

Slovak University of Technology Bratislava

Faculty of Chemical and Food Technology
Department of Biochemistry and Microbiology



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Coordinator of the EC Asia – Link 147-063 project:
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IN THE NAME
OF
GOD



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Preface

In this world we are facing with rapid changes in different scientific, cultural, social and economical fields. Among all the mentioned fields, science and technology are the ones that are developing most rapidly and taking more hasty actions for human's comfort. Science and technology cause the daily affairs coordination, universal development, and causes the people from all over the world to be closer.

Biochemistry, which is an important scientific branch, could be a good example. Currently, biochemistry has its special technology. Its study and research are interesting and profitable for those who want to be aware of chemical reactions and physiology. In addition, biochemistry would be interesting for the seekers of this knowledge to investigate in hundreds of other hidden secrets in living creatures like human, animals and plants.

According to the recent biochemistry discoveries and based on my experiences in teaching Biochemistry and Organic chemistry in Herat Medical and Science Faculties, I decide to use this opportunity from this fellowship to provide the gained knowledge for those who are interested in this field.

The issues that interest me more are new methods usable for the study of carbohydrates, lipids and protein metabolism and some new practical experiences in analytical methods for the metabolism intermediates determinations.

In almost two months of my stay in one of the Biochemistry labs at Faculty of Chemical and Food Technology, Slovak University of Technology in Bratislava, I have done my best to learn about these discoveries either theoretically or practically by doing technical and laboratorial experiments. My work was done under the directions of Professor Dr. Varečka, the head of the Department of Biochemistry and Microbiology and under the supervision of Dr. Boris Lakatoš and help of engineer Monika Vaneková.

Moreover, myself and two professors of Kabul PolyTechnical University participated in the 36th International Conference of Chemical Engineering held in High Tatras mountains located in Slovakia. Actually, we were the first Afghan representatives who have ever been invited in such conference. The conference was very interesting, well organized and also organizing committee was kindly and hospitable. Report from this conference you can find at the end of this report.

At last but not at least I would appreciate Dr. Haydary from Faculty of Chemical and Food Technology in Bratislava for his candid cooperation and inexhaustible efforts in preparing fields for scientific and cultural coordination between Afghanistan Islamic Republic and Slovak Republic and Professor Varečka, Dr. Boris Lakatoš and engineer Monika Vaneková coordination, generously guides and good manners. I wish them success and health and happiness for all time.

Dr. Pharmacist
Saleh Mohammad BAHER

Goal of the training stay:

The aim of my training stay at the Slovak University of Technology in Bratislava could be shortly described as follow:

1. Increasing of my knowledge and skill via collecting the new information and carrying out laboratory practices in biochemistry
2. Improving my knowledge in using of internet in science and technology research
3. Scientific research in chemical and biochemical technology
4. Participation in 36th International Conference on the Slovak Society of Chemical Engeneering held in High Tatras from May 25 to May 29, 2009.
5. Providing the collected informations and materials to interested people in Afghanistan
6. Hard working on improvement and strengthening the relationship between Herat University and Slovak University of Technology in Bratislava

Reference of supervisor

Certificate



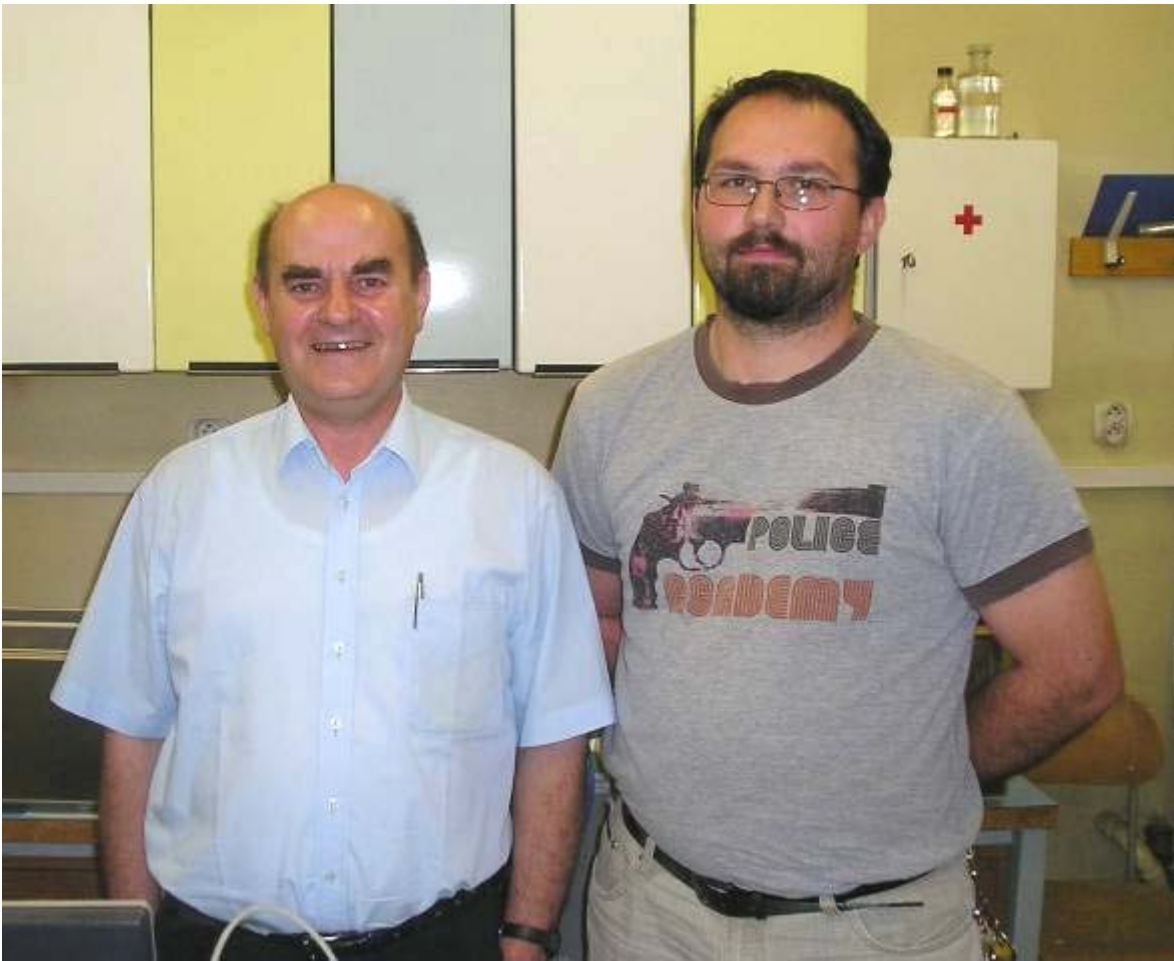
Picture № 1: Main entrance to the Faculty of Chemical and Food Technology in Bratislava



Picture № 2: Periodic table of elements and the inventor (D. I. Mendelejev) in artistic realization. In front of the main entrance to the Faculty of Chemical and Food Technology in Bratislava



Picture № 3: Dr. Juma Haydary, coordinator of the EC Asia – Link 147-063 project at the Faculty of Chemical and Food Technology in Bratislava.



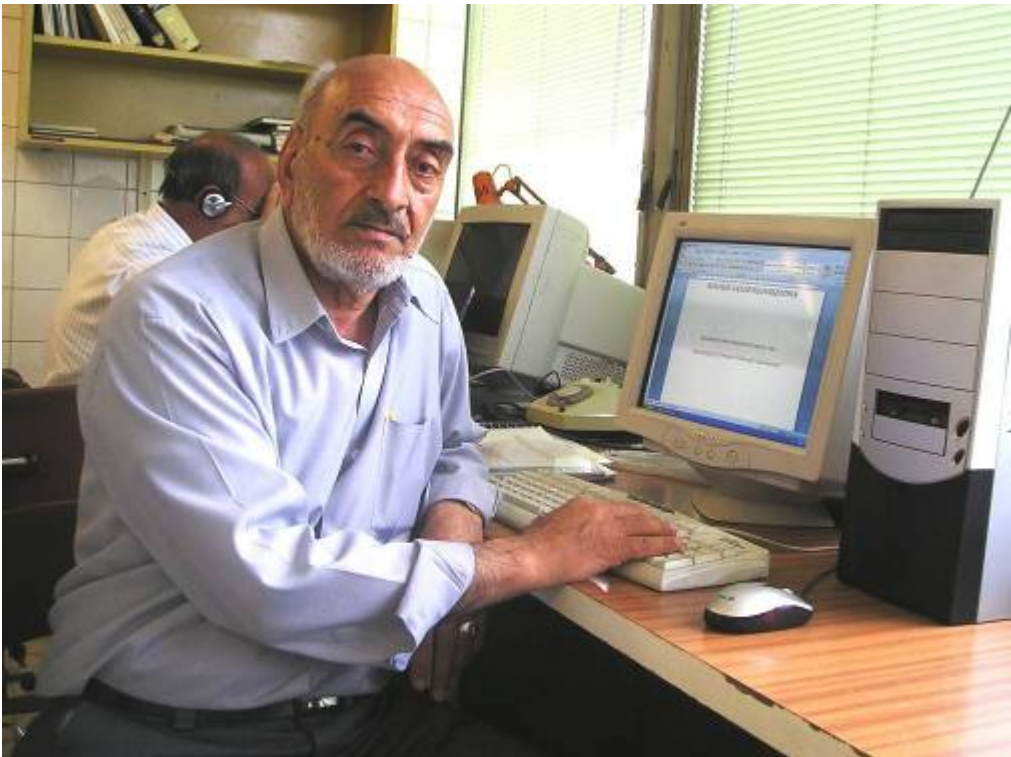
Picture № 4: Professor Varečka – director of Department of biochemistry and Microbiology (left side), Ing. Lakatoš – supervisor (right side)



Picture № 5:Picture Baher and Boris



Picture № 6: Pictures of Bahar and Monika



Picture № 7: Dr. Baher at work in the laboratory (upper photo) and in the office (lower photo)

Laboratory practice

Carbohydrates

Carbohydrates [α] or saccharides [β] are the most abundant of the four major classes of biomolecules. They fill numerous roles in living things, such as the storage and transport of energy (eg: starch, glycogen) and structural components (eg: cellulose in plants and chitin). Additionally, carbohydrates and their derivatives play major roles in the working process of the immune system, fertilization, pathogenesis, blood clotting, and development.

Roles of carbohydrates

First, carbohydrates serve as energy stores, fuels, and metabolic intermediates. Second, ribose and deoxyribose sugars form part of the structural framework of RNA and DNA. Third, polysaccharides are structural elements in the cell walls of bacteria and plants. Fourth, carbohydrates are linked to many proteins and lipids, where they play key roles in mediating interactions among cells and interactions between cells and other elements in the cellular environment.

Structure of carbohydrates

Chemically, carbohydrates are simple organic compounds that are aldehydes or ketones with many hydroxyl groups added, usually one on each carbon atom that is not part of the aldehyde or ketone functional group. The basic carbohydrate units are called monosaccharides, such as **glucose**, galactose, and fructose. The general stoichiometric formula of an unmodified monosaccharide is $(C \cdot H_2O)_n$, where n is any number of three or greater; however, not all carbohydrates conform to this precise stoichiometric definition (eg: uronic acids, deoxy-sugars such as fucose), nor are all chemicals that do conform to this definition automatically classified as carbohydrates.

Glucose (Glc), a monosaccharide (or simple sugar) also known as grape sugar, blood sugar, or corn sugar, is a very important carbohydrate in biology. The living cell uses it as a source of energy and metabolic intermediate. Glucose is one of the main products of photosynthesis and starts cellular respiration in both prokaryotes (bacteria and archaea) and eukaryotes (animals, plants, fungi, and protists).

The name "glucose" comes from the Greek word glukus ($\gamma\lambda\upsilon\kappa\acute{\upsilon}\varsigma$), meaning "sweet", and the suffix "-ose," which denotes a sugar.

Two stereoisomers of the aldohexose sugars are known as glucose, only one of which (D-glucose) is biologically active. This form (D-glucose) is often referred to as dextrose monohydrate, or, especially in the food industry, simply dextrose (from dextrorotatory glucose). This article deals with the D-form of glucose. The mirror-image of the molecule, L-glucose, cannot be metabolized by cells in the biochemical process known as glycolysis.

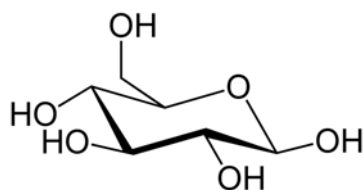


Figure: D-glucose

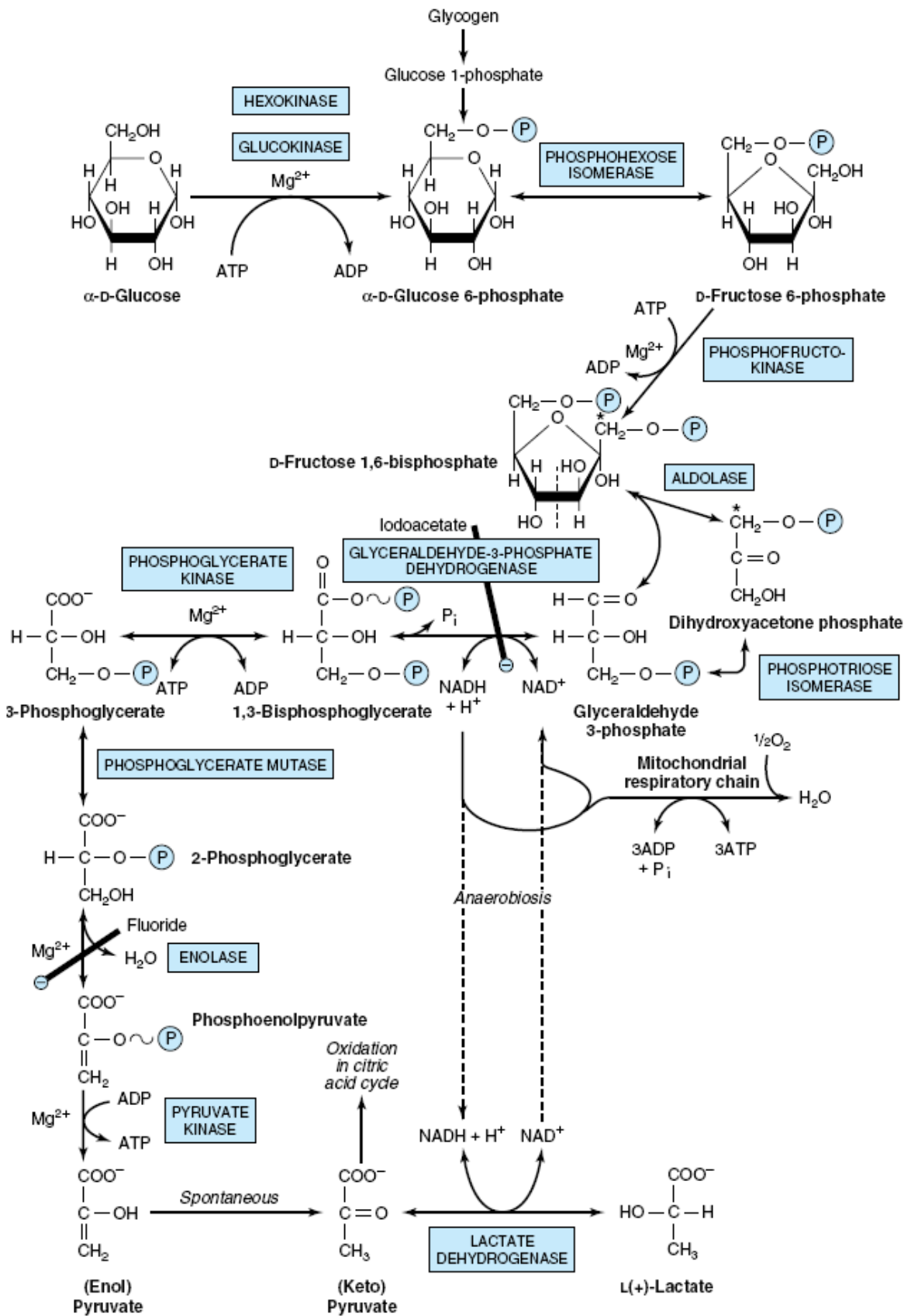


Figure: The metabolic pathway of glycolysis.

