ÚSTAV LÉKAŘSKÉ BIOCHEMIE A LABORATORNÍ DIAGNOSTIKY 1. LF UK

Reactions of Saccharides Polarimetry

General Medicine

Lenka Fialová

translated and edited by Jan Plášteník

2020/2021

1. Introduction

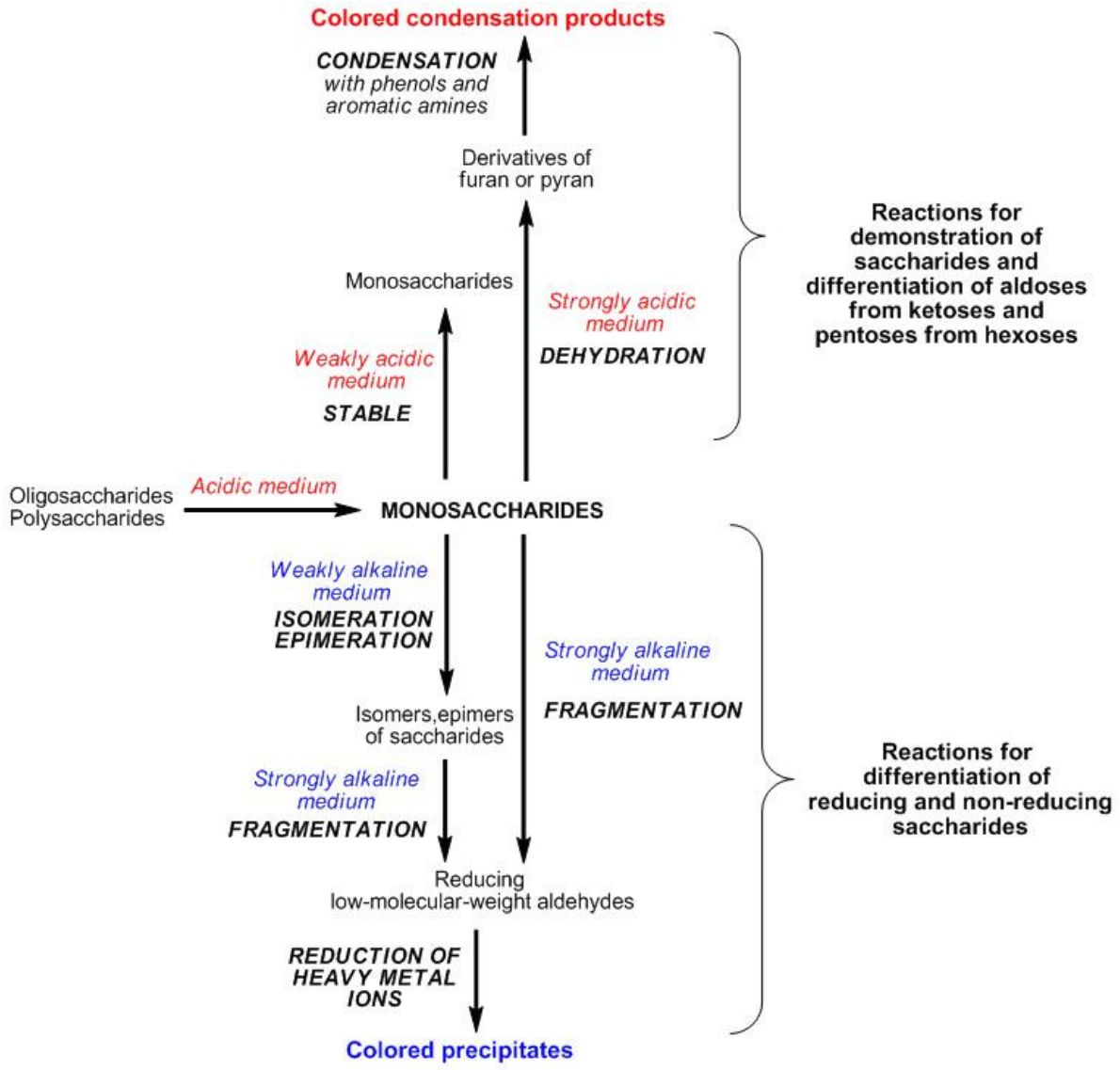
Saccharides are widespread organic compounds that are found both in plant and animal tissues. In our organism they represent the most readily available source of energy, but they are also important components of cell membranes and extracellular matrix.

According to the number of saccharide units these compounds are classified as mono-, oligo-, and polysaccharides. ***Monosaccharides contain*** just one monosaccharide unit, ***oligosaccharides*** 2–10 monosaccharide units, and ***polysaccharides*** consist of more than 10 monosaccharide units.

Monosaccharides are ***polyhydroxyaldehydes*** and ***polyhydroxyketones*** usually based on an unbranched chain 3–7 carbon atoms in length. According to the type of carbonyl functional group we classify them as ***aldoses*** or ***ketoses;*** according to the number of carbon atoms as ***trioses, tetroses, pentoses, hexoses, etc.***

2. Reactions of saccharides

Various chemical reactions exploit behavior of saccharides in acidic or basic medium and the reactivity of the carbonyl and hydroxyl functional groups.

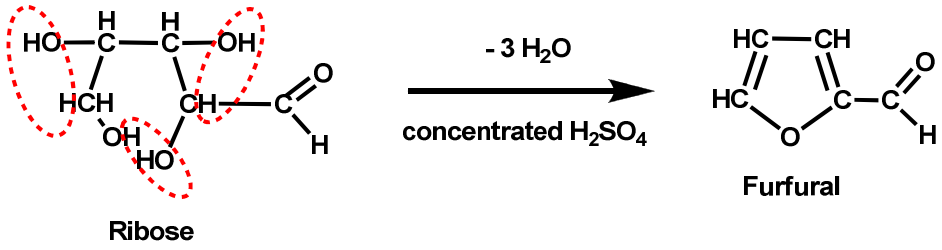


Simple chemical reactions can be used to differentiate saccharides from non-saccharide substances (Molisch test), pentoses from hexoses (Bial reaction), aldohexoses from ketohexoses (Selivanov reaction), reducing saccharides from non-reducing ones (Benedict and Fehling reactions), reducing monosaccharides from reducing disaccharides (Barfoed reaction), and starch from other polysaccharides (reaction with Lugol solution).

