DETERMINATION OF THE FLUORESCENCE EXCITATION SPECTRA OF PROTEINS

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Methods for determining true fluorescence excitation spectra by applying corrections for reabsorption and secondary fluorescence are described for different conditions of observation.The results are illustrated by fluorescence excitation spectra of horse-radish peroxidase, lysozyme, Triton X-100 detergent and peroxidase solutions containing fluorescein-isothiocyanate indicator.

1. *Introduction*

The fluorescence excitation spectrum

$$f_{exc}(\lambda', \lambda) = \frac{I_{fl}(\lambda', \lambda)}{I_0(\lambda)}, \tag{1}$$

i.e. the fluorescence intensity $I_{fl}(\lambda', \lambda)$ observed at a given wavelength λ' with unit intensity of the exciting light of wavelength λ, yields information about the properties of the fluorescent systems and the processes occurring in them. Until now, the uncorrected excitation spectra were generally given, *i.e.* the directly measured fluorescence intensities were published. Several authors [1—4] have called the attention to the importance of excitation spectra given for constant intensity of exciting light. In this case the shape of the excitation spectrum of a material of "not too high" extinction, containing a single absorbing component, will coincide with the absorption spectrum [5]. The excitation spectrum of aqueous tryptophan solution of extinction 0.1 is in good accordance with the absorption spectrum, while in solutions of higher extinction marked differences occured [6]. McDONALD and SELINGER [4] pointed out that excitation spectra depend on the geometry of observation and on the extinction of the solution. It has been generally recognized that the excitation spectrum may be distorted by reabsorption of fluorescence. If the reabsorption is significant, the secondary fluorescence may also be important and has to be taken into consideration. BUDÓ *et al.* [7], gave methods of corrections for secondary fluorescence in cases of different luminescence characteristics, therefore, the correction of the excitation spectrum can be given as an extension of the results of [7].

The aim of the present paper is to summarize our present knowledge concerning the determination of true excitation spectra, to describe the methods for the necessary corrections with special respect to reabsorption and secondary luminescence.

2. *Determination of true fluorescence excitation spectra*

a) *General remarks.* In order to obtain the excitation spectrum $f_{exc}(\lambda', \lambda)$ defined in Eq. (1), (i) the true fluorescence intensity $I_{fl}(\lambda', \lambda)$ at the wavelengths λ' and λ of observation and excitation, respectively, and (ii) the intensity of excitation $I_0(\lambda)$ for different exciting wavelengths λ have to be determined.

(i) For the determination of $I_{fl}(\lambda', \lambda)$ the values $I_{meas}(\lambda', \lambda)$ measured directly with the spectrofluorimeter have to be corrected a) for the spectral sensitivity $Q(\lambda')$ of the measuring instrument, including the sensitivity of the photomultiplier used, the band width, and changes in transmittivity of the monochromator [2, 8]; (b) for the factor $\varphi(\lambda', \lambda)$ taking into account reabsorption, and (c) for the factor containing $\varkappa$, which takes into account secondary luminescence according to [7] and [8],

$$I_{fl}(\lambda', \lambda) = \text{const} \cdot I_{meas}(\lambda', \lambda) \cdot Q(\lambda) \cdot \varphi(\lambda', \lambda) \cdot [1 - \varkappa(\lambda', \lambda)]. \tag{2}$$

(ii) The true fluorescence intensity obtained in this way has to be referred to unit exciting intensity, therefore the spectral energy distribution of the light source should be known. Replacing the fluorescent sample by a thermopile, the thermocurrents $I_T(\lambda)$ will be proportional to the incident energy of light, and independent of the wavelength; $I_T(\lambda) = \text{const} \cdot I_0(\lambda)$. Thus $I_{fl}(\lambda)/I_T(\lambda)$ is proportional to the exciting spectrum. It is more expedient to calibrate the multiplier of the apparatus for energy measurements. By reflecting the exciting light into the photomultiplier and measuring the photocurrent $I_{Ph}(\lambda)$, $E(\lambda) = I_T(\lambda)/I_{Ph}(\lambda)$ will give the energy value pertaining to the unit deflection of the galvanometer in the circuit of the photocell, *i.e.* I_T is obtained in the form $I_T(\lambda) = E(\lambda) \cdot I_{Ph}(\lambda)$. Thus the excitation spectrum is