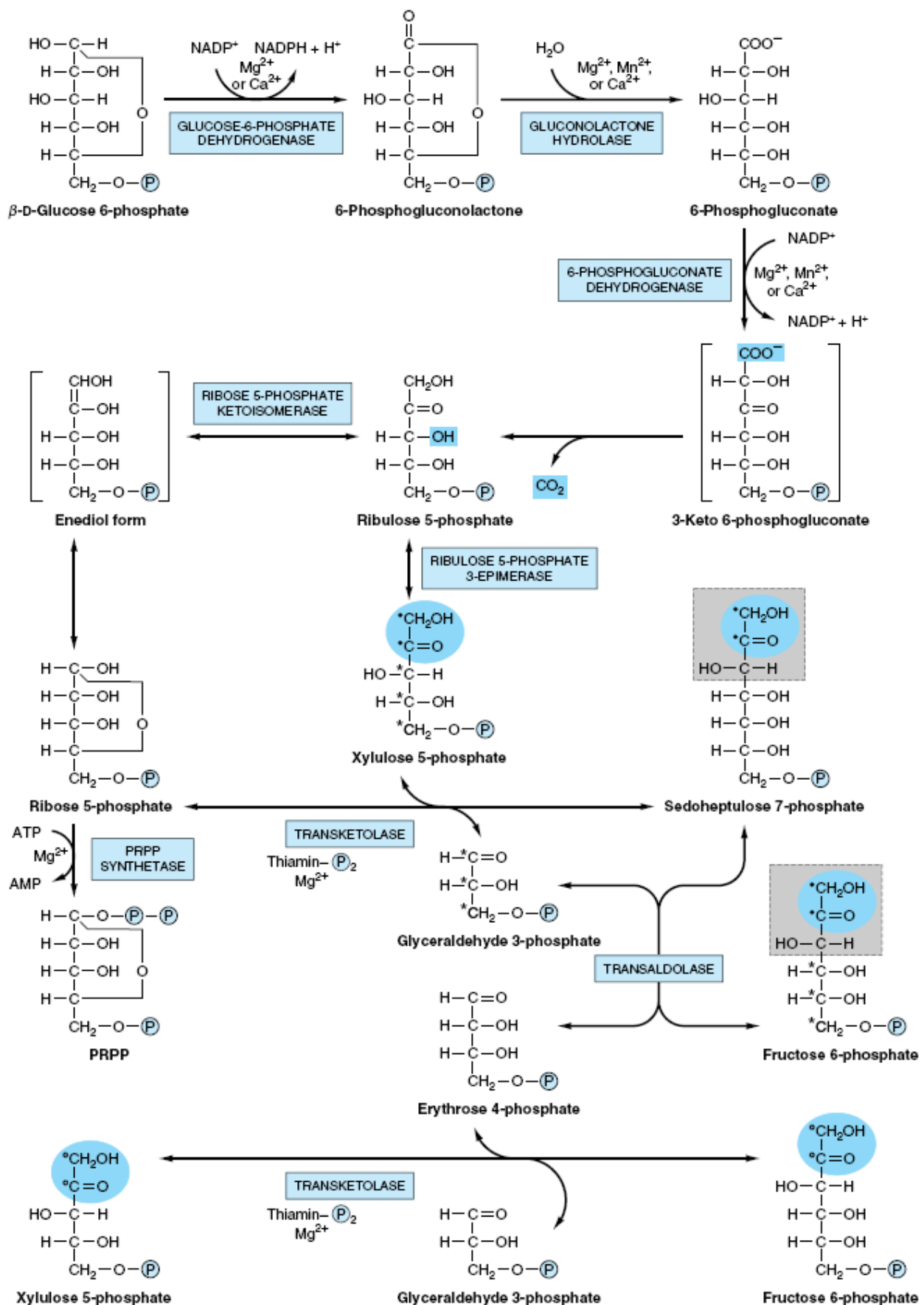


Figure: The pentose phosphate pathway.

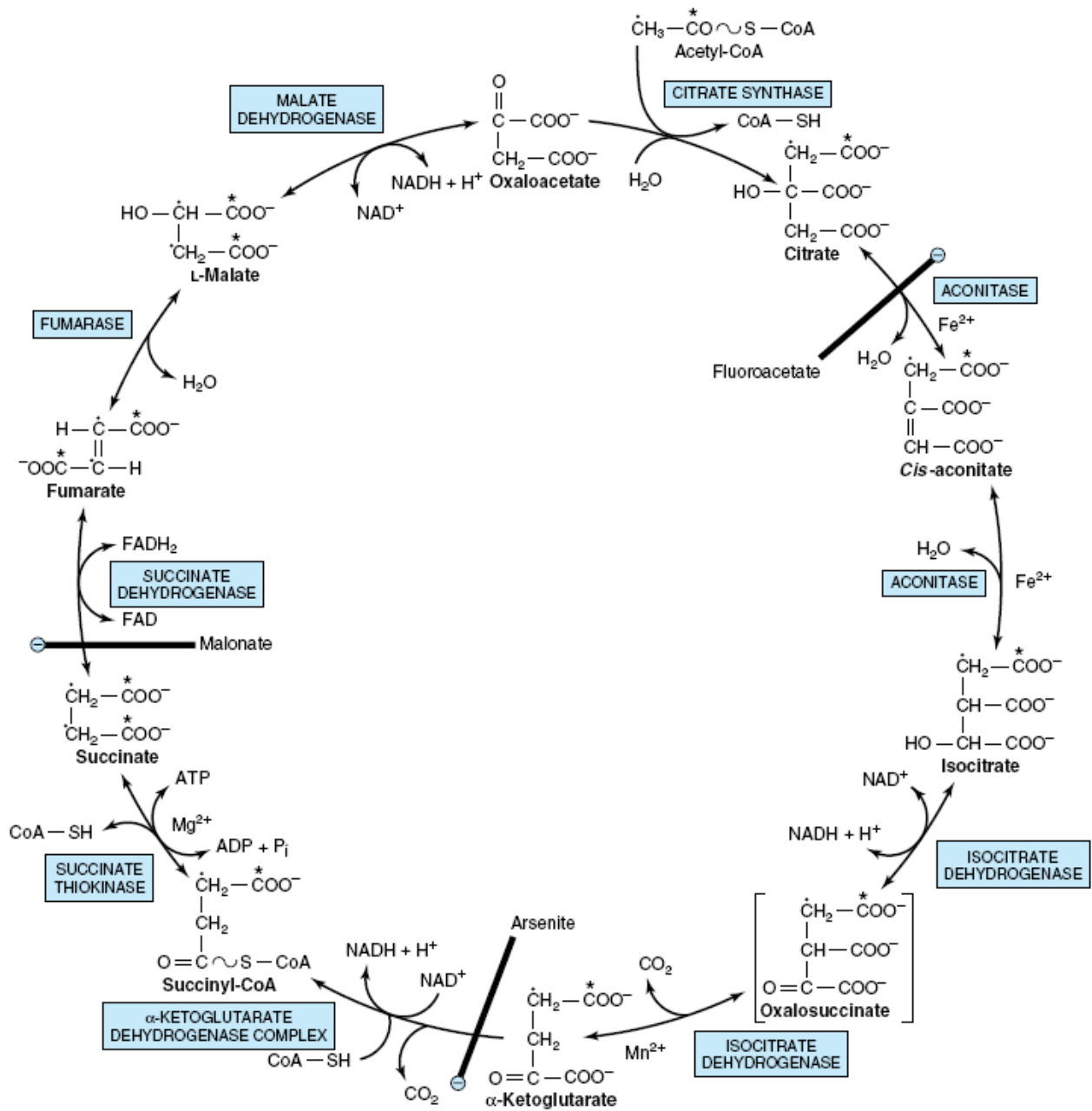


Figure: Reactions of the citric acid (Krebs) cycle.

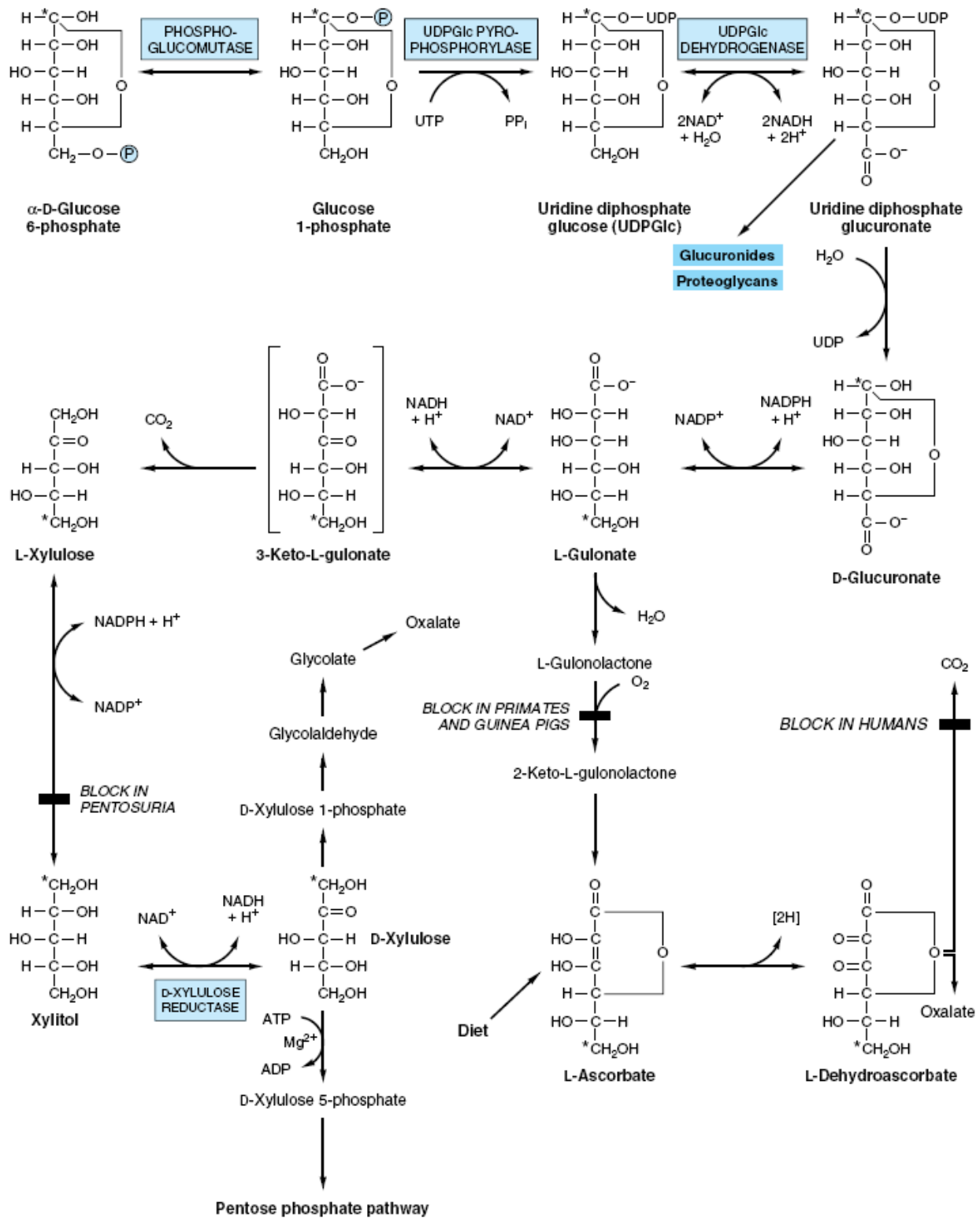
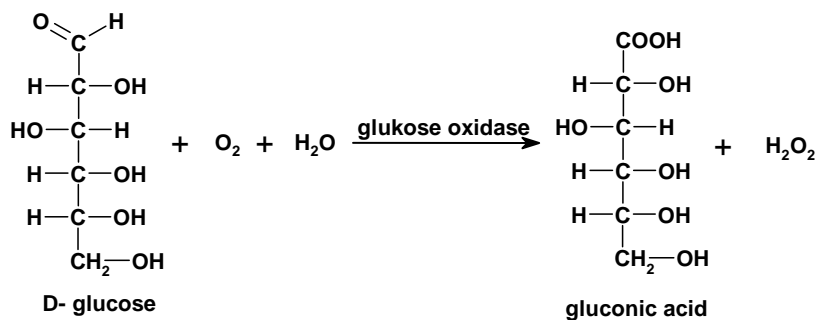


Figure: Uronic acid pathway. (Asterisk indicate the fate of carbon 1 of glucose)

Measurement of Glucose – coupled enzyme assay

The aim of the work: Determine the concentration of glucose in the sample of human blood serum.