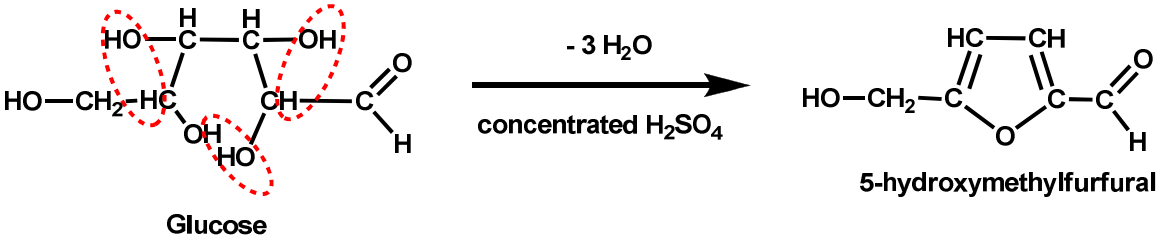
2.1. Reactions based on formation of furfural and its derivatives

Action of concentrated inorganic acids (HCl, H_2SO_4) results in ***dehydration of monosaccharides***. Successive dehydration gives rise to ***derivatives of furan***: removal of three water molecules produces furfural (2-furancarbaldehyde) from pentoses and 5-hydroxymethylfurfural (5-hydroxymethyl-2-furancarbaldehyde) from hexoses.

Origin of furfural from ribose



Origin of 5-hydroxymethylfurfural from glucose



The resulting aldehydes readily ***condense with phenols and aromatic amines*** (e.g. 1-naphthol, resorcinol) to colored products. Reactivity of different saccharides in these tests varies and that is why these reactions can be used e.g. to differentiate ketoses from aldoses, or pentoses from hexoses. Similar reactions are also observed with oligosaccharides and polysaccharides whose glycosidic bonds undergo hydrolysis by the acids presents in the reagents.

Condensation reactions of furfural and its derivatives are employed in some chemical reactions of saccharides, such as the Molisch, Selivanov, and Bial reactions.

Molisch reaction

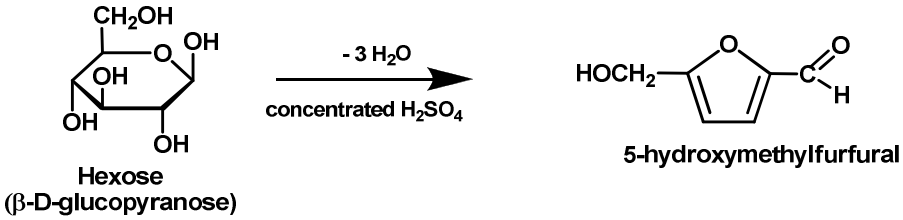
Significance

- The Molisch reaction is a ***general test for saccharides***. All monosaccharides, oligosaccharides and polysaccharides give positive results in this reaction.

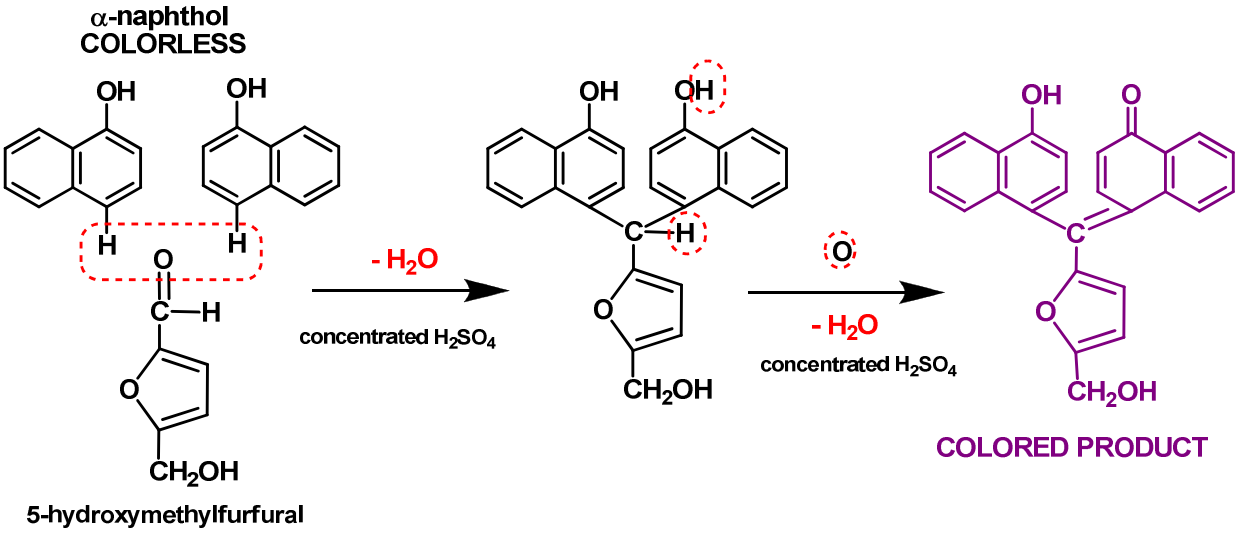
Principle

- In the first step action of ***concentrated sulfuric acid*** converts the monosaccharides containing 5 or 6 carbon atoms to furfural or 5-hydroxymethylfurfural, respectively.
- In the second step furfural or its derivatives condense with two molecules of ***α-naphthol*** to a colored product. In case of oligosaccharides and polysaccharides these reactions are preceded by cleavage of glycosidic bonds by acidic hydrolysis.

First step - dehydration of monosaccharide *with concentrated sulfuric acid*



Second step - condensation of furfural or its derivative with 2 molecules of *α-naphthol*

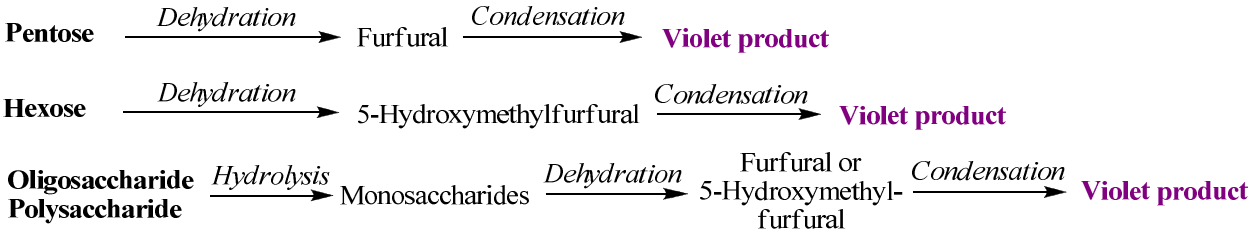


Reagent

- The ***Molisch reagent*** is a solution of α-naphthol in 96 % ethanol.

