INTERNATIONAL BIOLOGY OLYMPIAD e. V. IBO

Biochemistry Practical

English (Official)

Student Code	

International Biology Olympiad

July 10 - 18, 2022

Yerevan, Armenia

Practical Exam N 3

Biochemistry

Total points: 100

Duration: 90 minutes

Biochemistry Practical

IBO 33rd 2022 ARMENIA

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General instructions

1. Always wear gloves.

2. Check that you have received all the instruments and materials (see Instruments and accessories, Materials). If any are missing, let the lab assistant know by raising the red card during the first 10 minutes of the exam.

3. Use all the materials and instruments appropriately/handle equipment properly.

4. No spilt solution or broken instrument will be replaced, except gloves.

5. Write your Student Code in the given box on every page of your answer sheet.

6. Record your answers on your answer sheet. Only the answers recorded in the Answer Sheet will be evaluated.

7. Stop writing and put down your pen immediately when you hear the bell ring at the end of the exam.

8. Put all the papers in an empty envelope, and then close it.

9. Wait in your seat until the assistant comes to you to pick up your envelope.

10. No piece of paper, material, stationery, or instrument should be taken out of the lab.

WARNING!
YOU MUST ABSOLUTELY ABIDE BY ALL SAFETY RULES AND REGULATIONS INCLUDING WEARING APPROPRIATE PROTECTION, OTHERWISE THE LAB ASSISTANT WILL ESCORT YOU OUT FROM THE LAB.

GUIDELINES FOR FILLING OUT THE ANSWER SHEET

Answers to biochemistry assignments are of 3 types. They are:

1. **numbers (integer or decimal fraction)** in which **the decimal point must be marked with <.>, not <,>.** It is allowed **to write a maximum of 3 digits** after the decimal point (for example, whole number: 8; decimal fraction: 0.127), unless specifically requested in the text of the assignment.

2. **a multiple choice answer** indicated by marking **X** inside the squares with dark red borders,

3. **function graphs** to be drawn clearly and visibly.

An **ANSWER SHEET** consisting of 2 pages is provided for filling in the answers to the tasks. The answer forms are processed, checked and evaluated by the computer, that's why it is necessary to follow some rules to fill them:

1. Fill in the answers to the tasks **with a pen** in the answer sheets exclusively in rectangles and squares marked with dark red borders.

2. Write the answers to the tasks clearly and legibly.

3. It is not allowed to make deletions in the answer sheet.

ATTENTION. Only the answers you have recorded on the answer sheet will be considered and evaluated. First, fill your records in the corresponding places of the text part of the assignments, but **it is necessary** to transfer them to the answer sheet in the same way. Any marks made on the assignment sheets will **NOT** be considered.

Materials

1. Solution of alcohol dehydrogenase from yeast (marked **ADH**) in an Eppendorf tube

2. 0.1 M glycine buffer (marked **Gly**) with pH9.5 in a chemical vial
3. 36 mM solution of NAD+ (marked **NAD**) in an Eppendorf tube
4. 0.41 M solution of ethanol (marked **Eth**) in an Eppendorf tube
5. Solution of Ethylenediaminetetraacetic acid (marked **EDTA**) in an Eppendorf tube
6. Solution of para-chloromercuribenzoic acid (marked **PCMB**) in an Eppendorf tube. **Beware! This compound is dangerous. Handle with care**

Instruments and accessories

Name	Quantity
Spectrophotometer with opaque cuvette	1
Cuvettes	3
Pipette 5-50 µL	1
Pipette 20-200 µL	1
Pipette 100-1000 µL	1
Pipette tips – 100-1000 µl	1
Pipette tips – 5-200 µl	13
Eppendorf tube holder	1
Empty Eppendorf tubes	2
Filter paper	3
Gloves	1
Pen	1
Pencil	1
Eraser	1
Ruler	1
Marker pen	1
Clock (stopwatch)	1
Trash can	1
Calculator	1