$$f_{exc}(\lambda', \lambda) = \text{const} \frac{1}{I_{Ph}(\lambda) \cdot E(\lambda)} \cdot I_{meas}(\lambda', \lambda) \cdot Q(\lambda') \cdot \varphi(\lambda', \lambda) \cdot [1 - \varkappa(\lambda', \lambda)]. \tag{3}$$

The constant in the equation is determined by the geometry of the apparatus, but independent of the wavelength (λ) of exciting light. $Q(\lambda)$ has to be calculated with the method described for the determination of the emission spectrum (see *e.g.* [3]).

b) *Correction for reabsorption and secondary luminescence. Determination of* $\varphi(\lambda', \lambda)$ *and* $\varkappa(\lambda', \lambda)$.

The correction for reabsorption. $\varphi(\lambda', \lambda)$ depends on the wavelengths of the exciting and emitted light, and on the geometry of excitation and observation. As the fluorescence intensity has to be determined for the excitation spectrum, the value of φ will be the same as that used in determining the true fluorescence spectrum. According to FÖRSTER [8], the values of φ are as follows:

for front-surface observation (excitation and observation parallel)

$$\varphi = \frac{1}{\alpha} \cdot \frac{\alpha + \beta}{1 - e^{-(\alpha+\beta)}} \tag{4}$$

for in-line observation

$$\varphi = \frac{1}{\alpha \cdot e^{\alpha}} \cdot \frac{\beta - \alpha}{1 - e^{-(\beta-\alpha)}} \tag{5}$$

for right-angle observation

$$\varphi = \frac{1}{\alpha} \cdot \frac{\beta}{1 - e^{-\beta}} \tag{6}$$

where $\alpha = k(\lambda) \cdot l$ and $\beta = k(\lambda') \cdot l$; $k(\lambda)$ and $k(\lambda')$ are absorption coefficients and l is the layer thickness. It has been shown [9] that under certain conditions a simpler formula can be used.

The correction for secondary fluorescence is completed by giving the ratio of the intensities of secondary fluorescence S to the primary fluorescence P, *i.e.* the quotient $\varkappa = \frac{S}{P}$, $\varkappa$ is a constant depending on the properties of the solution, on the geometry of observation and that of the sample, as well as on the absorption and emission spectra. The calculation of $\varkappa$ is rather complicated [7], but several values are tabulated or can be determined with computer methods [10]. Therefore it is advantageous to choose experimental conditions for which the secondary fluorescence can be neglected. When α and β are less than 0.5, the error caused by neglecting the correction will be less than 1 %.

c) *The choice of adequate geometry* is very important. The excitation spectra are subject to changes not only by reabsorption, but also by absorption of the exciting light, which are not taken into consideration in the formulae (4)—(6).

The following limiting cases are possible.

In the case of strong absorption the exiting light will be absorbed in a thin layer, causing fluorescence only in this layer (surface fluorescence). In this case the intensity of the fluorescence for right-angle observation will be strongly reduced with increasing distance from the excited surface. If the reabsorption of fluorescence light is weak, the same fluorescence intensities will be obtained for front-surface and in-line observation. If the reabsorption is strong, higher intensities will be found for front-surface observation, because the path of light in the medium will be shorter. In this case front-surface observation or layer thicknesses corresponding to $k(\lambda)_{max} \cdot l < 0{,}1$ should be used for determining the excitation spectrum. In the case of right-angle observation the intensity of fluorescence will depend both on wavelength and on the observed part of the lateral face. Further, it is difficult to determine the path-length of fluorescence in Eq. (6). The errors resulting from these circumstances cannot be considered by calculation, therefore they have to be avoided by choosing proper experimental methods.

