European Olympiad of Experimental Science

Jahresbericht 2021/22

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Vom

Bundesministerium
Bildung, Wissenschaft
und Forschung

gefördert

Klagenfurt, 20. Mai 2022

European Olympiad of Experimental Science (EOES)

Die EOES ist ein naturwissenschaftlicher Teamwettbewerb der Europäischen Union für Biologie, Chemie und Physik. Österreich war 2022 zum schon dreizehnten Mal mit zwei Teams bei der EOES, die heuer in Hradec Králové in Tschechien stattfand, vertreten.

Das Credo der EOES

- begabten SchülerInnen die Möglichkeit geben, ihre Talente zu entfalten
- Das Interesse an Wissenschaft und des forschenden Lernens zu wecken bzw. zu fördern
- Durch die Eindrücke und Erfahrungen der EOES auf eine mögliche Teilnahme an weiteren Internationalen Olympiaden vorzubereiten

Ziel des Wettbewerbs

- Öffentliche Interesse auf die naturwissenschaftliche Ausbildung lenken
- Ermittlung der besten SchülerInnen der Europäischen Union im naturwissenschaftlichen Bereich
- Wertschätzung der Wissenschaft in der Allgemeinheit
- Intensivierung der Zusammenarbeit zwischen europäischen Bildungssystemen
- Individuelle Ideen und Konzepte innerhalb der gesamten Europäischen Union zu verbreiten
- Vorbereitung europäischer SchülerInnen auf die Internationalen Facholympiaden

Mehr dazu unter: www.eoes.science und www.eoes.at

Inhalt

1. Vorbereitungswoche an der BIKO mach MINT

31 SchülerInnen aus sechs Bundesländern, wurden, organisiert vom Regionalen Netzwerk Kärnten, vom 14. - 18. März 2022 an der Bildungskooperation BIKO mach MINT am Educational Lab des Lakeside Parks Klagenfurt auf den Teamwettbewerb in Tschechien vorbereitet.

Insgesamt beteiligte SchülerInnen im Auswahlverfahren

Abermann	Mia	BG/BRG Gmunden
Davis	Sophie	BG/BRG Gmunden
Delliehausen	Severjan	BG/BRG St. Martin
Dorfer	Felix	Gymnasium Tamsweg
Drexel	Jolina	BG/BRG St. Pölten
Fuchs-Eitel	Simon	BRG18
Gehrer	Linda	GRG2
Giulini	Donata	Sir Karl Popper Schule
Gasteiner	Maximilian	G/RG Sachsenbrunn
Hirschegger	Manuel	Herta-Reich Gymnasium Mürzzuschlag
Hofmann	Michael	Sir Karl Popper Schule
Holzfeind	Anna	Peraugymnasium Villach
Innerhofer	Bernhard	Sir Karl Popper Schule
Jahn	Karoline	Sir Karl Popper Schule
Kaiser	Moritz	BG/BRG St. Pölten
Klein	Valentina	Sir Karl Popper Schule
Knauder	Paul	Peraugymnasium Villach
Koch	Mara	Bischöfliches Gymnasium Graz
Kolmasch	Klara	BG/BRG Gleisdorf
Koschier	Elias	ERG-Donaustadt
Köttl	Clara	Wiedner Gymnasium
Lechner	Philipp	Sir Karl Popper Schule
Löw	Valentin	Modellschule Graz
Milojevic	Vasilije	Peraugymnasium Villach
Mitterdorfer	Jana	BRG St. Veit an der Glan
Pesendorfer	Zoe	Peraugymnasium Villach
Rath	Péter	BG/BRG Leibnitz
Ruisinger	Jonas	ERG-Donaustadt
Sehic	Emma	BG/BRG Gmunden
Tonner	Benjamin	Gymnasium Zell am See
Tschlatscher	Anna	Peraugymnasium Villach
Walia	Ishita	BG/BRG St. Martin

2. Trainingstage an der BIKO mach MINT in Klagenfurt

Sechs Jugendliche schafften es in die Qualifikation und somit zum Intensivtraining, das heuer wieder in Kooperation mit dem deutschen EOES-Nationalteam ebenfalls am Lakeside Park in Klagenfurt stattfand (4. – 8. April 2022). Diese Trainingstage mit den deutschen EOES KandidatInnen sind inzwischen zur Tradition geworden und werden von der Teamleitung beider Länder als äußerst produktiv gewertet.

Insgesamt beteiligte TrainerInnen in Klagenfurt

TrainerIn	Stamminstitution
Mag Brachtl Karl	RN Kärnten
Mag. Holub Peter	RN Kärnten
Dr. Morgenstern Christina	PH Kärnten, Bundeslehrerin
Mag. Winkler Dieter	Bischöfliches Gymnasium Graz, Bundeslehrer
Hohl Elias	Student und ehem. Teilnehmer
Christina Lassnig	Studentin und ehem. Teilnehmerin

3. EOES 2022 in Tschechien

Die Organisation in Tschechien war ausgezeichnet. Das Freizeitprogramm war sehr spannend, die Aufgabenstellungen fordernd, Fächer-übergreifend und gut vorbereitet, allerdings extrem schwierig. Vor allem, weil sie in der Zeit kaum bewältigbar waren. Für unsere noch recht unerfahrenen Teams war es eine Herausforderung.

4. Team AUSTRIA 2022

Delegationsleitung: Dr. Christina Morgenstern

Mentorin Biologie: Dr. Christina Morgenstern

Mentor Chemie: Mag. Karl Brachtl

Mentor Physik: Mag. Dieter Winkler

Team A: Benjamin Tonner, Mara Koch, Valentina Klein: *Bronzemedaille*

Team B: Péter Rath, Elias Koschier, Paul Knauder: *Bronzemedaille*

Österreich Team A,

von links nach rechts: Benjamin Tonner - Gymnasium Zell am See, Mara Koch – Bischöfliches Gymnasium Graz, Valentina Klein – Sir Karl Popper Schule

Österreich Team B

von links nach rechts: Péter Rath - BG/BRG Leibnitz, Elias Koschier – ERG Donaustadt, Paul Knauder – Peraugymnasium Villach

5. Zwei Mal Bronze in Hradec Králové!

Anlässlich der diesjährigen Europäischen Science Olympiade in Tschechien kann sich Österreich im guten Mittelfeld präsentieren. Beide Teams verpassten nur knapp die Silbermedaillen!

Gold und Silber Medaillenränge EOES 2022

	Land	Team
1.	Germany	A
2.	Estonia	A
3.	Bulgaria	A
4.	Luxembourg	A
5.	Germany	B
6.	Slovenia	B
7.	Hungary	A
8.	Czechia	A
9.	Latvia	A
10.	Croatia	A
11.	Lithuania	A
12.	Estonia	B
13.	Czechia	B
14.	Hungary	B
15.	Croatia	B
16.	Denmark	A
17.	Lithuania	B
18.	Ireland	A
19.	Luxembourg	B

Die österreichische Delegation in Tschechien 2022

Von links nach rechts: Dr. Christina Morgenstern - RECC für Naturwissenschaften der Pädagogischen Hochschule Kärnten, Mentorin Biologie, Péter Rath - BG/BRG Leibnitz, Team B, Paul Knauder – Peraugymnasium Villach, Team B, Valentina Klein – Sir Karl Popper Schule, Team A, Mara Koch – Bischöfliches Gymnasium Graz, Team A, Benjamin Tonner- Gymnasium Zell am See, Team A, Elias Koschier – ERG Donaustadt, Team B, Mag. Karl Brachtl – RN Kärnten, Mag. Dieter Winkler – Bischöfliches Gymnasium Graz

Sowohl die Trainingswoche im März als auch die Trainingstage mit den deutschen Nationalteams im April wurden vom Regionalen Netzwerk für Naturwissenschaften und Mathematik Kärnten koordiniert und fanden in den Experimentierräumen der Bildungskooperation BIKO mach MINT am Educational Lab, Lakeside Park Klagenfurt statt.
Zwei der fünf Goldmedaillen entsprangen dieser Trainingsgemeinschaft, in der als TrainerInnen auch Christina Lassnig und Elias Hohl ehemalige EOES-TeilnehmerInnen und Studierende, einen wesentlichen Teil zum Erfolg geleistet haben.

6. Unterstützung durch

Land Kärnten

Klagenfurt am Wörthersee

Industriellenvereinigung Kärnten

Regionales Netzwerk für Naturwissenschaften und Mathematik Kärnten

IMST- Innovationen machen Schulen Top

7. Anhang – Aufgabenstellungen 2022

T1

Task 1

Hyaluronic acid

10. 05. 2022.
Hradec Králové
Czechia

T1

Preface

Our body is a masterpiece. And not only our own, but of all the organisms. Nature is a gallery inhabited by an examples optimized by the creativity of the natural selection. The sculptor is therefore an evolution. It uses the available chemistry and physics to invent the optimized assemblies with a features which are hard to beat with the cutting edge technologies.

Molecular “gems” are not only constituent parts of our cells but are secreted as extracellular matrix to provide the multicellular organisms with striking mechanical properties. Collagen and elastin are well known examples of the proteins providing the tissues with the strength and elasticity. The same importance belongs to the molecules similar to carbohydrates, which are highly hydrated and in many modified variants enabling our body to behave in many aspects “smart” and cost effectively.

In our task you experience one of the most important extracellular matrix molecules – the hyaluronic acid –from different points of view to uncover it´s peculiar properties. It should be mentioned that hyaluronic acid is getting more and more interest in biomedicine as biochemical, which could be used to treat several pathologies, to assist *in vitro* cultivated tissues for regeneration medicine or even could be used for beautification or anti-aging interventions. To isolate and properly characterize the hyaluronic acid, chemical and physical methodologies need to be applied. Our task is using some of them with intention to blur the borderlines between the disciplines of the natural science. As already mentioned, biology, chemistry and physics joined together to become an evolutionary “sculptor” %.

Part A

Introduction

Skin – many layers, different composition

Mammalian skin consists of several layers and fulfills various roles. It covers and protects the body, allows communication with external environment, maintains homeostasis of the body, etc. The skin of a particular species is adapted to its lifestyle and habitat. We will look closer at the skin of a house mouse (*Mus musculus*, Fig. A1) and naked mole-rat (*Heterocephalus glaber*, Fig. A2). Both are rodents (Rodentia), and their bodies are of similar size. However, their lifestyles differ markedly.

Fig. A1 House mouse.

Fig. A2 Naked mole-rat.

T1

Material and chemicals for experiments in Task A1 and Task A2

Each team

- Microscope
- Distilled water in a wash bottle
- Plastic Falcon tube labeled H – with 8 mL hematoxylin dye
- Plastic Falcon tube labeled E – with 8 mL eosin dye
- Beaker as a rack for the Falcon tubes
- Eppendorf tube labeled M – with 1 mL mounting medium Rotihistokitt
- 4 plastic Pasteur pipettes
- 2 beakers for waste – one for liquid waste, second for solid waste
- Forceps enabling better manipulation with glass slides
- Petri dish containing 4 glass slides labeled A, B, C, D and two long glass coverslips
- Plastic box with two built-in glass tubes
- Paper towels or tissues

Shared in each laboratory

- Gloves, different sizes (pick your size)
- Wall clock
- Glass jars with lids containing:
    - Rotihistol (labeled Roti)
    - 100% ethanol (labeled EtOH 100)
    - 96% ethanol (labeled EtOH 96)
    - 80% ethanol (labeled EtOH 80)

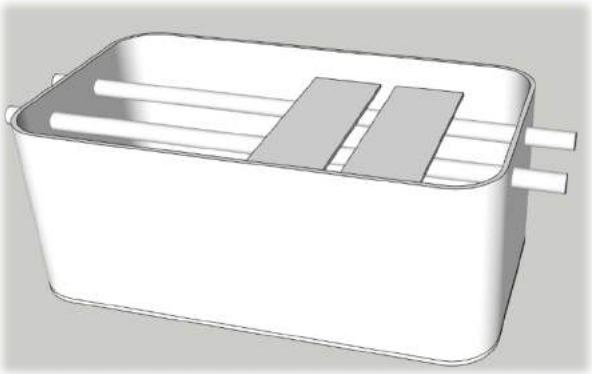
Task A1 (20.5 points)

For the first task, you will use two glass slides: slide A and slide B. There are several paraffin skin sections attached onto each slide. One slide contains skin sections from mouse, the other sections from naked mole-rat. Both skins were fixed, embedded in paraffin, and thin sections were cut on microtome.

Perform the same histology staining on both slides in parallel by following the protocol provided below. Take extra care not to touch the skin sections; they can be rubbed off or damaged very easily!

Experimental Procedure

1. Deparaffinize the sections in Rotihistol for 5 min. Insert the slide into the accordingly labeled glass jar. (There are grooves in the glass wall to hold the slides.) Insert the slides into the jar carefully. Neighboring slides must not touch each other.
2. Transfer the slides to 100% ethanol, incubate for 4 min.
3. Transfer the slides to 96% ethanol, let hydrate there for 4 min.
4. Transfer the slides to 80% ethanol, let hydrate there for 4 min.
5. Bring the slides back to your bench and place them on the glass tubes built in the plastic box, skin sections facing upward as in the diagram below. Rinse the slides with lots of distilled water at least 5 times. The water should flow gently to avoid washing away the sections. Continue with steps 6 – 9 also in the plastic box with the built in glass tubes.



6. Overlay all sections with approx. 3 mL of hematoxylin on each slide, incubate in hematoxylin for 3 min.
7. Carefully and gently rinse the slides with distilled water until you wash away the excess dye.
8. Overlay all sections with approx. 3 mL of eosin on each slide, incubate in eosin for 3 min.
9. Carefully and gently rinse the slides with distilled water until you wash away any excess dye.
10. Insert the slides into the glass jar with 80% ethanol, let dehydrate for 3 min.
11. Transfer them to 96% ethanol, dehydrate further for 3 min.
12. Transfer the slides to 100% ethanol, incubate for 4 min.
13. Incubate in Rotihistol for 5 min.
14. Bring the slides back to your bench. Place them on a paper towel and carefully cover the area with skin sections by the mounting medium (Rotihistokitt). Do NOT touch the sections. Cover with long coverslips, avoid bubbles. Wait several minutes before you put the slides under the microscope.
15. Observe first with 10x objective, later with 40x objective. Be careful, the mounting medium is still not solid!

T1

Draw a representative picture of your sections from slides A and B. Label them using terms from Table A1 below. Use as many terms as you can for these samples. However, you don't have to use all of them, and you should only draw and label structures you are actually able to see. Observe the section carefully and decide which slide contains mouse / mole-rat skin sections.

A	arrector pili (hair erector) muscle
B	basal membrane
C	blood vessel
D	cell wall
E	chondrocyte
F	connective tissue
G	red blood cell
H	dermis
I	epidermis
J	epithelium
K	glial cell
L	hair follicle
M	microvilli
N	striated muscle
O	ribosomes
P	sebaceous gland
Q	smooth muscle cells
R	vacuole

Table A1

Question A1.1 *Draw a representative section from Slide A in box 1.1 in the Answer sheet. Label with letters from the Table A1. (8 points)*

Question A1.2 *Draw a representative section from Slide B in box 1.2 in the Answer sheet. Label with letters from the Table A1. (8 points)*

Question A1.3 *Enter the correct letter (A or B) of your slides to the corresponding animal from which the skin sections were prepared into the proper box in the Answer sheet. (1 point)*

Question A1.4 *The outermost part of skin is called epidermis. In at least one of the samples, you may be able to distinguish its layers. The table A2 contains simplified descriptions of these layers. Label them with corresponding numbers correctly in one of your drawings from Question A1.1 or A1.2. (2 points)*

1	*Stratum granulosum*	*Cytoplasm of cells in this layer is filled with dense masses of filaggrin and other proteins associated with keratin tonofibrils, linking them into large cytoplasmic structures. The cells also contain small structures with many lamellae containing various lipids and glycolipids. The lamellar granules undergo exocytosis, producing a lipid-rich, impermeable layer around the cells in this layer.*
2	*Stratum basale*	*Characterized by intense mitotic activity. Contains most of the progenitor cells for all the epidermal layers.*
3	*Stratum corneum*	*Cells of this layer continuously shed off. The cells contain only amorphous, fibrillar proteins. Their plasma membranes are surrounded by a lipid-rich layer.*
4	*Stratum spinosum*	*Cells that very actively synthetize keratins in the cytoplasm. The keratin filaments assemble into bundles called tonofibrils, which hold layers together by maintaining cell–cell contact. During histological processing, the cells usually shrink slightly, leading to a fuzzy appearance.*

Table A2

Question A1.5 *Observe which parts of the sample are stained by hematoxylin most intensely. Take into consideration that hematoxylin is a basic dye, which means that it stains anionic cell components. Which biomolecule is most likely the target of hematoxylin staining? Indicate the corresponding molecule by a tick (√) in table 1.4 in the Answer sheet (select 1 answer). (1.5 point)*

cellulose
water
DNA
transmembrane proteins
phospholipids
simple sugars (oligosaccharides)

THE SLIDES A AND B MUST STAY ON YOUR BENCH. THEY WILL BE COLLECTED AND EVALUATED!

Task A2 (13 points)

Besides cells, skin also contains extracellular space filled with so called extracellular matrix (ECM). ECM consists of different molecules – proteins, water, ions, and polysaccharides. One such component is hyaluronic acid (hylauronan, HA). Naked mole-rat is known to produce very large HA molecules (high molecular weight of 6–10 MDa).

HA can be visualized in the skin sections by using labelled HA-binding protein. This protein sticks to the HA and stains the HA-rich region brown. On the other hand, the HA molecules can be degraded – both in skin *in vivo* as well as in the skin sections by enzymes called hyaluronidases.

In Task 2 you will examine slide C and slide D. Again, there are skin sections from mouse and naked mole-rat on those slides (not necessarily in the same order as in Task 1). Both slides were divided into two sections (labeled 1 and 2). One part of each slide was treated with hyaluronidase whereas the other part was mock treated and serves as control.

The slides were already stained for you with HA-binding peptide. In order to facilitate skin layer recognition, the sections were simultaneously stained with hematoxylin.

Observe all four slide sections (C1, C2, D1, D2) under the microscope. Focus on similarities and differences between the sections.

Question A2. 1 *Draw all four sections into corresponding parts of the box 2.1 in the Answer sheet. Your picture doesn´t need to be as detailed as in Task 1; try to capture the main differences between the sections. Indicate the HA-rich layer with prominent brown signal by an arrow. Do so in those sections where its clearly present by an arrow and label it correctly with name of the layer. (8 points)*

Question A2.2 *Analyze sections C1, C2, D1, D2 and decide which part was treated with hyaluronidase and which was only mock treated. Also asses the animal origin of each section. Write the code of the section (C1, C2, D1, D2) into the corresponding field in the Answer sheet. (2 points)*

Question A2.3 *Where do the extracellular matrix components come from? Choose the correct statement(s) regarding the origin of the protein and polysaccharide molecules found in the matrix and label by a tick (√) in the Answer sheet. (1 point)*

They are directly extracted from the surrounding environment. The animal incorporates selected molecules into both superficial and deeper layers of skin and keeps them deposited there for days or longer.	
They are largely synthetized by skin microbiome (mainly bacteria). Animals with different microbiome display different composition of extracellular matrix.	
They are synthetized solely by liver cells. Blood and lymph transport them to the skin.	
They are synthetized by cells directly in the tissue. Some molecules are made inside the cells and subsequently exported by exocytosis; others are synthetized by transmembrane enzymes.	

Question A2.4 *What is the role of HA in skin and other tissues? Indicate the correct answer(s) by a tick (√) in the Answer sheet. (2 point)*

It maintains sufficient hydratation of the tissue.	
It can serve as lubricant, e.g., in joints.	
It has a large space-filling capacity.	
It regulates migration of cells.	