spectrophotometer

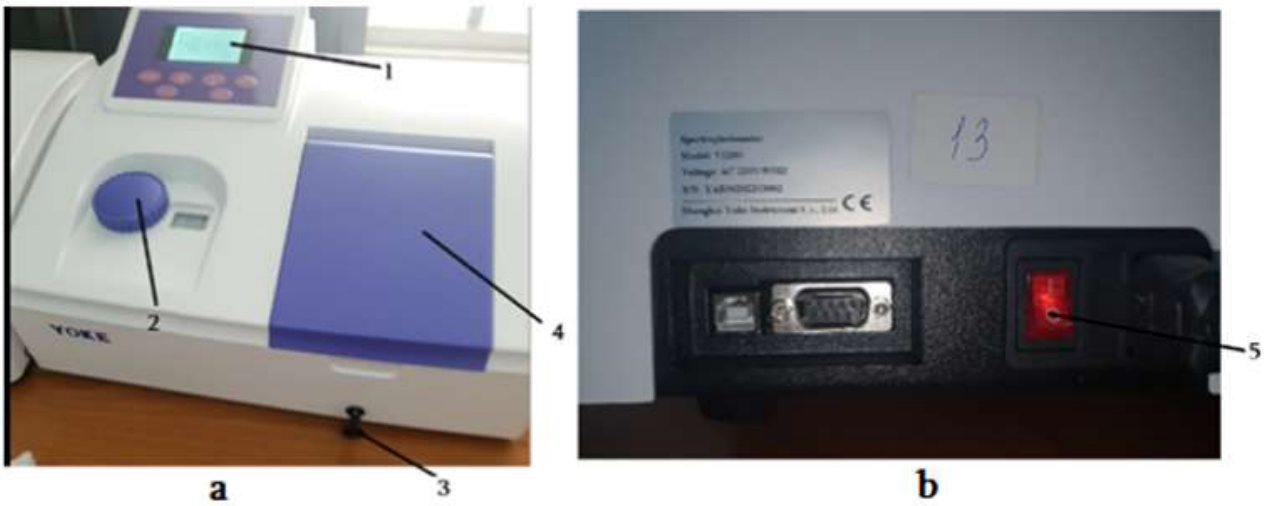

Fig. 1. View of the spectrophotometer from the front (a) and back (b). 1- spectrophotometer display (menu), 2- wavelength screw, 3- handle of the cuvette holder, 4- cuvette compartment cover, 5- spectrophotometer on/off button.

Switching on the spectrophotometer and bringing it to the working state

Switch **On** the spectrophotometer with the On/Off switch on the back wall (Fig. 1b-5).

Press Mode on the screen repeatedly until the ***T*** option appears (Fig. 1a-1). Open the cover of the cuvette compartment (Fig. 1a-4), and place the opaque black cuvette in the 4th position, farthest from you. Fully pull the handle of the cuvette holder (Fig. 1a-3) towards you, so that the fourth position of the holder with an opaque cuvette appears in front of the light window. Then install the required wavelength by turning the screw (Fig. 1a-2). Close the cover of the cuvette compartment and press the following button

on the screen. The display '0' will appear on the spectrophotometer screen (Fig. 1a-1). Move the cuvette holder one step so that there is nothing in the way of light (zeroing with air). Press the following button

Using a pipette

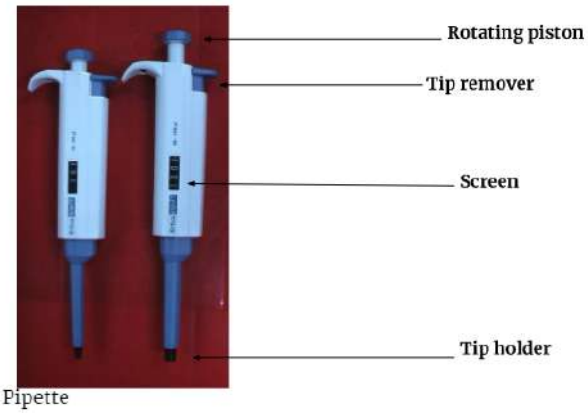

Fig.2 Pipette

To define the desired volume on a pipette, rotate the piston to see the target value on the **screen**.

Remember that every pipette has its defined limits written on the pipette. ***You should stay within those limits.***

How to use: Attach the **tip** to the **tip holder**. Gently push the **piston** until the first stop, and immerse the tip perpendicularly into the liquid at 2-4 mm depth. Slowly release the piston until it reaches the resting position. Move the pipette to the target container and push the piston to the end. To remove the used tip, bring the pipette closer to the trashcan on the table, and press the **tip remover**, after which the tip will fall into the trash. ***Install a new tip to take from the next solution.***

3. Clock (stopwatch)

Fig.3. Clock (stopwatch)

To run the stopwatch:

1. Press M once to enter the stopwatch mode.
2. Press D once to start the stopwatch.
3. Press D once, and the stopwatch stops.
4. Press S once, and the stopwatch resets.
5. Press M once to enter to clock mode.

Press **M** to toggle between stopwatch and clock modes.

Press **D** to start/pause the stopwatch.

Press **S** to reset the stopwatch (to 0:00:00).

Assignment

This assignment is composed of four parts.

