

Evaluation of Association Constants from Fluorescence Data: A Corrected Method*

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ABSTRACT

An equation is proposed for the evaluation of partition or association constants from fluorescence properties (such as spectrum shape or anisotropy) whose values are not linearly related to the solute distribution. The proposed procedure is applied to the binding of pyrene to β -cyclodextrin (employing the I_1/I_3 relationship and fluorescence anisotropy) and to the association of pyrene to dioctadecyldimethylammonium small unilamellar vesicles (sonicated) and large unilamellar vesicles (injected). The results obtained in the latter system indicate that the pyrene partition constant between the vesicles and the aqueous medium is nearly 10 times larger in the small vesicles.

INTRODUCTION

The extent in which one molecule is associated to microphases or macromolecules, as well as to dimer or small aggregate formation, can be followed spectrophotometrically if the characteristics of the emission and/or absorption spectra differ for "bound" and "free" molecules because for any property, A , which is linearly related to the solute concentration, the measured value of the property can be related to the fraction of bound molecules, α , by the relationship

$$\alpha = \frac{A^0 - A}{A^0 - A^c} \quad (1)$$

where A^0 and A^c correspond to the measured property value in the free and bound states, respectively.

Fluorescence measurements are particularly suited to evaluate the extent of binding of fluorescent solutes, due to their high sensitivity, that allows monitoring very low solute concentrations and its strong dependence upon the characteristics of the environment. The fluorescence properties that de-

pend on the probe environment are the lifetime, polarization and position, intensity and fine structure of the spectra. In principle, any of them could be employed to evaluate the extent of binding. However, this simple formula (Eq. 1) can only be employed when the value of the considered parameter varies linearly with the binding extent (*i.e.* the fluorescence intensity at very low absorbances). In particular, this equation cannot be employed when the fluorescence polarization or the spectrum shape (*i.e.* changes in relative intensity between different vibronic bands or band position) is measured. However, this limitation is frequently overlooked and formulas like Eq. 1 are employed to treat data obtained in these types of measurements.

Polycyclic aromatic hydrocarbons stand among the compounds whose properties and distribution are more frequently evaluated using fluorescence measurements (1-3). Pyrene (Py),[‡] whose lifetime and fluorescence spectra shapes are extremely sensitive to the medium, is one of the probes most extensively employed in these type of studies (4-6). In particular, the ratio between the intensities of the first and third vibronic bands (I_1/I_3 ratio) has been frequently employed to characterize the Py microenvironment (7,8) and to evaluate Py distribution and/or association (9-15) in spite of the fact that this property is not linearly related to the probe distribution, making the treatment of the data according to Eq. 1 unwarranted (12-15). In the present communication a simple correction to Eq. 1 is proposed, allowing its use in order to obtain the degree of solute association. We then apply the corrected equation to the distribution of Py between pseudophases and to its association to β -cyclodextrin (β -CD).

MATERIALS AND METHODS

Pyrene (Sigma Chemical Co.) was purified by crystallization in methanol. Dipalmitoylphosphatidylcholine (DPPC) (Sigma Chem. Co.) and β -CD (Sigma Chem. Co.) were used as received, without further purification. Dioctadecyldimethylammonium chloride (DODAC) (Herga Industria Química) was purified as previously described (16). All aqueous solutions were prepared in HPLC quality water, Omnisolv.

*This paper is dedicated to the memory of Giuseppe Cilento.

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‡Abbreviations: CD, cyclodextrin; DODAC, dioctadecyldimethylammonium chloride; DPPC, dipalmitoylphosphatidylcholine; LUV, large unilamellar vesicle; Py, pyrene; SUV, small unilamellar vesicle.

Fluorescence intensity and fluorescence polarization measurements were carried out in a Perkin-Elmer LS-50B luminescence spectrophotometer equipped with a thermostated cell housing. Temperature was kept at $25 \pm 0.1^\circ\text{C}$.

Solutions of Py/ β -CD inclusion compounds were prepared by addition of the appropriate quantity of β -CD to a stock solution of aqueous Py (below 10^{-7} M in Py). Equilibrium was achieved by shaking the mixture for at least 18 h in a water bath thermostated at $25.0 \pm 0.1^\circ\text{C}$.

Vesicles were prepared by controlled injection of DODAC/chloroform or DPPC/hexane solutions in water, with simultaneous vaporization of solvent (large vesicles) or ultrasonic irradiation of an aqueous suspension of the amphiphile (small vesicles) (16). Preparation of Py/vesicle solutions was performed in a similar way to Py/ β -CD solutions. However, in this case equilibrium was rapidly obtained (less than 10 min).

