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

Instructions for the practical lesson on biochemistry

Topic: **Lipids, lipoproteins, examination of lipid metabolism**

Task 1: Hydrolytic cleavage of fat with pancreatic lipase

Reagents:

1) Cow milk boiled and cooled to 37 °C
   (Boiling inactivates all enzymes in milk)

2) NaOH 0.02 mol/l
3) Extract of pancreatic lipase prepared from coated pills Pancreolan forte
   (30 pills of Pancreolan forte after removal of coating are dissolved in 600 ml of mixture glycerol-water in 1:1 ratio and filtered)

4) Solution of phenolphthalein in ethanol 2 g/l

5) Sodium deoxycholate 100 g/l

Procedure:

a) Pipette 30 ml of boiled milk to an Erlenmeyer flask. Add 3.5 ml of extract of pancreatic lipase and mix.

b) Prepare 4 test tubes, mark them 1, 2, 3, and 3D, and also one titration flask. Measure 5 ml of the milk-lipase mixture to each of the tubes and once more to the flask. Add 1 ml of sodium deoxycholate to the tube marked 3D and mix.

c) Place the four test tubes to thermo-block set to 37 °C and write down the time.

d) The mixture measured to titration flask will serve as a blank sample. Add few drops of phenolphthalein and immediately titrate the sample with NaOH 0.02 mol/l to the first permanent pink coloration (compare with the white color of the unused milk-lipase mixture in the Erlenmeyer flask). Record the consumption of NaOH and rinse the titration flask.

e) 20 minutes after putting the test tubes to thermo-block take out the tube No. 1, transfer its content to a titration flask and again titrate in the same way as in the step d). After another 20 minutes (i.e., 40 minutes from the beginning of incubation) titrate the content of the tube No. 2. In time 60 minutes perform two titrations: test tube No. 3 and also 3D.

Evaluation and conclusion:

Create a simple graph to show the course of hydrolysis of milk fat. Plot the time (min.) on the x axis and the consumption of NaOH 0.02 mol/l (ml) on the y axis.

Explain the increasing consumption of NaOH 0.02 mol/l during the incubation and consider whether your experiment has demonstrated a promoting effect of deoxycholate on fat digestion.

Task 2: Demonstration of unsaturated bonds in fatty acids

Reagents:
1) Palmitic acid (solid substance)
2) Oleic acid
3) Plant oil
4) $KMnO_4$  0.05 mol/l
5) Ethanol 

Procedure:

Prepare the reaction mixtures to tubes 1-4 according to the table:

Measure:		TEST TUBE 1	TEST TUBE 2	TEST TUBE 3	TEST TUBE 4
Palmitic acid		crumb	-	-	-
Oleic acid	ml	-	0.5	-	-
Plant oil	ml	-	-	0.5	-
Ethanol	ml	0.5	0.5	0.5	0.5
Mix contents of tubes.					
$KMnO_4$	drops	1-2	1-2	1-2	1-2
Mix contents of tubes.					

Evaluation and conclusion:

Summarize the observed color changes and explain them.

Dispose the contents of these tubes to the collection bottle for toxic waste.

Task 3: Estimation of malondialdehyde

Reagents:

1) Thiobarbituric acid reagent (2-thiobarbituric acid 29 mmol/l in 2.19 mol/l acetic acid)

2) Plant oil fresh
3) Plant oil expired

Procedure:

Prepare reaction mixtures to 3 long glass test tubes according to the table:

Measure:		TEST TUBE 1 (Fresh oil)	TEST TUBE 2 (Expired oil)	TEST TUBE 3 (Blank)
Deionized water	ml	2.0	2.0	2.0
Plant oil fresh		5 drops	-	-
Plant oil expired		-	5 drops	-
Thiobarbituric acid reagent	ml	1.0	1.0	1.0
Place the tubes to a water bath (beaker with water) and heat 20 – 30 minutes.				

Evaluation and conclusion:

Compare the color intensity in all the test tubes and try to explain the results.

Task 4: Estimation of serum concentration of total and HDL cholesterol

Reagents:

A commercial kit Bio-La-Test Oxochrom Cholesterol 2500 and HDL-Cholesterol – precipitation solution made by Erba-Lachema s.r.o.. is used for analysis.

