The 39th International Chemistry Olympiad

Chemistry: art, science and fun

PREPARATORY PROBLEMS

(Experimental)

July 15-24, 2007

Moscow, Russia

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RULES TO BE FOLLOWED IN LABORATORIES

As mentioned in the Preface, we pay great attention to safety of experimental work. Below you will find a list of rules to be followed during laboratory exam at IChO-2007. We also hope you will take this information into account while preparing for the Olympiad.

- Students have to bring their own laboratory coats.
- Prior to the exam, students will be given Safety instructions in their mother tongue. Each student must carefully read the text and then sign.
- When students enter the lab they must familiarize themselves with the locations of emergency exits, safety shower, fire blanket and eye wash.
- Laboratory coats, eye protections and closed shoes must be worn while staying in the laboratory.
- Coats and bags are forbidden in the laboratory. Those have to be deposited in the cloakroom.
- Eating, drinking or smoking in the laboratory or tasting chemicals are strictly forbidden.
- Pipetting by mouth is strictly forbidden.
- Organizers do their best to avoid harmful chemicals at the exam. All potentially dangerous materials (if any) will be labeled by international symbols. Each student is responsible for recognizing these symbols and knowing their meaning.
- Do not dispose of chemicals down the sink. Follow all disposal instructions provided by Organizers.
- Do not hesitate to ask your lab instructor if you have got any questions regarding safety issues.

Nobody can create rules that will cover all situations, which may happen in reality. We do rely on your common sense and responsibility.

Good luck during preparations and at the exam!

LIST of R- and S-PHRASES

for the reagents used in Experimental problems

R-PHRASES

R5: Heating may cause an explosion

R8: Contact with combustible material may cause fire

R9: Explosive when mixed with combustible material

R10: Flammable

R11: Highly flammable

R20: Harmful by inhalation

R22: Harmful if swallowed

R23: Toxic by inhalation

R25: Toxic if swallowed

R34: Causes burns

R35: Causes severe burns

R36: Irritating to eyes

R37: Irritating to respiratory system

R40: Limited evidence of a carcinogenic effect

R43: May cause sensitization by skin contact

R50: Very toxic to aquatic organisms

R61: May cause harm to the unborn child

R20/21/22: Harmful by inhalation, in contact with skin and if swallowed

R23/24/25: Toxic by inhalation, in contact with skin and if swallowed

R36/38: Irritating to eyes and skin

R36/37/38: Irritating to eyes, respiratory system and skin

R50/53: Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment

S-PHRASES

S2: Keep out of the reach of children

S7: Keep container tightly closed

S16: Keep away from sources of ignition - No smoking

S17: Keep away from combustible material

S22: Do not breathe dust

S23: Do not breathe gas/fumes/vapor/spray (*appropriate wording to be specified by the manufacturer*)

S24: Avoid contact with skin

S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice

S28: After contact with skin, wash immediately with plenty of ... (*to be specified by the manufacturer*)

S30: Never add water to this product

S35: This material and its container must be disposed of in a safe way

S36: Wear suitable protective clothing

S37: Wear suitable gloves

S38: In case of insufficient ventilation wear suitable respiratory equipment

S45: In case of accident or if you feel unwell seek medical advice immediately (show the label where possible)

S60: This material and its container must be disposed of as hazardous waste

S61: Avoid release to the environment. Refer to special instructions/safety data sheet

S1/2: Keep locked up and out of the reach of children

S36/37: Wear suitable protective clothing and gloves

S36/37/39: Wear suitable protective clothing, gloves and eye/face protection

S37/39: Wear suitable gloves and eye/face protection

Problem 29. TITRIMETRIC DETERMINATION OF FE IN DIFFERENT OXIDATION STATES

Some methods of iron determination in the oxidation states +2 and +3 are discussed in Problem 12**.** You are invited to test one more approach to solving that problem in practice.

Reagents and solutions required

KIO_3 (R9, R22, R36/37/38, S35), reagent grade, solid
Ascorbic acid, solid
KI (R36/38, R42-43, R61; S26, S36/37/39, S45), 5% aqueous solution
HCl (R34, R37, S26, S36, S45), conc. and 2 M
HNO_3 (R8, R35, S1/2, S23, S26, S36, S45), conc.
Sulfosalicylic acid, 25% aqueous solution
NH_3 (R10, R23, R34, R50, S1/2, S16, S36/37/39, S45, S61), 10% aqueous solution
EDTA (R36, S26), standard solution, about 0.05 M (the exact value will be given)

1. Preparation of a primary standard solution of KIO_3

1.1. Calculate with the accuracy of 0.0001 g the weight of KIO_3 necessary for the preparation of 200.0 mL of 0.01000 M KIO_3 solution.

1.2. Using analytical balance weigh out accurately a portion of KIO_3. The weight of the portion may differ from the calculated one no more than by 0.05 g and it should be measured with a 0.0001 g accuracy.

