Instructions for the practical lesson on biochemistry

Topic: **Amino acids – reactions and conversions. Nitrogen balance**

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1 Colored reactions of amino acids and proteins

These are qualitative reactions – use plastic droppers, not pipettes.

The tables below include both substances that are known to react in the given test (positive controls), and substances that do not react (negative controls).

In addition, reactivity of two proteins is compared: **egg albumin** and **gelatin**. The egg albumin contains all amino acids. On the other hand, gelatin (product of denaturation and partial hydrolysis of collagen) contains mostly glycine, proline, hydroxyproline and glutamic acid, whereas the content of aromatic amino acids phenylalanine and tyrosine is very low, and tryptophan as well as cysteine are absent.

1.1 Ninhydrin reaction

Reagents:

1. Tested samples: alanine 20 g/l, proline 20 g/l, solution of egg albumin, solution of gelatin
2. Ninhydrin 2 g/l in ethanol 

Procedure:

Prepare the following reaction mixtures in long test tubes:

	1 Alanine	**2** Proline	**3** Egg albumin	**4** Gelatin
Alanine	cca 0.5 ml	–	–	–
Proline	–	cca 0.5 ml	–	–
Egg albumin	–	–	cca 0.5 ml	–
Gelatin	–	–	–	cca 0.5 ml
Ninhydrin	several drops	several drops	several drops	several drops

Mix contents of the tubes, heat the tubes in water bath and record any color changes.

1.2 Xanthoproteic reaction

Reagents:

1. Tested samples: alanine 20 g/l, tyrosine 1 g/l, egg albumin, gelatin
2. Nitric acid concentrated 

Procedure:

Prepare the following reaction mixtures in long test tubes:

	1 Alanine	**2** Tyrosine	**3** Egg albumin	**4** Gelatin
Alanine	cca 0.5 ml	–	–	–
Tyrosine	–	cca 0.5 ml	–	–
Egg albumin	–	–	cca 0.5 ml	–
Gelatin	–	–	–	cca 0.5 ml
Nitric acid	cca 0.5 ml	cca 0.5 ml	cca 0.5 ml	cca 0.5 ml

Mix contents of the tubes and heat carefully only the test tube with egg albumin. Record any color changes.

1.3 Reaction of cysteine – proof of sulfur in protein molecules

Reagents:

1. Tested samples: alanine 20 g/l, cysteine 20 g/l, egg albumin, gelatin
2. Lead(II) acetate 50 g/l 
3. Sodium hydroxide 100 g/l (from the basic set) 

Procedure:

Prepare the following reaction mixtures in long test tubes:

	1 Alanine	**2** Cysteine	**3** Egg albumin	**4** Gelatin
Alanine	cca 0.5 ml	–	–	–
Cysteine	–	cca 0.5 ml	–	–
Egg albumin	–	–	cca 0.5 ml	–
Gelatin	–	–	–	cca 0.5 ml
Sodium hydroxide	cca 1 ml	cca 1 ml	cca 1 ml	cca 1 ml
Lead(II) acetate	2-3 drops	2-3 drops	2-3 drops	2-3 drops

Mix contents of the tubes and boil in water bath about 5 minutes. Record any color changes.

1.4 Biuret reaction

Reagents:

1. Urea (solid substance)
2. Tested samples: alanine 20 g/l, egg albumin, gelatin
3. Sodium hydroxide, 100 g/l (from the basic set) 
4. Copper sulfate 70 g/l (from the basic set) 

Procedure:

a. **Preparation of biuret:** Into one long glass test tube get about 0.1 g (one measure) of urea and carefully heat by keeping the bottom of the tube in direct contact with the hot plate of an electrical cooker (can be held in hand). The heated urea melts and changes to biuret. Stop heating once the melt hardens to white solid. Allow to cool and dissolve the biuret in about 1 ml of deionized water.

b. **Biuret reaction:**

Into 4 test tubes prepare the reaction mixtures according to the table:

	1 Biuret	**2** Alanine	**3** Egg albumin	**4** Gelatin
Biuret	cca 1 ml	–	–	–
Alanine	–	cca 1 ml	–	–
Egg albumin	–	–	cca 1 ml	–
Gelatin	–	–	–	cca 1 ml
Sodium hydroxide	cca 1 ml	cca 1 ml	cca 1 ml	cca 1 ml
Copper sulfate	1 drop	1 drop	1 drop	1 drop

Mix contents of the tubes and record any changes in color. Positive reaction manifests by violet coloration, better seen against a white background. Avoid excess of copper sulfate – it leads to a light blue precipitate of copper hydroxide that interferes with proper evaluation of the test.