Task 1. Determining the activity of alcohol dehydrogenase by spectrophotometry (42 points)

Task 2. The effect of different modifiers on alcohol dehydrogenase activity (32 points)

Task 3. Calculation of alcohol dehydrogenase activity in the presence of modifiers (16 points)

Task 4. Clarifying of the structure of the active site of alcohol dehydrogenase. (10 points)

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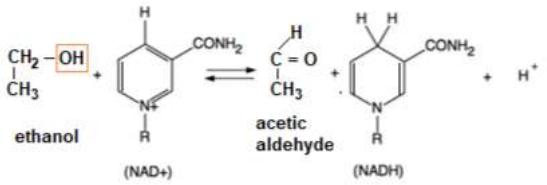
GENERAL INFORMATION ON ALCOHOL DEHYDROGENASE

Alcohol dehydrogenase (EC 1.1.1.1) is a representative of oxidoreductases and in different organisms, catalyzes the oxidation of ethanol to acetic aldehyde:



The coenzyme of alcohol dehydrogenase in this reaction is NAD+, which is reduced as a result of this reaction taking up two electrons and one proton, turning into NADH. The produced NADH absorbs light at a wavelength of 340 nm while the oxidized forms do not.

The interaction between the enzyme and coenzyme is mediated by tyrosine, histidine and aspartic acid residues, where the role of the acid-base catalyst is played by either the histidine residue or the divalent ion of a metal, e.g., zinc, manganese or others.

The primary structures of variants of alcohol dehydrogenase isolated from different sources strongly resemble each other. These enzymes are similar also in the structure of the active site.

As a rule, dehydrogenases are distinguished by their quaternary structures. They are oligomeric proteins, which are made up of different numbers of subunits in different organisms. The subunits separately do not have catalytic activity. Most dehydrogenases require the presence of bivalent metallic cations for catalytic activity, which stabilize the active conformation of the enzyme and take part in activating the substrate. The activity of dehydrogenases can be regulated by interaction with a modifier. The activity of most NAD-dependent dehydrogenases is altered by the sulfhydryl (SH-)-reactive agents such as para-chloromercuribenzoic acid (PCMB). Other modifiers of dehydrogenases include monoiodoacetic acid and diethylpyrocarbonate (DEP), which specifically interact with histidine residues. EDTA interacts with bivalent metal ions. Ninhydrin interacts with the arginine.

Part 1. Determining the activity of alcohol dehydrogenase by the spectrophotometric method.

Before you start your work!

1. Put on the gloves and **then read all the instructions.**
2. Turn on the spectrophotometer and set the wavelength at 340 nm (see Fig. 1 and 2).
3. An opaque, black cuvette is placed in the fourth position of the cuvette holder, which is necessary for zeroing the spectrophotometer indication.
4. Close the cuvette compartment.
5. Put one blue tip on the 1000-µL pipette and do not remove it until the end of the work. Use this pipette only to pipette the glycine buffer (Gly).
6. Before measuring alcohol dehydrogenase activity, you need to blank the spectrophotometer. For that, **prepare a control solution** in one spectrophotometer cuvette according to Table 1, using the appropriate pipettes.

	Gly	NAD	Eth
Control solution	2.7mL	0.1mL	0.1mL

7. Cover the cuvette with a lid, **hold it with your index finger and thumb from the top and bottom of the cuvette, and mix the contents of the cuvette, by inverting it once.**

8. If necessary, clean the transparent sides of the cuvette with filter paper.

9. Place the cuvette into any slot of the cuvette holder of the spectrophotometer, with the opaque side facing you (the transparent side facing the small window).

10. Position the cuvette holder so that the fourth position with an opaque cuvette appears on the path of light (in front of the light window).

11. Now close the cover of the cuvette compartment.

12. Press Mode on the display (fig.1) repeatedly until T mode appears.

13. Press 0%.

14. Move the cuvette holder so that the cuvette with the control solution appears in the light path.

15. Press Mode on the screen repeatedly until Mode A appears. The value on the screen is the absorption of the control solution and must be reset.

16. Press 100% T to reset the spectrophotometer.

17. Now open the cover of the cuvette compartment and remove the cuvette.

18. Prepare the clock, and set the stopwatch to the starting position.

19. Remove the cuvette lid and add 0.1 mL of the alcohol dehydrogenase solution (ADH) to the contents of the cuvette, and **immediately start the stopwatch**. Cover the cuvette with a lid and invert to mix as in point 7 (**hold it with your index finger and thumb from the top and bottom of the cuvette, and mix the contents of the cuvette, by inverting it once**).