Evaluation

- The condensation product has a violet color.
- Monosaccharides react rapidly, while reactions of disaccharides and polysaccharides are slower, as the dehydration and condensation steps are preceded by the cleavage of glycosidic bonds.



Bial reaction

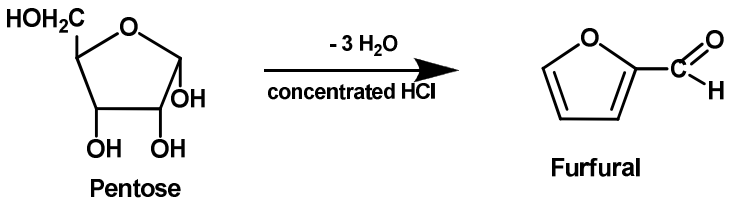
Significance

- The Bial reaction is used to ***distinguish pentoses from hexoses***.

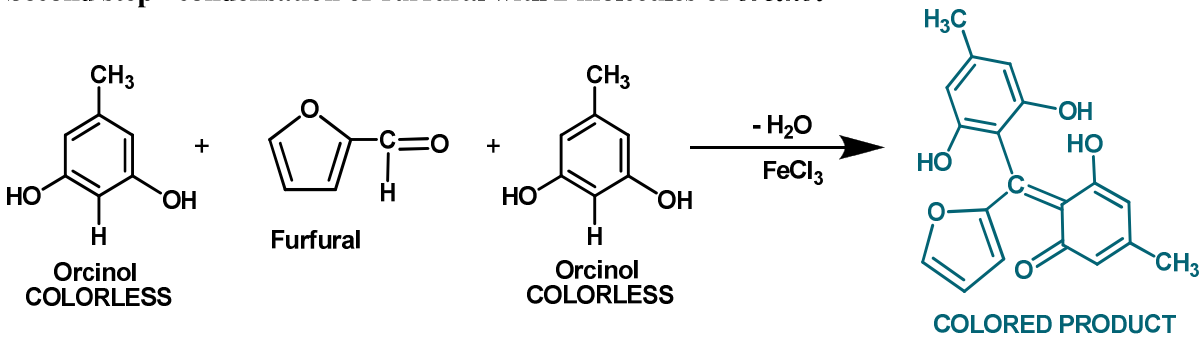
Principle

- Dehydration of pentoses (aldoses or ketoses) with ***hydrochloric acid*** produces furfural, which then reacts with ***orcinol*** (5-methyl-1,3-benzenediol) and ***ferric chloride*** giving a blue-green colored product.

First step - dehydration of pentose with concentrated *hydrochloric acid*



Second step - condensation of furfural with 2 molecules of *orcinol*



Reagent

- The ***Bial reagent*** contains hydrochloric acid, orcinol and ferric chloride.

Evaluation

- In the presence of ***pentoses*** a blue-green color develops. Only blue-green products are considered as the positive reaction result.
- **Hexoses,** from which dehydration produces 5-hydroxymethylfurfural, yield different colors – yellow to brown – and the reaction proceeds rather slowly. Oligo- and polysaccharides react in a similar manner, but more slowly.

Pentose —*Dehydration*→ Furfural —*Condensation*→ **Blue-green product**

Hexose —*Dehydration*→ 5-Hydroxymethylfurfural —*Condensation*→ **Brown product**

Selivanov reaction

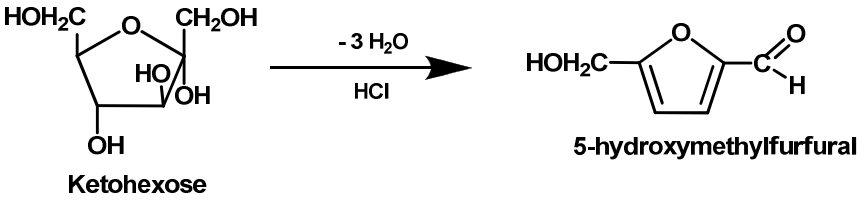
Significance

- Selivanov reaction serves for ***differentiation of aldohexoses from ketohexoses***.

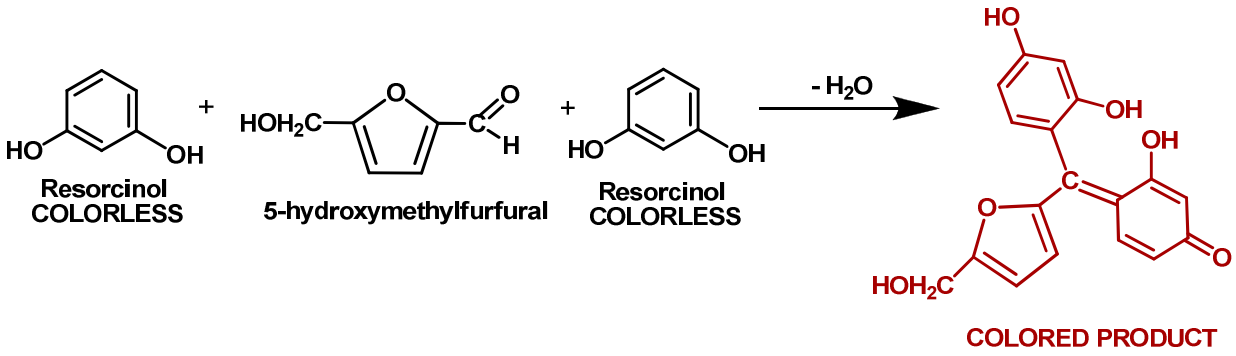
Principle

- Differentiation of aldohexoses and ketohexoses is based on different rate of their dehydration. As in the Bial test, ***hydrochloric acid*** is used for dehydration of the saccharide.
- The dehydration product 5-hydroxymethylfurfural in the Selivanov reaction condenses with ***resorcinol***.

First step - dehydration of ketohexose with *hydrochloric acid*



Second step - Condensation of 5-hydroxymethylfurfural with 2 molecules of *resorcinol*

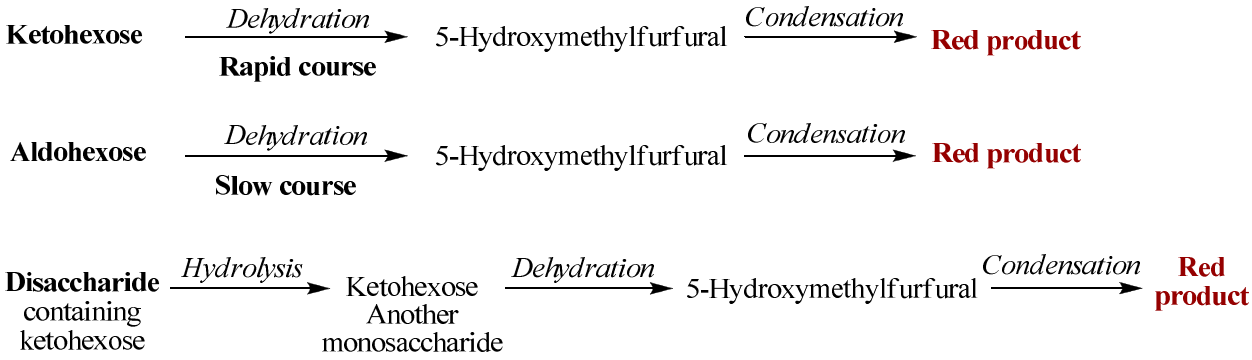


Reagent

- ***The Selivanov reagent*** is a solution of resorcinol in hydrochloric acid.

Evaluation

- ***Ketoses*** react rapidly and during 1-2 minutes yield a cherry red color.
- The reaction of ***aldoses*** is rather sluggish (several minutes) because dehydration of aldoses to 5-hydroxymethylfurfural proceeds in a much slower way than the reaction of ketoses.
- ***Disaccharides*** containing a ketohexose produce the Selivanov reaction as well. First, however, a hydrolysis of glycosidic bonds must take place. Therefore, development of color with such a disaccharide lasts a longer time than with a ketohexose, but still it is faster in comparison to aldohexoses.



2.2. Reaction with the Schiff reagent

Significance

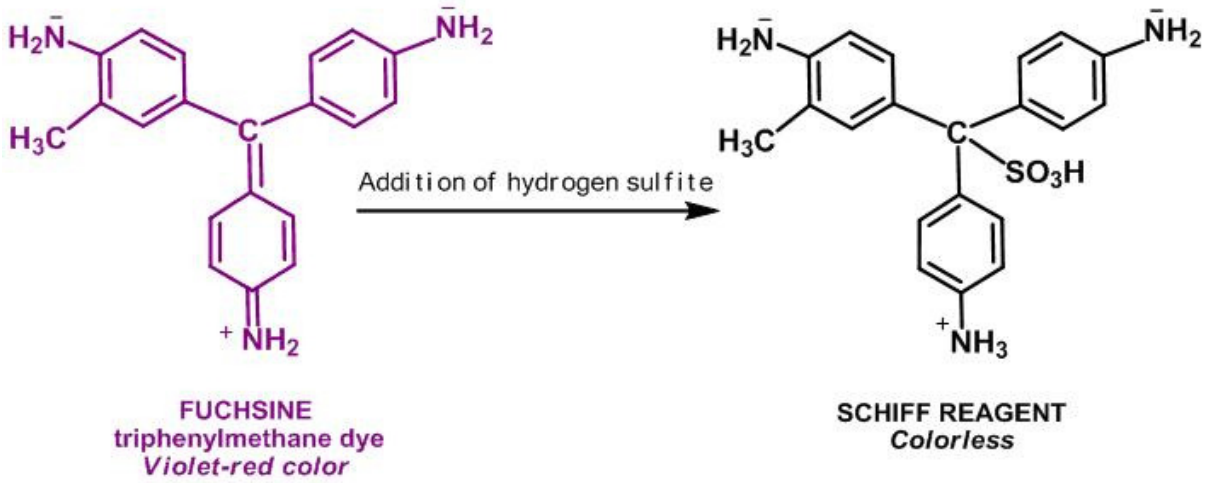
- The reaction with the Schiff reagent[1] is used for demonstration of ***free aldehyde group***. Certain ketones can give this reaction, too.

Principle and evaluation

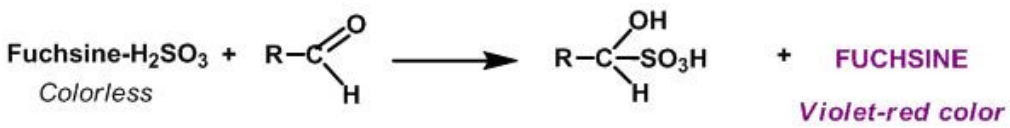
- The Schiff reagent is an aqueous solution of violet-red dye ***fuchsine***, to which hydrogen sulfite or sulfite is added. Hydrogen sulfite adds on the central carbon atom and disturbs the quinoid structure of the dye, necessary for the color. Colorless solution of fuchsine sulfurous acid results.
- After addition of an ***aldehyde*** the fuchsine is released from its interaction with sulfurous acid, which forms a stronger bond with the aldehyde. The quinoid structure of the fuchsine molecule is restored and the solution again acquires its ***violet-red color***.

Reactivity of ***aldoses*** with the Schiff reagent is different compared to the aldehydes, because during formation of the cyclic structures the aldehyde function makes the hemiacetal bond. In aqueous solutions the monosaccharides are present mostly in the cyclic forms that do not favor the addition reaction with hydrogen sulfite.

Preparation of reagent



Reaction with aldehyde



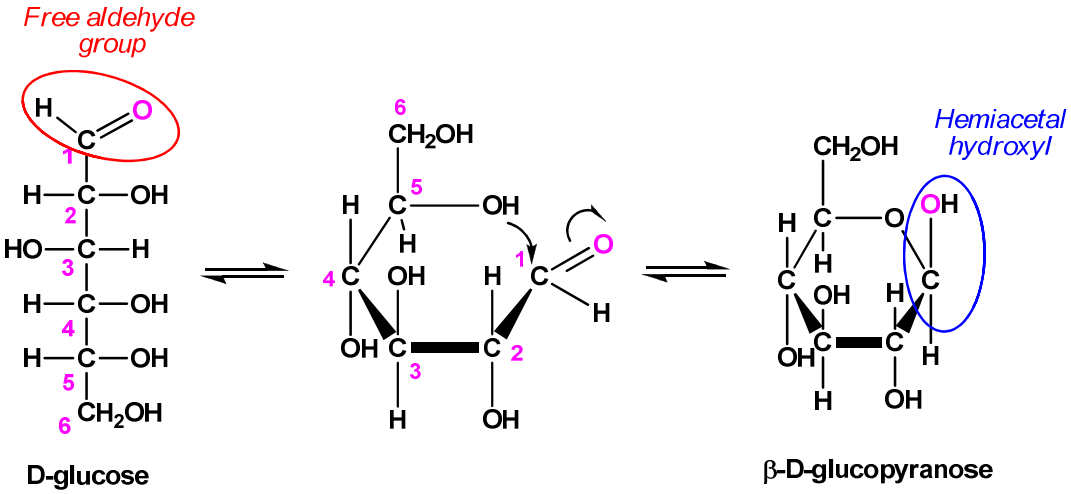
[1] The name of German chemist Hugo Schiff (1834 – 1915) is also connected to the term Schiff base. It denotes a product of condensation reaction between aromatic or aliphatic primary amine and an aromatic aldehyde or ketone.

2.3. Reactions for reducing properties of saccharides

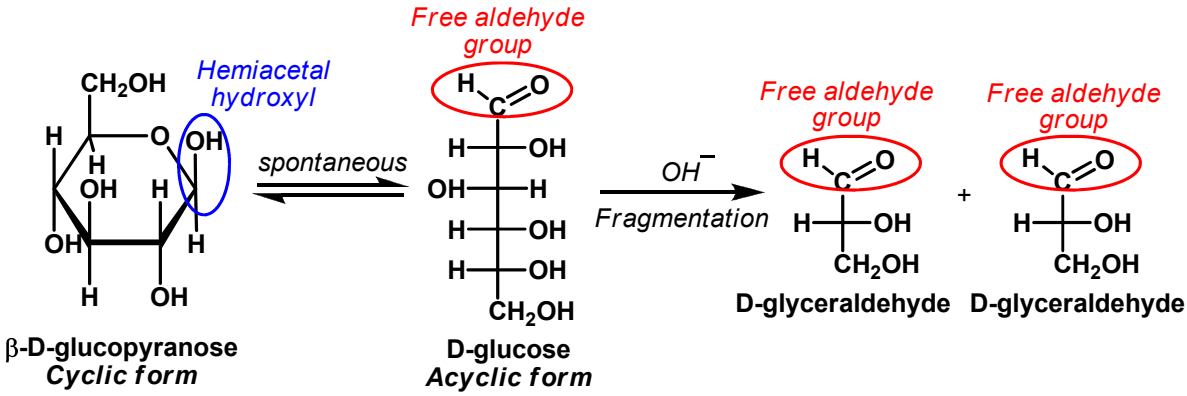
From the analytical point of view the classification of saccharides to reducing and non-reducing ones is important. The prerequisite for reducing properties of saccharides is presence of ***free or potentially free aldehyde functional group***.

Reducing properties of aldoses

- The presence of aldehyde functional group is typical for acyclic forms of ***aldoses***. However, monosaccharides with 4 and more carbon atoms give rise to cyclic forms, where one of the hydroxyl groups spontaneously adds on the carbonyl group, yielding an intramolecular hemiacetal or hemiketal.



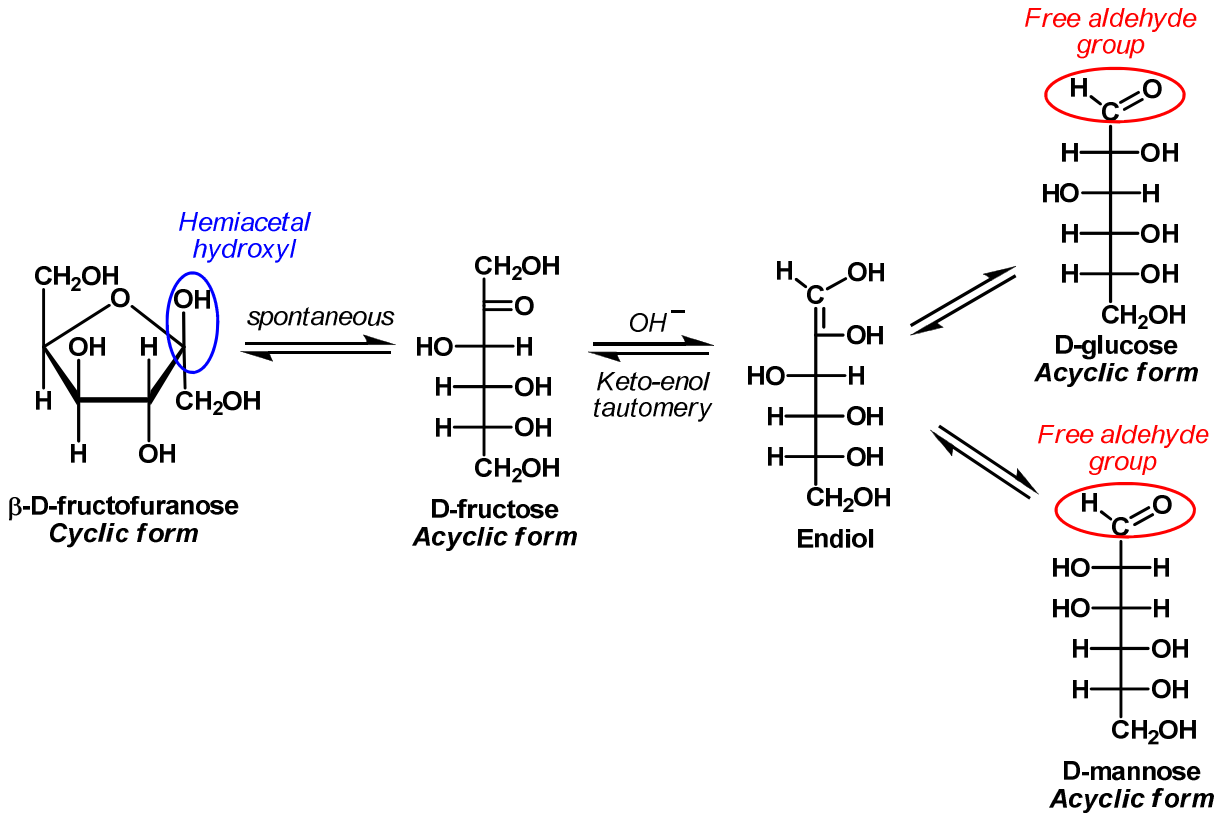
- In a monosaccharide solution the cyclic form, which cannot be oxidized, prevails. The cycle can however open in the solution and the liberated aldehyde group may undergo an oxidation. In addition, in a strongly alkaline medium and elevated temperature the monosaccharides decompose and produce strongly reducing low-molecular-weight aldehydes (e.g. formaldehyde, acetaldehyde, glyceraldehyde).



