Date ..... Name ..... Group .....

Instructions for the practical lesson on biochemistry

Topic: **Hemoglobin and its derivatives, iron**

Task 1: Estimation of hemoglobin in blood

Reagents:

1. **Cyanide reagent**  :

Potassium ferricyanide	0.20 g
Potassium cyanide	0.05 g
Potassium dihydrogen phosphate	0.14 g
Water	to 1000 ml

2. **Sample of heparinized blood** – unknown sample (**Infectious material**)

Procedure:
Measure 5.00 ml of the cyanide reagent from the dispenser into a test tube. Mix the vial with blood by brief vortexing or turning upside down. Measure 0.02 ml of the blood sample, add it to the cyanide reagent, wash (pipette in and out) the tip three times with the reagent, and mix contents of the tube. Allow to stand 10 minutes, and measure absorbance of the mixture at 540 nm using the cyanide reagent as the blank. The colored product is stable at least 24 hours.

Calculation is based on molar absorption coefficient:

$$\text{Hemoglobin (g/l)} = \frac{A \times Mr \times \text{Dilution of sample}}{\varepsilon \times \text{Cuvette thickness}}$$

A: absorbance of the sample
Mr: 1.61×10^4 (relative molecular weight of Hb per atom of Fe)
Dilution of sample: total volume of mixture / volume of sample (5.02 / 0.02)
ε: 1.1×10^3 l mol^{-1} mm^{-1} (molar absorption coefficient of cyanmethemoglobin at 540 nm)
Cuvette thickness: 10 mm

$$\text{Hemoglobin (g/l)} = \frac{A \times 1.61 \times 10^4 \times \text{Dilution of sample}}{1.1 \times 10^3 \times 10}$$

Task 2: Demonstration of blood and blood pigment in urine

Reagents:

1. **o-tolidine** (3,3'-dimethylbenzidine) 
2. **Heitz-Boyer's reagent**: colorless reduced phenolphthalein in alkaline medium, stored with several granules of zinc 
3. **Acetic acid** concentrated 
4. **Hydrogen peroxide** 30 g/l 
5. **Ethanol** 
6. **Diagnostic Strips** HemoPHAN or other multifunctional strips
7. **Urine samples**: Urine with blood (**Infectious material**)
   Normal urine (**Infectious material**)

Procedure:

Perform the following three tests with the urine with blood as well as with the normal urine.

In order to verify that hemoglobin, rather than a true peroxidase activity is detected, try the 'benzidine' test also with a portion of urine with blood that has been briefly boiled and cooled down. The pseudoperoxidase activity of hemoglobin is resistant to boiling, whereas a true peroxidase (e.g., from white blood cells) would be inactivated by thermal denaturation.

'Benzidine' Test:
Dissolve a few grains of o-tolidine in about 2 ml of ethanol and acidify with concentrated acetic acid (few drops). Add about 2 ml of hydrogen peroxide (the solution must not turn blue at this stage) and combine with 1-2 ml of the urine sample. If the solution turns blue the test is positive.

Heitz-Boyer's Test:
In a test tube combine about 1 ml of urine with equal volume of the Heitz-Boyer reagent. Carefully overlay with hydrogen peroxide: in the presence of hemoglobin a red-violet ring appears at the interface.

Diagnostic Strips:
Dip the test strip into urine sample, wipe any excess of urine from the strip and after 60 seconds assess color of the strip reaction zone by comparing with the color scales on the strip container. Notice that the strip tube bears a dotted scale for detection of intact red blood cells in urine, and also another homogenous scale for detection of free hemoglobin – decide which one seems more applicable to your case.

Task 3: Spectrophotometric examination of hemoglobin derivatives

Reagents:

1. Potassium ferricyanide $K_3[Fe(CN)_6]$
2. Sodium dithionite $Na_2S_2O_4$ 
3. Undiluted heparinized blood (**Infectious material**)
4. Undiluted heparinized blood saturated with carbon monoxide (**Infectious material**)

Procedure:

Using the diode array spectrophotometer, record the absorption spectra of the following hemoglobin derivatives in the **wavelength range 500 – 600 nm**. Distilled water serves as a blank.