1) Reagent solution [PIPES-buffer 50 mmol/l (pH 6.9), phenol 24 mmol/l, sodium cholate 0.5 mmol/l; 4-aminoantipyrin 0.5 mmol/l; cholesterol esterase ≥ 3.3 μkat/l; cholesterol oxidase ≥ 4.1 μkat/l; peroxidase ≥ 16.5 μkat/l] (all are the final concentrations in the reaction mixture)

2) Precipitating solution (phosphotungstic acid 0.56 mmol/l, $MgCl_2$ 30.0 mmol/l)
3) Standard cholesterol solution **7.0 mmol/l**
4) Serum for estimation of total cholesterol (infectious material)
5) Serum for estimation of HDL-cholesterol (infectious material)

Procedure:

a. Preparation of the serum sample for HDL determination:

Combine 0.1 ml of the serum sample for estimation of HDL cholesterol with 0.2 ml of the precipitation solution, mix and allow standing for 10 minutes at room temperature. Centrifuge for 10 minutes in the bench-top centrifuge. The supernatant must be clear.

b. Total cholesterol and HDL cholesterol will be assayed together. Prepare the reaction mixtures:

Measure in ml:	TEST TUBE 1 Total cholesterol	TEST TUBE 2 HDL cholesterol	TEST TUBE 3 Standard	TEST TUBE 4 Blank
Reagent solution	1.0	1.0	1.0	1.0
Serum	0.01	-	-	-
Supernatant	-	**0.10**	-	-
Standard	-	-	0.01	-
Distilled water	-	-	-	0.01

c. Mix and incubate the test tubes for 5 minutes at 37 °C (thermo block).

d. Within 20 minutes after the incubation measure the absorbances of the serum sample (tube 1), supernatant (tube 2) and standard (tube 3) against blank (tube 4) at 500 nm.

Calculation:

1. Concentration of total serum cholesterol:

$$\text{S-Total cholesterol (mmol/l)} = \frac{A_{sample}}{A_{standard}} \times c_{standard}$$

2. Concentration of HDL cholesterol:

$$\text{S-HDL-cholesterol (mmol/l)} = \frac{A_{supernatant} \times 3}{A_{standard} \times 10} \times c_{standard}$$

Task 5: Estimation of serum concentration of triacylglycerols

Reagents:

A commercial kit Bio-La-Test Triglycerols Liquid 250S made by Erba-Lachema s.r.o., is used for analysis (final concentrations in the reaction mixture are given).

1) Reagent solution [Good's buffer (pH 7.2) 49.5 mmol/l; 4-chlorophenol 3.96 mmol/l; adenosine-5-triphosphate (ATP) 1.98 mmol/l; 4-aminoantipyrine 0.495 mmol/l; Mg^{2+} 14.85 mmol/l; glycerol-3-phosphate oxidase $\geq$ 8.2 μkat/l; glycerol kinase $\geq$ 6.6 μkat/l; peroxidase 32.7 μkat/l; lipoprotein lipase 32.7 μkat/l]
2) Standard solution of triacylglycerols **2.3 mmol/l**
3) Serum – unknown sample

Procedure:

Prepare reaction mixtures to test tubes 1 – 3 according to the table:

Measure in ml:	TEST TUBE 1 Serum sample	TEST TUBE 2 Standard	TEST TUBE 3 Blank
Reagent solution	1.0	1.0	1.0
Serum	0.01	-	-
Standard	-	0.01	-
Distilled water	-	-	0.01

Mix and incubate 20 minutes at the ambient temperature (15–25° C). Within 60 minutes after the incubation measure the absorbances of serum sample and standard against the blank at 540 nm.

Calculation:

$$\text{S-Triacylglycerols (mmol/l)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times c_{\text{standard}}$$

Task 6: Calculation of LDL cholesterol, atherogenic index, and non-HDL cholesterol

$$\textbf{LDL cholesterol (mmol/l)} = \textbf{Total cholesterol (mmol/l)} - \textbf{HDL cholesterol (mmol/l)} - \frac{\textbf{Triacylglycerol (mmol/l)}}{\textbf{2.2}}$$

$$\textbf{Atherogenic index} = \frac{\textbf{Total cholesterol (mmol/l)}}{\textbf{HDL cholesterol (mmol/l)}}$$

Non-HDL cholesterol (mmol/l) = Total cholesterol (mmol/l) – HDL cholesterol (mmol/l)