Task A3 (20 points, no experimental work needed)

Question A3.1 *One of epidermal cell types are melanocytes found mostly in the stratum basale. Their main function is the production of pigments called melanins inside membrane-bound organelles called melanosomes. Melanosomes are transported to the periphery of the cell and into cytoplasmic extensions along microtubules. What type of molecular motors would you expect to be responsible for this transport? Write your answer in the Answer sheet. (1 point)*

A. Kinesins
B. Dyneins
C. Myosins

Tips of the cytoplasmic extensions break off and are phagocytosed by surrounding keratinocytes. Inside these cells, melanosomes form protective supranuclear caps, protecting DNA from mutagenic effects of UV radiation.

Two main types of melanins are present in mammals: eumelanins and pheomelanins. Eumelanins are black or dark brown in colour whereas pheomelanins are red, pink, or yellow. Relatively less eumelanins than pheomelanins are produced in people with red hair or lighter complexion, making them more susceptible to sunburn due to reduced effectiveness of pheomelanins against UV radiation. Below is a graph of absorbance of eumelanins and pheomelanins as a function of wavelength.

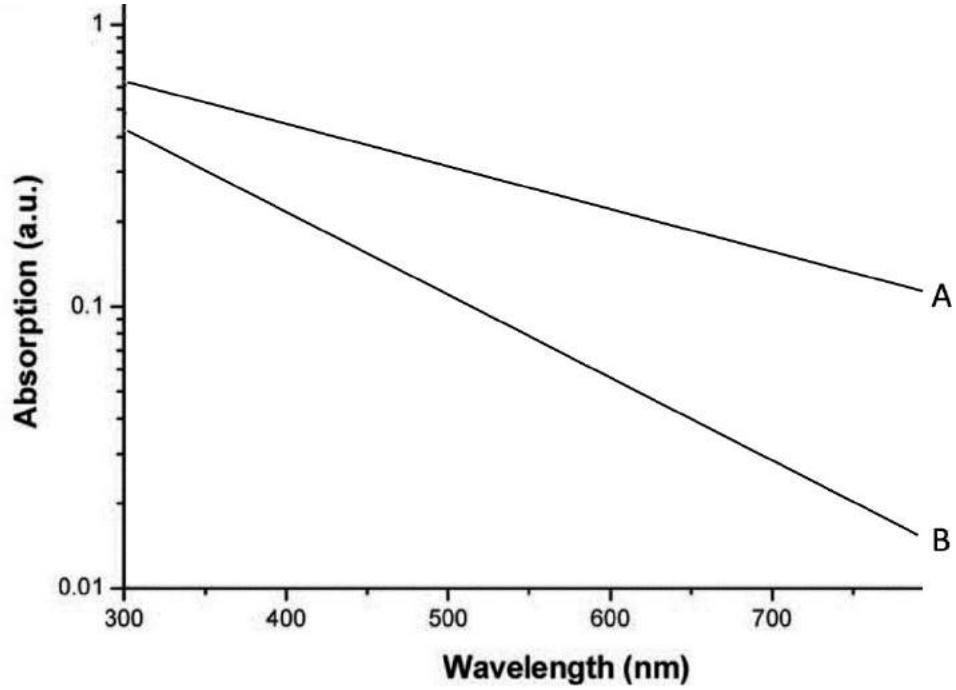


Fig. A3

Question A3.2 *Decide which curve corresponds to eumelanins and which to pheomelanins. Write your answers in table in the Answer sheet. (0.5 points)*

Question A3.3 *Which options below explain your decision? Choose all statements that apply and indicate them by a tick (√) in the table in the Answer sheet (select 0–3 answers). (1.5 points)*

a) *The overall amount of light that passes through eumelanins would be expected to be lower, therefore the curve will be lower on the graph.*
b) *Based on their known function, one could expect eumelanins to absorb more in the UV part of the spectra, therefore the absorbtion in the right part of the graph will be higher.*
c) *Based on the colour of pheomelanins, we could expect more light from the yellow to red part of the spectra to pass through, therefore the curve will be lower in the right part of the graph.*

In the next part we will be interested in the production of melanin, melanosome maturation, and regulation of these processes. Below is a scheme (Fig. A4) of the Raper–Mason pathway depicting the steps of melanin synthesis in mice. Some enzymes necessary for the production of pheomelanins or eumelanins are indicated in **red**.

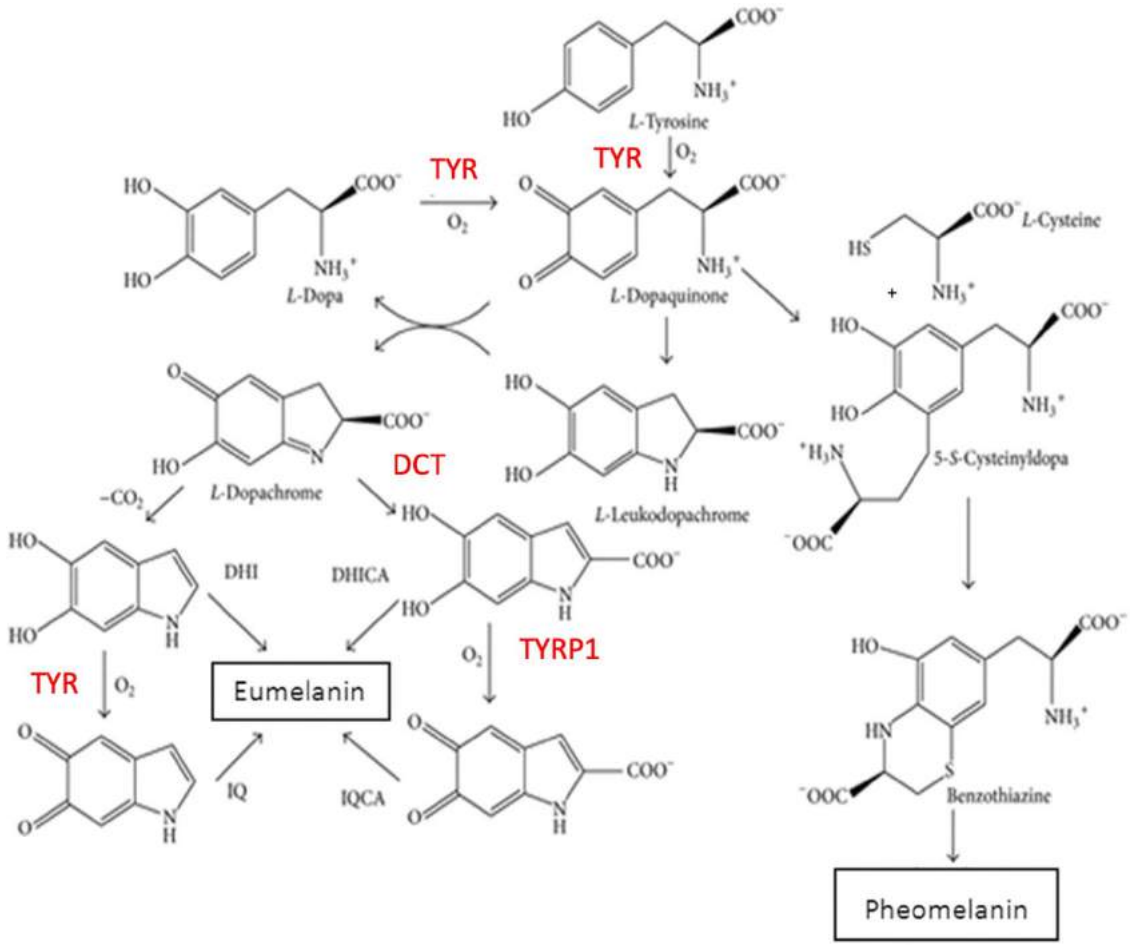


Fig. A4 Raper–Mason pathway of melanin synthesis.

In humans, this pathway as well as melanosome maturation is regulated based on UV light exposure of the skin. Below is a simplified scheme of this regulation. DNA damage in keratinocytes leads to activation of the protein p53 which activates expression of a peptide prohormone called proopiomelanocortin (POMC). POMC is further cleaved to different fragments such as adrenocorticotropic hormone (ACTH) which is then secreted by exocytosis. ACTH then binds to a protein receptor on melanocyte membrane, which leads to the activation of adenylyl cyclase via a signaling cascade. Adenylyl cyclase catalyses the production of cyclic adenosine monophosphate (cAMP) from ATP. cAMP binds to regulatory subunits of protein kinase A (PKA), causing them to dissociate, and thus activates the catalytic subunits of PKA. PKA then affects gene expression in two ways. First, it inhibits salt-inducible kinase (SIK) by phosphorylation. SIK, when active, phosphorylates one of CREB-regulated transcriptional coactivators (CRTC), preventing it from entering the nucleus. Unfosforylated CRTC migrates to the nucleus and forms a complex with CRE-binding protein (CREB). Second way by which PKA ensures expression of these genes is by direct activational phosphorylation of CREB. The complex of CRTC and phosphorylated CREB binds to specific DNA sequences, leading to expression of genes encoding enzymes of the Raper–Mason pathway, among others. These enzymes are tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1), and dopachrome tautomerase (DCT).

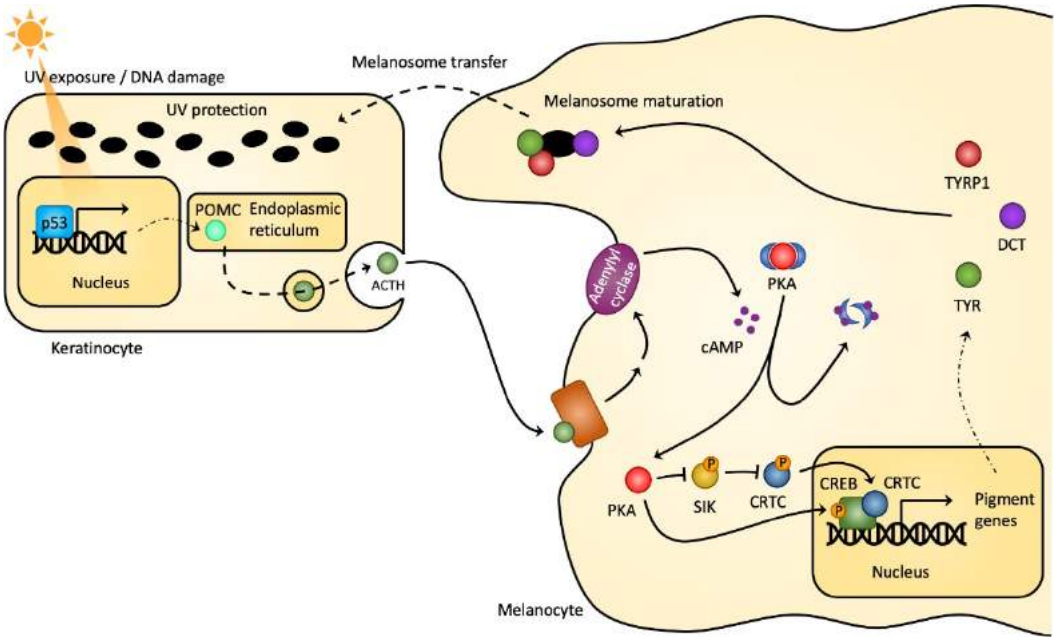


Fig. A5 Regulation of production of melanins.

Question A3. 4 *Decide whether the following statements are true based on information in the text and figures above. Choose all true statements and indicate them by a tick (√) in the table in the Answer sheet (select 0–10 answers). (5 points)*

a) POMC is likely translated on ribosomes bound to rough endoplasmic reticulum.

b) Large-scale deletion in the gene encoding POMC in melanocytes would lead to hypopigmentation.

c) A gain-of-function (=activating) mutation in the gene encoding adenylyl cyclase in melanocytes would likely lead to hypopigmentation due to less eumelanin being produced.

d) A successful inhibition of SIK could lead to hyperpigmentation due to more melanins being produced.

e) An inhibition of cAMP binding to regulatory subunits of PKA would lead to hyperpigmentation.

f) A loss-of-function (=inhibiting) mutation in PKA in melanocytes would lead to hyperpigmentation.

g) A mutation causing a substitution of the serine in CRTC that is phosphorylated by SIK for cysteine, which cannot be phosphorylated by SIK, would lead to hypopigmentation due to less eumelanin being produced.

h) The cAMP producing step of the signaling cascade in melanocytes could lead to signal amplification.

i) In case of a loss-of-function mutation in the gene encoding tyrosinase (TYR), eumelanin, but not pheomelanin would still be produced by melanocytes.

j) PKA activates SIK by phosphorylation.

Paracrine regulation of melanogenesis is not the only function of ACTH. It is also produced into the blood (endocrine secretion) by a specific population of cells in the anterior pituitary (adenohypophysis), and its main function is positive regulation of glucocorticoid production in adrenal cortex. However, in high concentration it can have the same effect on melanocytes in skin as described above. Adenohypophyseal ACTH secretion is regulated by at least two mechanisms: 1) positively by corticotropin-releasing hormone (CRH) from the hypothalamus, and 2) negatively by glucocorticoids from adrenal cortex through a negative feedback loop which also controls the secretion of CRH (Fig. A6).

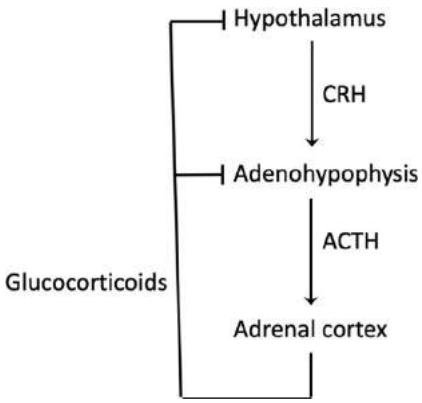


Fig. A6

Question A3.5 *Decide in which cases would you expect hyperpigmentation based on the text and scheme above. Choose all true statements and indicate them by a tick (√) in the table in the Answer sheet (select 0–4 answers). (2 points)*

a) After destruction of adrenal cortex by an autoimmune reaction
b) In case of a long-term medical administration of glucocorticoids
c) In case of an adrenal cortex tumor with glucocorticoid-producing activity
d) In case of a pituitary adenoma producing ACTH

Epidermal derivatives are also part of skin. They develop from epidermis, but are located within the dermis. These derivatives include hair, claws, and nails, amongst other things. Other important derivatives are skin glands, including sebaceous, aromatic, mammary, and sweat glands. In this section, we will turn our attention to the eccrine sweat gland. Pictured below is a scheme of the gland in the skin. It consists of a primary secretory part, sometimes called secretory coil, located in the dermis, and a secretory duct which leads the sweat to the epidermal surface but also reabsorps ions, resulting in the definitive sweat usually being hypotonic to interstitial fluid and blood plasma. The structure of both parts of the sweat gland is more complex, but for the purpose of this task assume both are formed by a single layer of epithelial cells.

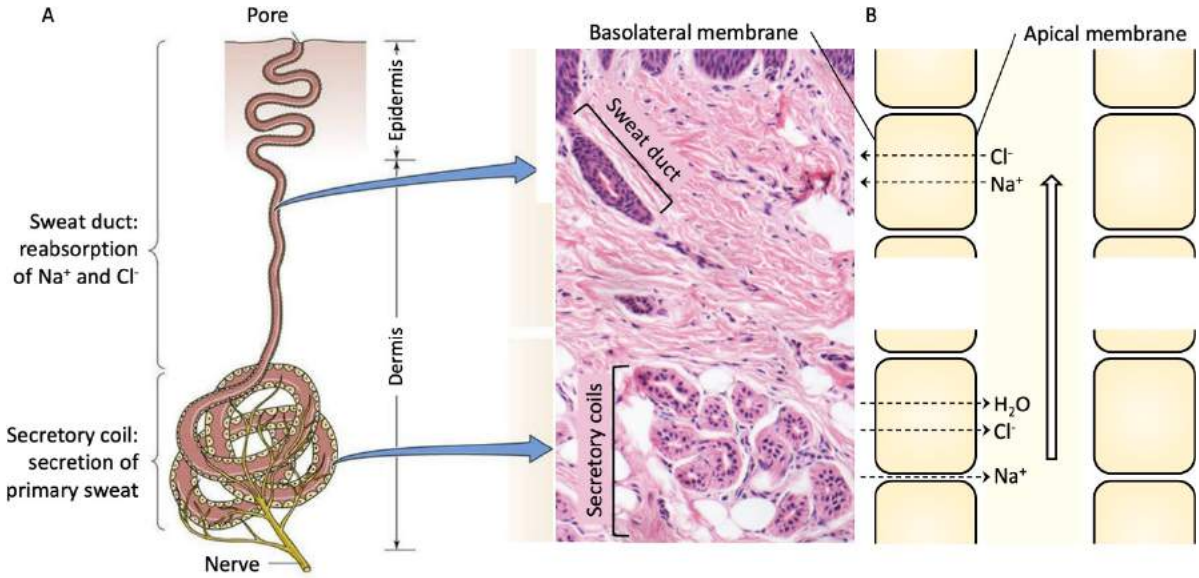


Fig. A7 Structure of eccrine sweat gland.

In the basolateral membrane of secretory coil cells, the protein Na^+/K^+ ATPase uses energy from ATP to transport Na^+ ions from the cytosol to the extracellular fluid, producing a transmembrane gradient of this ion. Sweat production begins when nerve stimulation activates cotransport (symport) into the cell through the basolateral membrane of Cl^- ions with Na^+ ions, utilising the mentioned gradient of Na^+ as source of energy. Cl^- ions then leave the cell through channels in the apical membrane into the lumen of the secretory coil, followed by Na^+ ions passing between the cells and water following the ionts osmotically through a different type of membrane channels called aquaporins. This leads to the production of isotonic primary sweat as compared to interstitial fluid or blood plasma (part B of Fig. A7).

Hypotonic sweat is then produced in the sweat duct by reabsorption of most Na^+ and Cl^- ions (Fig. 8). Na^+ enters the cells through the epithelial sodium channel (ENaC) on the apical membranes and exits into the surrounding tissue through the basolateral membrane, transported by the Na^+/K^+ ATPase. This creates the necessary electrochemical gradient for Na^+ reabsorption from the primary sweat. The excess K^+ transported by the Na^+/K^+ ATPase exits the cell through a K^+ channel in the basolateral membrane. The negative Cl^- ions follow the positive Na^+ ions from the lumen into the cells and exit into the surrounding tissue interstitium. Cl^- ions pass both the apical and basal membranes through a Cl^--specific channel called CFTR. However, water does not pass from the sweat duct throught plasmatic membranes of the lining cells because, unlike in the secretory coil, there are no aquaporins. The cells are connected by tight intercellular junctions, thus water cannot be reabsorbed neither through the cells nor around them, leading to the production of hypotonic sweat with relatively more water and less ions than the interstitial fluid or plasma membrane.

CFTR means **C**ystic **F**ibrosis **T**ransmembrane conductance **R**egulator. As the name suggest, it is a mutation in the gene encoding this specific protein that causes cystic fibrosis. This channel is found in many other epithelia throughout the body, including the lining of airways and the pancreatic ducts, where the mutation causes the most severe symptoms of cystic fibrosis. In sweat glands, a malfunction of CFTR results in less Cl^- being reabsorbed from sweat. This causes lower reabsorption of Na^+ as well because the negative charge of Cl^- cannot compensate its positive charge, meaning it is much harder to transport it across the membranes. Lower overall reabsorption of both Na^+ and Cl^- means higher concentration of NaCl in the definitive sweat. It is this salty taste of skin that is often noticed first in children with cystic fibrosis, usually by their parents when kissing them.

