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HIGH ACCURACY SPECTROFLUORIMETER FOR DETERMINATION OF RELATIVE QUANTUM YIELDS WITHIN A BROAD RANGE OF OPTICAL DENSITIES

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Summary

A spectrofluorimeter with an optical system gathering luminescence light with constant efficiency, irrespective of the optical density of samples, was constructed. The system permits accurate calculation of the corrections for re-absorption. Corrections for fluctuation of the light source intensity are introduced automatically. A description is given of the methods for measuring changes in quantum yields as a function of temperature and concentration. The statistical errors of these measurements in compounds of high quantum yield do not exceed 0.3%. The estimated systematic error in no case exceeds 0.5%.

Introduction

For a decade stacking interactions of nucleic bases have been the object of interest of many authors [1]. However, the measuring methods hitherto used are of low accuracy and in most cases permit only qualitative or semi-qualitative descriptions. It has been found that changes in the fluorescence quantum yield of purine and pyrimidine derivatives as a function of concentration and temperature can furnish valuable information on the association of these compounds [2, 3]. However, the accuracy of measurements taken on conventional fluorimeters is insufficient. Thus, the authors have developed a method enabling measurement of relative quantum yield of fluorescence with a relative error lower than 1%, of solution concentration (*i.e.* also its optical density) to be made over a range of three orders of magnitude.

In this paper, an apparatus of our own design and a measuring method developed to meet the above requirements are presented.

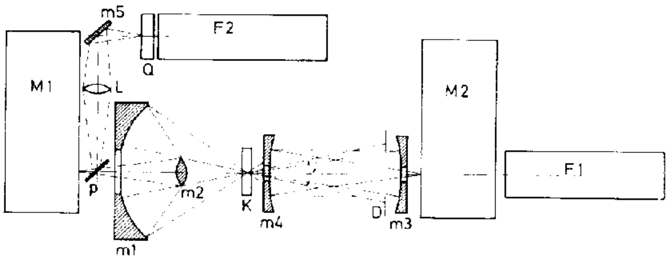

Fig. 1. Scheme of the optical system.

Optical system

The optical system shown in Fig. 1, is the essential part of the apparatus. The exciting beam passes through monochromator M_1 and plano-parallel plate P (situated at an angle of 45° to the optical axis), and falls on convex mirror m_2 which reflects the beam to mirror m_1. Mirror m_1 focuses the exciting beam on the sample. The solution to be examined is placed in a cuvette of 1 mm thickness. The image of the exit slit of monochromator M_1 is reproduced in the centre of cuvette K. Mirrors m_1 and m_2 are spherical; but because of appropriate selection of their radii the error of spherical aberration can be eliminated and a very large image aperture ratio (1:0.66) is obtained. The image of the slit is reduced five times and its height is 4 mm.

As shown in Fig. 1, inside the convergent exciting beam a dark cone with a solid angle φ is formed. Mirror m_3 gathers the luminescence light emitted by the sample and is situated in the dark area. In this way the exciting beam cannot directly reach the analyzing system. The luminescence light which is reflected from mirror m_4 is focused on the entrace slit of the analyzing monochromator M_2, and is detected by photomultiplier F_1.

The path of the exciting beam in the cuvette is illustrated by the broken line in Fig. 2. Within the cuvette an area of intense excitation (double shaded) and areas of weaker excitation (single shaded) can be seen. The continuous line shows the backward projection of the beam, *i.e.* the path of rays which would be observed in the system, if the entrace slit of monochromator M_2 were the source of light. Inside the emission beam there is an area within which the light emitted from every point is gathered by the analyzing system in the same yield because of the size of the entrance aperture of monochromator M_2. The excitation area lies entirely within the area of most efficient gathering of the luminescence light. Let us assume that the sample is completely transparent to light of wavelength λ; in this case, the intensity of luminescence light of this wavelength, reaching photomultiplier F_1, is strictly proportional to the intensity of the exciting light absorbed by the sample. In the case of optical densities exceeding 3, the signal of the photomultiplier changes identically as the quantum yield of the sample. If the

