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EXPERIMENT 1

Determination of the Stern-Volmer constant for fluorescein fluorescence quenching

Basic notions: absorption, fluorescence, fluorescence quenching, fluorescence quantum yield. Absorption spectrum versus fluorescence spectrum. Stern-Volmer equation. Reaction rate. Reaction rate constant. Kinetic equations. Reaction order. Stationary state. Relaxation time.

Introduction

A fluorophore is a molecule or a functional group capable of fluorescence. An example of fluorophore is fluorescein, showing green-yellow fluorescence in basic solutions. The aim of the experiment is characterisation of the fluorescein fluorescence quenching. Fluorescein is a fluorosensor sensitive to the presence of iodide ions. The structural formula of fluorescein molecule ($C_{20}H_{12}O_5$) is presented in Fig. 1.
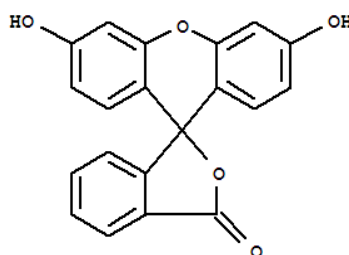

Fig. 1. Structural formula of the fluorescein molecule.

Theory

The radiation emitted by atoms or molecules that have been electron excited as a result of absorption of light is called luminescence. It accompanies the return of the atom or molecule from the excited electronic state to the ground electronic state. If the emission of radiation is related to a spontaneous transition from the first excited singlet state S_1 onto the ground singlet state S_0: $S_1 \rightarrow S_0 + hv$, then such a luminescence is called fluorescence. Fig. 2 presents the so-called Jabłoński diagram. It illustrates different types of radiative and nonradiative transitions that can take place in a multiatomic molecule after absorption of a photon.
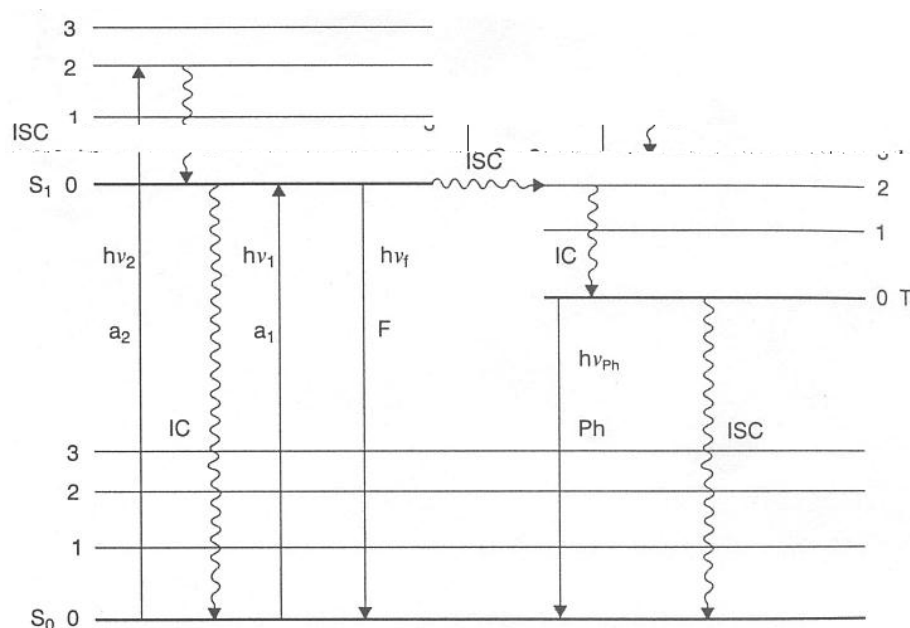
Fig. 2. The Jabłoński diagram; S_0, S_1 and T_1 stand for the singlet states and the triplet state; a_1, a_2 – show photon absorption, F – fluorescence, Ph – phosphorescence, IC – internal conversion, ISC – intersystem crossing. Straight arrows show radiative transitions, while wavy arrows show nonradiative transitions.