1.3. Transfer the portion into 200.0 mL volumetric flask, dissolve it in water, dilute to the mark and mix.

1.4. Calculate the exact concentration of the solution prepared in mol/L.

2. Preparation of the titrant solution – ascorbic acid

2.1. Calculate with the accuracy of 0.01 g the weight of ascorbic acid necessary for preparation of 200 mL of 0.1 M solution.

2.2. Using technical balance weigh out a portion of ascorbic acid. Its weight may differ from the calculated one no more than by 0.05 g.

2.3. Dissolve the portion in ~200 mL of water, mix well, transfer the solution into a vessel and close it tightly with a stopper.

3. Standardization of the ascorbic acid solution

3.1. Fill in a burette with the ascorbic acid solution.

3.2. With a pipette transfer 10.00 mL of standard KIO_3 solution into a 100 mL Erlenmeyer flask, add 20 mL of 5% KI solution and 5 mL of 2 M HCl.

3.3. Titrate the mixture with the ascorbic acid solution until the iodine color disappears.
Note. When titrating iodine with solutions of reducing agents, starch is usually added as an indicator. Here it is not recommended to do so because the reaction rate decreases significantly in presence of starch.

3.4. Repeat the titration until three titrant volumes differ no more than by 0.10 mL.

3.5. Calculate the average titrant volume.

3.6. Calculate the ascorbic acid concentration in the solution in mol/L.

Questions

1. Write down the balanced equations of all the reactions taking place during standardization of ascorbic acid solution. Ascorbic acid $C_6H_8O_6$ is being oxidized to dehydroascorbic acid $C_6H_6O_6$.

2. KIO_3 in presence of excess of KI can be used as a primary standard for HCl standardization as well. The method is similar to that described above with the exception that no HCl is added to the titrated solution in this case. Which compound(s) can be used as an indicator(s) for that titration:

☐ starch

☐ sulfosalicylic acid

☐ methyl orange

☐ methyl orange + $Na_2S_2O_3$ (in excess)

4. Determination of Fe(III) by ascorbimetric titration

4.1. From your instructor obtain a sample solution containing Fe(II) and Fe(III) (in 100.0-mL volumetric flask). Dilute the solution to the mark with water and mix.

4.2. Fill in the burette with the standardized ascorbic acid solution.

4.3. With a pipette place 10.00 mL of the sample solution into a 100 mL Erlenmeyer flask, add 40 mL of water and heat nearly to boiling.

4.4. Into the hot solution add 4-5 drops of 25% sulfosalicylic acid solution as an indicator.

4.5. Titrate the solution with the ascorbic acid solution until the violet color disappears. During the titration and especially near the end point the solution must be hot. You may need to heat it additionally, if necessary. Near the end point the ascorbic acid solution should be added slowly.

4.6. Repeat the titrations until three titrant volumes differ no more than by 0.10 mL.

4.7. Calculate the average titrant volume.

4.8. Calculate the weight of Fe(III) in the sample solution given to you.

Note. Ascorbic acid, especially in aqueous solutions, is instable and oxidizes with oxygen from the air. Therefore the standardization of ascorbic acid solution and ascorbimetric determination of Fe(III) must be carried out during one workday.

Questions

1. Write down the balanced equations of all the reactions taking place during Fe(III) determination. Ascorbic acid $C_6H_8O_6$ is being oxidized to dehydroascorbic acid $C_6H_6O_6$.

2. In what media does ascorbic acid exhibit its reducing properties most markedly?

☐ in acidic

☐ in neutral

☐ in alkaline

☐ reducing properties of ascorbic acid do not depend on the pH

5. Determination of total iron by complexometric titration

5.1. Fill in the burette with an EDTA standard solution.

5.2. With a pipette transfer 10.00 mL of the sample solution into a 100 mL Erlenmeyer flask. Add 5 mL of conc. HCl and 2 mL of conc. HNO_3 to oxidize Fe(II) present in the sample to Fe(III). Cover the flask with a watch glass, heat until boiling and continue heating for 3-5 min avoiding splashing.

5.3. Cool down the solution and neutralize it carefully adding 10% NH_3 dropwise until color changes from lemon yellow to yellowish brown and slight turbidity persists.

5.4. Add 1-2 drops of 2 M HCl to dissolve the precipitate, then 0.5 mL of 2 M HCl more, dilute up to 50 mL with distilled water and heat nearly to boiling.

5.5. Into the hot solution add 4-5 drops of 25% sulfosalicylic acid solution as an indicator.

5.6. Titrate the solution until color changes from violet to clear yellow. During the titration and especially near the end point the solution must be hot. You may need to heat it additionally, if necessary. Near the end point the EDTA solution should be added slowly.

5.7. Repeat the titrations until three titrant volumes differ no more than by 0.10 mL.

5.8. Calculate the average titrant volume.

5.9. Calculate the total weight of iron in the sample solution given to you.

5.10. Calculate the weight of Fe(II) as a difference between the results obtained in 5.9 and 4.8.

Questions

1. Write down the balanced equations of all the reactions taking place during total Fe determination.

2. One of the crucial items in the Fe(III) determination by complexometric titration is strict maintenance of solution acidity. What are the reasons for that?

☐ If the acidity is too low, $Fe(OH)_3$ precipitates

☐ If the acidity is too high, complex of Fe(III) with sulfosalicylic acid does not form

☐ If the acidity is too high, complex of Fe(III) with EDTA acid does not form

☐ If the acidity is too low and/or too high, the titrant decomposes

Problem 30. ASYMMETRIC AUTOCATALYSIS – THE NUMERICAL EXPERIMENT

Nature exhibits a curious asymmetry between the left and the right, which is generally called ‘chiral asymmetry’. Indeed, living organisms contain mostly L-amino acids and D-carbohydrates. One of the possible explanations of this phenomenon is based on the idea of autocatalysis. Chiral (asymmetric) autocatalysis is a reaction in which every chiral product serves as the catalyst of its own formation. In such reactions small initial excess of one of the enantiomers can increase exponentially in time.

Consider the kinetic scheme explaining this phenomenon. Two Enantiomers, X_L and X_D, are reversibly formed from achiral reagents T and S:

$$S + T \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} X_L \qquad (1)$$

$$S + T \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} X_D \qquad (2)$$

$$S + T + X_L \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} 2X_L \qquad (3)$$

$$S + T + X_D \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} 2X_D \qquad (4)$$

$$X_L + X_D \xrightarrow{k_3} P \qquad (5)$$

Enantiomers react with each other giving the product P. The reactions take place in an open system, where constant concentrations of reagents S and T are maintained.

The system of rate equations can be solved numerically using any of the mathematical packages, for example Mathematica, MathCad, etc. Alternatively, you may use the program KINET posted on the official website www.icho39.chem.msu.ru. Let us assume the following values of rate constants (in arbitrary units): $k_1 = 0.5$, $k_{-1} = 0.1$, $k_2 = 0.5$, $k_{-2} = 0.2$, $k_3 = 0.5$.

Procedure

For numerical solution of the systems of differential equations mathematical packages use different commands. In Mathematica it is done by the function NDSolve. The arguments are the list of equations, initial conditions and a time interval. For example, the system of equations

$$a'(t) = -a(t)\,p(t)$$
$$p'(t) = a(t)\,p(t) - 2 \cdot p(t)$$

with the initial conditions $a(0) = 2$, $p(0) = 0.5$ in a time interval from $t = 0$ to $t = 10$ is solved numerically by the command:

```
sol=NDSolve [{a ' [t] ==-a [t] *p [t], p ' [t] == a [t] *p [t]-2*p [t], a [0] == 2, p [0] == 0.5},
        {a, p}, {t, 0,10}]
```

The obtained solution is presented on the graph by the command Plot:
Plot [Evaluate [{a [t], p [t]}/.sol, {t, 0,10}], PlotRange-> All]

Questions

1. Compare equations 1 and 2 or 3 and 4 in the Scheme above. Why are the rate constants identical for enantiomers X_L and X_D?

2. The control parameter for this problem is the product of concentrations of reagents. Solve the system of kinetic equations numerically and draw on one graph the kinetic curves for X_L and X_D using the initial conditions: $[X_L]_0 = 0$, $[X_D]_0 = 0.01$. Consider two opposite cases: [S] [T] is small, [S] [T] is large. By varying the parameter [S] [T] determine its “break” value at which the shape of kinetic curve(s) changes drastically.

3. At fixed value [S] [T] = 5 study the influence of initial chiral asymmetry on kinetic curves. Consider two cases: $[X_D]_0 = 0.001$, $[X_D]_0 = 0.1$.

Let us determine which elementary reactions are essential for chiral asymmetry amplification.

4. Consider the role of reversibility. For this purpose, given the same initial concentrations compare kinetic curves for two mechanisms: with reversible ($k_{-1} \neq 0$; $k_{-2} \neq 0$) and with irreversible formation of the enantiomers ($k_{-1} = k_{-2} = 0$).

5. Consider the simplified scheme in which the first two reactions are absent. Whether or not amplification of chiral asymmetry is possible in such system?

6. Compare the open and closed systems. You have already treated the open system. In the closed system the reagents S and T are no more introduced to a reaction vessel during reaction, therefore they should be included in the system of kinetic equations. Whether or not amplification of chiral asymmetry is possible in a closed system?

Draw the conclusions. What conditions are necessary for amplification of chiral asymmetry to be observed? What elementary stages appear to be essential for it?

Problem 31. OSCILLATING REACTIONS

Introduction

In 1921 W. Bray published an article describing the oscillating reaction of oxidation of hydrogen peroxide with potassium iodate. However thorough investigation of oscillating reaction mechanisms has begun only in 1951, when B.P. Belousov discovered oscillations of concentrations of reduced and oxidized forms of cerium catalyzing oxidation of citric acid by bromate-ion. Later it was shown that oscillating reactions are possible in other redox systems. A.M. Zhabotinsky investigated the oxidation of malonic acid by bromate-ion in the presence of manganese ions. This reaction mechanism is very sophisticated and includes dozens of intermediate compounds.

We will investigate an oscillating reaction taking place in the malonic acid-iodate ion system in the presence of manganese salt and hydrogen peroxide.

Reagents and equipment

1) 40 % H_2O_2 (R5, R8, R20, R22, R35; S1/2, S17, S26, S28, S36/37/39, S45)
2) KIO_3 (R9, R22, R36/37/38, S35).
3) conc. H_2SO_4 (R23/24/25, R35, R36/37/38, R49, S23, S30, S36/37/39, S45)
3) $C_3H_4O_4$, malonic acid (R20/21/22, S26, S36/37/39)
4) $MnSO_4 \cdot 5H_2O$ (R20/21/22, R36/37/38, R40, S26, S36)
5) starch
6) KI, solution (R36/38, R42-43, R61; S26, S36/37/39, S45)
7) $AgNO_3$, solution (R34, R50/53, S1/2, S26, S45, S60, S61)
8) analytical balance
9) weighing dishes
10) flat-bottom flasks or beakers (250-500 ml), 4 items
11) stop-watch

Procedure

Prepare three solutions (may be prepared in advance):

1) solution of 80 ml 40 % H_2O_2 in 120 ml of water,
2) solution of 8.7 g KIO_3 and 0.9 ml conc. H_2SO_4 in 190 ml of water,
3) solution of 3 g $C_3H_4O_4$, 2.4 g $MnSO_4*5H_2O$ and 0.06 g starch in 195 ml of water.

Mix the solutions in the same vessel and observe the oscillating process. Evaluate the oscillation period and its change in time.

Split the mixture into two parts and place them into beakers.

To one of the parts add $AgNO_3$ solution (first – several drops, then ~3 ml). Observe changes of the oscillation period. Note the color of the solution upon completion of the oscillation reaction.

To the other part add KI solution (several drops). Observe changes of the oscillation period.

Questions

1. Oxidation of malonic acid by potassium iodate is an autocatalytic process. Write down the net equation of the reaction. Which product is the catalyst of the oscillating process? Explain the effect of silver nitrate.

2. B.P. Belousov used bromate-ion as an oxidizing agent. Suggest what would happen if we substitute iodate-ion by bromate-ion in the reaction with malonic acid. What role does hydrogen peroxide play in the oxidation of malonic acid with iodate-ion?

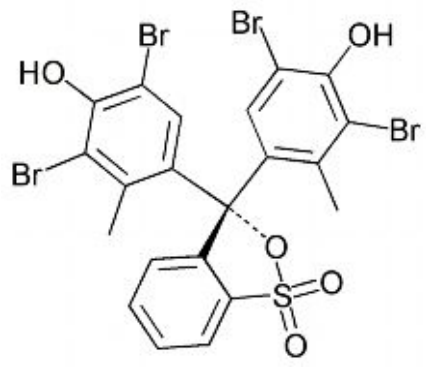
3. It is well known, that one of the stages of the oscillating process is formation of iodomalonic acid with its subsequent decomposition. How can we explain the fact that potassium iodide inhibits the reaction?

4. B.P. Belousov used the Ce^{4+}/Ce^{3+} redox couple to study oscillating reactions. Is it possible to use the following transient metal redox couples as a catalyst: Co^{3+}/Co^{2+}, Fe^{3+}/Fe^{2+}, Tl^{3+}/Tl^{1+}?

$E^o(Co^{3+}/Co^{2+})$ = 1.81 V, $E^o(Ce^{4+}/Ce^{3+})$ = 1.61 V,
$E^o(Mn^{3+}/Mn^{2+})$ = 1.51 V, $E^o(Fe^{3+}/Fe^{2+})$ = 0.77 V?

Problem 32. DETERMINATION OF THE ACIDITY CONSTANT OF BROMOCRESOL BLUE (3′,3″,5′,5″-TETRABROMO-M-CRESOLSULFONEPHTHALEIN, BCB)

Bromocresol blue (BCB)



is an organic dye, an acid-base indicator, a weak diprotic acid (H_2A). In aqueous solutions in the pH range of 3-6 BCB changes its color from yellow to blue due to dissociation of the second proton:

$$HA^-\ (\text{yellow}) \rightleftarrows A^{2-}\ (\text{blue}) + H^+$$

On the base of the absorbance of BCB solution measured as a function of the pH one can calculate the second acidity constant of BCB, pK_{a2}.

Reagents and solutions required

Bromocresol blue, 0.25% solution in 50% aqueous ethanol (R11, S2, S7, S16).
Mixture of acids for preparation of buffer solutions: an aqueous solution containing H_3PO_4, (R34, S1/2, S26, S45), CH_3COOH (R10, R35, S1/2, S23, S26, S45) and H_3BO_3 , (S22, S26, S36/37, S38, S45), 0.04 M each.
NaOH (R35, S1/2, S26, S37/39, S45), 0.2 M and 2 M solutions.
HCl (R34, R37, S26, S36, S45), 2 M solution.

1. Choice of the wavelength for the K_{a2} determination

1.1. Into each of two 50.0 mL volumetric flasks place 1.00 mL of the BCB solution and 10.00 mL of the mixture of acids (see reagent list). Then add 1.00 mL of 0.2 M NaOH

into the first and 6.00 mL of 2 M NaOH into the second flask. Dilute the solutions to the mark with water and mix.

1.2. Measure the pH of the solutions prepared. The first one must have the pH in the range of 2-3, the second – within 7-8. Under such conditions all BCB is in the form of either HA^- or A^{2-} respectively. If either of the pH is different from the required, adjust it by adding few drops of 2 M HCl or 2 M NaOH.

1.3. Measure the absorption spectra of the solutions in the range of 400-700 nm; 5-10 data points would be sufficient.

1.4. Choose the wavelength at which the absorbances of the solutions differ most greatly. Usually that wavelength corresponds to the maximum of absorbance of one of the species or close to it. Further carry out all the measurements at that wavelength.

2. Preparation of series of BCB solutions, measuring their absorbance and the pH

2.1. Into each of twelve 50-mL volumetric flasks place 1.00 mL of BCB solution and 10.00 mL of the mixture of acids. Then add 0.2 M NaOH to each flask in the amount indicated in Table below:

Flask number	0,2 M NaOH, mL
1	0.75
2	1.50
3	2.50
4	2.75
5	3.00
6	3.25
7	3.50
8	3.75
9	4.00
10	4.25
11	5.25
12	6.25

Dilute the solutions to the mark with water and mix.

Note. It is of essential importance that the concentrations of BCB be strictly the same in all the solutions. When preparing the solutions pay especial attention to that requirement!

2.2. For each solution measure the pH and the absorbance at the chosen wavelength.

2.3. Using the data obtained calculate $\log K_{a2}$ for each of the solutions unless fraction of either of the species involved in the acid-base equilibrium is negligible.

2.4. Calculate the average $\log K_{a2}$ value.

Questions

Denote as:
$[HA^-]$, $[A^{2-}]$, c – equilibrium concentrations of the corresponding BCB forms and its total concentration, respectively;
l – cuvette length;
K_{a2} – acidity constant of HA^-;
ε_{HA}, ε_A – extinction coefficients of the corresponding forms at the chosen wavelength;
A_{HA}, A_A, A – absorbances of BCB solution containing only HA^-, only A^{2-} and their mixture, respectively.

1. Write down the equations for A_{HA}, A_A and A as functions of $[HA^-]$, $[A^{2-}]$ and c.

2. Express A as a function of A_{HA}, A_A and $[H^+]$.

3. Write down the equation for calculation of K_{a2} from A_{HA}, A_A, A and $[H^+]$.

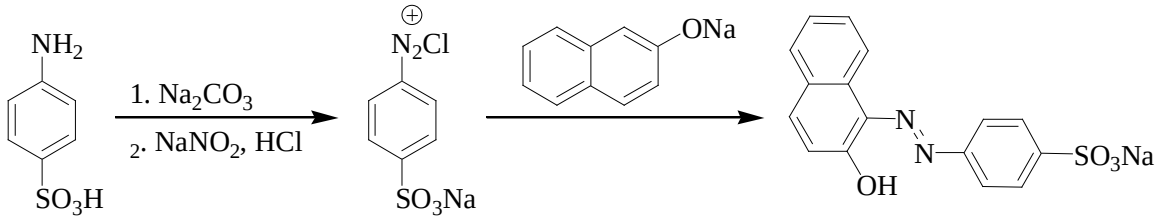
4. Consider the wavelength at which $\varepsilon_{HA} = \varepsilon_A$. It is called the isosbestic point.

a) Is it possible to determine K_a of a dye by measuring the absorbance at the isosbestic point?

b) What analytical information can be obtained from such measurement?

Problem 33. ACID ORANGE 7

A very popular azo-dye known under dozens of trade names and widely used in textile, leather, food, cosmetics, as well as other industries, Acid Orange 7 (Acid Orange II, Persian Orange, listed in the Color Index as No. 15510) can be readily obtained by azo-coupling of diazotized sulphanilic acid with 2-naphtholate



Materials and hardware

Sulfanylic acid (R36/37/38, R43, S24, S37)
2-Naphthol (R36/37/38, S26, S37)
Sodium carbonate (R36, S2, S22, S26)
Sodium nitrite (R8, R25, R36/37/38, R50, S26, S36, S45, S61)
Sodium hydroxide (R35, S1/2, S26, S37/39, S45)
Hydrochloric acid, conc. (R34, R37, S26, S36, S45)
Ice

Glass beakers (150, 200, 500 ml), thermometer, spatulas, magnetic stirrer and heating plate, vacuum filtration apparatus, desiccator.

The diazotization

Sulfanylic acid (8.66 g, 0.05 mol) is dissolved in the solution of 3 g of sodium carbonate in 50 ml water in a 150 ml glass beaker placed on a magnetic stirrer. 15 ml of concentrated HCl are added to this solution at vigorous stirring. After cooling to room temperature, the beaker is immersed in an ice bath (a couple of ice chunks can be added to the mixture to ensure good cooling) and the mixture is further cooled to 0 °C. A solution of $NaNO_2$ (3.45 g, 0.05 mol) in 20 ml of water is added dropwise (***warning!*** this

operation should be done in a hood because of evolution of nitrogen oxides). The rate of addition should be controlled to keep the temperature near 0 °C as accurately as possible (***warning!*** even a 2-3° increase leads to side-reactions which may lead to the formation of phenols giving unwanted azo-dyes which dramatically worsen the purity of color of the target dye). During the addition white precipitate of diazonium salt (diazotized sulfanylate is a betaine, an inner salt with zero net charge, therefore it is not well soluble in water) may sometimes form. The results of diazocoupling do not depend on whether the diazonium salt is in solution or suspension.

After the addition of all nitrite solution, stirring is continued for 10-15 min (***warning!*** temperature should be carefully controlled!). The diazonium salt solution (or suspension) should be used immediately after preparation.

The azocoupling

2-Naphthol (7.21 g, 0.05 mol) is dissolved in 40 ml of 5% NaOH solution. This solution is mixed with solution of 12.5 g Na_2CO_3 in 100 ml water in a 500 ml beaker. The resulting solution should be transparent, if any precipitate or suspension persists, it should be filtered off. The solution of naphtholate is cooled to 0 °C by ice (an ice bath + a few ice chunks inside). The diazonium salt solution is slowly poured to naphtholate solution under vigorous stirring by a spatula or a glass rod. Attention should be paid to keep the temperature below 8 °C throughout the addition. Afterwards, the mixture is left for an hour, preferably on a magnetic stirrer. The dye partially precipitates as golden plates.
After an hour, the solution is heated to completely dissolve the precipitate, filtered hot (*note*: this filtration can be omitted if a hot filtration funnel is not available), and saturated by 50 g of sodium chloride (50 g) while hot (it is necessary to keep temperature above 50° during saturation, so the beaker should be placed on a heating plate). Dye precipitate formed by salting-out is filtered off by vacuum filtration from hot solution (*note:* if the temperature of solution being filtered drops below 50°, sodium chloride partially co-precipitates with the dye). The dye is dried in a desiccator over $CaCl_2$. Orange solid, yield 25 g.

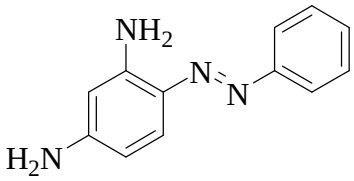
The quality of dye can be controlled by the UV/Vis spectroscopy. In aqueous solution λ_{max} 487 nm ($\log\varepsilon$ 4.87).

Questions

1. Under the name *tropaeolin 000* the dye is used as an acid-base indicator in aqueous solutions. Guess in which region of pH this dye changes its color:
☐ strongly acidic (pH<2); ☐ acidic (pH 2-6.5); ☐ neutral (pH 6.5-7.5) ☐ mildly alkaline (pH 7.5-9); ☐ strongly alkaline (pH 9-14).

2. Write the reaction equation which accounts for the color change.

3. Write the reaction equation of an azocoupling required to obtain *chrysoidine* dye.



4. Which pH region should be chosen for this azocoupling:
☐ strongly basic, ☐ weakly basic, ☐ weakly acidic, ☐ strongly acidic?

PROBLEM 34. DETERMINATION OF MOLECULAR WEIGHT OF A PROTEIN USING GEL FILTRATION

Gel filtration is a simple and reliable chromatographic method for separating molecules according to their size. Within a fractionation range chosen, molecules are eluted in a decreasing order of their size. Versatility of the method makes it applicable for purification and characterization of biological substances of all classes, including macromolecules not readily fractionated by other techniques.

Some gel forming organic polymers with a 3D network structure (usually referred to as gel filtration media, GFM) possess properties of molecular sieves and can separate molecules according to their size and shape. A chromatography column should be filled with swollen gel and equilibrated with corresponding buffer solution. The separation mechanism is non-adsorptive and independent of the eluent system used, thus being

fairly gentle. Liquid inside porous gel beads of GFM is the stationary phase, whereas eluent solution outside the beads is the mobile one.