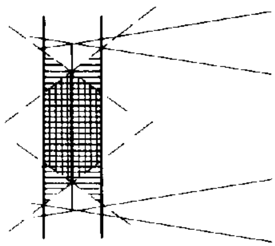

Fig. 2. Excitation of a sample and gathering of its luminescence light.

solution partly absorbs the light of wavelength λ, then a correction for re-absorption can be calculated with high accuracy. The method for calculating this correction will be given below.

A part of the exciting light reflected from plano-parallel plate P is focused by lens L, whereupon it is reflected from mirror m_5 and falls on a cuvette Q containing a solution of rhodamine B in ethylene glycol. Photo-multiplier F_2 situated behind the cuvette measures the intensity of rhoda-mine B fluorescence.

The source of light consists of xenon lamp XBO 150 or XBO 250 or else of mercury lamp HBO 200 situated in an illuminator of our own design, which permits gathering of light within a solid angle of about 3 sr.

In this system the amount of scattered excitation light is greater than that when observation of luminescence is at right angles. It is thus necessary to use a double monochromator in the excitation path. M_1 is a double grating monochromator of our own design, in which Jarrell–Ash gratings (50×50 mm, 1180 lines/mm, maximum blaze at 3000 Å) are used. M_2 is a Jarrell–Ash 82 - 410 monochromator. To reduce the level of parasitic light diaphragm D with a square aperture is placed in front of mirror m_3. The diaphragm is so selected that the image of the aperture should overlap the active surface of the grating of monochromator M_2. All lenses and plate P are made of Suprasil quartz glass. Because the system uses mirrors, the main optical line of the measuring system is completely achromatic. Both cuvette K and the quantum counter are thermostated with an accuracy of $\pm 0.1\,^{\circ}$C.

Electronic system

The electronic measuring system is typical; it has previously been used in various modifications [4, 5]. The main signal is measured with the use of photomultipliers EMI 9558 QB or EMI 6256 S. The reference signal is measured by photomultiplier EMI 9558 B. Both signals are amplified by lock-in nanovoltmeters Unipan 232. A rotating perforated disc situated in front of exciting monochromator M_1 modulates the signals with a frequency of about 250 Hz. The shaft of the motor rotating the disc is coupled with

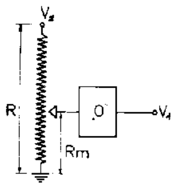

Fig. 3. Scheme of the system measuring the ratio of signals.

the shaft of another motor generating the signal which controls phase-sensitive detection.

For comparison of the signals, use is made of a very simple and accurate system operated manually: it is presented in Fig. 3. Signal V_2 from photomultiplier F_2 is applied to a potentiometer of resistance $R = 100\ k\Omega$. The main signal V_1 is compared with the voltage on the potentiometer slide. When the instrument "O" is zeroed, this indicates the equilibrium position and the equation:

$$\frac{V_1}{V_2} = \frac{R_m}{R}$$

is satisfied. The R_m/R ratio is read directly on the potentiometer scale. A compensation recorder with input resistance of 200 kΩ is used as zero instrument. As a result, the noise and drift of the measured signal ratio can be conveniently observed. The accuracy of the system, predetermined by the linearity of the potentiometer, amounts to 0.1%.

At present, we are completing the construction of an instrument designed to interface with a multichannel analyzer Didac 800; this will enable automatic recording of the excitation and luminescence spectra.

Evaluation of errors of the system

The non-linearity of photomultipliers and amplifiers as well as of the system measuring the signal ratio is a source of systematic errors. Upon measuring the non-linearity of amplifiers we found that if the height of the input signal remains within the range of 30 to 0.3 mV then the deviation from linearity is less than 0.1%. On the basis of the data published by N.B.S. [6] we estimate the error due to the photomultiplier non-linearity to be $< 0.3\%$, in case of changes in the measured signal over two orders of magnitude.

