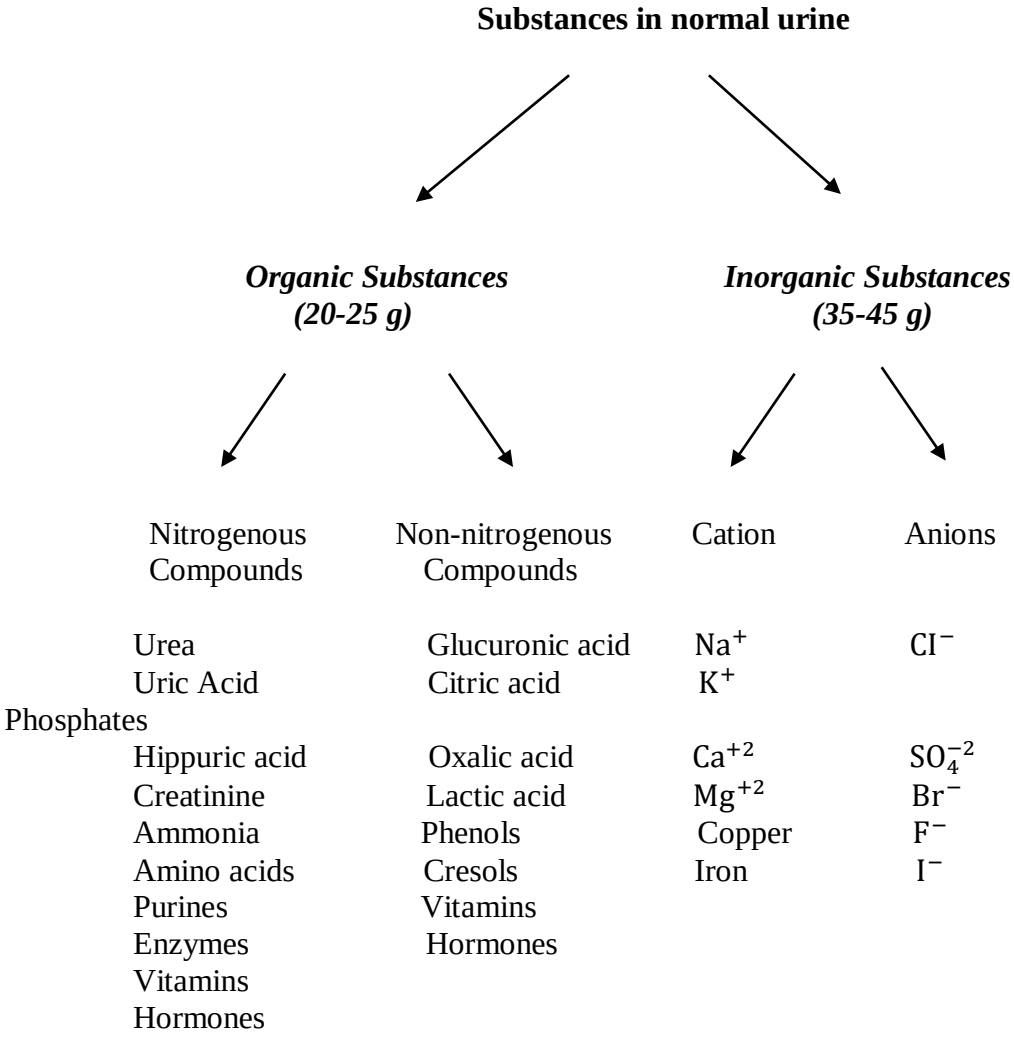
URINE

Urine is a liquid that removes many substances in the form of molten or suspended particles from the organism.



Phosphates

Specific reactions of some of the above-mentioned substances in a normal urine are known. Based on these reactions, it can be understood whether a fluid is a urine. Urine is: A liquid that gives chloride, sulfate, creatinine and urea reactions, and when the water is evaporated, leaves a rich residue of urea, uric acid and hippuric

acid.

Understanding whether a liquid is urine or not:

- •Determination of chloride, sulfate, urea, creatinine in liquid
- •Search for urea in the residual after the liquid has been evaporated

Experiments will be carried out by sampling 2-3 ml into the tube.

1-Determination of chloride

Liquid + a few drops of concentrated HNO_3+ 2-3 drops of 5% $AgNO_3$→ AgCl ↓ (white sediment)

Nitric acid (HNO_3 prevents the precipitation of phosphate and carbonate which could be precipitated by silver nitrate ($AgNO_3$).

2-Determination of sulphate

Liquid + several drops of 10% HCl + 2-3 drops $BaCl_2$→ $BaSO_4$↓ (white sediment)

3-Determination of urea

Searched by two experiments:

- Sodium hypobromite (NaOBr)
- Using the enzyme urease

a- Sodium hypobromite (NaOBr) test:

Liquid + 1-2 drops of alkaline NaOBr → take out white bubbles (nitrogen gas)

$$H_2N\text{-}CO\text{-}NH_2 + 3\ NaOBr \rightarrow CO_2 + 2\ H_2O + N_2 + 3\ NaBr$$

(NaOBr is obtained by dropping bromine to the NaOH solution, the excess CO_2 in the atmosphere during the reaction is retained by NaOH, so the gas bubbles seen in the tube belong to the nitrogen gas.)

b- Urease test

Experiment: Liquid + 1-2 drops phenolphthalein + 1 spatula tip powdered urease → (expected), pink color

Control: Liquid + 1-2 drops of phenolphthalein → color does not occur

Urease is an enzyme that breaks down urea. It converts urea to CO_2 and ammonia, resulting in an alkaline environment, and the phenolphthalein indicator which is colorless in neutral and acidic environments, turns pink in alkaline environment.

$$H_2N\text{-}CO\text{-}NH_2 + H_2O + \text{urease} \rightarrow 2NH_3 + CO_2$$

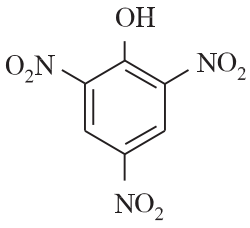
4- Determination of creatinine

Searched by two experiments:

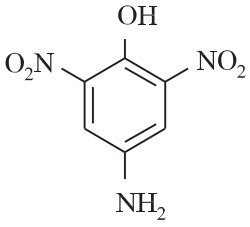
- Jaffe reaction
- Weyl-Legal reaction

a- Jaffe reaction

Liquid + 3-4 drops of saturated picric acid + 10% NaOH until alkaline → orange color



Picric Acid



Picramic Acid

b- Weyl-Legal reaction

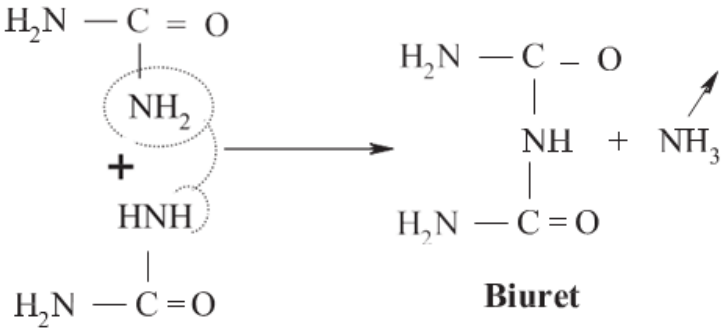
Liquid + 2-3 drops of sodium nitrosoprussiate $[Fe(CN)_5(NO)Na_2].2H_2O$ + 10% NaOH until alkaline → dark red color + a few drops of glacial acetic acid → red color disappears (turns to yellowish-greenish color).

5- Search of urea in the residue (Biuret test)

Residual: The remaining solid part of the liquid (urine) after the water has been evaporated in a porcelain capsule. In applications, urea powder will be used instead of "residual". A little urea powder is taken into a dry tube, heated in an open flame, urea is melted first, heating is continued. The smell of ammonia is heard. The tube is heated until this odor is not heard, while the liquid part evaporates, the material becomes solid. The tube is cooled and shaken with 1 ml of NaOH solution, 1-2 drops of 1% (dilute) $CuSO_4$ solution is added dropwise, violet color is seen.

During dry heating, two molecules of urea combine with the outlet of one molecule

of ammonia. This new substance is called biuret (biuret). This substance is combined with copper in an alkaline environment and a violet complex is formed.



2 moles of urea

CONCLUSION: If the reactions of all the substances required in the liquid and the residue are positive, we can postulate that the fluid is urine.

URINE ANALYSIS

In addition to the normal substances found in the urine, in some diseases, substances which should not normally be found, such as sugar, protein, appear in urine. The urine to be used in the analysis may be 24-hour urine (all or part of it is sent to the laboratory) or the first urine sample taken in the morning at awakening. The 24-hour urine is collected as follows: The first urine of the morning is discarded or taken apart, the others are collected in a bottle, and all and the first morning urine are brought separately to the laboratory.

Urine analysis is done in three sections:
• Examination of physical properties of urine
• Examination of chemical properties of urine
• Microscopic examination of urine

Examination of physical properties of urine
Determination of the physical properties of the urine constitutes the first stage in the examination of the urine for analysis. For this, the amount, color, clarity, odor, specific gravity and reaction of urine are examined.

The findings of the physical properties of the urine are analyzed in the Physical Examination section of the Urine Analysis Report.

Quantity: The amount of incoming urine for analysis is measured in a bladder. Daily urine average is 1500 ml in men and 1200 ml in women.

In cases where the amount of urine increases, is called **polyuria**.
Cases of polyuria:
• Drink a lot of water in the organism to expel certain substances that have increased a lot, and a lot of urine is removed (in the case of diabetes, when too salty substances are eaten),
• In some kidney diseases, where the kidney cannot thicken the urine,
•In cases where tissue retention ability is reduced (vasopressin deficiency: diabetes insipidus),
• Solving edema, drinking too much water.

When the amount of urine decreases, it is called **oliguria**.
Cases of oliguria:
• Drinking few water, sweat a lot, lose a lot of water in exile (diarrhea),
• Water retention (edema) in tissues and voids in kidney, heart and liver diseases.

No urine removal is called **anuria**. The condition of frequent urination is **pollakuria** and painful urination is called **dysuria**.

Color: The color of normal urine is amber yellow. It is given by the pigment urochrome, trace amounts of uroporphyrin, coproporphyrin, uroerythrin and urobilin, which are products of metabolism.
The color of the urine can sometimes be decolored, sometimes darker, the urine sometimes gets another color.
a-Decolorating of the normal color: If a large amount of urine is removed, the urochromogen, which is the precursor of the color in renal failure, cannot be oxidized by the kidney and transferred into a colored urochrome.

b- Darkening of the normal color: The urine becomes darker in color (colorless urochromogen is oxidized in the air), if very concentrated urine is removed,
c- It could be very important when the normal color is covered by other colors,
• Red color: Blood (hemoglobin), porphyrin, myoglobin
• Green color: Biliverdin, when methylene blue is used as urine antiseptic
• Yellow-orange or brown-red (tea color): Bilirubin
• Dark yellow and brown: Urobilin
• Blackening: Homogentizic acid and melanogen
• Pink-brown: in porphyria
• Milky-urine in the form of (chyluria): if urine has fat and prostatic secretion

In addition, some drugs may cause variation in urine color.