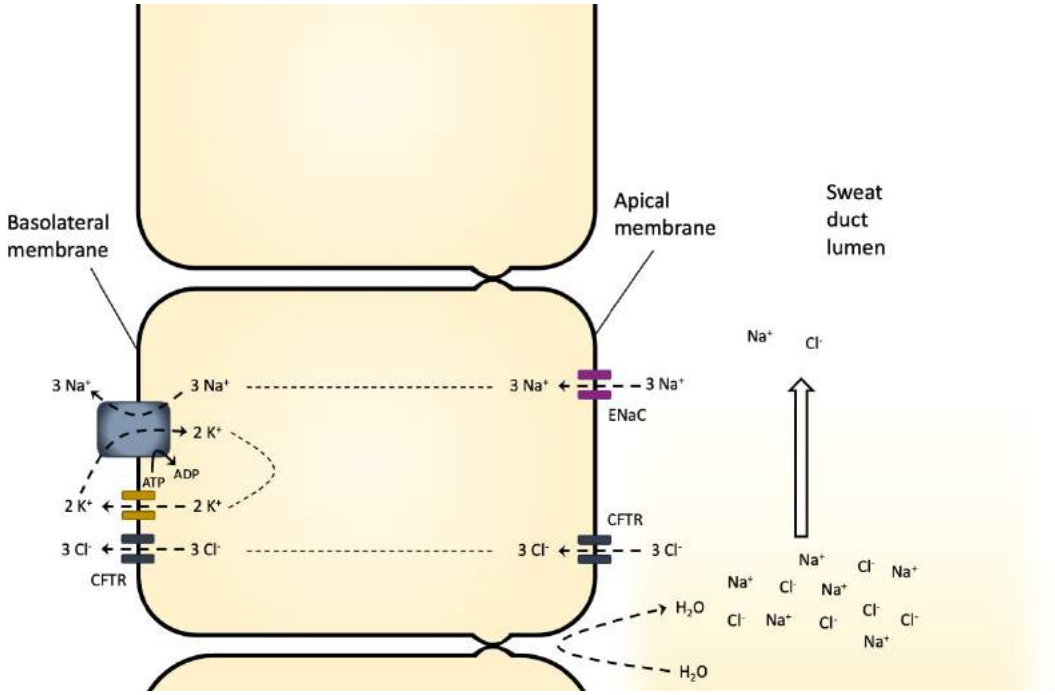


Fig. A8 Scheme of sweat duct ion reabsorption.

Question A3.6 *Based on the information above, which of the following statements are true? Indicate them by a tick (√) in the table in the Answer sheet (select 0–9 answers). (4.5 points)*

a) Cl- ions flow across the apical membrane of the epithelial cells into the lumen of the secretory coil following their electrochemical gradient.

b) Reduced function of the Na+/K+ ATPase in secretory coil cells would likely lead to hypertonic sweat production.

c) Reduced function of the Na+/K+ ATPase in sweat duct cells would lead to hypertonic sweat production.

d) In a healthy individual, it would be possible to encounter interstitial (blood plasma) Na+ concentration of 70 mmol/l and Na+ concentration in definitive sweat of 140 mmol/l

e) *In an individual with cystic fibrosis, Cl- concentration in definitive sweat can be expected to be up to twice as high as in the blood plasma/interstitial fluid.*

f) *Transport of Na+, Cl-, and water into the lumen of the secretory coil is directly or indirectly dependent on ATP in the epithelial cells surrounding the lumen.*

g) *Reabsorption of Na+ and Cl- from the primary sweat in the sweat duct is directly or indirectly dependent on ATP in the ductal epithelial cells.*

h) *The concentration of Na+ ions in the interstitial fluid or blood plasma is higher than in the cytosol of the epithelial cells lining the secretory coil.*

i) *The concentration of K+ ions in the interstitial fluid or blood plasma is lower than in the cytosol of the epithelial cells lining the sweat duct.*

Question A3.7 *When we are not sweating, the skin acts as a barrier preventing water loss. This function is ensured by the presence of hydrophobic contents of granules produced by cells in stratum granulosum and by sebaceous glands producing hydrophobic secretions onto the surface of the skin. However, naked mole-rats are adapted to living in relatively stable, warm, and very humid burrows their whole life. They completely lack both sweat glands and sebaceous glands, and the aforementioned granules of epidermal cells seem to contain much smaller amounts of hydrophobic products, especially in the skin on their abdomens. Which of the following statements make sense for the naked mole-rat in terms of thermoregulation and regulation of water loss? Indicate them by a tick (√) in the table in the Answer sheet (select 0–4 answers). (2 points)*

a) *In above-surface conditions of relatively low humidity and mild temperature, the evaporative water loss through skin would be much higher in mouse than in the naked mole-rat*
b) *The naked mole-rat developed an adaptation for situations of low humidity when it lies down on its back exposing its belly.*
c) *In conditions of low temperature, naked mole-rats huddle together to reduce heat loss through conduction and evaporation*
d) *During the day, naked mole-rats move to deeper parts of their burrows with higher humidity and lower temperature*

Part B

Mechanical properties of hyaluronic acid

Hyaluronic acid has several interesting physical properties, for which it is used in medicine. It decreases friction, so it is injected into the osteoarthritic joints (e.g., in the knees), where it partially replaces the worn cartilages, and the joint can then move better. Due to its optical characteristics, hyaluronic acid is used during ophthalmic surgery. Another mechanical property, surface tension, is important for this application. One of the common uses of hyaluronic acid is also the filling of wrinkles in aesthetic medicine (as the so-called dermal filler). Hyaluronic acid binds water very strongly and can thus increase its volume.

In this task, you will study the above properties either on models or directly on hyaluronic acid. Hyaluronic acid chains can vary in length. A unit called dalton (Da) can be used for the length specification. Dalton is de facto a unit of molar mass and is numerically identical to $g \cdot mol^{-1}$.

List of equipment for Task B

- Small beaker
- Five samples of water-binding material in plastic bag
- Caliper
- Weight scales
- Petri dish
- 3D-printed inclined plane with rectangular block
- 500 kDa hyaluronic acid in the syringe
- Syringe for distilled water
- Stopwatch

IMPORTANT: You will use the same hyaluronic acid with molar mass 500 kDa as in the task C1! It is important to use it in Task C1 BEFORE tasks B2 and B3!

Task B1: Water binding properties (20 points total)

IMPORTANT: This task is designed for three and a half-hour. You will repeat the measurement according to the Table in the Answer Sheet, and you will have the last half hour to process the data. Therefore, you should start Task B1 now and solve Task B2 and Task B3 in the meantime.

In aqueous solutions, hyaluronic acid forms specific stable tertiary structures, which allows it to bond with large amounts of water. For simplicity, you will use a material model that binds water like hyaluronic acid, only faster in this task.

Physics describes reality by making models. One very commonly used model is an *exponential* one. When the hyaluronic acid binds water, it can first bind water a lot and, over time, less and less.

In the exponential models, we can introduce a quantity known as *half-time*. During this time, the amount of water that hyaluronic acid can absorb is halved. You can see this in Fig. B1.

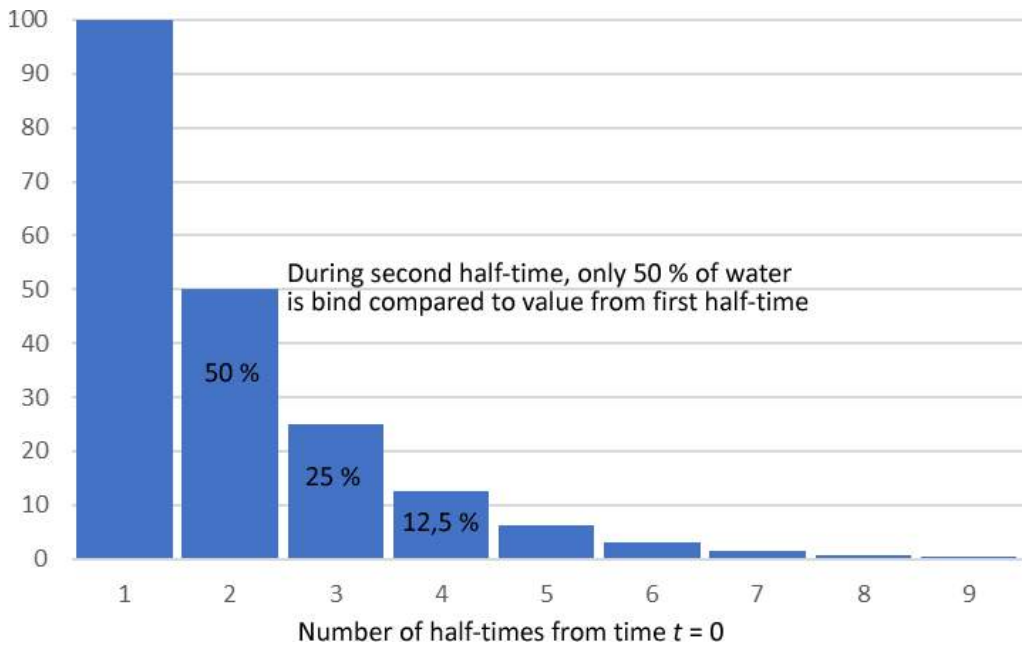


Fig. B1 Example of half-times.

Question B1.1 *According to Fig. B1, draw Graph B1 to the Answer Sheet of total water bound by the hyaluronic acid as the function of time (let the first increase be 1.0 unit, the second 0.5 unit and so on). Express time in half-times like in Fig. 1, use data points, not bar chart. (4 points)*

You will find material samples in the plastic bag. Measure its diameter with a caliper (you can measure just one sample). The mass of one sample in the dry state is 2.0±0.1 mg. Pour approximately 50 ml of distilled water into the beaker, throw in the samples, and write down the time. Then you will measure the diameter of the sample at the given times and use mass scales to determine its mass. You can use the timer. Fill in the results in the Table in the Answer Sheet.

IMPORTANT: Be careful when measuring the diameter because the sample is very fragile due to water content, and the sharp edge of the caliper can easily cut into it. Measure with the precision of tenths of a millimeter.

Question B1.2 *Based on the measurements and data in Table B1, draw Graph B2 as the time dependence of the sample diameter and Graph B3 as the time dependence of weight. Use data points, not bar chart. (12 points)*

Question B1.3 *Conclude if the exponential models described the water binding in the sample correctly and estimate the half-time for the weight of the sample. (4 points)*

TASK B2: Surface tension of hyaluronic acid (10 points)

Surface tension is the tendency of liquid surfaces at rest to shrink into the minimum surface area possible (e.g., to minimize so-called surface energy). For these reasons, the bubbles have the shape of a sphere. Hyaluronic acid is used in ophthalmology to treat glaucoma to adjust intraocular pressure. In this case, the surface tension of hyaluronic acid is essential. You can also consider that this quantity may not be correlated with the viscosity that is measured in Task C1. In this task, you will measure the surface tension on the hyaluronic acid using the stalagmometric (drop) method.

If a mass of liquid is stretched, the formation of drops occurs. If you imagine a dripping faucet (see Fig. B2), the water in the faucet is gaining mass until it is stretched to a point where the surface tension can no longer keep the drop to the faucet. The drop then separates, and surface tension forms the drop into a sphere.

Fig. B2 Formation of drop on the dripping water faucet.

Surface tension γ as physical quantity is defined either as force per unit length, or as energy per unit area:

$$\gamma = \frac{F}{l} = \frac{E}{S'}$$

so the unit is either $\mathrm{N \cdot m^{-1}}$ or $\mathrm{J \cdot m^{-2}}$. If you slowly push water from the syringe, you will see a drop forming at the outlet (needle adapter). A force of gravity acts on the drop downwards, but the surface forces keep the drop at the outlet of the syringe. When the force of gravity overcomes the surface forces, the drop separates from the syringe.

At that moment, this equation holds:

$$\gamma = \frac{mg}{\pi d},$$

where m is the mass of the drop, d = 2.4 mm is the diameter of the outlet (needle adapter) and g = 9.8 $m \cdot s^{-2}$ is acceleration due to gravity. Because the mass of one drop is usually very small, it is reasonable to count e.g., 50 drops, measure the mass of these 50 drops, and calculate the mass m of one drop.

Question B2. 1a *You will measure two samples: A solution of 500 kDa hyaluronic acid (Sample 1) and distilled water (Sample 2). (6.5 points)*

The procedure:

1) *Place an empty Petri dish on the digital weight scales. If you turn on the scales after placing the Petri dish on it, the scales will take the dish weight into account and show 0.00 g. If you put the Petri dish on the scales after, you must press the "TARE" button to reset the scale.*
2) *Slowly squeeze the drops of hyaluronic acid into a Petri dish and count them. Once you count 50 drops, write down the weight on the Answer Sheet.*
3) *You will repeat this procedure five times for Sample 1 (500 kDa hyaluronic acid) and five times for Sample 2 (distilled water). After every measurement, draw Sample 1 back to the syringe – you have only 10 ml of Sample 1, and you will need it for Task B3 also!*

Question B2.1b *Determine the observation error as the arithmetic mean of the deviations of individual measurements. (2.5 points)*

As the first step, you will write down the calculated values in the tables (Question B2.1.). Then you determine the absolute deviations of individual measurements $\Delta\gamma$ by calculating the absolute value of the difference between the calculated arithmetic mean $\bar{\gamma}$ and the individual measurements:

$$\Delta\gamma = |\,\bar{\gamma} - \gamma\,|.$$

So for the first measurement, we calculate the absolute value of the arithmetic mean minus the first measured value. For the second measurement, we calculate the absolute value of the arithmetic mean minus the second measured value and so on.

The final value of the absolute error $\Delta\bar{\gamma}$ is the arithmetic mean of the individual absolute errors from the last column of the tables in Question B2.1. Round this deviation to one significant digit and round the arithmetic mean to the same number of decimal places as the absolute error. Write down the measured value in the form of $\gamma = (\bar{\gamma} \pm \Delta\bar{\gamma})$.

Question B2.2 *(1 point)*

Decide according to your measurement and circle the right answer in the Answer sheet:
a) The surface tension of hyaluronic acid solution is higher than water.
b) The surface tension of hyaluronic acid solution is lower than water.
c) We cannot decide if this is case a) or case b).

TASK B3: Lubrication properties of hyaluronic acid (20 points total)

As was mentioned before, hyaluronic acid binds well to water, producing a viscous fluid. This viscous fluid provides lubrication and acts as a shock absorber within the joint, reducing inflammation caused by wearing the cartilage and bone in osteoarthritic joints.

The physical quantity that describes two surfaces just before they start to move on each other is the *static friction coefficient*, which you will measure in this task. For the friction force, we have the Coulomb-Amonont law of dry friction:

$$F_{\$} = fN,$$

where f is the coefficient of static friction and N is the normal force (force perpendicular to the surface). This friction force always acts in the opposite direction to that in which the block is pulled or pushed.

When an object rests on an incline that makes an angle α with the horizontal plane, the force of gravity $F_{\%}$ acting on the object is divided into two components: A normal component $F_{\#}$ acting perpendicular to the plane and a component acting parallel to the plane, which we denote here $F_{"}$ (see Fig. B4).

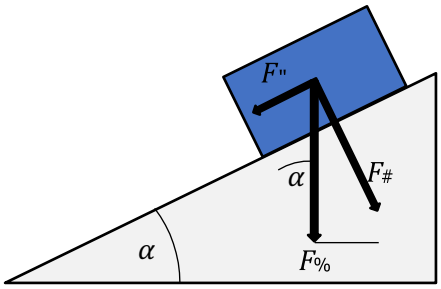


Fig. B4 Inclined plane.

If we put the block on the inclined plane and gradually increase the angle α, at the beginning, the block will not move (because the static friction force $F_{\$}$ is increasing as the component $F_{"}$ of the force is increasing). However, if we exceed the maximum angle $\alpha_{\&'(}$, the block begins to move down the inclined plane. For this angle, the following equation holds:

$$f = \tan\alpha_{\&'(}.$$

Question B3.1 (2 points)

Derive the equation $f = \tan\alpha_{\&'(}$ and explain what happens in terms of the forces acting on the block (draw picture if necessary).

Now you will measure the coefficients of static friction with the apparatus in Fig. B5. You can adjust the angle α of the inclined plane.

IMPORTANT: If the joint of the inclined plane comes loose, you can **gently** tighten it.

The block you have has two sides: one smooth and one rough. The inclined plane has only a smooth upper side. You will measure the coefficient of dry friction for these conditions:

1. The dry, rough side of the block on the smooth side of the inclined plane.
2. The dry, smooth side of the block on the smooth side of the inclined plane.
3. The rough side of the block lubricated with hyaluronic acid solution on the smooth side of the inclined plane.
4. The smooth side of the block lubricated with hyaluronic acid solution on the smooth side of the inclined plane.

For the measurement of coefficients of dry friction on lubricated surfaces use the syringe from the Task B2, dilute this sample 1 : 1 with distilled water using a beaker or Petri dish and draw back into the syringe. Then drip few drops of hyaluronic acid solution onto the the inclined plane, smear it and then put the block on.

IMPORTANT: It is crucial to set the angle as the first step, and then put the few drops on the top of the inclined plane and then put the block on it. When searching for the $\alpha_{\&'(}$, always put new drops on the top of the plane before each attempt!

Question B3.2 *Fill the Table in the Answer Sheet for these conditions (6 points):*

1. *The dry, rough side of the block on the smooth side of the inclined plane.*
2. *The dry, smooth side of the block on the smooth side of the inclined plane.*

Find the angle $\alpha_{\&'(}$ for which the block will start to move. In each case, adjust the angle five times and use the length of the inclined plane l and height h to calculate the angle $\alpha_{\&'(}$ and the corresponding coefficient of dry friction f.

Question B3.3 *Calculate the coefficient of the static friction for conditions from Question B3.2, calculate the observational error using the same procedure as in Task B2. (2 points)*

Question B3.4 *Fill the Table in the Answer Sheet for these conditions (6 points):*

3. *The rough side of the block lubricated with hyaluronic acid on the smooth side of the inclined plane.*
4. *The smooth side of the block lubricated with hyaluronic acid on the smooth side of the inclined plane.*

Find the angle $\alpha_{\&'(}$ for which the block will start to move. In each case, adjust the angle five times and use the length of the inclined plane l and height h to calculate the angle $\alpha_{\&'(}$ and the corresponding coefficient of dry friction f. C*alculate the observational error using the same procedure as in Task B2*

Question B3.5 *Calculate the coefficient of the dry friction for conditions from Question B3.4, calculate the observational error using the same procedure as in Task B2. (2 points)*

Question B3.6 *Express as a percentage how much the coefficient of friction has decreased in 1., 3. and 2., 4. from Questions B3.3 and B3.5 if the parts have been lubricated with hyaluronic acid. (2 points)*

Fig. B5 Inclined plane apparatus.

Part C

Task C1: Viscosity of polymer solution (19 points)

IMPORTANT: You will use the same hyaluronic acid with molar mass 500 kDa as in the task B2 and B3! It is important to use it in Task C1 BEFORE tasks B2 and B3!

The falling ball and a lengthy polymer

The Czech Republic counts as the world's producer of hyaluronic acid polymer and its medically applicable derivatives. Hyaluronic acid is polysaccharide consisting of molecules of D-glucuronic acid and *N*-acetyl-D-glucosamine connected by glycosidic bond, which form a repetitive structural unit of a polymer.

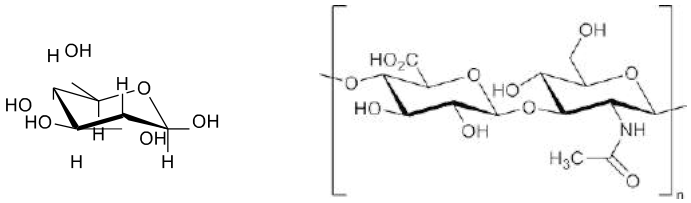


Fig. C1 Structural formula of glucose (left) and hyaluronic acid (right). Hydrogen atoms attached to carbons are omitted in hyaluronic acid for clarity.