RESULTS AND DISCUSSION

When the property being measured is not linearly related to the probe distribution, Eq. 1 must be modified in order to take into account differences in absorption coefficients and/or fluorescence quantum yields between the free and bound states. When the I_1/I_3 ratio or the anisotropy is the employed property (*i.e.* $A = I_1/I_3$ or $A = r$) it is easily shown that the value of α is related to the value of the measured property through Eq. 2. (See Appendix for equation deduction when $A = I_1/I_3$.)

$$\left[\alpha + (1 - \alpha) \frac{\Phi^0 \epsilon^0}{\Phi^\infty \epsilon^\infty} \right] \left[\frac{A^0 - A}{A^0 - A^\infty} \right] = \alpha \quad (2)$$

where Φ^0 and Φ^∞ , ϵ^0 and ϵ^∞ are the quantum yield of fluorescence and the extinction coefficient in the free (0) and bound (∞) states, respectively. When the property employed is the I_1/I_3 ratio, the above defined quantities correspond to the third vibronic band. When anisotropy is measured, they correspond to the wavelength of measurement.

In comparison with Eq. 2, Eq. 1 is correct only when

$$\Phi^0 \epsilon^0 = \Phi^\infty \epsilon^\infty \quad (3)$$

If this condition holds, I_3 remains constant and the I_1/I_3 ratio only measures the changes in the intensity of the first vibronic band, and the procedure is equivalent to measure the band intensity and not the change in the spectrum shape.

The above considerations are particularly relevant because changes in the fluorescence band shape are generally accompanied by changes in absorption coefficients and fluorescence yields. This implies that Eq. 1 will never be appropriate when parameters associated with the spectrum shape or the generalized polarization are employed. Furthermore, it must be noticed that the corrected Eq. 2 and the uncorrected Eq. 1 can only be employed at low absorbances and when the fluorescence quantum yields of the bound and free molecules are independent of the microphase (or macromolecule) concentration and, for the association to microphases, of the mean occupation number (17).

Association of pyrene to β -CD

The change in the I_1/I_3 ratio of Py fluorescence spectra with β -CD concentration is shown in Fig. 1. These data indicate that the I_1/I_3 ratio changes from 1.8 in bulk water to near 0.6 when the probe is totally associated to β -CD (8,12).

Under the conditions employed in the present work, the results obtained for the association of Py to β -CD suggest

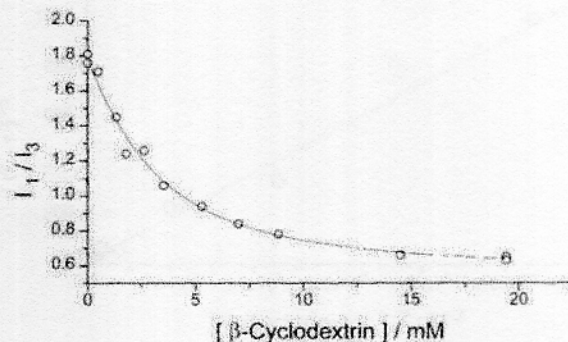
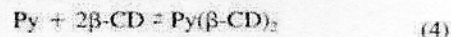


Figure 1. Change in the I_1/I_3 ratio of Py fluorescence spectra with β -CD concentration.

that the stoichiometry can be represented according to Eq. 4. The same stoichiometry has been proposed by Muñoz de la Peña *et al.* (12) and by Xu *et al.* (8) at high β -CD concentration.



The equilibrium can then be represented in terms of the association constant K , defined by

$$K = \frac{\alpha}{(1 - \alpha)[\beta - \text{CD}]^2} \quad (5)$$

Linearization of Eq. 5 by a double reciprocal plot, use of α values derived from Eq. 1 and the assumption that concentration of CD is much greater than the complex concentrations leads to

$$\frac{1}{A^0 - A} = \frac{1}{A^0 - A^\infty} + \frac{1}{(A^0 - A^\infty)K[\beta - \text{CD}]_0} \quad (6)$$

Fitting the data shown in Fig. 1 to Eq. 6 yields a best value of $K = (3.3 \pm 0.1) \times 10^5 \text{ M}^{-2}$. Previous results for the association constant, not corrected, exhibit marked differences. The value reported by Muñoz de la Peña *et al.* (12), $8.5 \times 10^5 \text{ M}^{-2}$, is in good agreement with our result without correction. That reported by Kusumoto (18), $1.16 \times 10^4 \text{ M}^{-2}$, is far from our uncorrected association constant, but it must be noticed that Kusumoto's I_1/I_3 ratio at high β -CD concentration, 0.9, is higher than 0.6, the value experimentally obtained by us and other authors (8,12).