Clarity: A urine that is fresh and slightly acid is clear. However, although the urine is normal, it may be blurred for some reason. Some substances (such as mucine) coming from the secretions from the surfaces of the urinary bladder and urinary tract walls, may produce a slight turbidity. The principal turbidity is mainly caused by the following reasons:

a- There are salts suspended in urine. If the turbidity is lost by heating, these salts are the urate salts (the urates melt in hot) and when urine is acidified with acetic acid, phosphate salts are lost.

$$Ca_3(PO_4)_2 + 4CH_3COOH \rightarrow 2\,Ca(CH_3COO)_2 + Ca(H_2PO_4)_2$$

If the turbidity doesnot disappear with acetic acid, the urine is acidified with some hydrochloric acid (HCl), when the turbidity is lost, the presence of oxalate salts is understood.

Calcium Oxalate **Calcium Chloride** **Oxalic Acid**

b- Many cellular elements (leukocytes, erythrocytes, epithelium, etc.) and pus from the kidney may interfere with urine. Bacterial contamination can also cause the urine to appear blurred.

Odor: Normal urine has a unique odor. This odor comes from the phenols found in the urine. You may smell ammonia in impaired urine. If there are some ketone bodies (heavy diabetes), the apple smell is heard.

• Sour smelling: Tyrosinemia
• Maple syrup or caramela smell: These amino acids and α-ketoacids increase in branched-chain amino acid metabolism disorder.
• Mouse or mildew smell in urine: Phenylketonuria
• Sweaty foot odor: Isovalericacidemia, glutaric acidemia.

Specific gravity (density): The urine is placed in the glass tape measure and the specific gravity is measured by the ureometer. The specific weight varies with the temperature, so there is also a thermometer in the ureometers. For all three degrees

above 15°C, 1 number is added to the density, 1 number is subtracted for each 3 degrees below 15°C.

The specific weight of normal urine is between 1,015-1,020. The specific weight of the urine is related to the substances present in the molten state. The most important of these are urea and NaCl.

Increases in specific weight:

• Increased melting substances of urine. Large amounts of NaCl, or some pathological substances such as glucose and protein.

• Removal of normal amounts of substances with little amount of urine (drinking less water, sweating). Specific gravity can reach up to 1,035-1,040.

In cases where specific gravity decreases:

• Failure to remove normally excreted substances (urinary urea in kidney diseases and increases in blood)

• Drinking a lot of water and removing too much urine can reduce the specific gravity up to 1,002-1,003. In a water metabolism disorder, diabetes insipidus (sugar-free diabetes) may have a specific gravity of 1,0005.

• There is a special case in Diabetes mellitus: Although the patient drank a lot of water and removed a lot of urine, the specific gravity is very high because there is a high amount of glucose in the urine.

Ureometer

Reaction: Determined by litmus paper. If the blue litmus is red, the urine is slightly acid, the red is the litmus blue, and if the color of the red and blue litmus papers does not change, it is neutral.

The reaction of urine in a normal diet is mild acid, pH is 5-7. The slight acid reaction of normal urine is due to primary phosphates ($H_2PO_4^-$). In unilateral feeding with vegetables alone, the urine reaction is alkaline, while it is acidic in the case of feeding only with proteins.
Normally the reaction of urine is mild acidic but if there is an acidosis in the organism, the pH could shift to acid due to acidic substances excreted. In these life-threatening conditions, the pH may drop to more than 5. Urine may be alkaline in infections with bacteria that break down urea and release ammonia.

Chemical analysis of urine
Pathological substances in urine; sugar, acetone, protein, hemoglobin, bilirubin, urobilin, urobilinogen is searched, if necessary, the amount of sugar, type and amount of protein can be determined.

Microscopic examination of urine
Sediment: Urine sediment is examined under a microscope. A 10 ml of urine is placed in a centrifuge tube to obtain the residue, the same amount of water is placed in another tube and the two tubes are equilibrated in the centrifuge scale. The tubes are centrifuged at 1300 rpm for 5 minutes. After centrifugation is stopped, in the tube containing urine, the clear supernatant portion is carefully discarded. The sediment deposited in the conical part of the tube is mixed by gently shaking the tube and a drop is placed in the microplate slide and covered with lamella. It is examined under a microscope. *As a result of scanning 10 areas in the microscope, the average number of cells and crystals detected in these areas is taken and processed in the Microscope Examination section of the Urine Analysis Report.*

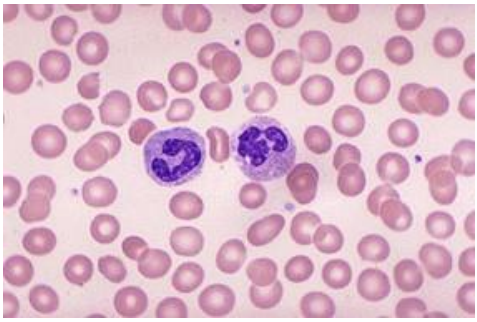
In the examination of the urine residue, the substances that can be seen can be grouped into three groups:
• Cells
• Cylinders
• Settleable chemicals

Erythrocyte	Leucocyte	Epithelial Cells
7-8μ	7-10μ, bigger than erythrocyte	Bigger than leucocyte
Very pale yellow	Colorless	Colorless
Homogeneous, round	Heterogeneous, round, cytoplasm-grained	Heterogeneous, often granular, in various forms
No nucleus	Have nucleus (lobed)	Have nucleus (round or oval)

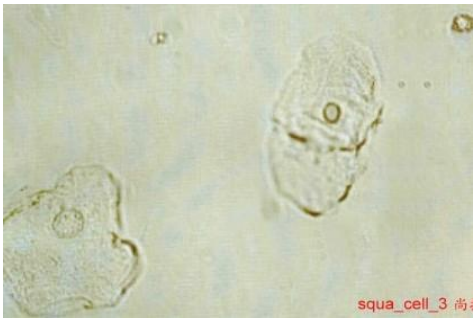
Normally, there is no erythrocyte in the urine. The presence of erythrocytes in the urine is called **hematuria**. Two types of hematuria can be seen: microhematuria and macrohematuria.
Microhematuria: The presence of erythrocyte in urine that can only be seen by microscope (kidney stone and kidney tuberculosis, etc.).
Macrohematuria: Including erythrocyte, which may change the color of urine (traumatic bleedings, tumors, severe infections of the urinary tract, etc.)

Properly structured erythrocytes (isomorphic) are a sign of bleeding in any part of the urinary tract. Deformation of erythrocytes (dysmorphic erythrocytes) is a sign of a disorder in glomeruli.

Normal urine does not contain leukocytes, but the presence of few may not be pathological. In women with vaginal infections, vaginal discharge can be seen in urine sediment and leukocytes in urine sediment. In inflammation of the kidney and urinary tract, acute glomerulonephritis, many or many leukocytes are present in the urine according to the degree of inflammation. Inflammation of urine is called **pyuria**.



Erythrocytes in the urine sediment and two leukocytes in the middle
www.opt.pacificu.educecatalog/0644



Epithelial cells in urine sediment
www.accuspeedy.com.tw

Epithelial cells of the pelvis, ureters, bladder (uretra) or tubular source according to the different shapes. They contain large oval or round nuclei. There are usually few epithelial cells in the urine sediment.

Cylinders

The cylindrical structures in the urine sediment are due to the collapse of the proteins in the tubules due to the disturbances in the glomeruli.

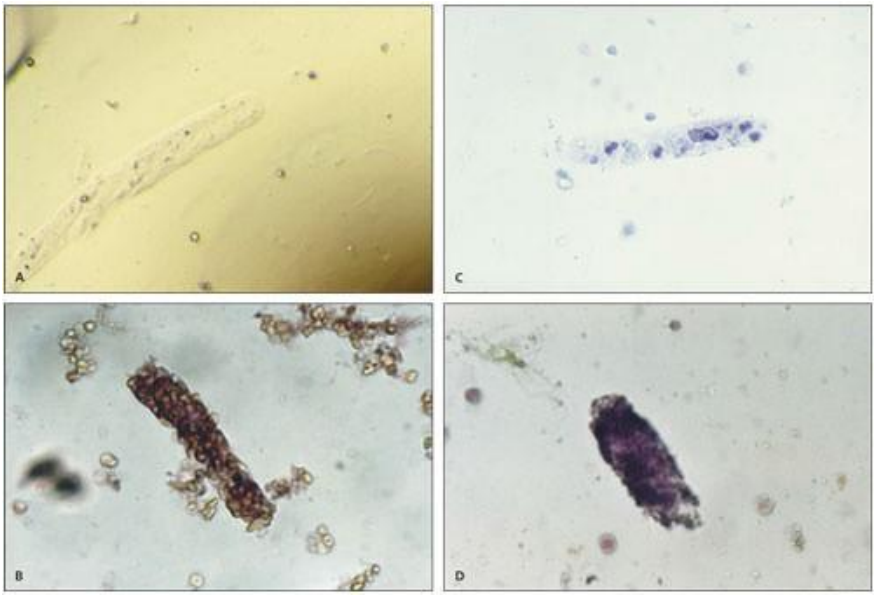
Major four types of cylinders are recognized:
• Hyaline transparent
• Waxy dull yellowish
• Granular dark colored
• Cellular erythrocyte, leukocytes or epithelial adhesions, they are called according to the cell they contain.

When the cylinders are seen in the urine, it is understood that an abnormal event occurs in the kidneys. This may be a simple irritation of the kidneys, an inflammation, or a severe deterioration.
• Hyaline cylinder, simple irritation,
• Granular cylinder, inflammation,
• Waxed cylinder, is a severe and chronic inflammation or renal amyloidosis.

The most important cellular cylinders are erythrocyte rollers. They show that the blood in the urine is caused by the kidney. The erythrocyte cylinders seen with dysmorphic erythrocytes suggest glomerulonephritis. Leukocyte cylinders are seen in pyelonephritis.

Urine may contain cylindroids other than cylinders. The cylindroids are longer and thinner than the cylinders, the edges are not parallel, most of them are pointed at

one end and rounded at the other end. They can be seen before or after the cylinder formation, they exhibit slight irritation.



Cylinders in urine sediment. A. Hyaline, B. Erythrocyte, C. Leukocytes, D. Grains.
www.aafp.org

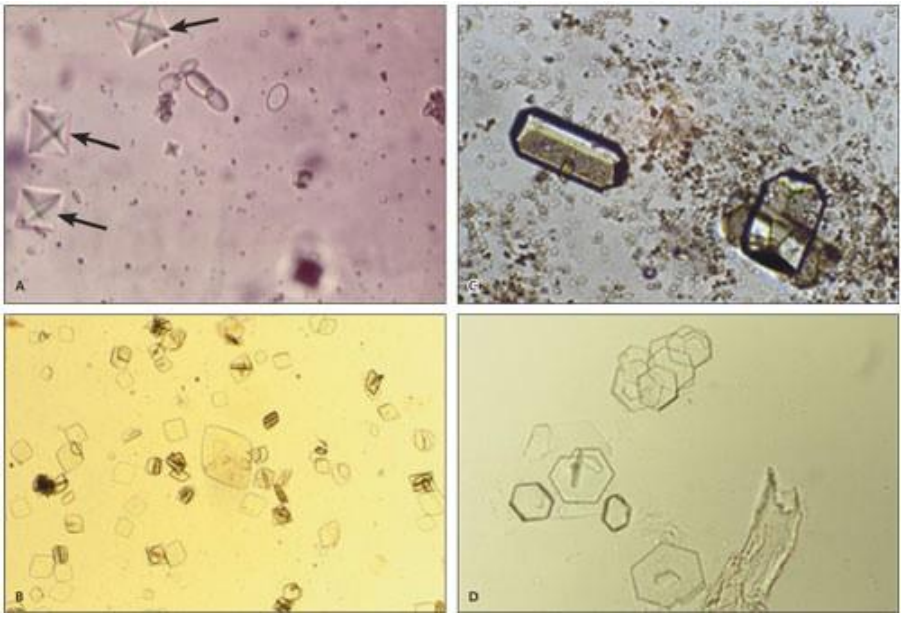
Settleable chemicals:
Most important ones,
• Salts
• Some amino acids
• Some drugs.
Salts: The most important are phosphate, urate and oxalates, either as crystals or amorphous (formless).