Hyaluronic acid chains can vary in length. A unit called dalton (Da) can be used for the length specification. Dalton is de facto a unit of molar mass and is numerically identical to $g \cdot mol^{-1}$.

Another way to express the length of a chain can be stating the number of structural units in the chain (value of *n* from Fig. C1). This is called degree of polymerization and is dimensionless.

Question C1. 1 *How many carbon atoms are in a structural unit of hyaluronic acid? Calculate the degree of polymerization of hyaluronic acid chain weighting 150 kDa (kilodalton). Write down the results into the Answer sheet. Molar masses: M(H) = 1 $g \cdot mol^{-1}$, M(C) = 12 $g \cdot mol^{-1}$, M(N) = 14 $g \cdot mol^{-1}$, M(O) = 16 $g \cdot mol^{-1}$. (1.5 points)*

List of chemicals for Task C1

- 10ml syringes with 2% hyaluronic acid solutions in water and metal ball:
  - one syringe: 500, 970, 1610 kDa hyaluronic acid
  - five syringes: 1900 kDa hyaluronic acid
- 2% solution of H_2O_2, 5 ml in a vial
- 0.02M solution of $ZnSO_4$, 5 ml in a vial
- 0.02M solution of $Fe(NO_3)_3$, 5 ml in a vial

List of equipment for Task C1

- 5 empty 20ml syringes
- 5 lock-lock syringe connectors
- holder for syringes (beakers or tumblers, 3 pieces)
- ruler
- marker
- 1 stopwatch
- 1 wall clock
- wash-bottle with distilled water

Viscosity of solution

The viscosity of solution, η, can be determined from speed of a ball falling (moving) through the solution using Stokes law. For laminar flow around a sphere it gives:

$$F_r = 6\pi \cdot \eta \cdot r \cdot v$$

where F_r is the resistance force (Stokes drag), π is pi (Archimedes constant), r the radius of the ball and v is speed of the ball. In our experiment, the speed of the falling ball will be small and more-less constant. Therefore, speed of the ball can be simply calculated from length of the path and time of the fall. Due to a slow movement of the ball, it is reasonable to assume that all forces acting on the ball are balanced.

The relationship between the molecular weight of a dissolved polymer and the viscosity of a solution is described by semi-empirical Mark-Houwink equation:

$$[\eta] = K \cdot M^{a}$$

where $[\eta]$ is the contribution of the dissolved polymer to viscosity of the whole solution (intrinsic viscosity), M is molar mass in suitable unit (usually in kDa, kilodaltones), and K and α are empirical constants. As the viscosity contribution of other species to the viscosity of the whole solution is negligible and as the concentration of the hyaluronic acid is identical in all syringes, we can assume that intrinsic viscosity of hyaluronic acid equals the viscosity of the whole solution.

Polymer degradation

Some bonds in long chain of polymer can be split under certain conditions. Such degradation can occur by different mechanisms. They are for example oxidative treatment (bond splitting by reaction with oxidation agent, e.g. O_2, H_2O_2, NaClO...), simple hydrolysis (reaction with water molecule), proton- or hydroxide-assisted hydrolysis (reaction with water molecule after preceding attack of H^+ or OH^- ions present in acid/alkaline media), metal-assisted hydrolysis (coordination of the metal ion to the polymer backbone can influence ability of some bonds in the chain for water/hydroxide ion attack; e.g. Zn^{2+} is utilized by some peptidases – enzymes hydrolysing peptides), etc.

Procedure

Viscosity measurement

In front of you, there are 4 syringes filled with 2.0% wt. hyaluronic acid solution in water labelled with molar mass of used polymer (500 kDa – white tip, 970 kDa – blue tip, 1610 kDa – red tip and 1900 kDa – blue-white tip) containing a steel ball.

Question C1.2 *If you flip the syringe over, the ball will fall. The time of fall is dependent on the viscosity of the solution, which is affected by the polymer chain length, as described above. Measure the time of fall of the steel ball in each syringe. You can repeat the measurement couple of times to obtain reproducible result. Write down the accepted times (t) into the Answer sheet. Measure the trajectory length of the balls (l) in individual syringes (should be approximately the same) and write down the values into the Worksheet. Calculate speed of the balls in individual solutions and write down the results into the Answer sheet. (1 point)*

Question C1.3 *Based on your results, calculate viscosity of individual solutions. Density of studied solutions is 1.0 $g \cdot cm^{-3}$, density of steel ball is 7.5 $g \cdot cm^{-3}$, diameter of the ball is 5.0 mm, gravitational acceleration is 9.8 $m \cdot s^{-2}$. Write down the viscosity values in $N \cdot s \cdot mm^{-2}$ into the Answer sheet and transfer the values to Pa·s units. (6.5 points)*

Question C1.4 *Using graph paper, try to determine empirical constants K and α by interpolation of your data. Find a way to linearize the Mark-Houwink equation, so the data could be fitted by a straight line. Use values of viscosity in Pa·s units. Choose suitable ruler of the graph. (3 points)*

Polymer degradation

Polymeric hyaluronic acid can be split in smaller fragments. You will test several methods suggested for such degradation. The proposed conditions/solutions to be tested are:

a. Oxidative treatment with H_2O_2 (*approx 1 ml of 2% H_2O_2 and approx 1 ml of water).*

b. Metal-assisted hydrolysis using $Fe(NO_3)_3$ (*approx 1 ml of 0.02M $Fe(NO_3)_3$ and approx 1 ml of water).*

c. Metal-assisted hydrolysis using $ZnSO_4$ (a*pprox 1 ml of 0.02M $ZnSO_4$ and approx 1 ml of water).*

d. Combined treatment with H_2O_2 and $Fe(NO_3)_3$ (*approx 1 ml of 2% H_2O_2 and approx 1 ml of 0.02M $Fe(NO_3)_3$).*

e. Combined treatment with H_2O_2 and $ZnSO_4$ (*approx 1 ml of 2% H_2O_2 and approx 1 ml of 0.02M $ZnSO_4$).*

For the measurement use five syringes with the polymer of the highest molecular mass (1900 kDa) – the one used for the previous experiment and other for nonlabeled syringes with the blue-white tip. To assure comparability of results, each polymer solution should be diluted with the same volume of selected agent. The volume of the agent will be measured using empty syringe in which the

individual solutions (a.– e. in Question C1.5) will be mixed, and the reagent will be mixed with the polymer solution using following procedure: connect the syringe with the reagent solution to the syringe with hyaluronic acid solution via lock-lock syringe connector. Mix the solutions by transporting the solutions from one syringe to the other for approx. 100 s. Start to measure the ball falling time with the stopwatches. If the ball does not move at all, it may get stuck to a wall of the syringe. Gentle pressing of the piston should help to start it. If this does not help, continue with mixing for ca 30 s, then repeat the measurement of falling time. Repeat the measurement each 3–5 min to obtain at least 5 data points for each mixed solution. Notice start of each of your experiments according to the wall clock. Do not disconnect the syringes.

Question C1.5 *Measure effect of individual reagents (conditions a–e) as simultaneously as possible to obtain as much data in less total time.*

Composition of reagent solutions is following:

a. Mix approx 1 ml of 2% H_2O_2 and approx 1 ml of water.

b. Mix approx 1 ml of 0.02M $Fe(NO_3)_3$ and approx 1 ml of water.

c. Mix approx 1 ml of 0.02M $ZnSO_4$ and approx 1 ml of water.

d. Mix approx 1 ml of 2% H_2O_2 and approx 1 ml of 0.02M $Fe(NO_3)_3$.

e. Mix approx 1 ml of 2% H_2O_2 and approx 1 ml of 0.02M $ZnSO_4$.

Write down times of ball fall in the Answer sheet. Notice starting time of your experiments. (5 points)

Question C1.6 *Choose from the reagents a–e the most suitable one for cracking the hyaluronate chains. Is the reaction mechanism electrophilic, nucleophilic, or radical? Justify your statement. (2 points)*

a) Electrophilic activation of the molecule of hydrogen peroxide before nucleophile attack of the sugar unit.

b) Catalytic role of the metal ion on peroxide decomposition – formation of HO· and HOO·radicals.

c) Electrophilic activation of the glycoside bond by coordination to the metal ion before nucleophilic attack of hydrogen peroxide.

d) Electrophilic activation of the glycoside bond by coordination to the metal ion before nucleophilic attack of water molecule.

e) Catalytic splitting of hydrogen peroxide to HO· radicals due to intermolecular hydrogen bonds formation with neighbouring hydroxide group after forming a cyclic intermediate.

Task C2 (16 points)

Volumetric determination of concentration and dissociation constant of D-glucuronic acid

Hyaluronic acid polymer is formed by a repeating disaccharide unit consisting of D-glucuronic acid and *N*-acetyl-D-glucosamine. Structural formula of D-glucuronic acid is shown on Fig. C2.

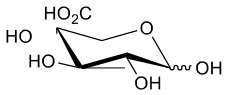


Fig. C2 Structural formula of D-glucuronic acid.

Concentration of glucuronic acid can be easily determined by acid-base titration. Moreover, a dissociation constant of the acid can be determined when using a suitable experimental setup.

List of chemicals for Task C2

- ca 0.01M NaOH
- ca 0.01M oxalic acid (exact concentration provided by lab assistants)
- sample solution of D-glucuronic acid
- phenolphthalein

List of equipment for Task C2

- 2 titration flask, 250ml
- burette, 25ml (+stand and holder)
- pipette, 10ml
- automatic pipette with tip, adjustable 1–5ml (can be common for few neighbouring groups)
- 2 vials with screw top, 20ml
- beaker, 250ml
- wash-bottle with distilled water
- pipetting balloon
- funnel
- pH-meter (for several groups)

Acid-base titration

During acid-base titration, an amount of the acid present in the sample is determined using titration with volumetric solution of the base (alkalimetry), or, vice versa, an amount of the base in the sample can be determined employing volumetric solution of the acid (acidimetry). In this Task, you will perform alkalimetric determination of concentration of organic acid with utilization of

phenolphthalein as an acid-base indicator. As a volumetric (titration) agent, solution of sodium hydroxide, NaOH, will be used.

Acid-base indicator is generally a compound, which behave as acid or base (i.e. its molecule can be protonated or deprotonated during the titration), and differently protonated forms thereof have different colours. In the case of titration of organic acid (usually weak) with strong alkaline hydroxide, the equivalence point lies in weakly basic region. [In equivalence point, a molar amount of the acid protons is equal to a molar amount of the base hydroxide ions, i.e., formally, a solution of the salt is formed. However, the solution needs not to be neutral (pH can differ from 7), as concentrations of H_3O^+ and OH^- ions need not be equal due to cation or anion hydrolysis.] Therefore, phenolphthalein is an appropriate indicator, as it shows colour change at pH ≈ 8.5–10. In the case of phenolphthalein, forms present in acid and basic solution have structures shown in Fig. C3:

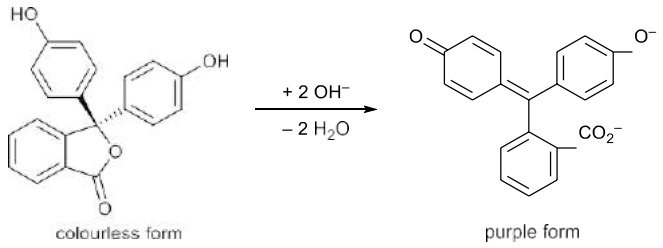


Fig. C3 Forms of phenolphthalein molecule in dependence on solution pH.

Determination of exact concentration of the volumetric solution of NaOH

Some substances form stable and well-defined solid phase, and their concentration can be derived from exact weight of the compound and from the total volume of its solution. Such substances are called “primary standards”. Unfortunately, solid NaOH cannot be used as a primary standard, because it is hygroscopic, and its surface is contaminated by water/moisture and some carbonate. Exact concentration of the stock solution of NaOH thus cannot be defined on the basis of the weight. Therefore, it has to be determined using so called factorization titration. For the purpose, oxalic acid can be used, as its dihydrate is well-defined in the solid state, $(CO_2H)_2 \cdot 2H_2O$.

During factorization of NaOH, following neutralization reaction occurs:

$$(CO_2H)_2 + 2\ NaOH \longrightarrow (CO_2Na)_2 + 2\,H_2O$$

When all oxalic acid is consumed, the first drop of NaOH titration solution steeply increase a value of pH and titrated mixture will change its colour from colourless to purple due to a phenolphthalein content.

T1

Acid-base equilibria

Arrhenius (and Brønsted) acids in aqueous solution dissociate according to equation:

$$HA + H_2O \rightleftharpoons H_3O^+ + A^-$$

(shortly also $HA \rightleftharpoons H^+ + A^-$)

For the equilibrium, corresponding *dissociation constant* K_A can be defined:

$$K_A(HA) = \frac{[H^+]\cdot[A^-]}{[HA]}$$

Values of dissociation constants can be of very different orders and, therefore, for practical reasons, they are presented in the form of negative common (decimal) logarithms, $-\log K_A = pK_A$. If the acid is in the solution almost fully dissociated, i.e. equilibrium in the equation written above is significantly shifted to the products H^+ and A^-, it is obvious, that $K_A >> 1$, and value of pK_A is negative. The strongest acids are e.g. $HClO_4$ and HI, whose pK_A is ≈ −10. Contrary, acids, which are dissociated only in small extent – i.e. the equilibrium shown above is shifted towards non-dissociated reactant HA – have $K_A << 1$, and the value of their pK_A is positive. Because of a huge number of different acids, the scale of pK_A values is continuous. Acids with $pK_A < 2$ are called strong, acids with pK_A in the range 2–4 are medium-strong, in the range pK_A = 4–9 are weak, and with $pK_A > 9$ are very weak. The lower value of pK_A is, the stronger acid is and, thus, higher degree of dissociation in the solution is.

By logarithming of the definition of dissociation constant we get:

$$\log K_A(HA) = \log[H^+] + \log[A^-] - \log[HA]$$

and, thus:

$$pH = pK_A + \log\frac{[A^-]}{[HA]}$$

This equation is called *Henderson-Hasselbalch equation*. From the relationship, it is obvious that if concentrations of dissociated and non-dissociated forms are equal, their ratio equals to one, and the logarithmic term in the equation is equal to zero. In such a situation, $pH = pK_A$.

Organic acids are usually weak acids. In such case, the concentration of dissociated form A^- coming from direct dissociation of HA if negligible compared to concentration of non-dissociated form HA. When a base is gradually added into the solution of the acid HA, the species A^- is generated only in the amount corresponding to molar amount of the added base, according to equation:

$$HA + OH^- \longrightarrow H_2O + A^-$$

i.e. $n(A^-) = n$(added OH^-). Condition $[HA] = [A^-]$ is thus fulfilled in the case, when exactly one half of the total amount of base needed for neutralization is added into solution of the acid (i.e. one half of acid is neutralized to salt A^-, and the second half of the acid remains in the form HA). For weak acids (**attention, only! for weak acids**), the following relationship is thus valid:

$$pK_A = \text{pH at consumption of } ½V(\text{eq.})$$

Typical course of alkalimetric titration of weak acid is shown on Fig. C4 together with corresponding distribution diagram of non-dissociated acid and its anion. When a strong base is added into a solution of the weak acid, gradual neutralization occurs and pH of the solution is slowly increased. The concentration of A^- is increased adequately. This part of the titration curve is called "buffering region" – the dissolved material buffers (moderates) – i.e. with addition of the titration agent (in general acid or base) is pH of the mixture changed only slightly. Close to the *equivalence point* – i.e. point, where added molar amount of the base equals to starting molar amount of the acid – a slope of the titration curve is increased significantly. In region with a large excess of the base, the pH is changed again only slowly. It can be seen in the distribution diagram, that at pH = pK_A the concentrations of HA and A^- are equal.

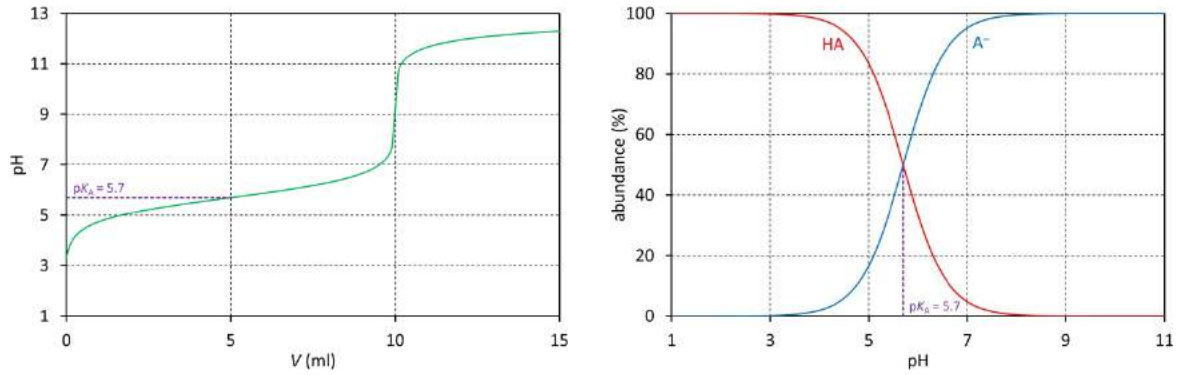


Fig. C4 Titration curve of titration of a weak monovalent acid HA with a strong base (left), and distribution of species HA and A^- in dependence on pH (right). Charts were simulated assuming pK_A = 5.7. For titration curve simulation, following conditions were defined: c(HA) = 0.1 M, V(HA) = 10 ml, titration solution was NaOH with c = 0.1 M. Equivalence point occurs at addition of 10 ml of titration agent.

Question C2. 1 *Value of pK_A of unsubstituted organic acids is ca 4.8 [e.g. pK_A(acetic acid) = 4.76, pK_A(propionic acid) = 4.88, pK_A(butyric acid) = 4.81].*

Mark the reason, why pK_A of D-glucuronic acid differs from those of mentioned organic acids into the Answer sheet. (1 point)

a) Electron withdrawing inductive effect of electronegative oxygen atoms bound to the α-carbon.

b) Electron donating inductive effect of free electron pairs of oxygen atoms from hydroxo groups present in the molecule.

c) Carboxylate deprotonation is stabilized by an intramolecular hydrogen bonding due to a presence of neighbouring hydroxo group.

d) Better delocalization of the negative charge in the anion of d-glucuronic acid when compared to mentioned examples.

e) Due to positive mesomeric effect of the hydroxo group bound to the β-carbon.

f) Due to a general Pauling-Bell rule of correlation of acidity with number of oxo/hydroxo groups present in the molecule.

Question C2.2 *In what pH region will solution of acetic acid work as the best buffer? Mark the right answer into the Answer sheet. (1 point)*

a) pH 0–2	*b) pH 2–4*	*c) pH 4–6*	*d) pH 6–8*	*e) pH 8–10*

Question C2.3 *In what pH region will lie pH of the 0.1M solution of sodium acetate? Mark the right answer into the Answer sheet. (1 point)*

a) pH 2–4	*b) pH 4–6*	*c) pH 6–8*	*d) pH 8–10*	*e) 10–12*

Procedure

Factorization of NaOH stock solution

The stock solution of NaOH has an approximate concentration ca 0.01 mol·dm^{-3}. You have also stock solution of oxalic acid with known exact concentration (ca 0.01 mol·dm^{-3} – exact concentration is given by lab assistants).

Fill the burette with the stock solution of NaOH. Use a small funnel; place a beaker under the burette. Remove the funnel after filling the burette and carefully lower a level of the stock solution in the burette to zero mark. Measure 10.0 ml of the oxalic acid stock solution using a pipette into a titration flask. Dilute the solution in the titration flask with ca 20 ml of distilled water and add 3–5 drops of indicator solution (phenolphthalein). Titrate the solution until first light purple colour, which will be stable for at least 5 s.