On the other hand, when the measured spectrum shape is related to α through Eq. 2, with the above-mentioned assumptions, then

$$\frac{1}{A^0 - A} = \frac{1}{A^0 - A^\infty} + \frac{\varphi}{(A^0 - A^\infty)K[\beta - \text{CD}]_0} \quad (7)$$

where φ is the correction factor that takes into account changes in absorbance and/or quantum yields between the bound and free states, $\varphi = (\Phi^0 \epsilon^0 / \Phi^\infty \epsilon^\infty)$. By comparison of Eq. 6 with 7, it can be shown that if the correction factor is neglected, the obtained partition constant (K') will be related to the corrected value (K) by the equation

$$K' = \frac{K}{\varphi} \quad (8)$$

and the introduced error will be proportional to the value of

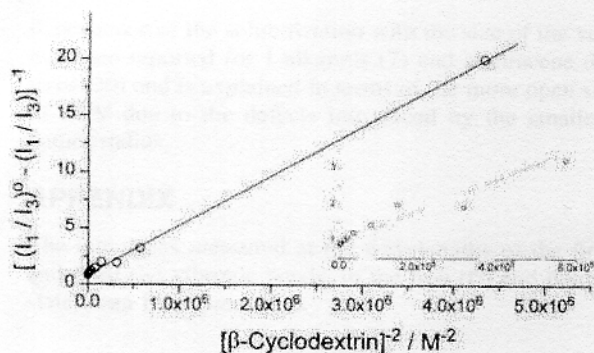


Figure 2. Plot of $[(I_1/I_3)^0 - (I_1/I_3)]^{-1}$ against $[\beta\text{-CD}]^{-2}$, for the association between Py and $\beta\text{-CD}$.

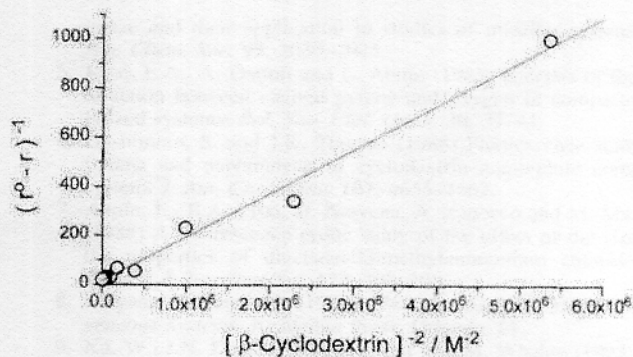


Figure 3. Plot of $(r^0 - r)^{-1}$ against $[\beta\text{-CD}]^{-2}$, for the association between Py and $\beta\text{-CD}$.

the neglected correction factor. When the I_1/I_3 ratio is employed, the correction factor can be calculated from the fluorescence intensities of the third vibronic band in the free and bound states at the same total Py concentration. These values were obtained from measurements in the bulk solvent and by extrapolation of the $\beta\text{-CD}$ data to infinite concentration, respectively. The value obtained by this procedure is $\psi = 0.35 \pm 0.02$. The experimentally determined data, plotted as $(A^0 - A)^{-1}$ against $[\beta\text{-CD}]^{-2}$, using the corrected Eq. 7 is shown in Fig. 2. The linearity of this plot supports neglecting the contribution of a 1:1 Py/ $\beta\text{-CD}$ complex, allowing the evaluation of $K = (1.2 \pm 0.1) \times 10^5 M^{-2}$, greater than $3 \times 10^4 M^{-2}$, the corrected value reported by Xu *et al.* (8). This difference can be attributed to the different mathematical method applied to fit the data.

The large difference between our corrected value and that obtained by a similar procedure but based on Eq. 1 (12) is associated with the greater quantum yield of the emission of the bound Py.

Attempts to take in account the effect of changes in fluorescence quantum yields upon partition constants evaluated from I_1/I_3 measurements have been carried out by Nakajima (10) in the Py/ $\beta\text{-CD}$ system and by Almgren *et al.* (19) in a study of Py/tetraalkylammonium ion associations. However, these treatments are not general because it is assumed that $\psi = 1$ (10) or that the extinction coefficients and the fraction of the intensity associated with the third vibronic bands are the same in the free and bound states (19).

The Py fluorescence anisotropy can also be employed to evaluate the extent of binding to the CD. In this case, Eq. 2 takes the form

$$[\alpha + (1 - \alpha)\psi] \left(\frac{r^0 - r}{r^0 - r^*} \right) = \alpha \quad (9)$$

where the correction factor, ψ , is the ratio between the fluorescence intensities for the free and totally bound molecules at the wavelength at which the emission is being observed ($\psi = I^0/I^*$).