- Phosphate crystals, coffin-lid shaped, colorless and snowflake-shaped (NH_4MgPO_4), yellowish or brown
- Ammonium urate crystals, large and small rounds or horse chestnut shaped, yellowish or brown
- Uric acid crystals, barrel, prism, needle, lemon, hexagonal, yellowish or brown
- Calcium oxalate crystals are in the form of letter envelope and colorless.

These crystals are often found in urine in small numbers, which is normal because urate, phosphate and oxalate are the substances normally found in urine. The fact

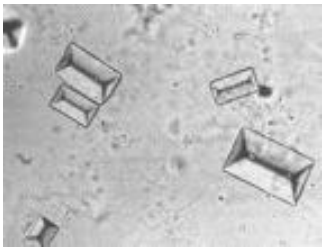
that they are too large may suggest a stone in the urinary tract.

In some cases urine leucine, tyrosine and cystine crystals can be seen. Crystals of sulfonamides are the most common drug substances.

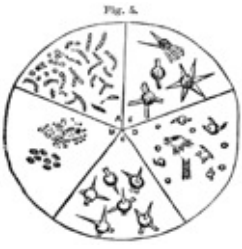


Crystals in urine sediment. A. Calcium oxalate, B. Uric acid, C. Phosphate, D. Cystine.
www.aafp.org

Oxalate crystals



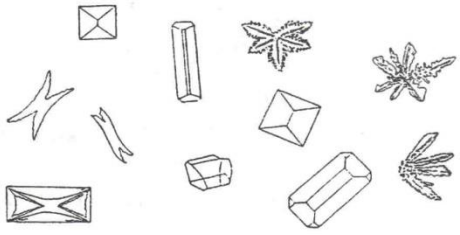
Phosphate crystals



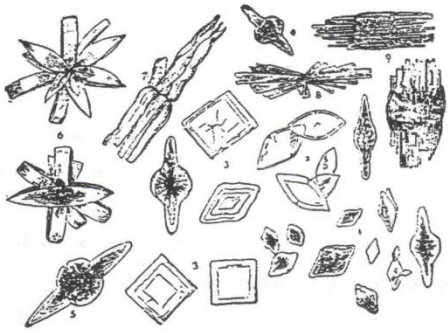
Urate crystals

Writing a microscope examination:
The average number of cells and crystals obtained by scanning 10 fields in a microscope is written to the report in a sequence. First the cells then the crystals are written. If there are erythrocytes which are the most important pathologically, they are written first. Leukocytes are written in second place.

The expression: “In each area ..... erythrocyte, ... leukocyte, ... cylinder, ...epithelial cell, ...crystal was observed” is used.



Fosfat kristalleri



Ürik asid kristalleri

Ürat kristalleri

Okzalat kristalleri

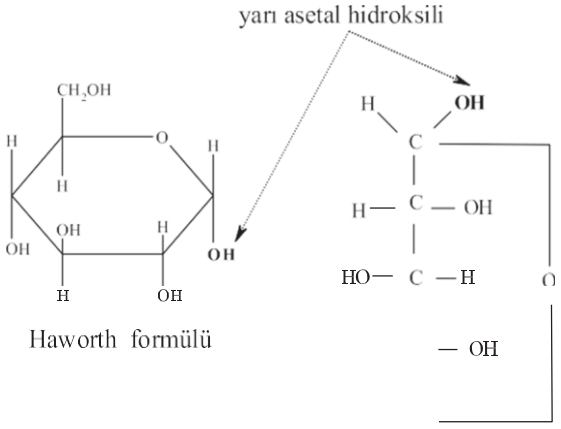
Hawk's Physiological Chemistry 1198-1200 (1965).

Main crystals that can be seen in urine sediment

DETERMINATION OF SUGAR, ACETONE IN URINE AND DETERMINATION OF THE SUGAR TYPE

Sugar determination in urine

Reduction reactions of monosaccharides are utilized in the search for sugar in urine. The free acetal and ketal hydroxyl groups in sugars show reducing properties in hot and alkaline environment. Aldehyde group, even in non-sugar simple substances have reducing properties. For example, formaldehyde (HCHO) is reducing. However, the keto group must be adjacent to alcohol groups in order to be reduced. For example, the keto group in acetone (CH_3COCH_3) is not a reducing agent.



α-D- glucopyranose

Fischer formülü

Two tests are done to determinate for sugar in urine:

1- Trommer test

The basis of this test is the reduction of Cu^{2+} to Cu^{+}.

Preparation: 1-2 ml of urine + equal volume 10% NaOH + 4-5 drops of 5% $CuSO_4$ (use Fehling I) → heated to open flame, shaking thoroughly → yellow precipitate is continued to heat → tile-red precipitate is formed if there is sugar in urine.

The following reduction reactions occur in the test.

$$CuSO_4 + 2\ NaOH \rightarrow Cu(OH)_2\downarrow + NaSO_4$$
(blue precipitate)

$$Cu(OH)_2 \rightarrow Cu\ OH\downarrow + H_2O + RCOOH$$
(yellow precipitate)

$$2\ CuOH + RCHO \rightarrow Cu_2O\downarrow + H_2O + RCOOH$$
(tile-red precipitate)

If there is no reducing agent in the test environment copper II ($Cu(OH)_2$) hydroxide is converted to copper II oxide (CuO), which is a black precipitate.

$$Cu(OH)_2 \rightarrow CuO\downarrow$$ (black precipitate)

2-Fehling's Test

In this experiment, the main reducing group is the reduction of the Cu^{2+} ion to Cu^{+}, resulting in the formation of a yellow colored copper I hydroxide (CuOH) followed by a red precipitate of copper I oxide (Cu_2O).

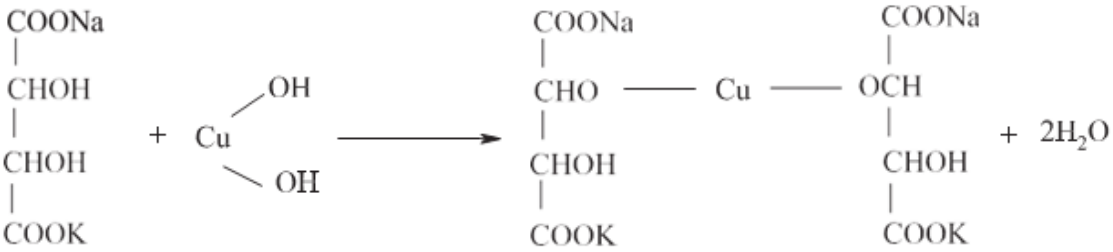
Preparation: 1 ml Fehling I + 1 ml Fehling II → tube is shaken, heated in open flame, blue color should not change + urine equal volume of (Fehling I + Fehling II) → heated, before the yellow, then the tile-red precipitate, if there is sugar in urine.

Fehling I: 7% $CuSO_4$

Fehling II: Seignette salt (Na-K tartarate) + NaOH

Caution: The Fehling reagent may well out of the tube when it is boiled. In order to prevent spurting from tube, the boiling process tube should be slowly and intermittently shaken. When the boiling begins, the tube is drawn from the flame and brought back to the flame. Be careful not to keep the tube towards yourself or your friend's face.

The superiority of this test towards the Trommer test is that the Seignette salt in Fehling II keeps the precipitate $Cu(OH)_2$ formed by the addition of $CuSO_4$ and NaOH dissolved in solution.



Sodium potassium tartarate (Seignette salt) **Cu II Hydroxide (blue precipitate)** **Seiginette-Cu II complex (dark blue melt)**

The advantage of $Cu(OH)_2$ to remain soluble is as follows: It is easier for the sugar to act as a reducing agent to a molten substance than to act on the precipitated material. Even if the sugar is very low, Cu^{2+} is reduced, but some of the undissolved Cu^{2+} can be reduced to CuO. For this, the tube should be shaken very well to ensure that the sugar acts on the Cu^{2+} ions in the Trommer test.

Sugars other than glucose in urine:

Lactose: It can be excreted in urine during the last months of pregnancy and the puerperium. In addition, those who are fed with only milk may have lactose in the urine.

Galactose: It is rare, but it is an indicator of an important congenital disease. In these children, galactose is seen in their urine because there is no enzyme converting galactose into glucose. This causes growth disorders and mental retardation. Such children must be fed with galactose-poor foods. (milk: lactose = galactose + glucose)

Pentoses: Too much cherry, plum ingestion and fruit juice prepared from these fruits, if you drink too much, urinary arabinose comes out, it is not pathological. In the absence of an enzyme involved in glucuronic acid metabolism, L-xylulose is excreted in the urine. It is a congenital disease.

Fructose: It rarely occurs in urine, it is a congenital but harmless anomaly.

The determination of the sugar type in urine

1- Fermentation test: The urine, which contains sugar, is placed in half of a 150 ml beaker and it should be mixed well by adding a little bit of brewer's yeast with a spatula. Then the urine is poured into a tube and closed with a middle hole cork stopper. The tube is inverted in the same urine sample, which is in the beaker, so that there are no air bubbles at the bottom of the tube. It is left at room temperature for one night (= 12 hours). If the gap forms at the bottom of the tube the next day, it is understood that fermentation occurred.

Here sugar is broken down with brewer's yeast, CO_2 and ethyl alcohol occurred.

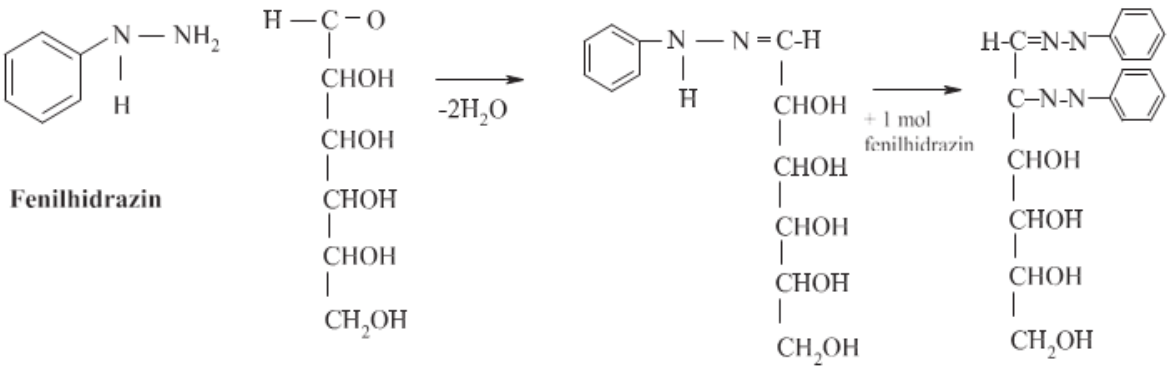
There is a gap in the bottom of the tube due to carbon dioxide.

$$C_6H_{12}O_6 \rightarrow CO_2 + 2\,C_2H_5OH$$

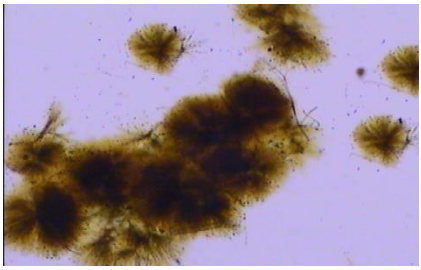
Not every sugar will be fermented. In order for a sugar to be fermented, the carbon number can be divided by 3, and the spatial states of the H and OH groups must be suitable for the ability of the enzymes to provide fermentation. According to this, hexoses are fermented, not pentoses. Among hexoses glucose and fructose could undergo fermentation but not galactose. Among the disaccharides, maltose could undergo fermentation but not lactose. Therefore, the positive fermentation point up to glucose, fructose or maltose.