- The different reactivity of saccharide carbonyl group in comparison to aldehydes can be demonstrated by ***reaction with the Schiff reagent***.

Reducing properties of ketoses

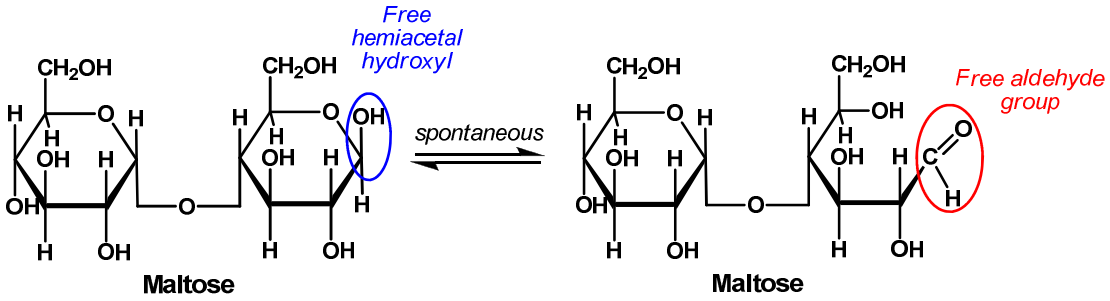
- ***Ketoses*** do not contain an aldehyde functional group, but in alkaline medium they isomerize to aldoses, which can further decompose to reducing fragments (see above).



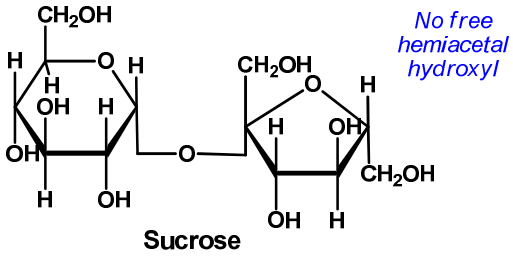
Reducing properties of disaccharides and polysaccharides

- Among ***disaccharides***, the reducing properties are found only in those that have a free hemiacetal hydroxyl (i.e., the hydroxyl group resulting from the hemiacetal bond formation) do not participating in the glycosidic linkage, such as in maltose and lactose. The cycle of monosaccharide with the free hemiacetal hydroxyl can open in the solution and participate in the redox reactions. In contrast, disaccharides in which the hemiacetal hydroxyl groups of both monosaccharides participate in the glycosidic linkage (such as in sucrose), do not display any reducing properties, because in this case neither of the monosaccharide cyclic forms can open in the solution.
- ***Starch*** is almost non-reducing. The only groups able to reduce are the terminal glucose units with free hemiacetal hydroxyl groups.
- Glycosidic bond in disaccharides and polysaccharides can be hydrolytically cleaved and the reducing properties then appear due to the released monosaccharides.

Reducing disaccharide



Non-reducing disaccharide



The reducing saccharides are able at higher temperature to reduce heavy metal ions, such as Ag^{1+}, Cu^{2+}, Bi^{3+}, bound to a complex. There are various tests for demonstration of reducing properties of saccharides. The ***Tollens reaction*** uses diamminsilver nitrate, from which in the presence of reducing saccharides metallic silver is reduced (see the practical lesson on organic reactions). Another possibility is the oxidation reagents containing Cu^{2+} in alkaline medium such as in the ***Fehling*** (see the practical lesson on organic reactions) and the ***Benedict tests,*** or in weakly acidic medium in the ***Barfoed test***. The reduction of Cu^{2+} to Cu^{+} is associated with oxidation of aldehyde groups to carboxylic ones.

The reducing tests are used as a simple examination of urine in cases of suspect inborn errors of saccharide metabolism.

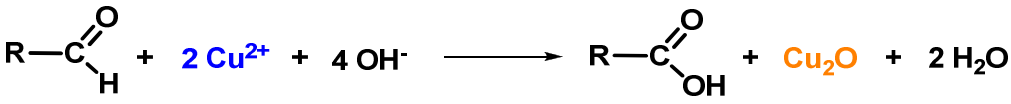
Benedict reaction

Significance

- The Benedict reaction serves for ***demonstration of saccharides with reducing properties***.

Principle

- In ***alkaline medium*** the saccharides with reducing properties ***reduce Cu^{2+} ions contained in the reagent to Cu^{1+}*** and simultaneously the free aldehyde group of saccharides or their fragments is oxidized to carboxylic functional group. The reaction takes place at higher temperature.



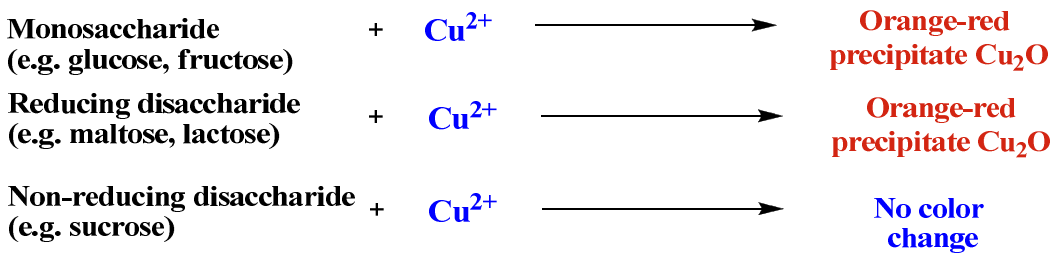
For the Benedict test a small amount of sample is sufficient, hence the test is suitable for examination of elevated levels of reducing saccharides in urine of the newborn in the diagnostics of the inborn errors of saccharide metabolism.