Question C2.4 *Write down the exact concentration of the oxalic acid stock solution into the Answer sheet. Write down the volume of consumed titration agent. Repeat the titration. In the case that results of both titrations differ by more than 0.2 ml perform the third titration. Write down consumed volumes of all titrations into the Answer sheet. Into the Answer sheet, write down accepted value of consumed volume (averaged consumption from individual titrations, or the value obtained with exclusion of outlying result). (3 points)*

Question C2.5 *Based on the accepted value of the consumed volume, calculate exact concentration of the NaOH stock solution. (1 point)*

Determination of concentration of D-glucuronic acid

Measure 2.50 ml of sample solution of D-glucuronic acid using an automatic pipette into the titration flask. Dilute the sample with ca 25 ml of distilled water and add 3–5 drops of phenolphthalein solution. Titrate the mixture with titration solution of NaOH according to procedure described above. Repeat the titration. In the case that results of both titrations differ by more than 0.2 ml perform the third titration.

Question C2.6 *Write down consumed volumes of all titrations into the Answer sheet. Into the Answer sheet, write down accepted value of consumed volume. (3 points)*

Question C2.7 *Based on the accepted value of the consumed volume, calculate concentration of the sample solution of D-glucuronic acid. (1 point)*

Determination of dissociation constant of D-glucuronic acid

Measure 2.50 ml of sample solution of D-glucuronic acid using an automatic pipette into two vials with screw top. Add appropriate volume of the NaOH stock solution using the burette (write the chosen volume into the Answer sheet). Choose the volume in such a way that pH of the final mixture will correspond to the value of pK_A of D-glucuronic acid. Close the vials and give them to a lab-assistant, who will measure pH of both solutions.

Question C2.8 *Write down chosen volume of NaOH stock solution and measured pH values into the Answer sheet and determine pK_A value of D-glucuronic acid. (5 points)*

Task C3 (15 points)

Spectrophotometric determination of concentration of *N*-acetyl-D-glucosamine

Hyaluronic acid polymer is formed by a repeating disaccharide unit consisting of D-glucuronic acid and *N*-acetyl-D-glucosamine. Structural formula of *N*-acetyl-D-glucosamine is shown on Fig. C5.

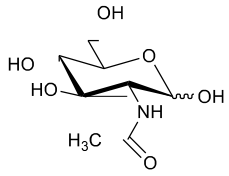


Fig. C5 Structural formula of *N*-acetyl-D-glucosamine.

Concentration of *N*-acetyl-D-glucosamine can be easily determined using spectrophotometry due to formation of a coloured products on reaction with 4-(*N*,*N*-dimethylamino)benzaldehyde (DMAB).

Spectrophotometry

Absorption spectroscopy in ultraviolet and visible region (UV-Vis region) studies an interaction of a matter with electromagnetic radiation in wavelength range ca 200–750 nm. The region can be divided to near ultraviolet part (200–400 nm, UV) and visible part of the spectra (400–750 nm, Vis). In the visible region, individual wavelengths correspond to different colours of the light (Fig. C6).

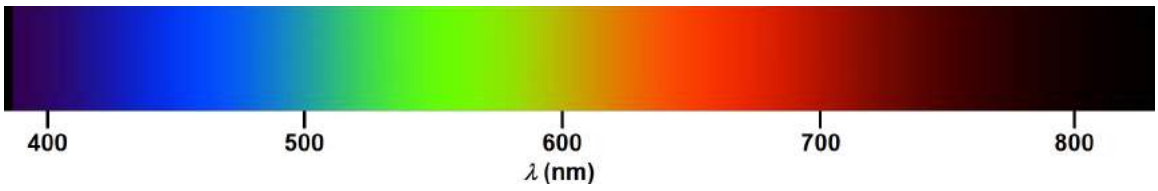


Fig. C6 Dependence of light colour on wavelength.

Absorption process in the UV-Vis region is associated with excitation of the molecule from the ground to the excited electronic state. UV-Vis absorption spectrum (dependence of absorption intensity on wavelength, frequency of the radiation or wavenumber) reflects an electronic structure of the molecule. If the compound absorbs photons from visible region, it is coloured, and it appears to our eyes in complementary colour to the colour of the absorbed light (Tab. C1).

λ (nm)	Colour	
	absorbed	complementary
400-435	violet	yellow
435-480	blue	orange
480-500	blue-green	red-orange
500-560	green	red
560-580	yellow-green	red-violet
580-600	yellow	violet
600-630	orange	blue
630-750	red	green

Tab. C1 Relationship between wavelength of the absorbed radiation and corresponding colour of the material.

Position of the absorption band is usually characterized by wavelength of maximum λ_{max} (in nm), by wavenumber $\tilde{\nu}_{max}$ (in cm^{-1}), or as frequency ν_{max} (in Hz). Relations between these quantities and photon energy E_{photon} are following:

$$\tilde{\nu} = \frac{\nu}{c} = \lambda^{-1}$$

and

$$E_{photon} = h \cdot \nu = h \cdot c \cdot \tilde{\nu} = \frac{h \cdot c}{\lambda}$$

where h is Planck constant (h = 6.626069·10^{-34} J·s) and c is speed of light in vacuum (c = 299792458 m·s^{-1}).

Question C3.1 *What is energy of photon of light used with λ = 585 nm for spectrophotometric measurement? Write the value into the Answer sheet as frequency (in Hz) as well as wavenumber (in cm^{-1}). (2 points)*

Change of intensity of light characterized by wavelength λ during passage through an absorbing material can be quantified by *absorbance*, *A*. Its relationship with concentration of the absorbing molecule in the sample and with length of the optical path (practically inner width of the cuvette) is expressed by Lambert-Beer law:

$$A(\lambda) = \varepsilon(\lambda) \cdot c \cdot l$$

where $\varepsilon(\lambda)$ is molar absorption (extinction) coefficient at given wavelength λ. Extinction coefficient is for given compound and chosen wavelength constant and is presented usually in units $dm^3 \cdot cm^{-1} \cdot mol^{-1}$. The c is molar concentration of the absorbing compound in the sample (in $mol \cdot dm^{-3}$) and l is optical path (in cm).

So, the Lambert-Beer law can be applied for quantification. Usually, at first, so-called *calibration line* is constructed, when values of absorbance are drawn versus known concentration of the solutions. Ideally, a line is obtained, which goes through origin of the Cartesian chart:

$A = k \cdot c$

Once absorbance of the sample with unknown concentration is measured, the concentration can be easily calculated from the equation of the calibration line or can be directly read from the graph.

List of chemicals for Task C3

- *N*-acetyl-D-glucosamine solution, $c = 1.00 \cdot 10^{-3}$ mol·dm^{-3}, 1 ml
- $Na_2B_4O_7$ solution, $c(Na_2B_4O_7) = 0.80$ mol·dm^{-3}, 5 ml (pre-heated solution common for several groups)
- 4-(*N*,*N*-dimethylamino)benzaldehyde reagent, ca 10% solution in 1:9 mixture of conc. HCl:conc. AcOH, 20 ml
- sample of *N*-acetyl-D-glucosamine solution of unknown concentration, 0.5 ml

List of equipment for Task C3

- automatic pipette with tip, adjustable 1ml (can be common for few neighbouring groups)
- automatic pipette with tip, adjustable 1–5ml (can be common for few neighbouring groups)
- magnetic hot plate with oil bath
- stand + holder
- 6 wires (paper clips)
- 6 vials with screw top, 4ml
- marker
- 6 plastic cuvettes
- beaker, 100ml
- wash-bottle with distilled water
- $Na_2B_4O_7$ solution, $c(Na_2B_4O_7) = 0.80$ mol·dm^{-3}, placed on magnetic hot plate and pre-heated to 80 °C (for several groups)
- Spectrophotometer (for several groups)

Procedure

During the Task you will construct spectrophotometric calibration line using four solutions with known concentration and a blank solution. In addition, you will prepare also sample of unknown concentration for spectrophotometric measurement. As the coloured product of reaction between *N*-acetyl-D-glucosamine with 4-(*N*,*N*-dimethylamino)benzaldehyde is not stable in time and its colour is developing, it is necessary to prepare all solutions for spectrophotometry simultaneously and all solutions should be measured during a short time (ca 10 min). Measurements should be performed at 15–60 min after standard/sample preparation.

Preparation of solutions for spectrophotometric measurements and data treatment

Prepare solutions of *N*-acetyl-D-glucosamine of known concentrations by defined dilution of a stock solution with concentration of $1.00 \cdot 10^{-3}$ mol·dm^{-3} according to Tab. C2. Use automatic pipette for measurement of required volumes, and mix the solutions in closable 4ml vials. Calculate concentrations of the standards 1–3 and write them into the Answer sheet.

standard	*c*(*N*-acetyl-D-glucosamine) [mol·dm^{-3}]	*V*(*N*-acetyl-D-glucosamine) [ml]	*V*(water) [ml]
0	0	0	0.400
1	?	0.100	0.300
2	?	0.200	0.200
3	?	0.300	0.100
4	$1.00 \cdot 10^{-3}$	0.400	0

Tab. C2 Dilution of the stock solution of *N*-acetyl-D-glucosamine.

Measure 0.400 ml of solution of the sample with unknown concentration into other closable vial.

Measure 0.150 ml of sodium tetraborate of concentration $c(Na_2B_4O_7) = 0.80$ mol·dm^{-3} pre-heated to 80 °C into the vials using automatic pipette. Close the vials and stir the content. Using a wire, hang the vials on a holder. Put the vials into an oil bath pre-heated to 95 °C by holder shift on a stand and let them heat for 10 min. After the period remove the vials from the bath and left them cool down to laboratory temperature. Open the vials and add 3.000 ml of DMAB agent solution (**attention!, the agent is dissolved in mixture of concentrated acetic and hydrochloric acids**) using automatic pipette. Close the vials, stir their content and left them for 15 min. After the period fill individual cuvettes for spectrophotometric measurements with the solutions and give them to the supervisors – they will measure absorbance of individual mixtures at 585 nm.

Question C3.2 *Write absorbance values into the Answer sheet. (8 points)*

Draw measured data points and construct a calibration line using a graph paper – choose suitable ruler. Absorbance of the standards were measured against pure water, therefore, absorbance of solution with zero concentration of *N*-acetyl-D-glucosamine (standard 0) can differ significantly from zero, as there are present also other absorbing species. Therefore, you can subtract the absorbance of standard 0 from absorbance of all other standards (and you will obtain the calibration line of formula $A = k \cdot c$). Alternatively, you can use uncorrected values. In such case you will obtain the calibration line with the constant term, i.e. in form $A = k \cdot c + l$. Both approaches are equivalent, it does not matter, which variant you will choose.

Question C3.3 *Read the concentration of the unknown sample from the calibration line chart. (5 points)*

T2

Task 2

DNA

12. 05. 2022
Hradec Králové
Czechia

Preface

To maintain the sustainability of the complex life forms, the continuous metabolic turnover is taking place – including the protein content. There must be an information storage media inside the cell and a way how to use it. Genetics as a scientific discipline dealing with the information and the organismal memory got a solid foundation by Gregor Johann Mendel, born just 200 years ago in small village Hynčice at the Moravian-Silesian border. Since the "Experiments on Plant Hybridization" published by Mendel in 1865 enormous effort was invested to understand the principles of genetics in a very detail, including the genomics, evolutionary genetics, molecular genetics or the link between genes and pathology.

Host-pathogen interactions are important drivers of evolution. These "star wars" escalating sophisticated strategies and contra-strategies on both sides are memorized in our genetic information. Without understanding evolution shaping the particular genome, we can´t understand it´s meaning, because in some aspects it simply does not make sense.

Important pathogens (and extremely important from the evolutionary point of view) are the viruses with the only way how to "survive" – infect the cell and highjack it´s metabolism to copy and amplify the new virions. Luckily, viruses can´t hide 100% from the body security having own proteins (which could be recognized by the immune system) and nucleic acid (RNA or DNA) often with specific marks we have the receptors for. Majority of viruses use their own enzymes for their own replication, which are often highly different from the host ones. It makes them a good target for antivirotics, which could be therefore highly specific.

Development of HIV inhibitors is an outstanding "case study", where chemistry and biomedicine joined to find a treatment for one of the most serious infectious disease. One of the heroes of such effort is a modest genius prof. Antonín Holý. His key contribution is the synthesis of nucleotide analogues, synthetic mimetics of the building blocks of RNA and DNA, that have found utility as inhibitors of viral replication. These include Tenofovir, Adefovir and Cidofovir highly effective against HIV or hepatitis B which helped to safe live millions of people.

To understand the principles of genetics in a very detail, we have to uncover it´s molecular principles. In a history of such effort -a single picture can become a gamechanger. It is the case of the famous Photo 51 taken by Raymond Gosling under supervision of Rosalind Elsie Franklin. Photo 51 (X-ray diffraction image of DNA) was a key piece of information for Francis Crick and James Watson to invent and support their DNA model. Diffraction phenomena are shared in between physics, chemistry and biology and from application of diffraction-based techniques many scientific disciplines.

Part A

List of equipment for Part A:

- DNA samples 1-3 (30 µL each): 3 small Eppendorf tubes – labelled 1, 2, 3. (Task A2)
- Immersion oil: Eppendorf tube – labelled O
- Molecular weight size marker (50 µL): 1 Eppendorf tube – labelled M. (Task A2)
- Electrophoretic apparatus with precast gel and running buffer (Task A2)
- 3 microscopic slides (numbered 1–3) with fixed red blood smears (Task A4)
- 2 microscopic slides (labelled A and B) with fixed red blood smears (Task A5)
- Box with glass tubes for Giemsa staining (same as used the first experimental day)
- Micropipette for loading DNA samples
- Pipette tips (5x)
- 1x Pasteur pipette for Giemsa staining
- 1x Pasteur pipette for dropping immersion oil
- Calculator
- Microscope equipped with 100x immersion objective
- Immersion oil
- Falcon tube with Giemsa stain (GIEMSA)
- Set of color pencils

All calculations in Task A should be accurate up to 2 significant digits.

GREGOR JOHANN MENDEL

CCR5 is a receptor for immunoregulatory substances present on the surface of white blood cells. As is typical for many genes involved in immune system function, it is involved in precise tuning of the immune response under specific conditions (e.g., fighting particular pathogens), though its function is not vital for the function of the immune system as a whole. Therefore, mutation in a CCR5 encoding gene is not linked to marked differences in phenotype. In case of the CCR5-Δ32 mutation (A deletion mutation of 32 nucleotides. Δ denotes the Greek letter delta.), a mild statistical phenotype is observed, manifesting as more severe symptoms during influenza and zika infections. In short, the lack of functional CCR5 attenuates the immune response. This may be beneficial in a situation when a too strong immune response is causing damage (immunopathology). Some medieval plagues are presumed to have shaped the recent population genetic polymorphism in European populations so that the number of individuals carrying the CCR5-Δ32 mutation was enriched. These individuals are descendants of plague survivors.

On the other hand, CCR5 is also a key co-receptor for HIV. When it is absent from the cell surface, the risk of viral infection is almost completely abolished. This strong antiviral phenotype is more frequent in Europeans. Even in CCR5 heterozygotes, the HIV infection is attenuated and takes significantly longer to manifest immunosuppressive symptoms.

During the academic year we collected buccal smear samples (containing detached cells from stratified mouth epithelium) from almost all Erasmus students at Charles University (to characterise the genetic

variability of the Europeans similar to the EOES competitors) as a material for genetic analysis. The overall results are:

- Dominant homozygotes: 155
- Heterozygotes: 25
- Recessive homozygotes: 1

Task A1 (3 points)

Question A1.1 *What is the overall frequency of wild-type CCR5 (e.g., "normal") and mutant CCR5-Δ32 alleles in "Erasmus human population" (which can be assumed to be in Hardy-Weinberg equilibrium)? The Hardy-Weinberg equation used to determine genotype frequencies is:*

$p^2 + 2pq + q^2 = 1$. *(1 points)*

Question A1.2 *Last two years were highly affected by the COVID-19 pandemic which was studied in detail, including studies taking its interaction with CCR5-Δ32 into account. Interpret the graphs below from the publication with heading on the right.*

On the x-axis is the frequency of the CCR5-Δ32 allele, and on the y-axis is the quantification of A) COVID-19 cases/million and B) COVID-19 deaths/million.

> Physiol Res. 2021 Dec 16;70(S2):S249-S252. doi: 10.33549/physiolres.934725.

Could the CCR5-Delta32 mutation be protective in SARS-CoV-2 infection?

N Starcevic Cizmarevic [1], M Kapovic, D Roncevic, S Ristic

Affiliations – collapse

Affiliation

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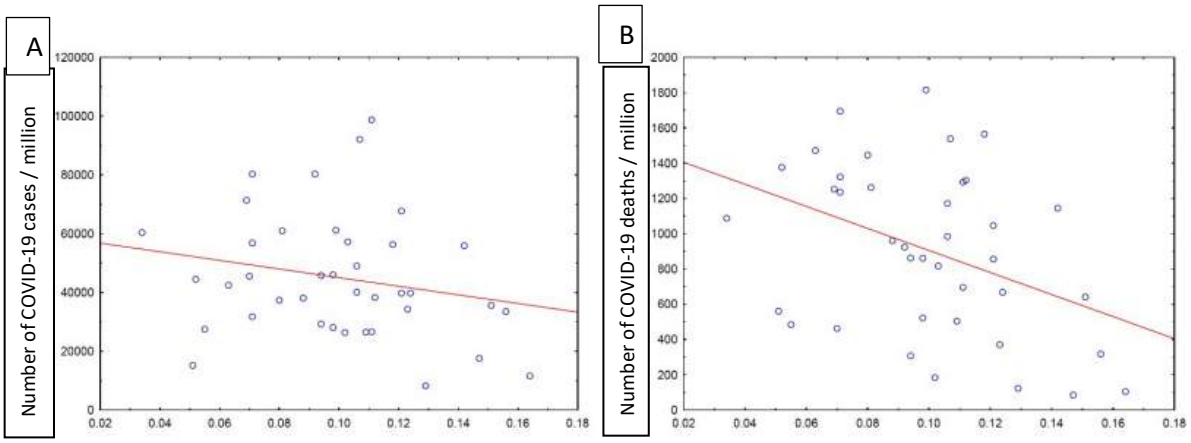


Fig. A1 Correlation between CCR5-Δ32 allele frequency and COVID-19 prevalence and mortality in Europe. (**A**) The number of COVID-19 cases per million inhabitants (r=-0,347, *p*=0,035). (**B**) The number of COVID-19-related deaths per million inhabitants (r=-0,444, *p*=0,006).

Which of the following statements A–D can be used as a correct interpretation of the plotted data? (1 point)

A) *There is no statistical correlation between the outcome and severity of SARS-CoV-2 infection and CCR5-Δ32 mutation frequency.*
B) *A stronger protective effect of the CCR5-Δ32 mutation can be seen in the number of deaths compared to the sum of infections.*
C) *The immune system actively contributes to the pathology of COVID-19.*

Question A1.3 *The most abundant monogenic disease in Europe is cystic fibrosis with approximately 1/30 frequency of the deleterious recesive allele (the particular frequency and identity of the mutation depend on geographic location and local genetic background). What is the corresponding average frequency of people who suffer from cystic fibrosis in Europe? Assume that the*

population is in Hardy-Weinberg equilibrium. The Hardy-Weinberg equation used to determine genotype frequencies is:

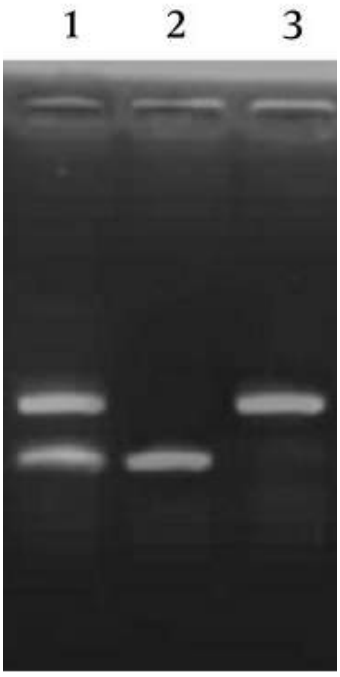
$p^2 + 2pq + q^2 = 1$. *(1 point)*

Task A2 (9.5 points + 5 points for loading and optimal running of electrophoresis –14.5 points total)

Our CCR5 genetic analysis was performed using a PCR reaction with specific primers flanking the mutated region. Different lengths of the PCR products correspond to the wild-type and mutant allele. You obtained three Eppendorf vials with typical results for recessive and dominant homozygote + heterozygote together with molecular weight size marker (DNA ladder).