2- Osazone test: Monosaccharides are reacted with phenylhydrazine, acetic acid and sodium acetate to form a precipitate of typical yellow crystals. These crystals are called osazone crystals, and by examining their shapes in the microscope, the sugar type can be distinguished. The shape of the osazone crystals of glucose, fructose and mannose is the same, but that of galactose is different.

Preparation: One tube is placed up to a height of 1 cm of phenylhydrazine HCl and equal quantity of Na acetate. 10 ml of urine is placed in another tube and acidified with 3% acetic acid. Acidic urine is added to the powder materials, heated in an open flame, after these substances are dissolved, urine is kept 30 minutes in a boiling water bath. The tubes are removed from the water bath and cooled to room temperature. The yellow crystals formed are examined under a microscope.
During this reaction, phenylhydrazine molecules enter the aldehyde groups and the second carbon atoms of the sugars, resulting in osazone crystals. The osazone of the glucose is called glucosazone and for lactose is called lactosasone.



Glucose **Osazone**

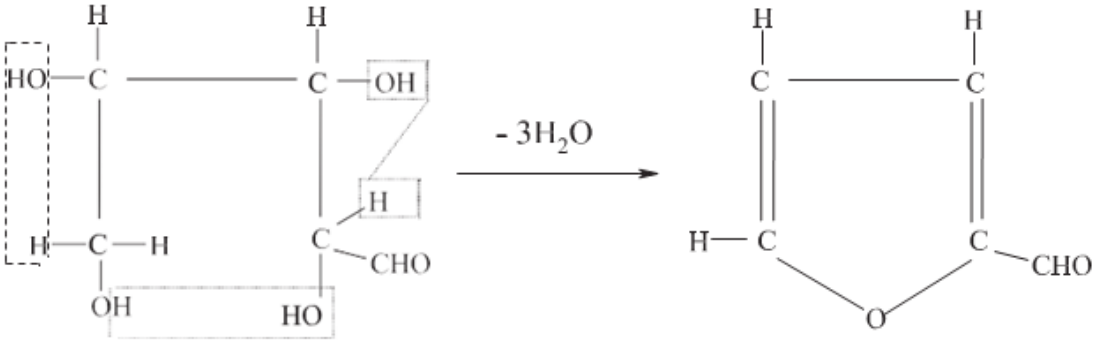


Glucosazone Crystals **Lactosazone Crystals**

Glucosazone crystals are yellow and in the form of crop bundle, broom branch and pine branch. Lactosazone crystals are yellow and in form of horsechestnut. In this way, the osazone assay distinguishes between lactose and glucose.

1- Experiments based on the formation of furfural derivatives:

Monosaccharides lose water when heated with non-oxidizing strong acids. As a result, furfural from pentoses and hydroxymethylfurfural from hexoses are formed. Furfural and hydroxymethylfurfural combined with some phenols in the acid medium, they give specific color condensation products for monosaccharides. Pentoses are green **(Bial test)** with orsinol, red color with fluoroglycine and ketoses with red color **(Seliwanoff reaction)** with resorcinol.



Pentose **Furfural**

a-Determination of fructose by Seliwanoff reaction:

This test is used to separate fructose from other sugars. Hot hydrochloric acid converts fructose to hydroxymethyl furfural. Hydroxymethyl furfural also combines with resorcinol to form a red complex. This test is sensitive up to 100

mg/dL on condition that there is no excess glucose in the medium. The reaction of fructose, a ketohexose, is faster than the reaction of the corresponding aldohexose glucose. At the same time interval, fructose produces a dark red colored precipitate, giving glucose only a light pink color.

Preparation: 5 ml Seliwanoff reagent + 0.5 ml (8 drops) of urine sample → heated until boiling. The color change in each tube is monitored for 15 minutes, the resulting color and time of occurrence are recorded. Red color formation in 30 seconds → Fructose

b-Determination of pentose in urine by Bial's orsinol reaction:

Pentose and nucleosides containing pentose can be detected by this experiment. Pentoses are converted to furfural when heated with HCl. Furfural forms a green complex with orsinol in the presence of iron ions. This test is not a specific test of pentoses. 2-deoxypentoses, 6-deoxypentoses, hexuronic acids, trioses and heptoses may also give a similar reaction.

Preparation: 5 ml of orsinol reagent + 0.5 ml (8 drops) of urine sample → heated until boiling. Green color formation in 30 seconds → Pentose

Assessment: This test is as sensitive as 100 mg /dL pentose. Xylose solution should be used as a control. This test is not specific for pentoses. If the heating process lasts long, some hexoses give the same color reaction. Therefore, the color formed during the 15 minutes of the heating process must be observed and recorded.

Molisch test: The solution containing carbohydrate is mixed with a 10% solution of α-naphtol in alcohol and stratified with concentrated sulfuric acid to form a violet colored ring between the two surfaces (all monosaccharides give this reaction).

In a normal human, fasting blood glucose concentration ranges from 80-120 mg % (70-110 mg % in some methods). After meals, the blood sugar begins to rise, in normal conditions can be increased up to 140-170 mg %, but rarely exceeds 160 mg %. . In a normal kidney, glucose reabsorption is complete and no sugar is found in normal urine. We defined the **renal threshold for glucose** reabsorption as the lowest blood glucose level that correlated with the first detectable appearance of urine glucose. The renal threshold of blood glucose level is approximately 170-180 mg %. The presence of sugar in urine is called **melituria**. The most common sugar in urine and important in terms of human pathology is glucose. The appearance of glucose in urine is called **glycosuria**. Glycosuria has various causes. The most

important types of glycosuria are:

- Diabetic glycosuria
- Renal glycosuria
- Dietary glycosuria

Diabetic glycosuria: It is seen in *diabetes mellitus*. In this disease, the blood sugar is above 170 mg %, the value of renal threshold, and therefore the sugar started to be excreted in the urine. Only urine analysis is not sufficient to detect diabetes, and a blood test and even oral glucose tolerance test should be performed with a urine analysis.

Renal glycosuria: Although the blood glucose is normal, urine contains glucose because the renal threshold is low in such people.

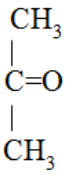
Dietary glycosuria: A food-dependent state. If too much carbohydrate-rich food is taken, urine could contain glucose. It is a temporary condition.

Acetone Determination in Urine

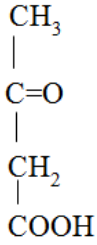
Weyl-Legal test: To 2-3 ml of urine, 2-3 drops of 10% sodium nitroprusside is added dropwise and 10% NaOH is added until alkaline medium. A red color appears. When the red color becomes darker by adding glacial (concentrated) acetic acid, there is acetone, if the color is opened (yellowish-greenish) there is no acetone. Acetone forms "isonitro acetone" with "NO" group of the sodium nitroprusside.

Acetoacetic acid (3-oxobutyric acid) is formed by the condensation of acetyl-CoA molecules corresponding to acetoacetyl-CoA and this acid formed by the reduction of **acetone** and **3-hydroxybutyric** acid is called ketone bodies. These amounts are normally 2-3 mg % in blood. The increase in the amount of these in the blood is called **ketonemia**. Ketonuria is the urination of these substances, and all the metabolic state is called ketosis. Two other substances, except acetone, are acidic and cause acidosis, which means the pH of the blood decrease.

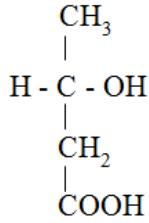
Clinical meaning: Ketone bodies are not normally found in urine, but when the body does not use carbohydrates as a fuel, it burns fat instead and results in acetyl CoA formation from where ketone bodies are synthetised. For example, in diabetes, excessive starvation, fever, such as internal toxicities such as pregnancy toxicosis, atropine, phosphorus, lead, carbon monoxide poisoning.



Acetone



Acetoacetic acid



3 - Hydroxy Butyric acid (3-Oxobutyric acid)

DETERMINATION OF PROTEIN IN URINE AND QUANTITATION

Before protein determination the urine must be filtered through a filter paper.

There are four experiments for protein determination in urine:
1- Boiling test
2- Heller's ring test
3- Sulfosalicylic acid test
4- Potassium ferrocyanide test

The most rough of these experiments is boiling, the most sensitive is the sulfosalicylic acid test.

1- Boiling test: Filtered urine is poured until 2/3 of the tube and the upper side of the liquid is boiled in open flame. A turbidity may be caused by the presence of carbonates and phosphates as well as from this protein. If the turbidity disappears after the addition of 3% acetic acid, it is understood that it comes from the carbonate and phosphates and if it does not disappear protein is present.

If the specific weight of the urine is less than 1,010, the turbidity is very low or not at all.
In this case, the specific weight of the urine should be increased to 1.010 by the addition of NaCl.

Proteins are denatured with heat, lose their natural properties, and consequently collapse by clotting. Acetic acid is also a substance that denats the proteins, so turbidity is not lost by the addition of acid. In the case of precipitation of phosphates and carbonates by heating, primary calcium phosphate [Ca(H2PO4)2] in urine and carbonates are present in the urine as calcium bicarbonate [Ca(HCO3)2], CO2, which keeps them in solution, flies during heating and occurs in secondary calcium phosphate (CaHPO3) and calcium carbonate (CaCO3) precipitates. By addition of acetic acid, phosphates and carbonates dissolve into primary phosphate and bicarbonate.

2- Heller's ring test: Three finger high of urine are poured in the tube and the tube is bent and layered with concentrated nitric acid. A ring of turbidity on the surface touched by acid and urine, and if this ring is not lost by heating, it is understood to be protein in the urine. The reason for turbidity is the collapse of proteins as a result of their denaturation with acid. Sometimes too much urea and uric acid amounts in the urine can be the cause of turbidity.
However, these rings can be easily distinguished from the ring of the protein because the ring of urea nitrate is bright but that of the protein is opaque. The ring

of urate salts is lost by heating, the ring of protein is not lost.