The phenomenon of signal drift, which results from a number of unexplicable causes, is the main source of accidental errors. Slight changes in the shape of the lamp's arc seem to be the chief cause of this phenomenon. Since the main measuring system and the reference system fail to be strictly equivalent (differences in the apertures, chromatic aberration of the reference system), the signal ratio varies with a change in the distribution of light intensity in the exciting beam. This phenomenon can be eliminated by placing plate P immediately in front of cuvette K, perpendicularly to the optical axis. In this case the light reflected from the plate can be transmitted to the quantum counter by way of a light transmitting rod.

If the apparatus is turned on for 2 h before the measurements, then the drift usually amounts to about 0.3%/h and does not exceed 0.5%/h. To minimize the errors caused by this phenomenon, measurements have to be taken in a definite sequence and from time to time the fluorescence of a standard sample must be determined for control purposes. Details of the measuring procedure and practical evaluation of errors are given below.

In case of weakly fluorescing solutions the accuracy of measurements is additionally reduced because of the scattering and fluorescence of the quartz cuvette. The former factor can be eliminated by way of using additional filters, whereas the latter one cannot be removed. In contrast to optical systems at right angles, in our apparatus the walls of the cuvette are observed by the analyzing system. Even Suprasil II quartz shows residual fluorescence corresponding to the signal of a solution completely absorbing the exciting light and having a quantum yield of about 10^{-5}. Clearly, the necessity of introducing a correction for the fluorescence of quartz reduces the accuracy.

Calculation of corrections for re-absorption

Let us consider layer dx of a solution placed in a cuvette of width l. It is reached by a beam of exciting light:

$$I = I_o \exp(-c\xi x \epsilon_{ln})$$

where I_o is the intensity of light entering the cuvette, c is the concentration of the solution, ϵ_{ln} is the extinction for the exciting light expressed in natural logarithms, x is the distance from the front of the cuvette. ξ represents the ratio of the effective path length of the exciting light to x.

In layer dx fraction dI of the exciting light is absorbed:

$$dI = -I\, c\epsilon_{ln}\xi\, dx$$

This, in turn, causes emission of a luminescence beam:

$$dI'_o = -\Phi\, dI$$

where Φ is the quantum yield. The intensity of luminescence light falling on the photomultiplier from layer dx is expressed by the equation:

$$dI' = dI'_o \text{ Const. } \exp\left[-c\xi'\epsilon'_{ln}(l-x)\right]$$

where ϵ'_{ln} is the extinction for the observed wavelength and ξ' is the ratio of the effective optical path length in the cuvette to the cuvette's width, for the light gathered by the analyzing system. Const. is an apparatus constant depending only on the wavelength of the analyzed light.

Finally we obtain:

$$dI' = I_o \, \Phi \text{ Const. } c \, \epsilon_{\ln} \xi \exp(-c \, \epsilon'_{\ln} \, \xi' l) \exp(c \, \epsilon'_{\ln} \, \xi' x - c \, \epsilon_{\ln} \, \xi \, x) dx$$

After integration within the limits of 0 to l:

$$I' = I_o \, \Phi \text{ Const. } \frac{\epsilon \xi}{\epsilon' \xi' - \epsilon \xi} \exp(-\epsilon'_{\ln} \, c \, \xi' \, l) - \exp(-\epsilon_{\ln} \, c \, \xi \, l)$$

If the solution does not absorb the analyzed light, then $\epsilon' = 0$ and we obtain, of course, the well-known dependence:

$$I'_o = I_o \, \Phi \text{ Const. } [1 - \exp(-\epsilon_{\ln} \, c \, \xi \, l)]$$

Thus, the correction by which the results have to be multiplied amounts to:

$$\frac{I'_o}{I'} = \frac{\xi A - \xi' A'}{\xi A} \, \frac{10^{A \xi l} - 1}{10^{(\xi A - \xi' A) \, l} - 1}$$

where A and A' are the absorbances of a solution of unit thickness for excitation and analysis, respectively.