2 Estimation of aspartate aminotransferase (AST) in serum

Reagents:

Commercial kit BioLaTest AST-UV L 500, made by ERBA-Lachema Diagnostika, is used.

1. **Working solution:** malate dehydrogenase ≥ 12.5 μkat/l, lactate dehydrogenase ≥ 66.6 μkat/l, Tris buffer (pH 7.8) 110.0 mmol/l, L-aspartate 340.0 mmol/l, pyridoxal-5'-phosphate 120.0 μmol/l.
2. **Starter:** 2-oxoglutarate 60.0 mmol/l, NADH 900.0 μmol/l
3. **Serum** – unknown sample

Procedure:

The starter solution, as well as the cuvette for the measurement, must be pre-warmed for at least 5 minutes at 37 ˚C.

Measure in ml directly to the cuvette:	
Working solution	1.000
Serum	0.100
Mix and pre-incubate for 5 – 10 minutes at 37 ˚C	
Starter	0.250
Mix and incubate for 1 minute at 37 ˚C	

During 1 minute incubation prepare photometer for measurement: set the wavelength to 340 nm, and zero absorbance using a cuvette filled with distilled water. After the 1 minute incubation place the cuvette with reaction mixture to the spectrophotometer and immediately read the absorbance. Then keep reading the absorbance in (exactly) one-minute intervals for 3 minutes.

Important!!!: once the starter is added, the reaction is running and the measurement must be really started immediately after the 1 minute incubation. In case of any unexpected delay do the experiment again from the beginning.

Calculation:

First calculate the average change of absorbance per minute (ΔA):

Time:		ΔA_{340}
0	A_0	
1 minute	A_1	$A_0 - A_1 \rightarrow \Delta A_1$ .....
2 minutes	A_2	$A_1 - A_2 \rightarrow \Delta A_2$ .....
3 minutes	A_3	$A_2 - A_3 \rightarrow \Delta A_3$ .....

$$\Delta A_{340} = \frac{\Delta A_1 + \Delta A_2 + \Delta A_3}{3}$$

Next, calculate catalytic AST concentration using the equation:

$$\text{S-AST (μkat/l)} = \frac{\Delta A_{340} \times 100 \times 1.35 \times 10^{-3} \times 10^{6}}{622 \times 1000 \times 0.1 \times 10^{-3} \times 60} = \Delta A_{340} \times 36.2$$

- ΔA_{340} is change in absorbance per minute
- number 100 converts 1 cm light-pass (cuvette thickness) to 1 m
- number 1.35×10^{-3} is total volume of reaction mixture
- 10^6 converts result in katals to microkatals
- 622 is molar absorption coefficient NADH at 340 nm ($m^2\ mol^{-1}$)
- number 1000 converts liters to m^3
- 0.1×10^{-3} is volume of serum sample
- number 60 converts one minute measuring interval to seconds

3 Estimation of alanine aminotransferase (ALT) in serum

Reagents:

Commercial kit BIOLATEST® ALT-UV Liquid, made by Erba-Lachema a.s., is used.

1. **Working solution:** lactate dehydrogenase ≥ 26.6 μkat/l, Tris buffer (pH 7.5) 110 mmol/l, L-alanine 567 mmol/l, pyridoxal-5'-phosphate 100.0 μmol/l, 2-oxoglutarate 17.0 mmol/l, NADH 0.21 mmol/l.
2. **Serum** – unknown sample

Procedure:

Cuvettes for the measurement should be pre-warmed for at least 5 minutes at 37 ˚C before use.

Measure in ml directly to the cuvette:	
Working solution	1.00
Serum	0.1
Mix and incubate for 1 minute at 37 ˚C	

During 1 minute incubation prepare photometer for measurement: set the wavelength to 340 nm, and zero absorbance using a cuvette filled with distilled water. After the 1 minute incubation place the reaction mixture in the cuvette to spectrophotometer and immediately read the absorbance. Then keep reading the absorbance in (exactly) one-minute intervals for 5 minutes.

Again, once the working solution and serum are mixed, the reaction is running and the measurement must be really started immediately after the 1 minute incubation. In case of any unexpected delay do the experiment again from the beginning.