3-Sulfosalicylic acid test: Two finger high of filtered urine is added to the tube and 3-4 drops of 20 % sulfosalicylic acid solution is added. If a turbidity occurs, it could be caused by protein. If the tube is heated and the turbidity disappears by heating, it results from albumose and peptones, which are small polypeptides which are the products of breakdown of proteins. If the turbidity is not lost by heating, it is understood that there is protein in the urine.
The cause of turbidity is that the amino groups of proteins become cation in the acid medium and bind sulfosalicylic acid with the sulfone group and collapse into the protein-sulfosalicylate compound. As the heating denaturates the proteins, the turbidity continues as soon as it is heated.

4 - Potassium ferrocyanide test: Two-finger high filtered urine is placed into the tube and acidified with 30% acetic acid and 5% potassium ferrocyanide solution is added. If turbidity occurs, protein is present.
When the urine is acidified, the proteins become cation and merge with the ferrocyanide anion which is the cause of the turbidity in urine.

When determining protein in urine, at least two of the mentioned experiments should be performed. Preferably the most sensitive sulfosalicylic acid assay and the most rough, boiling test are performed.

Types of protein in urine
1- Blood proteins (serum albumin, serum globulin, fibrinogen, hemoglobin)
2- Settleable proteins with acetic acid in cold
3- Mucins
4- Albumoses and peptones
5- Bence-Jones proteins

1- Blood proteins: The most common protein in urine is serum albumin and sometimes serum globulin. When the urine is filtered through the glomeruli, the glomeruli easily pass proteins with a molecular weight of less than 70,000, but not more than 70,000. If the kidney glomeruli have been destroyed for any reason, the glomeruli become permeable to albumin and the urine contains albumin. If the damage in the glomeruli is even more severe, the globulins also pass through the glomeruli and they are emitted in urine as well as albumin. In cases where the amount of globulins increases too much, they do not dissolve like albumin, they collapse in the renal tubules and take shape as the tubules. These structures, which have taken the shape of tubules because of their resemblance to the cylinder which are seen in microscope examination of the urine residue, are called urine

cylinders (see urine sediment). Albumin and globulins appear in the urine in kidney diseases such as **nephritis** and **nephrosis**.

2- Settleable proteins with acetic acid in cold: If some of the urine is added with some acetic acid (concentrated) at room temperature, turbidity occurs. The reason for this turbidity is settleable proteins in cold. Although the type of proteins are definitely not known, it has been determined that they involve proteins such as chondroitin sulfate proteins (lordotic proteinuria), bile acids proteins (in jaundice), nucleic acid proteins (pus), lipid globulins (in nephrosis) are excreted in the urine. Proteins that precipitate with acetic acid in the cold dissolve in excess of acetic acid.

3- Mucins: As it is known, mucopolysaccharide and mucoprotein mixture substances secreted from mucous membranes are called mucin. These are normally mixed with the mucus secreted from the mucosa of the lower part of the urethra. Normally, the mucus in the urine collapses in the cold with acetic acid, but they do not dissolve in excess of acetic acid, as opposed to proteins that are precipitated in cold with acetic acid.

The amount of mucin in the urine increases in inflammation of urinary epithelium.

4- Albumoses and peptones: They are small polypeptides which are the products of breakdown of proteins. They give positive results in all experiments for proteins except boiling test. However, unlike albumin and globulins in the sulfosalicylic acid experiment, the blurring of albumose and peptones is lost by heating. They are excreted into urine in large apces and some febrile diseases.

5- Bence-Jones proteins: This protein collapses at 50°C-60 ° C at and dissolves at 90° -100°C. They are excreted into urine in malignant bone marrow tumor called multiple myeloma. To search for Bence-Jones protein: The urine is heated, if there is a turbidity without more boiling, the Bence-Jones protein is present if this turbidity is dissolved by boiling and is observed again when the tube is allowed to cool.

Clinical meaning: Excretion of urine protein is called **proteinuria**. Because the most abundant protein is albumin, urine protein excretion is more commonly referred to as albuminuria.

There are three kinds of proteinuria:
1- Functional proteinuria
2- Organic proteinuria
3- Accidental (random) proteinuria

1. **Functional proteinuria:** These proteinurias are not pathological, in some cases temporarily seen. After some physical and mental activities or cold showers, it can be seen in high-protein-fed and pregnant women. Some

young people have a kind of proteinuria called lordotic (orthostatic) proteinuria. This proteinuria is associated with the person's position change. For example, proteinuria is lordotic when a person bends, takes a step, or puts a foot on a chair.

2. **Organic proteinuria:** Proteinuria in this group are also grouped into three subgroups.
   a- Pre-renal proteinuria
   b- Renal proteinuria
   c- Post-renal proteinuria

a- Pre-renal proteinuria: These proteinurias are not associated with renal impairment. kidney circulation becomes difficult, as a result of some diseases and disorders of some organs except kidneys. The kidney glomeruli become permeable to proteins, so the protein leaks in urine. Pre-renal proteinurias occur in pathological conditions such as heart diseases, circulatory disorders, abdominal disorders and edema, liver diseases, some febrile diseases and blood diseases.

b- Renal proteinuria: It is the most important type of proteinuria that occurs as a result of kidney disorder. These kidney disorders are nephritis and nephrosis. Chronic or acute nephritis are inflammatory conditions of the kidney. Nephrosis is a severe kidney disorder caused by the degeneration of the kidney.

c- Post-renal proteinuria: This type of proteinuria are not associated with kidney. Protein is seen in urine in inflammatory, traumatic, bleeding or tumor diseases of parts of the urinary tract after the pelvis. These diseases include pyelitis (inflammation of the pelvis), ureteritis (inflammation of the uretera), cystitis (inflammation of the bladder), urethritis (inflammation of the urethra) and prostatitis in men (inflammation of the prostate gland).

2- **Accidental (random) proteinuria:** These are not pathological. Prostate secretion, seminal sac secretion, vaginal secretion and menstruation leakage may cause proteinuria.

Determination of protein amount in urine

The amount of protein in urine in pathological conditions may be trace amount up to 20-30 grams per day. Protein determination in urine is usually performed to differentiate nephritis from nephrosis. In nephritis 1-5 g of protein per day could be seen in urine, this number can rise up to 12 g in nephrosis. One of the most

common methods of protein determination in urine and in the clinics is the Esbach method. In this method, a special tube called Esbach albuminometer (Esbach tube) is used which lines are determined experimentally. The figures in this tube start from 0.5 and continue until 12. There are also letters "U "and" R "above the figures. When the amount is determined, filtered urine is added to the U mark and the Esbach reagent until the" U "mark. Esbach reagent is a mixture of 1% picric acid and 2% citric acid. The tube is closed by a stopper, mixed for 5-10 times and left in the dark for 24 hours at room temperature.
After 24 hours, the height of precipitate is read, which gives the amount of protein in grams per liter. This amount is then converted to the amount of protein extracted in daily urine.

In this experiment, the principle is to precipitate the protein by denaturing with double acid. However, the Esbach method is not an ideal method. With this method less than 0.5 g of protein cannot be determined. In addition, there are some sources of error in the experiment, such as Esbach, which is one of the natural proteins of urine, other than proteins, such as creatinine, uric acid substances and drugs such as urotropin, quinine also precipitates. If the temperature at which the test is performed is higher than the room temperature, it causes incorrect results.

In urine with high specific weight, the urine should be quantified after dilution. Generally, urine with a specific gravity of 1,016 is diluted 2 times, 1 part of urine is diluted with 1 part of water. The urine with a specific gravity of 1,024 is diluted three times, 1 part of urine is diluted with 2 parts of water. Then, the amount read in the Esbach tube as g / L is multiplied by the dilution rate.

Esbach tube

HEMOGLOBIN, BILIRUBIN, UROBILINOGEN AND UROBILIN DETERMINATION IN URINE

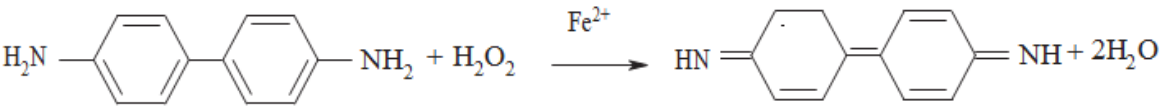
Hemoglobin (Hb) Determination:

Hemoglobin excretes urine inside or outside the erythrocytes. The presence of hemoglobin in urine in erythrocytes is called **hematuria**, the presence of hemoglobin in urine outside the erythrocytes is called **hemoglobinuria**. Whether in or outside the erythrocyte, the presence of Hb is first searched for by a chemical method, and then by physical methods it is determined that Hb in the urine is in or out of the erythrocyte. The presence of Hb in or outside the erythrocyte is important in the diagnosis of the disease because both occur in different pathological conditions.

The presence of Hb in the urine is determined by a chemical method, Benzidine.

Benzidine Test:
1-2 ml 1% benzidine (in concentrated acetic acid solution) + 1-2 ml 3% H_2O_2 (no green coloration in the mixture) + drop by drop of urine → dark green color (emerald green) if there is Hb
This color then turns from green to brown, color change is not important. The important thing is the green color that appears first. In addition, if urine is dripped so fast instead of drop by drop, benzidine precipitates in white and green color cannot be seen.

Test principle:



Benzidine Dehydrobenzidine

Benzidine is oxidized to dehydrobenzidine by the catalytic effect of Fe^{2+} ion in Hb, the passage mixture of benzidine-dehydrobenzidine is green in color whereas dehydrobenzidine is brown.

After detection of Hb by the benzidine test (chemical method), physical methods are used to determine whether this Hb is in or out of erythrocytes. For this purpose microscope and hand hand spectroscope are used.

Hand spectroscope

Looking at daylight, it is an instrument that shows the seven colors of the sun in other words the spectrum. As it is known, different colors consist of light that vibrates at different wavelengths.

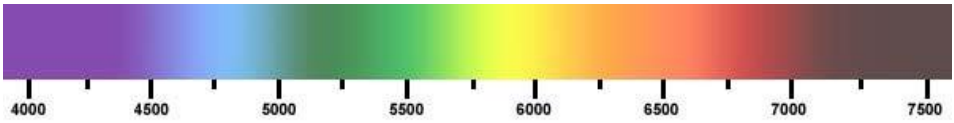
To examine urine by hand spectroscopy, the urine is drained and placed in a tube, the tube is exposed to daylight, and the tube is screened with a hand-held spectroscope. If black absorption bands (Fraunhofer lines) are seen on certain colors, Hb in the urine is understood to be unconjugated, so there is hemoglobinuria. Unconjugated Hb and different species absorb light from different wavelengths, so the color corresponding to the absorbed light also appears as a black band in the spectrum.
The urine usually exits oxyHb and gives two free black bands, yellow and green. In poisoning (nitrite, chlorate, permanganate), teh Fe^{2+} of haemoglobin is converted to Fe^{3+} of methemoglobin that gives three black bands, on yellow, green and red. Hb gives a single common black band in the area between yellow and green.

If any absorption band is not seen when the urine is examined by hand spectroscope, then the urine is centrifuged, the upper liquid part is poured and the sediment below is examined under microscope. If the erythrocyte is seen in the microscope, it is understood that the hemoglobin in the urine is in erythrocyte, so there is hematuria.