If the absorption of exciting light is weak, the reabsorption of fluorescence can be calculated with Eqs. (4)—(6), depending on the geometry of observation. In this case, right-angle observation will be the most favourable, while in the case of strong reabsorption front-surface observation should be used.

3. *Connection between excitation and absorption spectra*

The excitation spectrum is proportional to the intensity $I_{abs}(\lambda)$ of the absorbed light and the fluorescence yield $\eta(\lambda', \lambda)$. The intensity of fluorescence is

$$I_{fl}(\lambda', \lambda) = \text{const} \cdot I_{abs}(\lambda) \cdot \eta(\lambda', \lambda),$$

where

$$I_{abs}(\lambda) = I_0(\lambda) \cdot [1 - e^{-k(\lambda) \cdot l}].$$

Thus the excitation spectrum is as follows:

$$f_{exc}(\lambda', \lambda) = \frac{I_{fl}(\lambda', \lambda)}{I_0(\lambda)} = \text{const} \cdot [1 - e^{-k(\lambda) \cdot l}] \cdot \eta(\lambda', \lambda). \tag{7}$$

For the two limiting cases, this equation leads to the following consequences.

In the case of great layer thickness and solutions of high concentration $e^{-k(\lambda) \cdot l} \approx 0$; the excitation spectrum does not depend on the absorption, but on the other hand, the spectrum obtained will be false, therefore such conditions must be avoided.

For low concentrations and small layer thicknesses $k(\lambda) \cdot l \ll 1$ and $1 - e^{-k(\lambda) \cdot l} \sim k(\lambda) \cdot l$, *i.e.* the excitation spectrum is proportional to the active absorption spectrum $k(\lambda) \cdot \eta(\lambda', \lambda)$; the absorption spectrum and the excitation spectrum of solutions with a single fluorescent component have the same shape.

4. *Experimental results*

The excitation spectra of phosphate buffered neutral solutions of horse-radish peroxidase (HRP) and lysozyme with the detergent Triton X-100 and the protein indicator dye fluorescein isothiocyanate (FITC) were measured with a Perkin—Elmer—Hitachi Type PMF-3 spectrofluorimeter.

Fig. 1 shows the relative excitation spectrum of a $2 \cdot 10^{-3}$ M Triton X-100 solution at 305 nm with right-angle and with front-surface observation under an