Oxyhemoglobin (O_2-Hb)

It prevails in the blood samples standing in the air. Add 0.02 ml of the blood to 4.0 ml of distilled water, mix, and record the spectrum. If the absorbances are still too high, the sample can be diluted further.

Deoxyhemoglobin (Hb)

Prepare by reducing the oxyhemoglobin solution (diluted as above) with a small amount (tip of spoon) of sodium dithionite; after 2-5 minutes record the spectrum. Compare with the values for O_2-Hb. Notice also any difference in color of the solution.
N.B.: Only a really small amount of dithionite must be added, otherwise the protein precipitates – in that case prepare a new mixture for the measurement. On the other hand, if you do not see the expected change, i.e. the two peaks of oxyhemoglobin replaced with a single peak of deoxyhemoglobin, record the spectrum again later and/or try to add a bit more dithionite.

Methemoglobin (hemiglobin)

To 4.0 ml of distilled water add 0.02 ml of blood and also a small amount (3-4 grains) of potassium ferricyanide as the oxidation reagent. After 5-10 minutes record the absorption spectrum and compare with the values for hemoglobin. Notice also change in color.
N.B.: Add only a small amount of ferricyanide. If the solution has become yellow and/or precipitated, too much was added and it must be repeated. But if you do not see the expected change in spectrum, it means a longer incubation and/or more ferricyanide is needed.

Carbonyl hemoglobin

To 4.0 ml of distilled water add 0.02 ml of blood saturated with CO. Evaluate the color and measure the spectrum. Check whether there is indeed the expected shift of the absorption maximum to 570 nm compared to oxyhemoglobin that has it at 578 nm. This shift is used for estimation of carbonyl hemoglobin in CO poisoning.

Evaluation:

In each recorded spectrum, find the absorbances for selected wavelengths as directed by the table in your lab report form. Fill these values to the table. Find also the absorption maxima and assess whether they match the theoretical expectations.

Use a piece of graph paper or Excel to draw the absorption spectra of the hemoglobin derivatives.

Task 4: Colorimetric estimation of iron in serum

Reagents:

(Commercial kit IRON - FERROZINE, manufactured by Biosystems, is used)

1. **Solution A:** Guanidine hydrochloride 1 mmol/l
   Acetate buffer 0.4 mol/l pH 4.0

2. **Solution B:** Ferrozine sodium salt 8 mmol/l
   Ascorbic acid 200 mmol/l

3. **Working solution: Solution A + Solution B 4:1**

4. **Iron Standard** (Fe^{2+} concentration **20 µmol/l**)
5. **Serum** – unknown sample (**Infectious material**)

Procedure:

N.B.: in order to avoid contamination of the sample and reagent by iron from glassware, for this assay it is necessary to use plastic test-tubes, not the glass ones! Likewise, special high quality deionized water and new plastic cuvettes must be used.

Measure in ml:	Serum sample (Tube No 1)	Standard (Tube No 2)	Blank 1 (Tube No 3)	Blank 2 (Tube No 4)
Serum	0.2	-	-	0.2
Standard	-	0.2	-	-
Deionized water	-	-	0.2	-
Solution A	-	-	-	1.0
Working solution	1.0	1.0	1.0	-

Allow to stand 5 minutes at ambient temperature, and measure absorbance of the sample, standard as well as blank 1 and blank 2 against distilled water in 1 cm cuvette at 560 nm.

Calculation:

$$\textbf{S-Iron (µmol/l)} = \frac{A_{sample} - (A_{blank\,1} + A_{blank\,2})}{A_{standard} - A_{blank\,1}} \times c_{standard}$$