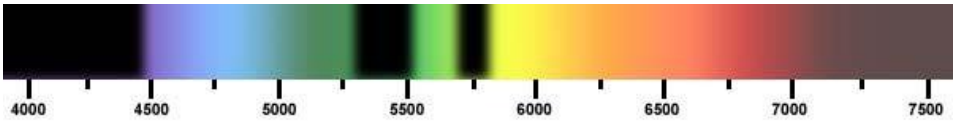
Hand spectroscope

www.grauhall.com

3900 to 7600 Angstrom Units (1×10^{-8} cm)



Visible light spectrum



Oxyhemoglobin bands in visible light spectrum

Clinical meaning: Hematuria occurs in haemorrhagic diseases of the kidney and urinary tract. Kidney stones, kidney tuberculosis, severe infections of the urinary tract, urinary tract traumatic bleeding. Hemoglobinuria occurs when large amounts of hemolysis are present in the organism.
Hemoglobinuria, especially after transfusions of stored blood, sulfamides, quinine, phenol derivatives and phosphorus poisoning, snake poisoning, some of the enzymes involved in erythrocyte metabolism are inherited deficiencies. Hemolytic anemia in which hemolysis occurs slowly, and hemoglobinuria in hemolytic icterus is not seen.

Determination of bilirubin

Rosin-Trousseau test: 1-2 ml of urine is put into the tube; the tube is held upright and iodine is stratified with 1% solution in alcohol. The formation of a green ring on the touch surface of the two liquids indicates the presence of bilirubin.

Gmelin test: 2-3 ml of urine is put into the tube and the tube is leaned from the edge of the tube by leaking the concentrated nitric acid. If there is bilirubin in the urine, a green colored ring is seen in the layer where the acid and urine touch each other (the brown ring given by nitric acid with normal urine should not be taken into account).

In the Gmelin test, bilirubin is oxidized by nitric acid, in the Rosin-Trousseau test by iodine it is oxidized to the green-colored pigment biliverdin.

Clinical meaning: Bilirubin in the blood is found in two ways; free bilirubin (indirect) and conjugated bilirubin (direct). Free bilirubin is bilirubin complexed

with albumin in the blood. The conjugated bilirubin is the ester bilirubin which is formed by the conjugation of glucuronic or sulfuric acids with bilirubin. Normally, there is 0.5-1% total bilirubin in the blood. Free (indirect) bilirubin is never found in urine. Conjugated (direct) bilirubin exits in urine and is called **bilirubinuria**. Urine with bilirubin shows mahogany color (dark tea color or brownish-red).

Bilirubinuria is seen in icterus.

1- Mechanical icterus (post-hepatic):, In the case of gallbladder stones or pancreatic head cancers (in the first, the bile duct is obstructed from inside, in the second the bile duct is blocked by external compression) conjugated bilirubin does not flow into the small intestine because the bile duct is clogged, and passes into the blood. When the amount of bilirubin in the blood exceeds 2% mg, it excrete into urine.

2- Hepatocellular icterus: As a result of the infected inflammation of the liver (hepatitis), or when the liver cells are destroyed by alcohol or drugs, bilirubin is often conjugated with glucuronide and sulfate, but it cannot flow into the gallbladder due to a disorder in the hepatic bile ducts and passes into the blood.

In addition, in pathological cases such as cirrhosis and liver tumors, conjugated bilirubin is also excreted into urine.

3 - Hemolytic icterus (pre-hepatic): In cases where hemolysis increases, the amount of bilirubin in the blood increases, bilirubin infiltrates the tissues and the body is seen in yellow, but there is no bilirubin in urine.

Determination of Urobilinogen (Ehrlich aldehyde reaction):
5-6 drops of Ehrlich reagent (20% HCl solution of p-dimethylamino benzaldehyde) are added dropwise on 3-4 ml of urine. The tube is shaken, there is urobilinogen if pink color is seen in 3-4 minutes.

Determination of Urobilin (Schlesinger test):
A volume equal to 5 to 6 ml of urine is added to the Schlesinger reagent (zinc acetate's suspension in alcohol) (***Caution!*** The bottle should be shaken well before use), mixed and filtered through filter paper. If the filtrate shows green fluorescence, it is understood that urobilin is present.

When urobilinogen and urobilin are searched for in urine containing bilirubin, bilirubin is first precipitated with 10% $CaCl_2$ and aqueous ammonia solution (bilirubin can be precipitated also with 10% $BaCl_2$ solution). The precipitate is discarded, urobilinogen and urobilin are determined in the supernatant after

acidification with acetic acid.

Clinical meaning: Urobilin and urobilinogen are never present in normal urine. Urobilin and urobilinogen are seen in urine in hepatic insufficiency and hepatocellular icterus. In these cases urobilinogen which is re-absorbed in the intestine and transported to the liver by the *vena porta* (enterohepatic circulation) cannot be re-oxidized to bilirubin, due to cell damage. Urobilinogen is usually found in fresh urine. Urobilin is present in the awaited urine (after 16 hours) because urobilinogen is oxidized to urobilin by the air oxygen.

URINE ANALYSIS

Physical examination

Sent amount …….	Color …….
24 hour quantity …….	Clarity…….
Reaction …….	Sediment
…….	
Density…….	Odor …….

Chemical examination

Protein	…….	Hemoglobin	…….
Sugar	…….	Bilirubin	…….
Aceton	…….	Urobilinogen	…….
Acetoacetic acid	…….	Urobilin	…….

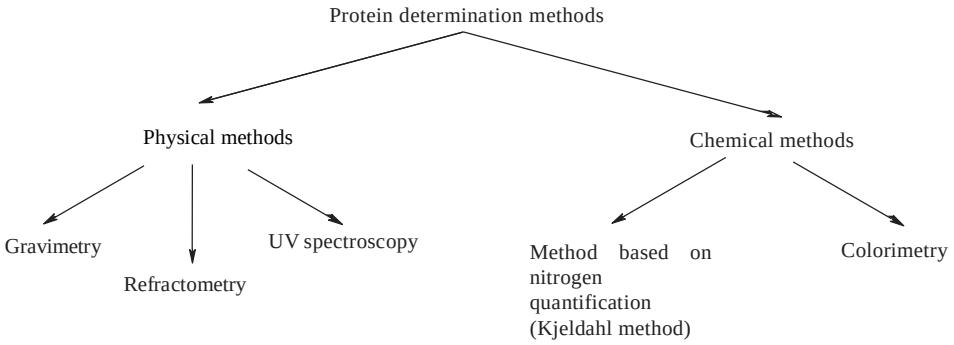
Microscope Inspection

……

……

……

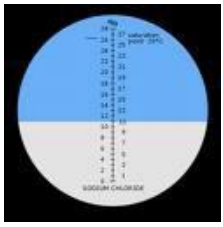
DETERMINATION OF PROTEIN AMOUNT IN SERUM



Physical methods

1- Refractometry

It is a method of determining the amount of light-breaking substances in a solution based on the refractive index. In this method, the refractive index of both the dissolved substance and the liquid dissolved therein is found together, then the refractive index of the liquid to which it is dissolved is read separately and removed from the first reading. Thus, the refractive index of the dissolved substance is found. The devices used in refractometry are called refractometers. The refractometer contains prisms. The angle of incidence of the beam is proportional to the velocity of the beam and the index of the medium. The angle of incidence, which allows the radiance to be broken at an angle of 90°, is called the critical angle. The beam we send passes through the sample and comes to the prism at different angles. If the angle is less than the critical angle, a bright area is formed. The boundary of the dark and light areas corresponds to the critical angle. The refractive index of a substance depends on the wavelength, concentration and temperature of the light used.



Refractometer

In the protein refractometer, the scale is adjusted for the refractive indices of the proteins so that the amount of the protein in g % can be read directly. A drop of serum is spread between the two prisms of the refractometer. Light is given by a mirror. From the oculars, the knob on the ocular is rotated until a sharp line is formed between the dark and open two sections, and the number on the scale in which this line meets is read.

Chemical methods

1- Method based on nitrogen quantification (Kjeldahl method)

Principle of the experiment: The amount of nitrogen contained in proteins is constant, ie 16% of each protein is nitrogen. Therefore, the amount of nitrogen in serum proteins can be determined by determining the amount of serum proteins. Therefore, the amount of nitrogen contained in proteins must first be determined, Kjeldahl method is used for this purpose.

Kjeldahl method:
1- Destruction
2- Distillation
3- Titration

It consists of these three steps.

In the first stage, serum proteins are destroyed in the presence of concentrated H_2SO_4 with -$CuSO_4$ and K_2SO_4 as catalysts, in the destruction ballon with a burner flame. Thus, the organic nitrogen contained in the proteins is converted into inorganic nitrogen as $(NH_4)_2SO_4$.

In the distillation step, the $(NH_4)_2SO_4$ is distillated in the Parnas-Wagner (Kjeldahl-Pregl) distillation apparatus and converted to NH_3. This NH_3 specific volume is retained in 0.1 N H_2SO_4.

In the titration step, to detect the NH_3 retained by the H_2SO_4 the free H_2SO_4 is titrated back until the color is green with 0.1N NaOH in the presence of the Tashiro indicator (a mixture of methyl red and methylene blue). Thus, the amount of nitrogen in the protein is calculated over NH_3 nitrogen.

0.1 ml serum is destroyed in order to determine serum total protein content. Globulins are precipitated at a concentration of 22.2% Na_2SO_4 to determine the amount of serum albumin and the amount of serum albumin remaining in the filtrate is determined. This is the amount of serum globulins.

Serum globulin nitrogen = % Total serum protein nitrogen - % Serum albumin nitrogen

However, there are also nitrogen-containing substances that are not proteins in the serum. These substances are called **non-protein nitrogen substances** and the nitrogen they contain is called **non-protein nitrogen** (**NPN**). The amount of NPN in the serum is about 35 mg %. NPN are **urea**, **uric acid**, **creatine**, **creatinine** and **free amino acid**s. The amount of nitrogen belonging only to the proteins is determined by deducting NPN amount from the above % nitrogen amounts.

For this: Add 13.5 ml of 20% trichloroacetic acid to 1.5 ml of serum, mix and filter. 10 ml of the filtrate is destroyed in the manner described in total protein damage.

15 ml volume 1.5 ml serum

10 ml filtrate x
x= 1 ml serum

Accordingly, the amount of serum to be used when calculating NPN is 1 ml.

% Total real serum protein nitrogen = % Total serum protein nitrogen - % NPN
% Serum real albumin nitrogen = % Serum albumin nitrogen - % NPN
% Serum real globulin nitrogen = % Serum globulin nitrogen - % NPN

The calculated real % nitrogen amounts are multiplied by 6.25 and the % total serum protein, % serum globulin and % serum albumin content are determined.

2- Biuret method (colorimetric)

Principle of the experiment: The amide bond (-CO-NH-) reacts with Cu II ions to form a violet colored complex. (See Biuret reaction, in urine analysis). Since the peptide bonds in the protein and its derivatives are also amide bonds, they give this reaction in an alkaline medium.

The protein-free sample is colored by the Biuret method and the color intensity is compared to the color intensity of a standard protein solution (a solution containing a certain amount of protein) in the spectrophotometer. As a result, the amount of protein is calculated.

Determination of serum total protein content:

Preparation: Take 3 tubes.

1 **Blank**	**2** **Standard**	**3** **Experiment**
-	0.1 ml standard serum	0.1 ml serum
1 ml 0.9 % NaCl	0.9 ml 0.9 % NaCl	0.9 ml 0.9 % NaCl
4 ml Biuret reagent	4 ml Biuret reagent	4 ml Biuret reagent

Mix well, incubate half an hour at room temperature. Absorbances are read on the spectrophotometer at 540 nm against the reagent blank.