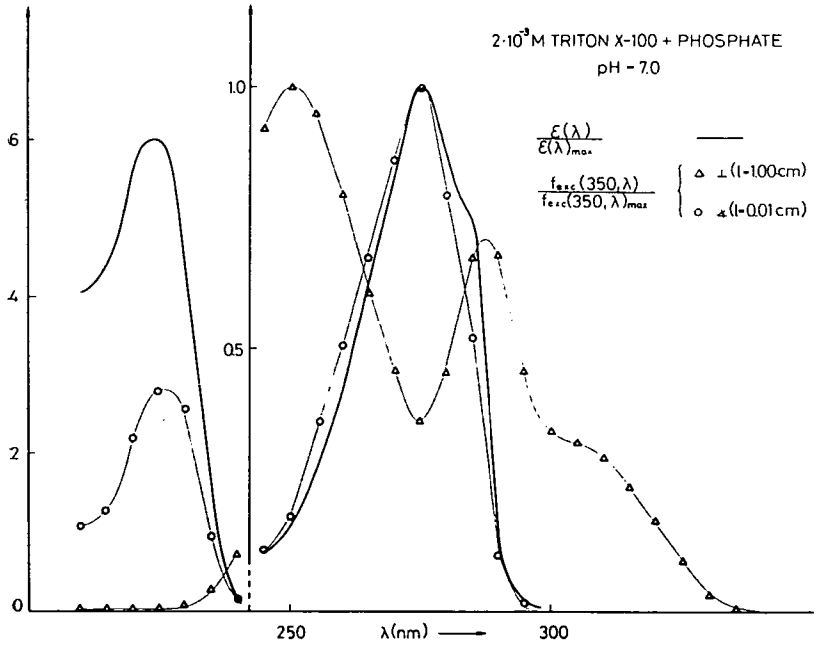

Fig. 1

angle of $\sim 7°$ ($l = 0.01$ cm), together with the relative absorption spectrum. In the case of right-angle observation, an excitation minimum is obtained where the absorption spectrum has a maximum, and reversely. The excitation spectrum obtained in the 250—300 nm band with front-surface observation shows good coincidence with the absorption spectrum, because the 305 nm fluorescence band of Triton X-100 is due to the 275 nm absorption band. The short wavelength bands of excitation and absorption do not coincide, because its participation in the fluorescence is less.

A similar picture is shown by a $5 \cdot 10^{-5}$ M lysozyme solution. Consequently the right-angle observation cannot be used for determining the excitation spectra of proteins, even at concentrations as low as $5 \cdot 10^{-5}$ M (Fig. 2).

Fig. 3 shows the relative absorption spectrum, the directly measured excitation spectrum, the relative excitation spectra corrected for unit exciting intensity and for reabsorption, respectively. The coincidence with the absorption spectrum is the best in the case of the curve corrected for reabsorption.

In Fig. 4 the relative absorption spectrum of the protein—dye complex HRP+FITC, the relative excitation spectra measured with two geometries of observation and corrected for reabsorption, as well as the absorption spectrum of the dye solution in the wavelength range 420—520 nm are presented. The participation of the dye in the fluorescence observed at the emission maximum of the FITC ($\lambda' = 525$ nm)