20. Place the cuvette into the cuvette holder so that the cuvette appears in the light path. The absorbance value (A) of the solution will appear on the screen. Record the absorbance of the solution at the 15th second.

21. Follow the reaction for 120 seconds in total by taking measurements at 15-second intervals of the absorbance of the NADH formed during the reaction. Record your results **in the table of Question 1.1 in the answer sheet** (as shown below).

Question 1.1

Reaction time, s	15	30	45	60	75	90	105	120
A								

Reaction time, s	

22. Remove the cuvette from the spectrophotometer and close the cuvette compartment cover. Leave the spectrophotometer in the working state.

23. Empty the cuvette in the trash can on the table, and put the used cuvette in the cuvette box.

24. Using the millimeter grid in Question 1.2/f in your answer sheet, plot a curve for the dependence of the absorbance of NADH as a function of reaction time (t) based on the data you receive. The reaction time is set on the X-axis and the absorbance on the Y-axis.

25. Draw a linear curve to fit the data points.

26. Derive a function equation for the curve to express the dependence of NADH absorbance on reaction time. You can use the clean pages of the task pages as rough paper.

27. Record your result in the "Function equation" field of Question 1.2 in your answer sheet. Enter the coefficient of the function equation to 3 decimal points, for example, 0.222.

Question 1.2

Function equation f(t) = [ ] t ADH [ ]

Function equation	

28. Determine the alcohol dehydrogenase activity (U/mL) using the arbitrary units based on this formula:

$$ADH(\frac{U}{mL}) = \frac{\triangle Absorbance * V_1}{6.22 * V_2 * \triangle t},$$

where:

ΔAbsorbance – is the change in absorbance (unitless) according to the curve in the arbitrary time range of your choice

6.22 – absorption coefficient, mM^{-1} * cm^{-1}

V_1 – the final volume of the control solution after adding enzyme, mL

V_2 – enzyme solution volume, mL

Δt – duration of the arbitrary time range of your choice, in minutes

29. Record the result in the “ADH” field of Question 1.2 in your answer sheet (see above).

Part 2. The effect of different modifiers on alcohol dehydrogenase activity

1. Label the two empty Eppendorf tubes “1” and “2” with the marker pen.
2. Place the tubes on the Eppendorf tube holder and open them.
3. Add 180 µl of the alcohol dehydrogenase solution (ADH) to Tube #1. Then add 20 µl EDTA solution. Close the tube lid tightly, and mix the contents by inverting the tube. Put the tube into the Eppendorf tube holder.
4. Pre-incubate the enzyme with the EDTA for 10 min. Set the time with the stopwatch given to you.
5. Before the pre-incubation time is over, take a clear cuvette and prepare the control solution according to Table 1 in Part 1.
6. Insert the cuvette into the spectrophotometer and reset it as in Task 1 (Points 6-7).
7. After the 10 min pre-incubation time is over, reset the stopwatch.
8. Remove the cuvette from the spectrophotometer, open the lid and place the cuvette on the table. Add 100 µl of enzyme solution from Eppendorf Tube #1 and **immediately start** the stopwatch.
9. Cover the cuvette with a lid and mix the contents, as described in Task 1 (Point 19). Put the cuvette into the cuvette holder.
10. Record the absorbance of the solution at each 15th second during the next 120 seconds.
11. Record the results in Question 2.1 EDTA of your answer sheet.

Question 2.1 EDTA

Reaction time, s	15	30	45	60	75	90	105	120
A								

Reaction time	

12. Remove the cuvette from the spectrophotometer and close the cuvette compartment cover. Leave the spectrophotometer in the working state.

13. Empty the cuvette in the trash can on the table, and put the used cuvette in the cuvette box.

14. Add 180 µl of the alcohol dehydrogenase solution to Eppendorf Tube #2. Then add 20 µl of PCMB solution. Close the tube lid tightly, and mix the contents by inverting the tube. Put the tube into the Eppendorf tube holder.

15. Pre-incubate the enzyme with a PCMB modifier for 10 min. Set the time with the stopwatch.

16. Before the pre-incubation time is over, take the clean cuvette and prepare the control solution according to Table 1.

17. Insert the cuvette into the spectrophotometer and reset it as in Task 1 (Points 6-7).

18. After the 10-min pre-incubation time is over, reset the stopwatch.

19. Remove the cuvette from the spectrophotometer, open the lid and place the cuvette on the table. Add 100 µl of enzyme solution from Eppendorf Tube #2 and run the stopwatch **immediately**.

20. Cover the cuvette with a lid and mix the contents, as described in Task 1 (Point 19), and place the cuvette into the cuvette compartment in the spectrophotometer.