Principle: Measurement of glucose in the samples by this method is based on formation of the gluconic acid (gluconate) by the action of enzyme glucose oxidase in the presence of oxygen (O_2). The by-product of this reaction H_2O_2 is a substrate for another enzyme - peroxidase, which in the presence of artificial substrate (e.g. orto-dianisidine = „Saifer Gernstenfield method“ or 4-aminoantipyrine and 3-methyl-phenol = „Trinder method“) leads to the formation of oxidized chromogen (orto-dianisidine) or to the formation of colored complex (4-aminoantipyrine with phenol). The intensity of the pink color measured at 540 nm (490 – 540 nm) is proportional to the original glucose concentration.



Indicator reaction

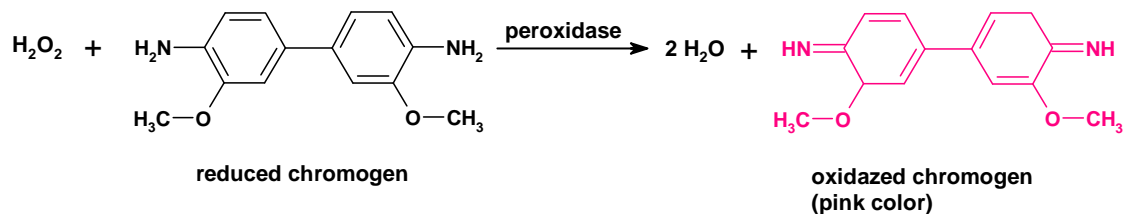


Figure: Saifer Gernstenfield Method with orto-dianisidine

Reagents:

- 1.) Enzymes: peroxidase, glucose oxidase, 4-aminoantipyrine (0,245 mmol/vial) – vial 1
- 2.) Buffer-chromogen: phosphate (0,14 mol/l), 3-methylphenol (10 mmol/l) – 250 ml – bottle 2

Incubation mixture:

Phosphate buffer, pH 8(25°C)	140 mmol/l
glucose oxidase	≥ 166 µkat/l
peroxidase	≥ 16 µkat/l
3-methylphenol	10 mmol/l
4-aminoantipyrine	1 mmol/l
Volume ratio serum/incubation mixture	1/101 (1/151)

Procedure:

Preparation of incubation mixture: Dissolve the contents of vial 1 in approximately 6 ml of distilled water and add it into the bottle 2. Mix well.

Measurement: Mix in the tubes incubation mixture (working solution) in the ratio 100:1 with serum (sample), standard (Glucose 5 mmol/l) and distilled water (control – blank). Incubate 15 minutes at 37 °C or 30 minutes at 25 °C (room temperature). Avoid exposure to direct light. Read the absorbance of sample (A_1) and standard (A_2) against the control.

Tubes	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
	Sample (serum)	Standard – Glucose (5 mmol/l)	Control – H ₂ O	Fructose (10 mmol/l)	Saccharose (10 mmol/l)
	20 µl	20 µl	20 µl	20 µl	20 µl
Working solution	2 ml	2 ml	2 ml	2 ml	2 ml

Measure after 30 minutes of incubation at 25 °C.

Results:

Tubes	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
Absorbance (530 nm)	0,555	0,250	0	0,001	0,000

Calculations:

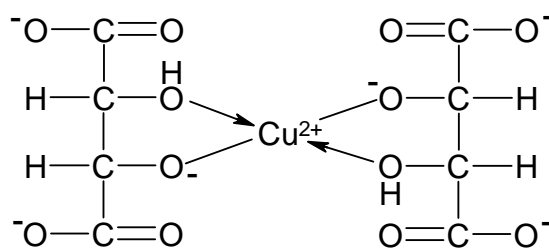
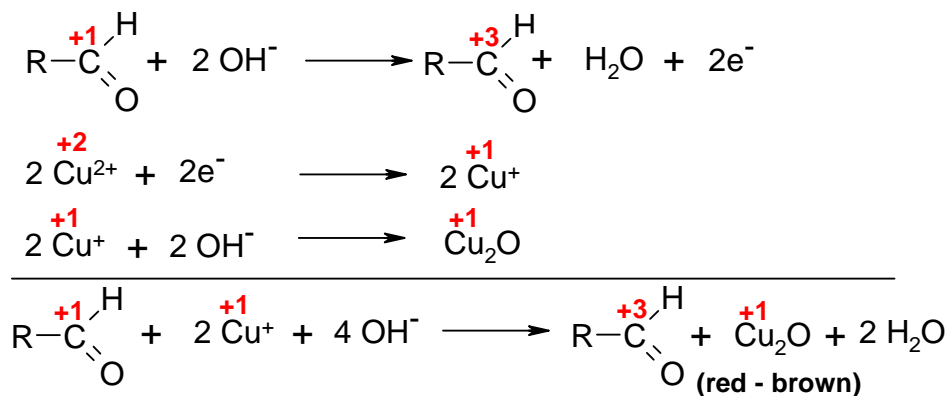
c - concentration of standard = 5 mmol/l

$$\text{Glucose in sample (mmol/l)} = c * \frac{A_1}{A_2}$$

Tubes	Tube 1	Tube 2	Tube 4	Tube 5
Glucose (mmol/l)	11,1	5	0	0

Fehling method

Principle: Fehling method of glucose detection is based on the reduction of Cu^{2+} cations to Cu^+ . In this reaction soluble CuSO_4 is reduced to insoluble Cu_2O , which form in the solution red-brown precipitate. This method is not specific for glucose because other reducing sugars (e.g. fructose, galactose) are able to reduce Cu^{2+} to Cu^+ . Moreover this method is also only semiquantitative and inaccurate.



Copper-tartrate complex

Figure: The reaction mechanism of Fehling detection of reducing sugars.

Positive result is indicated by the formation of the red-brown precipitate. Aldoses like other aldehydes are easily subjected to oxidation forming carboxylic acids. Copper ion is complexated with tartrate ion and forms Cu_2O . Without the tartate the copper ion could be reduced from the solution in the basal conditions.

Solutions (reagents)

Solution 1: 4 % CuSO_4 (w/v) – *Fehling solution 1*

Solution 2: 15 % sodium-potassium tartrate dissolved in 20 % NaOH – *Fehling solution 2*

Procedure:

1. To 1 ml of sample in the tube add 1 ml of Fehling solution 1 and 1 ml of Fehling solution 2.
2. Slightly mix the tube
3. Heat up the tube for few minutes (in boiling water) to the appearance of red precipitate.

Orto-tholuidine method

Principle: This method is based on the esterification reaction of o-tholuidine with glucose to form a Schiff base with green-blue color absorbing light at 630 nm.

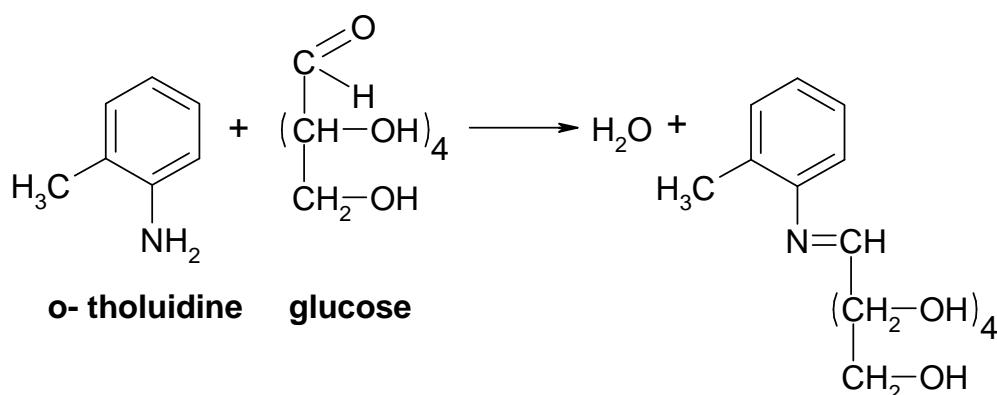


Figure: The reaction of o-tholuidine with glucose.

Reagents:

1. Solution of o-tholuidine – dissolve o-tholuidine in 0,75 mmol/l acetic acid. Final concentration of o-tholuidine is 20 mmol/l. This solution should be stabilized by the addition of small amount of thiourea (1 g /l of solution)
2. 5 % solution of trichloroacetic acid (TCA)

Procedure:

1. To the 0,025 ml of sample add 0,225 ml of TCA solution.
2. Mix the tube and add 2,0 ml of o-tholuidine solution
3. Boil the reaction mixture for 8 minutes
4. Chill the mixture and read the absorbance of sample against the control (prepared in parallel) (wavelength 630 nm)

Results:

Tube	Absorbance at 630 nm	Concentration of glucose (mmol/l)
Glucose standard (5 mmol/l)	0,163	5
Sample (serum)	0,301	9,23
Saccharose (10 mmol/l)	0,053	--
Fructose (10 mmol/l)	0,010	--

Calculations:

c - concentration of standard = 5 mmol/l

$$\text{Glucose in sample (mmol/l)} = c * \frac{A_1}{A_2} = 5 \text{ mmol/l} * \frac{0,301}{0,163} = 9,23 \text{ mmol/l}$$

A₁ – absorbance of sample

A₂ – absorbance of standard

Lipids

The lipids are a heterogeneous group of compounds, including fats, oils, steroids, waxes, and related compounds, which are related more by their physical than by their chemical properties. They have the common property of being (1) relatively insoluble in water and (2) soluble in nonpolar solvents such as ether and chloroform. They are important dietary constituents not only because of their high energy value but also because of the fat-soluble vitamins and the essential fatty acids contained in the fat of natural foods. Fat is stored in adipose tissue, where it also serves as a thermal insulator in the subcutaneous tissues and around certain organs. Nonpolar lipids act as electrical insulators, allowing rapid propagation of depolarization waves along myelinated nerves. Combinations of lipid and protein (lipoproteins) are important cellular constituents, occurring both in the cell membrane and in the mitochondria, and serving also as the means of transporting lipids in the blood. Knowledge of lipid biochemistry is necessary in understanding many important biomedical areas, eg, obesity, diabetes mellitus, atherosclerosis, and the role of various polyunsaturated fatty acids in nutrition and health.

TRIACYLGLYCEROLS (TRIGLYCERIDES) ARE THE MAIN STORAGE FORMS OF FATTY ACIDS

The triacylglycerols are esters of the trihydric alcohol glycerol and fatty acids. Mono- and diacylglycerols wherein one or two fatty acids are esterified with glycerol are also found in the tissues. These are of particular significance in the synthesis and hydrolysis of triacylglycerols.

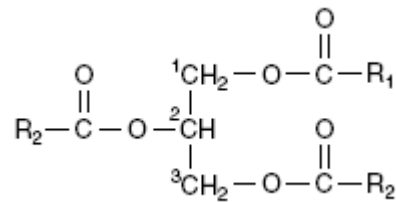
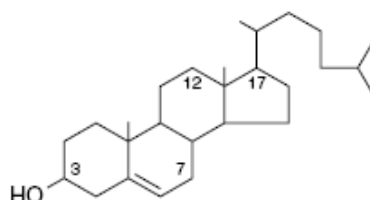


Figure: Triacylglycerol. R – fatty acids – may be the same or different kind

CHOLESTEROL

Cholesterol is present in tissues and in plasma either as free cholesterol or as a storage form, combined with a long-chain fatty acid as cholesteryl ester. In plasma, both forms are transported in lipoproteins. Cholesterol is an amphipathic lipid and as such is an essential structural component of membranes and of the outer layer of plasma lipoproteins. It is synthesized in many tissues from acetyl-CoA and is the precursor of all other steroids in the body such as corticosteroids, sex hormones, bile acids, and vitamin D. As a typical product of animal metabolism, cholesterol occurs in foods of animal origin such as egg yolk, meat, liver, and brain. Plasma low-density lipoprotein (LDL) is the vehicle of uptake of cholesterol and cholesteryl ester into many tissues. Free cholesterol is removed from tissues by plasma high-density lipoprotein (HDL) and transported to the liver, where it is eliminated from the body either unchanged or after conversion to bile acids in the process known as reverse cholesterol transport. However, its chief role in pathologic processes is as a factor in the genesis of atherosclerosis of vital arteries, causing cerebrovascular, coronary, and peripheral vascular disease.

Figure: Structure of cholesterol



Metabolism of lipids

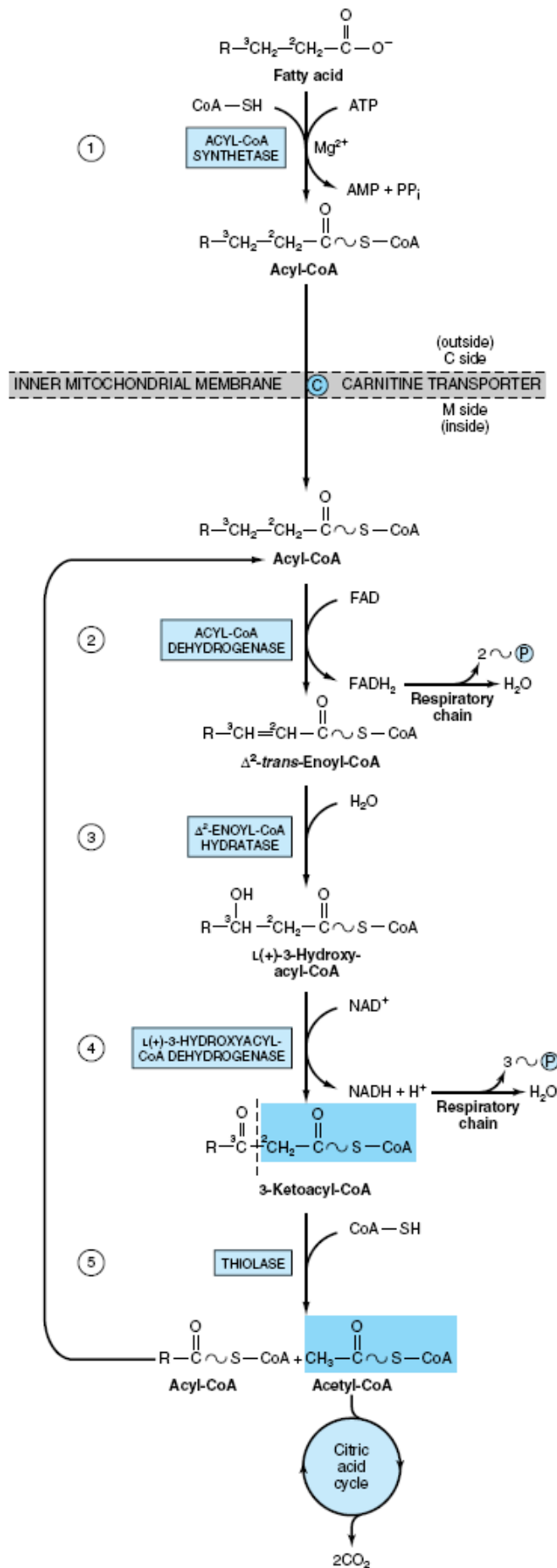


Figure: Degradation of fatty acids by β -oxidation

Sequence of reactions in the oxidation of unsaturated fatty acids, eg, linoleic acid. Δ^4 -cis-fatty acids or fatty acids forming Δ^4 -cis-enoyl-CoA enter the pathway at the position shown. NADPH for the dienoyl-CoA reductase step is supplied by intramitochondrial sources such as glutamate dehydrogenase, isocitrate dehydrogenase, and NAD(P)H transhydrogenase.