Anisotropy measurements were carried out at 372 nm. From the plot of anisotropy data according to Eq. 7, see Fig. 3, and employing an experimental correction factor of $\psi = 0.8$, an association constant of $(0.95 \pm 0.1) \times 10^5$ is obtained. This value agrees favorably with that obtained from the I_1/I_3 ratio measurements.

Association of Py to vesicles

The association of Py to DODAC or DPPC vesicles, inside the framework of the pseudophase approximation, can be given in terms of the partition constant

$$K = \frac{\alpha}{(1 - \alpha)x_{\text{Amph}}} \quad (10)$$

where x_{Amph} represents the molar fraction of the amphiphile in the aqueous pseudophase. The value of K can be obtained from the double reciprocal relation according to

$$\frac{1}{A^0 - A} = \frac{1}{A^0 - A^*} + \frac{55.6\psi}{(A^0 - A^*)K[\text{Amph}]} \quad (11)$$

where $[\text{Amph}]$ is the molar concentration of amphiphile.

The association of Py to DODAC large unilamellar vesicles (LUV) was evaluated using the I_1/I_3 ratio as the measured property. The obtained data, plotted as $(A^0 - A)^{-1}$ against $[\text{DODAC}]^{-1}$, are shown in Fig. 4. From this plot we obtain a partition constant, equal to $(7.1 \pm 0.4) \times 10^5 M^{-1}$, when an experimentally obtained correction factor, ψ , of 0.50 ± 0.02 is used. Applying the same procedure for DPPC LUV, with $\psi = 0.60 \pm 0.02$, a partition constant of $(6.0 \pm 0.5) \times 10^5 M^{-1}$ is obtained. For DODAC small unilamellar vesicles (SUV), with $\psi = 0.48 \pm 0.02$, the partition constant takes a value of $(4.4 \pm 0.3) \times 10^6 M^{-1}$.

These data are the first reported values regarding the partition of Py in vesicles and indicate that it is considerably more prone to incorporate into the smaller vesicles. Similar

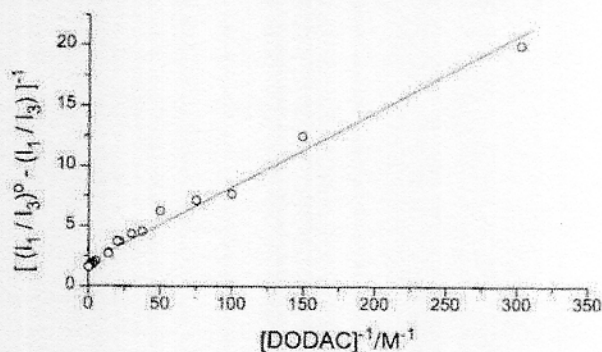


Figure 4. Plot of $[(I_1/I_3)^0 - (I_1/I_3)]^{-1}$ against $[\text{DODAC}]^{-1}$, for the association between Py and DODAC.

dependence of the solubilization with the size of the vesicles has been reported for 1-alkanols (7) and anthracene derivatives (20) and is explained in terms of the more open surface of SUV due to the defects introduced by the smaller curvature radius.

APPENDIX

The intensities measured at the wavelengths of the first (I_1) and third (I_3) vibronic bands, in the free (I^0) and bound (I^*) states, can be expressed as

$$I_1^0 = \beta \epsilon^0 \phi_1^0 \quad (12)$$

$$I_1^* = \beta \epsilon^* \phi_1^* \quad (13)$$

$$I_3^0 = \beta \epsilon^0 \phi_3^0 \quad (14)$$

$$I_3^* = \beta \epsilon^* \phi_3^* \quad (15)$$

where β is a proportionality constant between absorption and emission.

The observed emission in the first and third band, when a fraction α is complexed with β -CD, will be given by

$$I_1 = \beta \{ \epsilon^* \phi_1^* \alpha + \epsilon^0 \phi_1^0 (1 - \alpha) \} \quad (16)$$

$$I_3 = \beta \{ \epsilon^* \phi_3^* \alpha + \epsilon^0 \phi_3^0 (1 - \alpha) \} \quad (17)$$

because

$$\frac{A^0 - A}{A^0 - A^*} = \frac{I_1^0/I_3^0 - I_1/I_3}{I_1^0/I_3^0 - I_1^*/I_3^*} \quad (18)$$

the introduction in Eq. 18 of I_1 and I_3 values, given by Eq. 16 and 17, leads to Eq. 2.

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