Usually the excitation takes place from the lowest vibrational level of the ground electronic state, because at room temperature most of the molecules are at this level. The state for $v = 0$ corresponds approximately to the minimum of the potential energy of molecules. In Fig. 2 the straight arrows show the transitions from the ground state S_0 to the excited state S_1 taking place as a result of absorption of a quantum of light. The molecule transits from the zero vibrational level of the ground electronic state to the zero (a_1) or a higher (a_2) vibrational level of the first excited state. The molecule lifetime in the excited state S_1 depends on the following processes: 1) fluorescence, F, 2) chemical reaction, 3) nonradiative transition, IC (internal conversion) and 4) nonradiative intersystem crossing ISC, from S_1 to T_1. If internal conversion takes place, the excess energy produced in this process is dissipated to the environment as heat. The intersystem crossing is accompanied with spin inversion leading to formation of a triplet state. The lifetime of T_1 triplet state depends on the processes of 1) phosphorescence, Ph, which is a radiative transition from T_1 to S_0 2) chemical reactions, 3) intersystem crossing ISC, which is a nonradiative transition from T_1 to S_0. Phosphorescence and internal conversion (transition from T_1 to S_0) are forbidden processes, which explains the relatively long lifetime of the lowest excited triplet states.

The spectrum of fluorescence is always shifted towards longer wavelengths with respect to the absorption spectrum. The shape of the absorption and emission spectra depends on the distribution of energy of the vibrational levels of the states S_0 and S_1. Often this distribution is the same for both states and then the emission spectrum is almost a mirror reflection of the absorption spectrum. Fig. 3 presents the absorption and emission spectra of fluorescein.
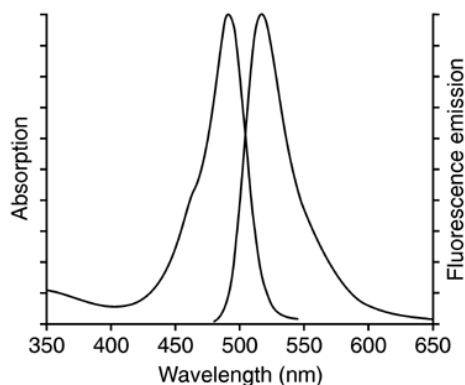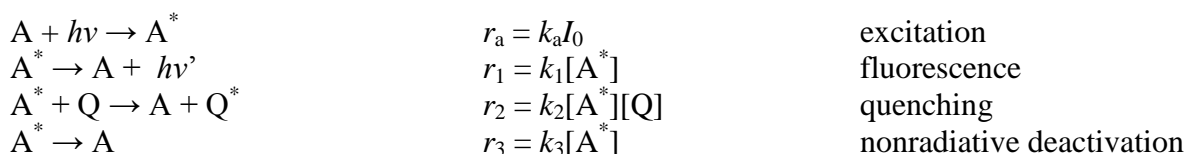
Fig. 3. The absorption (on the left) and fluorescence (on the right) spectra of fluorescein.

Let's consider the process of excitation of substance A as a result of absorption of a photon of incident light $h\nu$ and possible pathways of the loss of energy absorbed by the molecule A. The processes can be described by the following kinetic scheme:

$A + h\nu \rightarrow A^*$	$r_a = k_a I_0$	excitation
$A^* \rightarrow A + h\nu'$	$r_1 = k_1[A^*]$	fluorescence
$A^* + Q \rightarrow A + Q^*$	$r_2 = k_2[A^*][Q]$	quenching
$A^* \rightarrow A$	$r_3 = k_3[A^*]$	nonradiative deactivation

The symbols r_a, r_1, r_2, r_3 stand for the rates of particular processes, while k_1, k_2, k_3 are the rate constants of fluorescence of excited A^*, nonradiative energy transfer from A^* to the quencher molecules Q and nonradiative deactivation of A^*, respectively. The rate r_a of the process of excitation of molecules A is equal to the number of photons $h\nu$ absorbed by the molecules of A in a unit of time and in a unit volume, so it is equal to the number of molecules A undergoing excitation in given conditions. The Lambert-Beer law implies that the number of adsorbed photons is proportional to the intensity of incident radiation I_0 (the concentration of unexcited molecules remains practically constant in the normal excitation conditions) so $r_a = k_0 = k_a I_0$ (k_0 is the rate constant of zeroth order reaction, k_a is a constant in given experimental conditions).