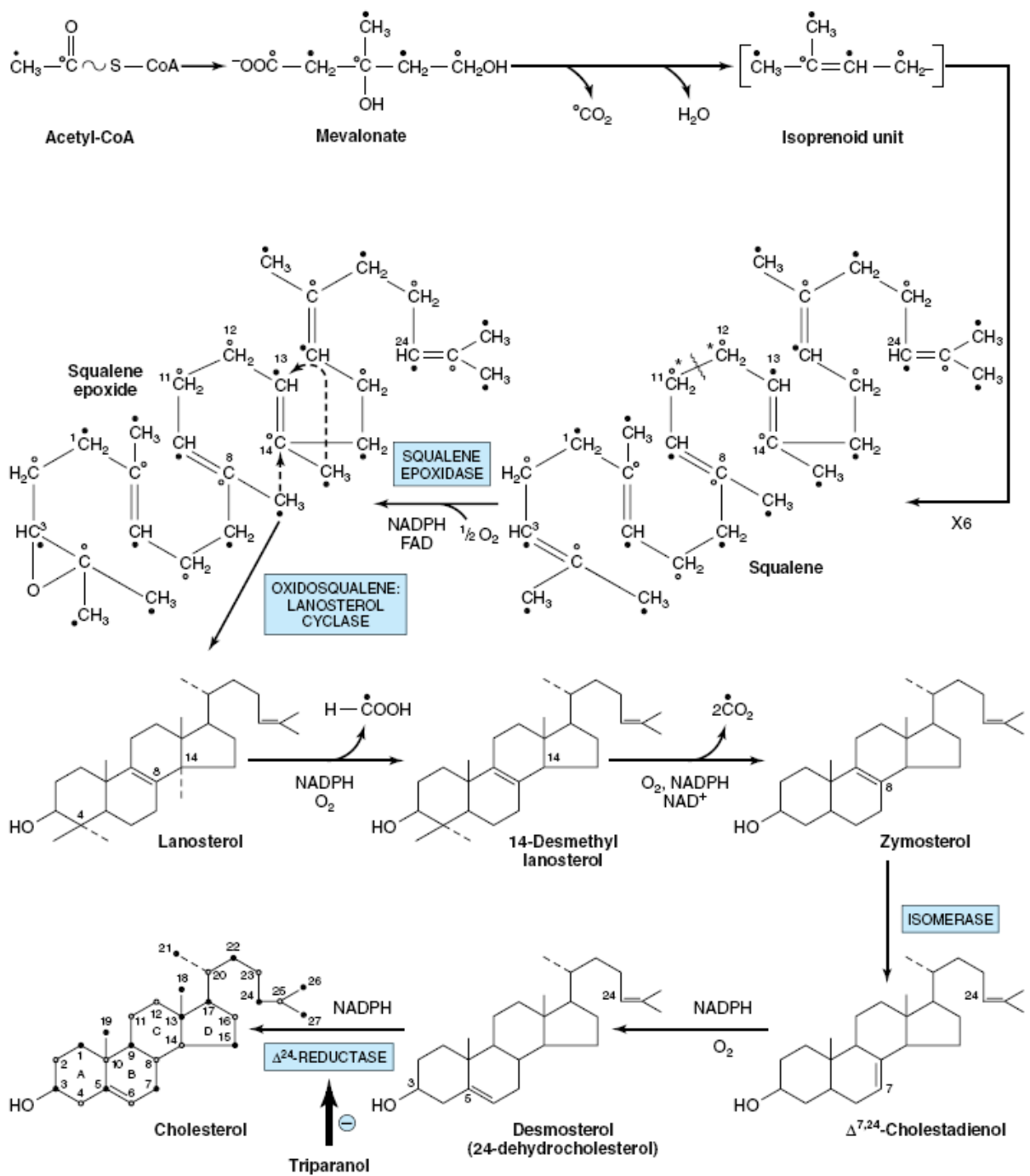


Figure: Biosynthesis of cholesterol. The numbered positions are those of the steroid nucleus and the open and solid circles indicate the fate of each of the carbons in the acetyl moiety of acetyl-CoA.

Measurement of cholesterol

Total cholesterol test (Liebermann-Burchard reaction)

Principle:

Cholesterol reacts with acetic anhydride and sulphuric acid to form green coloured product. The interference of proteins is suppressed by 2,5-dimethylbenzenesulfonic acid.

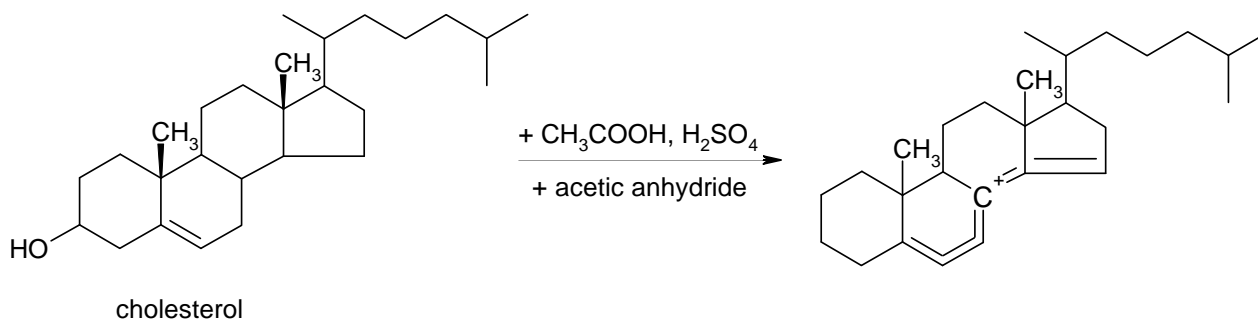


Figure: Liebermann-Burchard reaction of cholesterol with acetic anhydride in the presence of acetic acid and sulfuric acid. Product of the reaction absorbs the light at 560 – 590 nm.

Reagents:

1. Standard solution: cholesterol – 5,17 mmol/l, acetic acid – 17,5 mol/l
2. Acetic anhydride 6,5 mol/l, acetic acid 7,0 mol/l, 2,5-dimethylbenzenesulfonic acid
3. Sulphuric acid (pure)

Reaction mixture:

Acetic anhydride	5,3 mol/l
Sulphuric acid	3,0 mol/l
Acetic acid	5,7 mol/l
2,5-dimethylbenzenesulfonic acid	0,04 mol/l

Procedure:

In three test tubes mix Reagent 2 with serum (sample), with standard (Reagent 1) and with distilled water (control – blank) in the ratio 30:1. Incubate for 5 minutes in water bath (temperature 10 – 20°C). Then add into all test tubes 6 volume parts of Reagent 3 on each volume part of sample. Shake (mix) promptly and incubate for 10 minutes in water bath (10 – 20°C). Read the absorbance of the sample (A_1) and standard (A_2) against the control (blank) at 560 nm.

Reagents	Tubes	Sample	Standard	Control H_2O (blank)
Reagent 2		900 μ l	900 μ l	900 μ l
Serum		30 μ l	---	---
Standard – Reagent 1		---	30 μ l	---
H_2O		---	---	30 μ l
H_2SO_4 – Reagent 3		180 μ l	180 μ l	180 μ l

Results:

Absorbance of sample: $A_1 = 0,120$

Absorbance of standard: $A_2 = 0,197$

Calculations:

$$\text{Cholesterol in serum (mmol/l)} = 5,17 \text{ mmol/l} * \frac{A_1}{A_2} = 5,17 * \frac{0,120}{0,197} = 3,15 \text{ mmol/l}$$

HDL – cholesterol testHDL – **h**igh **d**ensity lipoproteins

Measurement of HDL-cholesterol is based on the same reaction as for total cholesterol (Lieberman-Burchard) but from the sample LDL-lipoproteins and VLDL lipoproteins have to be removed by the precipitating solution.

Precipitating solution: phosphothungstic acid 0,56 mmol/l, magnesium chloride 30 mmol/l.

Procedure:

Mix 1 volume part of serum (sample) with 2 volume parts of precipitating solution. Incubate for 10 minutes at 15 – 25°C. Centrifuge for 10 minutes at 3000 g or more. Into the new test tube replace the supernatant. For the measurement of HDL-cholesterol use the same volume of supernatant as for total cholesterol measurement and follow the procedure of total cholesterol measurement.

Note: For the calculations do not forget the dilution of sample (3x)

Results: Total and HDL-cholesterol measurement

Reagents	Tube	Sample 1 (original serum)	Sample 2 (supernatant of precipitated serum)	Standard	Control
Reagent 2		900 µl	900 µl	900 µl	900 µl
Original serum		30 µl	----	----	----
Supernatant		----	30 µl	----	----
Standard (Reagent 1)		----	----	30 µl	----
H₂O		----	----		30 µl
H₂SO₄ (Reagent 3)		180 µl	180 µl	180 µl	180 µl

Obtained values:

Tube	Sample 1	Sample 2	Standard
Absorbance (at 560 nm)	0,128 (A ₁)	0,01(A _{1HDL})	0,177 (A ₂)

Calculations:

$$\text{Total cholesterol in serum (mmol/l): } 5,17 \text{ mmol/l} * \frac{A_1}{A_2} = 5,17 * \frac{0,128}{0,177} = \underline{3,74 \text{ mmol/l}}$$

$$\text{HDL-cholesterol in serum (mmol/l): } 5,17 \text{ mmol/l} * \frac{A_{1HDL}}{A_2} * 3 = * \frac{0,01}{0,177} * 3 = \underline{0,87 \text{ mmol/l}}$$

LDL - calculations

Low-density lipoprotein (LDL) is a type of lipoprotein that transports cholesterol and triglycerides from the liver to peripheral tissues. LDL is one of the five major groups of lipoproteins; these groups include chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein, and high-density lipoprotein (HDL), although some alternative organizational schemes have been proposed. Like all lipoproteins, LDL enables fats and cholesterol to move within the water based solution of the blood stream. LDL also regulates cholesterol synthesis at these sites. It commonly appears in the medical setting as part of a cholesterol blood test, and since high levels of LDL cholesterol can signal medical problems like cardiovascular disease, it is sometimes called "bad cholesterol," (as opposed to HDL, which is frequently referred to as "good cholesterol" or "healthy cholesterol").

The lipid profile does not measure LDL level directly but instead estimates it using the Friedewald equation using levels of other cholesterol such as HDL:

Limitations for calculations: There are limitations to this method, most notably that samples must be obtained after a 12 to 14 h fast and that LDL-C cannot be calculated if plasma triglyceride is $>4,52$ mmol/L.

In mg/dl: $LDL\text{-}CH = TCH - HDL\text{-}CH - TG/5$ **Friedewald equation**
In mg/dl: LDL cholesterol = total cholesterol – HDL cholesterol – (0.20 × triglycerides)

In mmol/l: LDL cholesterol = total cholesterol – HDL cholesterol – (0.45 × triglycerides)

Normal ranges of LDL cholesterol

Level mg/dL	Level mmol/L	Interpretation
<100	<2.6	Optimal LDL cholesterol, corresponding to reduced, but not zero, risk for heart disease
100 to 129	2.6 to 3.3	Near optimal LDL level
130 to 159	3.3 to 4.1	Borderline high LDL level
160 to 189	4.1 to 4.9	High LDL level
>190	>4.9	Very high LDL level, corresponding to highest increased risk of heart disease

Measurement of triacylglycerol

Triglycerid (triacylglycerol) test

Triglycerides are formed from a single molecule of glycerol, combined with three fatty acids on each of the OH groups, and make up most of fats digested by humans. Ester bonds form between each fatty acid and the glycerol molecule.

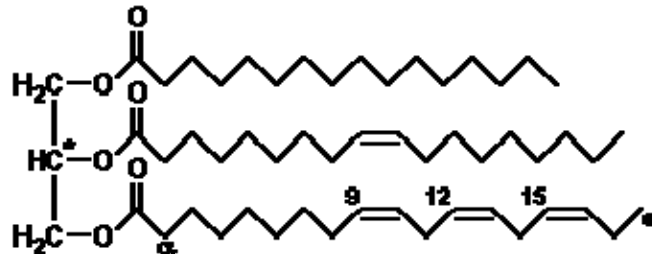


Figure: Example of an unsaturated fat triglyceride. Left part: glycerol, right part from top to bottom: palmitic acid, oleic acid, alpha-linolenic acid, chemical formula: $C_{55}H_{98}O_6$

Principle:

Triacylglycerols are saponified to glycerol by potassium hydroxide. The glycerol is oxidized to formaldehyde which forms with acetyl acetone and ammonium ions yellow 3,5-diacetyl-1,4-dihydrolutidine.