Usually condition $A \gg A'$ is satisfied, thus permitting the simplification:

$$\frac{I'_o}{I'} = \frac{\xi A - \xi' A'}{\xi A} \, 10^{\xi' A' l}$$

ξ is very close to unity and it can be evaluated by geometrical calculations. For the aperture of our analyzing system we obtained $\xi' = 1.002$. The value of ξ can be calculated similarly, if the angular distribution of the intensity of the exciting beam is known. This distribution can be determined by measuring the fluorescence of the sample by means of a diaphragm with variable diameter, situated in the plane of mirror m_1 (Fig. 1). In practice it is sufficient to use four different diaphragms for calculating ξ with an accuracy of $\pm$ 0.1%. By this method we obtained $\xi = 1.041$.

Measurement of function $\Phi = f(T)$

The cuvette used for measuring the quantum yield as a function of temperature is illustrated in Fig. 4. The solution contained in the cuvette is closed by a cold layer of the same solution cooled with ice. A plug in the form of a long glass rod prevents convection. Owing to this design, the solution in the cuvette can be heated to boiling without any risk of evaporation. In measurements taken at temperatures $< 15°$C, dry nitrogen is passed through the sample-containing chamber, in order to prevent fogging.

To exemplify the procedure used for determination of $\Phi = f(T)$, a typical experiment for aqueous solutions of 2-aminopurine is presented below. Since the fluorescence spectrum of 2-aminopurine is temperature-independent, the quantum yield can be determined by measurements taken at a selected wavelength. To avoid the oxygen-induced quenching effect, for de-aeration argon saturated with water vapour was bubbled through the solution. Beginning at 40 °C, measurements were taken at 5 °C intervals while the sample was gradually cooled to 0 °C, whereupon they were again taken at 5 °C

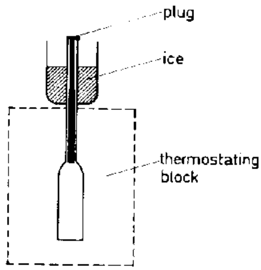

Fig. 4. Measuring cuvette.

intervals with the solution gradually reheated to 40 °C. The signal of the standard was measured at the beginning and at the end. Likewise, measurements were taken within the range of 40 - 90 °C. As the final result of the measurements the mean values for each temperature, divided by the mean signal of the standard, were obtained. Thus, the effect of signal drift was reduced to a minimum. After introducing corrections for thermal expansion and for changes in the index of refraction we obtained values directly expressing the change in quantum yield as a function of temperature. For diluted solution this function can be expressed as follows:

$$\frac{1}{\text{const.}\ \Phi} = \frac{1}{\text{const.}\ \Phi_o} + \frac{k_{on}}{k_\psi\ \text{const.}} \exp - \frac{E}{RT}$$

where Φ_o is the quantum yield extrapolated to $T = 0$, Φ is the quantum yield at temperature T, k_ψ is the fluorescence rate constant, E is the activation energy of radiationless transition and k_{on} is the frequency factor. The above parameters were fitted to experimental data by means of minimization program. The maximum deviation of the experimental points from the interpolation curve usually did not exceed 0.3% and the mean square error amounted to 0.04%. On account of the small range of changes in the signal measured, the systematic error did not exceed 0.1%.

