Laboratory Session 3: Fluorescence Spectroscopy

Objectives. The study is divided into two parts. In the first part, the absorption, fluorescence, and excitation spectra of a set of organic dyes are recorded. The intensity of the fluorescent emission will be correlated with the molecular structure of the dyes. In the second part, the phenomenon of bimolecular deactivation (quenching) of the fluorescent emission of the flavin mononucleotide by I^- ions is studied.

Related Concepts:

Electronic spectroscopy, excited states (singlet, triplet), photophysical processes, photochemical kinetics.

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PART I.- Effect of Molecular Structure on the Fluorescent Capacity of Organic Dyes

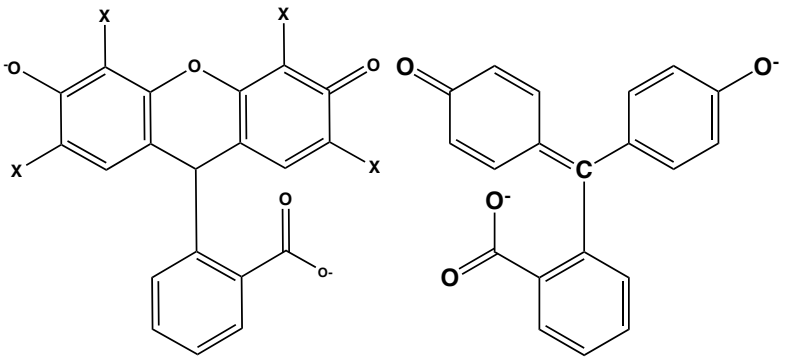
1. Introduction

The first part of the study aims to obtain the fluorescence spectrum of a series of dyes from the same family and to relate the intensity of fluorescence with the molecular structure.

The probability (velocity constant) of deactivation processes of excited electronic states is controlled by a series of aspects related to the molecular structure. Thus, the fluorescent velocity constant is governed primarily by the dipole moment of transition between states S_0 and S_1, and, therefore, it is directly related with the probability of absorption. Intersystem crossing, which involves a change in multiplicity, depends largely on the spin-orbit interaction, which is favored by the presence of heavy atoms. The internal conversion process, a non-radiative transition between electronic states of the same multiplicity, is induced by the electronic coupling terms neglected from the Born-Oppenheimer approach. This process is followed by vibrational relaxation to the lowest vibrational level of the electronic state.

The fluorescence intensity of fluorescein and its halogenated derivatives, as well as phenolphthalein, is examined. Their absorption, fluorescence, and excitation spectra (in this order) are recorded in the laboratory.

The differences observed between absorption and excitation spectra will be analyzed, considering instrumental factors. The fluorescent capacity of the dyes will be discussed based on the internal conversion processes (rigidity/flexibility of the molecules) and intersystem crossing (heavy atoms).



(A) **(B)**

Figure 1: Molecular structures of: (**A**) Fluoresceín (X=H), Eosin (X=Br) and Erythrosine (X=I). (**B**) Phenolphtalein.

2. Experimental

2.1. Solutions preparation

If the dye solutions are not prepared, prepare 1L of a 2×10^{-5}M solution of the following compounds using 0.01M NaOH as a solvent (pH = 12); the solutions to be prepared are:

- Fluorescein (7 mg/L at pH=12) Mr = 332.32 $C_{20}H_{12}O_5$.
- Yellowish eosin (14 mg/L at pH=12) Mr = 691.86 $C_{20}H_6Br_4Na_2O_5$.
- Erythrosine B (18 mg/L at pH=12) Mr = 879.92 $C_{20}H_6I_4Na_2O_5$.
- Phenolphthalein (20 mg/L a pH=12) Mr = 318.33 (6×10^{-5}). Warning: to record the spectra (absorption and emission) of phenolphthalein, fill the cell with 2/3 of the colorless solution and 1/3 of the solvent (0.01 M NaOH). You should see an intense violet color.
  Measure the spectrum immediately. (Remember the practical from the Lab. QFI: *Decoloration of phenolphthalein*).

Solution to dilute the dyes:

- 500 mL 0.01 M NaOH solution.

NOTE: To record the emission spectra, it is necessary to dilute the 2×10^{-5}M solutions by a factor of 1/100 for Fluorescein and 1/50 for Yellowwish Eosin and Erythrosine. To do this, take 1 mL of the solution (2 mL for the Yellowwish Eosin and Erythrosine) and to pour it in a volumetric flask of 100 mL using the 0.01 M NaOH solution as solvent to obtain the corresponding dyes solution. Absorption spectra are recorded from the concentrated solutions.