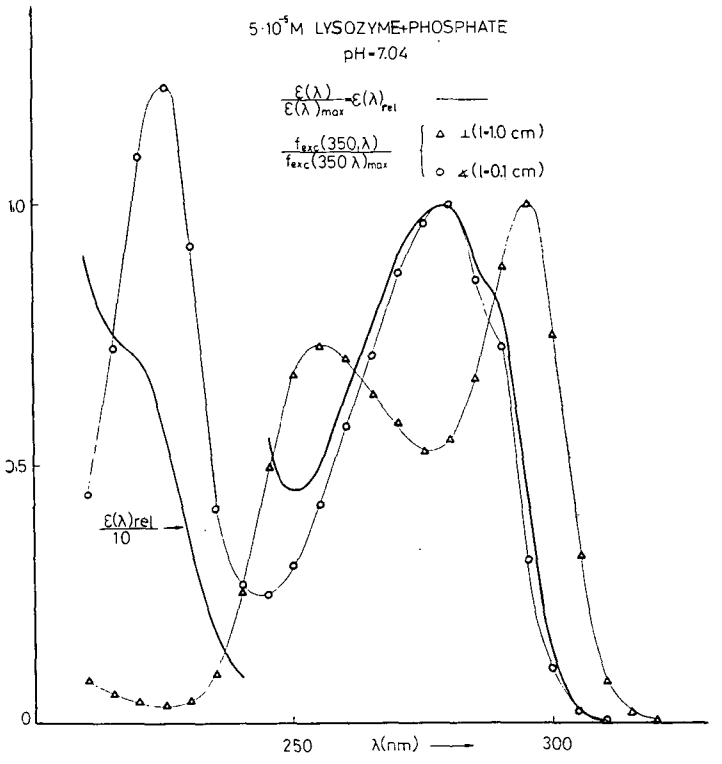

Fig. 2

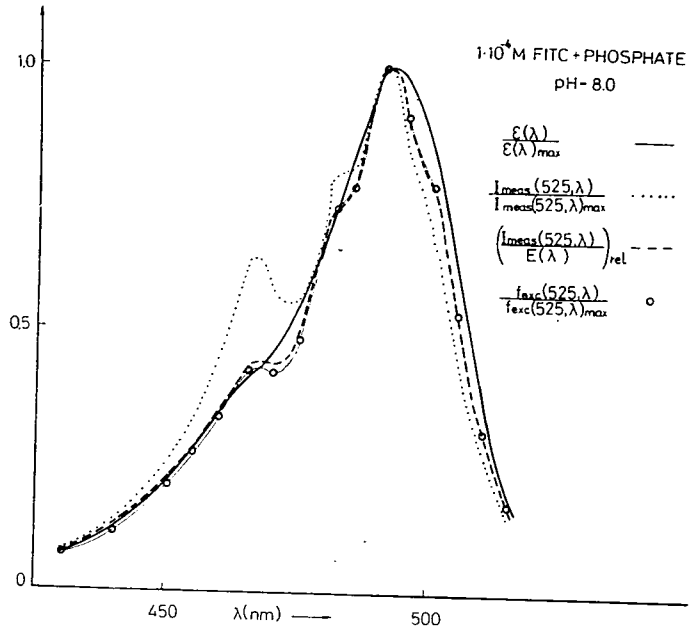
1·10⁻⁴M FITC + PHOSPHATE
pH - 8.0
ε(λ)/ε(λ)max
Imeas(525,λ)/Imeas(525,λ)max
(Imeas(525,λ)/E(λ))rel
fexc(525,λ)/fexc(525,λ)max
1.0
0.5
0
450
λ(nm)
500

Fig. 3

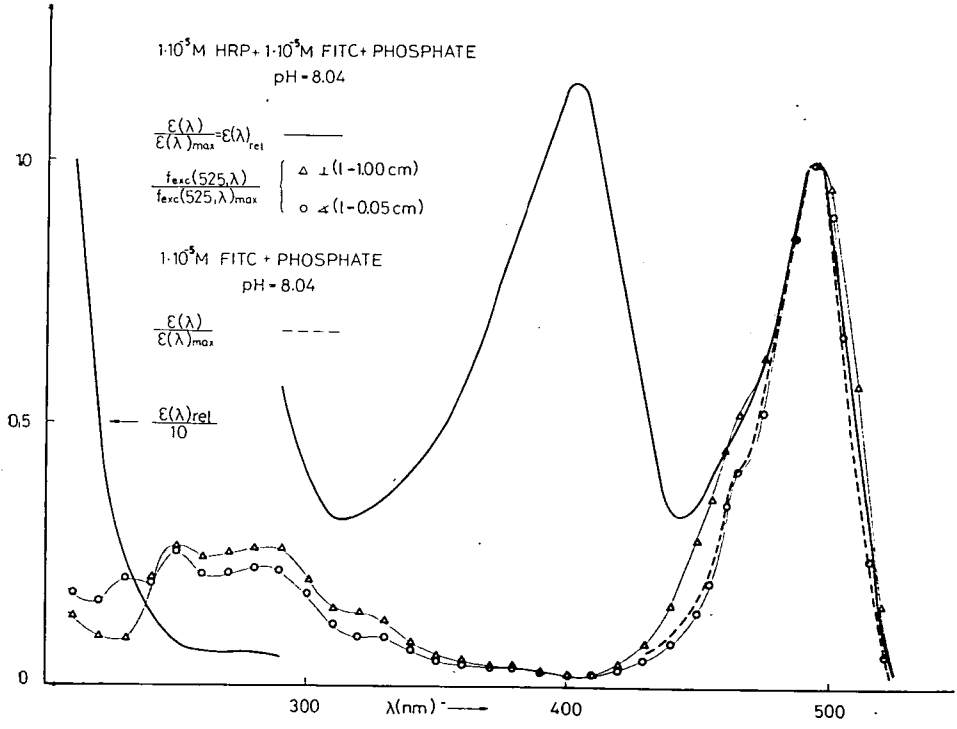
1·10⁻⁵M HRP + 1·10⁻⁵M FITC + PHOSPHATE
pH - 8.04
ε(λ)/ε(λ)max = ε(λ)rel
fexc(525,λ)/fexc(525,λ)max
△ ⊥ (l - 1.00 cm)
○ ∠ (l - 0.05 cm)
1·10⁻⁵M FITC + PHOSPHATE
pH - 8.04
ε(λ)/ε(λ)max
ε(λ)rel/10
1.0
0.5
0
300
λ(nm)
400
500

Fig. 4

is high, that of the HRP—protein is low and the contribution of the haem is insignificant despite its high absorption band at 405 nm. This means that, in accordance with our earlier results [11], the haem does not take part in the transfer of the exciting energy.