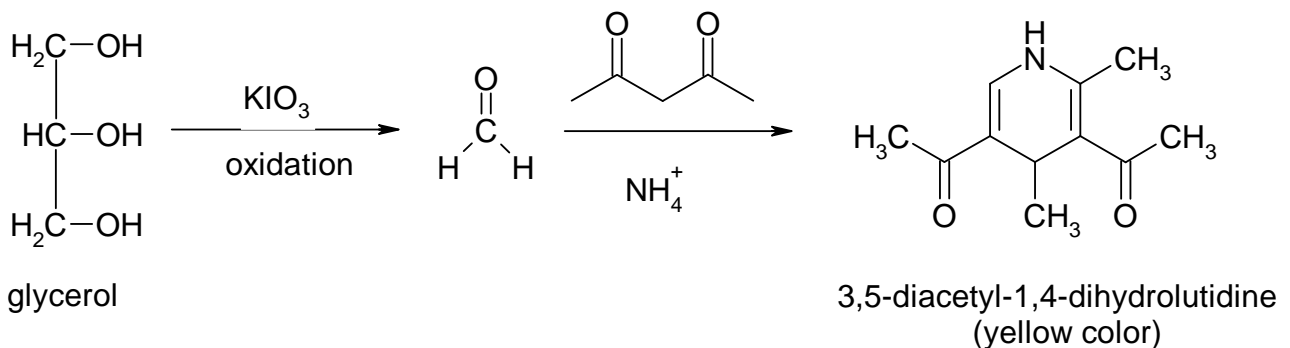
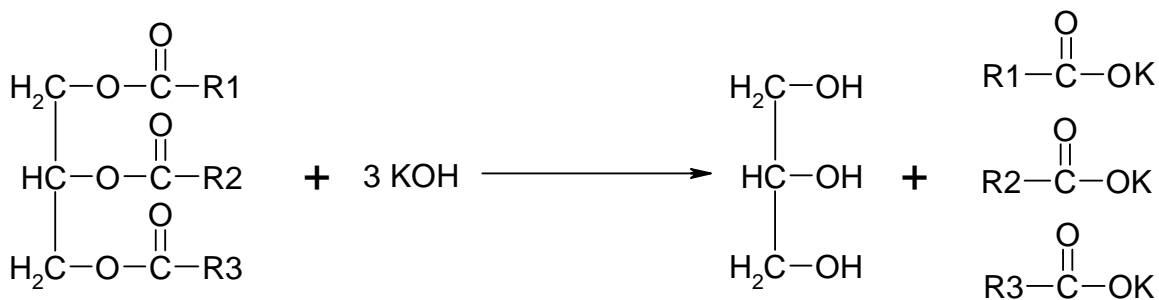


Figure: Reaction mechanism of triacylglycerol detection.

Reagents:

1. Standard solution (trioleine 3,39 mmol/l)
2. Acetyl acetone (0,75g/l in 20 % isopropanol)
3. Oxidizing solution (0,13 g KIO₃ in ammonium acetate buffer)
4. Potassium hydroxide (1 mol/l)
5. Izopropanol
6. Adsorbent (alumina)

Procedure:

In three tubes :

Pipette (ml)	Sample (serum)	Standard	Control (blank)
Serum	0,10	----	----
Reagent 1 (standard)	----	0,10	----
Distilled H ₂ O	----	----	0,10
Reagent 5 (izopropanol)	4,0	4,0	4,0

Shake the tubes and to all tubes add 0,4 g of Reagent 6 (adsorbent). Mix and shake the tubes for 10 minutes. Use shaking machine or your hands (use the stopper for the tubes).

Centrifuge the tubes for 5 minutes at 3000 rpm.

Pipette into dry test tubes:

Supernatant	2,0 ml	2,0 ml	2,0 ml
Reagent 4 (KOH)	0,5 ml	0,5 ml	0,5 ml

Mix, stopper test tubes and incubate for 10 minutes at 60±2 °C. Then cool for 5 minutes in cold water.

Add into all tubes 0,5 ml of Reagent 3 (oxidizing solution) and let stand for 10 minutes at room temperature.

Pipette into all test tubes 0,5 ml of Reagent 2 (Acetyl acetone), mix and incubate 30 minutes at 60±2 °C. Then cool in cold water. Read the absorbance of sample and standard against control at 405 nm.

Results:

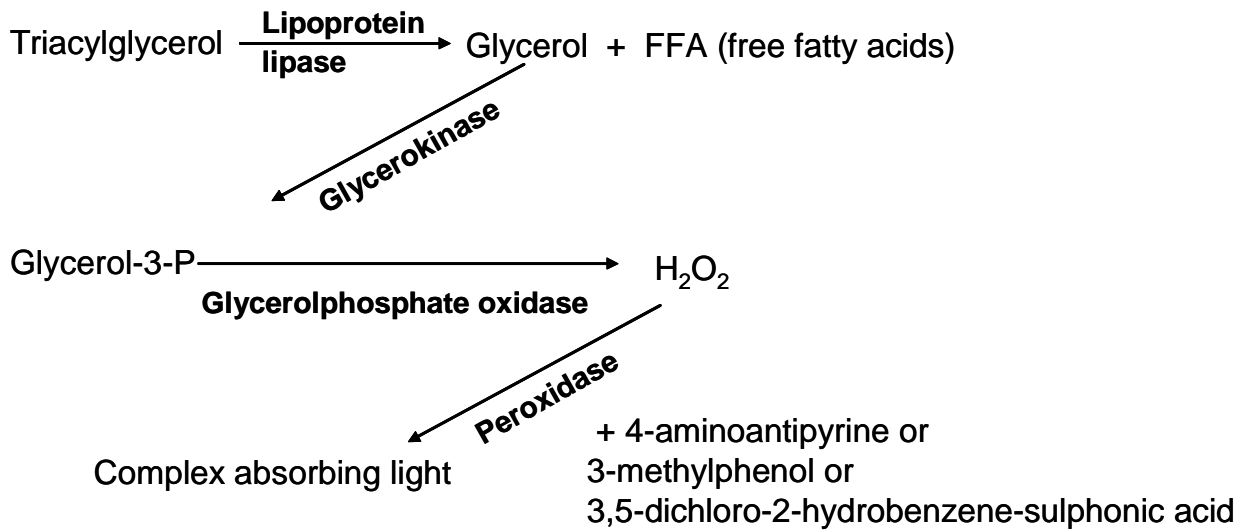
Tube	Sample (serum)	Standard
Absorbance (at 405 nm)	0,203	0,609

Calculations:

$$\text{Triacylglycerols in serum (mmol/l)} = 3,39 \text{ mmol/l} * \frac{0,203}{0,609} = \underline{\underline{1,13 \text{ mmol/l}}}$$

Another method for Triglycerid measurement – use of coupled enzyme assay

Principle:



Proteins

Functions of blood proteins

Blood proteins, also called serum proteins, are proteins found in blood plasma. Serum total protein in blood is 7g/dl, which in total makes 7% of total blood volume. They serve many different functions, including circulatory transport molecules for lipids, hormones, vitamins and metals enzymes, complement components, protease inhibitors, and kinin precursors regulation of acellular activity and functioning and in the immune system.

All the plasma proteins are synthesized in liver except gamma globulins.

60% of plasma proteins are made up of the protein albumin, which are major contributors to osmotic pressure of plasma which assists in the transport of lipids and steroid hormones. Globulins make up 35% of plasma proteins and are used in the transport of ions, hormones and lipids assisting in immune function. 4% is fibrinogen and this is essential in the clotting of blood and can be converted into insoluble fibrin. Regulatory proteins which make up less than 1% of plasma proteins are proteins such as enzymes, proenzymes and hormones.

Measurement of total blood proteins

Biuret method:

Principle: This method is most appropriate for the total blood proteins determination because it is not necessary to dilute the sample. Other methods (Lowry, Bradford) are very sensitive and the sample (serum) needs to be diluted.

Biuret method is the oldest method used for protein measurement and the principle is based on the reaction of $-\text{CO}-\text{NH}-$ (peptide bond) of proteins with the Cu^{2+} salts (e.g. CuSO_4) leading to the formation of the complex compound absorbing the light (at 555 nm), and thus enables spectrophotometric determination. Amount of proteins is calculated upon comparison with the absorbance of standard protein with known concentration.

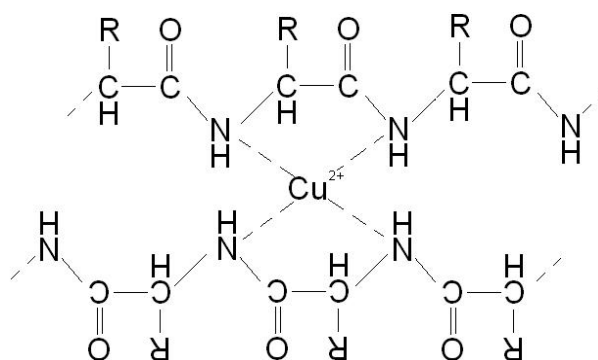


Figure: Light absorbing complex produced during the biuret reaction.

Reagent:

Biuret reagent: Dissolve 1,5 g $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ and 6 g potassium-sodium tartrate in 500 ml of distilled water and add under permanent mixing (stirring) 300 ml of 10 % NaOH prepared in distilled water. After the dissolution add water to the final volume 1000 ml and add 1 g of KI. This solution is ready for use.

Reagents:

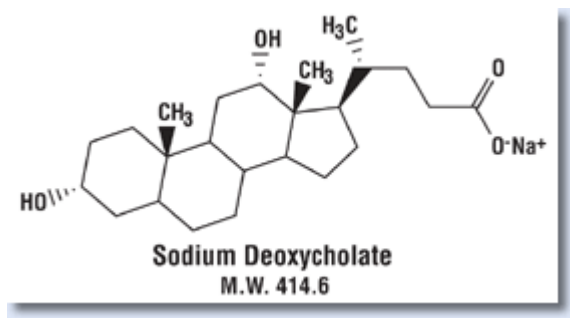
Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol, add 100 ml 85 % (w/v) phosphoric acid. Dilute to 1 liter when the dye is completely dissolved, and filter through Whatman #1 paper just before use.

sodium deoxycholate

standard protein solution of Bovine serum albumin (BSA), 1 mg/ml

testing samples

physiological solution : 160 mmol/l NaCl



Sodium deoxycholate is water soluble ionic detergent/bile salt commonly used in applications ranging from cell lysis (RIPA Buffer), liposome preparation, isolation of membrane proteins and lipids, preventing nonspecific binding in affinity chromatography and a cell culture media supplement. *Sodium deoxycholate can be added to the samples before the measurement for the precipitation of proteins and elimination of interfering substances.*

Procedure:

Preparation of calibration curve:

Standard protein weight (μg)	0	2	4	6	8	10	12	15
Volume of standard protein solution (μl)	0	2	4	6	8	10	12	15
Volume of physiological solution (μl)	1000	998	996	994	992	990	988	985
Volume of Bradford reagent (μl)	1000	1000	1000	1000	1000	1000	1000	1000
Total volume (μl)	2000	2000	2000	2000	2000	2000	2000	2000

Blank - control

Notes: 1. In some cases physiological solution can be replaced with distilled water.

2. Calibration curve (standard) and samples have to be treated at the same time !!!

Determination of protein concentration in sample:

- 1.) Pipette sample to the test tube and dilute to 1000 μl with physiological solution.
- 2.) Add 1000 μl of Bradford reagent, mix and incubate for 5 minutes at room temperature
- 3.) Read the absorbance of standards (calibration curve) and samples against the blank solution (Bradford reagent + physiological solution) at 595 nm.

Results:

Standard (μg)	0	2	4	6	8	10	12	15
Absorbance (at 595 nm)	0	0,108	0,144	0,269	0,335	0,342	0,415	0,482

Sample (serum) (μl)	1	2	10
Absorbance	0,914	1,018	1,163

Very high values. The sample was 10x diluted and the absorbance was measured as follow:

Sample (serum 10x diluted) (µl)	1
Absorbance	0,276

Calculations:

The protein concentration in samples is calculated from the calibration curve – linear regression of values obtained during the measurement of standard protein absorbances.

Least Squares method

Calibration curve can be obtained using some programmes (e.g. Microsoft Office – Excel, Origin Lab – for PC, Kaleidagraph – for Macintosh)

Manual calculation:

$Y = a + b.X$ General equation of linear line – regression line

$A = a + b.X$ A = absorbance
X = weight or concentration of protein (standard)
a, b = parameters of linear regression

$b = \frac{\overline{x.y} - \overline{x}.\overline{y}}{s_x^2}$ $a = \overline{y} - b.\overline{x}$ $\overline{x}, \overline{y}$ = average values of data

$s_x = \sqrt{\frac{1}{n} \sum_{i=1}^n (x_i - \overline{x})^2}$ s_x = standard deviation

	x (weight of standard protein)	y (absorbance)	x.y
	2	0,108	0,216
	4	0,144	0,576
	6	0,269	1,614
	8	0,335	2,68
	10	0,342	3,45
	12	0,415	4,98
	15	0,482	7,23
Average:	8,14	0,299	2,964

$s_x = 4,23, s_x^2 = 17,87$
 $a = 0,05751$
 $b = 0,02966$

$A = 0,05751 + 0,02966.x$ - equation of linear regression for our data

Sample (serum 10x diluted) (µl)	1
Absorbance	0,276
Weight of proteins in sample (µg)	7,36

Concentration of proteins in non-diluted (original) serum: 73,6 g/l

Nonprotein nitrogen compounds

Bulk of nitrogen compounds present in blood plasma is formed by proteins. However we can find in blood many different non-protein nitrogen compounds of which numerous have a diagnostic significance. Non protein nitrogen compounds differ in the concentrations, origin and significance. Some of them are waste products of metabolism, other are belonging to the biologically active substances. Some of those of diagnostic significance are mentioned below.

Urea, uric acid, creatinine – metabolism and functions

Urea is an organic compound with the chemical formula $(\text{NH}_2)_2\text{CO}$. Urea is highly soluble in water and has a pKa close to zero. Therefore it is an efficient way for mammals to expel excess nitrogen. Urea is, in essence, a waste product. It is found in and extracted from urine. However, it also plays a very important role in that it helps set up the countercurrent system in the nephrons. The countercurrent system in the nephrons allows for reabsorption of water and critical ions. Urea is reabsorbed in the inner medullary collecting ducts of the nephrons, thus raising the osmolarity in the medullary interstitium surrounding the thin ascending limb of the Loop of Henle. The greater the osmolarity of the medullary interstitium surrounding the thin ascending Loop of Henle, the more water will be reabsorbed out of the renal tubule back into the interstitium (and thus back into the body). Some of the urea from the medullary interstitium that helped set up the countercurrent system will also flow back into the tubule, through urea transporter 2, into the thin ascending limb of the loop of Henle, through the collecting ducts, and eventually out of the body as a component of urine. It is dissolved in blood (reference range of 2.5 - 7.5 mmol/liter) and excreted by the kidney as a component of urine. In addition, a small amount of urea is excreted (along with sodium chloride and water) in sweat.