21. Record the absorbance of the solution at each 15th second.

22. Follow the reaction for 120 seconds, recording the absorption of the resulting NADH every 15 seconds. Record the results in the table Question 2.2 PCMB of the answer sheet.

Question 2.2 PCMB

Reaction time, s	15	30	45	60	75	90	105	120
A								

Reaction time	

23. Remove the cuvette from the spectrophotometer and close the cuvette compartment cover. Turn off the spectrophotometer.

24. Empty the cuvette in the trash can on the table, and put the used cuvette in the cuvette box. Close the cuvette box.

25. Put the used tips, filter papers, and Eppendorf tubes into the trash can.

Part 3. Calculation of alcohol dehydrogenase activity in the presence of modifiers

1. Using the millimeter grid of Question 3.1/f in your answer sheet, plot a curve for the dependence of the absorbance of NADH in the presence of EDTA as a function of reaction time (t), as in the previous task. Draw a linear curve to fit the data points. .
2. Derive a function equation for the curve to express the dependence of absorbance of NADH on reaction time in the presence of EDTA.
3. Record your result in the "Function equation" field of Question 3.1 in your answer sheet. Enter the coefficient of the function equation to 3 decimal points, for example, 0.222.

Question 3.1

Function equation, EDTA, f(t) = [ ] t ADH [ ]

Function equation, EDTA	

4. Determine the activity of alcohol dehydrogenase during the enzymatic reaction after pre-incubation with various modifiers using the arbitrary units based on this formula:

$$ADH(\frac{U}{ml}) = \frac{\triangle\, Absorbance * V_1}{6.22 * V_2 * \triangle\, t}$$

where ΔAbsorbance – is the change in absorbance (unitless) according to the mean curve in the arbitrary time range of your choice

6.22 – millimolar absorption coefficient, mM-1*cm-1

V_1 – the final volume of the control solution after adding enzyme, mL

V_2– enzyme solution mixture, mL

$\triangle t$ – duration of the reaction, minutes

5. Record your results in the "ADH" field of Question 3.1 in your answer sheet.

6. Using the millimeter grid of Question 3.2/f in your answer sheet, plot a curve for the dependence of the absorbance of NADH in the presence of PCMB as a function of reaction time (t). Draw a linear curve to fit the data points.

7. Derive a function equation for the curve to express the dependence of absorbance of NADH on reaction time in the presence of PCMB. Record your result in the "Function equation" field of Question 3.2 in your answer sheet. Enter the coefficient of the function equation to 3 decimal points, for example, 0.222.

Question 3.2

Function equation, PCMB, f(t) = [ ] t ADH [ ]

Function equation, PCMB	

Using the equation above (as in Task 1) determine the activity of alcohol dehydrogenase during the enzymatic reaction after pre-incubation with PCMB using the arbitrary units based on this formula:

$$ADH(\frac{U}{ml}) = \frac{\triangle Absorbance * V_1}{6.22 * V_2 * \triangle t}$$

where ΔAbsorbance – is the change in absorbance (unitless) according to the mean curve in the arbitrary time range of your choice.

6.22 – millimolar absorption coefficient, mM^{-1}*cm^{-1}

V_1 – the final volume of the reaction mixture after adding enzyme, mL

V_2 – enzyme solution volume, mL

$\triangle t$ – duration of the reaction, minutes

8. Record your results in the “ADH” field of Question 3.2 in your answer sheet (3 points).

9. Based on the data you received, **calculate the percentage change** in alcohol dehydrogenase activity and record it in the corresponding cell of Question 3.3 in your answer sheet.

Question 3.3

Change on enzyme activity, %		
Modificator	EDTA	PCMB
Increases		
Decreases		

Change on enzyme activity, %	
Modificator	
Increases	
Decreases	

Part 4. Clarifying of the structure of the active site of alcohol dehydrogenase.

Choose which statement is correct from the given statements. Answer the questions by putting an X in the appropriate box of the answer sheet in Question 4.1.

1. Significant change of alcohol dehydrogenase activity by PCMB is due to the presence of histidine residues in the active site.
2. Sulfur-containing amino acids are part of the structure of the catalytic site of the enzyme alcohol dehydrogenase.
3. Bivalent metal ions are indispensable for the formation of the quaternary structure of the enzyme alcohol dehydrogenase.
4. A mutant of alcohol dehydrogenase with a mutation involving glutamic acid in subunits interaction to alanine performs like a native in the presence of EDTA.

Question 4.1

	True	False
Statement 1	☐	☐
Statement 2	☐	☐
Statement 3	☐	☐
Statement 4	☐	☐

Statement	
True	
False	

We wish you the best of luck!