The excitation spectra measured with different geometries of observation and corrected for reabsorption show marked deviations in the wavelength ranges 260—300 nm and 450—500 nm. These deviations are due to the high absorption of the

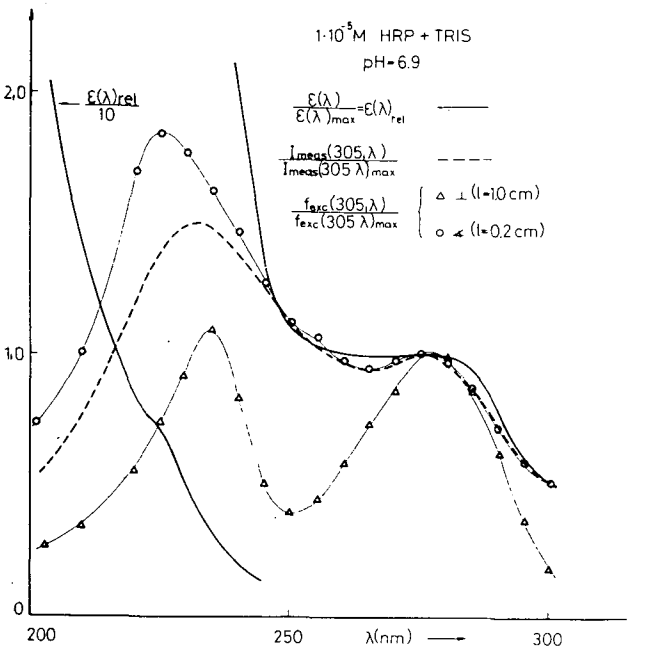

Fig. 5

protein-dye complex in this wavelength range. This explanation is confirmed by the fact that in the wavelength range 420—500 nm the excitation spectrum obtained with front-surface observation and corrected for reabsorption coincides with the relative absorption spectrum of the dye.

An interesting observation is shown in Fig. 5. The corrected excitation spectrum of the HRP fluorescence obtained at $\lambda' = 305$ nm by front-surface observation in the 250—300 nm range coincides with the absorption spectrum. This means that the 305 nm fluorescence is due to the 276 nm absorption maximum of tyrosine (see [11]). A contribution to this fluorescence originates from the component causing a shoulder in the absorption spectrum at 225 nm. This component can be found in the excitation spectrum only by front-surface observation and performing the corrections; otherwise the maximum of the curve shifts towards longer wavelengths and its ratio relative to the protein maximum becomes less.

Our remarks in connection with Figs. 4 and 5 point to the important role of the corrections and of the adequate geometry used for the measurements.

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* The paper under [12] has been published after the submission of the manuscript, therefore, the results of [12] could not be considered.

ОПРЕДЕЛЕНИЕ ДЕЙСТВИТЕЛЬНЫХ СПЕКТРОВ ФЛУОРЕСЦЕНЦИИ ВОЗБУЖДЕННЫХ БЕЛКОВ

З. Варкони, Л. Салаи

Описаны способы определения действительных спектров возбуждения, получаемые с применением коррекций на реабсорбцию и вторичную флуоресценцию, для разных методов возбуждения и наблюдения. Результаты иллюстрируются спектрами флуоресценции растворов пероксидазы (HRP) лизоцима, детергента тритона X—100 и пероксидазы содержащего в качестве индикатора флуоресцеин-изотиоцианат (FITC).