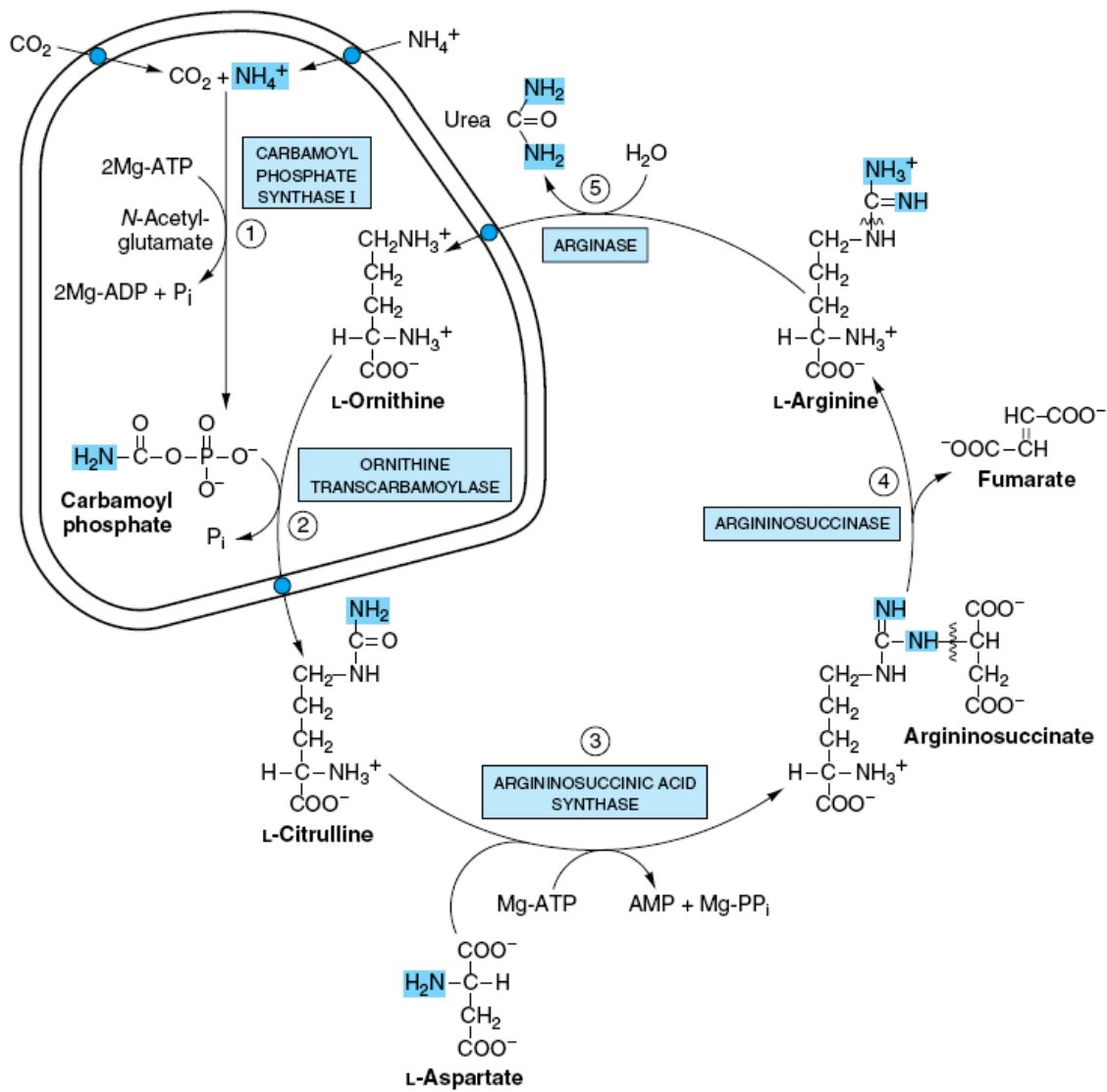


Figure: Reactions and intermediates of urea biosynthesis. The nitrogen-containing groups that contribute to the formation of urea are shaded. Reactions 1 and 2 occur in the matrix of liver mitochondria and reactions 3, 4, and 5 in liver cytosol. CO₂ (as bicarbonate), ammonium ion, ornithine, and citrulline enter the mitochondrial matrix via specific carriers (see heavy dots) present in the inner membrane of liver mitochondria.

Creatinine (from the Greek *kreas*, flesh) is a break-down product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body (depending on muscle mass). Chemically, creatinine is a spontaneously formed cyclic derivative of creatine. Creatinine is chiefly filtered out of the blood by the kidneys, though a small amount is actively secreted by the kidneys into the urine. There is little-to-no tubular reabsorption of creatinine. If the filtering of the kidney is deficient, blood levels rise. Therefore, creatinine levels in blood and urine may be used to calculate the creatinine clearance (CrCl), which reflects the glomerular filtration rate (GFR). The GFR is clinically important because it is a measurement of renal function. However, in cases of severe renal dysfunction, the creatinine clearance rate will be "overestimated" because the active secretion of creatinine will account for a larger fraction of the total creatinine cleared. Ketoacids, cimetidine and trimethoprim reduce creatinine tubular secretion and therefore increase the accuracy of the GFR estimate, particularly in severe renal dysfunction. (In the absence of secretion, creatinine behaves like inulin.)

A more complete estimation of renal function can be made when interpreting the blood (plasma) concentration of creatinine along with that of urea.

Men tend to have higher levels of creatinine because they generally have more skeletal muscle than women. Vegetarians have been shown to have lower creatinine levels.

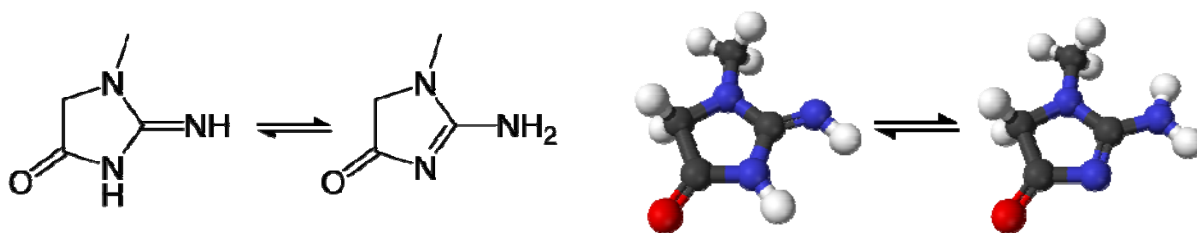


Figure: Tautomere isoform of creatinine.

Plasma creatinine (PCr)

Measuring serum creatinine is a simple test and it is the most commonly used indicator of renal function.

A rise in blood creatinine levels is observed only with marked damage to functioning nephrons. Therefore, this test is not suitable for detecting early stage kidney disease. A better estimation of kidney function is given by the creatinine clearance test. Creatinine clearance can be accurately calculated using serum creatinine concentration and some or all of the following variables: sex, age, weight, and race as suggested by the American Diabetes Association without a 24 hour urine collection. Some laboratories will calculate the CrCl if written on the pathology request form; and, the necessary age, sex, and weight are included in the patient information.

Urine creatinine (UCr)

Creatinine concentration is also checked during standard urine drug tests. High creatinine levels indicate a pure test while low amounts of creatinine in the urine indicate a manipulated test, either through the addition of water in the sample or by drinking excessive amounts of water.

Uric acid (or urate) is an organic compound of carbon, nitrogen, oxygen and hydrogen with the formula $C_5H_4N_4O_3$.

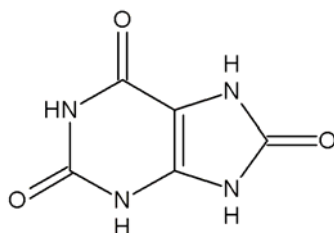
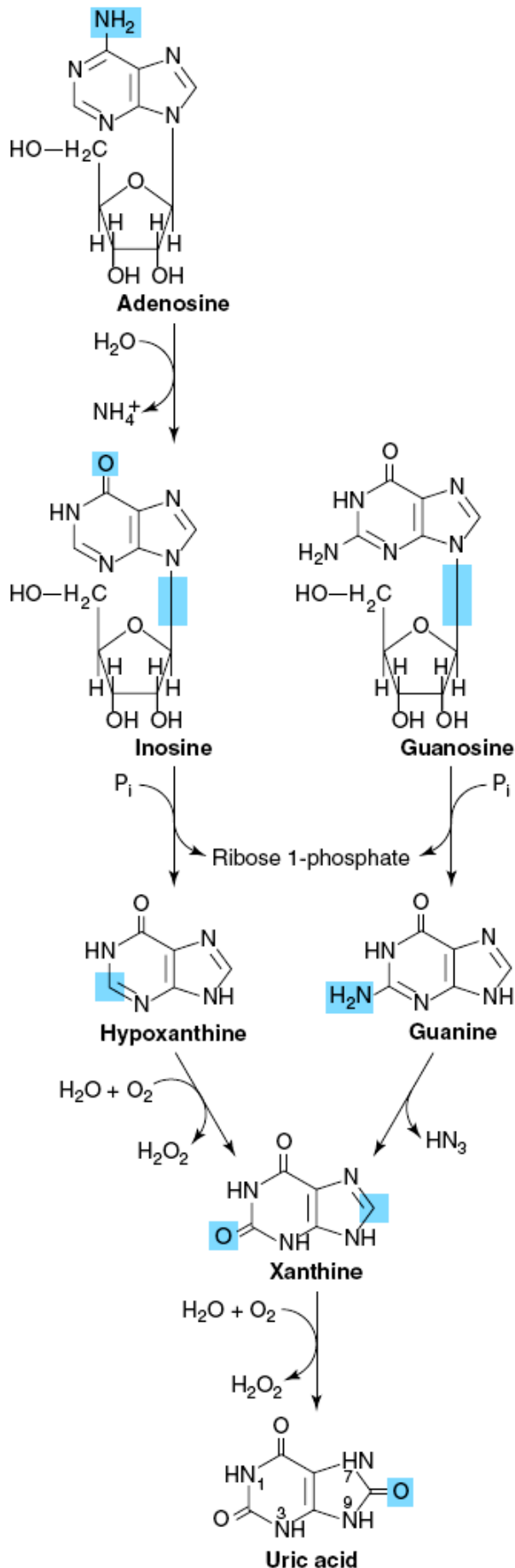


Figure: Structure of uric acid (7,9-dihydro-1H-purine-2,6,8(3H)-trione, 2,6,8-trioxopurine)

Uric acid is produced by xanthine oxidase from xanthine and hypoxanthine, which in turn are produced from purine. Uric acid is more toxic to tissues than either xanthine or hypoxanthine.



In humans and higher primates, uric acid is the final oxidation (breakdown) product of purine metabolism and is excreted in urine. In most other mammals, the enzyme uricase further oxidizes uric acid to allantoin. The loss of uricase in higher primates parallels the similar loss of the ability to synthesize ascorbic acid. Both uric acid and ascorbic acid are strong reducing agents (electron donors) and potent antioxidants. In humans, over half the antioxidant capacity of blood plasma comes from uric acid.

In humans, about 70 % of daily uric acid disposal occurs via the kidneys, and in 5-25 % of humans impaired renal (kidney) excretion leads to hyperuricemia.

Figure: Formation of uric acid from purine nucleosides by way of the purine bases hypoxanthine, xanthine, and guanine. Purine deoxyribonucleosides are degraded by the same catabolic pathway and enzymes, all of which exist in the mucosa of the mammalian gastrointestinal tract.

Measurement of serum urea

Principle: Urea reacts with diacetylmonoxime in strongly acidic medium in the presence of thiosemicarbazide and Fe(III) ions to form red coloured adduct suitable for the photometric determination. This method can be also used for the urea determination in urine. Urine has to be diluted prior to the analysis 1:50 or 1:100 with distilled water (result x dilution)

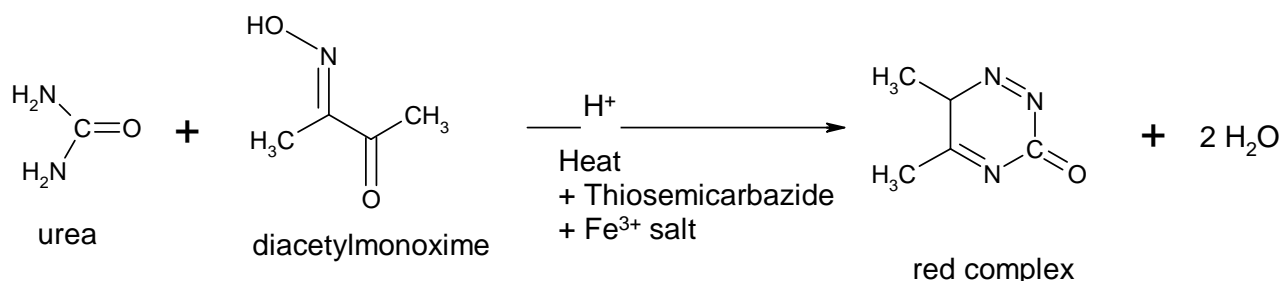


Figure: Reaction mechanism of urea determination.

Reagents:

1. Urea standard – 16,65 mmol/l
2. Diacetylmonoxime
1 tablet contains diacetylmonoxime 0,5 mmol, thiosemicarbazide 0,08 mmol and Fe(III) salt 2,5 µmol (Work carefully – POISON)
3. Sulphuric acid

Procedure:

1. Dissolve 1 tablet of Reagent 2 in 50 ml of distilled water
2. Prepare 50 ml of 10x diluted sulphuric acid : In about 30 ml of distilled water add 5 ml of concentrated (96%) sulphuric acid under continuous stirring. Add water to final volume 50 ml.
3. Prepare **Working solution** by mixing 1 volume of solution of Reagent 2 and 1 volume of diluted sulphuric acid. Prepare amount for daily consumption = prepare fresh working solution for every day
4. In three tubes mix 0,01 ml of sample/standard/distilled water with 2 ml of working solution.