Calculation:

$$g\ \%\ \boldsymbol{Protein} = \frac{\boldsymbol{Absorbance\ of\ experiment}}{\boldsymbol{Absorbance\ of\ standard}} \times g\ \%\ \boldsymbol{Standard}$$

With the help of the above-mentioned equation, the total protein content can be calculated for each sample, also a standard protein curve can be drawn by using

the protein concentration of known serum samples (regression curve). This graph is an accurate form of y=ax+b is expressed by the regression equation. Using this equation, the total protein amount of any unknown serum can be calculated.

Drawing the standard curve: The protein content of standard serum is determined by Kjeldahl method and diluted in various proportions, the absorbances of diluted serum samples with the Biuret reagent are plotted on the y-axis and the protein amounts (grams) are written on the x-axis and the standard curve is drawn. To draw the standard curve, the standard serum is diluted in 4 separate concentrations.

0.1 ml serum + 0.1 ml 0.9 % NaCl: 1/2 diluted serum

0.1 ml serum + 0.2 ml 0.9 % NaCl: 1/3 diluted serum

0.1 ml serum + 0.3 ml 0.9 % NaCl: 1/4 diluted serum

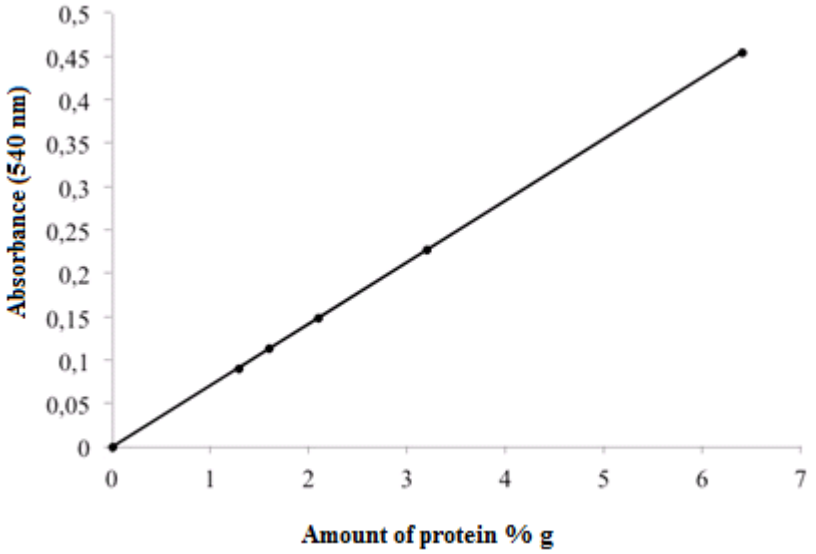
0.1 ml serum + 0.4 ml 0.9 % NaCl: 1/5 diluted serum

The Biuret assay is performed by taking 0.1 ml from each diluted serum, as described above and the standard curve (regression curve) is plotted by measuring the absorbances separately.

Clinical implications: The normal range of serum total protein is in the range of 6-8 g %. Increased serum total protein, may be due to dehydration of the body due to heavy water loss or increased γ-globulin levels due to infection. Serum albumin elevation is usually not seen due to a pathological condition, so the increase in total protein is not due to serum albumin. In cancer, there may be an increase in the amount of protein due to the synthesis of abnormal proteins by the tumor.

A lower than normal amount of protein can result from excessive protein loss, excessive protein utilization, low protein intake, or decreased protein synthesis. Patients with gastrointestinal cancers can cause significant protein losses in this way. Vitamin deficiency and malnutrition reduce the total protein amounts since it reduces the intake of amino acids. In glomerulonephritis, as the kidney's ability to retain proteins is reduced, the proteins are excreted in the urine and hence their blood levels are reduced. The following table shows the normal values of serum proteins and the pathological conditions related to the increase or decrease of these values:

	Normal value (g %)	Increase	Decrease
Total protein	6-8	Water loss Heavy body activity Infection Some cancers	Gastrointestinal cancer Liver diseases Malnutrition Glomerulonephritis
Albumin	3.5-5	Water loss Exercise Multiple sclerosis Hypothyroidism	Pregnancy Malnutrition Absorption disorders Liver and kidney diseases Heavy burns



Standard protein curve (regression curve)

DETERMINATION OF SUGAR (GLUCOSE) CONTENT IN BLOOD

Although blood glucose, called blood sugar, is one of the substances in the serum, its quantification is made in whole blood, not in serum. To obtain serum we need to wait for coagulation of the blood. But as soon as blood moves away from the vein, glucose is quickly degraded by glycolysis.

The blood is taken with a Hagedorn pipette (0.1 ml pipette) from the tip of the finger drilled with a sterile lancet.

Somogyi-Nelson method (colorimetric)

Principle of the experiment: It is based on the reducing property of the free acetal hydroxyl group of the glucose molecule. Glucose thus reduces the Cu^{2+} to Cu^{+}, resulting in a yellow-red copper I oxide. This copper I oxide, reduces arsenomolybdic acid and forms a constant green or blue colored arsenomolybdate complex. The color intensity is measured colorimetrically comparing with a standard with known glucose concentration.

Preparation: Deproteinization of blood. 0.2 ml of blood, 3 ml of distilled water and 0.4 ml of barium hydroxide solution are placed in a centrifuge tube. Gently mix until brown, than add 0.4 ml of zinc sulphate, mix well and centrifuge. The experiment is carried out with the above clear solution (supernatant). We will use directly this supernatant in our experiment.

Place in 25 ml Folin tubes:

1 **Blank**	**2** **Standard**	**3** **Experiment**
2 ml water	2 ml glucose standard	2 ml of supernatant
1 ml copper reagent	1 ml copper reagent	1 ml copper reagent
Mix		
Kept in a boiling water bath for 10 minutes and thoroughly cooled.		
1ml color reagent	1ml color reagent	1ml color reagent
Mix		
Wait for 2 minutes.		
Complete with distilled water to reach 25 ml.		

Absorbances are read at 500 nm on the spectrophotometer.

Calculation:

$$\%\, mg\ Glucose = \frac{Absorbance\ of\ experiment}{Absorbance\ of\ standard} \times \%\, mg\ standard$$

Clinical meaning: In a normal human, fasting blood glucose level is 80-120 % mg (70-110 % mg in some methods). The amount of sugar above these limits is called **hyperglycemia**. If the amount of sugar falls below these limits, it is called **hypoglycemia**.

Conditions with hyperglycemia: The most important is **diabetes mellitus**. Diabetes mellitus is a chronic disease characterized by increased blood glucose and urine sugar (glucosuria). Clinically, the etiology of the most common forms of diabetes is not well known. These are called **primary diabetes**. The reason for some forms of diabetes is known. These are called secondary diabetes.

Primary diabetes:

1-Insulin-Dependent Diabetes Mellitus (IDDM), Type-I Diabetes, Juvenile (Young) Diabetes: β cells of Langerhans islets in the pancreas are immunologically degraded. As a result insulin secretion is significantly reduced or none. The disease starts suddenly at an early age and progresses rapidly.

• Preliminary symptoms: Weakness, polydipsia (excessive water drinking), polyphagia (excessive appetite), polyuria (excessive urination).

• Clinical findings: glucosuria, negative nitrogen balance, low blood pressure, smell of ketones in the mouth, ketosis, ketonuria.

2-Non-insulin-dependent Diabetes Mellitus (NIDDM), type-II diabetes, adult diabetes: Previously known as old age diabetes or adult onset type. Plasma insulin levels may be normal or decreased, but the number of insulin receptors decreases, so insulin sensitivity to target organs is reduced (insulin resistance). The clinical onset is usually in middle age and the incidance increases with age. Inherited factors are important. There is a strong relationship between obesity and the disease.

• Preliminary symptoms: Power loss and decreases in work resistance. Polydipsia and polyuria may be mild for a long time.

• Clinical findings: Although it is the same as Type I diabetes, ketosis is rarely seen.

Secondary diabetes:

1- Pancreatic disorders: Acute and chronic pancreatitis, tumors (glucagonoma), pancreotectomy.

2- Diseases of the internal secretory system: Hyperthyroidism (thyrotoxicosis), Cushing's syndrome, gigantism and acromegaly, pheochromocytoma (adrenal medulla tumor), glucagonoma, hyperaldosteronism and adrenaline-induced conditions (adrenal gland tumors, adrenaline injection, stress).

3- Drug-induced: Thiazide group diuretics, phenytoin, steroids and ACTH, oral contraceptives.

4- Other causes: Central nervous system lesions, vitamin B1 deficiency (Wernicke encephalopathy).

Blood glucose values may be 140-300 mg % in a moderate level of diabetes. Blood glucose levels may increase up to 500-1200 mg % in severe diabetes. However, in pancreatitis, pancreatic cancers and in other cases, glucose value rarely exceeds 150 mg %.

Conditions with hypoglycemia: (more rare)

Insulin-forming pancreatic tumors (insulinoma), excessive insulin administration, adrenaline deficiency, Addison's disease, hypothyroidism (rarely), liver disease (acute infection, intoxications, cirrhosis), hunger and long-term exercise.

QUANTITATIVE CREATININE ANALYSIS IN SERUM

Jaffe method (colorimetric)

The principle of the experiment: In the alkaline environment, the absorbance of the orange color given by creatinine (in the serum filtrate that is separated from its proteins according to Folin-Wu method) with picric acid is determined colorimetrically.

Procedure: Serum is separated from its proteins according to Folin-Wu method: 2 ml of serum, 1 ml of 0.3 M Na-tungstate (= wolframate) and 3 ml of distilled water are mixed by shaking. Then 2 ml of 0.33 M H_2SO_4 is added ve mixed well (the total volume is 8 ml). The mixture is filtered through a dry filter paper. The filtrate should be colorless and clear. The experiment is done with this filtrate.

3 tubes are taken:

1 **Reagent blank**	**2** **Test**	**3** **Standard**
3 ml of distilled water	3 ml of filtrate	1 ml of standard (0.015 mg)
-	-	2 ml of distilled water
1 ml of picric acid	1 ml of picric acid	1 ml of picric acid
1 ml of NaOH	1 ml of NaOH	1 ml of NaOH

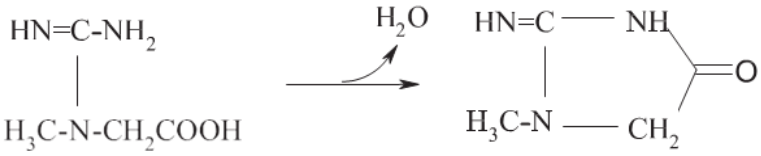
The solutions indicated in the table are mixed and allowed to stand at room temperature for 20 minutes. The absorbances of the experiment and standard are measured at 520 nm against the reagent blank on the spectrophotometer.

Calculation:

$$\%\ \text{mg Creatinine} = \frac{\text{Absorbance of the experiment}}{\text{Absorbance of the standard}} \times \frac{8^{*} \times 100}{2 \times 3} \times \text{0.015 mg Standard}$$

or

$$\%\ \text{mg Creatinine} = \frac{\text{Absorbance of the experiment}}{\text{Absorbance of the standard}} \times 2$$



Creatine **Creatinine**

Reference Intervals:	Males:	0.9-1.5 % mg
	Females:	0.7-1.3 % mg

Creatinine is the final product of creatine metabolism that most commonly occurs in muscles. Since creatinine is filtered out of glomeruli but not resorbed from renal tubules, serum creatinine level is an important indicator of renal function disorders. Serum creatinine level increases in the impairment of renal function. A marked increase only starts when the kidney is severely impaired, therefore it is recommended to perform clearance of creatinine for the diagnosis of renal dysfunction. Clearance is a measure of the kidney's ability to expel a substance and is defined as “the amount of blood that has been cleared from the measured substance in a given time unit”.

Urine samples are taken from the patient at specific time intervals for 24 hours, their creatinine amounts are measured and clearance is calculated from the following formula:

$$\text{Clearance (ml/min)} = \frac{\text{Urinary creatinine level} \times \text{Urine amount/Time}}{\text{Plasma creatinine level}}$$

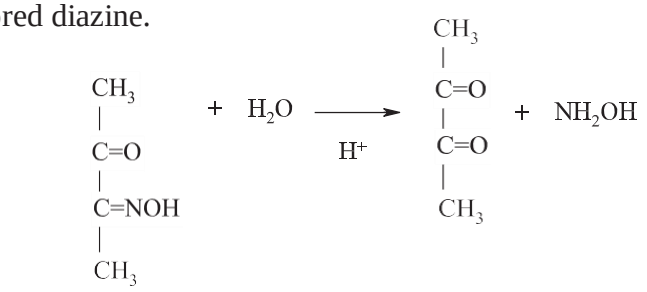
The normal clearance value of creatinine is 80-140 ml per minute.

*(8: the total volume of the Folin-Wu method; 3: the total volume of the taken

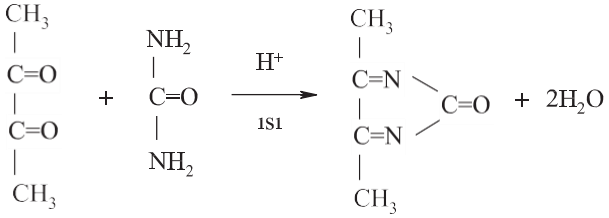
QUANTITATIVE UREA ANALYSIS IN SERUM

Diacetyl monoxime method (colorimetric)

The principle of the experiment: It is based on the colorimetric measurement of the absorbance of the pink colored compound formed by combining urea with diacetyl. Diacetyl is formed by combining the diacetyl monoxime with the acid as it is an unstable substance in the aqueous medium. Urea reacts with diacetyl to form colored diazine.



Diacetyl monoxime **Diacetyl** **Hydroxylamine**



Diacetyl **Urea** **Diazine**

Procedure: Serum is separated from its proteins according to Folin-Wu method. 1 ml of serum, 1 ml of 10% Na-tungstate, 7 ml of distilled water and 1 ml of 3% sulfuric acid are mixed and filtered (the total volume is 10 ml). The experiment is done with this filtrate.

3 tubes are taken:

1 **Reagent blank**	**2** **Standard**	**3** **Test**
1 ml of distilled water	1 ml of standard	1 ml of filtrate (or serum)
3 ml of oxime solution	3 ml of oxime solution	3 ml of oxime solution
3 ml of acid solution	3 ml of acid solution	3 ml of acid solution

The solutions in the amounts indicated in the table are mixed, kept in a boiling water bath for 15 minutes, and cooled. The absorbances are measured at 520 nm against the reagent blank on the spectrophotometer.

Calculation:

$$\% \text{ mg Urea} = \frac{\text{Absorbance of the experiment}}{\text{Absorbance of standard}} \times \% \text{ mg Standard}$$

In recent years, the term blood urea nitrogen (BUN) is used instead of % mg urea. This term is not mistaken because the amounts of urea in the serum, plasma, and whole blood are very close to each other and % mg urea nitrogen can easily be calculated from % mg urea. The ratio of nitrogen atoms to urea molecule,

$$\frac{28}{60} = 0.46666 \text{ as it is;}$$

$$\% \text{ mg Urea nitrogen} = \% \text{ mg Urea} \times 0.4666$$

Clinical meaning: Normally, the amount of urea in serum (plasma, whole blood) is 20-40 % mg. This value corresponds to 10-18 % mg BUN.

Increased situations: The increase of urea in the blood is called **uremia**. "Urea retention" is in question in uremia. Diseases that cause severe parenchymal cell destruction, such as kidney inflammation (nephritis and pyelonephritis), renal tuberculosis, and kidney tumors, cause insufficiency in the urea excretion of the kidney and thus increase the urea level in the blood. The serum urea level in acute nephritis can be as high as 250% mg. Apart from these, in cases of severe heart failure, myocardial infarction, severe infections, conditions causing large water loss such as diarrhea and vomiting, bleeding and after operations urea can be increased in the serum. Urea itself is not a toxic substance, an incorrect acceptance among the public that urea is the poisoning of blood by urea. Uremia is important in terms of being an indicator of renal impairment.

Decreased situations: Sometimes in nephrosis and severe liver failure.

ENZYMES

Most of the enzymes in body fluids are secreted from tissue cells. Therefore, an increase in enzyme activity in these fluids is an indication of tissue or cell damage and is important in the diagnosis of the disease.

Since the amounts of enzymes in tissues and liquids are very low, their blood levels are not expressed in grams or milligrams, as in other substances. "Unit" is defined for enzymes. In the identification of the unit, the amount of substrate and the amount of product formed during the activity of the enzyme (the substance affected by the enzyme) is in question. An international enzyme unit is described as "the amount of enzyme that catalyzes the modification of a micromole (10^{-6} mol) substrate under certain and specific conditions".

When the cell is damaged, the enzymes inside the cell move out of the cell. The serum levels of enzymes passing from extracellular fluid to systemic circulation increase rapidly. This sudden increase is a very important parameter used in the diagnosis of the disease. Main enzymes used in diagnosis are: pancreatic amylase, serum acid phosphatase, alkaline phosphatase (ALP), creatine kinase (CK), glutamate oxaloacetate transaminase (GOT=Aspartate transaminase; AST), glutamate pyruvate transaminase (GPT=Alanine transaminase; ALT), lactate dehydrogenase (LDH), γ-glutamyl transpeptidase (GGT).

DETERMINATION OF AMYLASE ACTIVITY IN SERUM

Wohlgemuth method

The principle of the experiment: Amylase is an enzyme which is secreted mainly by salivary glands and pancreas and hydrolyzes α(1→4) glycoside bonds of starch. As a result of the hydrolysis of starch with α-amylase, maltose, isomaltose, limit dextrins and a small amount of glucose are formed.

In the examination of amylase activity; the color of the starch degradation products (the result of the amylase activity) with iodine is used. These colors are respectively:

- Starch - blue
- Soluble starch - blue
- Amylodextrin- violet
- Erithrodextrin - red
- Acrodextrin - colorless
- Maltose - colorless

The method used to determine amylase activity in serum and urine is the Wohlgemuth method. In this method, a certain amount of starch solution is incubated at 37ºC, with a certain amount of diluted serum. After cooling, the minimum amount of serum is determined, which is sufficient to hydrolyze a certain amount of starch until it does not give off blue color with iodine.

The minimum amount of amylase in serum (or urine) that breaks down 1 ml of 0.1% starch solution in 30 minutes at 37ºC until it does not give off blue color with iodine is called "1 Wohlgemuth unit".

Procedure: 8 test tubes are taken, 1 ml of 0.9% NaCl solution is added to all except the first tube. 2 ml of serum is then added to the first tube. 1 ml serum is taken from this tube and placed in the second tube. It is mixed one or two times by pipetting up and down. 1 ml is taken from the second tube with the same pipette and transferred to the third tube. This process is continued until the eighth tube. 1 ml is discarded from the eighth tube. Thus, serum (= enzyme) is serially diluted in half. Then 2 ml of amylase reagent (= substrate containing starch) is added to all tubes and mixed. Tubes are incubated in a 37ºC water bath for half an hour. The reaction is stopped by adding 1 ml of N HCl. One drop of N/50 iodine solution is then added to each tube and the tubes are mixed.

Evaluation: The tube with the first blue color is detected, the number of the previous tube (yellow-red or brown-red) is taken and the tube number is put in the formula.

$$D_{30'}^{37^oC} = 2^x$$

x = first colorless tube number

	Tube No.							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
The amount of serum in 1 ml	1	0.5	0.25	0.125	0.062	0.031	0.016	0.008
Dilution rate	1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
The corresponding Wohlgemuth unit	2	4	8	16	32	64	128	256

Clinical meaning: The serum level of amylase is normally between 8-12 units. It is not pathological unless it is less than 6 and no more than 128. There is no amylase in the serum of newborns. It starts to appear in the 2-3 months and reaches the normal limit at the age of one year. Serum amylase is excreted in the urine and the enzyme activity is 2 to 6 times greater in urine than that in serum. The determination of amylase in the urine is done in the same way.

Increased situations: Acute pancreatitis (shows a significant increase in the diagnosis at the first 24-36 hours, decreases to the normal value on days 2-6), obstruction of the pancreas channel with stone, some pancreatic cancers. In cancers, amylase secretion by tumor was detected. There may be a moderate increase in acute conditions such as inflammation of of the salivary gland ducts, obstruction of the salivary glands with stones and mumps. However, the serum amylase level remains normal in chronic conditions. Various liver diseases, obstruction of the bile duct, and volvulus can also lead to elevated amylase levels in the serum.

Some drugs also increase serum amylase activity. Codeine, morphine, glucocorticoids, dexamethasone, oral contraceptives are among these drugs.

DNA ISOLATION FROM PERIPHERAL BLOOD

The three basic steps of DNA extraction are

1) lysis of cells,

2) separation of DNA from cellular debris (protein, RNA, other macromolecules) and

3) precipitation of DNA.

The basis of DNA isolation is lysis of blood cells, the release of DNA, RNA, and proteins, precipitation of proteins by ammonium acetate and precipitation of DNA by adding alcohol. Add 30 mL of erythrocyte lysis buffer and incubate for 15 min at +4°C.

- Centrifuge 10 min at 1500 rpm. Remove the supernatant.
- Suspense the cell pellet and add 15-20 mL erythrocyte lysis buffer.
- Incubate for 15 min at +4°C. Centrifuge 10 min at 1500 rpm. Remove the supernatant.
- Add 500 µL of 10% SDS solution, 75 µL of proteinase K (20 mg/mL), and 9.4 mL of leukocyte lysis buffer (WBL). Incubate for overnight at 56°C (or for one hour at 65°C).
- After incubation, add 3.7 mL of 9.5 M ammonium acetate solution and mix gently.
- Precipitate the proteins by centrifuge 25 min at 3000 rpm.
- Transfer the supernatant to a sterile test tube and collapse the DNA by adding absolute ethanol (1:2 supernatant:absolute ethanol). DNA should be visible. DNA is separated from the solution via the tip of the pipette.
- Wash DNA with 70% alcohol several times and dissolve in Tris-EDTA solution. Mix and store at +4˚C.

Clinical meaning: DNA is a hereditary material. It carries the genetic code necessary for the biological development of all organisms and some viruses with their vital functions. DNA stores the genetic information and transfers it to the next generation. DNA isolation is used in many molecular biology and genetic fields such as gene transmission, gene therapy, forensic medicine, fingerprint analysis, investigation of DNA-protein interaction, gene expression, and analysis of genome structure and hereditary diseases.