In a column, all sample molecules can be present in the liquid between the beads. The total volume of such “outside” liquid is referred to as *the void volume* in gel filtration and is equal to about 30% of the column volume. Sample molecules are partitioned between the eluent (the mobile phase) and the accessible part of bead pores (the stationary phase). This partitioning acts to establish a *dynamic equilibrium* of sample molecules between the mobile and stationary phases and is driven exclusively by diffusion. The mobile phase transports the sample molecules down the column. The molecules present in the pores are "stationary" and not subjected to transportation. Migration rate of a sample zone depends on the fraction of sample molecules present in the mobile phase. Separation of individual macromolecules can only be achieved in the case of their partial access to the pores of the GFM. *Applicable sample volume* is restricted to 0.5-5% of that of the column, since no concentration effect is active in gel filtration. *Flow rate* is kept low to avoid peak broadening due to incomplete mass transfer, whereas columns used are long to allow optimum resolution.

Materials

Blue dextran (molecular weight, MW=2 MDa), 4 mg
Proteins:
Ovalbumin (MW=43 kDa), 1.5 mg
Cytochrome C (MW=13 kDa), 0.4 mg
Bovine serum albumin (BSA) (MW=67 kDa), 2.2 mg
Chymotrypsinogen (MW=25 kDa), 1 mg
Hemoglobin (MW=64.5 kDa), 1.5 mg

0.1 M HCl (R34, R37, S26, S36, S45) 230 mL, KCl 22.35 g
Buffer: Tris (2-Amino-2-(hydroxymethyl)propane-1,3-diol; R36/37/38, S26, S36) 6.05 g
GFM: Toyopearl HW-50 (or HW-55), fine, 70 mL.

If the mentioned above proteins are partially inaccessible, those missing can be substituted by proteins with close MW, but not proteases. Toyopearl may be also replaced by a GFM with similar properties.

Apparatus

70 mL chromatography column; packing reservoir; stand; peristaltic pump; UV-cord connected to plotter; Eppendorf centrifuge; analytical balances; water-jet pump; one 1000 mL measuring cylinder; one 250 mL volumetric flask; one big Buchner funnel with glass filter; one 1000 mL Bunsen flask; one 1000 mL round-bottom flask; one 100 μL micropipette with tips; one 1000 μL micropipette with tips; one 2 mL syringe connected to 20 cm tubing; four Eppendorf tubes; one 100 mL measuring cylinder; one 200 mL flask; one 100 mL beaker; big steel spatula; small spatula; glass rod; filter paper.

Note: A UV-cord can be substituted by a UV-visible spectrophotometer and measuring test tubes.

Procedure

Step 1. Preparation of buffer solution

To prepare 0.2 M Tris buffer solution, dissolve 6.05 g of Tris in 250 mL of distilled water in the 250 mL volumetric flask. Mix 125 mL of 0.2 M Tris solution and 230 mL of 0.1 H HCl in the 1000 mL measuring cylinder. Add distilled water to 800 mL. Add 22.35 g of KCl to the Tris-HCl solution and stir thoroughly until the salt completely dissolves. Add water to 1000 mL (the final concentration of KCl is 0.3 M

Step 2: Preparation of a chromatographic column

Packaging the column is one of the most important stages in chromatography, as it determines the separation quality to a great extent. The column should be packed uniformly, and the upper and lower gel surfaces should be strictly horizontal.

1. Equilibrate gel material to room temperature.
2. Gently shake the bottle to make an even slurry.
3. Pour 70 ml of gel slurry into a beaker and dilute with buffer to 100 ml.
4. Stir with a glass rod to make a homogeneous suspension free from aggregates.
5. Add eluent buffer solution to the column to check for leaks, wet the walls of the column and remove air from the bed support. (It is better to fill the column bottom-up

using the water-jet pump). Drain buffer leaving about 1 cm above the gel surface. For columns with bottom glass porous filter, a filter paper circle with a diameter equal to the inner column diameter should be placed on the glass filter to prevent from gel leakage from the column.

6. Mount the column vertically and attach the addition packing reservoir firmly to the column. It should be twice shorter than the column.

7. Wash the gel with three portions (of about 100-120 mL) of Tris-buffer solution on Buchner funnel with glass filter attached to 1000 mL Bunsen flask using water-jet pump. Try not to dry Toyopearl. After each washing disconnect the water-jet pump when the upper gel surface just starts turning dry. Then add next portion of buffer, stir with big steel spatula to make a homogeneous suspension, and subject to suction.

8. Transfer the gel from the funnel into 1000 mL round-bottom flask, add 50 mL of buffer solution and connect the flask to water-jet pump using a connector. Vacuum degassing should proceed for at least 5 min.

9. Re-suspend and pour the gel slurry into the column in one continuous motion. Pouring down a glass rod held against the wall of the column prevents from air bubbles (Fig.1). Try gel slurry to flow along the column wall.

10. Carefully fill the reservoir to the top with buffer solution, disturbing the gel as little as possible. Connect the reservoir with the peristaltic pump, which should in turn be joined to buffer stock in the 200 mL flask. Turn on the pump and open the column outlet.