In the stationary state, the rates of the processes of activation and deactivation must fulfil the equation $r_a = r_1 + r_2 + r_3$, so

$$k_a I_0 = k_1[A^*] + k_2[A^*][Q] + k_3[A^*] \tag{1}$$

The quantum yield Φ of fluorescence is the ratio of the number of quanta emitted to the number of quanta of the exciting radiation absorbed:

$$\Phi = \frac{k_1[A^*]}{k_a I_0} \tag{2}$$

So that

$$\Phi = \frac{k_1}{k_1 + k_3 + k_2[Q]} \qquad (3)$$

In the absence of the quencher molecules Q, [Q] = 0, the quantum yield Φ_0 is

$$\Phi_0 = \frac{k_1}{k_1 + k_3} \qquad (4)$$

so

$$\frac{\Phi_0}{\Phi} = 1 + \frac{k_2}{k_1 + k_3}[Q] = 1 + k_2\tau[Q] \qquad (5)$$

This equation is known as the Stern-Volmer equation. Instead of rate constants of particular processes we often use the relaxation times defined as $\tau = 1/k$, which inform about lifetimes of particular states. In equation (5) the relaxation time $\tau = 1/(k_1+k_3)$ characterises the lifetime of the excited state of molecules A in the absence of quenchers. The quenching of fluorescence is the reduction of its intensity as a result of interactions of the fluorescent substance molecules with the molecules of the quenching substance, known as the quencher.

Experiment Part 1

Method
The aim of the experiment is determination of the effect of concentration of the quenching substance on the intensity of fluorescence (*I*) of fluorescein. In the experiment the fluorescence spectra of solutions containing fluorescein and potassium iodide in different concentrations are recorded and analysed.

Reagents and apparatuses
- Fluorescein solution of concentration 0.01 mol/dm^3 in the presence of 0.1 mol/dm^3 NaOH
- Potassium iodide solution of concentration 0.05 mol/dm^3
- 10 tubes of 10 ml in capacity
- automatic pipettes of 5 ml, 1 ml, 0.1 ml in capacity
- 3 beakers of 500 ml, 100 ml, 25 ml in capacity
- 1 wash bottle
- spectrofluorimeter

Procedure
1. Make solutions containing 0.1 cm^3 of fluorescein of concentration 0.01 mol/dm^3 and different volumes of water and a solution of potassium iodide of concentration 0.05 mol/dm^3. The volumes of KI solution are given in Table 1; total volume of each solution prepared should be 5 cm^3. The sequence of loading the above mentioned liquids is water as first, then a solution of potassium iodide and finally a solution of fluorescein. The contents of each tube should be well mixed so that fluorescein was uniformly distributed in the whole volume of the sample. Fluorescein solutions must be protected against light and directly prior to measurements they should be carefully stirred.
2. Record the fluorescence spectra of fluorescein in the range from 500 to 600 nm, at the excitation wavelength (λ_{ex}) of 492 nm, in the absence of potassium iodide (quencher) and in its presence in different concentrations. In order to obtain the fluorescence spectrum of the

dye, its molecules are excited with the light of the wavelength at which the dye absorption reaches maximum. Therefore, according to Fig. 3, for fluorescein the excitation wavelength is λ_{ex} = 492 nm.

3. From the spectra recorded read off the maximum intensity of fluorescence so the value of fluorescence at the maximum of the spectrum, i.e. at the wavelength from the range 525-530 nm.

4. Write the results of measurements in Table 1.

Table 1

No.	Volume of KI solution added [cm^3]	Volume of water [cm^3]	Concentration of KI, c [mol/dm^3]	Maximum intensity of fluorescence, I	I_0/I
1	0	4.9			
2	0.25	4.65			
3	0.5	4.4			
4	0.75	4.15			
5	1	3.9			
6	1.5	3.4			
7	2	2.9			
8	3	1.9			
9	4	0.9			
10	4.9	0			

Calculations

On the basis of the data from Table 1 plot I_0/I as a function of the quencher concentration, c; I_0 is the fluorescence intensity for c = 0. According to the Stern-Volmer equation, eq. 5, the plot of I_0/I versus c should be linear as $I_0/I = \Phi_0/\Phi$. At higher concentrations of the quencher, positive deviations from linearity can take place. Their appearance is related to the collision mechanism of quenching and formation of complexes between the molecules of fluorescent dye and the quencher. The proportionality coefficient, $k_2/(k_1+k_3) = k_2\tau$, known as the Stern-Volmer constant or the quenching constant, is the slope of the linear part of the dependence $I_0/I = f(c)$.

Discussion

1. Give the units of the Stern-Volmer constant and explain its physical meaning.

2. Estimate the quencher concentration to which the dependence $I_0/I = f(c)$ is linear.

Experiment Part 2

The Stern-Volmer equation is applied for dynamic quenching that takes place when the quencher (Q) collides with the fluorophore (A) in the excited state: $A^* + Q \rightarrow A + Q^*$.Eq. (5) can be written in the form:

$$I_0/I = \Phi_0/\Phi = 1 + K_{SV}[Q] \tag{6}$$

According to eq. (6) for dynamic quenching the dependence $I_0/I = f([Q])$ is linear. Another type of quenching is the so-called static quenching, when the quencher and the fluorophore in the ground state make a non-fluorescent complex $A + Q \rightarrow A\text{-}Q$

The complexation constant K_{kompl} is:

$$K_{kompl} = \frac{[A-Q]}{[A][Q]} \qquad (7)$$

The total concentration of fluorophore is
$$[A_0] = [A] + [A\text{-}Q] \qquad (8)$$

Inserting eq. (8) into eq. (7), we get:

$$K_{kompl} = \frac{[A_0]-[A]}{[A][Q]} = \frac{[A_0]}{[A][Q]} - \frac{1}{[Q]} \qquad (9)$$

After some transformations of eq. (9) we get a linear relation $I_0/I = f([Q])$ for the static quenching:

$$\frac{I_0}{I} = \frac{[A_0]}{[A]} = 1 + K_{kompl}[Q] \qquad (10)$$

Deviations form linearity of $I_0/I = f([Q])$ inform that both dynamic and static quenching processes take place. In such conditions the following formula is used:

$$\frac{I_0}{I} = (1 + K_{SV}[Q]) \cdot (1 + K_{kompl}[Q]) \qquad (11)$$

from which after some transformations and substitution of

$$\frac{I_0}{I} = 1 + K_{poz}[Q] \qquad (12)$$

We get a linear relation $K_{poz} = f([Q])$:

$$K_{poz} = \left(\frac{I_0}{I} - 1\right)\frac{1}{[Q]} = (K_{SV} + K_{kompl}) + K_{SV}K_{kompl}[Q] \qquad (13)$$

Procedure

The procedure of the second part of the experiment is similar to that of the first part, but the difference is that instead of a solution of potassium iodide of concentration 0.05 mol/dm³, use a solution of concentration 0.2 mol/dm³, and instead of water use a solution of potassium chloride of concentration 0.2 mol/dm³.

1. Make solutions containing 0.1 cm³ of fluorescein solution and the volumes of KI and KCl solutions given in Table 2.
2. Record the fluorescence spectra of fluorescein in the conditions specified in part 1 of the experiment. Read off the maximum intensity of fluorescence.

3. Write the results of measurements in Table 2.

Table 2

no.	Volume of KI solution added [cm^3]	Volume of KCl solution added [cm^3]	Concentration of KI, c [mol/dm^3]	Maximum intensity of fluorescence, I	I_0/I	K_{poz}
1	0	4.9				
2	0.25	4.65				
3	0.5	4.4				
4	0.75	4.15				
5	1	3.9				
6	1.5	3.4				
7	2	2.9				
8	2.5	2.4				
9	3	1.9				
10	3.5	1.4				
11	4	0.9				
12	4.9	0				

Calculations

For each KI concentration used find the K_{poz} values from eq. (12) and then by linear regression find the parameters of the line $K_{poz} = f(c)$ from eq. (13). Estimate the value of the complexation constant, K_{kompl} and Stern-Volmer constant, K_{SV}, knowing that K_{SV} is higher than K_{kompl}.

Discussion

1. Compare the values of Stern-Volmer constant, K_{SV}, obtained in the first and second part of the experiment.
2. Explain why in the second part of the experiment a solution of potassium chloride was used instead of water.

References

[1] P. W. Atkins, *Chemia fizyczna*, Wydawnictwo Naukowe PWN, Warszawa 2003, rozdz. 17.3.

[2] K. Pigoń, Z. Ruziewicz, *Chemia fizyczna. T2*. Wydawnictwo Naukowe PWN, Warszawa 2007, rozdz. 11.7.4-11.7.6.

[3] L. Sobczyk, A. Kisza, K. Gatner, A. Koll, *Eksperymentalna chemia fizyczna*, PWN, Warszawa 1982.

Related problems

The notions of fluorophore and fluorosensor, luminescence. The Jabłoński diagram. Radiative and nonradiative transitions. Internal conversion, intersystem crossing. Dynamic and static quenching.

Instruction of JASCO spectrofluorimeter operation

1. Switch on the spectrofluorimeter by pressing the button "ON" (on the back of the instrument on the left hand side). Wait about 5 minutes after the lamp lights on to stabilise the system.
2. Switch on the computer and the monitor. With the left button of the mouse choose the icon "pracownia".
3. From the ribbon "Programs" choose "JASCO" and "Spectra Manager".
4. Open the window "Spectra Manager" and click twice the left button on the mouse on the icon "Spectrum Measurement".
5. In the open window of "Spectrum Measurement", click the left button on the mouse on the icon "Measurement".
6. From the menu ribbon choose the icon "Parameter", and then in the open window of "Spectrum Measurement – Parameter" introduce the following parameters:
   - "Measurement Mode": "Emission Single",
   - "Excitation Band Width": 5 nm,
   - "Emission Band Width": 5 nm,
   - "Response": "Fast",
   - "Excitation Wavelength": as described in the experiment,
   - "Start" (the initial wavelength in the recorded fluorescence spectrum): as described in the experiment,
   - "End" (the final wavelength in the recorded fluorescence spectrum): as described in the experiment,
   - "Data Pitch": 1 nm,
   - "Scanning Speed": 60 nm/min or 125 nm/min,
   - "Display": "Auto",
   - "Sensitivity": "Medium".

   After introducing the above parameters close the window by clicking on "OK".
7. In the window "Spectrum Measurement" click the left button of the mouse on the icons with images of bulbs "Ex" and "Em"; then the colour of the bulbs will change from grey to yellow.
8. Fill the cuvette for spectrofluorimetric measurements (all the sides are transparent) with the solution to be studied. Choose subsequently the solutions of growing KI concentrations. After filling the cuvette with the solution to be studied, up to ¾ of its volume, carefully dry all the external walls of the cuvette so that they are clean and dry. Open the spectrofluorimeter chamber. Holding the cuvette by the upper parts of the brims, place it in the spectrofluorimeter and close the cover.

Caution: Do not leave open the cover of the spectrofluorimeter chamber. Open it only for placing the cuvette in or removing it.

9. In the open window of "Spectrum Measurement" click the icon "Start". At this moment the fluorescence spectrum recording begins. After recording of the full spectrum, the monitor shows the active window "Spectra Analysis" showing the spectrum and a red vertical line. Place the cursor on the red line and move it to the maximum of the spectrum. The bottom ribbon of the window "Spectra Analysis" shows the values of the wavelengths λ and the corresponding intensity of fluorescence, I. Read off these values and write them.
10. To begin the recording of a subsequent spectrum, go back to the window "Spectrum Measurement", which is under the window "Spectra Analysis".
11. After completion of the measurements and approval of the results by the tutor, close all the windows (without recording them) and leave the program by choosing the

option "Measurement" in the window "Spectrum Measurement" and then on the menu ribbon choose the icon "Exit", then close the window "Spectra Manager".