	Sample	Standard	Control
Serum	0,01 ml	----	----
Reagent 1 - standard	----	0,01 ml	----
Distilled water	----	----	0,01 ml
Working solution	2 ml	2 ml	2 ml

Stopper the tubes and set it into boiling water for exactly 10 minutes.

Cool the content immediately in the tap water and read the absorbance of sample and standard against control at 490 nm (490 -540 nm).

Results:

Tubes	Sample (A ₁)	Standard (A ₂)
Absorbance	0,079	0,407

Calculations:

$$\text{Urea in serum (mmol/l)} = 16,65 \text{ mmol/l} * \frac{A_1}{A_2} = 16,65 * \frac{0,079}{0,407} = \underline{\underline{3,23 \text{ mmol/l}}}$$

Measurement of serum creatinine

Principle: Creatinine reacts in an alkaline medium with picric acid to form an orange-red coloration, which is suitable for photometric determination. Serum creatinine is determined after deproteinizing the sample, the urine is analyzed after dilution with water. The method is unspecific and compounds with active methylene group and some of the reducing substances (glucose) interfere with the determination.

Reagents:

1. Creatinine standard 1 vial
creatinine 442,5 $\mu\text{mol/l}$
2. Albumin 1 vial
albumin 0,16 g/vial
3. Deproteinizing solution – trichloroacetic acid 1,22 mol/l
4. Picric acid solution – 0,04 mol/l
5. Sodium hydroxide solution – 0,75 mol/l

Composition of reaction mixture:

Trichloroacetic acid	0,15 mol/l
Picric acid	10,0 mmol/l
Sodium hydroxid	0,19 mol/l

Procedure:

Determination in serum

1. Prepare solution 1: Dissolve the content of the vial with Reagent 1 in 8,0 ml of distilled water. Solution contains creatinine 442,5 $\mu\text{mol/l}$
2. Prepare solution 2: Dissolve the content of the vial with Reagent 2 in 8,0 ml of distilled water. Solution contains albumin 20 g/l.
3. Prepare solution 3 by mixing solution 1 and solution 2 in the ratio 4:6. This solution contains creatinine 177 $\mu\text{mol/l}$.
4. Prepare solution 4 by mixing solution 2 with distilled water in the ratio 4:6. This is control solution

Volume (ml)	Sample	Standard	Control	Control 2
Serum	0,5 ml	---	---	----
Distilled water	1,0 ml	1,0 ml	1,0 ml	1,5 ml
Solution 3	---	0,5 ml	---	---
Solution 4 (Control solution)			0,5 ml	---
Reagent 3	0,5 ml	0,5 ml	0,5 ml	0,5 ml
Stir (shake) and the centrifuge 10 minutes at 3000 rpm				
Supernatant	1 ml	1 ml	1 ml	1 ml
Reagent 4	0,5 ml	0,5 ml	0,5 ml	0,5 ml
Reagent 5	0,5 ml	0,5 ml	0,5 ml	0,5 ml

Stir (shake) and let stand 20 minutes at room temperature in the dark. Then read the absorbance of the sample (A_1) and standard (A_2) against the control at 500 nm.

Results:

	Sample (A ₁)	Standard (A ₂)
Absorbance (500 nm) against control 1	0,151	0,229
Absorbance (500 nm) against control 2	0,16	0,230

Calculations:

$$\text{Creatinine in serum } (\mu\text{mol/l}) = 177 \mu\text{mol/l} * \frac{A_1}{A_2} = 177 * \frac{0,151}{0,229} \quad \text{for control 1}$$

$$\text{Creatinine in serum } (\mu\text{mol/l}) = 177 \mu\text{mol/l} * \frac{A_1}{A_2} = 177 * \frac{0,160}{0,230} \quad \text{for control 2}$$

Creatinine in serum measured against control 1	116,7 $\mu\text{mol/l}$
Creatinine in serum measured against control 2	123,1 $\mu\text{mol/l}$

Note: There is only a marginal difference in the result depending on control used for the measurements. Both types of control can be used for measurement.

Measurement of serum uric acid

Principle: Uric acid is oxidized by oxygen in a reaction catalyzed by the enzyme uricase forming hydrogen peroxide and allantoin. The hydrogen peroxide is determined by an oxidation coupling with 4-aminoantipyrine and 4-chloro-3-methylphenol, a peroxidase catalyzed reaction.

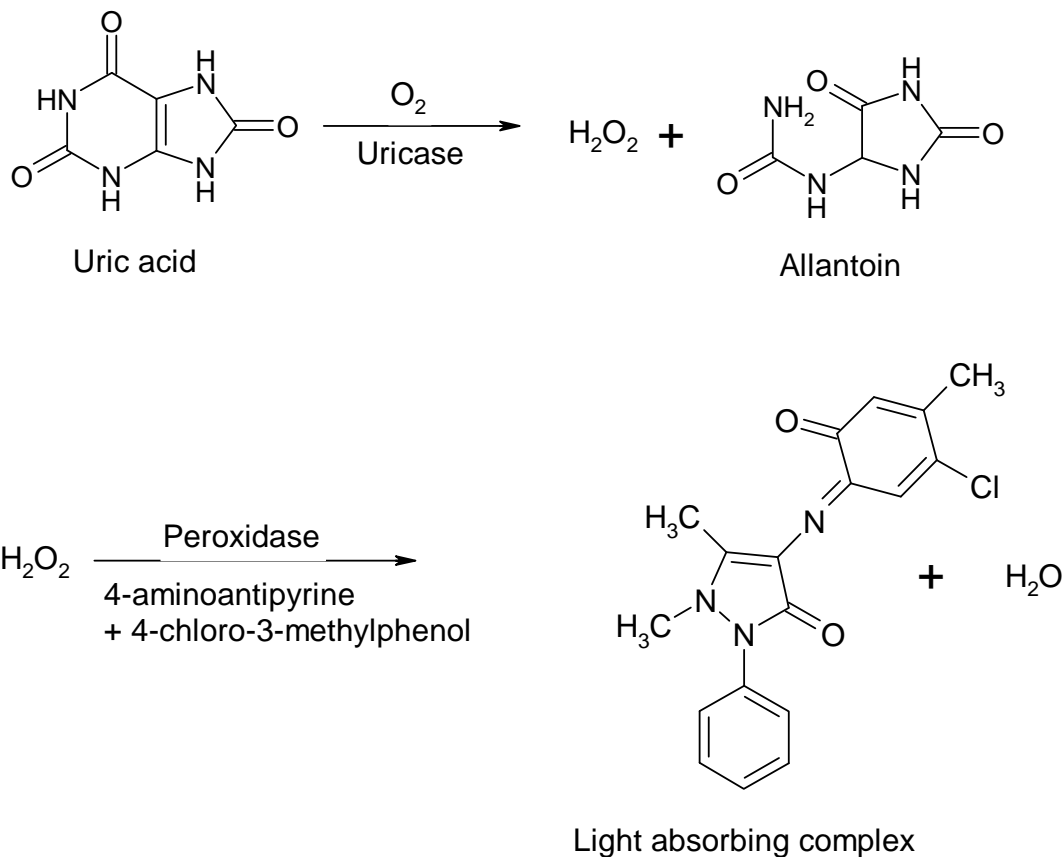


Figure: Reactions of uric acid determination

Reagents:

- | | |
|---|-----------|
| 1. Buffer – chromogen
phosphate buffer (0,1 mol/l), 4-chloro-3-methylphenol (2 mmol/l) | 200 ml |
| 2. Enzymes + 4-aminoantipyrine
peroxidase ($\geq 0,7 \mu\text{kat}$),
uricase ($\geq 0,088 \mu\text{kat}$),
4-aminoantipyrine ($\geq 42 \mu\text{mol}$),
potassium ferrocyanide (0,71 $\mu\text{mol/l}$) | in 1 vial |
| 3. Standard solution – uric acid (357 $\mu\text{mol/l}$) | 2 ml |

Reaction mixture composition:

phosphate bufeere, pH 7	100 mmol/l
Peroxidase	$\geq 15,8 \mu\text{kat/l}$
Uricase	$\geq 2,5 \mu\text{kat/l}$
4-aminoantipyrine	$\geq 0,9 \text{ mmol/l}$
4-chloro-3-methylphenol	2 mmol/l
Potassium ferrocyanide	14 $\mu\text{mol/l}$

Procedure:

1. Dissolve the content of vial with Reagent 2 in 2,5 ml of distilled water = Solution 1
2. Mix 1 volume part of Solution 1 with 20 volume parts of Reagent 1 = Solution 2
3. In three test tubes mix 20 volume parts of Solution 2 with one volume part of serum (sample), Reagent 3 (standard) or distilled water (control 1).
4. In the 4th test tube mix together Reagent 1 and serum in the same ration as mentioned above (control 2)

Notes: It is recommended to prepare the Solution 2 only in a quantity you can use the same day.

	Sample	Standard	Control 1	Control 2
Serum	0,1 ml			0,1 ml
Standard		0,1 ml		
Distilled water			0,1 ml	
Solution 2	2 ml	2 ml	2 ml	
Reagent 1				2 ml

Incubate 10 minutes at room temperature in the dark.

Then read the absorbance of the sample (A_1), standard (A_2), control 1 (A_3) and control 2 (A_4) at 500 nm against distilled water.

Results:

Tubes	Sample	Standard	Control 1	Control 2
	A_1	A_2	A_3	A_4
Absorbance	0,120	0,052	0,038	0,069

Calculations:

$$\begin{aligned} \text{Uric acid } (\mu\text{mol/l}) &= 357 \mu\text{mol/l} * \frac{A_1 - (A_3 + A_4)}{A_2 - A_3} = 357 * \frac{0,120 - (0,038 + 0,069)}{0,052 - 0,038} = 357 * \frac{0,013}{0,014} = \\ &= \underline{\underline{331,5 \mu\text{mol/l}}} \end{aligned}$$

Enzymes

Enzymes serve a wide variety of functions inside living organisms. They are indispensable for signal transduction and cell regulation, often via kinases and phosphatases. They also generate movement, with myosin hydrolysing ATP to generate muscle contraction and also moving cargo around the cell as part of the cytoskeleton.

An important function of enzymes is in the digestive systems of animals. Enzymes such as amylases and proteases break down large molecules (starch or proteins, respectively) into smaller ones, so they can be absorbed by the intestines. Starch molecules, for example, are too large to be absorbed from the intestine, but enzymes hydrolyse the starch chains into smaller molecules such as maltose and eventually glucose, which can then be absorbed. Different enzymes digest different food substances.

Several enzymes can work together in a specific order, creating metabolic pathways. In a metabolic pathway, one enzyme takes the product of another enzyme as a substrate. After the catalytic reaction, the product is then passed on to another enzyme. Sometimes more than one enzyme can catalyze the same reaction in parallel, this can allow more complex regulation: with for example a low constant activity being provided by one enzyme but an inducible high activity from a second enzyme.

Enzymes determine what steps occur in these pathways. Without enzymes, metabolism would neither progress through the same steps, nor be fast enough to serve the needs of the cell. Indeed, a metabolic pathway such as glycolysis could not exist independently of enzymes. Glucose, for example, can react directly with ATP to become phosphorylated at one or more of its carbons. In the absence of enzymes, this occurs so slowly as to be insignificant. However, if hexokinase is added, these slow reactions continue to take place except that phosphorylation at carbon 6 occurs so rapidly that if the mixture is tested a short time later, glucose-6-phosphate is found to be the only significant product. Consequently, the network of metabolic pathways within each cell depends on the set of functional enzymes that are present.

Measurement of serum alkaline phosphatase

Alkaline Phosphatases are a group of enzymes found primarily the liver (isoenzyme ALP-1) and bone (isoenzyme ALP-2). There are also small amounts produced by cells lining the intestines (isoenzyme ALP-3), the placenta, and the kidney (in the proximal convoluted tubules). What is measured in the blood is the total amount of alkaline phosphatases released from these tissues into the blood. As the name implies, this enzyme works best at an alkaline pH (a pH of 10), and thus the enzyme itself is inactive in the blood. Alkaline phosphatases act by splitting off phosphorus (an acidic mineral) creating an alkaline pH.

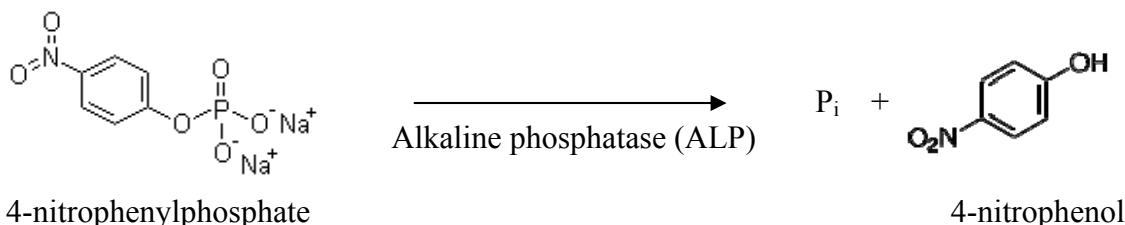
Alkaline phosphatase (ALP) (EC 3.1.3.1) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. The process of removing the phosphate group is called dephosphorylation.

In humans, alkaline phosphatase is present in all tissues throughout the entire body, but is particularly concentrated in liver, bile duct, kidney, bone, and the placenta.

The normal range is 20 to 140 IU/L. High ALP levels can show that the bile ducts are blocked. Levels are significantly higher in children and pregnant women. Also, elevated ALP indicates that there could be active bone deposition occurring as ALP is a byproduct of osteoblast activity (such as the case in Paget's disease).

Principle

Alkaline phosphatase splits 4-nitrophenylphosphate into 4-nitrophenol and phosphate in N-methyl-D-glucamine buffer. ALP is activated by sodium chloride. The enzyme activity is measured by the amount of liberated 4-nitrophenol which can be determined either by the kinetic method or by the constant time method using inhibitor blocking the active enzyme center.



