

# 1 INTRODUCTION TO LABORATORY INVESTIGATION

## INVESTIGATION OF GLUCOSE METABOLISM

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### Topics to be reviewed

Blood and its components. Blood plasma and serum. Anticoagulants. Metabolism of glucose. Glycemia and its regulation. Disorders of glucose metabolism. Glucosuria.

### Pre-lab questions

1. Which factors can induce hemolysis during blood collection and blood processing?
2. Give examples of disinfectants used during blood collection.
3. Why is NaF added to collected blood before glucose determination?
4. Draw structural formulas and names of ketone bodies.

## 1.1 Importance of laboratory biochemical investigations

For accurate appraisal of health state many information should be acquired. Results of laboratory examinations are considered as the source of valid information needed for the assessment of health state and the reflection of metabolism changes in body. According to World Health Organisation (WHO) laboratory examinations provides about 80 % information leading to the assessment of correct diagnosis.

The physician can use a variety of laboratory methods during a different phases of diagnostic and therapeutic process. However it is necessary to indicate rationally and to use effectively these methods and then to evaluate and correctly interpret obtained results There are known basic, specialized and highly specialized laboratory investigation. According to rate of carrying out we distinguish routine and urgent laboratory investigation. A small number of simple, mostly qualitative tests with direct informative results take place in the doctor's surgery or at the patient's bed (*bed-side diagnostics, point-of-care testing, near-patient testing*).

### 1.1.1 Handling biological material, blood collection

The largest and most important part of the clinical-biochemical examinations concerns blood, blood serum and plasma analyses. Another frequently analysed material is urine . Analyses of the other body fluids (gastric juice, duodenal juice, amniotic fluid, cerebrospinal fluid, saliva, transpiration, etc.) are demanded only in a chosen number of patients, their frequency is much lower, and they are often carried out in the special laboratories.

Blood is most frequently analysed biological material. Blood is easily accessible material and its composition reflects the range of biochemical processes blood is a good indicator of the physiological

conditions throughout the body. Blood for analysis may be taken from veins, arteries, or capillaries. Venous blood is usually the specimen of choice, for some analysis and especially in young children is taken capillary blood. The arterial puncture is used only exceptionally, especially for the blood gas analysis.

When blood is drawn without the addition of an anticoagulant and allowed to stand, it clots after several minutes, because soluble fibrinogen is transformed to the insoluble network of fibrin by the clotting mechanism. **Serum** is excluded from the blood clot after some time and is obtained from clotted blood by centrifugation. Sufficient time is needed for complete blood clotting (at room temperature at least 15–30 min).

Non-clotted blood is required for some clinical-biochemical examinations. Blood is taken to the tubes with the addition of the anticoagulants (sodium oxalate, sodium citrate, EDTA Na<sub>2</sub>, heparin). Centrifugation of non-clotted blood produces **plasma**. Blood may be centrifuged immediately after the collection. Plasma or serum should be separated from blood cells or coagulum within 2 hours at the latest (or 1 hour for the determination of potassium ions).

#### 1.1.1.1 Venous blood collection

With regard to the method and equipment used for the venipuncture are distinguished two ways of collection

- **Open collection system** - phlebotomist is in direct contact with the biological material

- blood is drawn through needle directly into a test tube

- blood is drawn into a disposable syringe and by back sticking into a test tube.

- **Closed collection system** – the sample handling is carried out after the collection directly in the collection syringe or test tube. Thus, the laboratory technician is protected against the contamination by the patient's blood, the biological material is protected against the external contamination, and also against breaking during transport and centrifugation. The individual syringes/evacuated test tubes are marked by colour according to the additive used (clotting accelerators, separation gel, heparin, EDTA, ...). The separation gel enables proper separation of serum from the blood coagulum after centrifugation (creation of interlayer). The collection syringe/test tube can be placed directly into the analyzer. The used material is easily burnt down.

The collection is carried out:

- *into the closed syringe* – the blood is drawn out with the help of a piston (Fig. 1-1a) or with immediately before the collection prepared vacuum (pulling the plunger into the base of syringe and locking it into place, Fig. 1-1b). After the collection, the piston is broken off and the syringe is transformed into the closed test tube. The method of collection is adapted to patients with problematic veins. Using piston we collect the blood samples in patients with thin veins. Vacuum is used in case of strong veins

- *into the evacuated test tube* (Fig. 1-1c)

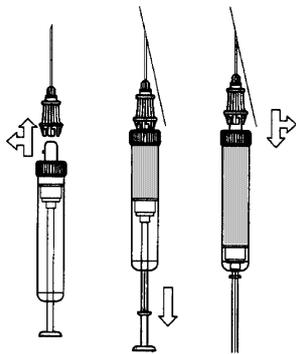


Fig. 1-1a

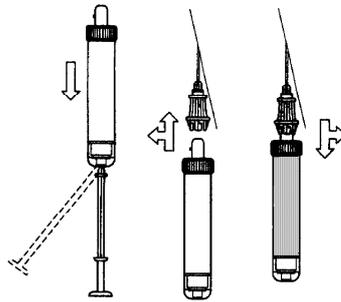


Fig. 1-1b

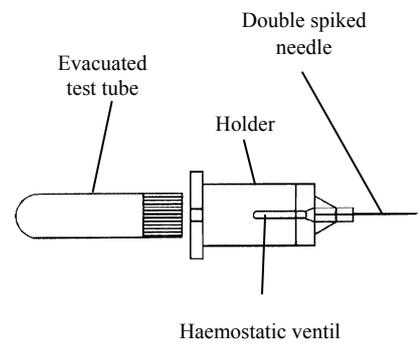


Fig. 1-1c

### Specimen collection general instruction

- 10–12 hours before the blood withdrawal patients are not allowed to eat; they have to exclude fat food and alcohol from their diet.
- In the morning before the blood withdrawal, patient can drink about ¼ L of plain water or bland tea.
- Before the blood withdrawal, the patient should be asking for any allergies to antiseptics, adhesives.
- Standard posture of patient at the blood withdrawal is sitting posture.
- Pumping of the fist before venipuncture should be avoided (results of many tests can be affected).
- The site of injection must be disinfected (Jodonal B, Persteril, Jodisol, Ajatin).
- If the veins are visible, the use of a tourniquet should be minimized.
- If the tourniquet is used (not more than 1 min), it should be released before withdrawal of blood begins.
- Press down on the gauze once the needle is out of the arm (to avoid formation of a hematoma). The gauze should be moisturized in disinfectants.
- If the non-clotted blood is drawn out, it is necessary to mix blood immediately after collection by rotating the tube at least five times.

#### 1.1.1.2 Capillary blood collection

Capillary blood is used in case only a small amount of the sample is needed. General instructions for specimen collection:

- Warm the site of injection (tip of the finger, an earlobe, the heel or big toe of an infant), ensure good blood supply. Avoid massaging the site.
- The injection site must be disinfected.

- Puncture should be directed onto the outer and upper region of the fingertip, halfway between the centre of the finger pad and the edge of the fingernail.
  - After puncture with the lancet, the first drop should be wiped off (dilution of blood with the tissue fluid). Gently apply intermittent pressure to the surrounding tissue. Avoid excessive squeezing or "milking" of the puncture site.
  - After puncture with the lancet, the first drop should be wiped off (dilution of blood with the tissue fluid). Gently apply intermittent pressure to the surrounding tissue. Avoid excessive squeezing or "milking" of the puncture site.
  - Blood is usually collected into the special capillary blood test tubes or into the small plastic or glass test tubes. For strip tests, the drop of blood is applied directly on the paper.
  - Following collection press clean gauze moisturized in disinfectants on the puncture side.
- ▶ See video recording and acquaint with the blood withdrawal ways.
  - ▶ Draw chemical formulas of common anticoagulants and explain how they act.
  - ▶ Describe the appearance of blood before and after being centrifuged
  - ▶ Give the main differences in composition of blood plasma and serum.
  - ▶ What does it mean, when we describe plasma/serum as a) hemolytic; b) icteric; c) chylouse?

## 1.2 Investigation of glucose metabolism

Glucose concentration in blood, plasma, or serum is the basic biochemical value of saccharide metabolism. The most frequent type of disorder of saccharide metabolism is diabetes mellitus (DM). This metabolic disease can occur, if fasting blood glucose is above 7 mmol/L. To confirm the diagnosis, if not determined from *glycemia*<sup>1</sup>, the glucose tolerance test is used. We use other biochemical examinations in order to monitor the course of diabetes and to control the therapy: the quantitative determination of glucose in urine, glycated hemoglobin in blood, the detection of microalbuminuria and ketone bodies in urine.

Diabetes mellitus is a condition characterised by absolute or relative insulin deficiency. DM is divided into several forms and stages, which must be distinguished from prognostic as well as therapeutic reasons. According to etiology, we recognize DM of the 1<sup>st</sup> type, DM of the 2<sup>nd</sup> type, gestational diabetes, and other specific forms of diabetes.

Diabetes mellitus of the 1<sup>st</sup> type is a polygenic autoimmune disease. It is a less frequent type of diabetes. Genetic predisposition combined with external factors (such as viral infection, toxins, stress) can induce pre-diabetic phase of the disease, which can last several years. In this time, the  $\beta$ -cells of the Langerhans' isles are slowly destroyed by activated T-lymphocytes and cytokines, a condition expressed as *insulitis* (the lymphocytic infiltration of the cells of the isles, inflammation). Insulitis gradually reduces the number of functional  $\beta$ -cells, which results in the disruption of synthesis and

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<sup>1</sup>The more accurate term for the description of the blood level of glucose is *glucosemia*. However, the literature commonly relies on the term *glycemia*.

secretion of insulin. Diabetes is clinically manifested, when 60–70 % of pancreatic  $\beta$ -cells are destroyed by the autoimmune inflammation.

**Diabetes mellitus of the 2<sup>nd</sup> type** results from insulin resistance and/or relative insulin deficiency (abnormal insulin, insulin antibodies). It is the most frequent type of DM. Insulin resistance can be the result of the decreased number of plasmatic membrane receptors on the target cells or the consequence of the post receptor blockade of the intracellular metabolism of glucose (decreased number of receptors or decreased affinity; decreased activity of the pyruvate dehydrogenase complex; abnormal signal transfer or abnormal phosphorylation reaction for the excessive production of  $\text{TNF}_\alpha$ ). The level of insulin insufficiency reflects the gradual loss of the potency of  $\beta$ -cells to respond to glucose.

### 1.2.1 Enzymatic determination of plasma glucose

Glucose oxidase (GOD) catalyses the oxidation of  $\beta$ -D-glucopyranose by air oxygen to  $\delta$ -lactone of gluconic acid and hydrogen peroxide:



In the presence of peroxidase (POD), hydrogen peroxide then reacts with the chromogenous reagent ( $\text{H}_2\text{A}$ ) to form the red coloured product (A):



Maintaining the prescribed reaction conditions, the amount of this product is proportional to the concentration of glucose. This reaction is sensitive and fast. The resulting colour is measured after reaching the final equilibrium (*end-point method*). It can be also measured continuously during the first few minutes after initiating of the reaction (*kinetic method*).

It is not necessary to adjust serum or plasma samples, if they are separated from whole blood immediately after blood collection. Otherwise, blood samples should be stabilized: the simplest way is blood cooling or adding of mannose (it is alternative substrate for hexokinase and acts immediately) or NaF (it inhibits glycolysis in red blood cells after entering into cell by diffusion, i.e. after about 2 hours).

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**Materials:** Kit Glu GOD (Erba Lachema\*): *Reagent-glucose* (containing 3-methylphenol 11 mmol/l; 4-aminophenazone 0,77 mol/l; glucose oxidase  $\geq 300 \mu\text{kat/l}$ ; peroxidase 18,3  $\mu\text{kat/l}$ ), *calibrator-glucose* (concentration is given on the label of vial). Specimen of blood serum. Pipettors 20  $\mu\text{l}$  and 2 ml, water bath at 37 °C. Spectrophotometer Spekol 1300 and software WinAspect or Helios Delta and software VisionLite Fixed.  
\*Alternatively, the kit of Roche-Diagnostics, Human or BioVendor can be used; the composition of reagents may be then different.

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### Procedure

☞ Deproteinization is not essential in the case of non-hemolytic blood serum or plasma. Pipette the components into clean test tubes according to the following table:

Reagens (µl)	Blank	Test	Standard
Reagent-glucose	1 000	1 000	1 000
Demi-water	20	-	-
Serum	-	20	-
Calibrator-glucose	-	-	20

Shake all the test tubes vigorously (important for saturating with oxygen).  
 Incubate all tubes for 30 min at room temperature (or 15 min at 37°C)  
 Avoid exposure to direct light.

Read the absorbances \* of sample ( $A_x$ ) and standard ( $A_{STD}$ ) at 495 nm against the blank within 40 min.

\*Measure absorbances according to the enclosed instructions to the spectrophotometers and use relevant software.

Calculation of the concentration of serum glucose: 
$$c_x = \frac{A_x}{A_{STD}} \times c_{STD}$$

where  $c_{STD}$  is the concentration of glucose in calibrator (it is given on the label of the vial).

## Evaluation

Reference values of plasma glucose concentration at fasting state in healthy adults (FPG-fasting plasma glucose): 3,9 – 5,6 mmol/l

For precise determination of glycemia, we should be aware of the fact that the reference levels differ according to the type of processed material. The levels of glucose in plasma are approximately about 10-15% greater than in whole blood. There are also differences between capillary and venous blood. The levels in capillary blood are greater than in the venous blood, at fasting about 5% and postprandial about 10-15%.

If the concentration is lower than the reference interval, the condition is recognized as **hypoglycemia**. It is a pathological state, during which the organism is not able to keep glucose homeostasis. Clinical symptoms of hypoglycemia are manifested, when the level of glucose drops in adults below 2.8 mmol/L. Hypoglycemia is dangerous mostly because of the insufficient energetic supply into the brain with symptoms like feeling hungry, headache, somnolence, mental perplexity, hallucinations and finally spasms and coma. The second group of symptoms results from the activation of the adrenergic system and the increase of the catecholamine secretion (palpitations, anxiety, tremble, perspiration). Clinically, we distinguish between hypoglycemia developing after starvation and postprandial hypoglycemia (develops after the meal). Hypoglycemia is evoked by a number of pathological conditions, e.g. pancreatic tumours, defects in the production of anti-insulinic hormones, hepatic cirrhosis, defects of enzymes metabolising glucose. Moreover, hypoglycemia develops after the inadequate dose of insulin or peroral antidiabetics.

Levels greater than reference are described as **hyperglycemia**. The most frequent and the most serious cause of hyperglycemia is DM.

The level of fasting plasma glucose (FPG) is a good parameter for

- diagnosis of diabetes mellitus
- seeking persons with high risk of diabetes mellitus

### **Glycemia for diagnosis of diabetes mellitus (DM)**

Diabetes is confirmed if glycemia exceeds:

- combination of clinical symptoms with random determination of plasma glucose  $\geq 11,1$  mmol/l
- the concentration of fasting plasma glucose  $\geq 7$  mmol/l
- the concentration of glucose in glucose tolerance test  $\geq 11,1$  mmol/l

To confirm the diagnosis of diabetes, it is necessary to check the result repeatedly during following days.

### **Seeking persons with high risk of diabetes mellitus**

The high risk of diabetes is characterised by the value of fasting plasma glucose in the range 5,6-7,0 mmol/l. This state (IFP-impaired fasting glucose) is known also as prediabetes.

Critical levels of fasting plasma glucose (FPG):

<b>FPG (mmol/l)</b>	<b>Interpretation</b>
$\leq 5,6$	excluding DM
5,6 – 7,0	prediabetes (increased FPG, IFG)
$\geq 7,0$	DM

## **1.2.2 Determination of glycemia by personal glucometer**

Quick and easy monitoring of glycemic profiles by patients themselves (*self-monitoring*) or directly at the patients' bed (*bedside diagnostics*) is carried out with the help of a large number of different types of glucometers. Nowadays, there are given preferences to glucometers with broadened functions such as: indication of measured values pre- and postprandial, setting sound signal for the remainder of next determination of glucose, saving of dates to the memory of glucometer, because it is convenient for monitoring of glycemia in diabetes. To compare measured values in capillary blood directly with laboratory values it is suitable to use glucometer calibrated directly to the plasma. Capillary blood is collected from the finger, sometimes alternatively collection is recommended (forearm, arm, calf etc.).

Glucometers are mostly based on reflectance photometry: the sequence of reactions with glucose oxidase and peroxidase proceeds on the indication zone of the diagnostic strip specific to the given type of glucometer. The concentration of formed colour product is determined by reflectance photometry. The new types of glucometers are based on electrochemical method when the weak electric current formed during oxidation of glucose is detected.

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**Materials:** Personal glucometer with diagnostic strips, blood collection strips, lancets

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## Procedure

- ☞ Strictly follow the rules for the proper collection of capillary blood (see Exp. 1.1.1.2) and the detailed instructions to glucometer.
- ☞ Capillary blood collection:
  - wash hands in warm water
  - disinfect the puncture site
  - point the puncture using of lancet from *lateral* side of finger
  - wipe off the first drop of blood
  - support formation of the other drops by gently pressure to the surrounding tissue
  - after blood collection apply a cotton wool wetted in disinfect solution on the puncture site
- ☞ Apply the drop of blood directly on the paper of test strip.
- ☞ Insert the test strip into glucometer and after the required time read the value on the display.

## Evaluation

Personal glucometers provide the patient the possibility to monitor and note down the glycemc profile during the whole day, which is important for the proper treatment. Levels, which do not exceed 7 mmol/L, are regarded as satisfactory.

The determination of glycemia by glucometer can be used for diabetes/prediabetes screening program. Generally, we may screen for elevated blood glucose with either a Fasting Plasma Glucose (venipuncture) or Fasting Capillary Blood Glucose (home/fingerstick) test. It is necessary to confirm the elevated blood glucose (determined randomly during day, independently on food intake) in capillary blood above 7 mmol/l in physician's office and then to confirm diagnose based on analysis of clinical symptoms together with the examination of glycemia in venous blood by standard method in laboratory.

### 1.2.3 Oral glucose tolerance test (oGTT)

If we find increased concentration of fasting glucose in the blood plasma, within 5,6–7 mmol/l, that is known as a prediabetes, we should verify the effectiveness of glucose metabolism by the functional test. However, oral glucose tolerance test is also performed when fasting plasma glucose is less than 5,6 mmol/l that is in this cases regarding persons with higher risk of diabetes or in the case of disorder of tolerance glucose from the previous measurements.

The glucose tolerance test monitors changes in glycemia after the peroral administration of the standard dose of glucose by mouth. The oGTT test is not necessary at higher glucose concentrations, especially if the characteristic symptoms of diabetes are present.

*Standard procedure of oGTT in adults.* Three days before the test saccharides are not restricted in the diet. After a night fasting (10–14 hours), a sample of venous blood is taken. Then the patient is asked to drink 75 g of glucose in approximately 300 mL of tea and venous blood sample is taken 2 hours after drinking the tea. Patients are not allowed to smoke, eat or drink or do any physical activity. Glycemia is determined in plasma of venous blood

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**Materials:** 75 g of glucose dissolved in 300 ml of weak tea. Personal glucometer with diagnostic strip, blood collection kit.

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## Procedure

Due to time-consuming and laboratory demands we will carry out oGTT only theoretically.

Despite this fact we will perform at least approximately this test for understanding of regulatory mechanism of glucose and we will determine glycemia by glucometer.

- ☞ Determine the concentration of glucose in capillary blood by personal glucometer (see Exp.1.2.2.) in healthy volunteer at the beginning of the lesson (ideally at fasting state).
- ☞ After drinking about 300 mL of tea with 75 g of glucose, repeat the determination of glucose concentration in capillary blood by personal glucometer after 2 hours. For better understanding of the regulatory mechanisms at healthy subjects there is recommended the determination of the glucose concentration in capillary blood 30 minutes after drinking the tea.

## Evaluation

Glycemia in plasma venous blood after 2 hours post-glucose (75 g) load (mmol/l)

Glucose tolerance	Glycemia 2 hours after glucose load
Normal (excluding DM)	< 7,8
Impaired glucose tolerance	7,8 - 11
Diabetes mellitus	> 11

To confirm diagnose the result of test has to be check repeatedly. When finding impaired glucose tolerance, o-GTT should be repeated at two-year intervals.

- ▶ Evaluate the theoretical oGTT values given by teacher.
- ▶ Evaluate values obtained in the practical lesson by glucometer and note the trend of the changes of glycemia during the time.

## 1.2.4 Test for glucose in urine

Under normal conditions, there is only very small amount of glucose and other saccharides (galactose, fructose, pentoses, lactose, and maltose) in urine, depending on the composition of the food eaten. This concentration is undetectable by routine tests. Glucose concentrations in urine higher than 0.8 mmol/L are considered as **glucosuria**. When the maximal reabsorption capacity of the cells of the proximal tubule for glucose is exceeded, the resulting hyperglycaemia is manifested as glucosuria. This value is usually given as 10 mmol/L (so-called *renal glucose threshold*), however, it may vary in relatively large range (2.8–18 mmol/L). Rarely *renal glucosuria* appears during normal glycemia and it is caused by disrupted tubular reabsorption.

Glucosuria usually reveals diabetes; however, if glucose is not present in urine, diabetes is not excluded. Quantitative determination of glucosuria is not so appreciated in recent days it is used as complementary test. It is important for evaluation of daily losses of glucose in urine.

Determination of glucosuria can be achieved by a *non-specific* test with Benedict's reagent (see Biochemistry II– practicals, Lesson 3, Exp.3.3.1) These tests are used only exceptionally. *Specific* proof for glucose in urine can be carried out by diagnostic strips, e.g. GlukoPHAN.

Quantitative determination of glucose in urine is performed using the same methods as for the determination of glucose in plasma.

### 1.2.4.1 Test for glucose by diagnostic strip GlukoPHAN

**Principle:** The principle is the same as that in the enzymatic determination of glucose (see Exp. 1.2.1). The indicator zone of the diagnostic strip contains the enzymes glucose oxidase and peroxidase and a chromogenous substrate, which is oxidized in the presence of glucose by forming peroxide to a coloured product.

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**Materials:** Diagnostic strips GlukoPHAN.

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#### Procedure

- ☞ Dip the test strips in urine samples for about 1 s and wipe off the excess liquid on the vessel edge.
- ☞ Put the strips onto the containers with urine (horizontal position of the strip).
- ☞ After 3 minutes, compare the colour of the indicator area with the colour scale on the container.

Verify the possibility of *false positive* result by repeating the test with a new strip and urine sample (with no proved glucose), which is measured into a test tube rinsed with a small amount of 3% hydrogen peroxide or 0.5% Persteril

#### Evaluation

The test is quite sensitive. The result is clearly positive by the glucose concentration of 2 mmol/L. It is very specific as well; other saccharides than D-glucose do not react.

*False negative results* are created during high concentrations of reducing substances (ascorbic acid or some spasmolytics slow down the development of the coloration).

*False positive results* can be created by peroxidase substrates present in the containers for urine (common disinfectant Persteril, i.e. peroxyacetic acid, or hydrogen peroxide). Therefore, the containers must be carefully washed by pure water after disinfection.

The use of the reduction test with Benedict's reagent: If any of the hereditary disorders of saccharide metabolism (e.g. galactosemia) are suspected, newborn departments request determination of other sugars than glucose. Comparison of glycosuria examined by the enzymatic and reduction tests is carried out. If any difference is found, specific examination of the presence of other monosaccharides must be done.

## 1.2.5 Test for ketone bodies in urine

Ketone bodies should be tested in urine of mainly diabetics of the 1<sup>st</sup> type. If a diabetic undergoes correct treatment, ketone bodies are not found in urine. Their presence, together with marked hyperglycemia and glucosuria, indicate diabetic ketoacidose.

**Principle:** Ketone bodies react with sodium nitroprusside in alkaline medium to form a violet coloured product. Test strips are based on this principle. We can use any of the test strips with a test zone for detection of ketone bodies, e.g. diaPHAN. This test proves especially acetoacetate in fresh urine, which during longer standing spontaneously decarboxylates to acetone (the test is less sensitive for acetone).

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**Materials:** Diagnostic strips diaPHAN.

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### Procedure

- ☞ Dip the strip in urine sample for about 1 s and wipe off the excess liquid on the vessel edge.
- ☞ After 1 minute, compare the colour of the indicator area with the colour scale on the container.

### Evaluation

The excretion of ketone bodies in urine (**ketonuria**) is negligible under normal conditions; it does not reach 0.5 mmol/day. A sharp increase in ketone bodies formation is a result of the increased utilization of lipids (without providing enough glucose to be metabolized), as may occur in diabetes mellitus, exhaustive physical activity without sugar delivery, prolonged starvation, reduction diet with protein domination. In such cases, the concentration of ketone bodies in the blood rises above 200 µmol/L and more than 100 mmol of ketone bodies may be excreted in urine daily. Acetone is relatively volatile and it is expelled relatively quickly from the body through exhalation. Ketonuria with concurrent glucosuria reflects metabolic decompensation of diabetics. Therefore, it is important to test ketonuria, especially in every case of detected glucosuria.