Reagent

- The basic component of the ***Benedict reagent*** is copper sulfate pentahydrate; it also contains sodium carbonate and sodium citrate.
- Sodium carbonate provides the alkaline medium that the reaction needs. However, without further addition the carbonate anions together with copper cations would form a precipitate of copper carbonate.
- Therefore, the next component of the reagent is sodium citrate that keeps Cu^{2+} ions in a soluble form as copper citrate. Likewise, in the Fehling reagent this function is provided by the sodium-potassium tartrate.

Evaluation

- Reduction of Cu^{2+} by **reducing saccharides** manifests as formation of orange-red precipitate of Cu_2O, or as yellow CuOH, which at higher temperature transforms also to Cu_2O.



- The positive test is obtained not only with the reducing saccharides, but also with ***other compounds with reducing properties***, such as ascorbic acid or homogentisic acid.

Barfoed reaction

Significance

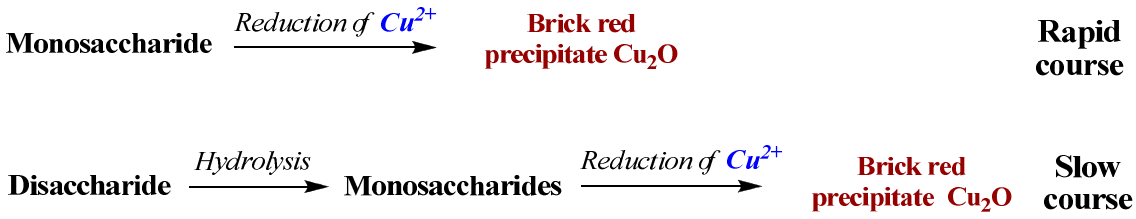
- By means of the Barfoed reaction it is possible to ***differentiate reducing monosaccharides from reducing disaccharides***.

Principle

- Monosaccharides ***reduce at higher temperature Cu^{2+} ions***, which are in the reagent in form of copper acetate. Unlike the Benedict test the redox reaction takes place in ***weakly acidic medium***. These conditions enable during the first reaction minutes faster reaction of monosaccharides in comparison to disaccharides.

Evaluation

- During the 3–4 minutes of heating **monosaccharides** react by producing the brick red precipitate of cuprous oxide.
- **Disaccharides** require a longer time of heating during which a cleavage to reducing monosaccharides can occur, to give a positive result.



Reagent

- ***The Barfoed reagent*** is a solution of copper acetate with acetic acid.

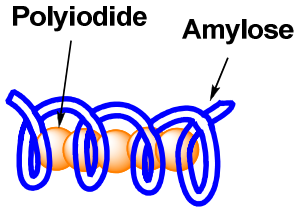
2.4. Reaction for demonstration of starch

Significance

- ***To test for starch*** a reaction with the Lugol solution is used.

Principle

- The starch contains amylose that consists of an unbranched chain of glucopyranose molecules connected with α-1,4-glycosidic bonds. The amylose chain is coiled to a helix. The central cavity of the amylose helix adopts linearly arranged polyiodide ions from the Lugol solution. The resulting complex of polyiodides with amylose yields a dark blue color that disappears after heating.



Evaluation

- The ***dark blue color of starch*** in the reaction with the Lugol solution is provided by **amylose**.
- **Amylopectin** produces with the Lugol solution a ***reddish-brown to red color***. Glycogen, which also contains branches like the amylopectin, reacts in a similar manner.
- The color produced by the **cleavage products of starch** (dextrins) depends on the length of polysaccharide chain. The resulting color can be violet (amylodextrin), purple to red (erythrodextrin) or no color at all (achrodextrin).
- Reactions of **monosaccharides and disaccharides** with the Lugol solution ***do not lead to color change.***

Reagent

- The Lugol solution is a solution of iodine in potassium iodide, which increases solubility of iodine in water. Molecules of iodine combine with iodide anions to linearly arranged triiodides and pentaiodides that are better soluble in comparison to iodine:

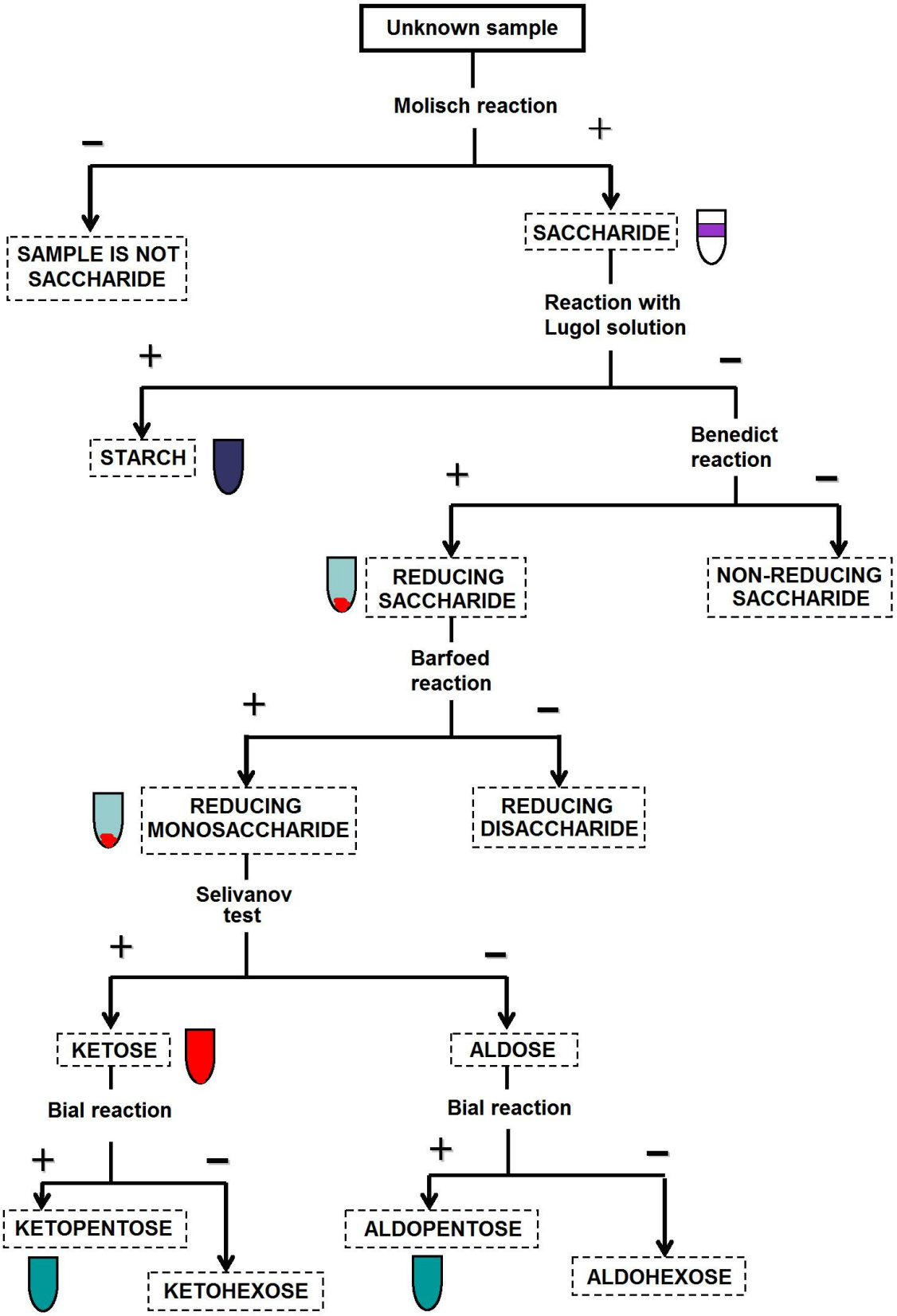
$$I_2 + I^- \longrightarrow [I_3]^- \longrightarrow I_2 + [I_3]^- \longrightarrow [I_5]^-$$

2.5. Analysis of unknown sample

The reactions described above can be employed for analysis of an unknown sample of saccharide. Evaluation and significance of the reactions is summarized in the table.

Reaction	Usage	Positive result	
Molisch reaction	Evidence of saccharide	Saccharide Violet ring	
Reaction with Lugol solution	Evidence of starch	Starch Dark blue color	
Bial reaction	Distinguishes pentoses from hexoses	Pentoses Blue-green color	
Selivanov reaction	Distinguishes ketohexoses from aldohexoses	Ketohexoses Red color	
Benedict test	Distinguishes reducing saccharides from non-reducing	Reducing saccharides Orange-red precipitate	
Barfoed reaction	Distinguishes reducing monosaccharides from reducing disaccharides	Reducing monosaccharides Red precipitate	

In analysis of the unknown sample we proceed according to the following scheme:



3. Thin layer chromatography of saccharides

The thin layer chromatography is one of the methods used in analysis of saccharides.

Saccharides are hydrophilic substances, which substantially affects their behavior during chromatographic separation. The R_f value of saccharides is determined by many factors such as the size of saccharide (monosaccharides, disaccharides), number of carbons in the molecule and also the number and spatial arrangement of the hydroxyl groups. High number of hydroxyl groups causes a strong interaction between the adsorbent and the saccharide.

For the subsequent detection of saccharides either their reducing properties or the conversion of aldoses and ketoses to the furfural derivatives can be used. The latter then give colored reactions with various aromatic amines and phenols, such as in e.g. the Molisch test.

The thin layer chromatography enables a more precise identification of saccharides in urine (e.g. galactose, fructose) in examination of patients with inborn errors of metabolism.

4. Polarimetry

4.1 Principle of the technique

Polarimetry is an optical analytical method that uses the fact that optically active compounds ***rotate the plane of polarised*** light. Compounds that are permanently optically active contain an asymmetrical carbon in its molecule, i.e. a carbon with four different substituents attached. The plane of polarised light can be rotated to right or to left by optically active compounds.

The angle α of rotation of the plane of polarised light by an optically active compound depends on several factors:

- concentration of optically active compound;
- optical length of solution through which the polarised light passes through;
- temperature of measured solution;
- wavelength of used polarised light;
- structure of optically active compound;
- character of used solvent.

Relationship between α and concentration of optically active compound is described by equation:

$$\alpha = [\alpha]_D^{20°C} \times \frac{l \times w}{100}$$

α ……..	rotation of the plane of polarised light in degrees
l …….....	length of polarimetric tube in meters
w …….……	mass concentration of compound in g/l
$[\alpha]_D^{20°C}$ …….....	specific rotation

The ***specific rotation*** characterises optically active compound. It is a constant for every given compound, temperature and wavelength of light. It is defined as the angle of rotation of polarised light by a solution in 1 m long tube containing 100 g of the compound in 1 liter. Usually, measurement is performed at 20 °C and yellow light of sodium lamp with wavelength 589 nm (spectral line D) is used.

$$[\alpha]_D^{20°C} = \frac{\alpha \times 100}{1 \times w}$$

Values of specific rotations are listed in tables.

The relation between concentration of optically active compound and rotation of the plane of polarised light is used to determine some biologically important compounds. If the specific rotation of tested compound is known and the angle α is measured, concentration of the compound can be calculated using a formula derived from the previous one:

$$w\ (g/l) = \frac{\alpha \times 100}{[\alpha]_D^{20°C} \times 1}$$

Polarimeters are used to measure the rotation α.

4.2 Inversion of sucrose

Sucrose (table sugar, cane or beet sugar) is widely used in foods because of its sweet taste as the commonest sweetener.

Sucrose can be hydrolyzed either in ***acidic medium***, or by the action of enzyme ***sucrase (invertase)***. The hydrolysis yields an equimolar mixture of D-glucose and D-fructose known as ***invert sugar***. This name refers to the change in optical activity that occurs during sucrose hydrolysis. Sucrose is dextrorotatory, whereas the invert sugar is levorotatory. The invert sugar is sweeter than sucrose.