Question A2.1 *Load individual DNA samples (vials 1–3) and molecular weight size marker (DNA ladder) (all 20 µL) into appropriate wells in the precast agarose gel and run the gel. Arrange the loading pattern of samples and molecular weight standards according to rational experimental practice. Draw your loading design in the box in the Answer sheet first. (1 point)*

Question A2.2 *The figure below shows the typical result of CCR5 genotyping performed according to the same methodology as we use here. (1.5 points)*



Write which of the samples 1–3 correspond to

Dominant homozygote, heterozygote and recessive homozygote in the Answer sheet.

Question A2.3 *The PCR used to prepare the samples was run for 20 amplification cycles. What is the resulting number of amplified molecules if the PCR started from DNA isolated from a single human somatic cell and the reaction conditions were ideal? For the definition of the amplified region, a single pair of primers was designed and used. Write the numbers of amplified molecules for Dominant homozygote, Heterozygote (specify the number of dominant/recessive copies), and Recessive homozygote in the Answer sheet. (2 points)*

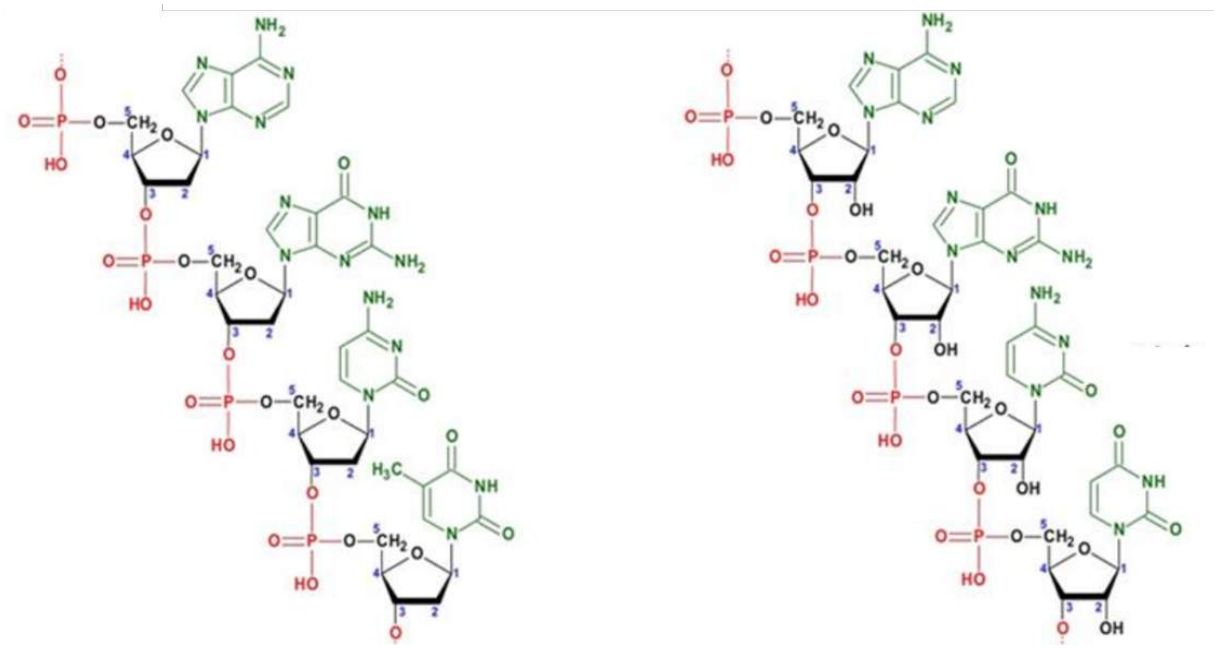
Question A2.4 *The number of nucleotides missing in the CCR5-Δ32 mutation is 32. Which of the following statements A–D) is/are true for the severity of the mutational outcome? Mark your answer in the Answer sheet. (1 point)*

A. *The result is shortening of the CCR5 by 8 amino acids and no shift in a reading frame, since human leucocytes use a 4-letter genetic code.*
B. *The result is a shortening of the CCR5 and a shift in a reading frame that typically leads to a premature termination of translation resulting in the substantial shortening of the protein product.*
C. *Shortening of the CCR5 gene leads to a shift in a reading frame that typically leads to inhibition of translation termination, which results in the production of a longer protein.*
D. *Similar severity of the phenotype could be observed in CCR5-Δ1 or CCR5-Δ2 mutation, but not in CCR5-Δ3.*

Question A2.5 *One of the recent major bioethical controversies is linked to CCR5. The first use of genome editing in viable human embryos was performed by a Chinese scientist He Jiankui in 2018. He altered the genomes of two human embryos – twin sisters known under the pseudonyms Lulu and Nana. The intention was to create HIV-resistant human beings by introducing the CCR5-Δ32 mutation in the genome. Two days after Lulu and Nana were born, whole genome sequencing of DNA from their blood samples confirmed the mutations. However, the available sources indicate that Lulu and Nana carry incomplete CCR5 mutations. Lulu carries a mutated CCR5 gene that has a 15-bp deletion on one chromosome 3 while the other chromosome 3 is normal (heterozygous allele). Nana carries a homozygous mutant gene with a 4-bp deletion and a single base insertion in close proximity.*

Which of the two sisters could most likely to be fully resistant to HIV? Indicate your answer in the Answer sheet. (1 points)

Question A2.6 *Distinguish the structure formulas of DNA and RNA from each other (indicate this by writing 'DNA' or 'RNA' in the boxes provided in the Answer sheet. Circle the difference in chemical structure between DNA and RNA in the sugar part in both drawings using a blue pencil. (3 points)*

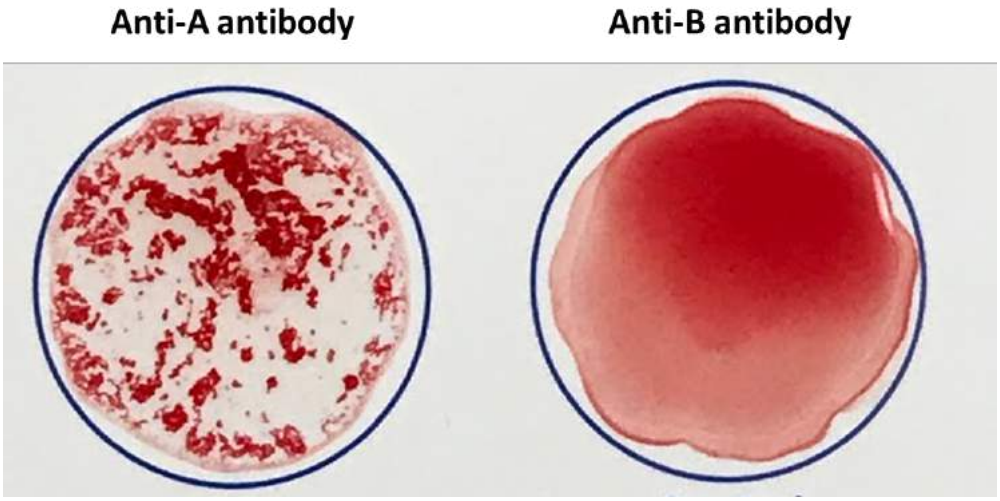


JAN JANSKÝ

G. J. Mendel's postulates were based on using an allelic pair of one dominant and one recessive allele. However, in reality, co-dominance of various alleles may also occur and contribute to phenotypic plasticity. A well-known example combining the concepts of dominance and co-dominance is genetics of the AB0 human blood group system. The underlying mechanism of blood group (in)compatibility, a phenomenon essential for safe blood transfusion, is based on the expression of variable glycosyltransferases that modify the amphipathic membrane components – glycolipids with different sugar moieties. An important contribution to the blood group classification was made by Jan Janský, a Czech serologist, neurologist, and psychiatrist who is credited for the classification of blood into four types (I, II, III, IV) including the AB blood group that remained unrecognised till Janský discovered it in 1907.

Task A3 (6 points)

The figure bellow provides a result of an AB0 blood group testing of an unknown sample with anti-A and anti-B antibodies. Use of the particular antibodies is indicated.



Question A3.1 *To which blood group/groups does the depicted sample belong? (1 point)*

A

B

AB

0

Question A3.2 *Blood groups, which can be identified by using antibodies, are the phenotypes. What would be genotype/genotypes for all 4 blood groups? Use A, B, and 0 to denote alleles. If more than one genotype exists, include all possible genotypes. Omit rare alleles of other genes interacting with the AB0 system, such as the h allele responsible for the Bombay phenotype. (1 point)*

A:

B:

AB:

0:

Question A3.3 *(1 point)*

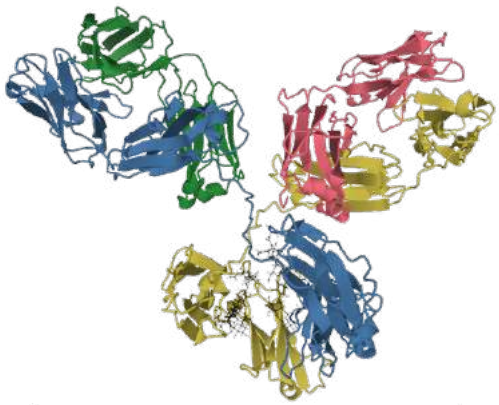
Which allele/s is/are codominant?

Which allele/s is/are recessive?

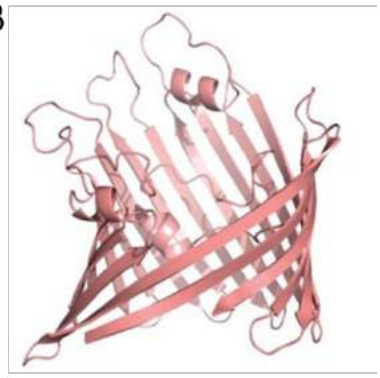
Question A3.4 *Draw a schematic explanatory figure depicting the role of antibodies in blood group identification for the sample from question 3.1. Red blood cells and antibodies don't need to be in scale. (1 point)*

Question A3.5 *Antibodies (immunoglobulins) are proteins produced by B-lymphocytes (B-cells) during immune response and optimised for high-affinity binding to particular antigens. Which of the pictures A–D corresponds to the 3D conformation of an antibody? Indicate the correct letter in the Answer sheet. (1 point)*

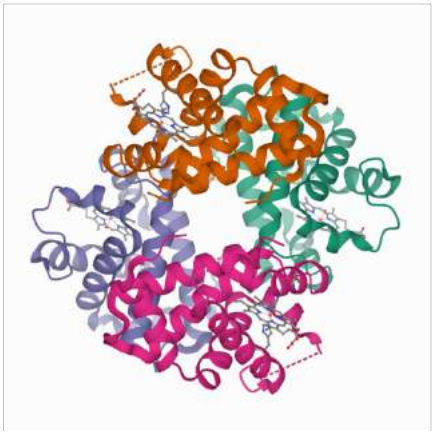
A



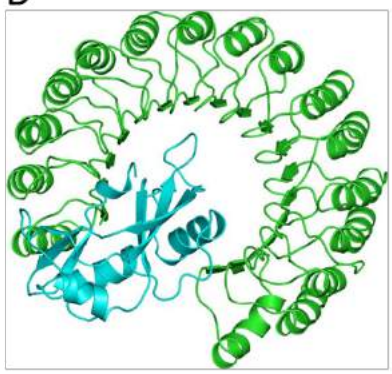
B



C



D



Question A3.6 *Imagine that a plasmatic cell (B-cell producing soluble antibodies specific for a particular antigen in large quantities) is used for animal cloning. What will be the immunological phenotype of the experimental animal? (1 point)*

A) There will be no difference between cloned and naturally born animal.
B) The animal will produce only one type of T-cells.
C) The animal will be able to produce only one type of antibody specific to one antigen.
D) Immunodeficiency.

JAN EVANGELISTA PURKYNĚ

Cell theory (*Omnis cellula e cellula*) is one of the major intellectual concepts in life sciences. In the middle of the 19th century, the definition of the cell as the smallest unit of life was independently formulated by J. E. Purkyně, a Czech physiologist, T. Schwann, a German physiologist, and M. J. Schleiden, a German botanist. Since then, cells have garnered the attention of many scientists. As a result, cell biology nowadays belongs to the fundamentals of not only biology, but also medicine. The hallmarks of life and cell structure and physiology are generally the same. Continuation of the complex life forms involves molecular and cellular turnover, energy consumption and dissipation, or the use of DNA or RNA as information storage media. However, defining life is not an easy task, not even today…

Task A4 (13 points + 6 points for blood smear specimens – 19 points total)

You are provided with 3 microscopic slides (numbered 1–3) with fixed red blood smears

(from mammal, bird, and amphibian). Prepare stained blood smear specimens using Giemsa stain (binding to negatively charged cellular components, incubate for 20 minutes in the provided Giemsa solution, use Pasteur pipettes to cover the smear with the staining solution.) and finally wash with distilled water in a wash bottle. Let the specimens dry up before observation. Wear gloves during the staining procedure.

Observe the specimens using immersion oil and immersion objective:

1. Rotate objective lens ring to a position between 40x and 100x objectives (neither lens will be locked in place.)
2. Remove slide from stage. Add a drop of immersion oil. Return slide (with oil) to stage or, leave the slide on the stage and add the oil drop directly.
3. Rotate the 100x objective lens into place.
4. Use coarse focus knob to raise the stage until the 100x lens just touches the oil.
5. While looking into the microscope, use fine focus knob (turn clockwise, to raise the stage) to find the plane of focus where the specimen is.

Question A4.1 *Draw representative images of all blood smear specimens prepared by yourself (use immersion oil and corresponding 100x magnification objective). (3 points)*

Question A4.2 *Identify the blood source of each sample and write the corresponding number of the slide in the proper box in the Answer sheet (1 point)*

Mammal blood:

Bird blood:

Amphibian blood:

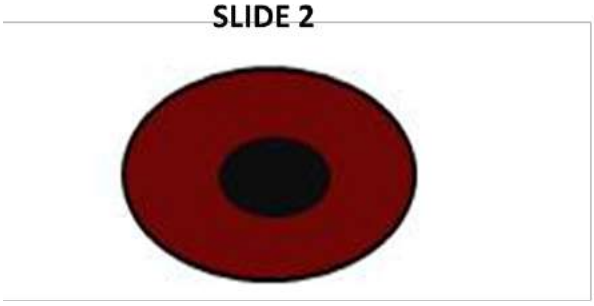
Question A4.3 *Draw a picture of the mammal to which the mammalian blood smear belongs. The best drawing will be awarded a special price! (0 point!!! but you can win special prize)*

Question A4.4 *You are provided with statements A–D. Write the corresponding letters of all statements that apply to the slides into the boxes provided in the Answer sheet. You may use the same statement more than once. (1 point)*

A) Erythrocytes contain haemoglobin as an oxygen carrier.
B) Erythrocytes constitutively produce proteins.
C) Erythrocytes could undergo oncologic transformation.
D) Erythrocytes develop from the precursor cell by mitosis.

Question A4.5 *The size of the cell is influenced by the size of the genome, and the size of the cell's nucleus also correlates with genome size. Test the hypothesis that the nucleo-cytosolic ratio is constant in the majority of cases, including red blood cells. The figure provided below shows sizes of cells and the sizes of the haploid genome from samples 2 and 3. Calculate the nucleo-cytosolic (genome-cytosolic) ratios for both cell types (consider the shape of the cells to be an ideal ellipsoid where b=c), state the units in the Answer sheet. Don't forget that the erythrocytes come from diploid organisms! (3 points)*

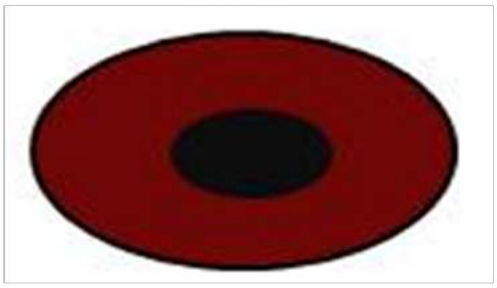
SLIDE 2



cell size 12x7 μm

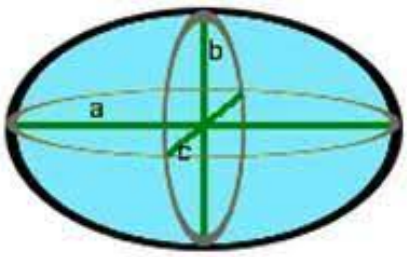
genome size (base pairs) $1{,}2 \times 10^9$

SLIDE 3



cell size 16x9,8 μm

genome size (base pairs) $3{,}1 \times 10^9$



VOLUME of ELLIPSOID

$\frac{4}{3}\pi abc$

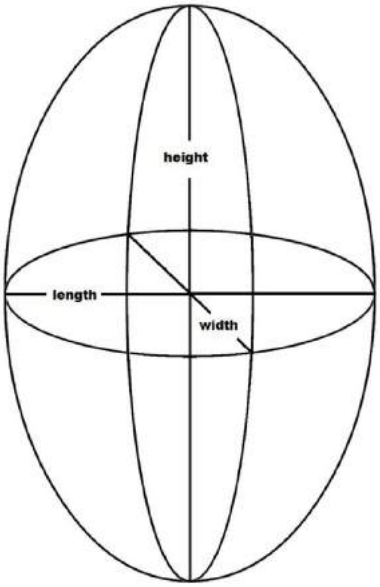
sample 2 ☐

sample 3 ☐

Based on your results, decide whether the hypothesis that the nucleo-cytosolic ratio is constant (close to an average value with difference less than 5%) is true for the red blood cells.

TRUE/FALSE

T2



Question A4.6 *The size of the nucleus corresponds to the size of the genome. The Australian lungfish (Neoceratodus forsteri) genome is the largest animal genome ever sequenced. With $43*10^9$ base pairs (in haploid state), it is 14 times larger than the human genome, and it exceeds the genome of the Mexican axolotl (Ambystoma mexicanum), the previous largest genome record holder in the animal kingdom, by an impressive 30%.*

What would be the volume of the lungfish red blood cells? What would be the height and length (length= width, b=c, height is 1,5x longer than length) of the lungfish red blood cells? Use the mean value of the nucleo-cytosolic ratio from your results for samples 2 and 3. (1 point)

Question A4.7 *The human red blood cell (RBC) exhibits remarkable deformability and durability. During its four-month lifespan, a human RBC circulates the body about a million times and undertakes a journey of about 500 kilometres. The deformability and durability of RBCs is determined by plasma membrane viscoelasticity, cytoplasm viscosity, and geometry of the cell. The resting RBC adopts a unique shape with a surface area to volume (SA:V) ratio ~1.5-fold greater than a sphere of the same volume. How is that achieved? Draw a side cross section (through the middle of the cell) view of the RBC. (1 point)*

Question A4.8 *What would be the surface volume ratio for the cells in the sample 2 and 3 and those of lungfish origin? (1,5 points)*

(SA:V) ratio (slide 2)

(SA:V) ratio (slide 3)

(SA:V) ratio (lungfish)

The formula for the calculation of ellipsoid surface area is: $4\pi[((ab)^{1.6}+(ac)^{1.6}+(bc)^{1.6})/3]^{(1/1.6)}$.