2.2. Experimental Work

1. Record the absorption spectra of all molecules between 250 and 700 nm using the corresponding concentrated solutions.

2. Record the fluorescence spectra up to 700 nm using as excitation wavelength a value between 10 and 15 nm less than that of the absorption maximum, more shifted towards the red obtained in the previous section, using the corresponding diluted solutions.

3. Record the excitation spectrum between 250 and 700 nm using the maximum fluorescence emission wavelength obtained in the previous section using the corresponding diluted solutions.

4. Overlay the excitation and fluorescence spectra for each substance. Overlay the fluorescence spectra for the first three substances.

5. Repeat the fluorescein fluorescence spectrum using an excitation wavelength of a shorter peak wavelength than that used in part (2) to validate compliance with Kasha's rule.

NOTE: In systems whose emission is very small or even null, the fluorescence of the sample may be affected by the fluorescence of possible impurities (from the sample, the solvent, and/or a previous sample). It is also possible that Raman signal appears from the solvent; remember that the position of a Raman signal changes as the excitation wavelength changes.

3. Results

3.1 Absorption Spectra

Prepare a table detailing the parameters of the absorption spectra for the dyes, including peaks, wavelength of maximum absorption, absorbance, and a description of the corresponding excitation. Arrange the peaks based on energy levels ($S_0 \rightarrow S_1$, $S_0 \rightarrow S_2$, ….). Conduct a comparative analysis of the various dyes.

3.2 Fluorescence Spectra

Create a table outlining the parameters of the fluorescence spectra for the dyes, specifying peaks, excitation wavelength, wavelength of maximum fluorescence, fluorescent intensity, and a description. Clearly differentiate between fluorescence signals and Raman signals. Compare and analyze the spectra across different dyes. How can one distinguish fluorescent emission from a Raman signal?

3.3 Kasha's Rule

Explain whether Kasha's Rule is observed or not in the studied dyes.

3.4 Excitation Spectra

1. Create a table summarizing the parameters of the excitation spectra, including peaks, emission wavelength, wavelength of maximum excitation, and intensity.

2. Compare the excitation and absorption spectra for different dyes, paying particular attention to the shape and position of the maxima.

3. Identify the electronic transitions evident in the excitation and absorption spectra of the various dyes.

3.5 Fluorescent Capacity and Molecular Structure of Dyes

1. Discuss the reasons behind the placement of the fluorescence band at longer wavelengths than the absorption band. Explore whether this phenomenon is consistent across different systems.

2. Correlate the fluorescent capacity of the dyes with their molecular structure and discuss the differences.

3.6 Stokes shift

Measure the Stokes shift in cm^{-1} between the absorption and fluorescence signals.

PARTE II. Energy Transfer of Excited Riboflavin Molecules. Equation of *Stern-Volmer*

Objective

This experiment aims to demonstrate the energy transfer of a colored excited molecule (riboflavin) to another non-excited and colorless molecule (KI).

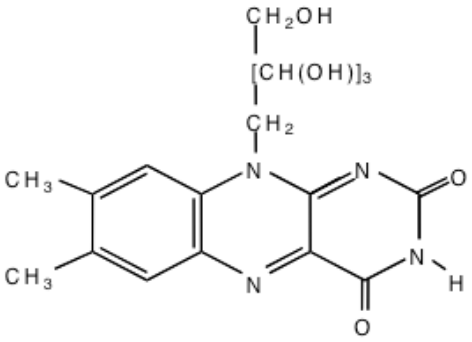


Figure 2: Flavin mononucleotide molecule

1. Introduction and Theoretical Background

When the flavin mononucleotide, represented with R, absorbs a photon and becomes an excited molecule, R*, reaction (1), a favorable path to return to the fundamental state is the emission of a photon or fluorescence, reaction (2).

$$R + h\nu \rightarrow R^* \qquad (1)$$

$$R^* \xrightarrow{k_F} R + h\nu_F \qquad (2)$$

In the presence of the I^- ion, the flavin mononucleotide can return to the ground state by transfer of energy to I^-, reaction (3), and, consequently, not by emitting a photon.

$$R^* + I^- \xrightarrow{k_Q} R + (I^-)^* \qquad (3)$$

In a solution with riboflavin and I^-, the fluorescence intensity, which we represent by I_F, can be considered in this way: if the excited molecule of riboflavin does not collide with I^- ions, most of the molecules emit a photon. If they collide with any I^-, they will be deactivated by non-photonic emission. The competition between these two processes, fluorescence and bimolecular deactivation ("quenching"), will be dependent on:

1. The average life time of the excited state (τ). Reaction (3) will be more favorable than reaction (2) when the molecule remains in its excited state for a longer period of time.

2. The number of collisions made. This will be related to the riboflavin and I^- concentrations.

3. The efficiency (k_Q) of I^- as a *quencher*. (Not all collisions are effective at transferring energy).

The quotient between fluorescence (I^0_F) and fluorescence with a *quencher* present (I_F) is related to these variables by means of the Stern-Volmer, equation (4),

$$\frac{I_F^0}{I_F} = 1 + k_Q \tau [Q] \qquad (4)$$

where [Q] is the *quencher* concentration, in this case the anion I^-.

2. Experimental Procedure

1. Prepare the following solutions (to share among the group):

- 500 mL 6.5×10^{-5}M of flavin mononucleotide.
- 200 mL 0.1 M of KI.

2. Prepare each of the solutions indicated in the table below in 25 mL volumetric flasks. Each solution will have the same concentration of riboflavin and different concentrations of KI.

3. Measure the fluorescence of each solution using as the excitation wavelength a value 15 nm lower than the maximum absorption wavelength (450 nm), as well as the area of the emission at the same interval of wavelengths.

Table 1. Maximum intensity and area of the emission band of riboflavin and I^- solutions.

Solution	*v*/mL (R)	*v*/mL (KI)	$I_{max,F}$	Band area
1	2.0	0.0		
2	2.0	1.0		
3	2.0	2.0		
4	2.0	3.0		
5	2.0	4.0		
6	2.0	5.0		

3. Results

3.1 Bimolecular deactivation (quenching) of excited riboflavin by unexcited iodide.

1. Draw a graph of $\frac{I_F^0}{I_F}$, taking into account the fluorescence intensity observed for each solution, against the concentration of KI.

2. Draw the same graph with the areas of the emission band.

3. Estimate the average fluorescence lifetime of the mononucleotide of flavin and the fluorescent rate constant.

4. Questions

4.1 Prelyminary questions

1. Indicate what is represented in the absorption, fluorescence and excitation spectra and whether they are absorption or emission spectra.

2. What is the Stokes shift?

3. State Kasha's rule, is it satisfied by all molecules?

4. Before starting the practice, read carefully the article cited in reference [8].

5. Deduce the equation (4).

6. Estimate a value for the bimolecular deactivation constant, k_Q, assuming that reaction (3) is controlled by diffusion.

4.2 Post-laboratory questions

1. How can the Rayleigh signal be distinguished from a Raman signal?

2. What are the factors that influence the intensity of an electronic transition?

3. Explain the concept of the Frank-Condon factor and how it manifests itself in the fluorescence spectrum of a molecule.

4. How does the fluorescence intensity change in different dyes and what is the reason for this?

5. Why are fluorescence spectra used to characterize the ground states of different dyes, while absorbance spectra are used to characterize their excited states? Relate this question to the Stockes shift.

6. Why is it more accurate to use the area under a fluorescence spectrum to estimate the fluorescence half-life of mononucleotide

Material and devices

Material for each pair:

- Four 100-mL volumetric flasks.
- Five 50-mL beakers.
- Four 1-mL pipettes.
- One 2-mL graduated pipette.
- One 5-mL graduated pipette.
- Six 25-mL volumetric flasks.

Material for the group:

- One 200-mL volumetric flask.
- One 500-mL volumetric flask.
- Two 100-mL beakers.

Devices:

- Spectrofluorimeter.
- Absorption spectrophotometer.

Reagents:

- Dyes: Fluorescein, erythrosine B, yellowish eosin, phenolphthalein..
- Riboflavin monofostat.
- KI.
- NaOH.

References

[1] J. Bertrán y J. Núñez. Química Física. Ariel Ciencia, 2004.
[2] Ira N. Levine. Fisicoquímica. McGraw-Hill, Madrid, 2004.
[3] M. Díaz Peña and A. Roig. Química Física, volumen 2. Alhambra Universidad,1972.
[4] A. Requena and J. Zúñiga. Espectroscopía. Pearson Educación, 2003.
[5] N.J. Turro. Modern Molecular Photochemistry. Benjamin Cummings, 1978.
[6] N.J. Turro. Modern Molecular Photochemistry. University Science Books, 1991.
[7] B. Valeur. Molecular Fluorescence. Wiley-VCH, 2001.
[8] R. J. Clarke and A. Oprysa *Journal of Chemical Education* **2004**, *81*, 705.