Calculation:

First calculate the average change of absorbance per minute (ΔA):

Time:		ΔA_{340}
0	A_0	
1 minute	A_1	$A_0 - A_1 \rightarrow \Delta A_1$ .....
2 minutes	A_2	$A_1 - A_2 \rightarrow \Delta A_2$ .....
3 minutes	A_3	$A_2 - A_3 \rightarrow \Delta A_3$ .....
4 minutes	A_4	$A_3 - A_4 \rightarrow \Delta A_4$ .....
5 minutes	A_5	$A_4 - A_5 \rightarrow \Delta A_5$ .....

$$\Delta A_{340} = \frac{\Delta A_1 + \Delta A_2 + \Delta A_3 + \Delta A_4 + \Delta A_5}{5}$$

Next, calculate catalytic ALT concentration using the equation:

$$\text{S-ALT (μkat/l)} = \frac{\Delta A_{340} \times 100 \times 1.1 \times 10^{-3} \times 10^{6}}{622 \times 1000 \times 0.1 \times 10^{-3} \times 60} = \Delta A_{340} \times 29.5$$

- ΔA_{340} is change in absorbance per minute
- number 100 converts 1 cm light-pass (cuvette thickness) to 1 m
- number 1.1×10^{-3} is total volume of reaction mixture
- 10^6 converts result in katals to microkatals
- 622 is molar absorption coefficient NADH at 340 nm ($m^2\ mol^{-1}$)
- number 1000 converts liters to m^3
- 0.1×10^{-3} is volume of serum sample
- number 60 converts one minute measuring interval to seconds

4 Estimation of urea in serum and urine

Reagents:
Commercial kit for kinetic estimation of urea Bio-La-Test UREA UV KIN 6x100 manufactured by Erba-Lachema s.r.o. is used.

1. **R1 reagent:**

   Tris buffer, 100 mmol/l

   2-oxoglutarate, 5.49 mmol/l

   Urease ≥ 166,6 µkat/l

   L-glutamate dehydrogenase (GLDH) ≥ 63.3 µkat/l
2. **R2 reagent:** NADH 1.66 mmol/l
3. **Working solution**: 4 volumes R1 mixed with 1 volume R2
4. **Standard solution of urea** (concentration will be provided).
5. **Serum** – unknown sample (infectious material)
6. **Diluted urine** – unknown sample (infectious material), dilution will be provided.

Procedure:

Working solution and spectrophotometric cuvettes need to be pre-warmed to 37 °C before analysis.

N.B.: Use distilled water as a blank. First process the cuvette No. 1 (serum sample): Prepare the mixture as directed by the table below, mix well by repeated pipetting, after 30 seconds measure the absorbance A_1, and after exactly 60 seconds measure the absorbance again, obtaining A_2. Then perform the same with the cuvette No. 2 (urine sample), cuvette No. 3 (standard) and finally with the cuvette No. 4 (reagent blank).

Measure in ml:	**Cuvette No. 1** Serum sample	**Cuvette No. 2** Urine sample	**Cuvette No. 3** Standard	**Cuvette No. 4** Reagent blank
Serum	0.01	-	-	-
Urine	-	0.01	-	-
Standard	-	-	0.01	-
Purified water	-	-	-	0.01
Working solution	1.0	1.0	1.0	1.0
Mix, after 30 seconds measure absorbance A_1 at 340 nm against distilled water.				
Leave the cuvette in the instrument and after 60 seconds measure the absorbance A_2.				

Calculations:

Concentration of urea in serum (S-Urea):

$$\text{S-Urea (mmol/l)} = \frac{(A_1 - A_2)_{serum} - (A_1 - A_2)_{reagent\ blank}}{(A_1 - A_2)_{standard} - (A_1 - A_2)_{reagent\ blank}} \times c_{standard}$$

Concentration of urea in the urine (U-Urea):

$$\text{U-Urea (mmol/l)} = \frac{(A_1 - A_2)_{urine} - (A_1 - A_2)_{reagent\ blank}}{(A_1 - A_2)_{standard} - (A_1 - A_2)_{reagent\ blank}} \times c_{standard} \times \text{Dilution of urine}$$

Daily output of urea into urine (dU-Urea):

dU-Urea (mmol/24 hrs) = U-Urea (mmol/l) × Volume of urine (liters/24 hrs)

5 Calculation of nitrogen balance

Use the values of urea in serum and urine from the previous experiment. Further data about your patient will be provided during the practical lesson.

Intake of nitrogen (g/24 hours) = Intake of proteins/amino acids × 0.16

Catabolic nitrogen (g/24 hrs) = Urea in urine (mmol/L) × V (L) × 0.028 × 1.2
+
Δ Urea in serum (mmol/l) × 0.028 × Body weight (kg) × Factor of body water
+
Loss of urea by stool and skin

Nitrogen Balance = Intake of Nitrogen (g/24 hrs) – Catabolic Nitrogen (g/24 hrs)