Question A4.9 *All living things have certain traits in common: cellular organisation, the ability to reproduce, growth and development, energy use, homeostasis, response to their environment, and the ability to evolve. Taking into account these hallmarks of life/cellularity – erythrocytes from which sample/samples are not alive? Mark the appropriate sample/s with a tick (✓) in the proper box in the Answer sheet. (1,5 points)*

Slide 1:

Slide 2:

Slide 3:

Task A5 (7.5 points)

You are provided with another two blood smear specimens stained with Giemsa stain (labelled A and B), containing a healthy person's blood and the blood of a patient afflicted with an unknown pathology.

Question A5.1 *Draw representative images of both samples under 100x magnification using immersion oil. (2 points)*

Question A5.2 *Write in the Answer sheet which of the two samples (A or B) is pathological. Write your answer in the box. (0.5 point)*

Question A5.3 *Which of the following pathologies correspond to the phenotype observed under the microscope? (1 point)*

A) Sickle cell anaemia
B) Polycythaemia vera (higher than normal erythrocyte count)
C) Anaemia caused by lack of iron
D) Leukaemia
E) Malaria
F) Thalassemia

After answering, raise your hand. A lab assistant will stamp your answer and provide you with the rest of the tasks for Part A5.

Part B

List of equipment for Part B:

- Red laser (wavelength of the laser $\lambda = 640\ \mathrm{nm}$)
- Clamps
- Optical rail
- Holder with hair
- Holder with helical spring
- Measuring tape
- Caliper
- Screen
- Pencil
- Calculator

Double helix structure of the DNA

The molecule of deoxyribonucleic acid (DNA) has a double helix structure, which was described in 1953 by James Watson and Francis Crick. This description is based on the X-ray diffraction image taken by Rosalind Franklin and her student Raymond Gosling. In this assignment, you will study diffraction theoretically and practically.

Diffraction is a phenomenon that occurs when a wave encounters an obstacle or opening. It can be a wave on the water surface, a wave of light, or the X-ray wave mentioned above. Diffraction is most clearly observable when the physical dimensions of the obstacle are comparable to the wavelength of the incident wave due to so-called interference.

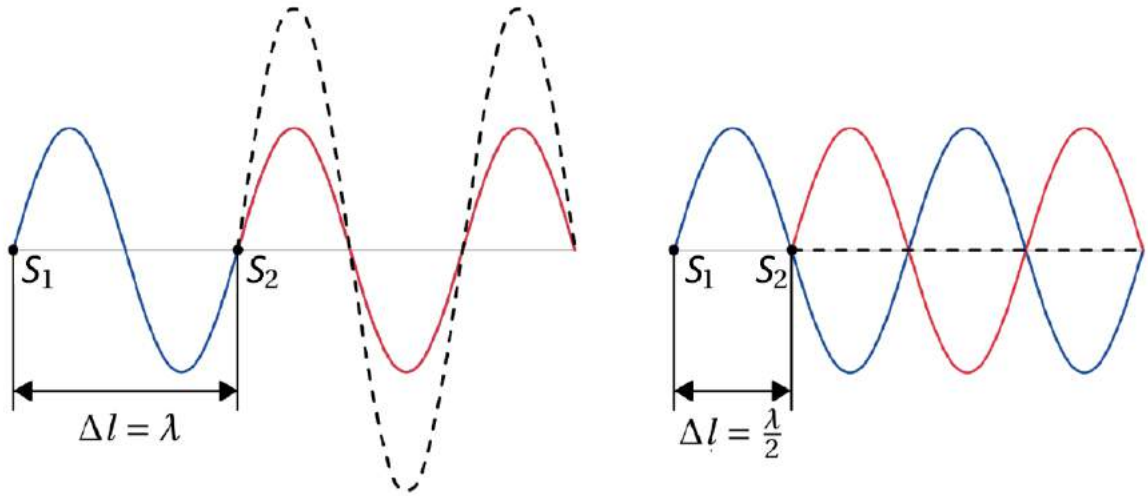


Fig. B1 Constructive and destructive interference.

To understand interference, let us consider two waves with the same wavelength λ propagating from the two sources S_1 and S_2 (Fig. B1). If these two sources are separated by the distance Δl (sometimes denoted *path difference*), the amplitudes of these two waves will add up at each point. If the path difference is the same as the wavelength λ, the maximum of the wave from the source S_1 will add to the maximum of the wave from the source S_2, resulting in greater amplitude. This will also happen if

the path difference will be $k\lambda$, where $k = 0, 1, 2, \ldots$ The constant k is usually called the *order of maximum,* and we can write

$$\Delta l_{max} = k\lambda = 2k\frac{\lambda}{2} \quad (1)$$

and this phenomenon is called *constructive interference*. In terms of light, this corresponds to a bright spot on the screen.

If the two sources are separated by the path difference Δl equalling $\frac{\lambda}{2}$, maximum of the wave from the source S_1 will add to the minimum of the wave from the source S_2, and so on. This will result in cancelling the waves out (if these waves have the same amplitude). This also applies to all situations where the path difference is a half-number multiple of the wavelength:

$$\Delta l_{min} = (2k - 1)\frac{\lambda}{2}, \quad (2)$$

where $k = 1, 2, \ldots$ and is the order of the minimum. This whole phenomenon is called destructive interference, and in terms of light, it corresponds to the dark spot on the screen.

The light used in these experiments must have special properties so that you will use the light of the red laser.

IMPORTANT: Do not shine a laser light on your eyes! Beware of even random reflections from shiny objects! The design of the experimental apparatus almost eliminates this, but please be careful anyway.

Task B1 Diffraction of light on the hair (10 points total)

In this task, you will familiarize yourself with the basic properties of light diffraction. When light strikes an obstacle, as shown in Fig. B2, the observed diffraction pattern is similar to the one observed after passing light through a single narrow slit. Just as in the one-dimensional case of Fig. B1, Δl between these two light rays can be calculated, in this case as

$$\Delta l = d \sin\theta, \quad (3)$$

where d is the width of the obstacle. Note however, that the conditions for constructive and destructive interference from the introductory text are opposite for the case of a single slit.

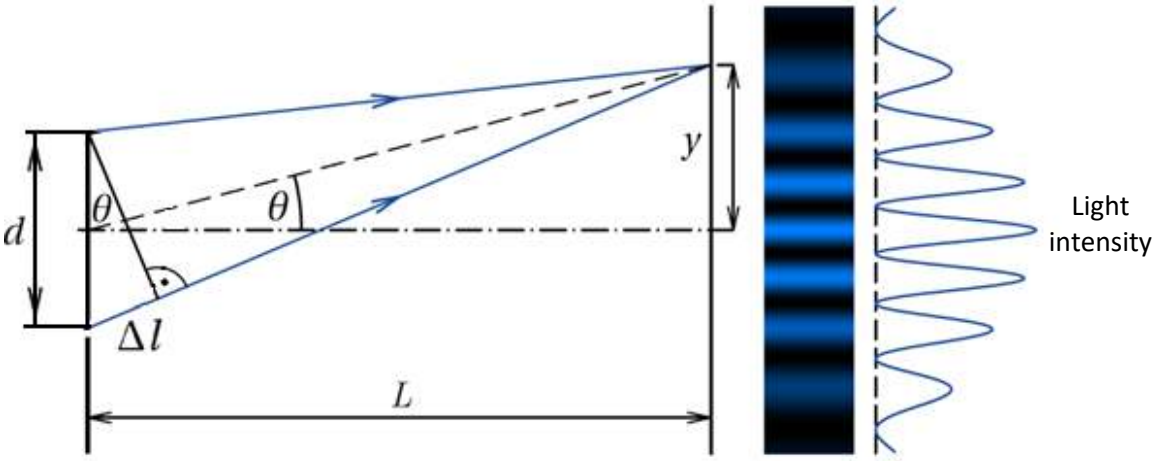


Fig. B2 Diffraction of light on an obstacle.

Question B1.1. *Write to the Answer Sheet the conditions for the diffraction minima and maxima during diffraction of light on the hair using equations (1) and (2). (3 points)*

As you can see from Fig. B2, the angle θ can be calculated from the geometry of the experimental apparatus due to the similarity of the triangles.

Turn on the laser with the button closer to the screen and observe the diffraction pattern. It should be similar to that one from Fig. 2: alternating light and dark stripes. The central bright spot is the zeroth maximum (for $k = 0$), then first minimum, first maximum, second minimum, and so on alternates. By measuring their distance from the zeroth maximum y you will find the corresponding angles θ_k and calculate the hair diameter d.

For the pattern to be large enough to measure the distances of the individual minima, it is necessary to place the screen at a distance of at least one meter. You will measure the distance from the hair sample to the screen and mark it as l. You can mark important points for measurement on the screen with a pencil and measure distances afterward, or measure straight from the screen using a caliper.

We denote the distance measured from the center of the zeroth maximum to the center of the first left minimum as y_{11}. The distance measured from the center of the zeroth maximum to the center of the first right minimum is denoted as y_{12}. Using the arithmetic mean of these two values, we find the distance y_1, which we will need for further calculations. Continue in a similar way to measure minima of the following two higher orders (also on the other side of the interference pattern). Record the measured values in the table in the Answer Sheet.

Question B1.2 *Fill in the table in the Asnwer Sheet and calculate the final value of d as the arithmetic mean of the all three d_k values. (7 points)*

Task B2 Diffraction of light on the helix (22 points)

IMPORTANT: Slide the screen off the rail, then slide off the holder with the hair. Then slide in the holder spring and slide back the screen. If you power up the laser, the diffraction pattern should look like in Fig. B3b.

If there is not one circular obstacle but a helical spring, the situation is slightly different. Helical spring with wire diameter d, spacing S, thread radius R, and perpendicular distance of wires P, which form an angle β with each other (see Fig. B3a) will produce an diffraction pattern that is depicted in Fig. B3b.

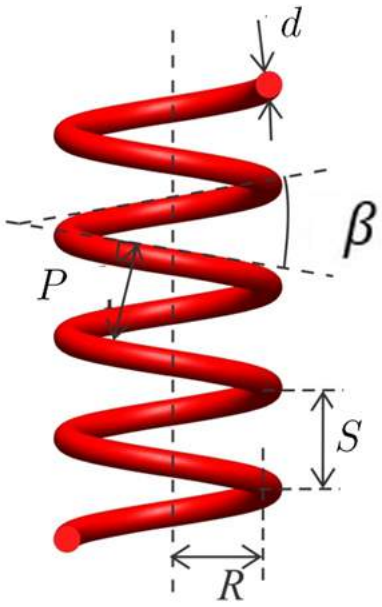


Fig. B3a Geometry of the helical spring.

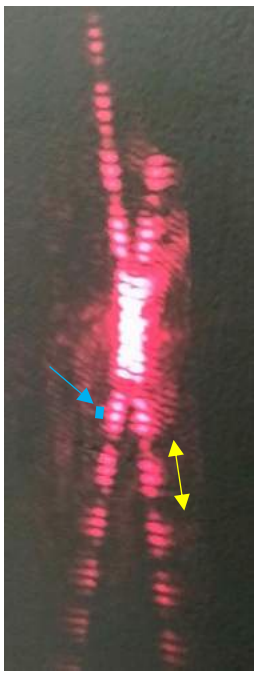


Fig. B3b Diffraction pattern.

The "X"-pattern of the diffraction maxima is an indication of a helical structure. The inclined segments which make the front view of a helix give rise to an inclined lines of diffraction peaks. The angle between the segments is equal to β.

You can observe two different structures: a coarser (see the yellow arrow in Fig. B3b) and a finer structure (see the light blue arrow and bar in Fig. 3b). The coarse structure corresponds to the smaller characteristic size of the helical spring d, whereas the fine structure corresponds to the larger length P. It can be shown that the distance x between two neighbouring minima of the coarse structure on the screen is connected to the wire diameter d by this equation:

$$d = \frac{\lambda L}{x}.$$

Measure the distance x ten times on the screen (between neighbouring minima), write it into the Answer Sheet, and use this data (along with the screen distance L) to calculate the values of d and β. Calculate the absolute errors of these quantities. We determine the observation error as the arithmetic mean of the deviations of individual measurements.

As the first step, you will write down the calculated values in the tables. Then you determine the absolute deviations of individual measurements Δd by calculating the absolute value of the difference between the calculated arithmetic mean $\bar{d}$ and the individual measurements:

$$\Delta d = \left| \bar{d} - d \right|$$

So for the first measurement, we calculate the absolute value of the arithmetic mean minus first measured value, for the second measurement we calculate the absolute value of the arithmetic mean minus second measured value and so on.

The final value of the absolute error $\Delta\bar{d}$ is the arithmetic mean of the individual absolute errors from the last column of the tables. Round this deviation to one significant digit and round the arithmetic mean to the same number of decimal places as the absolute error.

Question B2.1 *Fill in the tables in the Answer Sheet and Write down the measured value in the form of $d = (\bar{d} \pm \Delta\bar{d})$. Calculate the same for β. Write the value of L to the Answer Sheet. (16 points)*

For the fine structure a relationship similar as for the coarse structure can be used. The distance of wires P can be calculated by the distance b between neighbouring minima of the fine structure and the following equation:

$$P = \frac{\lambda L}{b}$$

Now you will estimate the quantity P. For this purpose count the number of minima of the fine structure that are present between two neighbouring minima of the coarse structure.

Hint: If you are not able to see the fine structure you can measure the distance between the two minima of the coarse structure and count the minima of the fine structure in Fig. B3b with no punishment.

Question B2.2 *Write down the number of the minima of the fine structure that are present between two neighbouring minima of the coarse structure in the Answer sheet. Also write down the distance between the used minima of the coarse structure. Calculate the distance b between the minima of the fine structure and the perpendicular distance of wires P and write your answer in the Answer sheet. Write also the value of L to the Answer Sheet. (6 points)*

Task B3 Diffraction of X-rays on the DNA helix (18 points)

Rosalind Franklin and her student Raymond Gosling took an X-ray diffraction image of a gel composed of DNA fiber in May 1952. This now-famous Photo 51 is in Fig. B5. Assume that the image is ten times larger than the original, which was captured using X-rays with a wavelength of $\lambda = 0{,}15\ \mathrm{nm}$. The screen was in the distance of 13.0 mm from the DNA sample.

Question B3.1 *Measure ten times the separation b between different neighbouring maxima using a caliper. Using only the caliper (and trigonometric formulas), determine the angle between diffraction patterns and calculate β. Draw everything you need in the image in the Answer Sheet to substantiate and explain your reasoning. Using formulas from Task B2, calculate the parameters P and β of the DNA helix. (16 points)*

DNA, in fact, is a double-helix structure, which can be proven with missing fourth maxima).

Question B3.2 *Why the X-rays have to be used to examine the structure of the DNA instead of light? State your answer using mostly mathematical symbols and physical quantities. (2 points)*

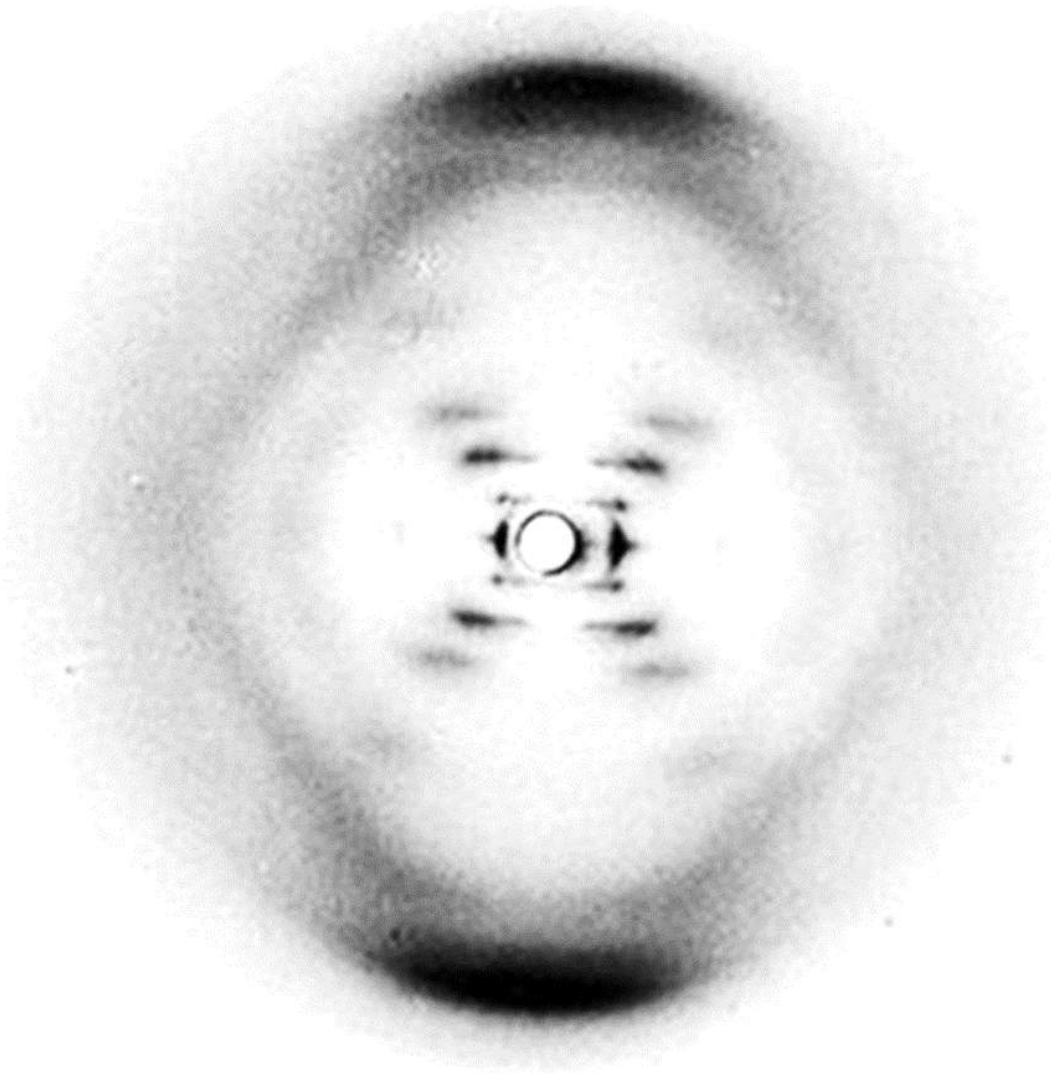


Fig. B5 Photo 51.

