Name, group No: Date: 

**Principle**
The solution of bathophenanthroline forms a stable red-coloured complex with Fe$^{2+}$ ions. This complex is suitable for spectrophotometric determination at its absorption maximum (535 nm). Using the measurement of absorbances of several different solutions of Fe$^{2+}$ with known concentrations we can create the analytical curve (dependence of $A_{535}$ on concentration). Concentration of unknown sample can be read from this curve or can be calculated.

![Structure of bathophenanthroline](image)

**Figure:** Structure of bathophenanthroline

**Reagents and accessories**
bathophenanthroline (4,7-diphenyl-1,10-phenanthroline-3,6-disulfonic acid) (0.46 mmol/l), natrium acetate CH$_3$COONa (2 mol/l), stock standard solution of Fe$^{2+}$ ions (18 μmol/l)

**Accessories and equipment**
spectrophotometer
Experimental procedure

<table>
<thead>
<tr>
<th>Test tube No</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>serum sample</th>
<th>reference sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard solution Fe^{2+} (ml) (17.9 µmol/l)</td>
<td>0.5</td>
<td>1.0</td>
<td>1.5</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H_{2}O (ml)</td>
<td>1.5</td>
<td>1.0</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>2.0</td>
</tr>
<tr>
<td>Serum sample (ml)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.0</td>
<td>-</td>
</tr>
<tr>
<td>Reagent (ml)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

$A_{535}$ is measured against reference sample within 5-60 min

| $A_{535}$ |  |  |  | - | |
| $c$ (Fe^{2+}) (µmol/l) |  |  | 18 | - | |

**Evaluation**

1. Construction of analytical curve using absorbances and concentrations in test tubes No. 1 – No. 4

2. Read the concentration of Fe^{2+} in serum from curve. (see Fig.)

3. Calculate the concentration of Fe^{2+} ions in serum using one of the known standard solutions:

$$c_{sam} = \frac{A_{sam}}{A_{st}} \cdot c_{st}$$

where $A_{sam}$ is absorbance of sample, $A_{st}$ is absorbance of standard solution, $c_{sam}$ is concentration of the sample and $c_{st}$ is concentration of the standard

4. Compare the determined concentration with physiological values:

- $fS$ (Fe^{2+}) males = 9,6 – 30,2 µmol.l^{-1}
- $fS$ (Fe^{2+}) females = 8,9 – 27,3 µmol.l^{-1}
- $fS$ (Fe^{2+}) children = 9 – 30 µmol.l^{-1}
Figure: Illustration, how to read the sample concentration from analytical curve

Calculations and conclusion
Free radicals are in the organism formed in many pathological conditions. They are derived mostly from oxygen, e.g. superoxide anion radical $\text{O}_2^-$ . The enzyme superoxide dismutase catalyzes its dismutation into oxygen and $\text{H}_2\text{O}_2$, and in this way it decreases its toxicity. Some metallic ions (e.g. Cu(II), Mn(III), Fe(III)) and mainly their biocoordination compounds have the ability to react with superoxide and thus to eliminate its increased production in the organism.

Superoxide formed by the system xanthine - xanthine oxidase reduces the detector of superoxide - a tetrazolium salt (INT) - into monoformazane that can be determined spectrophotometrically at 510 nm. Superoxide dismutase or Cu(II) complex scavenge superoxide, so they decrease its level and decrease the reduction of the INT.

$$X + \text{O}_2 + \text{H}^+ \xrightarrow{\text{XO}} \text{O}_2^- + \text{H}_2\text{O}_2 + \text{uric acid} \quad (1)$$

$$\text{O}_2^- + \text{INT} + \text{H}^+ \rightarrow \text{O}_2 + \text{INT} - \text{H} \quad (2)$$

$$2\text{O}_2^- + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{O}_2 + \text{H}_2\text{O}_2 \quad (3)$$

$$\text{O}_2^- + \text{Cu}^{\text{II}} \rightarrow \text{O}_2 + \text{Cu}^{\text{I}} \quad (4)$$

$$\text{Cu}^{\text{I}} + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{Cu}^{\text{II}} + \text{H}_2\text{O}_2 \quad (5)$$

Reagents
- 0.05 mol/l phosphate buffer solution (pH 7.8),
- xanthine ($5 \times 10^{-5}$ mol/l),
- xanthine oxidase (XO) (10 U/l), INT ($9.8 \times 10^{-5}$ mol/l),
- superoxide dismutase (SOD) ($1.33 \times 10^{-7}$ g/l),
- Cu(II) complex (N-salicylidene-L-glutamato)(2-methylimidazole)copper(II) complex ($4 \times 10^{-7}$ g/l)

Accessories and equipment
- spectrophotometer
Experimental procedure

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Test tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Buffer solution, pH 7.8 (ml)</td>
<td>1.2</td>
</tr>
<tr>
<td>Xanthine (ml)</td>
<td>0.1</td>
</tr>
<tr>
<td>SOD (ml)</td>
<td>-</td>
</tr>
<tr>
<td>Cu(II) complex (ml)</td>
<td>-</td>
</tr>
<tr>
<td>INT (ml)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Start the reaction by adding of xanthine oxidase in 20s intervals

| Xanthine oxidase (ml)         | 0.1| 0.1| 0.1| -  |

Incubation 10 minutes at room temperature. Measure absorbance at 510nm in 20s intervals, using buffer solution (pH = 7.8) (tube 4) as a reference

<table>
<thead>
<tr>
<th>A&lt;sub&gt;510&lt;/sub&gt;</th>
<th></th>
<th></th>
<th></th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of INT reduction</td>
<td>100</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>% of inhibition of INT reduction</td>
<td>0</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Units of dismutase activity (U)</td>
<td>0</td>
<td></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

Evaluation

a) Inhibition of INT reduction by the formed superoxide (in %) is calculated from measured absorbances:

\[
\text{INT reduction by superoxide (A}_1\text{)} \times 100 \%
\]

\[
\text{INT reduction by superoxide in the presence of SOD (or Cu(II) complex) (A}_2 \text{ or A}_3\text{)} \times x \%
\]

b) Calculate the % of inhibition of INT reduction \[I \text{ [\%]} = 100 - x\]

c) Calculate units of dismutase activity, if one unit of SOD activity (U) is defined as the ability to inhibit INT reduction by 50%.

d) Comparison of calculated dismutase activities (units of SOD activity) of SOD and Cu(II) complex.

Calculations and conclusion:
Name, group No:  
Date:  

Principle
Solution with concentration expressed as the ratio of the amount of substance concentration to the total volume of the solution is being prepared. The following formula is used:

\[ c = \frac{n}{V} \quad n = \frac{m}{M_r} \quad m = c \cdot V \cdot M_r \]

The solution will be prepared in the 250 ml volumetric flask.

Reagents
NaCl p.a. (Mr = 58)

Accessories and equipment
volumetric flask (250 ml),  
funnel,  
watch glass,  
scale

Experimental procedure
The mass of NaCl necessary to prepare the solution is calculated and the amount is weighed on the scale. This amount is transferred through the funnel into the volumetric flask and the watch glass is carefully washed with the distilled water into the volumetric flask. NaCl is dissolved in distilled water and the flask is filled with water to the graduation mark. The flask is equipped with a label stating composition and concentration of the compound and the date of preparation.

Calculations and conclusion:
PREPARATION OF 100 ml OF 75 AND 100 mmol.l⁻¹ NaCl SOLUTIONS
BY DILUTION OF 150 mmol.l⁻¹ NaCl SOLUTION

Principle

If the composition of solution is given as amount-of-substance concentration, the following balance equation holds for calculation of changes in solution composition by addition of a solvent:

\[ c_1 \cdot V_1 = c_2 \cdot V_2 \]

The equation is derived from balance of the amount of substance:

\[ n_1 = c_1 \cdot V_1 \]
\[ n_2 = c_2 \cdot V_2 \]

From the definition of amount of substance concentration it follows:

\[ c_1 \cdot V_1 = c_2 \cdot V_2 \]

*E. g.*, to prepare 100 ml of a 75 mmol.l⁻¹ solution NaCl by diluting physiological saline (150 mmol.l⁻¹) it holds that:

\[ c_1 = 150 \text{ mmol.l}^{-1} \]
\[ c_2 = 75 \text{ mmol.l}^{-1} \]
\[ V_1 = ? \text{ ml} \]
\[ V_2 = 100 \text{ ml} \]

\[ V_1 = \frac{c_2 \cdot V_2}{c_1} = \frac{75 \cdot 100}{150} = 50 \text{ ml} \]

In calculation of the amount of 150 mmol/l NaCl required to prepare 100 ml 100 mmol/l NaCl is proceed in the same way.

Reagents and accessories

NaCl solution, \( c = 0.15 \text{ mol.l}^{-1} \),
a set of 100 ml volumetric flasks,
pipettes,
graduated cylinder,
funnel,
wash bottle
Experimental procedure

Calculated volumes of physiological NaCl solution necessary for preparation of the diluted solutions are measured with a graduated cylinder, transmitted into 100 ml volumetric flasks, the flasks are filled with distilled water to the mark, closed with a stopper and well mixed. Flasks are identified with a label of composition and concentration of the compound, and date of preparation.

Calculations and conclusion
Comenius University, Bratislava, Faculty of Medicine  
Institute of Medical Chemistry, Biochemistry and Clinical Biochemistry

SOLUTIONS

PRACTICAL EXERCISE No5

EXAMINATION OF HYPOTONIC HEMOLYSIS (OSMOTIC RESISTANCE) OF ERYTHROCYTES

Name, group No:  
Date:

Principle

In hypotonic environment, erythrocytes undergo hemolysis. Osmotic resistance (or, alternatively, osmotic fragility) is examined by monitoring of erythrocytes hemolysis in hypotonic environment. The maximal osmotic resistance is determined at the concentration of NaCl, where the beginning of hemolysis is observed (staining of the supernatant over erythrocyte sediment is slightly pink or clear due to the small amount of released hemoglobin). Minimal osmotic resistance is measured at that concentration of NaCl, where hemolysis is maximal (erythrocytes are completely hemolyzed, the solution has red color and no sediment is observed at the bottom of the test tube, similarly to the control tube with distilled water).

Examination of osmotic resistance (fragility) is of diagnostic value. It is used for diagnosis as well as for differentiation of hemolytic disease.

In clinical practice, as well as in research, osmotic fragility is the more frequently used term. Not to be confused of these two terms, one has to recognize that maximal osmotic resistance indicates minimal osmotic fragility, and minimal osmotic resistance indicates maximal osmotic fragility. As it turns out, the more osmotically resistant (more stable against hemolysis) the erythrocyte is, the smaller its osmotic fragility (sensitivity to hemolysis) is.

Reagents and accessories

Suspension of washed erythrocytes in an isotonic NaCl solution (20%, v/v), NaCl solutions with concentrations 0.075, 0.1 and 0.15 mol.l\(^{-1}\) prepared in previous practical, test tubes of the same size, glass and automatic pipettes, aluminium foil, centrifuge, spectrophotometer

Experimental procedure

20% suspension of erythrocytes preparation: Erythrocytes are isolated from blood by centrifugation (200rpm for 10 min). After sucking off the plasma erythrocytes are three times washed with isotonic NaCl solution and subsequently centrifuged at the same conditions. Sediment with erythrocytes is resuspended at five times their volume of physiological solution.

Osmotic fragility examination: Prepared NaCl solutions in different concentrations are pipetted into a set of centrifuge tubes, according to the table:
Test tube No | 1 | 2 | 3 | 4
---|---|---|---|---
erthrocyte suspension (ml) | 0.1 | 0.1 | 0.1 | 0.1
0.15 mol/l NaCl (ml) | 5 | - | - | -
0.1 mol/l NaCl (ml) | - | 5 | - | -
0.075 mol/l NaCl (ml) | - | - | 5 | -
distilled water (ml) | - | - | - | 5

1. incubation 10 min/ 37°C
2. centrifugation 5 min/ 2500 r.p.m.
3. measurement of $A_{540}$ in supernatant against water

<table>
<thead>
<tr>
<th>$A_{540}$</th>
</tr>
</thead>
</table>
| % of hemolysis | 100

The mixtures in tubes are carefully mixed (cover the test tubes with aluminium foils and turn them upside down), incubated in a water bath at 37 °C for 15 minutes. After centrifugation (10 min at 2000 rpm) supernatant is transferred into a cuvette and absorbance is measured against water at 540 nm. The values are recorded into the table.

**Evaluation**

After centrifugation we observe that the volume of sediment (erythrocytes) in test tubes is inversely proportional to NaCl concentration. In the test tubes No. 2, 3, 4 (hypotonic medium) hemolysis is observed (supernatant is red colored due to hemoglobin presence). The solution in the test tube with physiological NaCl solution is isotonic with the inner environment of erythrocytes and hemolysis is not observed (clear supernatant).

Hemolysis is evaluated quantitatively by calculating percentage of hemolysis at different NaCl concentrations. The $A_{540}$ value in the tube with water (tube No. 4) is taken as 100 %, because in water hemolysis is complete. The bar graph is constructed.
Conclusion:
Compare behavior of erythrocytes (percentage of hemolysis) in solutions of NaCl with different concentrations.

Figure: Illustration of the bar graph of evaluation of hemolysis at different NaCl concentrations
ACIDS AND BASES. pH. BUFFERS

PRACTICAL EXERCISE No6

DETERMINATION OF BODY AND NATURAL FLUIDS pH

Name, group No: Date:

Principle

pH determination of clear body and natural fluids is technically simple. In routine practice it is sufficient to determine pH with a universal indicator strip or Phan strip. A universal indicator is a pH indicator composed of a solution of several compounds that exhibits several smooth color changes over a pH value range from 1 to 14 to indicate the acidity or alkalinity of solutions.

Reagents

Different solutions of body and natural fluids (lemon juice, vinegar, tap water, milk, saliva and distilled water)

Accessories and equipment

beakers, universal indicator Phan strips

Evaluation

Indicator strip is immersed into the examined liquid until color development is complete and taken out. The developed color is compared to the sequence chart on the package, and pH is read. Measured values are recorded into the table and compared with tabulated data.

<table>
<thead>
<tr>
<th>Liquid</th>
<th>pH (expected)</th>
<th>Measured pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lemon juice</td>
<td>2.2 – 2.4</td>
<td></td>
</tr>
<tr>
<td>Vinegar</td>
<td>2.6 – 2.7</td>
<td></td>
</tr>
<tr>
<td>Tap water</td>
<td>5.5 – 6.0</td>
<td></td>
</tr>
<tr>
<td>Fresh milk</td>
<td>6.3 – 6.6</td>
<td></td>
</tr>
<tr>
<td>Saliva</td>
<td>6.5 – 7.0</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>7.0</td>
<td></td>
</tr>
</tbody>
</table>
ACIDS AND BASES, pH. BUFFERS

PRACTICAL EXERCISE No7

DETERMINATION OF ACIDITY CONSTANTS OF WEAK MONOBASIC ACIDS BY TITRATION

Name, group No: Date:

Principle
Solution of a hydroxide (NaOH, KOH) is gradually added into a known amount of acid, and pH is measured throughout. Dependence of pH on the amount of added hydroxide solution is displayed graphically. After adding 0.5 of the hydroxide per 1 equivalent of the acid, concentrations of free acid and its conjugate base will be equal and from the equation it follows that pH of this solution will be equal to the pKₐ value of the acid. The value of pKₐ is determined from the graph, and the value of acidity constant of a weak acid, Kₐ, is calculated.

Reagents
solution of weak acid CH₃COOH (c = 0.1mol/l, K = 1.8.10⁻⁵) and strong base KOH (c = 0.1mol/l)

Accessories and equipment
burette,
pipette,
beakers,
pH meter,
electromagnetic stirrer

Experimental procedure
20 ml of acetic acid is added to the titration beaker and is filled to 70 ml by water. Stirring the content of the beaker 0.5 ml of potassium hydroxide is added and changed pH value is marked to the table.

<table>
<thead>
<tr>
<th>VₖOH (ml)</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VₖOH (ml)</td>
<td>18</td>
<td>18,5</td>
<td>19</td>
<td>19,5</td>
<td>20</td>
<td>20,5</td>
<td>21</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Evaluation
a) Measured values are plotted as a dependence of pH changes on the amount of added KOH solution. The slope of the curve changed at the midpoint of the titration. At this
point \([A^-] = [HA]\) and the pH values is equal to \(pK_a\) (\(pH = pK_a\)). At the equivalence point all the weak acid molecules HA have been converted to the conjugate base \(A^-\).

b) Calculate \(pK_a\) and \(K_a\) values for the given weak acid and compare to published data.

*Figure: Illustration, how to read the \(K_a\) value from constructed graph*

*Calculations and conclusion*
ORGANIC CHEMISTRY

PRACTICAL EXERCISE No8

DETERMINATION OF UREA CONCENTRATION IN SERUM AND URINE

Name, group No: ___________________________ Date: ___________________________

Principle
In strongly acidic environment, urea forms in the presence of thiosemicarbazide and Fe$^{3+}$ ions a red-coloured complex with diacetyl monoxime that is suitable for spectrophotometric detection.

Reagents
standard solution of urea ($c = 20$ mmol.l$^{-1}$),
reagent solution (contains 5.0 mmol.l$^{-1}$ diacetyl monoxime, 0.9 mmol.l$^{-1}$ thiosemicarbazide, 0.9 mmol.l$^{-1}$ H$_2$SO$_4$, 25 μmol.l$^{-1}$ Fe$^{3+}$)

Accessories and equipment
beakers,
boiling water bath,
spectrophotometer

Experimental procedure

<table>
<thead>
<tr>
<th>Test tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard solution of urea (ml)</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Serum (ml)</td>
<td>-</td>
<td>0.01</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urine – 100x diluted (ml)</td>
<td>-</td>
<td>-</td>
<td>0.01</td>
<td>-</td>
</tr>
<tr>
<td>H$_2$O (ml)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.01</td>
</tr>
<tr>
<td>Reagent (ml)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Stir, heat 10 minutes in boiling water bath, then cool down and measure $A_{525}$ against reference solution (tube 4)

$A_{525}$ |

$C$ (urea in samples (mmol.l$^{-1}$)) | 20 | - |
Evaluation:

1. Determination of urea concentrations in serum:

\[ c_{\text{serum}} = \left( \frac{A_{\text{serum}}}{A_{\text{st}}} \right) \times 20 \] (mmol/l)

2. Determination of urea concentrations in urine:

\[ c_{\text{urine}} = \left( \frac{A_{\text{urine}}}{A_{\text{st}}} \right) \times 20 \times 100 \] (mmol/l)

3. Determination of amount of urea excreted in urine per 24 hours:

\[ n_{\text{urea}} = c_{\text{urine}} \times \text{diuresis} \quad \text{(mmol/24 hours)} \quad \text{(diuresis = volume of daily urine)} \]

4. Compare the calculated concentrations with physiological values:
   
   \[ c \text{ (serum)} = 2.5 - 8.3 \text{ mmol/l} \]
   
   \[ c \text{ (urine)} = 320 - 568 \text{ mmol/24 hours} \]

Conclusion

Comparison of calculated values with physiological ranges.
ORGANIC CHEMISTRY

PRACTICAL EXERCISE No 9

DETECTION OF KETONE BODIES IN URINE

Name, group No: ___________________________ Date: ___________________________

Principle

Acetone and acetoacetic acid give a sensitive reaction with sodium nitroprusside \( \text{Na}_2[\text{Fe(CN)}_5\text{NO}].2\text{H}_2\text{O} \) in alkaline environment, in the course of which a coloured product is formed (Legal’s and Lestradet’s test)

Legal’s test

Reagents

sodium nitroprusside (\( c = 0.2 \text{ mol.l}^{-1} \))
NaOH (\( w = 10 \% \)),
CH\(_3\)COOH (\( w = 98 \% \)),
fresh urine,
acetone as positive control

Experimental procedure

Several drops of fresh sodium nitroprusside are added to a 2 ml urine sample. The solution is alkalized with several drops of NaOH. 1 ml of concentrated acetic acid is added to the red-coloured reaction product to eliminate false positive reaction with creatinine.

Evaluation

Formation of red-coloured complex is a proof of ketone bodies in the sample. Creatinine, which is a normal component of urine, gives a positive nitroprusside reaction as well. Reaction of creatinine can be discerned from that of ketone bodies by addition of acetic acid into the reaction mixture. Violet colour after acidification with acetic acid is a proof of ketonuria, decolorization of the red solution points to reaction of creatinine. Acetone and acetoacetic acid can be detected by the nitroprusside reaction, while \( \beta \)-hydroxybutyric acid does not give a positive reaction and usually is not determined in urine.

Conclusion
SACCHARIDES

PRACTICAL EXERCISE No10

DETECTION OF GLUCOSE IN URINE

Name, group No: ____________________________ Date: ____________________________

**Principle**

Detection of glucose in urine is based on its reducing properties. Fehling’s or Benedict's tests can be made as preliminary tests. These reactions are not specific for glucose and are positive with other reducing saccharides as well, thus, in case of a positive reaction a test specific for glucose has to be performed with the detection strip *Glucophan*, which is based on action of a specific enzyme *glucose oxidase*.

**a) Fehling’s test**

**Reagents and equipment**

Fehling’s I and Fehling’s II reagents,
fresh urine,
glass pipettes,
boiling water bath

**Experimental procedure**

2 ml of Fehling's reagent (Fehling I and Fehling II in a 1:1 ratio) is prepared in a test tube. An equal volume of examined urine is added and the reaction mixture is heated for 2 minutes in the boiling water bath. Reaction is positive, if an orange-red (ginger) precipitate of cuprous oxide forms. Sensitivity is about 10 mmol.l⁻¹. Reducing tests have to be performed in a fresh, non-turbid urine. In case of present proteins, urine has to be deproteinated before the test.

**b) Detection and semiquantitative determination of glucose with diagnostic strips "GLUCOPHAN"**

**Principle**

Diagnostic strips serve for fast and specific detection as well as for semiquantitative determination of glucose in urine. Their indicating zone contains the enzymes *glucose oxidase* and *peroxidase* together with a special chromogenic system that is oxidized in the presence of glucose to a red-colored product. This enzymatic reaction is specific only for glucose.

**Reagents and equipment**

Fresh urine,
diagnostic strips Glucophan (stored in a well closed bottle, in a dry and cool place)
Experimental procedure

The strip is immersed into the examined urine and immediately taken out. After three minutes the test is evaluated by comparing intensity of the color of indicating zone that has been formed with the scale on the case. Color frames on the scale correspond approximately to concentrations of glucose given in the table:

<table>
<thead>
<tr>
<th></th>
<th>Urine 1</th>
<th>Urine 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fehling test</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose concentration (mmol/l)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Principle

Glucose is catalytically oxidized by air oxygen by effect of the enzyme glucose oxidase, giving hydrogen peroxide and gluconic acid as products. Hydrogen peroxide formed in this process is determined by oxidative copulation with a substituted phenol and 4-aminophenazone, reaction is catalyzed by peroxidase. Hydrogen peroxide, with the help of the peroxidase, oxidizes a suitable hydrogen donor - 3-methylphenol, which then copulates with 4-aminophenazone into a coloured product. The amount of produced color is proportional to the amount of glucose. The method is used to determine glucose in biological material - in blood, serum, urine, and it is also suitable for microanalysis.
**Reagents**

Bio-La-Test kit - reagent for glucose determination contains: phosphate buffer solution (0.14 mol.l⁻¹), glucose oxidase (160 μmo.l⁻¹), peroxidase (16 μcat.l⁻¹), solution of 3- methylphenol (0.01 mol.l⁻¹), 4-aminophenazone solution (0.001 mol.l⁻¹), standard solution of glucose (0.01 mol.l⁻¹)

**Experimental procedure**

During glucose determination in blood or hemolytic serum, biological material has to be deproteinaited using trichloroacetic acid. 0.5 ml of a deproteinizing solution is added to 0.05 ml of the sample. Formed precipitate is centrifuged (5 min at 3000 rpm). The supernatant is pipetted for determination of glucose, and the procedure given in the table is used:

<table>
<thead>
<tr>
<th>Test tube</th>
<th>Sample</th>
<th>Standard solution</th>
<th>Reference solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (ml)</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard solution (ml)</td>
<td>-</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
</tr>
<tr>
<td>Reagent (ml)</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Incubate 30 minutes at room temperature or 20 minutes in the dark (the incubation mixture is protected from direct light)

| A₄₉₈               |        | -                 |

**Evaluation**

Blood glucose level is hormonally kept in a constant range. It is changed at pathologic conditions, thus estimation of glucose level - glycaemia - is of diagnostic value (in diabetes). Calculate the concentration of glucose according to the formula:

\[
c = \frac{A_{sam}}{A_{st}} \times 10 \left( \frac{mmol}{l} \right)
\]

Compare the calculated concentration of glucose in serum with physiological values

*serum* - 3.9-6.1 mmol.l⁻¹
*whole blood* - 3.3 - 5.6 mmol.l⁻¹

**Calculations and conclusion**
LIPIDS

PRACTICAL EXERCISE No12

DETERMINATION OF CONCENTRATION OF TOTAL SERUM LIPIDS

Name, group No: ___________________________ Date: ___________________________

Principle
After hydrolysis with concentrated sulfuric acid blood serum lipids (including non-esterified fatty acids) react with vanillin and phosphoric acid, forming a red-colored product. Its intensity is proportional to the amount of total lipids in serum.

Reagents
Bio – La Test kit (vanillin, c = 10 mmol.l⁻¹, phosphoric acid, c = 11.5 mol.l⁻¹), H₂SO₄, concentrated serum, standard solution of total lipids, c = 8 g.l⁻¹

Accessories and equipment
water bath, spectrophotometer, burner

Experimental procedure
Determination of total serum lipids concentration is performed according to the table:

<table>
<thead>
<tr>
<th>Test tube</th>
<th>Serum sample</th>
<th>Standard solution</th>
<th>Reference solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (ml)</td>
<td>0.02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard lipid solution (ml)</td>
<td>-</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td>H₂SO₄ (ml)</td>
<td>1.5</td>
<td>1.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Mixture is stirred and heated in thin – walled tubes for 10 minutes in a boiling water bath. The tube contents are cooled down under a stream of cold water and from the hydrolysate (in the case of reference – solution of H₂SO₄) following amounts are pipetted into dry test tubes

<table>
<thead>
<tr>
<th>Hydrolysate (ml)</th>
<th>0.10</th>
<th>0.10</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂SO₄ (ml)</td>
<td>-</td>
<td>-</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent (ml)</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
</tbody>
</table>
The tube contents are stirred, let stand for 10 – 15 min at room temperature and absorbances of the sample and standard are measured at 530 nm against the reference solution.

<table>
<thead>
<tr>
<th>$A_{530}$</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipids (g.l$^{-1}$)</td>
<td>8</td>
</tr>
</tbody>
</table>

**Evaluation**

Concentration of total serum lipids is calculated from sample ($A_{sam}$) and standard ($A_{st}$) absorbances according to the formula:

$$c_{sam} = \frac{A_{sam}}{A_{st}} \times 8 \text{ (g/l)}$$

Compare the calculated concentration of total lipids with physiological values.

*fs total lipids: 4 – 8 g/l*

(*f = fasted, s = serum*)

**Calculations and conclusion**
PRACTICAL EXERCISE No13

DETERMINATION OF MALONDIALDEHYDE CONCENTRATION IN BLOOD SERUM

Name, group No: ___________________________ Date: ___________________________

**Principle**

Lipoperoxides present in serum are hydrolyzed in diluted phosphoric acid. Malondialdehyde (MDA), one of the end products of lipid peroxidation, reacts with thiobarbituric acid (TBA) and forms a pink to red product, suitable for spectrophotometric determination at 535 nm. Tetraethoxypropane (TEP), which liberates a stoechiometric amount of MDA after hydrolysis, is used as a standard.

**Reagents**

- H\(_3\)PO\(_4\), c = 0.44 mol.l\(^{-1}\)
- Thiobarbituric acid (TBA), c = 42 mmol.l\(^{-1}\)
- 1, 1, 3, 3 – tertraethoxypropane (TEP) standard solution, c = 100 µmol.l\(^{-1}\)

**Accessories and equipment**

- water bath,
- spectrophotometer

**Experimental procedure**

Determination of malondialdehyde concentration in serum is performed according to the table:

<table>
<thead>
<tr>
<th>Test tube</th>
<th>Serum sample</th>
<th>Control serum</th>
<th>Standard solution</th>
<th>Reference solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (ml)</td>
<td>0.10</td>
<td>0.10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard solution (ml)</td>
<td>-</td>
<td>-</td>
<td>0.10</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water (ml)</td>
<td>0.40</td>
<td>0.40</td>
<td>0.40</td>
<td>0.50</td>
</tr>
<tr>
<td>H(_3)PO(_4) (ml)</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>TBA (ml)</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Tubes covered with aluminium foil are heated for 20 min in a boiling water bath. The tube contents are cooled down under a stream of cold water and absorbances of the sample, control and standard are measured at 535 nm against the reference solution.

\[ A_{535} \]

**Concentration of MDA (µmol. l\(^{-1}\))**

- 100

**Evaluation**
Concentration of malondialdehyde in the blood serum of the patient and control is calculated according to the formulae:

\[ c_{\text{sam}} = \frac{A_{\text{sam}}}{A_{\text{st}}} \times 100 \ (\mu\text{mol/l}) \]

\[ c_{\text{control}} = \frac{A_{\text{control}}}{A_{\text{st}}} \times 100 \ (\mu\text{mol/l}) \]

Comparison of MDA concentrations in sample of patient to control sample (healthy person).

*Calculations and conclusion*
AMINOACIDS, PROTEINS

PRACTICAL EXERCISE No14

THIN – LAYER chromatography SEPARATION OF AMINO ACIDS

Name, group No: Date:

Principle

The principle of TLC (thin – layer chromatography) method is distribution of the components of the sample between the mobile and the solid phase (stationary) of the thin layer based on the different adsorption forces of the components of the sample. The stationary phase is formed by a thin layer of fine adsorbent which is either intersperse or, preferably, fixed on a base (aluminum foil, a glass plate). The mobile phase is formed by a mixture of organic solvents. The components of the sample are separated according to their different affinities to the adsorbent. Separation of compounds is based on the competition of the solute and the mobile phase for binding places on the stationary phase. E.g., if as the stationary phase the polar one is used and we separate two compounds that differ in polarity, the more polar compound has a stronger interaction with stationary phase and the less polar compound moves higher up the plate.

Detection of separated components is carried out by spraying an appropriate visualizing agent (ninhydrine), which gives a positive reaction with free alpha – amino acids. Each component is characterized by their retention factor.

\[
\begin{align*}
\text{ninyhydrine} & \quad \text{aminoacid} \\
\text{purple product}
\end{align*}
\]

Reagents

solutions of amino acids: leucine, lysine, glycine, glutamate, their mixture and unknown samples of amino acids (w = 1%),
detection spray [solution of ninhydrine in acetone (w = 0,4)],developing system [n-butanol – acetic acid - water (4:1:5)]

Accessories and equipment

silufol plate,
chromatographic chamber

Procedure

Samples of the known amino acids (leucine, lysine, glycine, and glutamate), their mixture and samples of unknown amino acids are applied in the form of small spots (about 10-20 ml) on the thin layer silufol plate. The plate is dried out, placed and closed in a chamber saturated with vapors of a developing system. The separation proceeds until the front of the mobile phase...
reaches a distance of about 1-2 cm from the top of the plate. The plate is taken out, the front of the mobile phase is marked by the pencil and the plate is dried out in an oven at 100 °C. Detection of the samples is carried out by spraying with ninhydrine solution to form a purple complex which is detectable as color spots. Retrace the outline of the stains and mark the center of each spot.

Figure: Illustration of thin layer chromatography procedure and evaluation

**Evaluation**

From the position of the center of the spot of amino acid calculate the value of retention factor $R_f$ according to the formula

$$R_f = \frac{b}{a}$$

where $(b)$ is the distance of the center of the spot from the start (cm), and $(a)$ is the distance of the front of solvent from the start (cm).

Write the values of retention factors to table:

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>$R_f$ value</th>
<th>$R_f$ Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample No 1</td>
<td>Sample No 2</td>
</tr>
<tr>
<td>Leucine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The identity of $R_f$ of known amino acids (standards) with $R_f$ for the unknown samples determines the presence of the corresponding amino acids.

*Calculations and conclusion*
AMINO ACIDS, PROTEINS

PRACTICAL EXERCISE No 15

SEPARATION OF HEMOGLOBIN FROM POTASSIUM HEXACYANOFERRATE (III) BY GEL PERMEATION CHROMATOGRAPHY

Name, group No: Date:

Principle
The gel chromatography is based on the separation of compounds passing through the swollen gel according to their molecular size. In the glass column filled by Sephadex G 100 are separated compounds with a molecular weight of about 100 000. If the molecules passing the gel are larger than the inner pores of gel, particles cannot diffuse into the pores and remain in the interstitial fluid. They are drifted by it, and elute from the column as first. The pores retain molecules of a smaller size, their movement through the column is stunted, and thus elute later. The first elutes the hemoglobin followed by potassium hexacyanoferrate. The separation efficiency of both components is evaluated by the elution diagram obtained by measuring the absorbance of the eluates at 420 nm. Absorbance is also the measure of the concentration of substances in the eluent.

Reagents
Sephadex G 100 swollen in water,
solution of hemoglobin (w = 2%),
solution of $K_3[Fe(CN)_6]$ (w = 2%)

Accessories and equipment
glass chromatographic column,
test tubes,
spectrophotometer

Procedure
Wash the column filled with swollen Sephadex G 100 with the distilled water. Before the separation of a mixture slightly remove the distilled water from the surface of Sephadex. By the pipette gently apply a mixture of 0.2 ml of hemoglobin solution and potassium hexacyanoferrate (1:1) to the surface of the gel and let it soak into the column. Add small amount of water and prevent the swirl. Connect with the water tank to enable the water to flow continuously through the column. We observe the separation of a mixture into two components.

Attention! Column surface must always be under the level of water to prevent the crack of the gel!!!

The first elutes out of the column the hemoglobin (brown to red band) as the second potassium hexacyanoferrate (yellow band). The eluent of the compounds are taken up in 1.5 ml fractions into calibrated tubes (10-15 samples) until both compounds of the mixture will not flow out the column.
Wash the column with distilled water. Read out the absorbances of all fractions at 420 nm against distilled water. Write the values of elution volumes and corresponding absorbances to the table:

<table>
<thead>
<tr>
<th>V (ml)</th>
<th>A_{420}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>V (ml)</th>
<th>A_{420}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Evaluation:**

The tubes containing pure hemoglobin and those with K₃[Fe(CN)₆] are determined from the graph. The efficiency of separation is evaluated.

**Figure:** Illustration of construction of elution diagram of separation of hemoglobin from potassium hexacyanoferrate (III)

**Conclusion**
ENZYMES

PRACTICAL EXERCISE No16

EFFECT OF SUBSTRATE CONCENTRATION ON ENZYMATIC ACTIVITY. DETERMINATION OF MICHAELIS CONSTANT ($K_M$) OF THE LACTATE DEHYDROGENASE (LDH)

Name, group No:  
Date:

**Principle**

Lactate dehydrogenase is an enzyme that catalyzes the reaction of lactic acid oxidation utilizing NAD$^+$ as coenzyme:

\[ \text{H}_3\text{C}-\text{HC}^{-}\text{COOH} + \text{NAD}^+ \rightarrow \text{H}_3\text{C}-\text{C}^{-}\text{COOH} + \text{NADH} + \text{H}^+ \]

lactate  \hspace{1cm} \text{pyruvate}

At different substrate (lactate) concentrations, the enzymatic reaction proceeds at different rates. The rate is directly proportional to the amount of the created product (pyruvate). In an alkaline environment, pyruvate gives with 2,4-dinitrophenylhydrazine (DNPH) a brown–orange pyruvate hydrazone, which is suitable for spectrophotometric determination.

From the values of measured absorbance at 505 nm, the graphical dependence of enzymatic reaction rate on substrate concentration is constructed, and the value of Michaelis constant $K_M$ is determined either approximately according to Michaelis and Menten, or exactly according to Lineweaver and Burk.

**Reagents**

- Solution of 2,4-dinitrophenylhydrazine (DNPH) ($w = 0.02\%$),
- lactate solution, ($c = 3\, \text{mmol.L}^{-1}$),
- NaOH solution ($c = 0.1\, \text{mol.L}^{-1}$),
- Tris-HCl buffer solution ($c = 0.05\, \text{mol.L}^{-1}$) pH 8.5 – for dilution of lactate and for use in a reference solution.

**Accessories and equipment**

- glass and automatic pipettes,
- spectrophotometer,
- water bath

**Experimental procedure**

Different concentrations of lactate are prepared as follows:
Enzymatic reaction at the different lactate concentration will be set according to the table:

<table>
<thead>
<tr>
<th>Test tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate (ml)</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>LDH (ml)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Resulting substrate concentration</td>
<td>2</td>
<td>1.5</td>
<td>1.0</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Incubate for 5 min at room temperature

<table>
<thead>
<tr>
<th>Test tube No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNPH (ml)</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>0.5</td>
</tr>
</tbody>
</table>

Stir and let stand for 10 min at room temperature

<table>
<thead>
<tr>
<th>Test tube No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH (ml)</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>5</td>
</tr>
</tbody>
</table>

Stir and let stand for 5 min at room temperature. Measure absorbance at 505 nm against the reference solution (tube No 5)

\[ A_{505} \]

\*buffer solution, pH 8.5

**Evaluation**

Absorbance values are proportional to the amount of generated product of the enzymatic reaction –pyruvate; therefore reaction rate can be plotted directly as absorbance values. The results are recorded into the table:

<table>
<thead>
<tr>
<th>Test tube No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resulting concentration ([S]) in the reaction mixture (mmol/l)</td>
<td>2</td>
<td>1.5</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Reaction rate (\dot{v}) ((A_{505}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1/[S])</td>
<td>0.5</td>
<td>0.67</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>(1/\dot{v}) ((1/A))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The tabulated values are used to create a plot for determination of $K_M$ of LDH on a millimeter paper:

1. According to Michaelis-Menten for approximate $K_M$ determination:

   \[ v = f ([S]) \]

2. According to Lineweaver-Bürk for precise determination of $K_M$:

   \[ \frac{1}{v} = f \left( \frac{1}{[S]} \right) \]

From the values plotted in the value of $K_M$ is calculated:

\[ -\frac{1}{K_M} = -x \quad \text{(mmol/l)} \]

\[ K_M = \frac{1}{x} \quad \text{(mmol/l)} \]

**Conclusion:**

Compare the obtained $K_M$ values
ENZYMES

PRACTICAL EXERCISE No17

EFFECT OF ACTIVATORS AND INHIBITORS ON ARGINASE ACTIVITY

Name, group No: __________________________ Date: __________________________

Principle

Arginase catalyzes hydrolytic cleavage of arginine into urea and ornithine in the urea cycle:

\[
\begin{align*}
H_2C\text{-}NH\text{-}C\text{-}NH_2 & \quad \xrightarrow{\text{arginase}} \quad NH_2 \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{HCO}_2\text{-}NH_2 & \quad \text{COOH} \\
\text{arginine} & \quad + H_2O \\
\end{align*}
\]

The amount of formed urea is directly proportional to the activity of arginase. Urea concentration is determined by reaction with diacetyl monoxime in strongly acidic conditions in the presence of thiosemicarbazide and ferric ions. A red complex suitable for spectrophotometric determination at 525 nm is formed.

Urea cycle is a series of biochemical reactions, which remove the toxic ammonia released during the degradation of proteins. Mammalian arginase is active as a trimer localized in the cytoplasm of hepatocytes. Each subunit contains the active site with two Mn\(^{2+}\) ions. Activators of the enzyme are Mn\(^{2+}\), Mg\(^{2+}\), Ca\(^{2+}\), Ni\(^{2+}\), and Co\(^{2+}\) ions. In their absence the activity of the enzyme decreases. The competitive inhibitors of arginase include L-ornithine and L-lysine, competing with arginine for binding to the active site of the enzyme. The high concentration of the substrate suppresses the inhibitory effect of competitive inhibitors.

Reagents

BIO-LA-TEST set for urea concentration determination, TRIS-HCl buffer (c = 0.05 mol.l\(^{-1}\), pH 9.7), MnCl\(_2\) (activator) in buffer pH 9.7 (c = 0.1 mol.l\(^{-1}\)), L-lysine in water (inhibitor) (w = 1%), liver homogenate as a source of arginase (w = 5%), L-arginine in TRIS-HCl buffer (c = 0.05 mol.l\(^{-1}\), pH 9.7), urea standard solution (c = 8.3 mmol.l\(^{-1}\)), diluted H\(_2\)SO\(_4\) for working solution preparation (stock solution from the kit with H\(_2\)SO\(_4\) in 1:1 ratio), trichloroacetic acid (TCA) (w = 5%)

Accessories and equipment

water bath (37°C and 100°C), centrifuge, spectrophotometer
### Experimental procedure

Proceed according to the following table:

<table>
<thead>
<tr>
<th>Test tube No</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer solution, pH 9.7 (ml)</td>
<td>1.0</td>
<td>1.4</td>
<td>0.4</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td>Homogenate (arginase) (ml)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>MnCl₂ (activator) (ml)</td>
<td>0.4</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Inhibitor (L-lysine) (ml)</td>
<td>-</td>
<td>-</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Substrate (L-arginine) (ml)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard urea solution (ml)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
</tr>
</tbody>
</table>

Incubation at room temperature, 5 min

| TCA (ml) | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |

Centrifugation (3000 rpm), 5 min

| Supernatant (ml) | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Working solution (ml) | 2   | 2   | 2   | 2   | 2   |

Heat 10 min in a boiling water bath, cool down and measure absorbance at 525 nm against the reference solution (tube No. 5)

\[ A_{525} \]

### Evaluation

The activity of enzyme is represented by the rate of substrate conversion and unit of enzymatic activity *katal* is defined as activity of enzyme that converts 1 mol of substrate per 1 second. Since arginase converts 1 mol of arginine to 1 mole of urea (see the reaction equation), the activity can be calculated from the amount of the product - urea. Urea concentration is calculated from the measured absorbances and the known concentration of the standard solution of urea according to the equation:

\[ c_{\text{sam}} = \frac{A_{\text{sam}}}{A_{\text{St}}} \times 8.3 \]  

(mmol/l of homogenate)

Calculated concentration corresponds to the concentration of converted substrate - arginine (mmol/l of homogenate). From the quantity of arginine converted per 5 minutes the amount of converted arginine per 1 second and the activity in kat/l of homogenate are calculated. From the activity of arginase in a 5% liver homogenate the specific activity (kat/g tissue) is calculated.
The effect of activators and inhibitors on arginase activity is evaluated by calculation of the activity and inhibition of the enzyme. The value of the absorbance of the enzyme without inhibitor and with activator (tube # 1) is considered as the 100% enzyme activity. The activity is proportional to the amount of converted substrate. Inhibition (%) can be calculated directly from the activity of enzyme as follows:

\[ I (\%) = 100 - \text{activity (\%)} \]

Results of calculations are recorded in the table:

<table>
<thead>
<tr>
<th>Test tube No</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>E + MnCl₂</td>
<td>E - MnCl₂</td>
<td>E + I</td>
</tr>
<tr>
<td>A₅₂₅</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activity (%)</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c (mol/l of homogenate/10 min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c (mol/l of homogenate/1s = katal/l of homogenate= katal/50g of tissue)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>katal/g of tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Conclusion**

Explain the effect of used activator and inhibitor on activity of arginase.