11. Buffer solution should be pumped through the column until the gel stops settling. After two bed volumes remove the gel reservoir and insert flow adaptor.

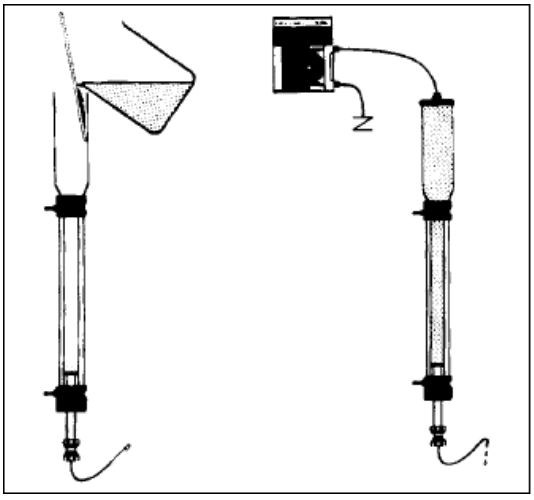


Fig. 1. Packaging the column with GFM.

Step 3: Preparation of solutions

Weigh blue dextran and proteins using balance and small spatula. Prepare solution of Blue dextran by dissolving it in 1 mL of Tris-buffer solution in an Eppendorf tube. Prepare two solutions of standard proteins in Eppendorf tubes. The first solution contains Ovalbumin, Cytochrome C, 0.07 mL of blue dextran solution and 0.93 mL of Tris-buffer solution. The second solution contains Bovine serum albumin, Chymotrypsinogen, 0.07 mL of blue dextran solution and 0.93 mL of Tris-buffer solution. Prepare solution of Hemoglobin (unknown protein) in 1 mL of Tris-buffer solution. Centrifuge two solutions with standard proteins and the solution of unknown protein for 5 min.

Step 4: Application of samples

1. Apply sample solutions carefully, trying not to disturb the gel. To make it easier, filter paper circle could be placed at the top of gel (still take into account possible protein absorption on the paper). Remove flow adaptor, disconnect the peristaltic pump and open the column outlet. Let the buffer soak into the gel (the gel surface should be free of buffer but not dry) and close the column outlet. Add sample solution slowly using pipette with wide tip or 2 mL syringe connected to 20 cm tubing, open the column outlet and allow the solution flow inside the gel. Close the column outlet and add buffer solution (about 1 mL) slowly and carefully (as during the sample application). Open the column outlet and let the buffer soak in the gel. Repeat the procedure. This allows the sample solution flowing deeper inside the gel and prevents from backward diffusion. Close the column outlet and carefully make a buffer layer with height of about 2 cm over the gel.

2. Connect the peristaltic pump to the column inlet and the UV-cord to the column outlet (the tube length should be as short as possible) and start elution.

Step 5: Column chromatography

1. Carry out calibration of the column in two steps:

A. Apply the first solution of standard proteins containing Blue dextran, Ovalbumin and Cytochrome C to the column. Start elution with the rate of about 1-2 mL/min, collecting

the eluate into 100 mL measuring cylinder. The elution process is monitored by following the eluate absorbance at 280 nm, which is registered by the UV-cord. Measure Elution volumes for Blue dextran and proteins using cylinder (record the volumes corresponding to maxima of the eluate absorbance).

Note: in the case of using a spectrophotometer and test-tubes, the procedure should be modified as follows. Collect the eluate in a measuring cylinder up to 25% of the column volume. Then continue collecting the eluate in test-tubes in portions of 1 mL. Determine the eluate absorbance at 280 nm in each test-tube by using a spectrophotometer and record the total volumes corresponding to maxima of the eluate absorbance).

After the three peaks are registered, the column should be washed with the buffer solution until the total elution volume becomes equal to that of the column.

B. Apply the second solution of standard proteins and proceed as described above.

2. Apply the solution of unknown protein. After the peak is registered, stop the peristaltic pump, close column outlet and turn off the UV-cord.

Questions

1. Correlate chromatographic peaks with substances you applied to the column. Complete the table:

Standard solution number	Number of peak (in the order of appearance)		
	1	2	3
1			
2			

2. What is the void volume of your column? Explain.

3. Calculate the volume of the chromatographic column.

4. Calculate the availability coefficient K_{av} for all proteins using formula

$$K_{av} = \frac{V_r - V_0}{V_c - V_0}$$

V_r is elution volume for sample molecule, V_o is the void volume, V_c is the column volume.

5. Plot the calibration curve as the dependence of K_{av} on log(MW) using the data obtained for four standard proteins.

6. Determine MW for the unknown protein.

7. Another important characteristic of a column is *the exclusion limit*, M_r, which is defined as the molecular mass of the smallest molecule excluded from the pores. Calculate this parameter by finding the intercept of the extrapolated linear part of the calibration curve with the log(MW) axis.

8. Estimate the elution volume for low molecular weight substances if applied to the column under consideration. Provide an explanation.