Part C

Antivirals

List of laboratory equipment:

- glass pipettes
- 20 TLC plates (silica gel, specifications)
- 'IPAV' solvent
- TLC cells or small beakers covered with Petri glass (at least 4 per team)
    - permanganate solution for TLC visualisation
- 10 unknown samples (marked A-J)
- Sodium periodate solution
- 12 Eppendorf vessels for reacting the samples with reagent solutions
- 10ml and 20ml graduated cylinder
- UV lamp (for more teams)
- Ammonia water (for more teams)
- 2-propanol (for more teams)
- distilled water (for more teams)
- Acidic solution (concentrated hydrochloric acid, for more teams)
- tweezers for TLC plates
- automated pipettes
- pencil, marker
- calculator
- plastic pipettes
- tape

T2

Antonín Holý (1936-2012)

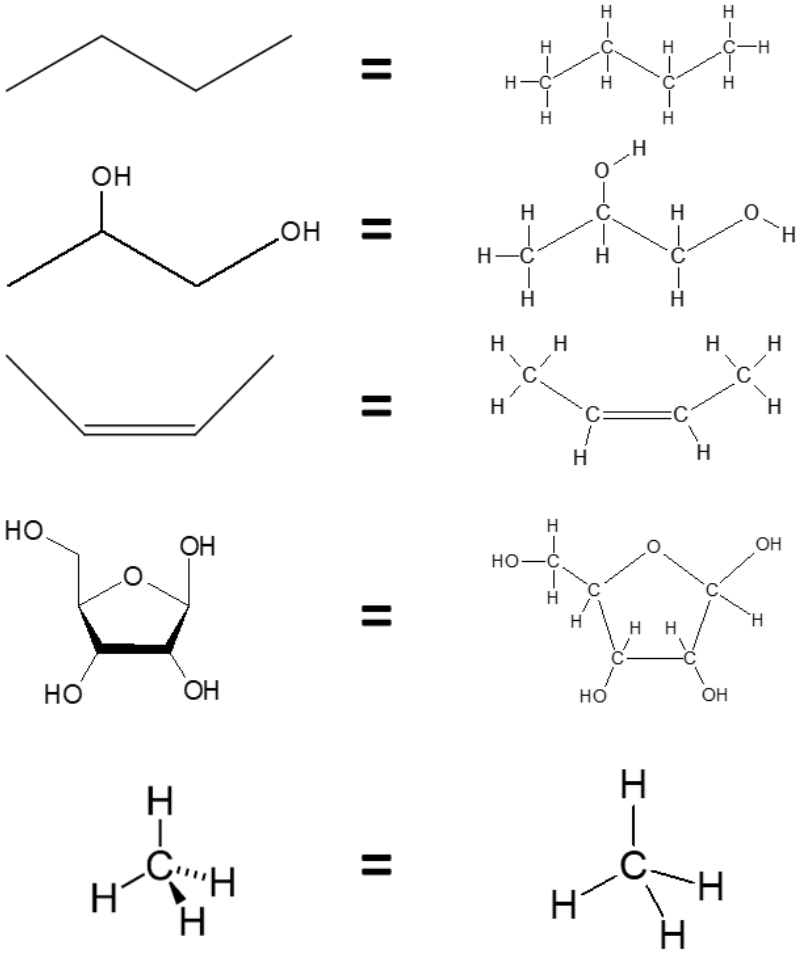
Nucleotide analogues (NAS) were synthesised by the team of Antonín Holý in the Institute of Organic Chemistry and Biochemistry (Prague, Czech Republic). NAS are used worldwide to treat various viral infections. Some of these derivatives such as Adefovir, sold as Vireon or Hepsera, are used to treat chronic hepatitis B infections. Tenofovir, in the form of tenofovir disoproxil or alafenamide, is used in combination with other antivirals to suppress HIV effectively. DHPA was the active ingredient of Duviragel, a treatment for the herpes simplex virus.

In this task you are provided with 10 unmarked samples labelled A-J, which contain different nucleotide analogues and natural products. You will try to identify the compounds by considering their chemical properties, followed by chromatographic investigation.

Task C1 (11 points)

Drawings of structures of organic compounds are often simplified to make them more compact or to leave out the elements that are not as essential in certain cases.

Below you will find a small guide on some of the structures included in the task.



Element is pointed into the planar plane (away from the viewer)

Element is pointed out of the planar plane (towards the viewer)

N.B. A wiggly or wavy (~) bond should be interpreted as either a dashed bond pointing away from the viewer or a wedged bond pointing towards the viewer.

Below a set of molecule structures corresponding to samples A-J is provided. Some of them are natural products, and some are antivirotics prepared by the team of Prof. Holý. The molecular structure of these compounds (Figure C1.1) can be used to predict specific properties, such as molecular mass, optical rotation, reactivity, and UV absorbance.

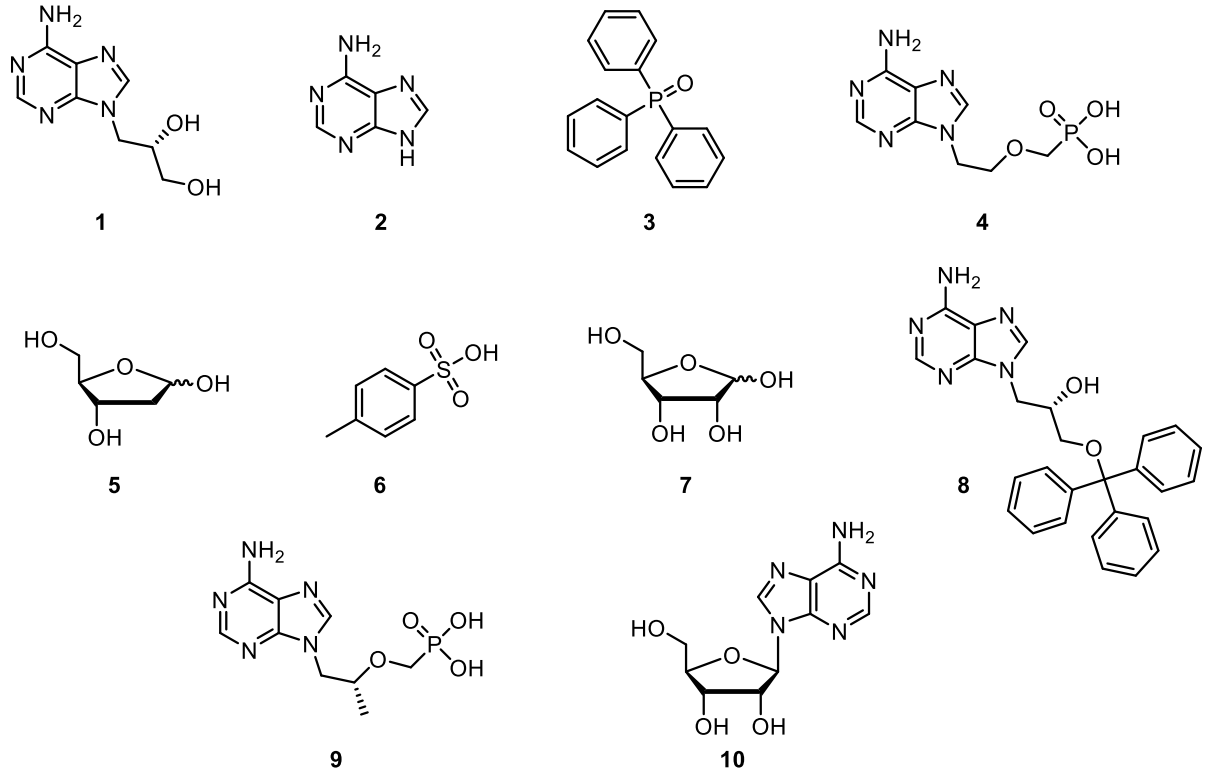


Figure C1.1 - The molecular structures of compounds 1-10

Systematic and trivial (in brackets) names for the compounds:

I — (2R,3R,4S,5R)-5-(hydroxymethyl)oxolane-2,3,4-triol (ribose)

II — 9H-purin-6-amine or 6-aminopurine (adenine)

III — {[2-(6-amino-9H-purin-9-yl)ethoxy]methyl} triphenylmethyl ether

IV — (2S)-3-(6-amino-9H-purin-9-yl)-1,2-propandiol

V — {[2-(6-amino-9H-purin-9-yl)ethoxy]methyl}phosphonic acid (Adefovir)

VI — (2R,3R,4S,5R)-2-(6-amino-9H-purin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol (adenosine)

VII — ({[(2R)-1-(6-amino-9H-purin-9-yl)-2-propanyl]oxy}methyl)phosphonate (Tenofovir)

VIII — 2-deoxy-D-ribose (deoxyribose)

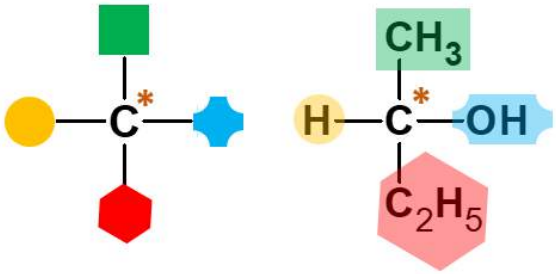
IX — triphenylphosphine oxide

X — 4-methylbenzenesulfonic acid (PTSA, Paratoluen sulphonic acid)

Question C1.1 *Match each compound (1-10) to its name (I-X, either trivial or systematic). (4 points)*

Optically active substances can change the orientation of plane polarised light. To decide whether a compound should be optically active, one needs to look for optically active centres. In organic substances these are mainly carbon atoms. Such atoms are called **chiral**.

Chiral carbon C atom is a C atom with 4 different *groups connected to it (as carbon forms 4 possible covalent bonds).* Below you will find an example of a chiral carbon atom in a carbohydrate molecule



chiral carbon = asymmetric carbon

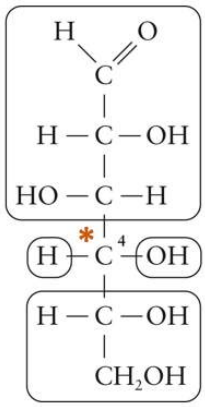


Fig. Cx. Example of a chiral C atom in glucose molecule.

Question C1.2 *State which of the given structures (1-10) have optical activity (e.g. they can change the orientation of plane polarised light, circle the chiral carbon atom, at least one in the answer sheet). (5 points)*

Question C1.3 *Compounds with conjugated double bonds can absorb light in the UV area. On the Answer sheet, indicate which of the compounds can be detected by UV light. (2 points)*

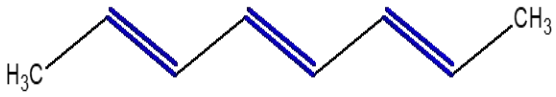


Fig. Cx. Example of a structure with conjugated (=multiple alternating) double bonds

Task C2 (39 points)

Thin Layer Chromatography

Structural analysis is an essential part of organic chemistry. Any analytical method can be described by the information it can provide, its accuracy, length of analysis, and very importantly its cost (of the analytical instrument, solvents, etc.).

In this task, you will get acquainted with thin-layer chromatography (TLC). This technique is used to separate non-volatile mixtures. The analysis is performed on a sheet of an inert substrate (such as aluminium foil) which is coated with a thin layer of adsorbent material, usually silica gel.

A glass pipette is used to transfer a minute amount of the analysed mixture and mark a spot on the "Start" line on the TLC plate (Fig. 6). The spots should preferably be as small as possible. A developing chamber (a beaker with a Petri dish on top of it) is filled with a desired eluent (or 'mobile phase') so that the level of the solution is 2-3 mm high from the bottom. The spotted TLC plate is then placed vertically into the TLC chamber using tweezers and the chamber is covered with the Petri dish (Fig. 7). When a sample has been applied to the plate, the mobile phase is drawn up the plate *via* capillary action. Because different analytes ascend the TLC plate at different rates, separation is achieved. With silica gel, compounds with lipophilic groups (alkyl, aryl) move faster (further) on TLC. On the contrary, ions and molecules with polar functional groups (OH, =O, SH, $-O^-$) move slower on TLC. The interaction grows stronger with an increasing number of polar groups.

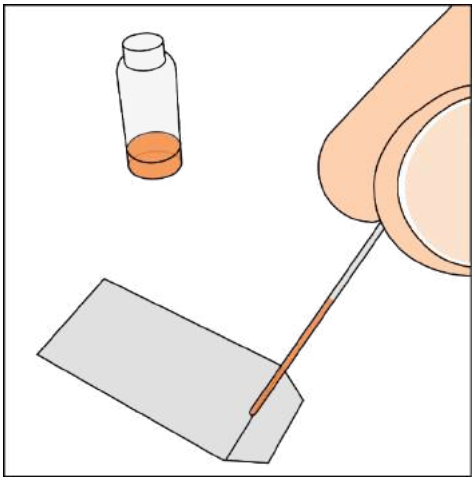


Figure X. Spotting of a TLC plate

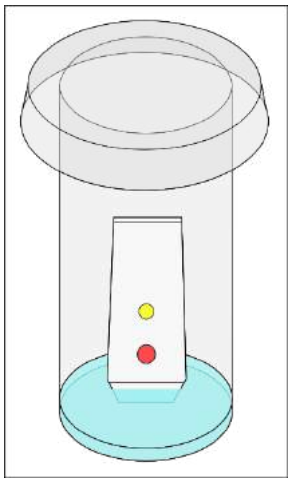


Figure Y. Development of a TLC plate

During the experiment the eluent will travel up the plate. When the eluent reaches the "Finish" line the plate must be removed from the chamber. The retardation factor (R_f) for every spot on the TLC plate can then be calculated.

R_f is the ratio of the distance travelled by the centre of the spot to the distance travelled by the solvent front. For example, if a particular substance travels 2.5 cm and the solvent front travels 5.0 cm, the R_f would be 0.50. Every chemical compound has a unique R_f value which is dependent on the eluent.

After the experiment, the spots have to be visualised. One way is to simply by projecting ultraviolet light onto the sheet. The TLC plates are doped with a fluorescent indicator. If a compound with conjugated double bonds is present, it absorbs the UV light and the spot looks dark under the UV lamp.

Contrast reagents can also be used to visualise the spots - dipping the TLC plate in permanganate solution causes oxidation of the sample. Reduced permanganate can then be observed as yellow spots on a brown background.

The set of 10 unknown samples labelled A-J that you have been provided with, correspond to the compounds 1-10 in Figure C1.1. Your objective is to find out which sample corresponds to which compound. In this task, you will employ TLC and some specific chemical reactions to help you answer this question.

As the mobile phase, you will use a mixture of isopropanol (propan-2-ol or 2-propanol) and dilute ammonia, known as 'IPAV'.

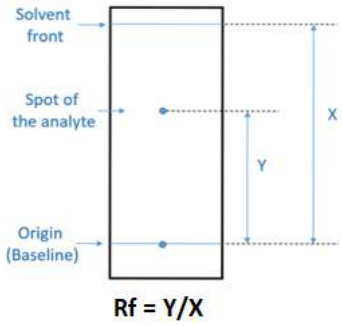
Question C2.1 *Spot compounds A-J on a TLC plate in a 2x5 grid (you are not going to use this plate for chromatography, so you can use the full plate). Make sure to label each spot (by pencil!!) with its corresponding letter. Allow the spots to dry completely, since wet spots will give false positives under UV-light and with the permanganate stain. Place the plate under the UV lamp and observe which compounds absorb UV light. Write down the letters of the compounds absorbing UV light on the answer sheet.*

Then, use the tweezers to briefly submerge ('dip') the TLC plate in the potassium permanganate solution. If you cannot completely submerge it at once, take it out, and dip it in the solution upside down. Let any remaining liquid drip from the plate. Lay it down on a paper towel or tissue and let it rest for 5 minutes. Some of the spots will turn a clear yellow; the compounds in these spots have been oxidised by the permanganate. (Some other spots may turn slightly yellow over time as well, but you can disregard those.) Write down the letters of the compounds that clearly turn yellow on the answer sheet. (5 points)

Paste the plate on the last page (labelled ´TLC plates´) of the answer sheets.

Question C2.2 *Perform TLC experiments with samples A-J. Use a suitable detection method (UV lamp or permanganate solution). Calculate the Rf values (see the picture below) and write them down into the Answer sheet. Use a pencil to mark the spots on the TLC plates and paste them on the last page (labelled ´TLC plates´) of the answer sheets and write down the sample letters (A-J). You can add up to 3 spots on one TLC plate. (10 points)*

Measurement of Retention Factor (R_f) after TLC Plate Development



Rf = Y/X

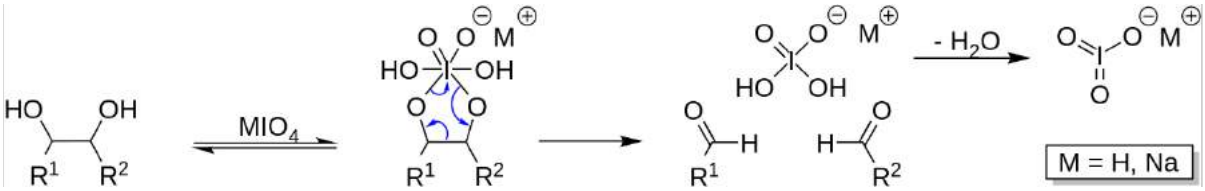
Question C2.3 For running a reliable and reproducible TLC you need to know the exact composition of the solvent. Unfortunately the composition of IPAV is not available. Try to make an approximation to that exact composition by preparing 5 solutions:

90% 2-propanol plus 10% ammonia
80% 2-propanol plus 20% ammonia
70% 2-propanol plus 30% ammonia
50% 2-propanol plus 50% ammonia
30% 2-propanol plus70% ammonia.

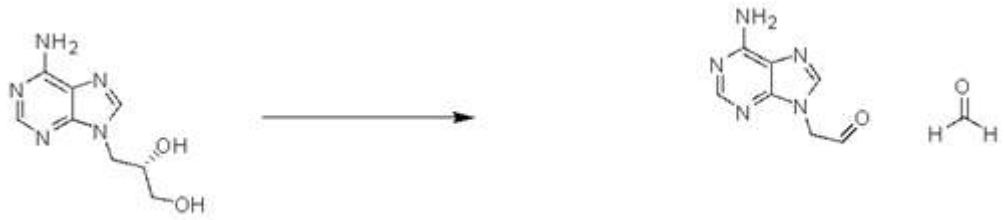
Choose 2 suitable compound (A - J) (not too fast and not too slow) and run individual TLCs with your prepared mixtures. Write Rf values for the different mixtures into the table. *Use a pencil to mark the spots on the TLC plates and paste them on the last page (labelled ´TLC plates´) of the answer sheets and write down the letters (A-J) of the samples that zou used.*

Indicate which mixture corresponds to IPAV in the answer sheet. (6 points)

Reaction with periodate ion can be used to detect the presence of vicinal diols (hydroxy groups on two adjacent carbon atoms). The general reaction is as follows:



And this is an example of the oxidation of compound 1:



Question C2.4 *Some samples can react with periodate anion by the mechanism explained before. Using a pipette, mix 10 drops of sample with 3 drops of periodate solution in an Eppendorf tube and let it stand for 10 minutes. Then use TLC again for analysis, similar to what you did earlier. Use a pencil to mark the spots on the TLC plates and paste them on the last page (labelled ´TLC plates´) of the answer sheets and write down the sample letters (A-J). Based on your observation, which samples reacted with sodium periodate solution? (8 points)*

Question C2.5 *One of the samples A-J is a precursor of DHPA. Acidic treatment of this sample provides the antiviral compound. The precursor can be revealed in the vapours of hydrochloric acid. Alternatively, you can mix a few drops of the sample with concentrated HCl and perform TLC. Which sample is the precursor? And which sample is DHPA? (10 points)*

T2

Trityl moiety in organic compounds can be cleaved with the help of strong acid:

- *All 10 samples (A - J) should be spotted on a single TLC plate at a reasonable distance from each other and described with letters Mark the spots on the TLC plate with the corresponding letters (A - J).*

- *The TLC is given to the lab assistant to put it in the chamber with hydrochloric acid vapors.*

- *Spot of tritylated DPHA will change the color to yellow the rest will stay unchanged. Mark the coloured spot and paste the plate on the last page (labelled ´TLC plates´) of the answer sheet.*

- *Lab assistant will add few drops of concentrated hydrochloric acid to your sample with tritylated DPHA. Wait for 1 minute and apply a dot of resulting mixture on TLC plate.*

- *Develop the plate in IPAV mobile phase. Compare the retention facter with the retention factor of compounds in experiments before. The sample with the same retention factor as your resulting mixture is DHPA. Use a pencil to mark the spots on the TLC plate and paste them on the last page (labelled ´TLC plates´) of the answer sheets*