Measurement of function $\Phi = f(c)$

It should be most convenient to use, for testing the system, a compound exhibiting no concentration quenching, e.g. 9,10-diphenylanthracene. However, highly structured absorption spectrum of this compound can give rise to serious errors in the determination of the absorption of the exciting light. Therefore, use was made of ethanolic solutions of 2-aminopurine and 9-ethyl-2-aminopurine, which satisfy the Stern–Volmer relation. Measurements were taken at a selected wavelength, and signal originating from the solution was compared each time with that of the standard. The optical density of the solutions of 2-aminopurine and 9-ethyl-2-aminopurine varied within the range of 10 — 0.02 and 30 — 0.04 per 1 mm of layer thickness, respectively. When calculating the relative quantum yields it must be borne in mind that the effective pathlength of the exciting beam in the solution is ξl, and that correction for the reflection from the rear wall of the cuvette has to be made. For concentrated solutions of 9-ethyl-2-aminopurine, additional corrections for re-absorption had to be introduced. Deviations of the experimental points from the interpolating curve $\Phi_o/\Phi = 1 + \text{const.}\ c$ did not exceed 0.3%. Moreover, a systematic error approaching 0.5% enters into account.

Fluorescence spectra and excitation spectra

The described apparatus, used as a spectrofluorimeter, presents a number of noteworthy advantages.

(1). The large effective aperture of the system and the possibility of using solutions which completely absorb the exciting light permit much higher signals to be obtained, compared with fluorimeters analyzing emission at right angles.

(2). The use of optically dense solutions prevents the entrance of the exciting light into the analyzing system and enables elimination of the scattering effects. Thus, the system can be particularly valuable for studying opaque, strongly scattering solutions.

(3). Corrections for the re-absorption of fluorescence can be calculated with high accuracy.

(4). The system enables measurement of light absorption in the sample. To this end, it is necessary to replace cuvette K (Fig. 1) by a focusing screen of quartz glass, and to place in front of the latter a cuvette filled with pure solvent and then a cuvette with the solution studied. The wavelength of the light of the analyzing monochromator has to be adjusted to a maximum signal. The error in absorption measurement accounts for a substantial part of the errors made when measuring the excitation spectra, because the wavelength and spectral width of the exciting beam are different, as compared with the absorption-measuring spectrophotometer. In the present apparatus this error can be eliminated.

(5). The excitation spectra are often compared with the absorption spectra, with a purpose of detecting the possible impurities. This procedure usually consists of the determination of whether the quantum yield does or does not depend on the wavelength of the exciting light. In this case the use of a concentrated solution, completely absorbing the exciting light within the investigated wavelength range, renders the result independent of absorption measurements and greatly improves the accuracy.

Corrections for the excitation spectra are determined by the following procedure. Measurement is made of the excitation spectrum of a rhodamine B solution in ethylene glycol; its concentration is selected in such a manner that within the range of 200 - 600 nm the exciting beam is completely absorbed. On the commonly accepted assumption that rhodamine B has a constant quantum yield, the corrections are obtained. This measurement is indispensable, since the distribution of the light beam on plate P depends on the wavelength. Thus, the system does not automatically make corrections with a sufficient accuracy. Systems being much superior in this respect are reported in the literature. However, in our opinion experimental determination of the corrections is simpler and more accurate. The results are as a rule treated using a computer, and thus the introduction of corrections is not very time-consuming.

Corrections for the fluorescence spectra are determined by Parker's method [7], with this difference that instead of a magnesium oxide screen we employ a quartz glass focusing screen in place of cuvette K.

In this apparatus the fluorescence spectra were operated manually which result in approximately a 2% error in the reproducibility, especially at the slopes of the spectra. In near future we expect to automate the measurements and to attain consequently considerable improvement in reproducibility.

Conclusions

To our best knowledge the only apparatus permitting measurements of the fluorescence of solutions within a wide concentration range has been described by Eisinger [8]. However, the data reported in his work do not allow us to carry out a comparison of both systems. Nevertheless, our design seems to have two important advantages: (1) more efficient utilization of the exciting light, owing to the achievement of very large apertures; and (2) precise and reproducible fitting of the cuvette entirely affects the reproducibility in Eisinger's system. In our apparatus, deviations within the range of 0.2 mm do not affect the measurements and thus fitting of the cuvette is less critical.

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