Procedure:

Constant time method:

1. Prepare **Solution 1** containing 4-nitrophenylphosphate (92 mmol/l)
2. Prepare Solution 2 = Buffer containing: N-methyl-D-glucamine (426 mmol/l)
NaCl (85,2 mmol/l)
Mg₂Cl (0,61 mmol/l)
3. Prepare Inhibition solution containing: EDTA 30 mmol/l
NaOH 1 mol/l

Composition of reaction mixture:

N-methyl-D-glucamine	0,35 mol/l
NaCl	70 mmol/l
Mg ₂ Cl	0,5 mmol/l
4-nitrophenylphosphate	15 mmol/l

	Sample	Control
Solution 2	1 ml	1 ml
Serum	0,02 ml	
Mix and incubate 5 minutes at 37 °C and then add:		
Solution 1	0,2 ml	0,2 ml
Mix and incubate 10 minutes at 37°C and then add:		
Inhibition solution	0,5 ml	0,5 ml
Serum	-----	0,02 ml

Mix the tubes and measure the absorbance of the sample (A₁) and control (A₂) against distilled water at 420 nm. Calculate $\Delta A = A_1 - A_2$

Results:

Tube:	Sample 1	Sample 2	Control
Absorbance at 420 nm	0,386	0,393	0,271

Calculations:

Catalytic activity of ALP in the sample is calculated following:

$$\text{ALP } (\mu\text{kat/l}) = 10,263 * (\Delta\text{A})$$

$$\text{For sample 1: ALP } (\mu\text{kat/l}) = 10,263 * (0,386 - 0,271) = 1,18 \mu\text{kat/l}$$

$$\text{For sample 2: ALP } (\mu\text{kat/l}) = 10,263 * (0,393 - 0,271) = 1,25 \mu\text{kat/l}$$

NOTE: Never use as anticoagulant for blood samples EDTA!!! EDTA is an inhibitor of ALP activity. As anticoagulant sodium citrate/citric acid or heparine should be used!!!

Vitamins – introduction and function,

A vitamin is an organic compound required as a nutrient in tiny amounts by an organism. A compound is called a vitamin when it cannot be synthesized in sufficient quantities by an organism, and must be obtained from the diet. Thus, the term is conditional both on the circumstances and the particular organism. For example, ascorbic acid functions as vitamin C for some animals but not others, and vitamins D and K are required in the human diet only in certain circumstances. The term vitamin does not include other essential nutrients such as dietary minerals, essential fatty acids, or essential amino acids, nor does it encompass the large number of other nutrients that promote health but are otherwise required less often.

Vitamins are classified by their biological and chemical activity, not their structure. Thus, each "vitamin" may refer to several vitamer compounds that all show the biological activity associated with a particular vitamin. Such a set of chemicals are grouped under an alphabetized vitamin "generic descriptor" title, such as "vitamin A," which includes the compounds retinal, retinol, and many carotenoids. Vitamers are often inter-converted in the body.

Vitamins have diverse biochemical functions, including function as hormones (e.g. vitamin D), antioxidants (e.g. vitamin E), and mediators of cell signaling and regulators of cell and tissue growth and differentiation (e.g. vitamin A). The largest number of vitamins (e.g. B complex vitamins) function as precursors for enzyme cofactor bio-molecules (coenzymes), that help act as catalysts and substrates in metabolism. When acting as part of a catalyst, vitamins are bound to enzymes and are called prosthetic groups. For example, biotin is part of enzymes involved in making fatty acids. Vitamins also act as coenzymes to carry chemical groups between enzymes. For example, folic acid carries various forms of carbon group – methyl, formyl and methylene - in the cell. Although these roles in assisting enzyme reactions are vitamins' best-known function, the other vitamin functions are equally important.

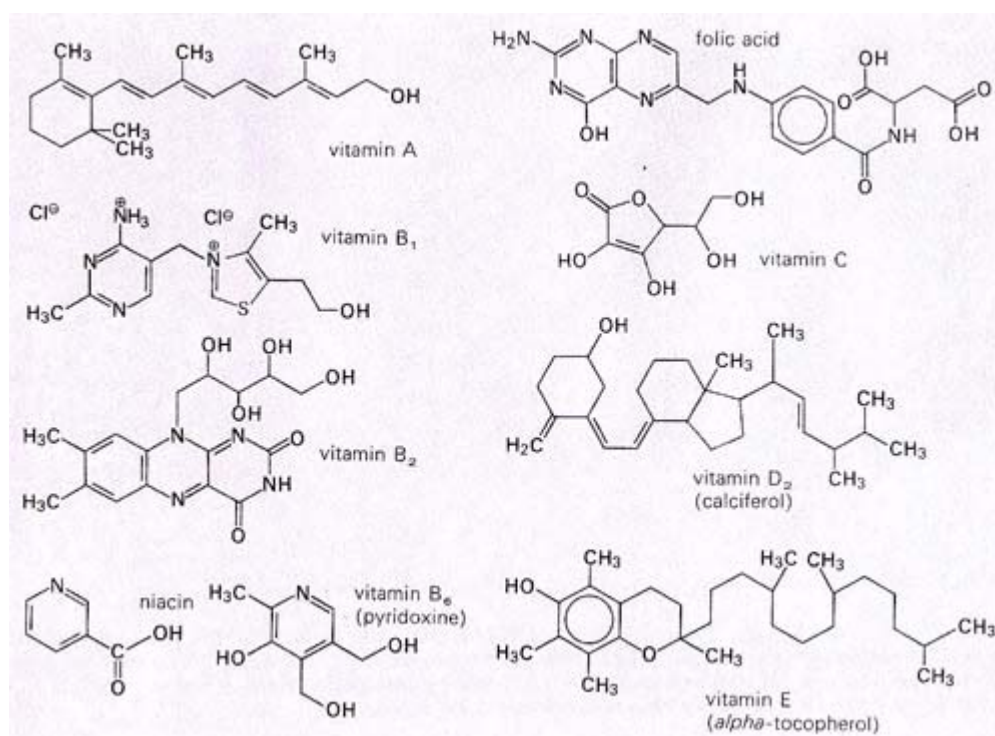


Figure: Chemical structures of vitamins.

Vitamic C measurement

Principle: Proof and determination of vitamins in samples are belonging to basic analyses in pharmacia, medical biochemistry and food analysis. Vitamins differ in their chemical structure and therefore their determination can not be assessed by a universal approach but the determination of each vitamin is based on its chemical nature.

For the determination of vitamic C (L-ascorbic acid) we use its property to be easily oxidated to dehydroascorbic acid.

One way to determine the amount of vitamin C is to use a redox titration. The principle of this method is a titration with dichlorophenolindophenol (or phenol-indo-2:6-dichlorophenol, also known as DPIP). DPIP is commonly used as an indicator for. If vitamin C, which is a good reducing agent is present, the blue dye, which turns pink in acid conditions and is reduced to a colorless compound by ascorbic acid.

They react in a 1:1 fashion, so if a known quantity of DPIP solution reacts with the plant tissue extract, the quantity of DPIP used gives a direct measure of the quantity of ascorbic acid present.

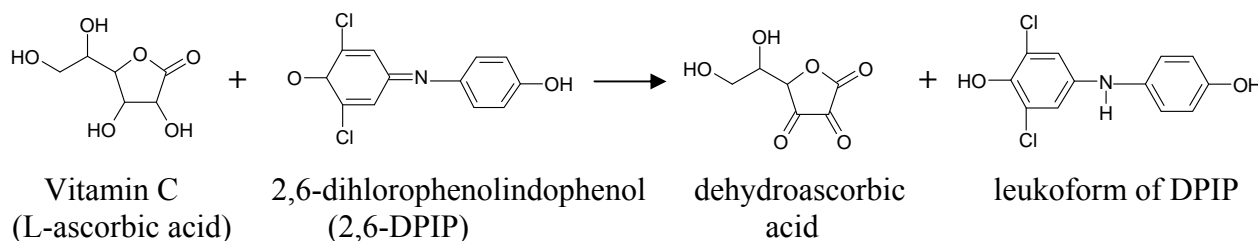


Figure: Reaction of L-ascorbic acid with 2,6-DPIP during the redox titration.

Reagents:

1. 2 % hydrochloric acid (HCl)
2. 1 mmol.l⁻¹ 2,6-DPIP dissolved in 30 mmol.l⁻¹ phosphate buffer, pH = 7
3. Phosphate buffer: Prepare: Solution 1: 150 mmol/l Na₂HPO₄
Solution 2: 150 mmol/l KH₂PO₄

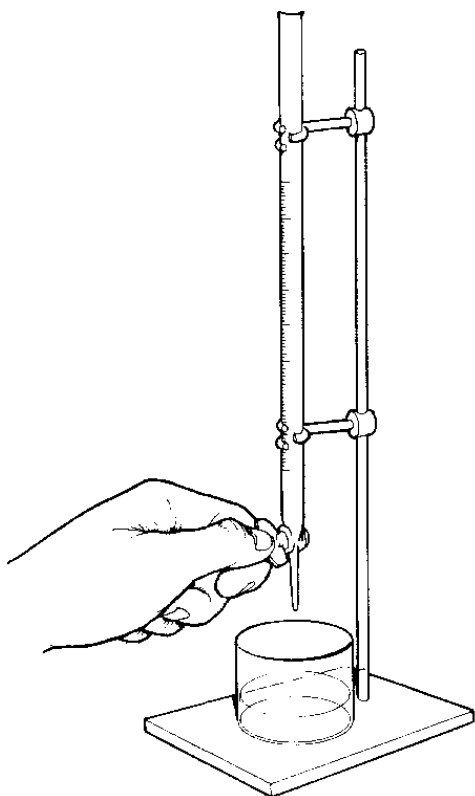
Mix solution 1 and solution 2 in the ratio 6:4 (e.g. : 6 ml + 4 ml, or 60 ml + 40 ml) and add adequate volume of distilled water (e.g.: for 100 ml of buffer add 90 ml of water, for 1000 ml of buffer add 900 ml of water)

Instruments:

1. mortar and pestle
2. burette
3. titration flasks
4. scales
5. samples = fruits (e.g.: lemon, apple, orange), vegetables (e.g.: potatoes), juice



mortar and pestle



burette

Procedure:

1. Sample of fruit or vegetable (approx. 25 g) is crushed in a mortar with a pestle in 2 % HCl
2. The suspension of sample is filtrated through the mull (mesh) (or can be centrifuged 5 minutes at 1000 g).
3. Filtrate (or supernatant) is replaced to the 50 ml volumetric flask and the volume is adjusted to 50 ml exact with 2 % HCl. The content of flask is mixed.
4. Fill up the burette with 2,6-DPIP solution.
5. 5 ml of sample is replaced to the dry titration flask and titrated with 2,6-DPIP. If the consumption of 2,6-DPIP is to low or to high the volume of sample can be adequately changed.

Results:

weight of Lemon = 7,6 g , volume of sample after the crushing and filtration = 50 ml
 weight of Apple = 33,5 g , volume of sample after the crushing and filtration = 50 ml

Volume of DPIP consumed for titration	Sample	Lemon – 0,5 ml (volume taken for titration)	Apple - 1 ml (volume taken for titration)
Titration 1		9,2 ml	2,4 ml
Titration 2		7,2 ml	2,4 ml
Titration 3		7,9 ml	---
Average:		8,1 ml	2,4 ml

Calculations:

The **equivalence point**, or stoichiometric point, of a chemical reaction occurs during a chemical titration when the amount of titrant (in this work = 2,6-DPIP) added is equivalent, or equal, to the amount of analyte present in the sample (in this = ascorbic acid).

The **end point** (similar, but not the same as the equivalence point) refers to the point at which the indicator changes color in a colorimetric titration.

In this work end point = equivalence point , because 2,6-DPIP is the titrant and also indicator.

From the reaction: $n(\mathbf{2,6-DPIP}) = n(\mathbf{Ascorbic\ acid})$ at the equivalence point.

$$M_r(2,6-DPIP) = 290,08 \text{ g/mol}$$

$$M_r(\text{ascorbic acid}) = 176,12 \text{ g/mol}$$

Lemon:

$$n(2,6-DPIP) = c(2,6-DPIP) * V(2,6-DPIP) = 1 \text{ mmol/l} * 8,1 \text{ ml} = 8,1 \text{ } \mu\text{mol}$$

$$8,1 \text{ } \mu\text{mol} = n(\text{ascorbic acid}) \text{ in } 0,5 \text{ ml of Lemon sample} \rightarrow$$

$$\text{in } 50 \text{ ml of lemon sample: } 8,1 \text{ } \mu\text{mol} * 100 = 810 \text{ } \mu\text{mol}$$

$$m(\text{ascorbic acid in original lemon sample}) = n(\text{ascorbic acid}) * M_r(\text{ascorbic acid}) = 810 \text{ } \mu\text{mol} * 176,12 \text{ g/mol} = 146,4 \text{ mg in } 7,6 \text{ g of lemon} = 18,7 \text{ mg/1 g of lemon}$$

Report from the 36th International Conference of Slovak Society of Chemical Engineering

25-29 May 2009, High Tatras, Slovakia

36th International Conference of Slovak Society of Chemical Engineering held in High Tatras mountains for 5 days from 25 May to 29 May 2009.

The conference is organized by the Slovak Society of Chemical Engineering with assistance of the Institute of Chemical and Biochemical Engineering of the Slovak University of Technology.

The aim of the conference is presentation of recent results of research and technological - engineering solutions in the area of chemical and biochemical engineering, mechanical engineering in chemistry, control of processes in chemical and food industry, with the focus on rational use of energy sources, ecology and risk management.

At the official opening of the conference the organizing committee of the conference welcomed me and my colleagues from Khabul Polytechnical University as the first Afghan representatives invited in such a conference.

There was about 280 participants from 27 countries and they presented 320 scientific works. All lectures of the conference were very interesting and useful. These lectures concerned about chemistry, chemical technology, biotechnologies using microbes for the production of different products (biogas, biofuel, food additives...), food technology, public health, environmental engineering and technology, prevention of pollution and many other fields.

It was done in three sections and at the end of every lecture proper answers were given to the questions which were considered to be very helpful.

Among the mentioned lectures there were three lectures of Dr. Juma Haydary an Afghan citizen, who is an assistant professor at the Faculty of Chemical and Food Technology of Slovak University of Technology in Bratislava, and he is a coordinator of cooperation projects between the University and Afghanistan Ministry of Higher Education. His lectures were really scientific and interesting and were worthwhile.

Each participant was given a Book of abstracts edited by the Faculty of Chemical and Food technology including a CD. In my idea these lectures are useful for professors and students for study and for new ideas in research.

Useful internet links: