+} + 5\text{OH}^- \\
\text{deep blue} & \rightarrow \\
\text{R - C - O}^- & + \text{Cu}_2\text{O}_x + 3\text{H}_2\text{O} \\
\text{aldose} & \rightarrow \text{carboxylate}
\end{align*}
\]
Oxidation Using Tollen's Reagent

\[
\begin{align*}
\text{aldose} & \quad + 2\text{Ag}^+ + 3\text{OH}^- \\
\text{colorless} & \quad \rightarrow \\
\text{carboxylate} & \quad \text{silver mirror} \\
\text{R} \quad \text{C} \quad \text{H} & \quad + 2\text{Ag}^+ + 3\text{OH}^- \\
\end{align*}
\]

Oxidation of Ketoses

Fructose is an example of a ketose.

Oxidation Using Benedict's or Fehling's Solution

\[
\begin{align*}
\text{ketose} & \quad + 2\text{Cu}^{2+} + 5\text{OH}^- \\
\text{deep blue} & \quad \rightarrow \\
\text{hydroxy carboxylate} & \quad \text{brick red} \\
\text{R} \quad \text{C} \quad \text{CH}_2\text{OH} & \quad + \text{Cu}_2\text{O} + 3\text{H}_2\text{O} \\
\end{align*}
\]

Oxidation Using Tollen's Reagent

\[
\begin{align*}
\text{ketose} & \quad + 2\text{Ag}^+ + 3\text{OH}^- \\
\text{colorless} & \quad \rightarrow \\
\text{hydroxy carboxylate} & \quad \text{silver mirror} \\
\text{R} \quad \text{C} \quad \text{CH}_2\text{OH} & \quad + 2\text{Ag} + 3\text{OH}^- \\
\end{align*}
\]
Experiments

1. Molisch’s Test:
Molisch’s Test is a sensitive chemical test for all carbohydrates, and some compounds containing carbohydrates in a combined form, based on the dehydration of the carbohydrate by sulfuric acid to produce an aldehyde (either furfural or a derivative), which then condenses with the phenolic structure resulting in a red or purple-colored compound.

Procedure:
- **Apply this test to two different carbohydrate solutions of your own choice**, preferably to one monosaccharide and one polysaccharide.
- Place 2 mL of a known carbohydrate solution in a test tube, add 1 drop of Molisch’s reagent (10% α-naphthol in ethanol).
- Pour 1-2 mL of conc. H$_2$SO$_4$ down the side of the test tube, so that it forms a layer at the bottom of the tube.
- Observe the color at the interface between two layers and compare your result with a control test.

* A brown color due to charring must be ignored and the test should be repeated with a more dilute sugar solution.

2. Anthrone Test

**Principle:** Conc.H$_2$SO$_4$ hydrolyses glycosidic bonds to yield monosaccharides which in the presence of acid get dehydrated to form furfural & its derivatives. These products react with anthrone to give a bluish-green complex.

**Reagents**

1. Conc.H$_2$SO$_4$
2. Anthrone: 0.2% (w/v) in Conc.H$_2$SO$_4$, prepare fresh.
3. Boiling water-Bath
4. Test tube
Method:
1. Add 2ml of anthrone reagent to 1ml of test solution.
2. Keep the test-tube in a boiling H₂O-bath for 10 min.
3. Appearance of bluish-green color indicates the presence of carbohydrate in the sample.

3. Benedict's Test

Benedict's test determines whether a monosaccharide or disaccharide is a reducing sugar, and is hence similar in purpose to the Tollen’s test. To give a positive test, the carbohydrate must contain a hemiacetal which will hydrolyse in aqueous solution to the aldehyde form. Benedict's reagent is an alkaline solution containing cupric ions, which oxidize the aldehyde to a carboxylic acid. In turn, the cupric ions are reduced to cuprous oxide, which forms a red precipitate. This solution has been used in clinical laboratories for testing urine.

\[
RCHO + 2Cu^{2+} + 4OH^- \rightarrow RCOOH + Cu_2O + 2H_2O
\]

Reagents

- Set up 1% solutions of: glucose, sucrose, starch, maltose, fructose, lactose.
- Benedict's reagent

Procedure

1. Place 15 drops of the following 1% carbohydrate solutions in separate, labeled test tubes: glucose, fructose, sucrose, lactose, maltose, and starch.
2. Also place 1 ml of distilled water in another tube to serve as a control.
3. To each tube, add 1 ml of Benedict's reagent and heat the tubes in a boiling water bath for 5 minutes. Remove the tubes from water bath and note and record the results. In the presence of a reducing sugar a precipitate which may be red, yellow or green will form.
4. Barfoed's Test

Barfoed's test is similar to Benedict's test, but determines if a carbohydrate is a monosaccharide or a disaccharide. Barfoed's reagent reacts with monosaccharides to produce cuprous oxide at a faster rate than disaccharides do:

$$RCHO + 2Cu^{2+} + 2H_2O \rightarrow RCOOH + Cu_2O + 4H^+$$

Reagents

- 1% solutions of: glucose, maltose, fructose, lactose, sucrose
- Barfoed's reagent.

Procedure:

1. Place 15 drops of the following 1% carbohydrate solutions in separate, labeled test tubes: glucose, fructose, sucrose, lactose, and maltose.
2. To each tube, add 1 ml of Barfoed's reagent, and heat in a boiling water bath for 10 minutes.
3. Remove the tubes from water bath. Note and record your observations. A red precipitate will form if the test is positive.

5. Picric Acid Test

Picric acid (2,4,6-trinitrophenol) or TNP reacts with reducing sugars to give a red colored picramic acid $\text{C}_6\text{H}_2\text{OH.NH}_2(\text{NO}_2)\text{2}$.
Reagents

- Set up 1% solution of: maltose, sucrose
- Saturated solution of picric acid
- 1 N NaOH solution

Procedure

1. Into a test tube add 1 ml of maltose solution, into the second tube, 1ml of sucrose solution.
2. Add into each tube 1 ml of saturated solution of picric acid, and then add into each tube 0.5 ml of sodium hydroxide solution. Heat both samples in a boiling water bath. In the presence of reducing sugars, the solution stains red; a sodium salt of picric acid is formed.

6. Tollen’s Test (Silver Mirror)

Is used to test for the presence of aldehydes. In this reaction, an aldehyde is oxidized to a carboxylic acid while the Ag⁺ is reduced to silver metal, which deposits as a thin film on the inner surface of the glass. The reaction is as follows:

![Reaction Equation]

Materials:
0.1 M AgNO₃,
0.8 M KOH,
0.5 M Dextrose,
Conc. HNO₃
Conc. NH₄OH
Deionized water, large test tube, rubber, stopper, beaker.

**Prepare Tollen’s reagent as follows:**
- Add 50 mL of 0.1 M AgNO₃ to the beaker and add NH₄OH to this.
- A brown precipitate will form. Continue adding NH₄OH until the solution becomes clear.
- To this, add 25 mL of 0.8M KOH.
- Again, add NH₄OH until solution becomes clear.

**Procedure**

1. Clean the test tube to be used by rinsing with concentrated nitric acid and washing well with hot water.
2. Add 1 mL of sample solution to the test tube.
3. To this add 5 mL of Tollen’s reagent. The solution will turn yellow and brown then become cloudy and dark before silver begins to form on the inside of the test tube. This should take a couple of minutes.
4. Remove the contents from the test tube and rinse the tube with water. The tube with a “silver mirror” can now be observed.

**7. Formation of Osazones (Phenyl Hydrazine Reaction)**

**Background**
- Phenyl hydrazine reacts with monosaccharides.
- Thus monosaccharides can be determined by the reaction with phenyl hydrazine.
- It is possible to isolate the hydrazone of an aldose or ketose.
- During reaction with monosaccharides, additional phenyl hydrazine is consumed in oxidizing the adjacent OH-group to carbonyl group which then forms a second phenyl hydrazone.
• Such bisphenyl hydrazones are called osazones.
• Osazones crystals have characteristic shapes & melting point which assist in the identification of the reducing sugars. The following reaction is known as phenyl hydrazine reaction:

\[
\text{D-glucose} + 3 \text{H}_2\text{NNNH} \rightarrow \text{the osazone of D-glucose}
\]

• Since only C1 & C2 of a saccharide are involved in osazones, sugars with the same configuration at the remaining carbon atom gives the same osazone.
• Osazones are insoluble yellow compounds.
• Depending on the time required to form the insoluble yellow osazone, different sugars can be classified into the following:
  - Mannose: 1-5 min
  - Fructose: 2 min
  - Glucose: 5 min
  - Xylose: 7 min
  - Arabinose: 10 min
  - Galactose: 20 min
• The mechanism of the osazone formation is not fully understood yet.
• Initially the sugar and phenylhydrazine form a phenylhydrazone (glucose → glucose phenyl-hydrazone).
• Upon addition of a second and a third equivalent of phenylhydrazine, the osazone is formed as a yellow solid.
Experiment: Formation of Osazones

Reagents:
- 1% solutions of glucose, galactose, lactose, maltose, mannose, and xylose
- Phenyl hydrazine mixture (2 parts phenyl hydrazine hydrochloride are mixed with 3 parts sodium acetate).

Procedure
1. To 300 mg of phenyl hydrazine mixture add 5 ml of the tested solution,
2. Shake well, and heat on a boiling water bath for 30 – 45 min.
3. Allow the tubes to cool slowly (not under tap) and examine the crystals microscopically, draw the shapes of the crystals.
4. Carefully note the time when the osazone is precipitated and also whether it is formed in hot or cold solution.

8. Bial's (Orcinol) Test For Pentoses

Bial's reagent contains orcinol (5-methylresorcinol) in concentrated HCl with a small amount of FeCl₃ catalyst. Pentoses are converted to furfural by this reagent, which form a blue green color with orcinol. This test is used to distinguish pentoses from hexoses.
Reagents

- Set up 1% solutions of: xylose, arabinose, glucose, fructose, and maltose.
- Bial’s reagent (0.1% orcinol in concentrated HCl containing 0.1% FeCl₃·6H₂O).

CAUTION!
Be extremely careful when heating the test tubes. Bial's reagent contains concentrated hydrochloric acid. Be sure to heat the tube slowly, tilting it slightly and heating along the entire side of the tube. Do not point the tube toward yourself or any of your fellow lab workers.

Procedure

1. Add about 2 ml of 1% xylose, glucose, fructose, maltose, arabinose, and xylose solution to their respective labeled test tubes.
2. Add 3 ml of Bial's reagent to each tube and mix well.
3. Carefully heat each tube (with some agitation) directly over the burner flame. Hold the tube at a diagonal and heat along the sides of the tube rather than at the bottom to prevent eruption of the liquid from the tube. Move the tube diagonally in and out of the flame, until the mixture just begins to boil. Stop heating when the mixture begins to boil.
4. Record your observations. A blue-green color indicates a positive result. Prolonged heating of some hexoses yields hydroxymethyl furfural which also reacts with orcinol to give colored complexes.

9. Aniline Acetate Test For Pentoses

The furfural produced by the reaction of hot dilute HCl on pentoses forms a bright red color with aniline acetate in a test paper held over the mouth of the reaction flask.

Reagents

1% solution of glucose, fructose, lactose, xylose, & arabinose.
aniline solution is prepared as follows:
- shake 5 ml of aniline with 5 ml of water and add 5 ml of glacial acetic acid to adjust clear the emulsion.

**Procedure**

1. Place 5 ml of solution to be tested and 20 ml water in 250 ml erlenmeyer flask.
2. Add 20 ml of conc. HCl and boil gently for about 1 min.
3. Cease heating, hold a filter paper moistened with a few drops of aniline acetate over the mouth of the flask.
4. Bright red color on a filter paper indicates a positive result.

10. **Seliwanoff’s (Resorcinol) Test**

The Seliwanoff’s reagent contains resorcinol in 6 M hydrochloric acid. Hexoses undergo dehydration when heated in this reagent to form hydroxymethylfurfural that condenses with resorcinol to give a red product. Ketohexoses (such as fructose) and disaccharides containing a ketohexose (such as sucrose) form a cherry-red condensation product. Other sugars may produce yellow to faint pink colors.

**Reagents**

- Set up 1% solution of: glucose, sucrose, fructose, lactose, and maltose.
- Seliwanoff’s reagent (0.5% resorcinol in 3N HCl).

**Caution:** Seliwanoff’s reagent is caustic, rinse thoroughly with water if you get this solution on your skin or clothing.
Procedure

1. Add about 3 ml of Seliwanoff’s reagent to each labeled test tube.
2. Add 1 drop of the respective sugar solution to the appropriate test tubes and mix well.
3. Place all the test tubes in the boiling water bath at the same time and heat for 3 min after the water begins to boil again.
4. Record your observations. A positive result is indicated by the formation of a red color with or without the separation of a brown-red precipitate.

Polysaccharides

1. Iodine Test for Polysaccharides
   - Iodine forms colored adsorption complexes with polysaccharides
   - Blue color with starch
   - Red-blue or purple color with dextrin
   - Red color with glycogen
   - With cellulose it gives no color

Reagents
   - 1% of solutions
   - Iodine solution (2% KI containing sufficient I₂ to color it deep yellow)

Procedure

1. In a suitable plate place a small amount of the tested solution.
2. Add 1 to 3 drops of a dilute solution of Iodine solution.
3. Compare the color obtained with that of water and iodine solution.
2. **Hydrolysis of Starch**

**Reagents:**
- 1% starch solution,
- Benedict’s solution
- Conc. HCl

**Procedure**
1. Place 25 ml of 1% starch solution in a small beaker, add 10 drops of conc. HCl & boil gently.
2. At the end of each min transfer 1 drop of the solution to the test plate and make the regular iodine test.
3. Also at the end of each min transfer 3 drops of mixture to 5 ml portions of Benedict’s solution in a series of test tubes.
4. As the test proceeds, the reaction with iodine should become weaker & finally be negative.
5. At this point place all test tubes containing Benedict’s solution in a boiling water-bath for 3 min.
6. Remove the tubes then allow to cool.
7. Note the degree of reduction in each case compare with the rate of the disappearance of iodine reaction.

3. **Mucic Acid Test For Galactose**

Galactose & any sugar gives galactose upon hydrolysis such as lactose, raffinose and some gums give mucic acid on oxidation with HNO₃. Conc. HNO₃ oxidizes galactose to mucic acid (a fine white gritty crystals separate, insoluble in water, but readily soluble in alkaline or ammonium carbonate solution & re-precipitated on the addition of nitric acid.

![Galactaric Acid](image)

**galactaric acid**
Reagents

- 2% solution of Galactose
- Conc. HNO₃

Procedure

1. To 10 ml of 2% of galactose solution in porcelain evaporating dish add 5ml conc. HNO₃
2. Place the dish on top of a beaker filled with 2/3 distilled water.
3. Evaporate (under fume hood) the contents of the dish on the boiling water bath to about a volume of 2 ml (it takes about 45 min boiling to reach the 2 ml).
4. Remove the hot dish with tong and allow to cool.
5. add 5 ml of distilled water to the dish and mix well with a string rod.
6. Note the insoluble mucic acid crystals from the oxidation of galactose.
7. Transfer one drop of the liquid plus crystals to a clean microscope slide, examine with a low power microscope (10X).
8. Note the gritty crystals of mucic acid.
Lipids

Background

Lipids are a large and diverse group of naturally occurring organic compounds that contain fatty acids or a steroid nucleus. They are soluble in organic solvents. Can be extracted from cells using organic solvents.

Lipids includes:
   a. Fats and oils (triglycerides)
   b. Waxes
   c. Fat-soluble vitamins (A, D, E, K)
   d. Phospholipids
   e. Steroids
   f. Mono- and di-glycerides

These are soluble in non-polar organic solvents (e.g. ether, chloroform, acetone & benzene) and generally insoluble in water. Lipids can be classified as:
1. Simple lipids (esters of fatty acids with alcohols) e.g. triglycerides like fats and oils, waxes.
2. Complex lipids (e.g. phospholipids and Glycolipids).

Chemical Tests for Lipids

1. Solubility in Polar and Non-polar Solvents: Lipids are insoluble in polar solvents like water and soluble in non-polar solvents like petroleum ether, benzene and mineral oil.

Reagents

- Olive oil (vegetable oil), butter (animal fat),
- stearic acid (saturated fatty acid), oleic acid (unsaturated fatty acid),
- Solvents: dilute acid and alkali solutions, cold alcohol, hot alcohol, benzene, chloroform, ether and carbon tetrachloride.
Procedure

1. Add a few drops of the liquid fat or 0.5 g of the solid fat in labeled test tubes.
2. To each test tube add a 5 ml of solvent and write down your observations.
3. Repeat the experiment with a different solvent and make your observations.

2. Emulsification

Reagents

- Neutral olive oil (may be prepared by shaking ordinary olive oil with 10 % solution of sodium carbonate, this mixture should then be extracted with ether and the ether should be removed by evaporation).
- 0.5 % Na_2CO_3,
- Rancid olive oil (to prepare rancid olive oil add 5 drops of oleic acid to 10 ml of olive oil, mix well).

Procedure

1. Shake up a drop of neutral olive oil with a little water in a test tube, the fat becomes finally divided forming an emulsion, upon standing fat separates and rises to the top.
2. To 5 ml water in a test tube add 2 to 3 drops of 0.5 percent Na_2CO_3 introduce into this alkaline solution, a drop of neutral olive oil and shake, the emulsion is not permanent and is not so transitory.
3. Repeat step 2 using rancid olive oil, in this case: the alkali combines with the free fatty acids to form soap and the soap being an emulsifying agent, emulsifies the fat.

3. Oxidation of Unsaturated Fatty Acids

Unsaturated fatty acids have one or more double bonds. The examples of most important unsaturated fatty acids are: palmitoleic acid (Δ^9), oleic acid (Δ^9), nervonic acid (Δ^15), linoleic acid (Δ^9,Δ^12), linolenic acid (Δ^9,Δ^12,Δ^15), arachidonic acid (Δ^5,Δ^8,Δ^11,Δ^14).
Procedure

1. Into a test tube place one drop of oil and 3 ml of Na₂CO₃ solution.
2. Warm it slightly and add drop by drop solution of KMnO₄ (potassium permanganate).
   After each drop of KMnO₄ the violet color disappears. The end of a reaction is recognized by precipitation of brown solid MnO₂.

4. Lecithin as Emulsifying Agent

Lecithin (phosphatidyl choline) is present in nerve, brain and other tissues of animals, plants. Egg yolk contains lecithin. It has a polar head (ionic) and 2 nonpolar hydrocarbon tails (fatty acid units) which make it an excellent emulsifying agent.

Procedure

- A. Preparation of lecithin:
  - Place an egg yolk in a 250 ml Erlenmeyer flask, add 50 ml of ether, shake vigorously.
  - Filter the mixture through a dry paper into a beaker and evaporate the ether on a steam-heated sand bath or water-bath.
  - Cool the solution, then add about 25 ml of acetone, stir until the precipitate clumps and sticks to the beaker, then pour off the acetone.
  - Transfer ½ of the precipitate to a test tube, add 1 ml of water and 1 ml of vegetable oil, stopper the tube and shake very vigorously.
  - Repeat the above step in another test tube but without lecithin. Compare the two test tubes after 5 min. Make your comments
5. Rancidity
Development of unpleasant smells in fats and oils often accompanied by changes in their texture and appearance. Two types:
- Hydrolytic rancidity
- Oxidative rancidity

Reagents
Fresh olive oil, rancid olive oil, oleic acid, litmus paper

Procedure
Try the reaction of fresh olive oil, oleic acid to litmus paper.
Repeat the test with rancid oil.
What is the reaction of a fresh lipid and that of rancid lipid?

6. Acid Value (free fatty acids) Determination

Acid value is a measure of the extent to which the glycerides have been hydrolyzed by a lipase action. Hydrolysis is accelerated by heat and light. Acid value is an index of the efficiency of oil refining during which the free fatty acids are removed and the acid value falls to very low values. Rancidity is usually accompanied by formation of free fatty acids (indication of a deterioration of oils in storage conditions). Acid value is expressed as mgs of KOH required to neutralize the free fatty acid in 1 g of the fat.
Result is often expressed as the percentage of free fatty acids and the free fatty acid is usually calculated as oleic acid.

1 ml 0.1 N KOH = 0.0282g oleic acid

Materials
Oil sample, 0.1 N KOH, 1% phenolphthalein solution, Ether, Ethanol, 250 Erlenmeyer flask, burette.
Procedure
1. Mix 25 ml of ether with 25 ml of ethanol, and 1-2 drops phenolphthalein in an erlenmeyer flask.
2. Dissolve 25 g of the oil in the mixed neutral solvent.
3. Titrate with 0.1 N KOH, shaking constantly until pink color which persists for 15 sec.
4. Calculate the acid value and the percentage of free fatty acid in the tested sample.

Calculations

\[
\text{Acid value} = \frac{\text{Titration (ml of 0.1 N KOH)} \times 5.61}{\text{Wt. of sample used}}
\]

\[
\% \text{ F.F.A} = \frac{\text{Titration (ml of 0.1 N KOH)} \times 0.0282 \times 100}{\text{Wt. of sample used}}
\]

Peroxide Value Determination
Peroxide value is the concentration of (-O-O-) groups in edible oils. It is a measurement of the decomposition of the product. In many countries, official standards specify a maximum peroxide number beyond which the oil is unfit for human consumption. Formation of peroxide during storage of oil or fat may occur after few weeks to several months according to the conditions of storage.

The peroxide number is therefore measured by oil manufacturers during production and after storage to check its preservation. International standards use a redox titration in non-aqueous media. Results are generally expressed in μg of peroxide (or active oxygen) per gram of product. But mmoles/kg or meq of O₂/kg are also used. Peroxide value is determined volumetrically. Reaction of KI in acid solution with the bound oxygen, followed by titration of the liberated I₂ with sodium thiosulfate. Chloroform is used as a solvent.
Fresh oil has peroxide value below 10 meq/kg, while rancid taste often begins to develop when the peroxide value is between 20 & 40 meq/kg.

Peroxide number determination involves a two-step redox reaction:

1) Reaction of peroxide group with an excess of iodide ion according to:

\[
R\text{-}O\text{-}O\text{-}R + 2I^- + 2H^+ \rightarrow 2ROH + I_2
\]

2) Titration of I\(_2\) with Na\(_2\)S\(_2\)O\(_3\) solution 0.002N, according to:

\[
I_2 + 2S_2O_3^{2-} \rightarrow 2I^- + S_4O_6^{2-}
\]

**Materials**

- 250 ml Erlenmeyer or volumetric flask, chloroform, fresh saturated aqueous KI solution (15g /10 ml H\(_2\)O) store in dark, glacial acetic acid, 0.1 M thiosulfate, starch, and oil sample.

**Procedure**

1. Weigh 1 to 4 g oil sample into 250 ml flask.
2. Add 10 ml chloroform, dissolve the oil by swirling
3. Add 15 ml of glacial acetic acid
4. Add 1 ml of a fresh saturated aqueous KI solution
5. stopper the flask, shake for 1 min and place the flask for 5 min in dark
6. Add about 75 ml distilled, mix and titrate (Vml) the formed I\(_2\) with 0.002 N solution of thiosulfate using starch solution (1%) as indicator.
7. Carry out a blank titration (V\(_0\)ml) which should not exceed 0.5 ml of 0.002 N thiosulfate solution.

**Calculation**

\[
\text{Peroxide value} = \frac{(V - V_0) \times 1000 \text{ meq/Kg}}{\text{weight of sample (g)}}
\]

- Where T is the exact molarity of thiosulfate solution.
Formation of Acrolein

**Reagents**
Olive oil, melted butter, potassium bisulfate $\text{KHSO}_4$, Schiff’s reagent.
Schiff's reagent is a solution that will combine chemically with aldehydes to form a bright red product.

![Acrolein Formation Reaction](image)

**Procedure**
1. Place about 1 g powdered $\text{KHSO}_4$ in a clean test tube.
2. Add 3–4 drops of olive oil (0.5 g melted butter) on the salt and heat.
3. Note the irritating odor of acrolein aldehyde or aldehyde which will color a filter paper moistened with Schiff’s reagent bright red.

Saponification (Formation of a soluble soap & insoluble soap)

**Reagents**
Olive oil, cow fat, 5% KOH solution, 2% MgSO$_4$ solution.

**Procedure**
1. Add 10 ml of olive oil or 10 g of cow fat in a 250 ml beaker.
2. Add 50 ml KOH solution.
3. Add 150 ml dist., water.
4. Hydrolyze the lipids by heating the beaker nearly the boiling point for 3–5 min.
5. Transfer a few amount of the beaker content into about 30 ml of dist., water.
6. Observe if any saponification has occurred (indicated by the complete solubility of the solution when fall into a dist., water).
7. To form insoluble soap, add a few mls of 2% MgSO$_4$ solution to the soap solution, while precipitate indicate formation of magnesium salts of fatty acids (insoluble soap).
Amino Acids

Background

The food we consume is divided into three main classes: carbohydrates, the body’s most readily available energy source; lipids, the body’s principal energy reserve; and proteins, the body’s source of energy for growth and cellular maintenance. Proteins also make up the second largest portion of cells, after water. They are large polymeric compounds that cells synthesize from various building blocks called amino acids. The general structural formula for an amino acid is shown in figure 1. Note that all amino acids contain carboxylic acid groups (-COOH), amino groups (-NH₂), and substituent, or replaceable, side chains (-R).

![Figure 1: General structural formula for an amino acid](image)

Twenty different amino acids, which differ only in the structure of their side chains, are used by human cells to build proteins. The side chain structure determines the class of the amino acid: non-polar, neutral, basic, or acidic. Human cells can synthesize most the amino acids needed to build proteins. However, about 9 amino acids, called essential amino acids cannot be synthesized by human cells and must be obtained from food. Amino acids incorporated into proteins are covalently linked by peptide bonds. Peptide bonds are amide bonds formed between the carboxylic acid group of one amino acid and the amino group of a second amino acid. Figure 2 shows a peptide linkage formed between two amino acids. The peptide bond is indicated. Proteins are composed of hundreds of amino acids linked by peptide bonds, forming a peptide chain. We define the direction in which the amino acids link by referring to the two ends of the chain as the N-Terminus and the C-Terminus. The N-Terminus is the terminal amino acid in the
chain that contains the only amino group not part of a peptide bond. The C-Terminus is the other terminal amino acid in the chain, containing the only carboxylic acid group not part of a peptide bond. The number of constituent amino acids and the order in which they are linked from the N-terminus, are referred to as the protein’s primary structure.

Figure 2: Formation of a peptide bond, dipeptide, and polypeptide

Chemical Reactions of Amino Acids and Protein Functional Groups

Certain functional groups in amino acids and 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 experiment, various color-producing reagents (dyes) will be used to qualitatively detect the presence of certain functional groups in amino acids and proteins.
1. Ninhydrin Test

Amino acids contain a free amino group and a free carboxylic acid group that react together with ninhydrin to produce a colored product. When an amino group is attached to α-carbon atom on the amino acid’s carbon chain, the amino group’s nitrogen is part of a blue-purple product, as shown in figure 4. Proteins also contain free amino groups on the α-carbon and can react with ninhydrin to produce a blue-purple product. Amino acids that have secondary amino groups also react with ninhydrin. However, when the amino group is secondary, the condensation product is yellow. For example the amino acid proline, which contains a secondary amino group, reacts with ninhydrin, as shown in figure 4. Blue-purple and yellow reaction products positively identify free amino groups on amino acids and proteins.
Reagents

6 clean test tubes, 2 % glycine solution, 1 % tyrosine solution, 2 % proline solution, 2 % casein solution, 2 % gelatin solution, 2 % albumin solution, 0.5 % ninhydrin in ethanol solution.

**Caution:** Ninhydrin-ethanol reagent is flammable, toxic, and irritant. Keep away from Bunsen burner flames. Prevent eye, skin, clothing contact. Avoid inhaling the vapors or ingesting the reagents.

Procedure

1. Label 6 cleaned, drained test tubes with the names of the following solutions: 2 % glycine, 1 % tyrosine, 2 % proline, 2 % casein, 2 % gelatin, 2 % albumin.
2. Place 15 drops of each solution in the corresponding test tube.
3. To each of the test tubes add 5 drops of 0.5 % ninhydrin reagent solution.
4. Place the test tubes into the boiling-water bath for 5 minutes. Remove the test tubes from the water bath and place them in a test tube rack. Record your observations.

2. Biuret Test

The copper atoms of Biuret solution (CuSO$_4$ and KOH) will react with peptide bonds, producing a color change. A deep violet color indicates the presence of proteins and a light pink color indicates the presence of peptides. Figure 5 shows the general Biuret complex with protein.

![Biuret complex with protein](image)

Figure 5: Biuret complex with protein
3. Millon’s Test

Millon's test is given by any compound containing a phenolic hydroxyl group. Consequently, any protein containing tyrosine will give a positive test of a pink to dark-red color. The Millon’s reagent is a solution of mercuric and mercurous ions in nitric and nitrous acids. The red color (either a precipitate or a solution, both positive results) is probably due to a mercury salt of nitrated tyrosine. Proteins that contain tyrosine will therefore yield a positive result. However some proteins containing tyrosine will initially form a white precipitate that turns red when heated, while others form a red solution immediately. Note that any compound with a phenol group will yield a positive test, so one should be certain that the sample being tested does not contain any phenols other than those present in tyrosine.

Caution: Millon’s reagent contains mercury and HNO₃ and is very toxic, corrosive, a strong oxidant, an irritant, and can cause burns.

Procedure

1. Place 2 ml of casein, 2% egg albumin, and 0.1 M tyrosine into separate labeled test tubes.
2. Add 3 drops of Millon's reagent and immerse the tubes in a boiling water bath for 5 minutes.
3. Cool the tubes and record the colors formed.

4. Xanthoproteic Test

Some amino acids contain aromatic groups that are derivatives of benzene. These aromatic groups can undergo reactions that are characteristic of benzene and its derivatives. One such reaction is the nitration of a benzene ring with nitric acid. The amino acids tyrosine and tryptophan contain activated benzene rings and readily undergo nitration. The amino acid phenylalanine also contains a benzene ring, but the ring is not activated and therefore does not
undergo readily nitration. The nitration reaction, when used to identify the presence of an activated benzene ring, is commonly known as the xanthoproteic test, because the product is yellow. The intensity of the yellow color deepens when the reaction occurs in basic solution. The xanthoproteic test for tyrosine is shown in figure 6. The reaction is one of these that occur if one spills a concentrated solution of nitric acid onto someone’s skin. The proteins in skin contain tyrosine and tryptophan, which become nitrated and turn yellow.

![Figure 6: Nitrated Tyrosine (a) and Tryptophan (b)](image)

**Reagents**

1% egg albumin solution, 1% peptone solution, 1% gelatin solution, 1% casein solution.

Concentrated HNO$_3$ (or 65%)

6 N sodium hydroxide solution.

**Procedure**

1. In a test tube containing 2 ml of a protein solution, add 1 ml of a concentrated HNO$_3$.
2. The formed white precipitate, will turn yellow upon heating, and finally will dissolve giving a yellow color to the solution.
3. Cool the solution down. Carefully add 3 ml of 6 N NaOH. Note that the yellow color turns orange.

**Comment**

Using a concentrated nitric acid the aromatic rings of amino acids like tyrosine and tryptophan are nitrated. The nitro derivate shows an intensely yellow color. Because nearly all proteins contain aromatic rings it is taken as a protein-test either.
5. Hopkins-Cole Test (Glyoxylic Acid Reaction)

The Hopkins-Cole test is specific for tryptophan, the only amino acid containing indole group. The indole ring reacts with glyoxylic acid in the presence of a strong acid to form a violet cyclic product. The Hopkins-Cole reagent only reacts with proteins containing tryptophan. The protein solution is hydrolyzed by the concentrated H$_2$SO$_4$ at the solution interface. Once the tryptophan is free it reacts with glyoxylic acid to form violet product.

Reagents

- Solutions of 2% egg albumin, 2% gelatin, 2% Casein, 2% peptone, 2% tryptophan, 2% tyrosine.
- Concentrated H$_2$SO$_4$
- Hopkins-Cole reagent

Procedure

1. To 2 ml of the solution under examination add an equal volume of Hopkins-Cole reagent in a test tube and mix thoroughly.
2. Incline the tube and let 5 to 6 ml of conc. H$_2$SO$_4$ acid flow slowly down the side of the test tube, thus forming a reddish-violet ring at the interface of the two layers.

6. Sulfur Test

The presence of sulfur-containing amino acids such as cysteine can be determined by converting the sulfur to an inorganic sulfide through cleavage by base. When the resulting solution is combined with lead acetate (CH$_3$COOPb), a black precipitate of lead sulfide is formed.

Sulfur-containing protein → NaOH → S$^{2-}$ ---- Pb$^{2+}$ → PbS
Procedure

1. Place 1 ml of 2% casein, 2% egg albumin, 2% peptone, 2% gelatine and 0.1 M cysteine into separate, labeled test tubes.
2. Add 2 ml of 10 % aqueous sodium hydroxide. Add 5 drops of 10 % lead acetate solution.
3. Stopper the tubes and shake them. Remove the stoppers and heat in a boiling water bath for 5 minutes. Cool and record the results.

7. Sakaguchi Test for Arginine

The Sakaguchi test is a specific qualitative test for the detection of a specific type of protein with the amino acid containing the guanidinium group (e.g. arginine). In basic conditions, α- naphthol and sodium hypobromite/chlorite react with the above mentioned compound to form red orange complexes. To avoid subsequent oxidation (which is responsible for losing color), conc. Ammonia is added to the solution.

Reagents

- Solutions of 1 % albumin, 1% casein, 1% gelatin, 1% peptone.
- 3 N NaOH solution,
- 0.1 % α-naphthol solution,
- sodium hypobromite solution NaOBr (2 g Br₂ in 100 ml of 5 % NaOH - Keep in dark colored bottle).

Procedure

2. To 1 ml of the protein solution add 1 ml of 3 N NaOH solution, 0.5 ml of 0.1 % α-naphthol solution, and a few drops of 2 % hypobromite solution.
3. The presence of a guanidinium group in the compound under examination will be confirmed by the formation of a red color.
1. Precipitation of Proteins at Isoelectric Point

A. Protein solubility

The solubility of proteins in aqueous buffers depends on the distribution of hydrophilic and hydrophobic amino acid residues on the protein’s surface. Proteins that have high hydrophobic amino acid content on the surface have low solubility in an aqueous solvent. Charged and polar surface residues interact with ionic groups in the solvent and increase solubility. Knowledge of amino acid composition of a protein will aid in determining an ideal precipitation solvent and method.

B. Isoelectric point precipitation

Isoelectric point (pI) is the pH-value of a solution at which the total net charge of a protein equals zero. At a solution pH that is above the pI the surface of the protein is predominantly negatively charged and therefore like-charged molecules will exhibit repulsive forces. Likewise the surface of the protein is predominantly positively charged at a solution pH that is below the pI, and repulsion between proteins occurs. However, at the pI the negative and positive charges are eliminated, repulsive electrostatic forces are reduced and the dispersive forces predominate. The dispersive forces will cause aggregation and precipitation. The pI of most proteins ranges between the pH 4 to 6.

When microorganisms grow in milk, they often produce acids and lower the pH of the milk. The phenomenon of precipitation or coagulation of milk protein (casein) at low pH as milk becomes spoiled is one of the common examples of protein isolation due to changes in the pH.

Reagents & Instruments

- Casein powder, solutions of 1 N NaOH, 1 N CH₃COOH, H₂O₂, Volumetric flask
Procedure

1. Into a 50 ml volumetric flask add 20 ml of water.
2. Add 0.25 g of pure casein, followed by the addition of 5 ml of 1 N NaOH solution.
3. Once casein is dissolved, add 5 ml of 1 N acetic acid solution, then dilute with H₂O to 50 ml and mix well. The resulted solution is a 0.1 N casein acetate sodium.
4. Setup a series of 9 test tubes as shown in the table below.
5. In the first test tube put 3.2 ml 1 N CH₃COOH, and 6.8 ml H₂O and mix thoroughly.
6. In each of the other test tubes (2-9) put 5 ml H₂Oₐ.
7. From the test tube 1 transfer 5 ml to the test tube 2, and mix thoroughly.
8. Repeat step 7 for the rest of test tubes (3 - 9).
9. Now to each test tube (1 -9) add 1 ml of the casein acetate sodium solution, and shake the test tubes immediately.
10. Let the samples stand for 30 min, and note the turbidity in the 9 test tubes.
11. Use + and – signs to describe the turbidity in the different test tubes.
12. You should observe the most precipitation in the test tube which has the pH around 4.7 (close to the isoelectric point of casein).

<table>
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<th>2</th>
<th>3</th>
<th>4</th>
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2. Isolation of Casein from Milk

Background

Milk is a food of exceptional interest. Not only is milk an excellent food for the very young, but humans have also adapted milk, specifically cow’s milk, as a food substance for persons of all ages. Many specialized milk products like cheese, yogurt, butter, and ice cream are staples of our diet. Milk is probably the most nutritionally-complete food that can be found in nature. This property is important for milk, since it is the only food young mammals consume in the nutritionally significant weeks following birth. Whole milk contains vitamins (principally thiamine, riboflavin, pantothenic acid, and vitamins A, D, and K), minerals (calcium, potassium, sodium, phosphorus, and trace metals), proteins (which include all the essential amino acids), carbohydrates (chiefly lactose), and lipids (fats). The only important elements in which milk is seriously deficient are iron and Vitamin C. Infants are usually born with a storage supply of iron large enough to meet their needs for several weeks. Vitamin C is easily secured through an orange juice supplement. There are three kinds of proteins in milk: caseins, lactalbumins, and lactoglobulins. All are globular. The average composition of the milk of each of several mammals is summarized in the following table.

<table>
<thead>
<tr>
<th></th>
<th>Cow</th>
<th>Human</th>
<th>Goat</th>
<th>Sheep</th>
<th>Horse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>87.1</td>
<td>87.4</td>
<td>87.0</td>
<td>82.6</td>
<td>90.6</td>
</tr>
<tr>
<td>Proteins</td>
<td>3.4</td>
<td>1.4</td>
<td>3.3</td>
<td>5.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Fats</td>
<td>3.9</td>
<td>4.0</td>
<td>4.2</td>
<td>6.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>4.9</td>
<td>7.0</td>
<td>4.8</td>
<td>4.5</td>
<td>5.9</td>
</tr>
<tr>
<td>Minerals</td>
<td>0.7</td>
<td>0.2</td>
<td>0.7</td>
<td>0.9</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Casein

Is a phosphoprotein, which has phosphate groups attached to some of the amino acid side chains. These are attached mainly to the hydroxyl groups of the serine and threonine. Actually, casein is
a mixture of at least three similar proteins, which differ primarily in molecular weight and amount of phosphorus they contain (number of phosphate groups).

<table>
<thead>
<tr>
<th>Casein</th>
<th>MW Kd</th>
<th>Phosphate groups/molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>27.3</td>
<td>9</td>
</tr>
<tr>
<td>β</td>
<td>24.1</td>
<td>4-5</td>
</tr>
<tr>
<td>κ</td>
<td>8.0</td>
<td>2</td>
</tr>
</tbody>
</table>

Casein exists in milk as the calcium salt, calcium caseinate. This salt has a complex structure. It is composed of α, β, and κ caseins which form a micelle, or a solubilized unit. Neither the α nor the β casein is soluble in milk, singly or in combination. If κ casein is added to either one, or to a combination of the two, however, the result is a casein complex that is soluble owing to the formation of the micelle. A structure proposed for the casein micelle is shown in the following figure. The κ casein is thought to stabilize the micelle. Since both α and β casein are phosphoproteins, they are precipitated by calcium ions.

![Protein structure](image)

The κ casein protein, however, has fewer phosphate groups and a high content of carbohydrate bound to it. It is also thought to have all its serine and threonine residues (which have hydroxyl groups), as well as its bound carbohydrates, on only one side of its outer surfaces. This portion of its outer surface is easily solubilized in water since these polar groups are present. Calcium caseinate has its isoelectric (neutrality) point at pH 4.6. Therefore, it is insoluble in solutions of pH less than 4.6. The pH of milk is about 6.6; therefore casein has a negative charge at this pH and is solubilized as a salt. If acid is added to milk, the negative charges on the outer surface of the micelle are neutralized (the phosphate groups are protonated) and the neutral protein precipitates:

\[ \text{Ca}^{2+}\text{Caseinate} + 2\text{HCl} \rightarrow \text{Casein} \downarrow + \text{CaCl}_2 \]
The calcium ions remain in solution. When milk sours, lactic acid is produced by bacterial action (see below), and the consequent lowering of the pH causes the same clotting reaction.

The casein in milk can also be clotted by the action of an enzyme called rennin. Rennin is found in the fourth stomach of young calves. However, both the nature of the clot and the mechanism of clotting differ when rennin is used. The clot formed using rennin, calcium paracaseinate, contains calcium.

\[
\text{Ca}^{2+}\text{Caseinate} + \text{rennin} \rightarrow \text{Ca}^{2+}\text{Paracaseinate} + \text{a small peptide}
\]

Rennin is a hydrolytic enzyme (peptidase) and acts specifically to cleave peptide bonds between phenylalanine and methionine residues. It attacks the κ casein, breaking the peptide chain so as to release a small segment of it. This destroys the water-solubilizing surface of the κ casein, which protects the inner α and β caseins and causes the entire micelle to precipitate as calcium paracaseinate. Milk can be decalcified by treatment with oxalate ion, which forms an insoluble calcium salt. If the calcium ions are removed from milk, a clot will not be formed when the milk is treated with rennin. The clot, or curd, formed by the action of rennin is sold commercially as cottage cheese. The liquid remaining is called the whey. The curd can also be used in producing various types of cheese. It is washed, pressed to remove any excess whey, and chopped. After this treatment, it is melted, hardened and ground. The ground curd is then salted, pressed into molds, and set aside to age.

**Reagents**

20 ml milk, Glacial acetic acid (100%), Ethanol (95% v/v), Ether, Thermometer to 100 °C.

**Procedure**

1. Place 20 ml (20 g) of milk into a 125 ml flask and heat at 40 °C in a water bath.
2. Add 5 drops of glacial acetic acid and stir for about 1 min.
3. Filter the resulting mixture through 4 layers of cheesecloth held in a funnel and gently squeeze out most of liquid.
4. Remove the solid (casein and fat) from the cheesecloth, place it into a 100 ml beaker and add 10 ml of 95% ethanol.

5. Stir well to break up the product. Pour off the liquid and add 10 ml of 1:1 ether-ethanol mixture to the solid. Stir well and filter through 4 layers of cheesecloth.

6. Let the solid drain well, then scrape it into a weighed filter paper and let it dry in the air.

7. Calculate the casein percentage in milk as follows:

\[
\text{% Casein} = \frac{\text{grams of casein}}{\text{grams of milk}} \times 100
\]

Casein is used to make white glue, so it is important that you don’t leave it on the filter paper or it will become glued to it! Calculate the % weight of casein isolated from the powdered milk.
Protein Precipitation

Proteins are precipitated from solution by:

1. Certain acids some of which are alkaloidal reagents such as picric-, and tannic acid
2. Salts of heavy metals such as HgCl$_2$, AgNO$_3$, CuSO$_4$, Pb(C$_2$H$_3$O$_2$)$_2$ etc.).
3. By conc. Solution of (NH$_4$)$_2$SO$_4$, Na$_2$SO$_4$, NaCl,
4. By dehydrating agents such as alcohol (ethanol, methanol), acetone.
5. Isoelectric point.

Experiments

1. Effect of strong Acids and Alkali

   **Reagents:**
   - 1% solution of egg albumin,
   - conc. HNO$_3$, H$_2$SO$_4$, HCl, and acetic acids,
   - conc. NaOH

   **Procedure**

   1. Place 2 ml of conc. HNO$_3$ in a test tube, incline the tube and add the dilute albumin slowly from a pipette, allowing the solution to run down the side of the tube and form a layer over the nitric acid.
      Note the appearance of a protein precipitate at the zone of contact between the two fluids.
   2. Now mix the contents of the tube thoroughly by careful shaking. Is protein precipitated by conc. HNO$_3$?
   3. Repeat the above steps using conc. H$_2$SO$_4$, conc. HCl, acetic acid, and NaOH.
      Observe what happens in each case, then compare it with the first experiment with HNO$_3$.

2. Precipitation by Metallic Salts

   **Reagents**
   1% Solution of egg albumin, 5% solution HgCl$_2$, CH$_3$COOPb, CuSO$_4$, 1% solution of AgNO$_3$. 
Procedure

1. Prepare 4 test tubes, each containing 2 to 3 ml of dilute albumin sol., to the first test tube add HgCl₂ sol., drop wise slowly until an excess of the reagent has been added.
2. Record your observations.
   Unless the reagent is added very slowly, the formation of the precipitate may not be noted, due to its solubility in excess of the reagent.
3. Repeat the above exp. With CH₃COOPb, CuSO₄, and AgNO₃

3. Precipitation by Alkaloidal Reagents

Reagents
1% albumin solution, sat. sol. Of picric acid, 10% trichloroacetic acid solution (TCA), tannic acid sol., Phosphotungstic acid sol.

Procedure

1. Prepare 4 test tubes each containing 2 to 3 ml of albumin sol.
2. To the first test tube add picric acid drop wise, until an excess of the reagent has been added.
3. Note any change that may occur.
4. Repeat the exp. With Trichloroacetic acid sol., Tannic acid sol., phosphotungstic acid sol.
5. Are these precipitates soluble in excess of the reagent? (acidify with hydrochloric acid before testing with the last 2 reagents).

4. Precipitation by K₄[Fe(CN)₆] · 3H₂O

Reagents
1% Albumin sol., 1% potassium ferrocyanide sol.
Procedure

1. To 5 ml of albumin sol., in a test tube add 5 to 10 drops of acetic acid, mix well and add potassium ferrocyanide drop by drop, until a precipitate forms.
2. Write down your observation

5. Fractional Precipitation of Proteins by Conc. Salt Solutions

Reagents
Dilute egg white solution (mix 1 volume of raw egg with 4 volumes of 1% NaCl and filter). Saturated sol. of (NH₄)₂SO₄, 1% acetic acid solution.

Procedure

1. To a portion of the dilute egg-white sol. add an equal volume of a saturated sol. of (NH₄)₂SO₄ and mix. Does the egg white contain a portion which precipitates by half-saturated (NH₄)₂SO₄?
2. If a precipitate is formed, then filter the content, and to the filtrate add an excess of solid (NH₄)₂SO₄ and stir until the solution is saturated with salt.
3. Record what happens? If a precipitate formed, filter and to the filtrate add 2 to 3 drops of 1% acetic acid sol. And heat to boiling.
4. If there is no precipitate formed, it indicates the absence of protein in the last filtrate.
**Enzymes**

**Background**
An enzyme is a protein molecule that is a biological catalyst with three characteristics:

- **First**, the basic function of an enzyme is to increase the rate of a reaction. Most cellular reactions occur about a million times faster than they would in the absence of an enzyme.
- **Second**, most enzymes act specifically with only one reactant (called a substrate) to produce products.
- **Third** and most remarkable characteristic is that enzymes are regulated from a state of low activity to high activity and vice versa.

**Classes of Enzymes**

<table>
<thead>
<tr>
<th>Group Name</th>
<th>Type of Reaction catalyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidases or Dehydrogenases</td>
<td>Oxidation-reduction reactions</td>
</tr>
<tr>
<td>Transferases</td>
<td>Transfer of functional groups</td>
</tr>
<tr>
<td>Hydrolases</td>
<td>Hydrolysis reactions</td>
</tr>
<tr>
<td>Lyases</td>
<td>Addition to double bonds or its reverse</td>
</tr>
<tr>
<td>Isomerases</td>
<td>Isomerization reactions</td>
</tr>
<tr>
<td>Ligases or Synthetases</td>
<td>Formation of bonds with ATP cleavage</td>
</tr>
</tbody>
</table>

**Comparative Activity of Enzymes and Nonbiological Catalysts**

Enzymes are different from other nonbiological catalysts (metals, acids, and salts) in the fact that they exhibit a high catalytic efficiency, specificity of action, and ability to accelerate reactions under mild conditions.
Experiment: Comparison of Action Exerted by Salivary $\alpha$-Amylase and Hydrochloric Acid on Starch Hydrolysis Reaction

Reagents & Materials
1% solution of Starch in 0.3% aqueous NaCl solution
Iodinated potassium iodide solution,
Benedict’s solution.
Test tube stand with a set of test tubes, a funnel, glass rod, eye pipettes, a thermometer, pipettes of 5 ml capacity.

Preparation of dilute Saliva
• Rinse your mouth thoroughly to remove eventually food remnants.
• Take a portion of distilled water (about 20 ml) in your mouth and keep it in for about 2 min. to allow it to mix the salivary secretion; use your tongue as a stirrer. Let the salivary liquid out into a beaker
• Pour the contents into a funnel with a cotton wad in it for a filter and filter off the liquid.
• Set aside the filtrate to be used in further exp.

Procedure
1. Transfer 1 ml of distilled water to a test tube, 1 ml of hydrochloric acid solution to another test tube, 1 ml of dilute saliva to a third test tube.
2. Add 5 ml of starch solution to each of the three test tubes, stir the contents with a glass rod.
3. Place the first and the third test tubes in a water bath at 38 C, and the second test tube in a boiling water bath.
4. In 15 min let the test tubes cool.
5. Sample 5 drops from each test tube into three clean test tubes.
6. Add 1-2 drops of iodine solution and compare the coloration developed in the samples.
7. To test for maltose sample 3 ml from each test tube, add 1 ml of Benedict’s solution and heat the upper layer of the mixture to boiling.
8. Note the formation of a red cuprous oxide precipitate in the samples.

**Experiment 2: Identification of Enzymes of Different Groups**

**Identification of Oxidoreductases in Biological Material**

**Identification of Aldehyde Oxidase (Aldehyde: Oxygen Oxidoreductase; EC1.2.3.1) in milk**

For most enzymes of this group, the recommended names are dehydrogenases and reductases. When O\(_2\) is the acceptor, the term oxidase is used; if the oxygen is involved in the reaction, makes part of the substrate, the enzyme is named oxygenase.

**Reagents & Materials**

0.4% aqueous solution of Formaldehyde, 0.01% aqueous solution of Methylene Blue

Test tube stand with test tubes, Water bath, Thermometer, Pipettes of 1 and 5 ml capacity.

**Identification of Aldehyde Oxidase (Aldehyde: Oxygen Oxidoreductase; EC1.2.3.1) in milk**

The method is based on visual observation of Methylene Blue (MB) decoloration by binding the hydrogen abstracted from the substrate through the aid of aldehyde oxidase. Aldehyde Oxidase is a catalyst for the dehydrogenation reaction of a variety of aldehydes, for example formaldehyde. Hydrogen is transferred onto FAD which is a coenzyme for the given enzyme, and then onto the final acceptor (oxygen) according to the scheme.

\[
\begin{align*}
\text{H}_2\text{C}=\text{O} + \text{H}_2\text{O} + \text{FAD} & \rightarrow \text{HCOOH} + \text{FADH}_2 \\
\text{FADH}_2 + \text{O}_2 & \rightarrow \text{FAD} + \text{H}_2\text{O}_2 \\
\text{MB} \text{ as a model hydrogen acceptor, on its addition to the system studied, is converted to a reduced form (leucoform), MBH}_2: \\
\text{H}_2\text{C}=\text{O} + \text{H}_2\text{O} + \text{MB} & \rightarrow \text{HCOOH} + \text{MBH}_2 \\
\text{The colorless methylene Blue solution on vigorous shaking regains the initial blue color.} \\
\text{MBH}_2 + \text{O}_2 & \rightarrow \text{MB} + \text{H}_2\text{O}_2
\end{align*}
\]
Procedure

1. Transfer 5 ml of fresh milk to 2 test tubes.
2. Add 2 ml of distilled water to one test tube and an equal volume of formaldehyde solution to the other test tube.
3. Pour 0.5 ml of Methylene Blue into each test tube, mix the contents with shaking and add 3 to 4 drops of vaseline oil (or paraffin oil) to prevent contact of liquid mixture with ambient oxygen.
4. Place the test tubes in a water bath at 37°C. Within 10 – 15 min note a change in sample color. Shake vigorously the test tubes and observe again a change in color.
Metabolism

Reaction Of Steroid And Determination Of Cholesterol In Blood Serum

Experiment 1: Qualitative Reactions For Steroids

Steroids:
Are tetracyclic organic compounds widely spread in animal and plant organisms. Typical representative of animal steroids is cholesterol. Found in tissues and biological fluids in a free or an esterified state with fatty acids such as palmitic, stearic, and oleic acids. Nervous tissue is especially rich in cholesterol, where the cholesterol conc., ranges 20 – 30g/kg. Highest conc., of cholesterol (40-55 g/kg) found in the white substance of brain and spinal marrow. Cholesterol maybe extracted from biological tissues with solvents such as chloroform, diethyl ether, acetone, and other organic solvents. Cholesterol swells in water and forms an emulsion. Plant sterols or phytosterol, include ergosterol, stigmasterols, β-sitosterols, and fucosterols which are contained in vegetable oils, fruits, algae, etc.,

Reagents & Materials

- Calcium sulfate (a hygroscopic), Chloroform, Conc. Sulfuric acid, Acetic anhydride
- Test tube stand with test tubes
- Analytical balance
- A mortar & a pestle
- 10x10 cm glass plates
- Spatula
- A funnel with a filter paper
- A drying cabinet with temp. of 60 C
- A scalpel
- A pipette of 5 ml capacity
- Eye pipette
Isolation Of Cholesterol From Calf Brain

Brain sample from a calf

1. To isolate cholesterol, triturate thoroughly 3 g of brain sample with 6 g of calcium sulfate added to the mortar using a spatula.
2. Spread the thick homogenized paste on a glass plate and place it in the drying cabinet at 60 C.
3. Scrape the dried mass off the glass plate with a scalpel into a clean mortar, grind thoroughly and transfer the contents to a dry test tube.
4. Add 6 ml of chloroform to the test tube and mix with shaking for 5 min.
5. Filter the chloroform extract through a dry filter paper into a dry test tube to be used in the color reaction as specified below.

Salkowski Reaction

The method is based on the dehydration of cholesterol molecule by concentrated sulfuric acid as dehydrating agent to yield cholesterylene colored red.

Procedure

1. Transfer 1 ml of chloroformic brain sample extract to a test tube and cautiously add 1 ml of concentrated sulfuric acid by allowing it to flow down the inner wall of a tilted test tube to form a layer on top of the chloroform solution.
2. Observe the appearance of a red colored ring at the interface of the two phases.

Liebermann Test

The method is based on the dehydration of cholesterol followed by a coupling of two dehydrated cholesterol molecules to yield bicholestadiene. Bicholestadiene gives a sulfunated green colored derivative in the presence of acetic anhydride and sulfonic acid.
Procedure

1. Transfer 2 ml of chloroformic brain sample extract to a clean test tube and add 10 drops of acetic anhydride and 2 drops of concentrated sulfuric acid.
2. Note the appearance of a red color which turns green-blue to green over time.

Determination Of Cholesterol Conc. In Blood Serum by Ilca’s Method

The method is based on the Liebermann reaction. The intensity of emerald-green coloration is proportional to the cholesterol concentration.

Reagents & Materials

- Ilca’s reagent
- A pipette, a thermostat set at 37°C, micropipettes, a test tube stand with a set of test tubes,
- A photometer
- Blood serum sample

Procedure

1. Transfer 4.2 ml of Ilca’s reagent to a test tube and add 0.2 ml of nonhemolyzed blood serum in small portions allowing them to flow down the inner wall of the test tube.
2. Shake vigorously 10-12 times and place the test tube in the thermostat at 37°C for 20 min.
3. Measure the absorbance on a photometer at 630 – 690 nm (red light filter) against a blank of Ilca’s reagent.
Vitamins

A. Fat-soluble Vitamins

The Detection Of Vitamins A And D

The vitamins A, D, E, and K are described as fat-soluble vitamins. They are absorbed in our organism together with lipids and can be cumulated in liver or kidneys as well as adipose tissues. They participate in many biological processes and play a role of anti-oxidative substances (vitamin A and E), protect an organism to damage by free radicals. Calciferols, the group of chemical compounds with chemical structure typical for steroids, with vitamin D activity, as well as carotenoids and retinol (vitamin A and its pro-vitamins) in reaction with SbCl₃ (Antimony Trichloride) form colored complexes. As a result of this reaction a blue color of carotenoids and vitamin A is observed, and in the presence of vitamin D violet-red color appear.

Reagents

- Saturated solution of SbCl₃ in chloroform
- Vitamin A, Vitamin D, Butter, Extract of hen egg yolk

Procedure

1. To 6 dry tubes add 0.5 ml saturated solution of SbCl₃ in chloroform.
2. Using a dry pipette, add chloroform solutions of:
   a. Vitamin A and D (1 drop)
   b. Vitamin A (1 drop)
   c. Vitamin D (1 drop)
   d. Butter (5 drops)
   e. Extract of hen egg yolk (5 drops)
   f. Chloroform (1 drop) – blank
3. Compare the colors and describe the results.
B. Water-soluble Vitamins

Determination Of Vitamin C (Ascorbic Acid) Concentration By A redox Titration With Potassium Iodate

Background

Vitamin C (ascorbic acid) deficiency leads to scurvy, a disease characterized by weakness, small hemorrhages throughout the body that cause gums and skin to bleed, and loosening of the teeth. Sailors that were out at sea for months on end would often develop scurvy unless the captain had the foresight to pack limes and other citrus fruits. Vitamin C is a water soluble antioxidant, and plays a vital role in protecting the body. Oxidizing species attack the body from many directions. Smog and cigarette smoke both contain high levels of oxidizing molecules that cause tissue damage. The body makes oxidizing molecules in response to an infection, and these molecules both kill the infecting organism and cause tissue damage. The body absorbs extra vitamin C in response to an infection. Because it is a water soluble vitamin, any unused vitamin C is excreted. The formula for ascorbic acid is C₆H₈O₆ and the structures for the reduced form and for the oxidized form (dehydroascorbic acid) are shown below:

The iodometric titration with potassium iodate (KIO₃) is a suitable method for the determination of vitamin C (C₆H₈O₆) quantities. Potassium iodate is used as a titrant and it is added to an ascorbic acid solution that contains strong acid and potassium iodide (KI). Potassium iodate reacts with potassium iodide, liberating molecular iodine (I₂)

\[ \text{KIO}_3 + 5\text{KI} + 6\text{H}^+ \rightarrow 3\text{I}_2 + 6\text{K}^+ + 3\text{H}_2\text{O} \]  (1)
As long as the solution contains ascorbic acid, the iodine produced in (1) is used up in a rapid reaction with ascorbic acid, during which dehydroascorbic acid and iodide ion are formed.

\[ \text{C}_6\text{H}_8\text{O}_5 + \text{I}_2 \rightarrow \text{C}_6\text{H}_6\text{O}_6 + 2\text{I}^- + 2\text{H}^+ \]  

(2)

Potassium iodide must be added in excess to keep iodine dissolved. Once all the ascorbic acid has been consumed, any excess iodine will remain in solution. Since aqueous iodine solutions are brown in color, iodine can act as its own indicator. However, it is quite difficult to detect endpoints using iodine coloration alone, and it is more usual to add starch, which forms an intensely blue colored complex with iodine but not with the iodide ion.

According to the above reactions, each mole of potassium iodate added corresponds to 3 moles of ascorbic acid dehydrogenated in the sample.

**Reagents & Instruments**

- 500 ml 0.011 M ascorbic acid solution
- 500 ml orange or lemon juice
- 100 ml 2M HCl
- 100 ml 0.006 M KI
- 100 ml 1% starch solution: (mix 1 g soluble starch with 5 ml distilled water, pour the starch into 95 ml boiling distilled water with stirring. Continue heating the solution until it is nearly transparent. Cool the solution to room temperature before use. This solution should be prepared on the day it is to be used (alternatively add ZnCl$_2$ as preservative).
- 1 L 0.0038 M KIO$_3$
- Conical flasks, Burettes
- Glass rod

**Procedure**

1. Pipette 25 ml of the provided ascorbic acid solution into a 250 ml conical flask,
2. Add 4 ml of 2M HCl,
3. Add 5 ml of potassium iodide (KI) solution and 3 ml starch solution.
4. Then titrate with the standard potassium iodate (KIO₃) solution until the solution turns intense blue. Write down the standard potassium iodate (KIO₃) solution volume.

5. Pipette 25 ml of an unknown ascorbic acid sample, a kind of juice, into a 250 ml conical flask, then follow the same procedure of steps 1-5 and write down the volume of the standard KIO₃ solution determine the concentration (mol/ml) of ascorbic acid in the selected sample.

**Note:** The end point is reached when the solution turns a permanent, dark blue color, due to the complex formed between starch and iodine. During an iodometric titration an intermediate dark blue iodine-starch complex may form momentarily, before the iodine reacts with ascorbic acid. However, if the color disappears upon mixing, the end point has not yet been reached. Thus, magnetic stirrers or glass rod are employed in the titration to ensure proper mixing and to facilitate the reaction of iodine with the ascorbic acid.

Tabulate your results as follows:

<table>
<thead>
<tr>
<th></th>
<th>Standardization Titration</th>
<th>Unknown sample Titration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final reading/ ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial reading/ ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Titre/ ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>